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Original Article

Angiotensin-converting enzyme - human amniotic mesenchymal stem cells improve pulmonary vascular remodeling in rats with pulmonary hypertension by promoting angiogenesis and counteracting inflammation

Changfang Wu[1], Hao Liu[2], Mingchuan Ba[2], Jie Zha[3], Zhen Gao[3], Lijun Li[2], Peiyuan Xu[2], Minfei Li[2], Fusheng Cai[2], Mingjie Chen[2], Xiaona Wu[2], Lin Guo[2], Hongzhe Zhang[2,3]

[1]Department of Cardiovascular Medicine, The Second People's Hospital of Guiyang, Guiyang, Guizhou, China; [2]Department of Cardiology, The Seventh Affiliated Hospital of Southern Medical University, Foshan, Guangdong, China; [3]The Graduate School, Guizhou Medical University, Guiyang, Guizhou, China

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Abstract: Objectives: Human Amniotic Mesenchymal Stem Cells (hAMSCs) have strong multidirectional differentiation ability. Studies have found that transfection of target genes into target cells by lentivirus can enhance the differentiation potential of the cells. Angiotensin-Converting Enzyme 2 (ACE2) was found to improve vascular remodeling. Research is lacking on ACE2-hAMSCs. Therefore, this study aimed to investigate the ability to improve pulmonary arterial hypertension using ACE2-hAMSCs. Methods: Lentiviruses overexpressing ACE2 were mixed with hAMSCs. Then, ACE2-hAMSCs and hAMSCs with good growth in logarithmic growth phase were collected. We detected their migration and angiogenesis. RT-qPCR technology was used to detect the expression levels of genes related to angiogenesis, and inflammation in the two cell lines, and western-blotting was used to detect the expression levels of ACE2. As an animal study, 21 rats were randomly divided into four different groups. Right heart hypertrophy, pulmonary angiogenesis, and serum inflammatory factors were measured before dissection. H&E staining was used to observe the inflammatory infiltration of lung tissues. Results: The migration and angiogenesis of ACE2-hAMSCs were strongerthan that of hAMSCs alone. The expressions of genes in ACE2-hAMSCs were higher, and the expression of ACE2 protein in ACE2-hAMSCs was less. H&E staining showed that the inflammatory infilration of lung tissue in ACE2-hAMSCs groups was significantly improved. In addition, the ACE2-hAMSCs group had stronger pro-angiogenesis and anti-inflammatory effects. Conclusion: These results suggest that ACE2-hAMSCs can repair pulmonary vascular endothelial cell injury caused by pulmonary hypertension by promoting angiogenesis and anti-inflammatory ability. This shows that ACE2-hAMSCs have stronger ability to improve pulmonary vascular remodeling than hAMSCs alone.

Keywords: Angiotensin-converting enzyme 2 in human amniotic mesenchymal stem cells, pulmonary vascular remodeling, angiogenesis, inflammation, pulmonary artery hypertension rats

Introduction

One of the causes of right heart failure is pulmonary artery hypertension (PAH). During the course of the disease, excessive proliferating and remodeling of pulmonary artery smooth muscle cells, fibroblasts, and other cells, vascular endothelial dysfunction, imbalance of vasomotor substances, accumulation of inflammatory cells around blood vessels, and formation of orthotopic thrombosis cause progressively increased pulmonary artery pressure, thus inducing right heart failure and functional decline [1]. Damage to pulmonary vascular endothelial cells is considered the initiating link and necessary condition for pulmonary vascular remodeling. The renin-angiotensins system (RAS) plays an essential role in impaired endothelial function and vascular remodeling [2]. In recent years, it has been found that apart from

the angiotensin-converting enzyme (ACE)/angiotensin II/angiotensin receptor subtype AT1 axis, RAS also has the ACE2/Ang1-7/Mas axis that can relax blood vessels and possesses negative regulatory effects such as anti-proliferation, anti-inflammatory, and anti-oxidative stress. ACE2 is a key enzyme [3]. ACE2 can improve endothelium-dependent vasodilation and has anti-proliferation and anti-inflammatory effects [4]. Studies have demonstrated that PAH and pulmonary fibrosis induced by bleomycin are related to decreased ACE2 activity [5]. Recombinant human ACE2 replacement therapy can improve the severity of lung injury in ACE2 knockout rats [6]. Previous studies on stroke have shown that with ACE2, the therapeutic effect on endothelial cells was significantly improved [7]. Therefore, increasing the expression of ACE2 might be an effective way to treat pulmonary hypertension.

Moreover, MSCs possess multidirectional differentiation and paracrine effects and the characteristic of directional homing to injured lung tissue. This could directly repair damaged endothelium and improve the endothelial function [8, 9]. Kim et al. [10] found that hAMSCs directly transplanted into the ischemic hindlimbs of rats not only achieve a high survival rate but also remarkably promote angiogenesis, indicating a significant effect on improving hindlimb ischemia. Transplanting hAMSCs into a rat model of liver cirrhosis induced by carbon tetrachloride could promote liver cell regeneration and improve liver fibrosis [11]. Previous studies have demonstrated that MSCs could express foreign genes for a long time after genetic modification [12], which meets the needs of gene therapy and serves as an ideal carrier. Takemiya et al. [13] found that injection of MSCs transfected with prostaglandin synthase (PCS) could remarkably reduce pulmonary artery pressure, and improve pulmonary artery remodeling and right ventricular hypertrophy compared with MSC injection alone. hAMSCs cells possess numerous advantages such as abundant sources and the potential to differentiate into various tissue cells, hence they might be an ideal source of stem cells for clinical research and application [14, 15].

In summary, the effect of MSCs combined with gene therapy surpasses that of MSCs alone. ACE2 combined with amniotic mesenchymal stem cells might be the best option for PAH, but there is still a lack of relevant research. Therefore, the present study applied ACE2 lentiviral vector to transfect hAMSCs to prepare ACE2-hAMSCs, aiming to observe whether there is a synergistic effect between the two and the intervention effect on the monocrotaline (MCT) induced PAH rat model. This could provide a new theoretical basis for PAH treatment.

Materials and methods

Materials

Main reagents and instruments

Reagents: DMEM (BI), α-MEM medium (Procell, USA), fetal bovine serum (Gibco, USA), antibodies CD29-APC and CD45-PE (Ebioscience, USA), ACE2 antibody (Wuhan Sanying, China), monocrotaline (Ald, USA), and ELISA kit (Yitlaier, USA). Main instruments: real-time fluorescence quantitative PCR (ABI, USA) and microspectrophotometer (Hangzhou Aoshengyi Doris Instrument System Co., Ltd., China).

Animals

Ethics statement: The present study was preapproved by the Laboratory Animal Ethics Committee of the Third Affiliated Hospital of Guizhou Medical University, the approval number being 2016A005. Experimental animals were provided by Liaoning Changsheng Biotechnology Co., Ltd. (License No. SCXK (Liao) 2020-0001, China).

Methods

Preparation of hAMSCs overexpressing ACE2

Primary human amniotic membrane mesenchymal stem cells were purchased from Shanghai Cybertron, China. Lentivirus overexpressing the ACE2 gene was constructed, packaged, and tested by Wuhan Baffir Biotechnology Service Co., Ltd., China. The third-generation hAMSCs with good growth status were selected with 3-5 × 10^4 cells per milliliter, and the suspension with a volume of 90 ul was inoculated into a 96-well plate and co-cultured with the lentivirus carrying the ACE2 gene for 48-72 h. The distribution of green fluorescent protein was observed under a fluorescence microscope to determine the optimal multiplicity of infection

Table 1. Retrotranscriptional response system

Reagent	Volume (μl)
RNA	2.6 μg
Oligo (dT) 18 (10 μM)	2
dNTP (2.5 mM)	4
5 × Hiscript Buffer	4
Hiscript Reverse Transcriptase	1
Ribonuclease Inhibitor	0.5
ddH_2O (Rnase free)	Up to 20

Table 2. Reaction conditions

Temperature	Time
25°C	5 min
50°C	15 min
85°C	5 min
4°C	10 min

and transfection efficiency. The lentivirus vectors used in this study were composed of vector plasmid (PFV:PLVX), helper plasmid psPAX2 (pHelper1), and helper plasmid pMD2G (pHelper2). The most important of these is the vector plasmid PFV vector which contains HIV basic components 5'LTR and 3'LTR and other auxiliary components. This plasmid is convenient for transfection of genes.

Flow cytometry for surface antigen detection of ACE2-hAMSCs

Cells that grew well at the logarithmic growth phase were collected with each tube containing 1 ml 0.5% bovine serum albumin (BSA) PBS, and antibodies CD29-APC and CD45-PE were added. Meanwhile, a negative control group was set, which was protected from light after being incubated for 30 minutes, and washed twice with PBS containing 0.5% BSA. After it was resuspended in PBS, flow cytometry was adopted for detection and analyses.

ACE2-hAMSCs functional test

ACE2-hAMSCs migration experiment: Well-growing cells at the logarithmic growth phase were collected, and a transwell assay was applied to detect cell migration [14].

Tube experiment of ACE2-hAMSCs: Cells at the logarithmic growth phase and in good growth condition were collected. A single-cell suspension was prepared with serum-free medium, and after being counted, the cells were evenly seeded into 24-well plates pre-lined with matrix gel with 1.5 × 10^5/cells per well and incubated overnight for 8-15 hours. The cells were observed and photographed under a microscope.

Detection of ACE2 mRNA and protein expression levels in ACE2-hAMSCs

RT-qPCR detection of ACE2, Ang-1, eNOS, TNF-α, FGF-2, VEGF-A, and SDF-1 gene expression in ACE2-hAMSCs and hAMSCs: The total RNAs of hAMSCs and ACE2-hAMSCs were extracted and reversed into cDNA for real-time reverse transcriptase-polymerase chain reaction (RT-PCR) (**Tables 1**-**3**).

Western blot detected the expression level of ACE2 in ACE2-hAMSCs and hAMSCs: The total protein of hAMSCs and ACE2-hAMSCs was extracted with lysate where BCA was quantified and incubated with ACE2 antibody (1:1000) overnight, and β-actin (1:500) was selected as the internal control. ACE2 antibody dilution ratio was 1:1000. The secondary antibody was HRP-labeled sheep antibody and rabbit secondary antibody.

Grouping and handling of experimental animals

All animal experiments followed the ARRIVE guidelines and were conducted in line with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines as well as EU Directive 2010/63/EU for animal experiments and Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

The experimental rats were randomly divided into 4 groups, i.e. normal group (3), PAH model group (6), PAH model + AMMSC cell group (6), and PAH model + ACE2 overexpression lentivirus transfected hAMSC cell group (6). The experiment was started after the rats were adaptively fed for 7 days. Rats in the model groups were intraperitoneally injected with monocrotaline MCT (50 mg/kg) while the normal group was injected with the same amount of normal saline. After 3 weeks of administration, the corresponding cells were injected into the tail vein in groups 3 and 4 while physiological saline was injected in groups 1 and 2, once a week and twice in total. Two weeks later, the indicators were measured and the rats were sacrificed (lung tissue, myocardial tissue, serum).

Table 3. Primers were detected by PCR

Gene	Primer	Sequence (5'-3')	PCR Products
Homo β-actin	Forward	CCCTGGAGAAGAGCTACGAG	180 bp
	Reverse	CGTACAGGTCTTTGCGGATG	
Homo ANG1	Forward	GGACAGCAGGAAAACAGAGC	216 bp
	Reverse	GCCCTTTGAAGTAGTGCCAC	
Homo eNOS	Forward	CCTGACAACCCCAAGACCTAC	112 bp
	Reverse	TAACATCGCCGCAGACAAAC	
Homo TNF-α	Forward	TCAGAGGGCCTGTACCTCAT	220 bp
	Reverse	GGAAGACCCCTCCCAGATAG	
Homo FGF2	Forward	GAGCGACCCTCACATCAA	222 bp
	Reverse	CGTTTCAGTGCCACATACC	
Homo VEGFA	Forward	GGAGGAGGGCAGAATCATCA	247 bp
	Reverse	CTTGGTGAGGTTTGATCCGC	
Homo SDF-1	Forward	GCTGGTCCTCGTGCTGAC	212 bp
	Reverse	TCCACTTTAGCTTCGGGTCA	
Homo ACE2	Forward	AGTGGATGAAAAAGTGGTGGG	101 bp
	Reverse	ATGGAACAGAGATGCGGGGTC	
Homo IGF-1	Forward	ATCAGCAGTCTTCCAACCCA	196 bp
	Reverse	TGTCTCCACACACGAACTGA	

Mean pulmonary artery pressure and right ventricular hypertrophy index were measured in each group

Two weeks after administration, the mean pulmonary artery pressure of the rats was recorded physiologically by Medlab. Afterward, the rats were sacrificed, and the right ventricle (RV) and left ventricle plus ventricular septum (LV+S) were separated and weighed. The ratio of RV/(LV+S) was applied to calculate the right ventricular hypertrophy index (RVHI). Afterward, the lung tissue and the right ventricle were fixed with 10% formaldehyde solution for 1 d and embedded and fixed in paraffin for H&E staining.

HE staining of rat lung tissue in each group

After being fixed with 10% formaldehyde, embedded in paraffin, sliced, and stained with hematoxylin and eosin, the pulmonary artery lumen and inflammatory cell infiltration were observed under the light microscope.

Western blot detection of ACE2, Ang II, and Apelin, and protein expression in lung tissue of rats in each group

β-actin (1:500) was selected as the internal reference. The lysate was applied to extract the total protein of each group of lung tissues and perform BCA quantification, electrophoresis, membrane transfer, and color development following the kit instructions. Ang II, Apelin, and ACE2 antibodies (1:1000) were incubated overnight. The gray values of ACE2/β-actin, Ang II/β-actin, and Apelin/β-actin represent the relative expression of ACE2, Ang II, and Apelin proteins respectively.

RT-qPCR detection of Ang II and Apelin gene expression levels in lung tissue of rats in each group

The total RNA in each group of lung tissues was extracted and reverse-transcribed into cDNA for real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

ELISA test

ELISA detected the expression of TNFα, IL-6, IL-10, IL-17, and IL-23 in the serum of rats in each group. The blood samples of each group were centrifuged for 10 minutes, and the supernatant was collected for further investigation.

Statistical processing

SPSS 22.0 statistical software was employed for data analysis, and the measured data of each group were expressed as mean ± standard deviation ($x \pm s$). The mean of multiple samples was analyzed by the analysis of variance named one-way ANOVA. The uniform variance was tested through the LSD method, and uneven variance was tested through the Tamhane method. $P<0.05$, was considered significant.

Results

Green fluorescent protein expression of hAMSCs transfected with ACE2

The hAMSCs were transfected with a lentiviral stock solution with a multiplicity of infection (MOI) of 10, and the expression of the fluorescent protein was observed under a fluorescence microscope 72 hours after transfection (**Figure 1A**).

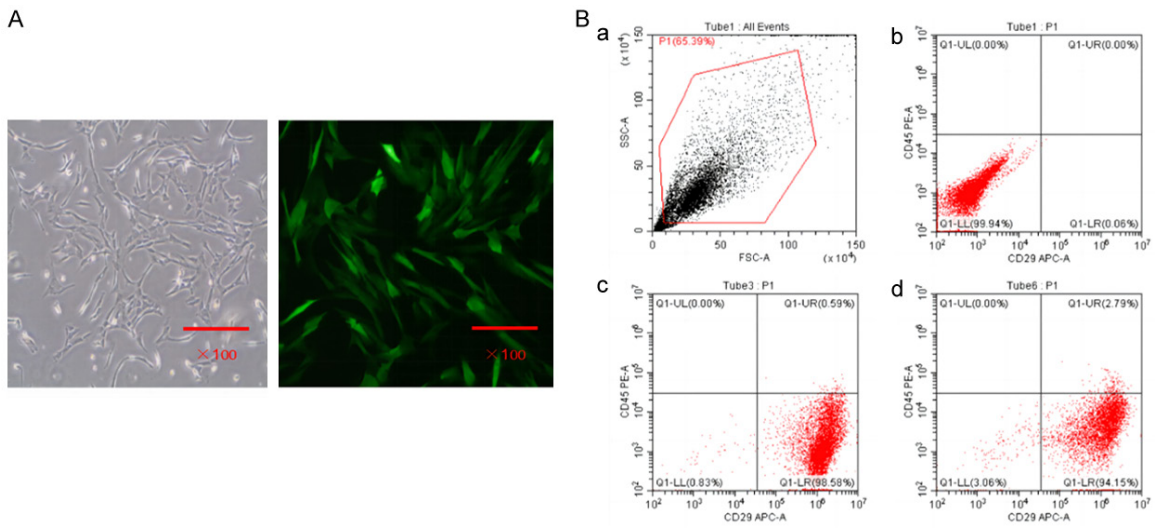


Figure 1. Expression of green fluorescent protein in hAMSCs transfected with ACE2 and Flow cytometry detection. (A) Expression of green fluorescent protein in hAMSCs transfected with ACE2 (× 100). (B) Flow cytometry detection of hAMSCs and ACE2-hAMSCs surface markers. (a and b) are blank control groups, (c) is the detection result of hAMSCs, (d) is the detection result of ACE2-hAMSCs.

FACS detection

FACS analysis revealed that hAMSCs and ACE2-hAMSCs displayed MSC-specific characteristics, expressing CD29 surface antigen but not CD45 surface antigen (**Figure 1B**).

ACE2-hAMSCs promote cell migration and blood vessel formation

The cell migration test indicated that after 24 hours of cell culture, the cell migration ability of ACE2-hAMSCs was enhanced compared to hAMSCs alone (**Figure 2A**).

Tube formation experiments indicated that after 15 hours of cell culture, ACE2-hAMSCs markedly improved the angiogenesis ability of cells compared to hAMSCs alone (**Figure 2B**).

ACE2-hAMSCs show high expression levels of ACE2 protein

To determine the secretion of ACE2 in ACE2-hAMSCs from the protein level, western blot was performed and compared to hAMSCs alone. ACE2 protein levels were increased in ACE2-hAMSCs ($P<0.05$) (**Figure 2C**).

ACE2-hAMSCs exhibit angiogenesis, anti-inflammatory, and vasodilatory properties

Compared to hAMSCs alone, the mRNA expression levels of pro-angiogenic factors VEGF-A, FGF-2, and Ang-1 were higher in ACE2-hAMSCs ($P<0.05$). Chemokines such as stromal cell-derived factor (SDF-1) play a role in promoting neovascularization and were up-regulated in ACE2-hAMSCs ($P<0.01$) while the expression of pro-inflammatory factor TNF-α mRNA was reduced ($P<0.01$). Moreover, compared to the hAMSC group, the expression level of vasodilator eNOS in the ACE2-hAMSC group also was increased ($P<0.01$). Overall, the data indicate that ACE2-hAMSC express higher levels of pro-angiogenic, anti-inflammatory, and vasodilator factors (**Figure 2D**).

In a rat model of pulmonary hypertension, ACE2-hAMSCs exerted a significant therapeutic effect

To investigate the therapeutic effect of ACE2-hAMSCs in PAH, 1×10^6 cells were injected through the tail vein into a rat model of monocrotaline-induced pulmonary hypertension. Two weeks after cell transplantation, the results indicated that compared with the control group, the mPAP and RV/LV+S in the PAH group remarkably increased ($P<0.01$) (**Table 4**). Compared with the PAH group, the mPAP and RV/LV in the ACE2-hAMSCs group and the hAMSCs group remarkably decreased ($P<0.01$). Compared with the hAMSCs group, mPAP and RV/LV+S in the ACE2-hAMSCs group declined (**Figure 3A**).

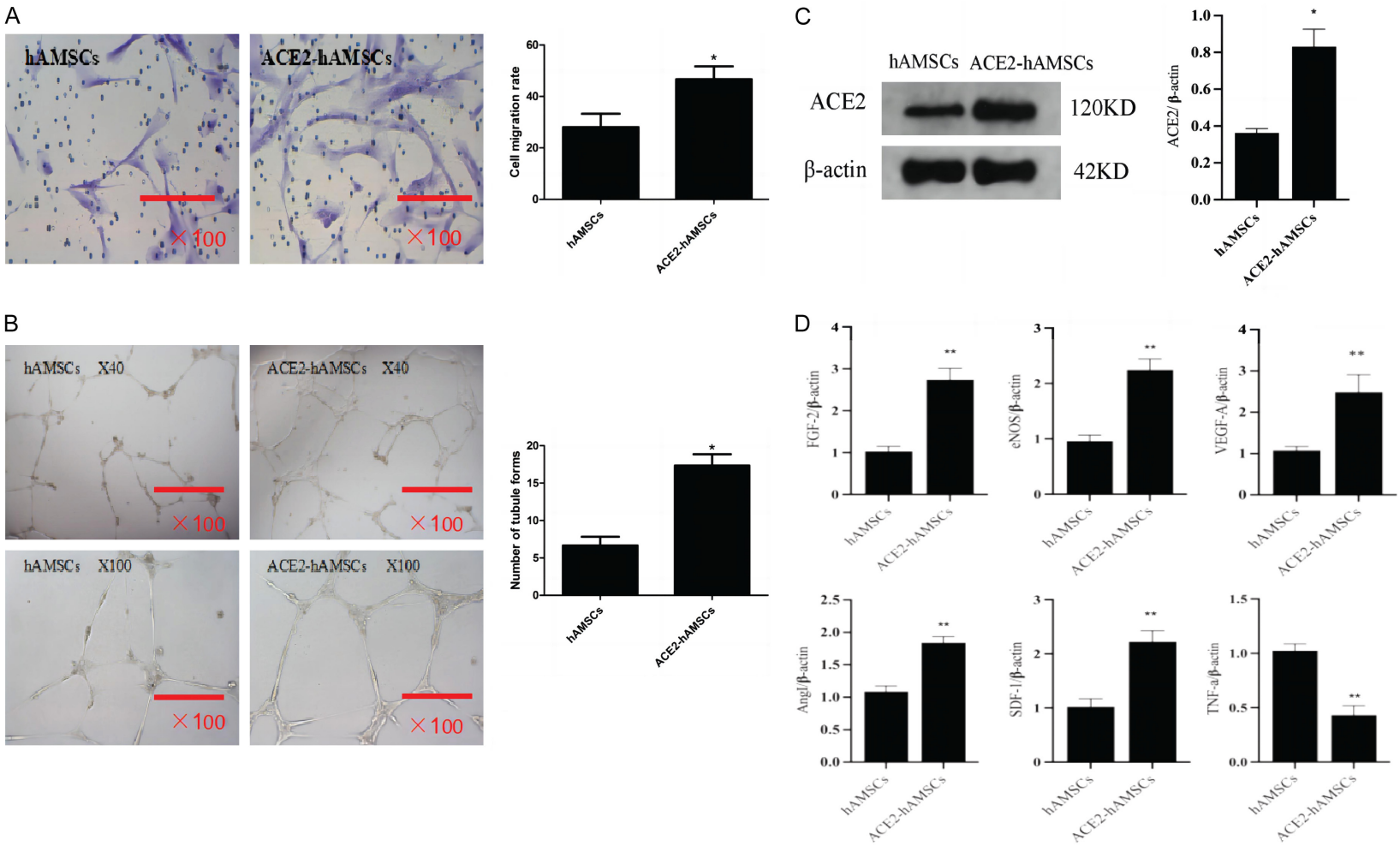
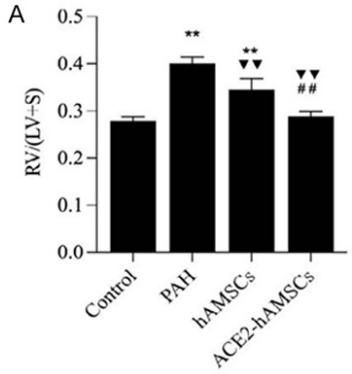


Figure 2. The role of ACE2-hAMSCs in cell migration and angiogenesis, protein, and gene expression test results. A. Detection of the migration ability of ACE2-hAMSCs. The number of cell migration in the ACE2-hAMSCs group was remarkably higher than that in the hAMSCs group. B. Detection of the migration ability of ACE2-hAMSCs (× 40, × 100). Cells were cultured for 24 hours, the tubule formation ability of the ACE2-hAMSCs group was substantially higher than that of the hAMSCs group. C. Western blot to detect the expression level of ACE2 protein in each group of cells (n=3). The expression level of ACE2 protein in the ACE2-hAMSCs group was significantly higher than that in the hAMSCs group. D. Gene expression of various pro-angiogenesis, pro-vasodilation substances, and pro-inflammatory factors in ACE2-hAMSCs (n=3). Compared with the control group, various angiogenic factors and pro-vasodilation substances were up-regulated while pro-inflammatory factors were down-regulated in the ACE2-hAMSCs group with $^{*}P<0.05$ and $^{**}P<0.01$ respectively.

Table 4. Pulmonary artery pressure

	Control (n=3)	PAH (n=6)	hAMSCs (n=6)	ACE2-hAMSCs (n=6)	P
mPAP (mmHg)	17.3-18.3	38.0-38.6	27.0-27.6	21.3-21.9	<0.01



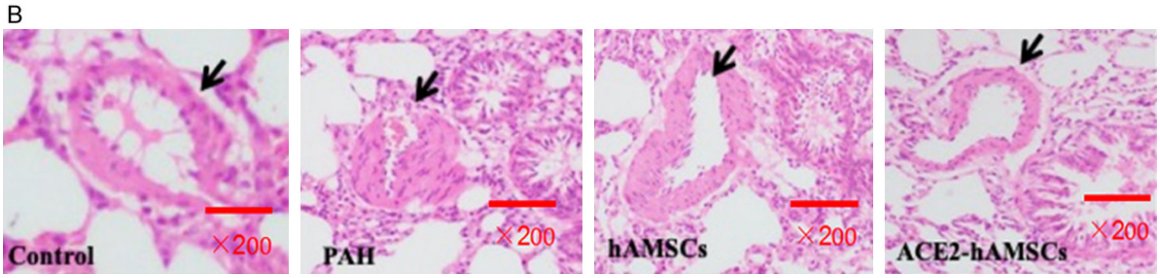


Figure 3. Pulmonary arterial pressures, right ventricular hypertrophy index, and HE staining of lung tissue specimens in each group. A. Right ventricular hypertrophy index. *P<0.01 compared with the control group, ▼P<0.01 compared with the PAH group, and ΔP<0.01 compared with hAMSCs group. B. HE staining of lung tissue specimens from four groups of rats (× 200).

Observation of rat lung tissue specimen under the HE staining microscope

HE staining of lung tissue specimens: Under an optical microscope (× 200), the PAH group showed obvious inflammatory cell infiltration, alveolar collapse, alveolar parenchyma, pulmonary arteriole wall thickening, and luminal narrowing compared with the control group. Compared with the PAH group, the inflammatory cell infiltration in the ACE2-hAMSCs group was remarkably reduced, the thickness of the pulmonary arterioles was reduced, and the lumen was enlarged, which was still worse than the control group, the difference being statistically substantial. The inflammatory infiltration and the thickness of the pulmonary arterioles of rats in the hAMSCs group were remarkably reduced and the lumen was enlarged, which was still inferior to the control group (**Figure 3B**).

Detection of ACE2, Ang II, and Apelin protein expression in rat lung tissues

The protein expression of ACE2, Ang II, and Apelin in each group was detected through the western blot method, and β-actin was detected as an internal control to correct the loading amount or expression error. Compared with the control group, the ACE2 and Apelin in the PAH group were remarkably reduced (P<0.01) while Ang II was remarkably increased (P<0.01), the difference being statistically substantial. ACE2 and Apelin in the ACE2-hAMSCs group were remarkably increased (P<0.01) while Ang II was remarkably decreased (P<0.01) compared with the PAH group, the difference being statistically substantial (**Figure 4A**).

Detection of Ang II and Apelin mRNA expression in rat lung tissues

RT-qPCR detected the expression of Ang II and Apelin mRNA in the lung tissues of rats in each group. The results indicated that compared with the control group, Apelin in the PAH group was remarkably reduced (P<0.01) while Ang II was significantly increased (P<0.01), the difference being statistically substantial. Regarding scientific significance, Apelin in the theACE2-hAMSCs group was remarkably increased (P< 0.05) while Ang II was remarkably decreased (P<0.01) compared with the PAH group, the difference being statistically substantial (**Figure 4B**).

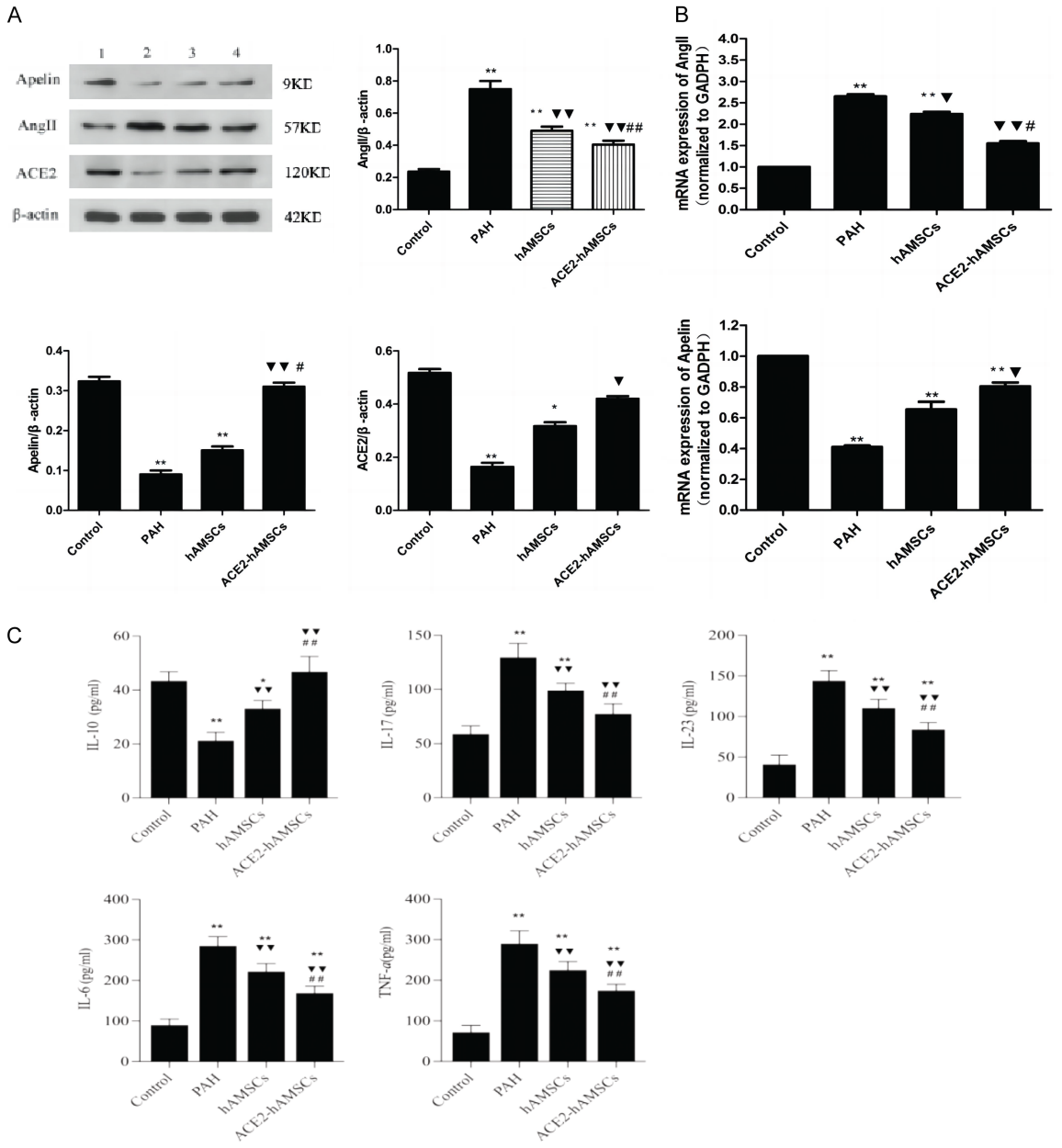


Figure 4. Detection results protein, mRNA expression, and ELISA test results. A. Western blot detection of the protein expression levels of ACE2, Apelin and Ang II in lung tissues of each group. B. Detection results of Ang II and Apelin mRNA expression in rat lung tissues. C. ELIAS test results in the serum cytokine protein expression results of each group. *P<0.05 compared with the control group, **P<0.01 compared with the control group, ▼P<0.05 compared with the PAH group, ▼▼P<0.01 compared with the PAH group, #P<0.05 compared with the hAMSCs group, and ##P<0.01 compared with the hAMSCs group.

ELISA test results

Regarding the ELISA test results in the serum of each group of rats, TNFα, IL-6, IL-23, and IL-17 in the PAH group were remarkably increased (P<0.01) while IL-10 was remarkably reduced (P<0.01) compared with the control group, the difference being statistically substantial. Compared with the PAH group, TNFα, IL-6, IL-23, and IL-17 in the ACE2-hAMSCs group were remarkably reduced (P<0.01) while IL-10 was substantially increased (P<0.01), the difference being statistically significant. Compared with the PAH group, IL-10 in the hAMSCs group

was higher while TNFα, IL-6, IL-23, and IL-17 were remarkably reduced ($P<0.05$), the difference being statistically substantial (**Figure 4C**).

Discussion

The main results of the present study are as follows. The migration and angiongenesis of ACE2-hAMSCs were significantly increased. In the monocrotaline-induced pulmonary hypertension rat model, the lung tissues overexpress ACE2 and down-regulate the expression of Ang II after transplantation of hAMSCs overexpressing ACE2. ACE2 enhances the paracrine effect of hAMSCs and promotes vascular growth. The expression of factors like VEGF-a, bFGF, Ang-1, and TGF-β reduces inflammatory factors.

The pathogenesis of pulmonary arterial hypertension (PAH) remains unclear, and endothelial injury is considered as the initiating link of PAH. Therefore, repairing the pulmonary vascular endothelium serves as a key link in the treatment of pulmonary hypertension [16]. Mesenchymal stem cells are extensively applied in cell transplantation due to their multi-directional differentiation ability, immune inertness, convenience in vitro acquisition and culture [17]. Studies have indicated that mesenchymal stem cell transplantation could reduce acute ventricular remodeling and improve heart function [18, 19]. As the key peptide of the ACE-Ang II-ATIR axis in RAS, the abnormal activation of Ang II causes excessive pulmonary vasoconstriction, oxidative stress, inflammation, cell proliferation, and fibrosis, and promotes the progress of PAH. In recent years, numerous studies have found that the ACE2-Ang (1-7)-Mas axis mainly antagonizes the effect of the ACE-Ang II-ATIR axis. ACE2 has a powerful effect, and its agonist DIZE can also reduce pulmonary vascular injury through the ACE-Ang II-AtIR pathway [20]. Meanwhile, Sini decoction can also reduce acute pulmonary vascular injury induced by escherichia coli and delay pulmonary vascular remodeling by balancing ACE-Ang II-AtIR pathway [21]. In the present study, it was found that the mPAP and RV/LV+S of the rats in the hAMSCs and ACE2-hAMSCs group remarkably declined compared with the PAH group, implying that amniotic mesenchymal stem cells impose a protective effect on pulmonary hypertension to some extent, which is consistent with previous reports in the existing literature. Moreover, the mPAP and RV/LV+S in the hAMSCs-ACE2 treatment group were remarkably lower than those in the PAH group and the hAMSCs group, and the treatment effect was better than that of the hMASCs group. Our study is consistent with previous studies, suggesting that the addition of ACE2 of hAMSCs does have stronger cytogenesis and anti-inflammatory ability [21].

In vitro experiments, we found that the ACE2 protein in the ACE2-hAMSCs group was remarkably higher than that in the simple hAMSCs group, indicating that the strong multidirectional differentiation ability of hAMSCs promote the formation of ACE2. This is consistent with previous research [21]. In animal experiments, ACE2 overexpressed after ACE2-hAMSCs transplantation antagonized the effect of ACE and hydrolyzed Ang I and Ang II to produce Ang (1-9) and Ang I (1-7), which resulted in a decrease in Ang II, inhibited cell proliferation, and achieved anti-inflammation, anti-fibrosis, and pulmonary artery relaxation, thereby reducing pulmonary artery pressure and pulmonary artery stenosis and improving pulmonary vascular remodeling. Similarly, our results are consistent with previous studies. The ACE2-Ang (1-7)-Mas axis does mitigate lung injury and promote pulmonary vascular remodeling [22]. In vitro experiments in the present study found that ACE2-hAMSCs promoted ACE2, vascular growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1), endothelial nitric oxide synthase (eNOS), and stromal cell-derived factor (SDF-1) expression, which reduced the expression of tumor necrosis factor (TNF-α). Our results are consistent with previous studies, suggesting that ACE2 can promote cell secretion, promote cell genesis and inhibit the production of inflammatory factors [22]. Nowadays, studies have confirmed that MSCs could express and paracrine VEGF, bFGF, IGF, Ang-1, transforming growth factor (TGF) β, hepatocyte growth factor (HGF), other angiogenic cytokines, and homing factors like stromal cells derivative factor (SDF-1) to promote the proliferation of vascular endothelial cells, wound angiogenesis, and increase blood vessel density [23]. In our study, the expression of VEGF, bFGF, IGF, ANG-1, etc., which promoted the angiogenesis in the ACE2-hAMSCs group, was promoted. These data indicate that the overexpression of ACE2 promotes the paracrine effect of hAMSCs, which is con-

ducive to cell migration, promotion of angiogenesis, protection of vascular endothelium, etc., thereby helping repair damaged endothelial cells. A research also found that the supernatant of hAMSCs could inhibit LPS-stimulated THP-1 to produce TNF-α and IL-1β [24]. Similarly, LPS-induced pneumonia damage is inhibited by ACE2-Ang-(1-7)-Mas axis. This is consistent with our results [25]. Salvucci et al. [26] found that the SDF-1/CXCR4 axis is closely related to the occurrence and formation of microvessels. SDF-1 enhances the expression of Matrix Metallo Proteinase (MMP), thereby indirectly regulating angiogenesis and inducing the formation of microvessels at the same time. SDF-1 stimulates endothelial cells to express vascular endothelial growth factors that up-regulate the expression of the SDF-1/CXCR4 axis, thus resulting in a synergistic effect in promoting angiogenesis and forming a positive feedback pathway. Interestingly, we found that over-expression of ACE2 down-regulated the expression of TNFα and up-regulated the expression of eNOS in the ACE2-hAMSCs group, which inhibited endothelial cell inflammation and vasodilation, thereby repairing endothelial cells. Cell studies have also demonstrated the anti-inflammatory effects of ACE2, similar to our results [27]. In the present study, the expression of SDF-1 was enhanced in the ACE2 + hAMSCs group, and the overexpression of ACE2 promoted the expression of SDF-1. In previous studies, it was found that a hypoxic environment aggravated the proliferation of rat pulmonary artery smooth muscle cells by inducing autophagy. Exogenous apelin inhibits autophagy under hypoxic conditions through APJ-mediated activation of PI3K/AKT/mTOR signals, thereby inhibiting the proliferation and migration of rat pulmonary vascular endothelium [28]. Apelin/APJ signaling pathway could activate the AMPK-KLF2-eNOS-NO axis, promote vascular endothelial NO production, restore pulmonary vascular endothelial function, and maintain pulmonary vascular homeostasis [29]. Apelin positively regulates ACE2 and plays a cardiovascular protective role. Studies have indicated that ACE2 has a high catalytic activity on the vasoactive peptide (hydrolase apelin), which is related to body fluid balance. Therefore, the expression of Apelin in the ACE2-hAMSCs group was enhanced, and it was also involved in the repair of endothelial cells, reducing pulmonary hypertension, and relaxing the pulmonary artery.

Apart from the participation of growth factors in the development of pulmonary hypertension, inflammatory factors are also essential. Previous studies have confirmed that serum levels of the pro-inflammatory factors IL-6, IL-1, IL-17, IL-23, and TNF-α are elevated in PAH. IL-6 is a pleiotropic pro-inflammatory cytokine. The level of IL-6 in the serum and lungs of PAH patients is increased [30]. Inflammatory factors have indeed been shown to be closely associated with vascular damage in the study of pulmonary hypertension related cells [31]. As a powerful pro-inflammatory factor secreted by Th17, IL-17 stimulates epithelial cells, endothelial cells, and fibroblasts to produce various cytokines like IL-6, IL-8, granulocyte-macrophage stimulating factor (GM-CSF), chemical activin, and cell adhesion molecule 1 (CAM-1), which cause inflammation [32]. Moreover, studies have indicated that IL-17 could also affect the vascular microenvironment by stimulating the expression of adhesion-related molecules like ICAM in fibroblasts, endothelial cells, and epithelial cells [33]. Pridgeon [34] pointed out that IL-23 is also involved in the synthesis of IL-17. In the present study, in the hAMSCs and ACE2-hAMSCs treatment groups, the growth factor pro-inflammatory factors that promote the occurrence and development of PAH were remarkably decreased, and the anti-inflammatory factor IL-10 was remarkably increased. Observed from the research findings, MSC could migrate to the injured site after transplantation and play a certain role in the repair of tissues. Nowadays, its mechanism of action is mainly considered to promote angiogenesis through paracrine action and secrete factors [35] and participate in immune regulation to inhibit inflammation. It could reduce the expression of inflammatory factors like tumor necrosis factor-α (TNF-α), IL-1, and IL-6 and increase the anti-inflammatory factor due to IL-10, thereby reducing the damage caused by excessive inflammation [36]. In this in vitro experiment, it was found that the pro-inflammatory factor TNF-α was remarkably decreased in the ACE2-hAMSCs group compared with the hAMSCs group. Therefore, it was considered that the expression of ACE was enhanced to play a more significant anti-inflammatory effect in the treatment of pulmonary hypertension.

One limitation of this study is that it does not explain whether stem cell therapy for pulmonary hypertension in rats with different sex is consistent.

In conclusion, this study demonstrated that ACE2-hAMSCs could promote angiogenesis and inhibit inflammation in rats, thereby delaying the progression of pulmonary vascular remodeling.

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Disclosure of conflict of interest

None.

Address correspondence to: Hongzhe Zhang, Department of Cardiology, The Seventh Affiliated Hospital, Southern Medical University, Desheng Road Section 28, Foshan 528200, Guangdong, China. Tel: +86-0757-85631264; E-mail: 193936-3232@qq.com

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