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Original Article

Inhibition of survivin promotes pulmonary arterial smooth muscle cell apoptosis in high blood flow-induced pulmonary arterial hypertension in rats

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Abstract: The precise pathogenesis of high pulmonary blood flow-induced pulmonary arterial hypertension (PAH) remains unclear. The purpose of this study was to explore the expression and function of survivin in pulmonary arterial smooth muscle cell (PASMC) in a rat model of high pulmonary blood flow-induced PAH. Sprague-Dawley (SD) rats were randomly divided into the following groups: Control group, Sham group, Shunt group, and Shunt+siRNA group. The rats in the Shunt and Shunt+siRNA groups were used to establish the high pulmonary blood flow-induced PAH model. The pulmonary arteries were isolated for the primary culture of PASMCs. PASMC in the Shunt+siRNA group underwent siRNA-survivin transfection. The survivin gene and protein expression levels were analyzed, and PASMC proliferation and apoptosis were assessed in each group. We found that survivin gene and protein expression levels were increased in PASMCs in the Shunt and Shunt+siRNA groups compared with the Control and Sham groups ($P<0.05$). PASMC proliferation and apoptosis were significantly increased and inhibited, respectively, in the Shunt group relative to the Control and Sham groups ($P<0.05$). In the Shunt+siRNA group, survivin gene and protein expression significantly decreased, PASMC apoptosis increased, and proliferation was inhibited relative to that in the Shunt group ($P<0.05$). Our results indicate that survivin is expressed in PASMC in a rat model of high pulmonary blood flow-induced PAH and both attenuate Kv1.5 and Kv2.1 channel expression and inhibits caspase-3 and caspase-9 activity. Targeting survivin may represent a potent therapeutic measure to promote PASMC's apoptosis and inhibit its proliferation in PAH, helping to reverse pulmonary arterial remodeling.

Keywords: High pulmonary blood flow, survivin, pulmonary arterial hypertension

Introduction

Congenital heart disease (CHD) is the most common pathogenesis of pulmonary arterial hypertension (PAH) in children [1]. The pathogenesis of PAH is largely unknown [2]; the cellular and molecular mechanisms of high pulmonary blood flow induced-PAH remain unclear, and further research is urgently needed [3-5]. If a CHD patient does not undergo surgery at an early stage of the disease, the risk of developing PAH is approximately 30%. Even when the heart defect can be repaired, the prognosis is poor due to the continuous increase in pulmonary arterial pressure and pulmonary vascular resistance. Therefore, PAH is a serious, life-threatening disease. The pathogenesis of PAH involves inflammation [6, 7], proliferation [8], vascular contraction and vascular remodeling [9], and neovascularization [10] of the pulmonary vasculature. The pathophysiology of high pulmonary blood flow-induced PAH differs from that of other types of PAH and involves high shear stress, decreased vascular reactivity, and increased vascular resistance [11]. However, the precise mechanisms underlying the proliferation and apoptosis of pulmonary arterial smooth muscle cells (PASMCs) remain unclear [12]. Although some treatments can decrease pulmonary arterial pressure, conventional treatments for reversing PASMC proliferation and pulmonary vascular remodeling in PAH remain unsatisfactory [13, 14]. A novel therapeutic approach to inhibiting proliferation and inducing apoptosis in PASMCs is needed [15].

Survivin is an inhibitor of apoptosis protein family member and controls cell reproduction,

division, and apoptosis. Survivin protein is expressed differentially between many cancer cells relative to their normal counterparts [16]. Survivin represents an attractive therapeutic target due to the restriction of its expression primarily to the developmental period and in the onset of malignancy; no expression is observed in normal terminally differentiated tissue [17]. Survivin inhibitors are often used in tumor therapy research. Growing evidence suggests the presence of survivin expression in a hypoxia-induced PAH rat model [18, 19] and a monocrotaline (MCT)-induced PAH rat model [20]. Therefore, therapies aimed at survivin are prominent in the current treatment of PAH. To date, research on survivin expression and function in PASMCs in high pulmonary blood flow-induced PAH is limited. Voltage-dependent potassium channels (Kvs), such as Kv1.5 and Kv2.1, are involved in maintaining the membrane potential and resting cytosolic calcium concentration ($[Ca^{2+}]$) of PASMCs in normal pulmonary arteries [18, 21, 22]. Kv channels, particularly Kv1.5 and Kv2.1, in vasoconstriction and proliferation and/or the apoptosis of PASMCs contribute to the remodeling of pulmonary arterial walls via regulation of the proliferation and apoptosis of PASMCs [11].

In this study, we hypothesized that survivin is positively expressed in PASMCs and could promote proliferation and inhibit apoptosis in PAH-PASMCs in vitro through the regulation of Kv1.5 and Kv2.1, ultimately reversing pulmonary arterial remodeling in PAH induced by high pulmonary blood flow.

Materials and methods

Ethics

The animal study was conducted in accordance with the Guide to the Care and Use of Experimental Animals issued by the Ministry of Health of the People's Republic of China and was approved by the Animal and Human Ethics committees, Guangxi Medical University, China.

Animal model preparation

Forty male adult Sprague-Dawley (SD) rats weighing 180-250 g were selected and provided by the Animal Research Centre of Guangxi Medical University (Nanning, Guangxi, China; license No. SCXK 2009-0002). Ten rats were randomly assigned to the control group without any treatment. Another ten rats were assigned to the sham group; these rats were anesthetized with 10% chloral hydrate at a dose of 2-4 ml/kg, followed by opening of the abdominal cavity, exposure of the abdominal aorta (AA) and inferior vena cava (IVC), and blockage of AA blood flow with a vascular clamp. After 10 minutes, the vascular clamp was released, and the abdominal cavity was closed. The remaining 20 rats were randomly assigned to either the Shunt group (n = 10) or the Shunt+siRNA group (n = 10), in which we created an AA to inferior vena cava shunt model to mimic the high pulmonary arterial blood flow state of a congenital heart defect, as previously described [5, 12, 23, 24].

After 11 weeks, the rats were sacrificed for further experimentation; alternatively, rats were scarified when they exhibited symptoms of 15% weight loss, dyspnea, and lethargy [25]. The rats in the Shunt and Shunt+siRNA groups were checked for a fistulous tract between the AA and IVC by color Doppler blood flow imaging. Three rats in the Shunt group and two in the Shunt+siRNA group failed to exhibit establishment of the left-to-right shunt model due to the closure of the fistulous tract from the AA to the IVC and were eliminated, whereas the other rats in these two groups exhibited shunt fistulous tract patency, a high blood flow speed, and a high pressure differential between the AA and IVC. During the 11-week period, none of the rats in the Control group died, one rat died in the Sham group, three rats died in the Shunt group, and two rats died in the Shunt+siRNA group. Therefore, the replacement rats were supplemented at the same surgery and raised under the same conditions to achieve ten rats in each group according to the study protocol.

Measurements of pulmonary arterial pressure and RV hypertrophy

The pulmonary arterial pressure was measured, and the right ventricle (RV) and left ventricle (LV) with the septum (S) were isolated and weighed after water was absorbed with filter paper. The weight ratio of the RV to the LV with S was measured as previously described [24, 26].

HE staining and immunohistochemistry

The left lung was excised, fixed in 10% formalin, embedded in paraffin, and cut into 5-μm sec-

tions. Sections were stained with hematoxylin-eosin (HE) as previously described [6].

Cell preparation and culture

Primary PASMCs were isolated from the pulmonary arteries of the right lung in this study. Right lung tissue was excised from each rat in each group and immediately placed in cold (4°C) phosphate-buffered saline (PBS) and dissected using a dissecting microscope (10×). Peripheral muscular pulmonary arteries (diameter range, 300-500 μm) were isolated from the rat lung tissues. The adventitia was stripped, and the endothelium was removed. The smooth muscle tissue was cut into small explants (0.1-0.5 mm) and planted on 25-cm^2 cell culture flasks, which were incubated at a humidified atmosphere of 5% CO_2 and 95% atmosphere at 37°C with 20% FBS high-glucose DMEM culture medium and 50 μg/ml penicillin/streptomycin. The culture medium was changed every 3 days. PASMCs were cultured for 10-14 days and then subcultured for 5-7 days. The PASMCs were harvested for subsequent experiments after passage for 5-10 generations of culture. The cultural cells were identified as PASMCs with an α-smooth muscle actin antibody through immunocytochemistry. The morphology of the cells was examined using an inverted phase contrast microscope attached to a digital camera.

Small interfering RNA lentivirus construction and infection

In the Shunt+siRNA group, PASMCs received siRNA-survivin lentivirus transfection for knockdown of survivin expression. We cloned the siRNA sequence (5'-ATGTGTAGGTGTTGGTTTA-3') into a lentiviral vector. A non-targeting siRNA negative control (5'-TTCTCCGAACGTGTCACGT-3') was inserted into a separate lentivirus vector. Based on the coding region of survivin, the DNA sequences were synthesized (SHANGHAI GENECHEM CO., LTD, China) as follows: 5'-CCATGTGTAGGTGTTGGTTTACTCGAGTAAACCAACACCTACACATGG-3'.

PASMCs were infected with lentiviruses in the presence of polybrene (10 μg/ml) for 24 h in a 6-well plate at a density of 1×104 cells/well, washed after 24 h by a medium change, and harvested for further culture or experimentation following the manufacturer's instructions.

Quantitative reverse transcription real-time polymerase chain reaction

Total RNA was extracted from primary PASMCs by using a Trizol Kit according to the manufacturer's instructions. Total RNA concentrations were determined by the ultramicro nuclear acid analyzer. RNA integrity was assessed by visual inspection of ethidium bromide-stained agarose gels. Genomic DNA was removed with RNase-free DNase. Reverse transcription was performed for 10 min at 25°C, 30 min at 42°C, and 5 min at 85°C, according to the manufacturer's instructions. For quantitative real-time polymerase chain reaction (qRT-PCR), sense and antisense primers were designed using the Primer 3 program for survivin, voltage-gated potassium channel (Kv1.5 and Kv2.1), and GAPDH. To avoid inappropriate amplification of residual genomic DNA, intron-spanning primers were selected when exon sequences were known.

Potassium voltage-gated channel 1.5 (KCNA5, Kv1.5): Sense: 5'-CCTCCGACGTCTGGACTCAATAA-3'; Antisense: 5'-CCTCATCCTCAGCAGATAGCCTTC-3'. Potassium voltage-gated channel 2.1 (KCNB1, Kv2.1): Sense: 5'-ACACGGGAGCACTAGGGATCAG-3'; Antisense: 5'-CTCAGTGGCAGCAAGCCAAG-3'. Survivin: Sense: 5'-CTTCATCCACTGCCCTACCG-3'; Antisense: 5'-CAGGGGAGTGCTTCCTATGC-3'. GAPDH: Sense: 5'-TGCTGAGTATGTCGTGGAG-3'; Antisense: 5'-GTCTTCTGAGTGGCAGTGAT-3'.

For each sample, the amplification reaction was performed in triplicate using the SYBR-GREEN PCR Master Mix (TaKaRa, Japan), specific primers, and diluted template cDNA. Analysis was performed using an Applied Biosystems R 7500 Real-Time PCR system (Thermo Fisher, USA). The cDNA samples were amplified in a DNA thermal cycler under the following conditions: the mixture was pre-denatured at 95°C (10 s) for 1 cycle, and PCR was performed at 95°C (30 s) and 60°C (60 s) for 40 cycles. GAPDH was used as an internal control to normalize the mRNA levels of survivin, Kv1.5, and Kv2.1. The relative change in expression was calculated. Means ± SEMs were calculated and plotted as column graphs for comparison.

Western blotting

Western blotting was performed using standard procedures in the present report, as fol-

lows. Total protein was extracted from PASMCs by the protein extract reagent kit (VazymeInc., Nanjing, China) according to the manufacturer's instructions. The concentration of total protein was determined using the Bradford protein assay before gel electrophoresis. Proteins were transferred to a nitrocellulose membrane by semidry electroblotting for 1 h. The membranes were blocked with 5% non-fat milk in 0.1% Tween-TBS for 1 h at room temperature and incubated overnight at 4°C with a primary goat rabbit anti-rat survivin antibody (ZSGB Inc., Beijing, China) and rabbit anti-rat Kv1.5 and Kv2.1 antibodies (Biolead Inc., Beijing, China). Bound antibody was detected with a secondary goat anti-rabbit antibody (Longislandbio Inc., Shanghai, China) and visualized using an electrochemiluminescence (ECL) system according to the manufacturer's directions. Protein levels were quantified by laser densitometry, and relative quantification was performed by normalization with GAPDH (Vazyme Inc., Nanjing, China). Protein expression levels are presented in arbitrary units (Thermo Fisher Scientific Co., Ltd., Shanghai, China).

Cell proliferation analysis

PASMCs were seeded into 96-well plates at approximately 5000 cells in 100 μl of medium per well and incubated for 24 h in culture medium. After 24 h, 10 μl of CCK8 solution was added to each well and incubated for 2 h in the absence of light. Cell viability was then measured with the CCK-8 cell count kit following the manufacturer's instructions as previously described [27], and experiments were repeated twice. The absorbance of the solubilized product at 450 nm was measured using an ELISA reader (BIO-TEK, USA).

Cell apoptosis analysis

The annexin/PI apoptosis detection kit was used for flow cytometry experiments to detect apoptotic cells according to the manufacturer's instructions. PASMCs were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×106 cells/ml. The cell suspension (100 μl, ~1×105 cells) was transferred to a 5-ml culture tube and mixed with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI). The cells were gently vortexed and incubated for 15 min at room temperature (20-25°C) in the dark. Subsequently, 400 μl of 1× binding buffer was added to each tube and analyzed by fluorescence-activated cell sorting (with a FACS Calibur system) using CellQuest software (Becon Dickinson, Mountain View, CA).

Enzyme-linked immunosorbent assay

Caspase-3 and caspase-9 concentrations in the supernatant of cultured PASMCs were examined with Quantikine rat caspase-3 and caspase-9 enzyme-linked immunosorbent assay (ELISA) kits (USA), respectively, according to the manufacturer's instructions. Caspase-3 and caspase-9 concentrations were obtained by referring to a standard curve obtained in parallel. The results represent the mean value of two separate measurements performed in duplicate and are expressed as pg/ml, according to the instructions of the ELISA kit. All experiments were performed three times.

Data analysis

All data are presented as the means ± standard deviations. The data were analyzed using the unpaired Student's t test. Statistical analysis was performed using SPSS 19.0 software, and $P<0.05$ was considered statistically significant.

Results

Successful establishment of a shunt-PAH rat model and the hemodynamic changes

Eleven weeks after undergoing shunt surgery in the Shunt and Shunt+siRNA groups, enlargement and pulsation of the IVC and an approximately 1.94±0.16-mm anechoic tubular tract between the AA and IVC verified the shunt fistulous tract patency of rats in the PAH and Shunt+siRNA groups, as determined by two-dimensional ultrasonography. The closure of the fistulous tract was observed in three rats in the Shunt group and two in the Shunt+siRNA group. Under color Doppler flow imaging, multicolored shunt blood flow signals were observed from the AA to IVC. Spectrum Doppler explored the continuity of the shunt blood flow signals, and the flow velocity was 0.81±0.15 m/s. All of the results suggested the successful establishment of shunt fistulous tracts from the AA to IVC (**Figure 1A**).

The mean pulmonary artery pressure (mPAP) and pulmonary arterial systolic pressure (PASP) were measured. The mPAP and PASP were sig-

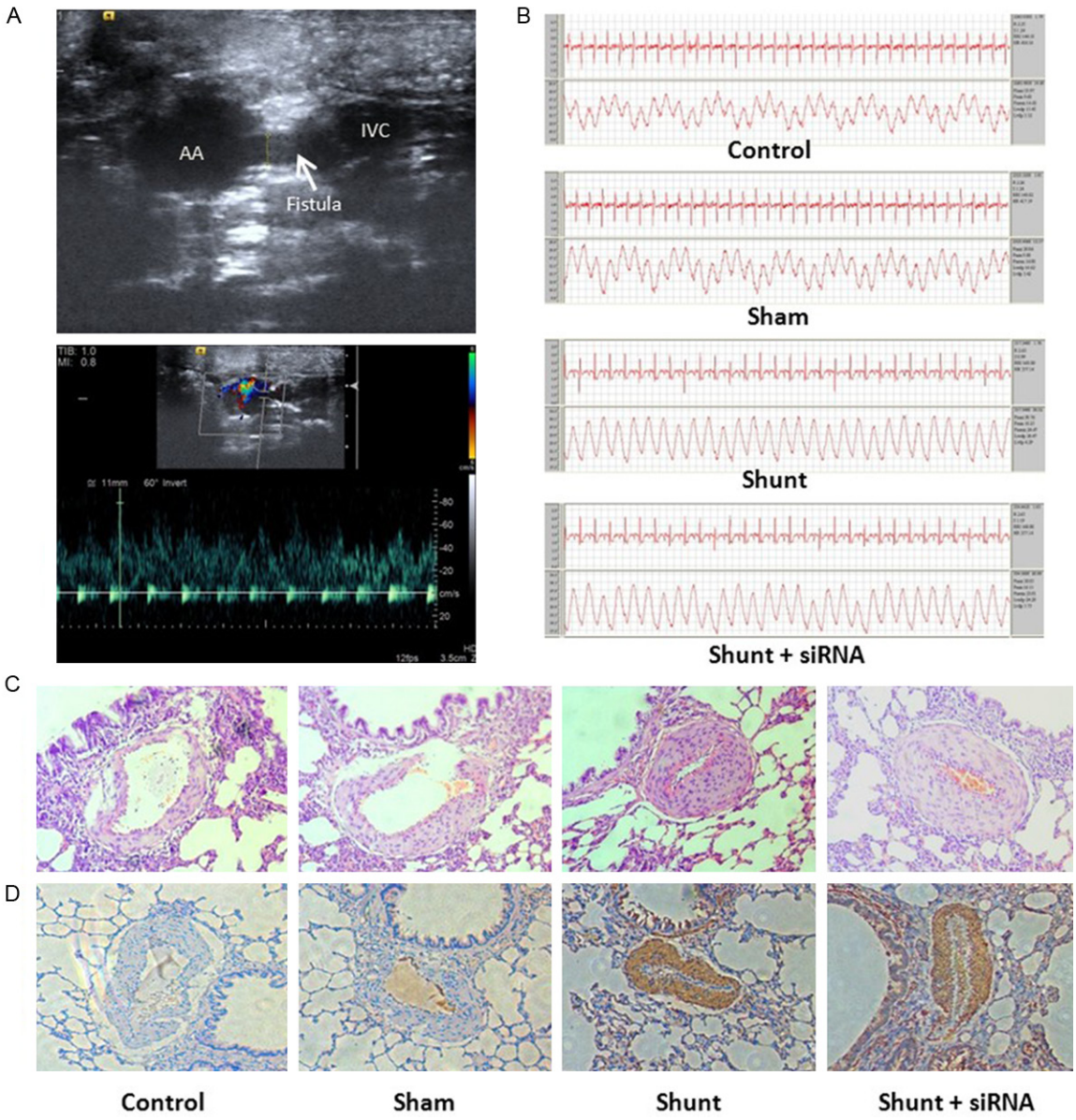


Figure 1. Establishment of high pulmonary blood flow-induced PAH rat model. A. Shunting between abdominal and inferior vena cava was confirmed by two-dimensional ultrasonography and blood flow velocity was measured by spectrum Doppler. B. Pulmonary arterial pressure curve and the corresponding Electrocardiograph. C. Hematoxylin-eosin (HE) staining of pulmonary artery in Shunt group and Shunt+siRNA group, presenting with narrower lumen, thicker vascular wall. D. Survivin expressed in the pulmonary arteries of the Shunt and Shunt+siRNA group instead of the Control and Sham group using immunohistochemistry, which was mainly located in muscular layer of the pulmonary arteries.

nificantly higher in the Shunt and Shunt+siRNA groups compared with the Control and Sham groups ($P<0.05$). However, no difference was observed in the mPAP or PASP between the Control and Sham groups ($P>0.05$) or between the Shunt and Shunt+siRNA groups ($P>0.05$). During the study period, the RV was significantly thicker and the ratio of the RV to the LV+S was significantly higher in the Shunt and Shunt+siRNA groups compared with the Control and Sham groups ($P<0.05$). No significant differences between the Control and Sham groups in RV/LV+S were observed. The above results indicated that the left-to-right shunt rat models had been successfully established (**Table 1**; **Figure 1B**).

Table 1. Effect of shunt surgery on hemodynamics and RV/LV+S

Group	mPAP (mmHg)	PASP (mmHg)	RV/LV+S (g/g)
Control	18.04±2.43	23.89±3.24	0.20±0.012
Sham	20.18±2.72	22.21±2.65	0.21±0.065
Shunt	25.35±2.38*,#	32.77±3.83*,#	0.25±0.043*,#
Shunt+siRNA	24.01±3.81*,#	30.62±3.16*,#	0.26±0.059*,#

The data were presented as mean ± SD (n = 10). *P<0.05 compared with the Control group, #P<0.05 compared with the Sham group.

Pulmonary arterial remodeling after shunt operation

Stenosed lumens and thicker vascular walls of the pulmonary arteries and muscular arteries in the rat lung tissues were demonstrated by hematoxylin and eosin (HE) staining of the lung tissue sections in the Shunt and Shunt+siRNA groups. The Sham group rats exhibited similar pulmonary vascular histologies relative to the Control group (**Figure 1C**). To study survivin expression in the remodeled pulmonary vasculature, we measured the pulmonary expression of survivin by immunohistochemistry. Robust staining in the media smooth muscle cells, muscularized vessels, and lumen-occluding lesions was found in the Shunt and Shunt+siRNA groups. However, in the Control and Sham groups, no staining was found in the pulmonary arterial vessel wall. Therefore, survivin expression was observed in the Shunt and Shunt+ siRNA groups according to immunohistochemical staining. However, little expression of survivin was found in the Control and Sham groups (**Figure 1D**).

Pulmonary arterial smooth muscle cell culture

After 5-7 days of culture of pulmonary arterial explants, fusiform and irregular-shaped cells were observed to be growing around these explants (**Figure 2A1**). Immunocytochemistry results with α-smooth muscle actin antibody were positive based on the specific myofilament structure of PASMCs (**Figure 2C**). The PASMCs in the siRNA-survivin group were successfully transfected with siRNA-survivin after 3 generations of culture and continued to grow with the other groups under the same culture conditions (**Figure 2B**). After passage for 5-10 generations, PASMCs fully satisfied further experimental requirements and were harvested (**Figure 2A2**).

Positive survivin mRNA expression in PAH-PASMCs and the down-regulation of Kv1.5 and Kv2.1 mRNA expression

To identify whether survivin was expressed in the Shunt PASMCs, PASMCs from each rat were cultured to extract total RNA. Quantitative RT-PCR revealed that survivin was positively expressed in the PASMCs of the Shunt group but was not expressed in the PASMCs of the Control or Sham groups (P<0.01). These results were consistent with immunohistochemistry findings. Our results demonstrated that survivin levels were markedly increased in the PAH rat model but not in the Control or Sham groups of rats. In the Shunt+siRNA group, siRNA-survivin successfully interfered with survivin mRNA expression in the PASMCs, and the level of survivin mRNA expression decreased significantly relative to that in the PASMCs of the PAH group. These findings indicated that siRNA-survivin had an inhibitory effect on survivin mRNA expression in PAH-PASMCs and that survivin is a major factor in the remodeling of the pulmonary vasculature (**Figure 3A**).

Finally, the Kv1.5 and Kv2.1 mRNA expression levels were measured in all groups. No significant difference was observed between the Control and Sham groups. However, the PASMCs in the PAH group exhibited significantly lower Kv1.5 mRNA levels compared with those inthe control and sham groups (P<0.05). Importantly, Kv1.5 mRNA levels in PASMCs were significantly increased after interference with siRNA-survivin, which confirmed that the stable knockdown of survivin mRNA levels is correlated with the significant up-regulation of Kv1.5 and Kv2.1 expression. However, in the negative control non-targeting siRNA group, no significant difference in survivin mRNA expression was observed (P>0.05). However, a significant difference in survivin mRNA expression was detected between siRNA-survivin interfering PAH-PASMCs and non-targeting siRNA interfering PAH-PASMCs in the siRNA-survivin PAH group (P<0.05) (**Figure 3A**).

Survivin protein expression and regulation of Kv1.5 and Kv2.1 protein expression

After the PASMCs in each group grew sufficiently for total protein extraction, survivin protein expression was detected by Western blot analysis. The survivin protein was positively expre-

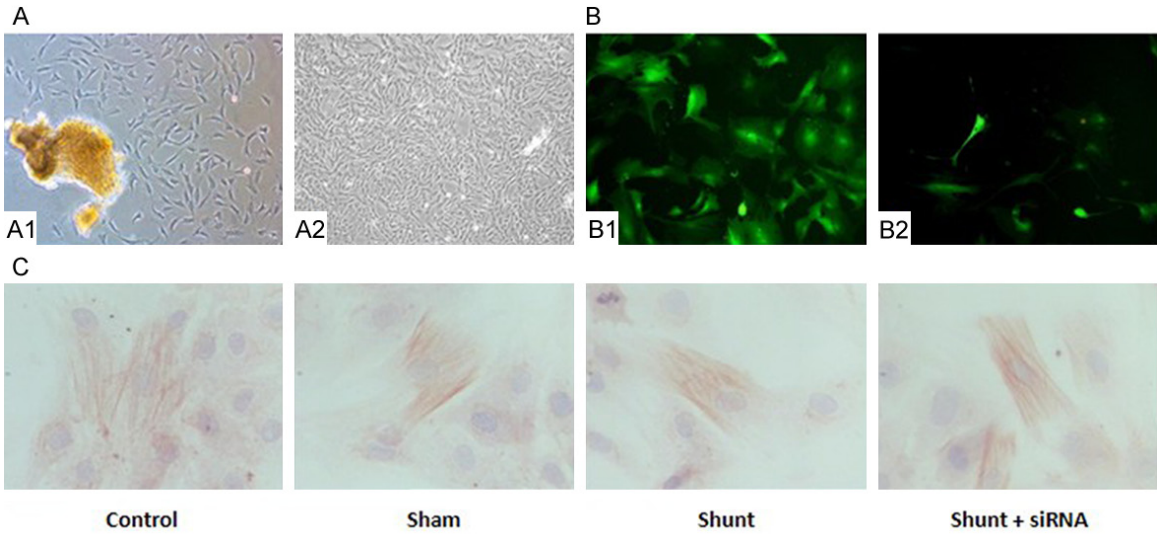


Figure 2. Culture, transfection and identification of PASMCs. A1. Primary cultured PASMCs. A2. PASMCs of vigorous growth. B1. Lentivirus transfection of survivin negative control non-targeting siRNA. B2. lentivirus transfection of siRNA-survivin. C. Specific marks of PASMCs, α-smooth muscle actin positive expressed in each group.

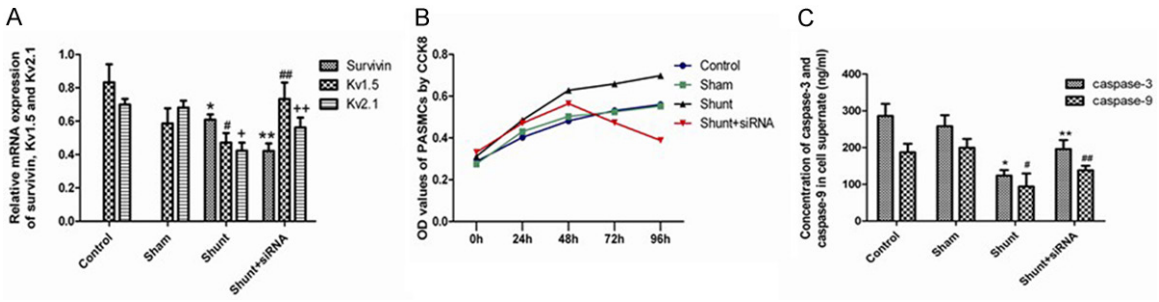


Figure 3. Relative mRNA expression, PASMCs proliferation curve and concentration of Caspase-3, caspase-9 in PASMC supernatant. A. Relative expression of survivin, Kv1.5 and Kv2.1 mRNA in each groups by qRT-PCR. No expression of survivin mRNA in the Control group and the Sham group, survivin mRNA expression decrease in the Shunt+siRNA group compared with the Shunt group (*P<0.05). B. PASMC growth curve on 0-, 24-, 48-, 72-, 96 h after 24 h of seeded into plates. C. The concentration of caspase-3 and caspase-9 in PASMCs supernatant. *P<0.05, #P<0.05 vs. the Control and Sham group, respectively. **P<0.05, ##p<0.05 vs. the Shunt group.

ssed in PAH-PASMCs but was barely expressed in the PASMCs of the control or sham-operated groups at any time point according to the Western blot analysis. These results are consistent with the PASMC survivin protein expression levels assessed by immunohistochemistry.

The relative optical density (OD) value of the survivin protein expression was 1.509±0.231 in the PASMCs of the Shunt group. In the Shunt+siRNA group, after siRNA-survivin transfection, the relative OD value of the survivin protein expression was 0.845±0.193, whereas that of the negative control siRNA interference was 1.675±0.357 (**Figure 4A**). Meanwhile, regarding the PASMCs in the Control and Sham groups, the relative OD values of the Kv1.5 protein expression were 0.792±0.181 and 0.863±0.162, respectively, and the expression of Kv2.1 was 0.708±0.146 and 0.635±0.172, respectively. Regarding the PAH-PASMCs, the relative OD values of the Kv1.5 and Kv2.1 protein expression were 0.492±0.148 and 0.115±0.051, respectively. The relative OD values of the Kv1.5 and Kv2.1 protein expression were 0.665±0.151 and 0.257±0.097, respectively, after siRNA-survivin interference (**Figure 4B**). All of the results indicated that the protein expression exhibited a significant decrease in the Shunt group compared with the control and Sham groups (P<0.05) and that the Kv1.5 and

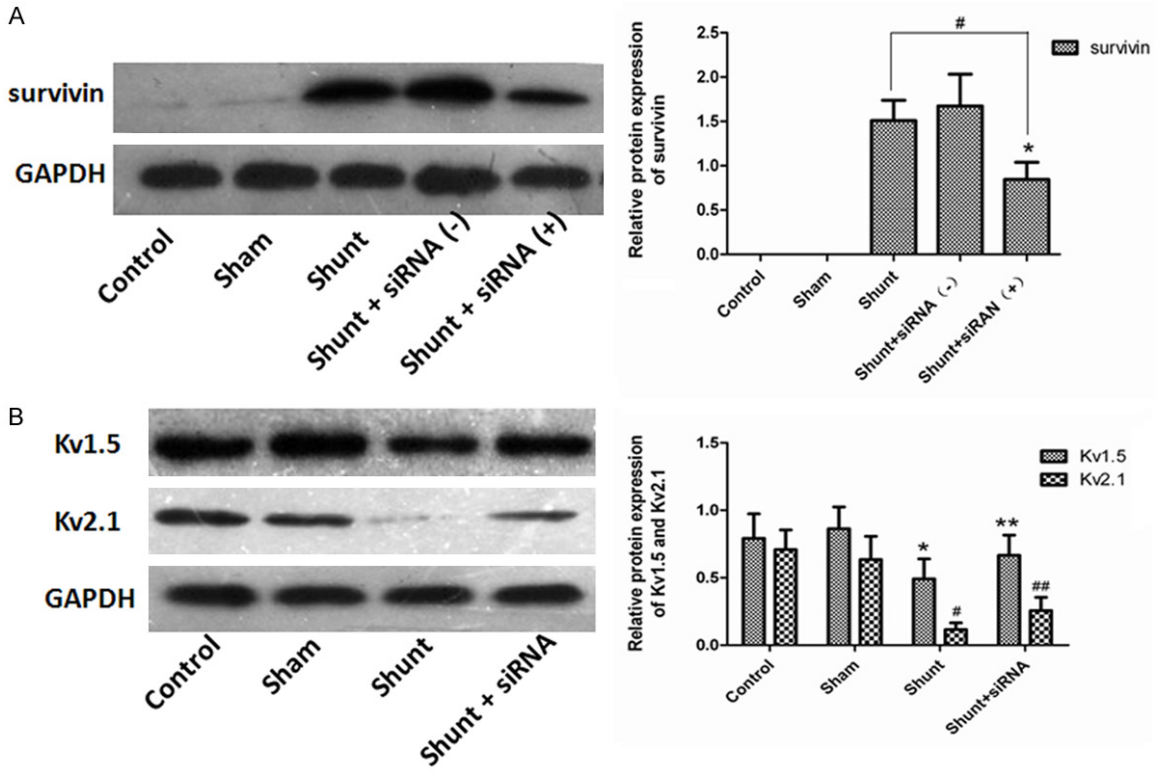


Figure 4. Relative protein expression of survivin, Kv1.5 and Kv2.1. (A) survivin was not expressed in the Control and Sham group. Survivin protein expression increased in the Shunt group. Survivin was significantly inhibited in the Shunt+siRNA group compared with the Shunt group (P>0.05). No obvious difference between the Shunt+siRNA group and the negative siRNA transfection group (B) Relative protein expression of Kv1.5 and Kv2.1 in each group. No significant difference in the Control group and the Sham group (P>0.05). In the Shunt group, Kv1.5 and Kv2.1 protein expression decreased compared with the Control group and the Sham group, respectively (*P<0.05, #P<0.05). Kv1.5 and Kv2.1 protein expression increased in the Shunt+siRNA group compared with the Shunt group (##P<0.05, ++P<0.05).

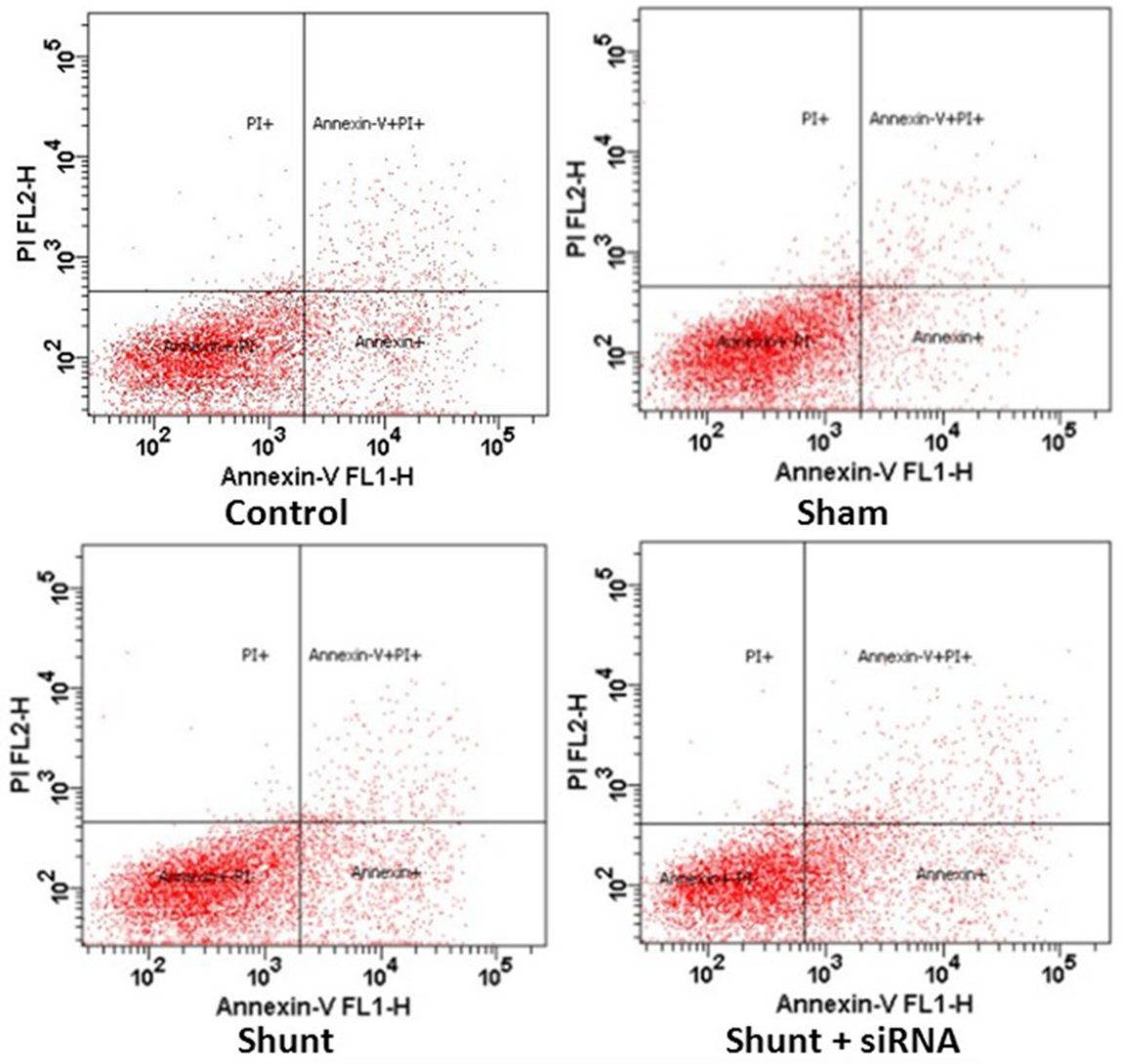
Kv2.1 protein expression was significantly increased after siRNA-survivin interference in the Shunt+siRNA group compared with the Shunt group (P<0.05).

PASMC proliferation

PASMC proliferation in the four groups was assessed using the CCK-8 Cell Counting kit. The PASMC proliferation rate in the Shunt group was significantly increased compared with that in the Control and Sham groups at different time points. The optical density (OD) value of the PASMCs in the Shunt group at the 0-h time point was 0.312±0.081, whereas the OD values of the PASMCs in the control and Sham groups were 0.288±0.062 and 0.276±0.055, respectively. In the Shunt+siRNA group, following the interference of the survivin expression in the PASMCs, the OD values at the 0-, 24-, 48-, 72-, and 96-h time points were 0.332±0.093, 0.471±0.129, 0.564±0.103, 0.473±0.079, and 0.388±0.072, respectively. The inhibitory effect on the PASMCs in the Shunt+siRNA group was observed 48 h after infection; specifically, the PASMC proliferation rate was decreased relative to that in the Shunt group (P<0.05). Together, these results demonstrate that survivin is most closely associated with the promotion of PASMC proliferation in our PAH model (**Figure 3B**).

Survivin inhibits PASMC apoptosis

We next investigated the effect of survivin on PASMC apoptosis. To assess whether the inhibitory effect of survivin expression led to an increase in apoptosis in PAH-PASMCs, apoptosis was measured in the PASMCs in the Shunt+siRNA group after siRNA-survivin interference. We observed an increase in apoptosis in these PASMCs relative to that in the PASMCs in the Shunt group. The specific apoptosis of PASMCs on day 14 was evaluated as 7.98±



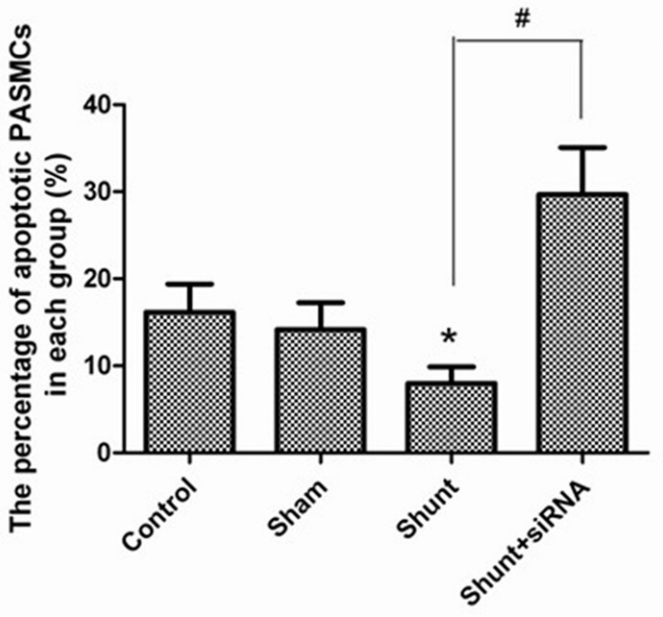


Figure 5. The percentage of apoptotic PASMC in each group by flow cytometry. Apoptotic percentage of PASMC decreased compared in the Shunt group compared with the control group and the Sham group, respectively (*P<0.05). In the Shunt+siRNA group, the percentage of apoptotic PASMC increased compared with the Shunt group (#P<0.05).

1.90% in the Shunt group and 29.69±5.40% in the Shunt+siRNA group. The PASMC apoptosis in the Shunt group was calculated relative to the Control group ($P<0.05$) and the Sham group ($P<0.05$). These results indicate that siRNA-survivin causes an increase in the spontaneous apoptosis rate of PAH-PASMCs in vitro (**Figure 5**).

Survivin induced apoptosis by increasing caspase-3 and caspase-9 levels

To further characterize the mechanisms of apoptosis, the supernatant levels of caspase-3 and caspase-9 were examined. There was a significant decrease in caspase-3 and caspase-9 levels in the supernatants of PASMCs in the Shunt group compared with the Control group ($P<0.05$) and the Sham group ($P<0.05$). Obvious difference of caspase3 and caspase-9 levels was observed between the Shunt+siRNA group and the Control group ($P<0.05$). These data indicate that the caspase pathway of apoptosis participates in survivin-induced apoptosis. Therefore, survivin down-regulated caspase-3 and caspase-9 expression (**Figure 3C**).

Discussion

This study is the first demonstration that survivin is positively expressed by PASMCs and that it inhibits PASMC apoptosis and promotes PASMC proliferation in a high pulmonary blood flow-induced PAH rat model. This study revealed that the potential pathway through which survivin regulates shunt-PAH PASMCs is the inhibition of Kv1.5 and Kv2.1 expression and the further inhibition of caspase-3 and caspase-9 activity.

The etiologies of PAH are varied, and the pathogenesis is complex [28]. Human PAH is related to an increase in pulmonary vascular resistance of unknown cause that impedes the ejection of blood by the RV [10]. Pulmonary vascular structural remodeling is the major pathological basis of high pulmonary blood flow-induced PAH, characterized by medial hypertrophy, the muscularization of small peripheral arteries, and the increased deposition of extracellular matrix components [29]. Pulmonary arterial medial hypertrophy [1] and plexiform damage are the main characteristics of PAH attributed to congenital heart disease, and the neointima is thin [23, 30], which is consistent with our study. Endothelial dysfunction and inflammation are recognized as some of the earliest abnormalities in PAH, resulting in a recognized imbalance of endothelium-derived vasoactive factors, with an increase in vasoconstrictors in MCT-PAH [8]. Flow-induced PAH reduces the biological activity of NO and the production of cGMP in the PA wall [23]. Increased pulmonary blood flow may increase pulmonary pressure and promote medial hypertrophy [5]. The histological criterion of medial hypertrophy is fulfilled when the diameter of a single medial layer, delineated by its internal and external elastic lamina, exceeds 10% of the artery's cross-sectional diameter [31]. The isolated hypertrophy of the medial layer may be considered an early and even reversible event [31]. The redistribution of blood flow in the left-to-right shunt rat model led to irregular flow, swirling flow, reciprocating flow, turbulent flow, and blood flow recirculation. These hemodynamic changes can lead to vessel bed enlargement, increased pulmonary blood, vessel stenosis or obstruction, and hemodynamic forces. However, these changes can also result in specific vascular and cellular responses to the remodeling of the vascular wall. Systemic-to-pulmonary artery shunting reduces coronary blood flow, which may precipitate myocardial ischemia [32].

An understanding of the pathological vascular anatomy is indispensable for the successful research of the clinical, biological, and pharmaceutical aspects of pulmonary hypertension [31]. No animal model can completely replicate the pathogenesis and histopathological progression of human PAH. Our establishment of a high pulmonary blood flow-induced PAH rat model was an ideal model with which to study PAH as a consequence of congenital left-to-right shunt heart diseases because of its ability to mimic human PAH disease progression and lead to right ventricular dysfunction and death [2]. In our rat shunt-PAH model, the increased mPAP, thickened RV, and thickened pulmonary arterial middle layer were significantly increased compared with those of the control and sham-operated groups. Significantly thickened medial formation was observed according to HE staining, which is a distinct characteristic of shunt-PAH. These findings demonstrate the successful establishment of the shunt-PAH rat model.

Our work primarily focused on high pulmonary blood flow-induced PAH PASMCs and explored the potential mechanism of the regulation of the unbalance between proliferation and apoptosis in PAH-PASMCs. Survivin is a member of the inhibitor of apoptosis protein family, which is a key regulator of cell death and mitosis and is up-regulated in many malignant cancers. Anti-survivin antibodies have been detected in the serum of patients with lung and colorectal cancer [33]. Survivin is expressed at high levels or is overexpressed in other experimental animal models of PAH and is an important protein in the pathogenesis of PAH [8]. Survivin is also expressed in patients with left-to-right shunt congenital heart disease [34]; however, survivin function and its mechanism of regulation in PAH induced by high pulmonary blood flow have not yet been elucidated. In our study, survivin gene expression and protein expression in shunt-PAH PASMCs were observed by quantitative RT-PCR and confirmed by Western blotting, whereas survivin expression was undetectable in the PASMCs in the control and sham-operated groups. This change was not seen in PAH-PASMCs transfected with a negative control siRNA. High survivin expression has also been positively associated with the progression and severity of pulmonary arterial remodeling [20] and was correlated with poor prognosis. To study survivin function further, a siRNA against survivin was constructed to suppress survivin expression in PASMCs associated with shunt-PAH. After the interference of siRNA-survivin, both survivin mRNA and protein levels were significantly decreased in PAH-PASMCs. Western blot analysis after siRNA-survivin in PAH-PASMCs confirmed the siRNA-mediated interference of survivin with a 20% reduction in protein levels. The role of survivin may be to increase vascular wall thickness, distal non-muscular artery muscularization, and pulmonary vascular stenosis. The results confirmed that the targeted treatment with survivin could prevent the proliferation and induce apoptosis of PASMCs to modify pulmonary vascular remodeling. Survivin may be involved early during malignant transformation or following disturbances in the balance between cell proliferation and death.

We next explored whether siRNA-survivin interference in PAH-PASMCs would have deleterious effects on the viability of PAH-PASMCs. PASMCs treated with siRNA-survivin consistently exhibited a significant reduction in viability of approximately 20%. Our results indicate that survivin plays a major role in the regulation of cell survival and the inhibition of cell apoptosis in PASMCs. Survivin also promotes cell survival by interfering with cell cycle-related kinases and microtubule networks. Survivin inhibits cell apoptosis, promotes cell transformation, and participates in cell division, vascularization, and the generation of tumor cell drug resistance. Survivin enters cells under the stimulation of the Fas gene and cell proliferation, where it binds cyclin CDK4, leading to CDK2/cyclin E activation and Rb phosphorylation. These processes promote DNA replication and shorten the G1/S phase [35]. The formation of the survivin/CDK4 complex allows p21 wafl/cipl to be released from the p21 wafl/cipl-CDK4 complex. The released P21 wafl/cipl then interacts with procaspase-3 to prime the inactivation of caspase-3, thereby inhibiting Fas-mediated apoptosis. After the interference of survivin gene expression, PASMCs in the high pulmonary blood flow-induced PAH model exhibited inhibited proliferation. These results demonstrate that siRNA-mediated inhibition of survivin expression can further inhibit the proliferative activity of PASMCs.

Previous studies have demonstrated that apoptosis of PASMCs is necessary for the regression of pulmonary vascular changes and PAH. Survivin plays a role in mitotic progression, and the loss of survivin can lead to cell death through mitotic catastrophe. Therefore, inhibition of survivin expression causes cell cycle arrest of PASMCs in the G2 phase and apoptosis and may have anti-proliferative and pro-apoptotic effects in high pulmonary blood flow-induced PAH.

We explored the survivin regulatory pathway in high pulmonary blood flow-induced PAH. After PASMCs were subjected to siRNA interference of survivin, Kv1.5 and Kv2.1 mRNA and protein expression levels were up-regulated in PAH-PASMCs. Kv1.5 and Kv2.1 are associated with PASMC proliferation and vascular remodeling in a variety of animal models, including the chronic hypoxia PAH rat model [36]. In MCT-induced PAH rats, Kv1.5 and Kv2.1 channel protein up-regulation was observed, which may represent a protective mechanism to compensate for increased RVSP [37]. Inhibition of survivin expression can increase Kv channels, thereby inhibiting PAH-PASMC proliferation and resistance to apoptosis in the distal pulmonary

artery remodeling process [8]. Survivin inhibitsKv1.5 and Kv2.1, opens voltage-gated calcium channels and increases intracellular calcium influx, and causes PASMC contraction and proliferation. The down-regulation of the Kv1.5 and Kv2.1 channels was attributed to the development of PAH by promoting pulmonary arterial vasoconstriction and remodeling.

To further explore the mechanism by which survivin inhibits apoptosis, we measured the protein levels of caspase-3 and caspase-9 in the culture medium of PASMCs. The result further confirmed that survivin caused apoptotic inhibition by reducing the expression of potassium channel expression, inhibiting pro-caspase activity, reducing the levels of caspase-3 and caspase-9, inhibiting the mitochondrial release of caspases to the cytoplasm, and inhibiting the exogenous apoptotic pathway; thus, survivin inhibits caspase-mediated apoptosis. The activation of caspase-3 and caspase-9 is due to the leakage of cytochrome C out of the mitochondria into the cytosol, suggesting the activation of mitochondria-dependent apoptosis [38]. Survivin serves as the direct repressor of caspase-3 and caspase-9 and blocks cell apoptosis [39]. Caspases transduce apoptosis signals or directly serve as apoptosis effector molecules by hydrolyzing their substrates to promote cytoskeleton degradation and DNA fragmentation.

Together, these results indicate that survivin down-regulated the expression of Kv1.5 and Kv2.1 in the membranes of PASMCs in the pulmonary arteries, thereby promoting pulmonary hypertension and pulmonary vascular structural remodeling in shunt-PAH. Similar to cancer tissues, survivin was not expressed in differentiated normal tissue. Thus, we have identified a useful molecule that is differentially expressed in PAH and normal lung tissue that may benefit novel therapeutic approaches through the regulation of PASMCs [6] and may represent a new therapeutic measure for the treatment of high pulmonary blood flow-induced PAH.

Study limitations

Our study failed to design an in vivo experiment or to explore the relationship between mPAP and survivin gene and protein expression levels at different time points in the experimental model of high pulmonary blood flow-induced PAH. Further studies should explore the effect of survivin on proliferation and apoptosis using human PAH-PASMCs from congenital heart disease tissues.

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Disclosure of conflict of interest

None.

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