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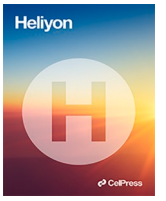

Research article

EPO regulates the differentiation and homing of bone marrow mesenchymal stem cells through Notch1/Jagged pathway to treat pulmonary hypertension

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ABSTRACT

Purpose: To investigate whether erythropoietin (EPO) can treat pulmonary arterial hypertension (PAH) in rats by regulating the differentiation and homing of bone marrow mesenchymal stem cells (BMSCs) through Notch1/Jagged signaling pathway.
Materials & methods: BMSCs were isolated from the bone marrow of 6-week-old male SD rats by whole bone marrow method and identified. BMSCs were treated with 500 IU/mL EPO, and the proliferation, migration, invasion and differentiation ability, and the expression of MMP-2 and MMP-9 protein of BMSCs were detected *in vitro*. After the establishment of the pulmonary hypertension model in rats, BMSCs were intervened with different concentrations of EPO and injected into the rats through intravenous injection. The levels of TNF-α, IL-1β and IL-6 in lung tissue, the expression of SRY CXCR4, CCR2, Notch1 and Jagged protein in lung tissue, and the levels of TGF-α, vascular endothelial factor (VEGF), IGF-1 and HGF in serum were detected. Immunofluorescence (IF) staining was used to detect the co-localization of CD34.
Results: EPO promoted the proliferation, migration, and invasion of BMSCs by inhibiting Notch1/Jagged pathway *in vitro*, and induced BMSCs to differentiate into vascular smooth muscle cells and vascular endothelial cells. EPO inhibited Notch1/Jagged pathway in PAH rats, induced BMSCs homing and differentiation, increased the levels of TGF-α, VEGF, IGF-1 and HGF, and decreased the levels of TNF-α, IL-1β and IL-6.
Discussion & conclusion: EPO can inhibit the Notch1/Jagged pathway and promote the proliferation, migration, invasion, homing and differentiation of BMSCs to treat pulmonary hypertension in rats *in vitro* and *in vivo*.

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1. Introduction

Pulmonary arterial hypertension (PAH) is a progressive respiratory disease characterized by persistent vasoconstriction and excessive vascular remodeling, ultimately resulting in a dismal prognosis. As the disease advances, sustained pressure overload precipitates right ventricular dysfunction, culminating in right ventricular failure and the eventual demise of the patient [1,2]. The main clinicopathological features of PAH include significant thickening of distal pulmonary arteries, obstructive remodeling of pulmonary arterioles, and peripheral vascular inflammation and metabolic changes. Studies have demonstrated that these lesions are primarily caused by excessive proliferation, migration, and apoptosis resistance of pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs) [3]. The phenotypic plasticity of smooth muscle cells (SMCs) is closely regulated by Notch signaling pathway. Smooth muscle cells activated by Notch pathway have been identified as the source of occlusive neointimal lesions in PAH. These results suggest that inhibition of Notch pathway can alleviate PAH [4,5].

Mesenchymal stem cells (MSCs) are a kind of adherent fibroblast-like cells derived from different tissues and organs, including adipose tissue, bone marrow, umbilical cord blood, lung, heart, etc. Bone marrow mesenchymal cells (BMSCs) are multipotent cells with the ability to proliferation and differentiation. Their repair, regeneration, and immunomodulatory properties make them good candidates for cell therapy and tissue regeneration [6]. The homing ability of MSCs allows them to navigate to the site of injury, thus enabling the systemic administration of MSCs in clinical practice. Homing involves transendothelial migration guided by certain chemokines from the site of injury. However, only a small fraction of MSCs are detected in target tissues, which is a major bottleneck for MSC-based therapies [7].However, the main disadvantages of BMSCs therapy are the lack of specific homing of cells after intravenous infusion, and the early death of injected cells due to the damaged microenvironment caused by various factors such as local hypoxia, oxidative stress, and inflammation, resulting in the low therapeutic effect of BMSCs [8]. If the migration and invasion ability of BMSCs can be enhanced by pretreatment in advance, the effect of repair and regeneration of organs can be improved. In this study, we decided to use the sex determining region Y(SRY) gene to track BMSCs and determine the homing efficiency of BMSCs.

Erythropoietin (EPO), as a hematopoietic hormone, is directly involved in erythropoiesis in mammals, and its synthesis region is located in the kidney and liver [9]. EPO has been clinically used to treat a variety of diseases, such as chronic renal failure and anemia. The coordinated regulatory effects of EPO on angiogenesis have also been demonstrated [10]. Recent studies have found that EPO can significantly improve the homing ability of BMSCs and help BMSCs differentiate specifically, which makes the idea of enhancing the specificity and vitality of BMSCs a reality [11]. Notch signaling pathway is closely related to BMSCs. Inhibition of Notch signaling pathway in BMSCs can increase the expression of CXCR4 and enhance the homing ability of BMSCs. Studies have found that CXCR4, rise in MMP-2 and MMP-9 can let HP-BMSCs penetration ability strengthen, can pass through blood vessels/organizational barriers into the injury, this could be the increase of capability of homing of BMSCs research direction [12].However, whether EPO can enhance the homing and differentiation ability of BMSCs by regulating Notch1/Jagged pathway is still unknown.

Therefore, this study aims to investigate whether EPO can treat PAH by regulating the differentiation and homing ability of BMSCs through Notch1/Jagged pathway.

2. Materials and methods

2.1. Isolation and culture of rat bone marrow mesenchymal stem cells (BMSCs)

Bone marrow of 6-week-old male SD rats was collected, and BMSCs were isolated from bone marrow according to SOP: The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg), and immediately after the epiphysis of the rat femur was collected, the rat femoral epiphysis was given DMEM medium with 1% penicillin/streptomycin, 2 mmol/L-glutamine and fetal bovine serum containing 10% (FBS; HyClone, Logan, UT, USA) was used to wash the bone marrow. The filtered extracts were collected using a 200-mesh cell filter and centrifuged at 1000 r/min for 5 min. The centrifuged substances were re-suspended in the medium. The cells were incubated in a 5% CO_2 incubator at 37 °C. When the cells reached 80–90%, the cells were fused for subculture, and subculture was performed every 3–4 days. The 2nd ~ 4th generation cells were used for experiments.

2.2. Cell groups and treatment

The BMSCs were divided into control group (0 IU/mL EPO), EPO group (500 IU/mL EPO), and EPO + Jagged1 group (500 IU/mL EPO+50 ng/ml Jagged1), The EPO concentration was selected according to Zhang et al. [13]. The corresponding concentrations of EPO and Jagged1 were added into BMSC culture for 48 h. After the intervention, Immunofluorescence (IF) staining and cell scratch test were performed. Cell invasion assay, Western Blot.

2.3. Animals

Male SD rats (6 weeks old) and female SD rats (8 weeks old) were purchased from Chengdu Dashuo Experimental Animal Co., LTD. (Chengdu, Sichuan Province). The temperature was (25 ± 2) °C, the relative humidity was 50%–60%, the light/dark cycle was 12 h a day, and the animals were free to eat and drink. Experimental animals and protocols were performed in accordance with the guidelines and ethical standards established by Laboratory Animal Ethics Committee, West China Hospital, Sichuan University (20,230,806,001).

2.4. Animal groups and treatment

Female rats were randomly divided into six groups according to body weight: control group (normal saline), PAH model group (PAH), PAH + BMSCs group (BMSCs-PAH), L-EPO-BMSCs group (L-EPO-BMSCs), PAH + BMSCs group EPO-BMSCs medium dose group (M-EPO-BMSCs) and pulmonary hypertension model + EPO-BMSCs high dose group (H-EPO-BMSCs), with 6 rats in each group. The PAH group were tail vein injected with 60 mg/kg Monocrotaline (MCT) and 1 mL PBS. BMSCs-PAH group were tail vein injected with 60 mg/kg MCT and 1 mL BMSCs (2×10^6 cells/mL). L-EPO-BMSCs group was tail vein injected with 60 mg/kg MCT, 1 mL BMSCs and 10 IU/mL EPO. M-EPO-BMSCs group was tail vein injected with 60 mg/kg MCT, 1 mL BMSCs and 100 IU/mL EPO. H-EPO-BMSCs group was tail vein injected with 60 mg/kg MCT, 1 mL BMSCs and 500 IU/mL EPO, and all rats were injected with BMSCs and EPO on the third day after MCT injection. The control group were tail vein injected with the same volume of PBS. After 14 days of intervention, sodium pentobarbital (50 mg/kg) was injected intraperitoneally to induce anesthesia, and abdominal aortic blood was collected. After the animals were sacrificed, lung tissues were collected for subsequent experiments.

2.5. Cell migration assay

The effect of EPO on the migration ability of BMSCs was examined by incubating 1×10^5 cells/well in 6-well plates at 37 °C overnight. The monolayer cells were scratched with the tip of a pipette, washed with serum-free medium to remove exfoliated cells, and then cultured with complete medium containing different concentrations of EPO. After scratch creation, the cells were continuously cultured for 0 h and 24 h, and the cells were photographed by an inverted microscope (TE2000, Nikon). Migration distances were measured using ImageJ analysis software (National Institutes of Health, Bethesda, MD).

2.6. Cell transwell assay

Serum-free medium precooled at 4 °C was filled to a transwell filter (BD Biosciences, San Jose, CA, USA) to form the chamber. BMSCs (3.0×10^5/mL) were inoculated into the upper transwell chamber, and 10% fetal bovine serum (FBS) was added to the basolateral chamber. The cells were incubated for 24 h at 37 °C in a 5% CO_2 incubator. After culture, the transwell filters were washed twice with sterile PBS and fixed with methanol at room temperature for 30 min. Finally, 0.1% crystal violet was added to stain the cells for 30 min. The cells were observed and photographed under an Olympus X51 inverted microscope (Olympus, Tokyo, Japan).

2.7. Flow cytometry

Cell survival and apoptosis were detected by Annexin V-FITC and PI staining (Annexin V-FITC Apoptosis Detection kit, Shanghai, China). First, the BMSCs that had been intervened were digested by trypsin without EDTA, washed 3 times with PBS, added with 5 μL Annexin V-FITC and 5 μL PI, mixed evenly, and incubated at room temperature in the dark for 5 min. The apoptosis rate was detected by flow cytometry (Annexin V-FITC, Ex/Em: 488nm/525 nm; PI, Ex/Em: 561nm/575 nm).

2.8. If staining

Rat lung tissues and were fixed in 4% paraformaldehyde, and lung tissue paraffin sections were prepared according to the SOP. After dewaxed and hydrated, the sections were incubated in QuickBlock™ blocking buffer (Beyotime, Shanghai, China) for 30 min at room temperature. Similarly, BMSCs after intervention were prepared as cell slides. Sections of lung tissue were subjected to anti-Sexdetermining Region Y (SRY; Abcam ab140309; 1/100) and anti-CD34 antibody (Abcam, ab4648; 1/100), the cell slides were subjected to anti-SRY, α-actin (abclonal, A7248; 1/100), calponin3 (Abcam, ab204365; 1/100), SM-MHC (Abcam, ab133567; 1/100), CD34 (Abcam, ab81289; 1/100), CD105 (Abcam, ab2529; 1/100), overnight incubation at 4 °C, and washed three times with phosphate buffered saline (PBS) after incubation. Secondary antibody incubation was performed with HRP-labeled goat anti-rabbit IgG (Servicebio, GB23303, 1/100) and Cy3-labeled goat anti-rabbit IgG (Servicebio, GB21303, 1/100), Nuclei were counterstained with DAPI (Sigma-Aldrich, St Louis, MO). After all staining was completed, the lung tissue staining was observed using a BX53 fluorescence microscope (Olympus, Tokyo, Japan) at 400× magnification.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The serum levels of IGF-1, SDF-1, VEGF, HGF, MCP-1, TNF-α, IL-1β and IL-6 in each group were detected. The procedure is about the instructions of the kit. Kits for IGF-1, SDF-1, VEGF, HGF, MCP-1, TNF-α, IL-1β, and IL-6 were purchased from Shanghai ZCIBIO Biotechnology Co., LTD. (Shanghai, China).

2.10. Hematoxylin and eosin (H&E) staining

The lung tissue of rats was fixed in 4% paraformaldehyde, and the lung tissue sections were prepared according to SOP and stained with H&E. Finally, the degree of lung tissue lesions and arterial changes were observed under a light microscope (Olympus BH2, Tokyo, Japan) at 400× magnification.

2.11. Western Blot (WB)

Lung tissue was resolved using RIPA tissue lysate (Signaling Technology, Inc.), and total tissue protein was extracted. BCA kit (Sigma-Aldrich; Merck) for protein quantification. Total proteins (30 μg/sample) were separated by electrophoresis on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Using 5% skim milk powder to block excess vacancies on the membrane, antibody incubation was performed at 4 °C. The corresponding protein antibodies were as follows: CXCR4 (Abcam, ab124824; 1/1000), CCR2 (Abcam, ab223366; 1/1000), Notch1 (Cell Signaling Technology, #3608; 1/1000), Jagged (Abcam ab300561; 1/1000), MMP-2 (Abcam, ab92536; 1/1000), MMP-9 (Abcam, ab76003; 1/1000), β-actin (Abcam, ab8226; 1/1000). After antibody incubation, the cells were washed 3 times with Tris-buffered saline/0.1% Tween (TBST) and incubated with HRP goat anti-rabbit IgG (Abcam, ab6721; 1/5000) were incubated at room temperature for 2 h and washed three times with TBST. Finally, the protein bands were visualized using the ECL system (Affinity Biosciences, Cincinnati, Ohio, USA) and β-actin was used as an internal reference.

2.12. Statistical

Data are expressed as mean and standard deviation. All data were analyzed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). One-way ANOVA with Tukey's post hoc mean test was used for multiple group comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. BMSCs from SD rats were isolated, cultured and identified

In this experiment, we first used the whole bone marrow separation method to extract and isolate the BMSCs of SD rats. Flow cytometry showed that compared with the cells in the Control group, the BMSCs showed negative expressions of CD34 and CD45, and positive expressions of CD44 and CD90 (Fig. 1B), which was consistent with the characteristics of BMSCs. The cell morphology of BMSCs was spindle-shaped, adhered to the wall, and radiate neatly and orderly. No changes in cell morphology were observed after more than 10 successive passages of culture (Fig. 1A), indicating that the extraction and isolation of rat BMSCs were successful.

EPO promotes the migration and invasion of BMSCs by inducing MMP-2 and MMP-9 protein expression through Notch1/Jagged pathway.

Cell migration and invasion are the key indicators to evaluate cell activity, and are the basis for the maturation and differentiation of BMSCs. The results of cell scratch assay showed that the migration distance of BMSCs treated with EPO increased within 48 h, while the effect of EPO on cell migration was weakened by Jagged1 intervention (Fig. 2A and B). The results of cell transwell invasion assays were also consistent (Fig. 2C and D). WB results showed that EPO could increase the expression levels of MMP-2 and MMP-9 proteins in BMSCs, and Jagged1 could attenuate the induction of MMP-2 and MMP-9 proteins by EPO in BMSCs (Fig. 2E–G).

EPO promotes the differentiation of BMSCs into vascular endothelial cells and vascular smooth muscle cells by inhibiting Notch1/Jagged pathway.

BMSCs need to differentiate into different types of cells to function, and the cell differentiation ability is closely related to Notch1/Jagged. Our experimental results showed that the expression of Notch1 and Jagged proteins in BMSCs decreased after EPO intervention, and Jagged1 reversed the inhibitory effect of EPO on the reduction of Notch1 and Jagged proteins (Fig. 3K-M). BMSCs expressed α-actin, calponin3, SM-MHC, CD34 and CD105 in the presence of Notch1/Jagged inhibition by EPO, while Jagged1 reversed the inhibitory effect of EPO on Notch1/Jagged pathway. Thus, the positive expression levels of the above proteins were reduced, and the differentiation of BMSCs into vascular smooth muscle cells and vascular endothelial cells was hindered (Fig. 3A–J).

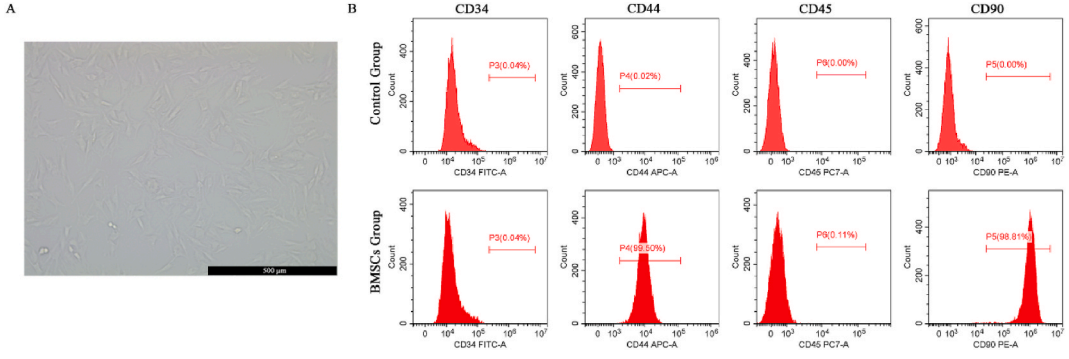

Fig. 1. Culture and identification of BMSCs from SD rats. A Cell morphology diagram of BMSCs after isolation and culture, magnification of 200×. B The positive expression of CD34, CD44, CD45 and CD90 in BMSCs.

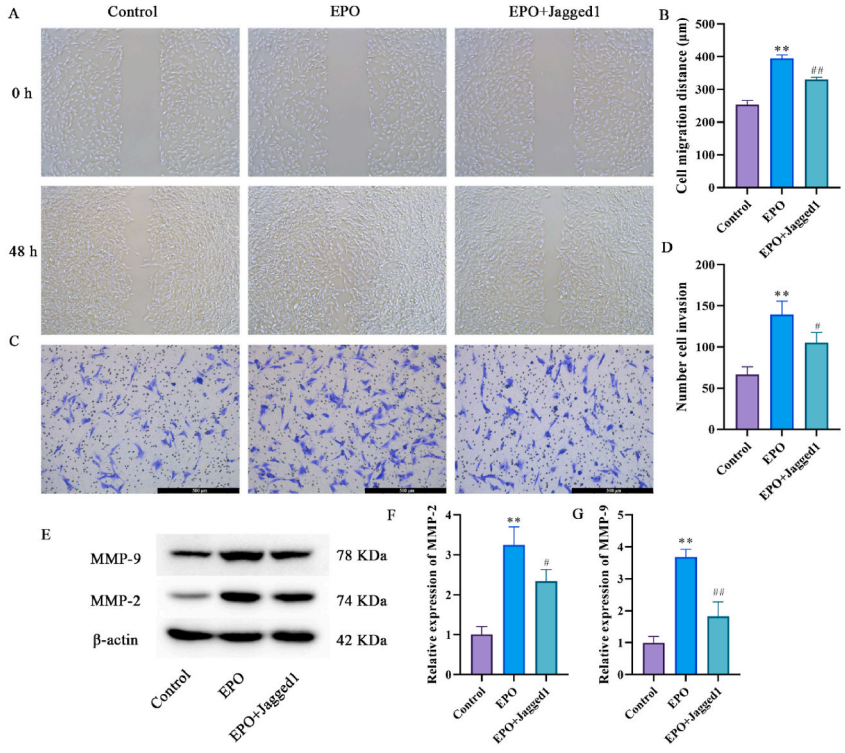

Fig. 2. EPO enhanced the migration and invasion of BMSCs by inhibiting the Notch1/Jagged pathway and inducing MMP-2 and MMP-9 protein expression in BMSCs. A-B Changes in migration distance of BMSCs after 48 h of culture. Magnification was 200×. C-D Changes in the number of cells stained at the bottom of the transwell chamber after 48 h of BMSCs culture. The cells were stained with crystal violet at a magnification of 200×. E-G Relative protein expression levels and Western blot changes of MMP-2 and MMP-9 in BMSCs cultured for 48 h. Data are presented as mean ± standard deviation, $^{**}P < 0.01$ vs. Control; $\#P < 0.05$, $\#\#P < 0.01$ vs. EPO.

3.2. EPO combined with BMSCs can effectively reduce tissue damage in pulmonary hypertension rats

The lung tissue of rats in each group was stained with HE, and the vascular wall thickness (VWT) and total vessel area (TVA) were measured. The results showed that the VWT and TVA in the lung tissue of the rats with pulmonary hypertension were severe (Fig. 4A and B), and the results were also consistent under the microscope (Fig. 4C). The intervention of BMSCs alone or EPO combined with BMSCs reduced the VWT and TVA in the lung tissue of the rats with pulmonary hypertension. The effect was enhanced with increasing EPO (Fig. 4A–C). And the results also showed that the levels of IL-1β, IL-6 and TNF-α in the lung tissue of rats with pulmonary hypertension were increased, while the levels of IL-1β, IL-6 and TNF-α in the lung tissue of BMSCs intervention alone or EPO + BMSCs intervention could reduce the levels of Il-1β, IL-6 and TNF-α in the lung tissue. The effect was enhanced with increasing EPO (Fig. 4D–F), indicating that EPO and BMSCs co-intervention exerted a protective effect by reducing the thickness and area of vascular wall and the level of inflammatory factors in lung tissue.

3.3. EPO can effectively promote the homing and differentiation of BMSCs

The expression of SRY in lung tissue was detected by immunofluorescence. The results showed that SRY fluorescence was not detected in the control group and the PAH group because BMSCs were not added. In the groups with SRY fluorescence intensity detected, the SRY fluorescence intensity of all the EPO added groups was higher than that of the BMSCs-PAH group, indicating that EPO had the ability to induce BMSCs homing, and the effect was enhanced with the increase of EPO concentration. (Fig. 5A and B). Then we investigated the ability of EPO to promote the differentiation of BMSCs. Elisa results showed that SDF-1 and MCP-1 levels were increased in the lung tissues of the PAH rats. EPO combined with BMSCs intervention significantly increased SDF-1 levels and decreased MCP-1 levels in the lung tissues (Fig. 5C and D). Therefore, we speculated that EPO could effectively promote the differentiation of BMSCs. Western blot analysis revealed a significant increase in the relative expression levels of CXCR4 and CCR2 proteins in lung tissues under pulmonary hypertension conditions. Exogenous BMSCs slightly alleviated this state, while EPO-stimulated BMSCs significantly inhibited the expression of CXCR4 and CCR2 proteins (Fig. 5E–G).

IF colocalization assay was used to further confirm the ability of EPO to promote BMSCs homing and differentiation. The results showed that the relative expression level of CD34 was reduced compared with normal rats (Fig. 6A–C). In addition, SRY fluorescence was observed only in the lung tissues of rats exogenously injected with BMSCs (Fig. 6B); The relative expression level of CD34

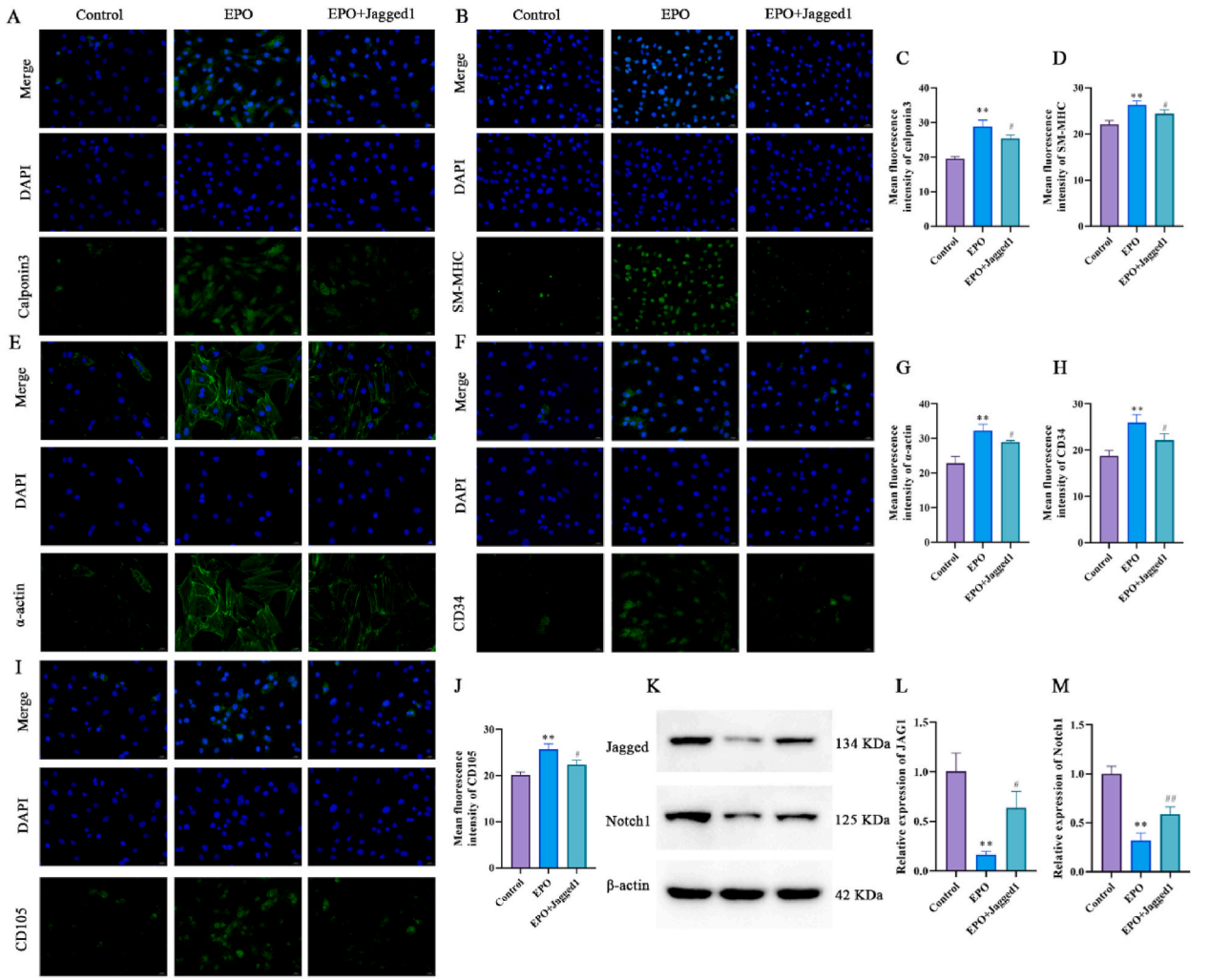

Fig. 3. EPO promotes the differentiation of BMSCs into vascular endothelial cells and vascular smooth muscle cells by inhibiting the Notch1/Jagged pathway. A-J The positive expression of vascular smooth muscle cell markers (α-actin, calponin3, SM-MHC) and vascular endothelial cell markers (CD34, CD105) in BMSCs. Immunofluorescence staining was performed at 400× magnification. K-M Relative protein expression levels of Notch1 and Jagged in BMSCs and changes in Western blotting. Data are presented as mean ± standard deviation, $^{**}P < 0.01$ vs. Control; $\#P < 0.05$, $\#\#P < 0.01$ vs. EPO.

(Fig. 6A–C) in the lung tissue of rats with pulmonary hypertension was significantly increased after EPO intervention. (Fig. 6A–C).

3.4. EPO and BMSCs can inhibit Notch1/Jagged pathway and promote angiogenesis

After confirming the ability of EPO to promote the homing and differentiation of BMSCs, we began to explore the underlying mechanism. Elisa results showed that the levels of HGF, IGF-1, TGF-α and VEGF in lung tissue of PAH group were slightly increased, but the difference was not statistically significant. After EPO and BMSCs co-intervention, compared with PAH group, HGF in lung tissue of M-EPO-BMSCs group and H-EPO-BMSCs group, HGF in lung tissue of M-EPO- BMSCs group and H-EPO-BMSCs group were significantly increased, but the difference was not statistically significant. IGF-1 and VEGF levels were increased, and a significant increase in TGF-α was only observed in the H-EPO-BMSCs group (Fig. 7A–D). We subsequently examined the impact of EPO-treated BMSCs on the Notch1/Jagged pathway in PAH rats. Western blot analysis revealed that compared to the control group, there was a significant increase in relative expression levels of Jagged and Notch1 proteins in lung tissue from the PAH group, which were significantly reduced following co-intervention with EPO and BMSCs (Fig. 7E–G).

4. Discussion

In this study, we revealed that EPO-BMSCs enhanced cell migration, invasion and differentiation by interfering with Notch1/

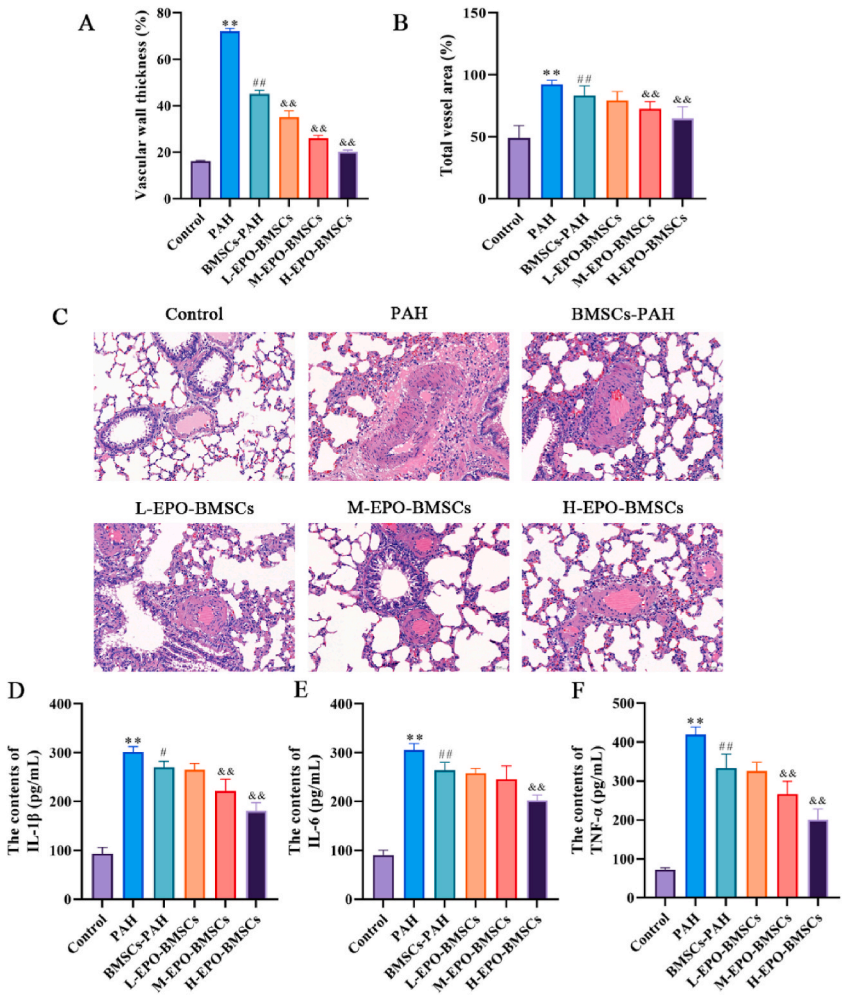

Fig. 4. Co-intervention of EPO and BMSCs can effectively reduce tissue damage in a rat model of pulmonary hypertension. A and B to investigate the effects of erythropoietin (EPO) and bone marrow mesenchymal stem cells (BMSCs) on the thickness and area of pulmonary vascular wall in rats with pulmonary hypertension. C HE staining was used to observe lung tissue injury and pulmonary artery changes (200× magnification). D-F The levels of IL-1β, IL-6 and TNF-α in lung tissue were detected by Elisa. Data are presented as mean ± standard deviation, $^{**}P < 0.01$ vs. Control; $\#P < 0.05$, $\#\#P < 0.01$ vs. PAH; $\&\&P < 0.01$ vs. BMSCs.

Jagged pathway, and the therapeutic effect and mechanism of EPO-BMSCs on PAH rats. After 500 IU/mL EPO intervention, the migration and invasion ability of BMSCs were enhanced within 48 h. α-actin, calponin3, MMP-2, MMP-9, SM-MHC in cells were detected. The up-regulation of CD34 and CD105 indicated that BMSCs were specifically differentiated into vascular smooth muscle cells and vascular endothelial cells, and these changes occurred under the condition of inhibition of Notch1/Jagged pathway. If Jagged1 was used to actively activate the Notch1/Jagged pathway, the effect of EPO-BMSCs was weakened. Our *in vivo* results also showed that EPO enhanced the homing ability of BMSCs, and the increased expression of CD34 indicated that EPO induced the differentiation of BMSCs into rVECs. The activation of CXCR4 and CCR2 protein is one of the causes of PAH in rats. BMSCs treated with EPO can effectively reduce the expression of CXCR4 and CCR2 proteins in lung tissue and reduce the levels of inflammatory mediators such as IL-1β, IL-6 and TNF-α by inhibiting the Notch1/Jagged pathway. It can promote the transport of TGF-α, VEGF, IGF-1 and HGF in serum to the lung tissue to repair the lesion, thus improving the abnormal thickening of blood vessel walls and reducing angiogenesis in PAH rats.

As a new treatment for a variety of lung diseases, stem cell transplantation has attracted great interest for its potential therapeutic effect [14]. In bleomycin and endotoxin-induced lung injury in rats, intravenous or intra-alveolar injection of BMSCs can reduce the severity of lung injury, reduce inflammation and improve the alveolar structure to protect the hyperoxic lung injury [15,16]. MSCs transplantation can help the lung and its cells to produce anti-inflammatory, anti-immune or anti-apoptotic effects, and prevent the growth arrest of alveoli and blood vessels, thereby improving lung structural lesions [17]. The main disadvantage of MSCs therapy is that it is difficult for MSCs to reach the target tissues due to the lack of specific homing after early lung entrapments and systemic infusion, or even after MSCs reach the target organs, the local microenvironmental conditions are unsuitable, such as hypoxia,

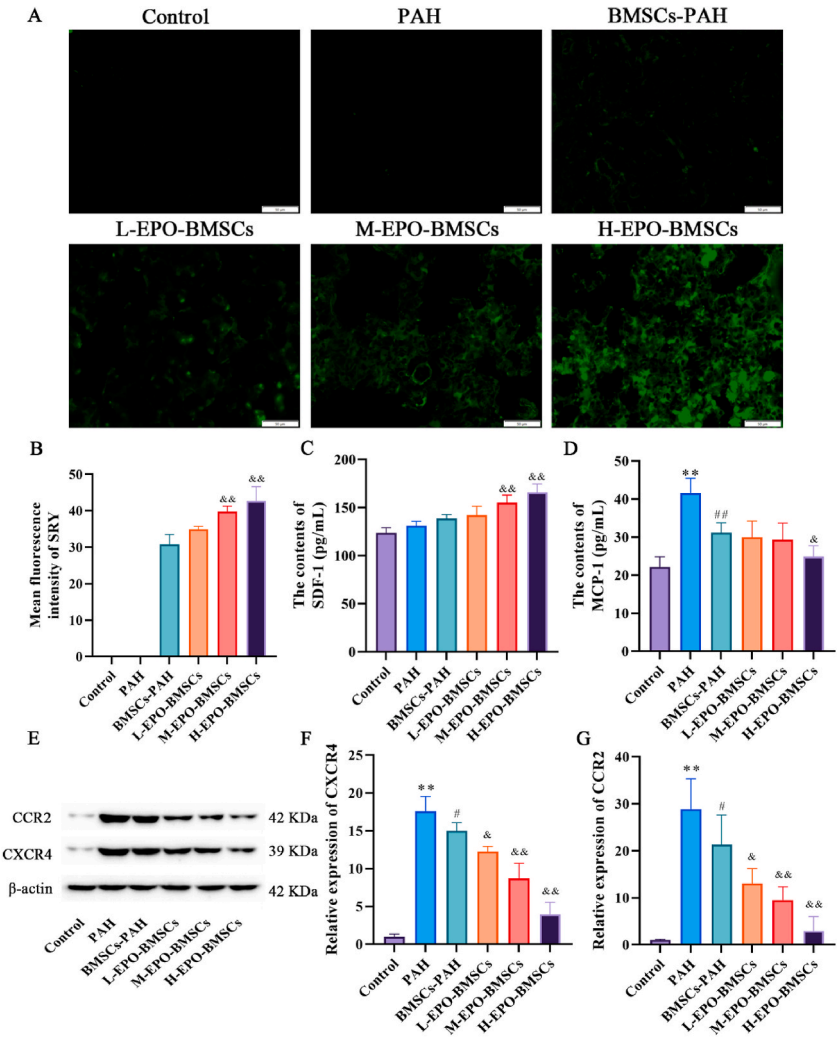

Fig. 5. EPO promotes the homing and differentiation of BMSCs in the lung tissue of rats with pulmonary hypertension. A and B The expression of SRY protein in rat lung tissue was detected by immunofluorescence (IF) stain (200× magnification). C and D The levels of SDF-1 and MCP-1 in lung tissue were detected by Elisa. E-G WB was used to detect the relative expression levels of CXCR4 and CCR2 protein in rat lung tissue. Data are presented as mean ± standard deviation, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control; $\#P < 0.05$, $\#\#P < 0.01$ vs. PAH; $\&P < 0.05$, $\&\&P < 0.01$ vs. BMSCs.

oxidative stress, and inflammation, which reduce the therapeutic effect of MSCs [18,19]. EPO has tissue-protective and anti-inflammatory effects, promotes neurogenesis and angiogenesis, repairs neuronal damage, promotes the proliferation and differentiation of endothelial progenitor cells, and accelerates wound healing. Recent studies have also found that EPO can promote the recruitment of BMSCs, which in turn trigger bone formation and angiogenesis of BMSCs [20,21]. These results indicate that EPO-pretreated BMSCs have the basis for the treatment of PAH. In this study, the expression of SRY protein in rat lung tissue was detected to reflect the aggregation of BMSCs in lung tissue, which proved that EPO had the effect of improving the homing ability of BMSCs, providing a basis for subsequent experiments.

The transfer of MSCs from the blood to a specific organ requires several steps, starting with the attachment of circulating MSCs to endothelial cells through the interaction of CD44 on MSCs and selectin on endothelial cells. Second, VLA4 of MSCs interacts with VCAM1 of endothelial cells, leading to rolling and arrest of MSCS. Finally, MSCs were subjected to endothelial basement membrane invasion and extracellular matrix cleavage in response to MMP-2 [22]. Among them, MMPs are a class of proteolytic enzymes that degrade extracellular matrix, and MMP-9 mainly hydrolyze type IV collagen, which is the main collagen component of basement membrane. Previous studies have found that MMP-9 plays an important role in stem cell mobilization, migration, and homing after myocardial infarction [23]. Another recent study reported that MMP-9 also promotes proliferation and migration of implanted MSCs [24]. In addition, MMPs are involved in cell migration, wound healing, bone development, angiogenesis, and embryonic development. MMP-9 is highly expressed in the airway epithelium and during wound healing [25].Our *in vitro* experiments showed that EPO enhanced the migration and invasion of BMSCs by promoting MMP-2 and MMP-9 expression in BMSCs at appropriate concentrations. EPO also enhanced the differentiation ability of BMSCs, as indicated by the increased expression of vascular smooth muscle cell

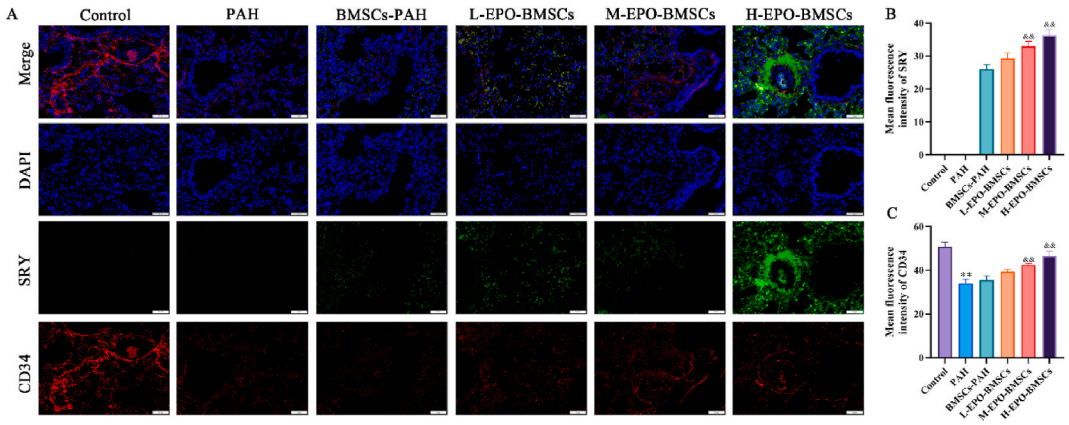

Fig. 6. Effect of EPO on BMSCs homing and CD34 differentiation proteins in rat lung tissue. A-C The expression of SRY and CD34 protein in rat lung tissue was detected by immunofluorescence (IF) stain (200× magnification). Data are presented as mean ± standard deviation, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control; $\#P < 0.05$, $\#\#P < 0.01$ vs. PAH; $\&P < 0.05$, $\&\&P < 0.01$ vs. BMSCs.

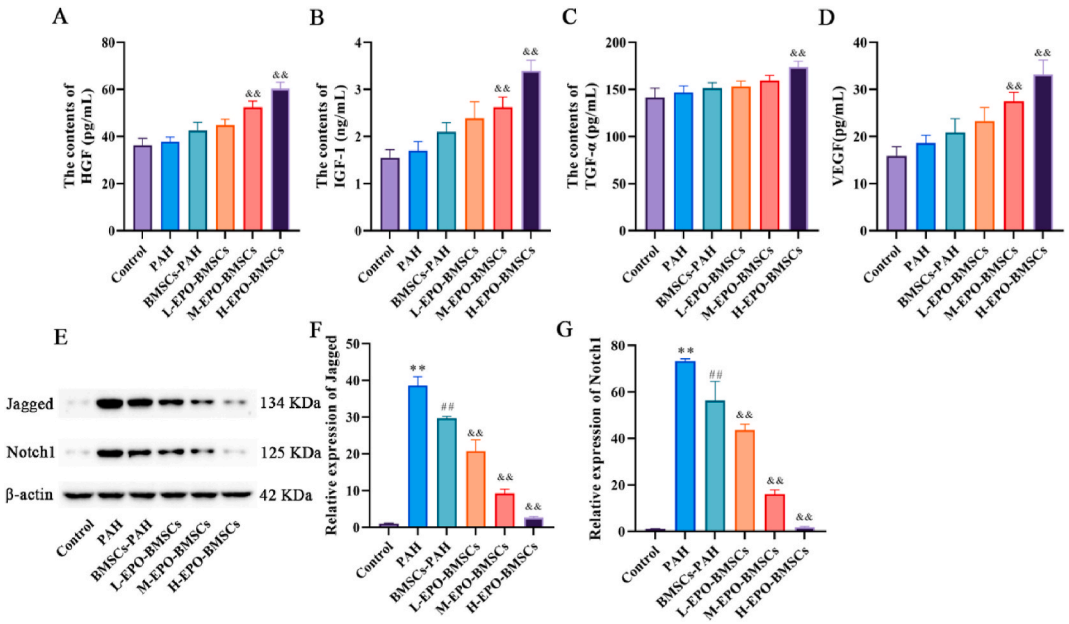

Fig. 7. Co-intervention of EPO with BMSCs inhibited the Notch1/Jagged1 pathway and promoted angiogenesis. A-D the levels of HGF, IGF-1, TGF-α and VEGF in rat lung tissues were detected by Elisa. E-G Western blot was used to detect the relative expression levels of Notch1 and Jagged proteins in lung tissue of rats. Data are presented as mean ± standard deviation, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control; $\#P < 0.05$, $\#\#P < 0.01$ vs. PAH; $\&P < 0.05$, $\&\&P < 0.01$ vs. BMSCs.

markers (calponin3 and SM-MHC) and endothelial cell markers (CD34 and CD105). It is well known that EPO is an important growth factor that can promote the recruitment of BMSCs, which in turn triggers bone formation and angiogenesis in BMSCs [21]. Chemokines and cytokines are important factors that regulate stem cell mobilization, migration, and recruitment [26]. Specifically, the SDF-1/CXCR4 axis plays a crucial role in the migration of BMSCs. Wynn et al. [27]. demonstrated a dose-dependent migration of human BMSCs to SDF-1 using Transwell assay, supporting the conclusion that SDF-1/CXCR4 axis is involved in BMSC migration. Similarly, we also detected high concentrations of SDF-1 in lung tissue, suggesting that SDF-1/CXCR4 axis may play a role in PAH rats, which still needs further investigation.

Next, our *in vivo* results also showed that the expression of CXCR4 and CCR2 protein in the lung tissues of PAH rats was increased, which was caused by the damage of the pulmonary artery endothelium. The chemokine receptor CXCR4 and it's cognate signaling ligand CXCL12 play a major role in regulating the homing of hematopoietic progenitor cells and their mobilization to the periphery. Disruption of either the CXCL12 or CXCR4 genes leads to embryonic or perinatal death, which reminds us of the significance of CXCL12/CXCR4 in cell homeostasis, organogenesis, and angiogenesis [28]. Recent studies have shown that hypoxia can induce

overexpression of CXCR4 in a variety of tumor cells, and the overexpression of CXCR4 is associated with tumor progression and invasion [29]. It is important to note that greater BMSCs differentiation capacity is not better because one of the histopathological features of vascular remodeling associated with pulmonary hypertension is a significant medial thickening of the distal pulmonary arteries, which are generally non-muscularized. Pathological media thickening is caused by preexisting smooth muscle cells that undergo dedifferentiation, distal migration, proliferation, and redifferentiation [30]. This leads to the possibility that if a large number of BMSCs differentiate into vascular smooth muscle, it may continue to thicken the vascular wall after repairing vascular damage and aggravate the symptoms of PAH. Our *in vivo* results showed that BMSCs treated with EPO could significantly reduce the expression of CXCR4 and CCR2 protein in lung tissue, which was because the lung tissue was damaged due to pulmonary hypertension at this time. If the high expression of CXCR4 and CCR2 protein in the tissue was not degraded, the exogenous BMSCs would differentiate rapidly and aggravate PAH. However, these BMSCs still have differentiation ability, and it is meaningless to improve PAH if BMSCs are not induced by homing and directional differentiation in other ways.

The Notch signaling pathway is an evolutionarily conserved regulatory system that plays an important role in cell proliferation, differentiation and survival. Members of Notch proteins are expressed on the cell surface and are activated by the interaction of the Notch receptor (Notch1-4) with ligands (Jagged1-2 and delta-like 1,3,4). Among Notch receptors, only Notch1, 2 and 3 are expressed in blood vessels and play a key role in the regulation of vascular morphogenesis and function during development and disease [31,32]. Our *in vitro* results showed that the Notch1/Jagged pathway was inhibited after EPO treatment of undifferentiated BMSCs, and the Notch1/Jagged pathway was reactivated after the addition of Notch1 pathway agonist. Taken together with the results of vascular smooth muscle and VEGF markers, it is suggested that EPO regulates the chemotaxis of BMSCs by inhibiting Notch1/Jagged pathway. Returning to our *in vivo* experiments, PAH activated the Notch1/Jagged pathway in rat lung tissues and slightly increased the levels of TGF-α, VEGF, IGF-1 and HGF in serum. The vascular endothelial growth factor is a chemotactic factor for monocytes, macrophages, and vascular endothelial cells, and its expression is regulated by hypoxia, IL-1, IL-6, IGF-1, and TGF-β. Some studies have shown that VEGF can reduce the occurrence of pulmonary hypertension [33]. There is also evidence that chronic hypoxia increases the expression of VEGF in lung tissue, and VEGF may regulate chronic hypoxia-induced pulmonary vascular remodeling [34]. In our *in vivo* study, EPO treated BMSCs inhibited the Notch1/Jagged pathway and reduced the expression of Notch1 and Jagged proteins in the lung tissues of PAH rats. Different from previous studies, we found that the serum levels of TGF-α, VEGF, IGF-1, and HGF in PAH rats treated with EPO and BMSCs did not decrease, but instead increased. This may be because the lung tissue is under hypoxic conditions. These vascular endothelial cell chemokines simply stay locally in the distal blood vessels, resulting in accumulation and abnormal thickening of the blood vessel wall, while BMSCs treated with EPO can specifically homing to the site of vascular injury and differentiate into rVECs to repair blood vessels and tissues. At this stage, a large number of endothelial growth factors and chemokines are produced, which does not cause distal pulmonary artery thickening and vascular remodeling.

5. Conclusion

In summary, we comprehensively evaluated the therapeutic effect of EPO-BMSCs on PAH rats by *in vitro* and *in vivo* experiments. Our results showed that EPO treatment improved the homing and differentiation ability of BMSCs, reduced the apoptosis of BMSCs, and enhanced the chemotactic ability of BMSCs by inhibiting the Notch1/Jagged pathway, which helped to repair the lung tissue damage specifically and reduce distal pulmonary artery thickening and vascular remodeling in PAH rats. It has the potential to treat PAH.

Ethics statement

Experimental animals and protocols were performed in accordance with the guidelines and ethical standards established by Laboratory Animal Ethics Committee, West China Hospital, Sichuan University (20,230,806,001).

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Date availability

The initial data used to support the findings of this study are available from the corresponding author upon request.

Consent for publication

All authors agree to publish.

CRediT authorship contribution statement

Kang Li: Writing – original draft, Data curation, Conceptualization. **Chongyang Shen:** Methodology, Data curation. **Nianchi Wen:** Formal analysis. **Yicen Han:** Formal analysis. **Lu Guo:** Writing – review & editing, Project administration, Funding acquisition,

Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25234.

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