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Evaluating the Effect of Circ-Sirt1 on the Expression of SIRT1 and Its Role in Pathology of Pulmonary Hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is a disease that plagues a major portion of the world's population, and there is currently no effective cure for this ailment. The proliferation and migration of pulmonary artery smooth muscle cells (PASMC) are known to be the pathological basis of pulmonary vascular remodeling in pulmonary hypertension. Studies in the past have shown involvement of CircRNA in the pathology of pulmonary as well as cardiovascular diseases. However, there are very few studies that have analyzed the relationship between CircRNA and PAH. The aim of this study was to explore this relationship by using rat PAH model. A hypoxic, PAH rat model was constructed for this study and the subsequently produced hypoxia-induced rat PASMC cells were utilized to demonstrate the reduction in expression of circular RNA of Silent information regulator factor 2-related enzyme 1 (circ-Sirt1) and SIRT1 mRNA in response to hypoxia, through cell function tests, cell rescue tests, and physical tests. We found that the expression of circ-Sirt1 and SIRT1 decreased in the PAH rat model induced by hypoxia. It was also revealed that the overexpression of circ-SIRT1 increased SIRT1 levels, but inhibited the expression of transforming growth factor (TGF)-β1, Smad3, and Smad7, and weakened PASMC cell vitality, proliferation, and migration ability. The findings of the present study indicate that circ-Sirt1 regulates the expression of SIRT1 mRNA and inhibits TGF-β1/Smad3/Smad7 mediated proliferation and migration of PASMC. This provides a new insight into the molecular mechanism of pulmonary artery vascular remodeling in PAH and may aid in the development of novel therapeutic options for management of PAH.

Keywords

hypertension, pulmonary artery, RNA, smooth muscle, vascular remodeling, vasoconstriction

Introduction

Pulmonary arterial hypertension (PAH) is characterized by long-term hypoxia in the lung tissue leading to pulmonary vasoconstriction and pulmonary artery vascular remodeling with a progressive increase in pulmonary vascular resistance, ultimately culminating into right heart failure in many cases[1]. The reported incidence of PAH ranges from 2.0 to 7.6 cases per million adults per year[2]. Although there are several clinical modalities that can alleviate the symptoms, the incurable nature of this pathology makes it impossible to reverse the progression of the disease, and thereby the prognosis of PAH patients is poor[3,4]. Due to the lack of effective treatment options for this condition, this field has been a hot spot of research for ages.

The pathogenesis of PAH is complicated, but the current consensus supports hypoxia and pulmonary vascular remodeling as the chief pathological mechanisms and pulmonary artery smooth muscle cells (PASMC) are supposed to be the key cells affected by this condition. Under hypoxic conditions, proliferation, and migration of PASMC play an important role in the reconstruction of pulmonary vascular structure[5]. There are multiple internal factors that play an active role in proliferation and migration PASMC; studies have shown that DNA methylation, histone modification,

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and non-coding RNA dysregulation may play an important role[1,6], but the exact molecular mechanism is unclear.

CircRNA is a highly stable, covalent closed loop structure with hardly protein coding ability. Studies have progressively shown that CircRNA has a variety of biological functions and is involved in the pathology of some lung diseases as well as cardiovascular diseases[7]. Silent information regulator factor 2-related enzyme 1 (sirtuin 1, SIRT1) has been reported to participate in the inflammatory cellular response of blood vessels, while transforming growth factor (TGF)-β1, Smad3, and Smad7 have been shown to play a crucial role in vascular remodeling[8,9]. In addition, studies have shown that circ_0006872 regulates the miR-145-5p/NF-κB pathway in chronic obstructive pulmonary disease, promoting pulmonary vascular cell apoptosis, inflammation, and oxidative stress[10]. However, there are hardly any studies that have explored the relationship between CircRNA and PAH.

In the present experiment, we constructed a hypoxic, PAH rat model and utilized the subsequently produced hypoxia-induced rat PASMC cells to demonstrate the reduction in expression of circ-Sirt1 and SIRT1 mRNA in response to hypoxia, through cell function tests, cell rescue tests, and physical tests. This finding indicated that circ-Sirt1 and SIRT1 may be related to PAH.

Materials and Methods

Research Ethics

Animal care and use were done in compliance with the "Guidelines for the Care and Use of Laboratory Animals" (National Institutes of Health Publication 85-23, revised in 1996). All animal experiments were performed in accordance with NIH guidelines, and all procedures involving animals were approved by the Institutional Laboratory Animal Management and Ethics Committee of Bengbu Medical College (Bengbu, Anhui, China).

Establishment of the Hypoxic Animal Model

Twelve, specific pathogen free (SPF) grade, Sprague-Dawley (SD) male rats weighing 150–200 g were obtained from the Experimental Animal Center of Bengbu Medical College. All rats underwent a 12-hour light/dark cycle at 25±1°C and were provided food and water freely. The rats were randomly divided into two groups: normal group and hypoxia group and subsequently kept under conditions of normoxia and hypoxia as per the allotted group. The feeding environment temperature was set as 22°C to 24°C and relative humidity was set as 50%. The rats in the normal group were kept under normoxia (21% O_2) for 40 days, while those in hypoxia group were kept in the hypoxia incubator (10% O_2) for the same time duration. Thereafter, all the rats were euthanized, their lung tissues were isolated and stored appropriately for later use.

Hemodynamic Experiments

Hemodynamic parameters were measured by right ventricular systolic pressure (RVSP) through right heart catheterization of the right jugular vein, using a YPJ01 pressure transducer (Chengdu Instrument Factory, Chengdu, China) and a RM6240 series physiological signal acquisition and processing system (Chengdu Instrument Factory, Chengdu, China). After analyzing the hemodynamic data, the chest cavity was opened. The heart and lungs were transferred to sterile petri dishes containing cold phosphate buffered saline (PBS). Right ventricular hypertrophy index was assessed by analyzing the ratio of right ventricle/left ventricle+septum (RV/LV+S). Half of the lung tissue was fixed in 4% paraformaldehyde, and the other half was stored at -80°C for subsequent experiments.

Hematoxylin and Eosin Staining and Immunofluorescence assay

Rat lung tissue samples were dehydrated, cleared, and embedded in paraffin. The paraffin blocks were cut into 4-μm-thick sections and used for H&E staining analysis. Rat lung specimens (rat PASMC) were fixed with 10% formalin, processed, embedded in paraffin, and sliced at a thickness of 4 μm. The sections were treated with 3% H_2O_2 for 15 min to block the endogenous peroxidase activity, followed by overnight incubation with the primary antibody. All primary antibodies used were purchased from Affinity Biosciences, Cincinnati, OH, USA and were listed as follows:α-SMA (AF1032), VCAM-1(DF6082), PCNA (AF0239), ICAM-1(AF6088), and Vimentin (AF7013). After removing the primary antibody, the sections were incubated with the secondary antibody IgG (A0516, Beyotime Biotechnology Co., Ltd., Shanghai, Chinese) at 37°C for 30 min, followed by dyeing with diaminobenzidine. Non-specific binding was blocked by application of 5% normal goat serum for 10 min. Finally, the sections were stained with hematoxylin. The resulting sections were then treated with anti-fluorescence quencher and observed under a fluorescence microscope.

Obtaining Primary Cells

Six-week-old SPF, male SD rats were taken and anesthetized by intraperitoneal injection of urethane (0.6 mL/100g); after anesthesia, the rats were sacrificed and disinfected. The rat's chest cavity was opened, heart and lung tissues were taken out and placed in pre-cooled sterile phosphate buffer sodium (PBS). After separating the left and right small pulmonary arteries, these were cut along the longitudinal axis of the arterial tube with ophthalmic scissors. A small blade was used to gently scrape the inner wall several times to remove endothelial cells and then the tube wall was cut into 1×1 mm^2 tissue pieces.

Cell Culture and Treatment

The cut tissue pieces were placed in a culture flask to which 20% fetal bovine serum (FBS) was added in Dulbecco's Modified Eagle's Medium (DMEM), and cultured using the tissue patch method. After the cells had grown and merged to about 80%, these were trypsinized for subculturing. Immunofluorescence was used to detect α-SMA in order to identify the cell purity of the cultured PASMC. Exposure to normoxia was done by keeping the cells in in 5% CO_2 and 21% O_2 at 37°C. For hypoxic exposure, PASMC were cultured in a hypoxic incubator (YCP-80/S, CHAOHONG, China) (3% O2) for 24 h.

RNA Interference and Cell Transfection

The PASMC were inoculated in a 6-well cell culture plate (1×10^5 density), and cultured in DMEM medium containing 10% FBS for 24 h. After the cells were fused to about 80%, the culture medium was replaced with 0.5% FBS in DMEM medium and cellular synchronization was allowed for the next 12 hours. The circ-Sirt1 overexpression plasmid and SIRT1 mRNA-specific RNAi oligonucleotides (siRNA) were designed and synthesized by Hanbio Co. Ltd, Shanghai, China. For the sequence of siRNA, see the supplementary material.

Adenovirus Expression Vector and Plasmid Constructs

The expression plasmid of circ-Sirt1 was created by putting the entire rat circ-Sirt1 sequence into pcDNA3.1circRNA Mini Vector (Addgene). Part of the green fluorescent protein (GFP) fragment was amplified by PCR and cloned into pcDNA3.1 circRNA Mini Vector as a control plasmid. The adenovirus vector encoding the circ-Sirt1 and the GFP control were both designed and synthesized by Hanbio Co. Ltd, Shanghai, China.

Quantitative Real-Time PCR

According to the instructions of the TRIzol Kit (15596018, Thermo Fisher, Rochester, NY, USA), the cell pellets were collected, RNA was extracted and purified, genomic DNA was removed and reacted to obtain cDNA template for fluorescence quantification. Thereafter, fluorescence quantitative PCR was performed under the reaction system and conditions reaction. PCR amplification was performed with Real-Time PCR System (Applied biosystems, Foster city, CA, USA). The expressions of mRNA and circ-Sirt1 were normalized with β-actin, and $2^{-\Delta\Delta CT}$ was used for relative quantification. Refer to the supplementary material for each reaction primer.

Western Blot Analysis

After collecting pulmonary artery tissues and PASMC, 100 uL of NP-40 cell lysate (BL504a, BioSharp, USA) was added per well of the 6-well culture plate and centrifuged at 12,000 rpm for 15 min. The resulting protein samples were collected, and 5x SDS (S8010, Solarbio Science&Technology Co.,Ltd., Beijing, China)-PAGE (T8090, Solarbio Science&Technology Co.,Ltd., Beijing, China) was added to these. After boiling and cooling, the protein samples were loaded into the sample hole. Following membrane transfer, 5% skimmed milk powder was added, and the samples were sealed at room temperature for 2 h. Thereafter incubation with the primary antibody (ZB-2305, Zs-BIO, Beijing, China) and the secondary antibody (ZB-2301, Zs-BIO, Beijing, China) was carried out as per the manufacturer instructions. Following incubation with secondary antibody for 2 h, washing solution PBST was added and all samples were washed 3 times (10 min each). Lastly, a 1:1 mixture of ECL A solution and ECL B solution (34094, Thermo Fisher, Rochester, NY, USA) prepared in a dark room was added to the plate and allowed to fully react for 1–2 min, after which the remaining solution was removed, and the plate was exposed.

Cell Proliferation CCK8

After rat PASMC were digested, 100 μl of culture medium was added to each well of the culture plate in order to resuspend the cells, the cell density of each group being 1 x 10^5, and the plate was placed in an incubator overnight to incubate in 5% CO_2 at a temperature of 37 . After culturing for a different time, 10 μl of CCK8 (BB-4202-01, Bebo, Shanghai, China) was added to each well and culturing was continued for another 1 h. The absorbance of each well was measured at the optical density of 450 nm of the enzyme-linked immunoassay.

Scratch Test

A marker pen was used to draw evenly placed horizontal lines on the back of the 6-well plate, with an interval of 1 cm between each line, and at least 5 lines were drawn across each hole. About 5 x 10^5 cells were added to each hole and scratched the next day with a pipette tip kept perpendicular to the horizontal lines drawn on the back of the plate. The cells were washed 3 times with PBS. The marked cells were removed and a serum-free medium was added to these cells. Finally, these were put it in an incubator at 37°C in 5% CO_2, and cultivated. Sampling was done at 0, 24, and 48 h, and corresponding photographs were taken.

Flow Cytometry

The cells were treated with trypsin (C0201, Beyotime Biotechnology Co., Ltd., Shanghai, Chinese) and PBS (SH30256.01, Hyclone, Logan City, Utah, USA), fixed, washed twice with pre-cooled PBS and centrifuged at 2000 rpm for 5 min. This was followed by addition of 500 μl PBS

and moving the cells to a 1.5 ml Eppendorf (EP) tube, after which 20 μl RNase (BB-4104, Bebo, Shanghai, China) (storage concentration 25 mg/ml, PBS diluted to 1 mg/ml, working concentration 50 ug/ml) was added to the EP tube. After resuspending the cells in a water bath, 40 μl PI staining solution was added to the resuspended cells and staining was done at 4 degrees in the dark. The cells were thoroughly mixed, filtered with a 200-mesh filter, and cell cycle detection was done using a flow cytometer.

Animal Testing

Eighteen, six-week-old, male SD rats, weighing 150-200g, were randomly placed into three groups: the hypoxia group, the hypoxia+ empty adenovirus group (pcDNA3.1-NC), and the hypoxia+circ-Sirt1 adenovirus group (pcDNA3.1-circ-Sirt1). For the hypoxia group, 1 mL of normal saline was injected into the tail veins of the rats with a microsyringe. For the hypoxia+ empty adenovirus group, $1*10^8$ CFU of empty adenovirus was injected into the tail veins of the rats with a microsyringe. Lastly, for the hypoxia+circ-Sirt1 adenovirus group, $1*10^8$ CFU of adenovirus was taken with a microsyringe and injected into the rat tail vein. The virus titer was 14–20 days, the injection was performed every 20 days, and the experimental treatment was carried out after 40 days. Feeding conditions, experimental methods of hemodynamics, and right ventricular hypertrophy index are the same as above. After euthanasia, the lung tissue and pulmonary blood vessels were removed and kept for future use.

Statistical Analysis

All statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). The data were presented as mean ± standard deviation (SD). The P value was calculated by applying Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test as appropriate. P values <0.05 were considered statistically significant.

Results

Decreased Circ-Sirt1 and SIRT1 in Rats With Hypoxia-Induced Pulmonary Hypertension

Right heart catheterization was performed after the hypoxia-induced pulmonary hypertension model was established (Fig. 1A). Compared with the normoxia group, the RVSP and right ventricular hypertrophy index increased in hypoxia-induced rats (Fig. 1B, C). Pulmonary artery vascular thickening and lumen stenosis were also observed in the hypoxia-induced PAH rat models (Fig. 1D), which showed that the animal model was successful. Western blot and RT-PCR analysis revealed a reduced expression of circ-Sirt1 and SIRT1 in hypoxia-induced rat PAH model compared to the normal group (Fig. 1E, F). Based on this we speculated that circ-Sirt1 and SIRT1 may be related to PAH.

Acquisition and Cultivation of Primary Pulmonary Artery Smooth Muscle Cells

In our experiment, we were able to successfully isolate primary rat PASMC, and culture these using the tissue patch method (Fig. 2A). We also observed the adherent growth of PASMC. The immunofluorescence method was used to detect α-SMA, in order to confirm cell purity. Most of the cells in the field of view stained positively for α-SMA (Fig. 2B).

Overexpression of Circ-Sirt1 Inhibited the Proliferation and Migration of Pulmonary Artery Smooth Muscle Cells

Rat PASMC were cultured under hypoxia, and after transfection with circ-Sirt1 overexpression plasmid, western blot, and RT-PCR detection, these were compared with the control group. It was seen that the overexpression of circ-Sirt1, up-regulated the expression of circ-Sirt1 in rat PASMC. In addition, SIRT1 mRNA and protein levels also increased correspondingly, while the expression levels of TGF-β1, Smad3, and Smad7 decreased. The expression levels of PASMC markers, VCAM-1, and α-SMA also decreased (Fig. 3A, B). Flow cytometry was used to detect the cell cycle of rat PASMC, and it was found that the cellular components decreased significantly in G2/S phase, while the number of cells increased in the G0/G1 phase. This shows that overexpression of circ-Sirt1 inhibits the cell proliferation of rat PASMC (Fig. 3C). The CCK8 method was used to detect cell viability at 24 hours, 48 hours, and 72 hours, and the cell viability of rat PASMC overexpressing circ-Sirt1 was significantly reduced (Fig. 3D). The scratch test was used to determine the migration ability of cells after 24 hours and 48 hours and it was noted that when circ-Sirt1 is overexpressed, the migration ability of rat PASMC is significantly inhibited (Fig. 3E). Immunofluorescence experiments showed that α-SMA and VCAM-1 in rat PASMC were reduced after overexpression of circ-Sirt1 (Fig. 3F). These results show that circ-Sirt1 may have a role in the control of the proliferation and migration of rat PASMC by affecting the mRNA of SIRT1 through TGF-β1/Smad3/Smad7 regulated pathways, thereby participating in the progress of pulmonary hypertension.

SiRNA Inhibition of SIRT1 Reverses the Effect of Overexpressed Circ-Sirt1 on the Proliferation of Pulmonary Artery Smooth Muscle Cells

In order to confirm the mechanism of the influence of circ-Sirt1 on rat PASMC, we conducted a rescue test to inhibit

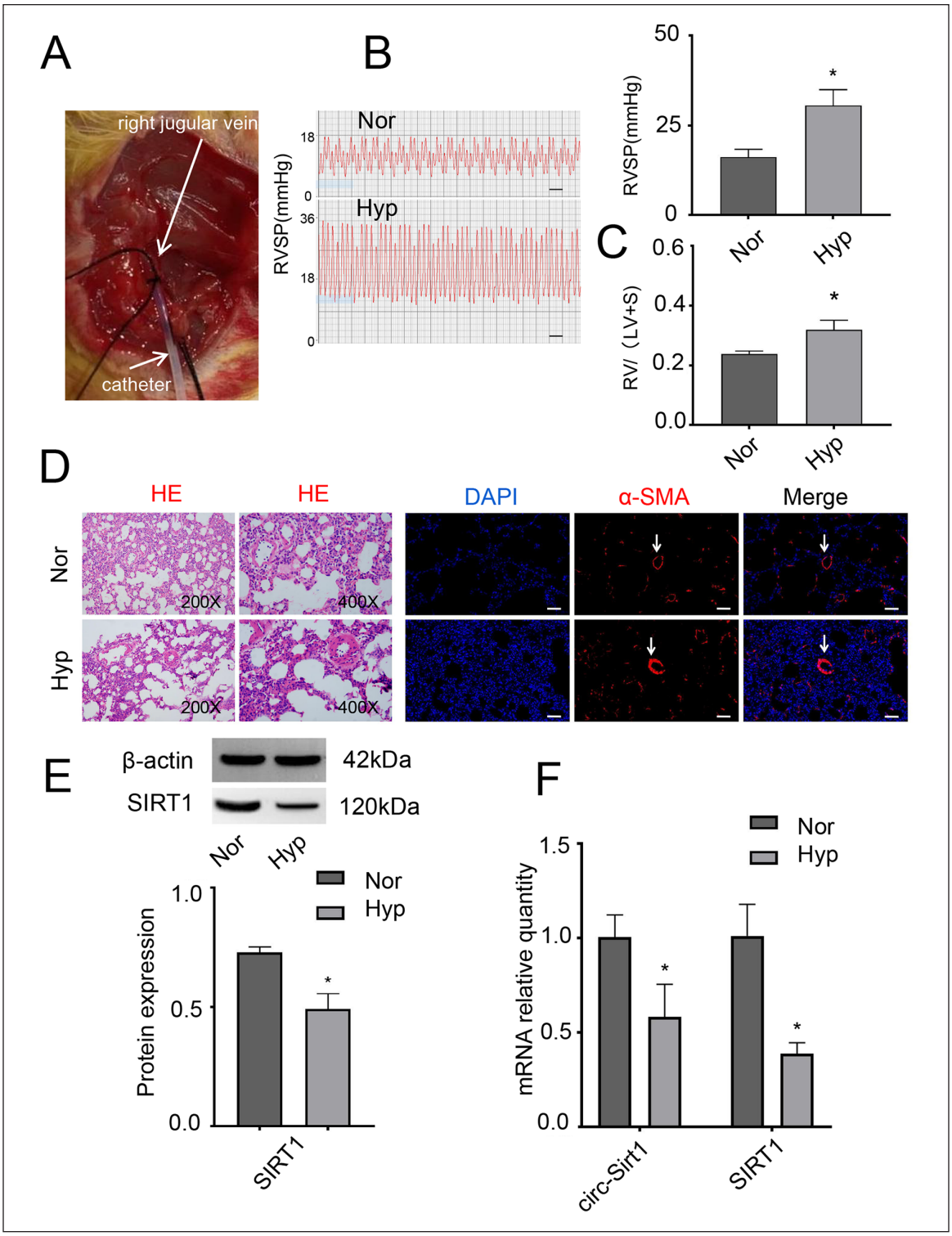

Figure 1. circ-Sirt1 and SIRT1 decreased in hypoxia-induced pulmonary hypertension in rats. (A) Operation diagram of heart catheterization into the right jugular venous cannula. (B) The right ventricular systolic pressure (RVSP), (Scale bar = 2.5s, n = 5). (C) The right ventricle/left ventricle plus septum (RV/LV+S) ratio (n = 6). (D) H&E staining and α-SMA showed the morphological changes of pulmonary small blood vessels in each group (original magnification, ×200, ×400; Scale bar=50 μm; n = 6). (E) Western blot was used to detect the changes of SIRT1 protein in lung tissue (n = 3). (F) qRT-PCR was used to detect the mRNA expression of circ-Sirt1 and SIRT1 in lung tissue (n = 6). The levels of expression were normalized to β-actin. Values are represented as the mean ± SD. H&E: Hematoxylin & eosin staining; DAPI: 4′, 6-diamidino-2-phenylindole, 2-(4-amidinophenyl)-1H -indole-6-carboxamidine; α-SMA: a-smooth muscle actin; qRT-PCR: quantitative real-time polymerase chain reaction; Nor: Normal; Hyp: Hypoxia. *$P < 0.05$, compared with normal group.

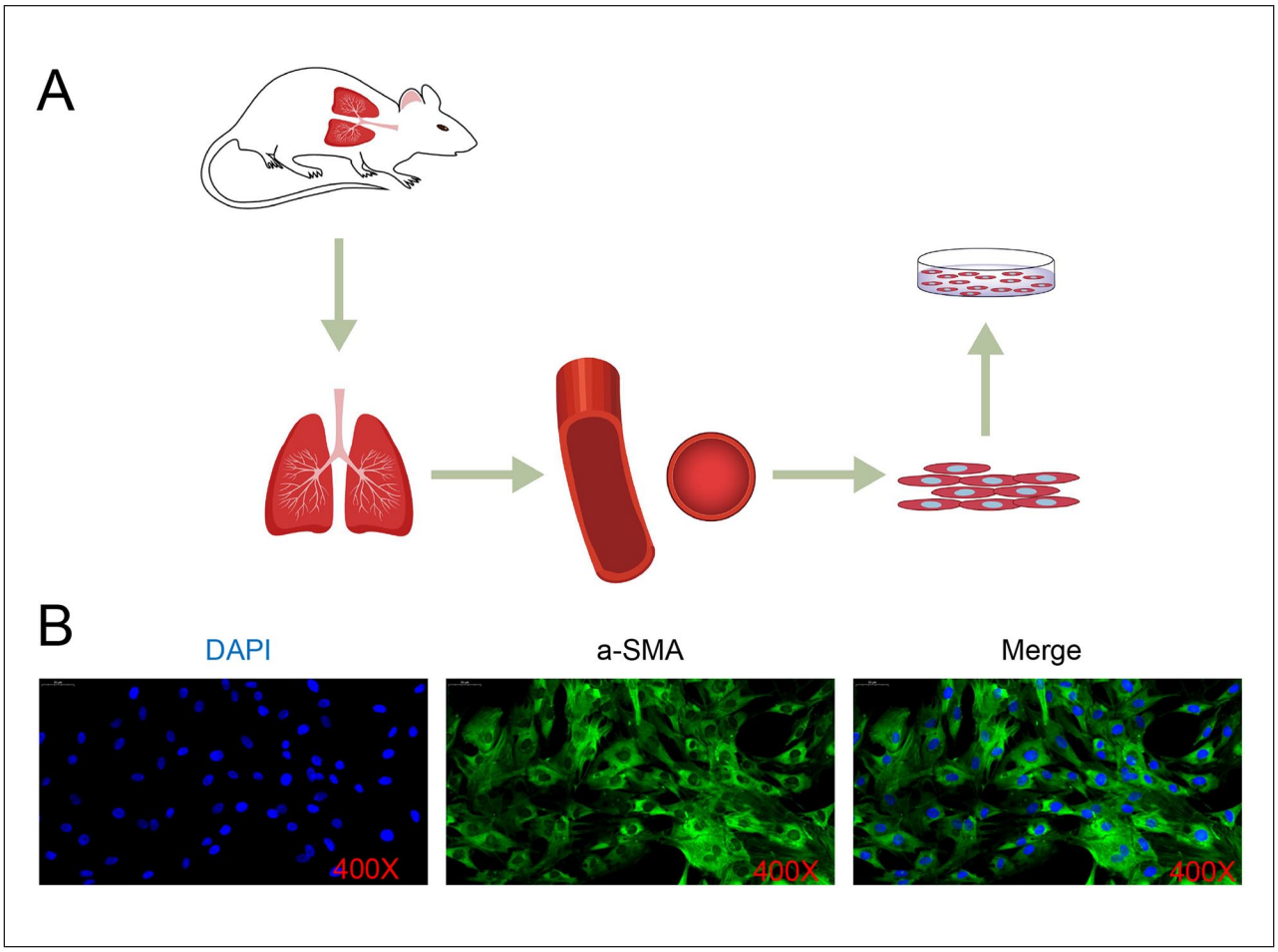

Figure 2. Acquisition and cultivation of primary cells of pulmonary artery smooth muscle cells. (A) Schematic diagram of the acquisition and culture of primary pulmonary artery smooth muscle cells. (B) Immunofluorescence method was used to detect the expression of α-SMA (green), a molecular marker of rat PASMC (original magnification, ×400). α-SMA: a-smooth muscle actin; DAPI: 4′, 6-diamidino-2-phenylindole, 2-(4-amidinophenyl)-1H -indole-6-carboxamidine.

SIRT1 by siRNA in cells transfected with circ-Sirt1 overexpression plasmid. RT-PCR and western blot tests revealed that si-SIRT1 can significantly inhibit the increasing effect of circ-Sirt1 on SIRT1. Similarly, si-SIRT1 can significantly restore the inhibitory effect of circ-Sirt1 on the levels of TGF-β1/Smad3/Smad7 as well as PASMC markers, VCAM-1 and α-SMA (Fig. 4A, B). Flow cytometry, CCK8 method, and scratch experiment showed that overexpression of circ-Sirt1 inhibited the cell proliferation, cell viability and migration of rat PASMC, and si-SIRT1 reversed this effect (Fig. 4C–E). Immunofluorescence experiment revealed that after overexpression of circ-Sirt1, α-SMA and VCAM-1 in rat PASMC were reduced, and SIRT1-siRNA inhibited this effect (Fig. 4F). The above findings show that circ-Sirt1 positively regulates the transcription process of SIRT1 mRNA, thereby inhibiting TGF-β1/Smad3/Smad7 to participate in the control of proliferation and migration of rat PASMC, and participating in the progression of pulmonary hypertension.

Role of Overexpressed Circ-Sirt1 in Rat Models of Pulmonary Hypertension

In order to verify the underlying mechanism of pulmonary hypertension, we injected circ-Sirt1 adenovirus or empty adenovirus into the tail vein of rats in a hypoxia-induced pulmonary hypertension arterial model. After 40 days of hypoxia induction, right heart catheterization measured RVSP and analyzed the ratio of RV/LV+S. Compared with the hypoxia group, the hypoxia+circ-Sirt1 adenovirus group had lower RVSP (Fig. 5A, B) and right ventricular hypertrophy index (Fig. 5C). RT-PCR and Western blot were used to detect lung tissue. When compared with the hypoxia group, the lung tissue of the hypoxia+circ-Sirt1 adenovirus (pcDNA3.1-circ-Sirt1) group showed significantly increased level of circ-Sirt1, and the expression of SIRT1 was also increased (Fig. 5D–F). The mRNA and protein expression levels of TGF-β1, Smad3, and Smad7

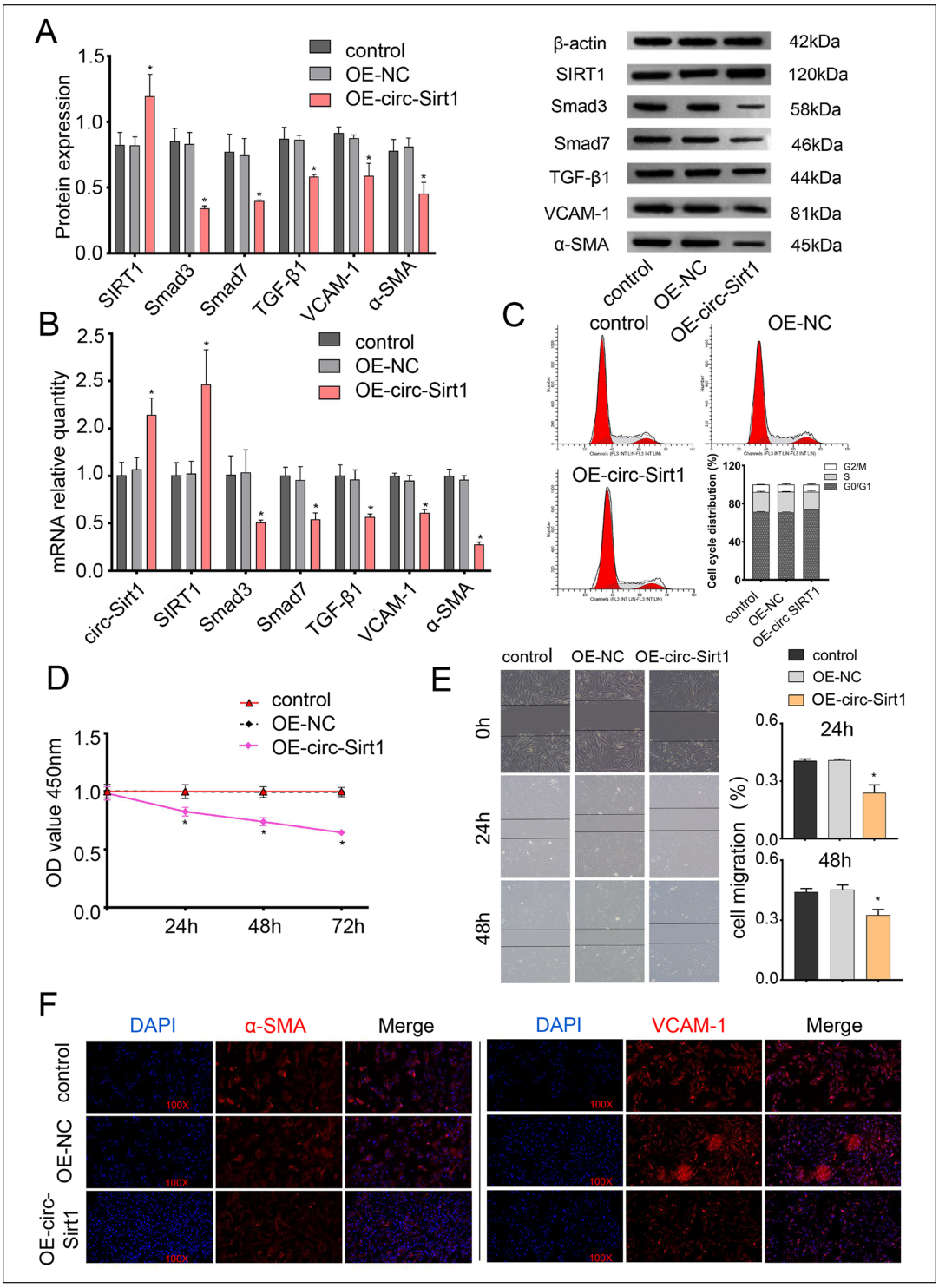

Figure 3. Effect of overexpressed circ-Sirt1 plasmid in pulmonary artery smooth muscle cells. (A) After circ-Sirt1 was overexpressed, western blot was used to detect the protein changes of SIRT1, TGF-β1, Smad3, Smad7, VCAM-1, and α-SMA in rat PASMC. (B) qRT-PCR was used to detect the mRNA expression of circ-Sirt1, SIRT1, TGF-β1, Smad3, Smad7, VCAM-1, and α-SMA in rat PASMC. (C) The cell cycle of PASMC was measured by flow cytometry. (D) The cell viability of PASMC detected by CCK-8 method. (E) The scratch-wound assay was used to determine the migration ability of cells after 24 h and 48 h. (F), α-SMA, VCAM-1 immunofluorescence staining of pulmonary artery in rats. (Original magnification, ×100). The levels of expression were normalized to β-actin. All values are represented as the mean ± SD (n = 3). TGF-β1: Transforming growth facter-β1; VCAM-1: recombinant Human Vascular cell adhesion molecule 1; α-SMA: a-smooth muscle actin; PASMC: pulmonary artery smooth muscle cells; qRT-PCR: quantitative real-time polymerase chain reaction; CCK-8: cell counting kit-8; OE: overexpression; NC: negative control; DAPI: 4', 6-diamidino-2-phenylindole, 2-(4-amidinophenyl)-1H -indole-6-carboxamidine; OD: optical density. *$P < 0.05$, compared with control group.

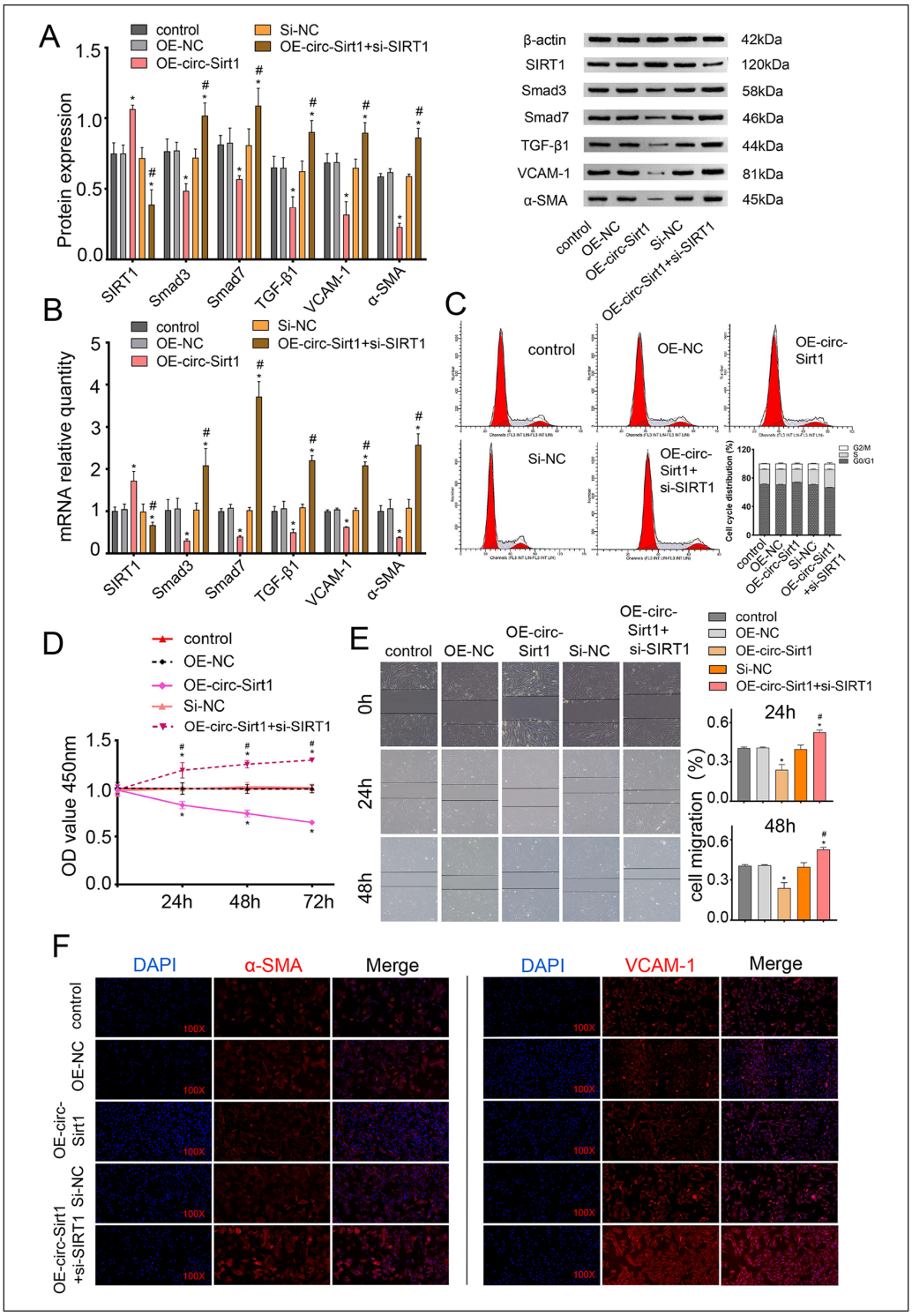

Figure 4. Effect of siRNA inhibition of SIRT1 in rat pulmonary artery smooth muscle cells. (A) Western blot was used to detect the protein changes of SIRT1, TGF-β1, Smad3, Smad7, VCAM-1, and α-SMA in rat PASMC. (B) qRT-PCR detects the mRNA expression of SIRT1, TGF-β1, Smad3, Smad7, VCAM-1, and α-SMA in rat PASMC. (C) The cell cycle of PASMC was measured by flow cytometry. (D) The cell viability of PASMC detected by CCK-8 method. (E) The scratch-wound assay is used to determine the migration ability of cells after 24 h and 48 h. (F) α-SMA, VCAM-1 immunofluorescence staining of pulmonary artery in rats. (Original magnification, ×100). The levels of expression were normalized to β-actin. All values are represented as the mean ± SD (n = 3). TGF-β1: Transforming growth facter-β1; SiRNA, Small interfering RNA; VCAM-1: Recombinant Human Vascular cell adhesion molecule 1; α-SMA: a-smooth muscle actin; PASMC: pulmonary artery smooth muscle cells; qRT-PCR, quantitative real-time polymerase chain reaction; CCK-8: cell counting kit-8; OE: overexpression; NC: negative control; OD: optical density; DAPI: 4', 6-diamidino-2-phenylindole, 2-(4-amidinophenyl)-1H -indole-6-carboxamidine. *$P < 0.05$, compared with control group; #$P < 0.05$, compared with OE-circ-Sirt1 group.

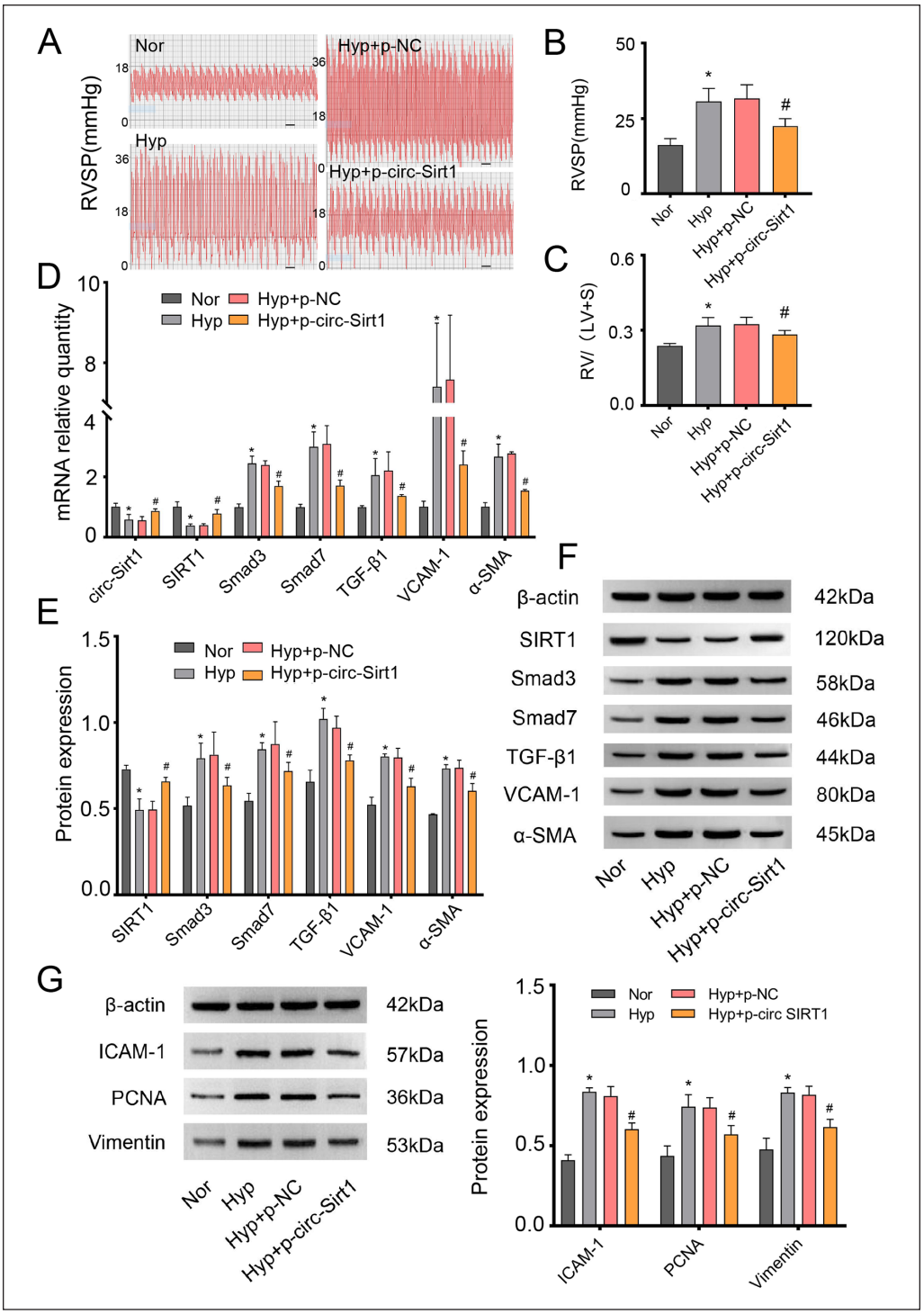

Figure 5. Overexpression of adenovirus circ-Sirt1 delayed the progression of hypoxia-induced PAH in rats. (A) and (B) Changes in RVSP, (Scale bar=5s, n = 5, 5, 5, 6). (C) Changes in the right ventricle/left ventricle plus septum (RV/LV + S) ratio (n = 6). (D) qRT-PCR detects the mRNA expression of circ-Sirt1, SIRT1, TGF-β1, Smad3, Smad7, VCAM-1, and α-SMA in lung tissue (n = 6). (E) and (F) Western blot was used to detect the protein changes of SIRT1, TGF-β1, Smad3, Smad7, VCAM-1, and α-SMA in lung tissue (n=3). (G) Western blot was used to detect the protein changes of ICAM-1, PCNA and vimentin in lung tissue (n = 3). The levels of expression were normalized to β-actin. Values are represented as the mean ± SD. PAH: Pulmonary arterial hypertension; TGF-β1: Transforming growth facter-β1; RVSP: right ventricular systolic pressure; RV: right ventricle; qRT-PCR: quantitative real-time polymerase chain reaction; VCAM-1: Recombinant Human Vascular cell adhesion molecule 1; α-SMA: a-smooth muscle actin; ICAM-1: intercellular cell adhesion molecule-1; PCNA: proliferating cell nuclear antigen; Nor: Normal; Hyp: Hypoxia; p: pcDNA3.1; NC: negative control; LV+S: left ventricle+septum. *$P < 0.05$, compared with normal group; #$P < 0.05$, compared with hypoxia group.

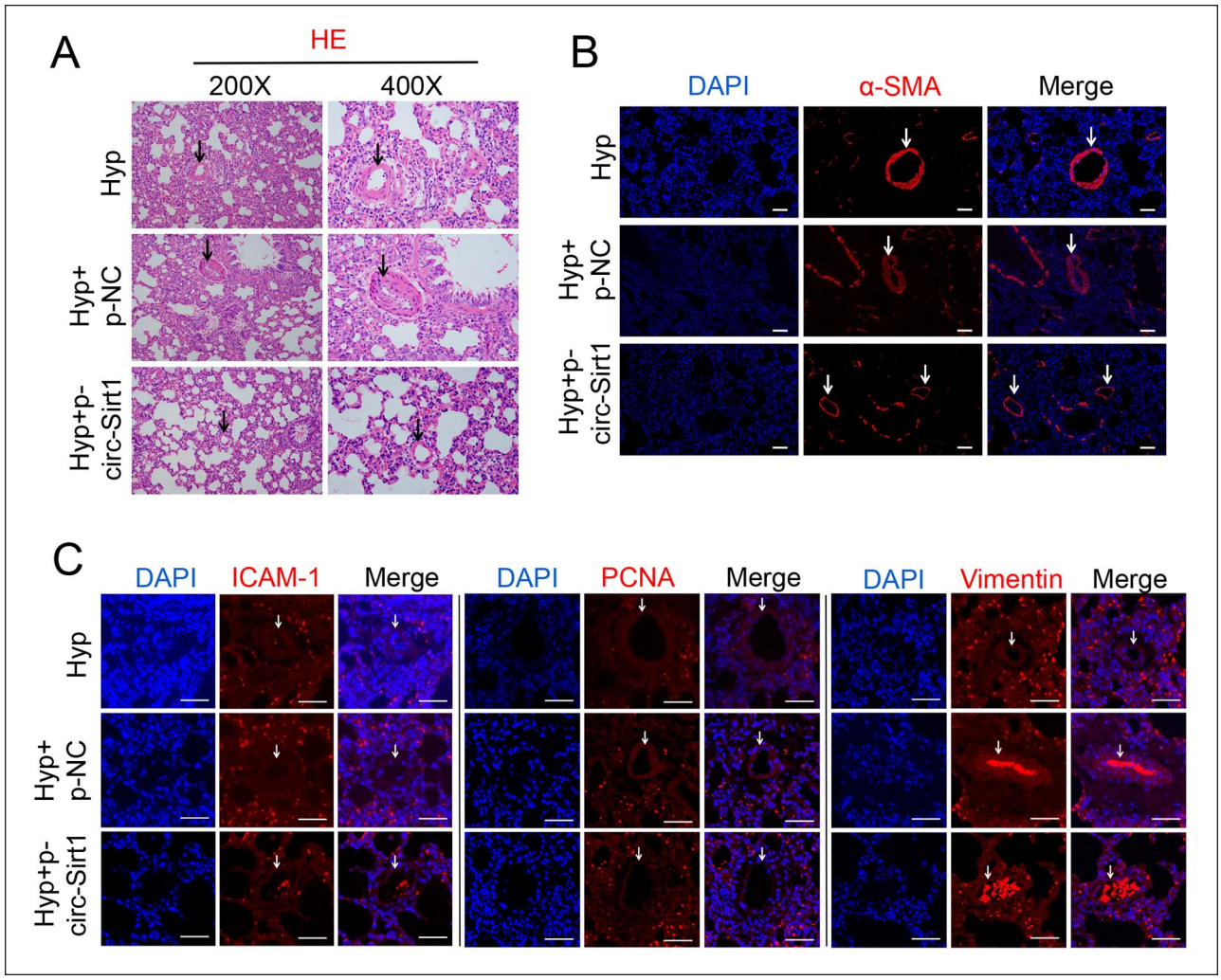

Figure 6. H&E staining and immunofluorescence of rat lung tissue in each group. (A) H&E staining showed the morphological changes of pulmonary small blood vessels in each group (original magnification, ×200, ×400). (B) α-SMA immunofluorescence staining of pulmonary arteries in rats (scale bar = 50 μm). (C) ICAM-1, PCNA, and vimentin immunofluorescence staining of pulmonary arteries in rats (scale bar = 50 μm). H&E: Hematoxylin & eosin staining; DAPI: 4′, 6-diamidino-2-phenylindole, 2-(4-amidinophenyl)-1H -indole-6-carboxamidine; α-SMA: a-smooth muscle actin; ICAM-1: intercellular cell adhesion molecule-1; PCNA: proliferating cell nuclear antigen; Nor: Normal; Hyp: Hypoxia; p: pcDNA3.1; NC: negative control; (n = 6).

decreased along with expression levels of PASMC markers (α-SMA and VCAM-1) (Fig. 5D–F). Protein expression levels of ICAM-1, PCNA and vimentin also decreased in lung tissue (Fig. 5G). Hematoxylin and eosin staining showed that the pulmonary arteries of rats in the hypoxia group were thickened and the lumen was narrowed, while the hypoxia + circ-Sirt1 adenovirus group revealed significantly reduced blood vessel thickness and lumen stenosis (Fig. 6A). The pulmonary artery of the rat was detected by immunofluorescence and compared with the hypoxia model group, it was found that the hypoxia + circ-Sirt1 adenovirus group had a decrease in α-SMA, ICAM-1, PCNA, and vimentin (Fig. 6B, C). It shows that in hypoxia-induced pulmonary hypertension animal models, increasing the expression of circ-Sirt1 can delay the proliferation and migration of pulmonary artery smooth muscle cells, thereby alleviating the process of pulmonary hypertension.

Discussion

As a key cell implicated in PAH pathogenesis, PASMC is known to participate in pulmonary vascular remodeling. However, the correlation of circRNA with PAH and its role in vascular remodeling have been rarely studied. We found in the hypoxia-induced PAH rat model that the expression of circ-Sirt1 and SIRT1 decreased, and thereby, we predicted that circ-Sirt1 and SIRT1 were related to PAH. This study was conducted to explore this relationship. First, primary PASMC were isolated from rats and cultured for cell function experiments. After overexpression of circ-Sirt1, it was

noted that the levels of SIRT1 mRNA and protein were upregulated. However, the expressions of TGF-β1, Smad3, and Smad7 were inhibited, and the expressions of VCAM-1 and α-SMA were also reduced. Overexpression of circ-Sirt1 weakened PASMC cell viability and proliferation, and inhibited cell cycle progression and migration ability. Second, through rescue experiments, it was found that circ-Sirt1 positively regulated the translation process of SIRT1 mRNA, thereby inhibiting TGF-β1/Smad3/Smad7, thus weakening the proliferation and migration of rat PASMC. Finally, it was verified via the experimental rat model that increasing the expression of circ-Sirt1 could inhibit the proliferation and migration of PASMC in rats with PAH.

Studies have shown that the TGF-β1/Smad signaling pathway is involved in the pathogenesis of heart and lung diseases[9,11]. Previous research by Tingting et al[12] demonstrated that Qiliqiangxin may inhibit the TGF-β1/Smad2/3 signaling pathway to weaken atrial remodeling and improve cardiac function in certain heart diseases. Wang et al[13] found that enzyme 8-oxoguanine DNA glycosylase-1 regulates cell transformation induced by TGF-β1, thereby promoting bleomycin-induced pulmonary fibrosis in mice and activating p-Smad2/3 during the process of pulmonary fibrosis and also by partly interacting with Smad7. Our study findings showed that the expressions of TGF-β1, Smad3, and Smad7 are consistent with the expression changes of VCAM-1 and α-SMA. TGF-β1/Smad3/Smad7 may mediate the changes of PASMC.

Studies have found that circRNA plays a key role in a variety of diseases, so it may emerge as a new diagnostic or therapeutic target for many diseases in the future. For example, Zheng et al[14] found that the expression of circ-000595 increased in the tissues of patients with aortic aneurysm, and further studied that circ-00059 inhibited the expression of miR-19a in cells and promoted the apoptosis of human aortic smooth muscle cells induced by hypoxia. Xu et al[15] knocked down rno-circ_005717 (circDiaph3) in their study and found that it can up-regulate the level of diaphanous-related formin-3, thereby promoting the differentiation of vascular smooth muscle cells (VSMC) to contraction and resisting arterial intimal hyperplasia. In a study on heart failure by Gorski et al,[16] it was revealed that the pharmacological activation of SIRT1 can promote the deacetylation of lysine 492 and restore the activity of sarco-endoplasmic reticulum Ca2+-APase (SERCA2a), thereby reducing chances of heart failure. Kong et al[17] found that circ-Sirt1 binds to miR-132/212, interferes with SIRT1 mRNA and promotes the expression of the host gene SIRT1, thereby affecting the inflammatory phenotype transition of VSMC. This showed that circ-Sirt1 plays an important role in pathogenesis of atherosclerosis. This previous study showed that the overexpression of circ-Sirt1 significantly increased the expression of SIRT1 at the protein level, which is consistent with our research finding. In addition, we found that up-regulation of the expression of circ-Sirt1 can inhibit the proliferation and migration of PASMC, which is a novel finding of our research.

A number of studies have shown that, among ncRNAs, microRNAs and lncRNAs are involved in the causation of PAH. Kang et al[18] found that miR-124 transactivates NFAT by targeting multiple genes including NFATc1, CAMTA1, and PTBP1, thereby weakening the proliferation of human PASMC. Xing et al,[19] in their research, found that the up-regulation of lncRNA-maternally expressed gene 3 (lncRNA-MEG3) promoted hypoxia-induced PASMC proliferation, cell migration, cell cycle progression and reduced PASMC apoptosis. In their study, Li et al[20] found that the peptide RPS4XL encoded by lnc-Rps4l is involved in PASMC proliferation induced by hypoxia. As a post-transcriptional regulatory factor, circRNA has been receiving increasing attention in the development of myocardial injury, myocardial fibrosis, myocardial hypertrophy, heart failure, and PAH. Zhou et al found that hsa_circ_0016070, miR-942 and CCND1 were differentially expressed in PAH patients. Further research in this area revealed that hsa_circ_0016070 induces PASMC proliferation through miR-942-5p/CCND1 axis and participates in PAH vascular remodeling[21]. Yang et al[22] found up-regulated mmu_circ_0000790 in both hypoxic pulmonary hypertension pulmonary vascular tissue and hypoxic PASMC, which can competitively bind miR-374c and up-regulate the target gene FOXC1, promote the proliferation of hypoxic PASMCs and reduce their apoptosis, thereby accelerating pulmonary vascular remodeling in PAH mice. The above studies have shown that ncRNAs play an important role in the formation of PAH. The most important finding of our research was that overexpression of circ-Sirt1 in hypoxic rats was shown to significantly slow down the proliferation of PASMC, thus highlighting the fact that circ-Sirt1 may be a new therapeutic target for PAH.

In addition, many studies have shown that circRNAs can regulate the expression of mRNAs[23,24]. Therefore, the specific mechanism of circ-Sirt1 regulating SIRT1 mRNA expression is our next research focus.

Although this experiment proved that circ-Sirt1 plays a key role in pathogenesis of PAH in rats, the next step of clinical application of this finding still needs experiment verification. Therefore, further research is required in this area to explore the clinical value and applications of circ-Sirt1 in PAH.

Conclusion

Our research findings indicate that circ-Sirt1 regulates the expression of SIRT1 mRNA and inhibits the proliferation and migration of PASMC. This effect may be mediated by TGF-β1/Smad3/Smad7 (Fig. 7). These results provide a new revelation regarding the molecular mechanism of pulmonary artery vascular remodeling in PAH and may help in the

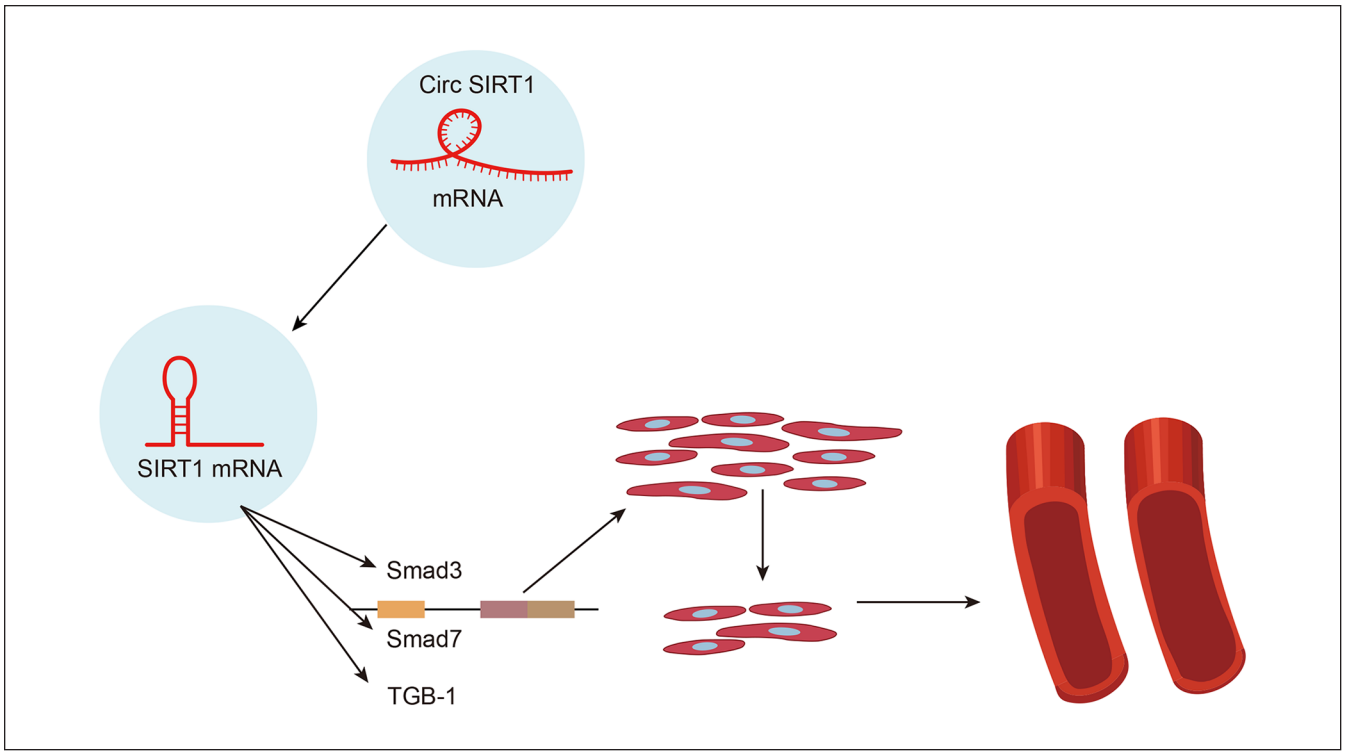

Figure 7. Circ-Sirt1/SIRT1 regulates the expression of TGF-β1/Smad3/Smad7 pathway, thereby affecting the proliferation and migration pattern of pulmonary artery smooth muscle cells induced by hypoxia.

development of new and effective treatment options for PAH.

Ethical Approval

Our study was approved by the Ethics Review Board of Bengbu Medical College, Bengbu, Anhui Province, China (Protocol number 2017081).

Statement of Human and Animal Rights

All of the experimental procedures used in this study were conducted in accordance with the Institutional Animal Care Guidelines of Bengbu Medical College, China and were approved by Institutional Experimental Animal Management and Ethics Committee of Bengbu Medical College, Anhui Province, China.

Statement of Informed Consent

There are no human subjects in this article. Consequently, informed consent is not applicable.

Study Association

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Declaration of Conflicting Interests

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Supplemental Material

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