Synemin promotes pulmonary artery smooth muscle cell phenotypic switch in shunt-induced pulmonary arterial hypertension

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Abstract

Aims Although considerable progress has been made in the diagnosis and treatment of congenital heart disease-associated pulmonary heart hypertension (CHD-PAH), the clinical prognosis and overall survival of patients with CHD-PAH remain poor. Therefore, the molecular pathogenesis of CHD-PAH requires further investigation. The intermediate filament protein synemin (SYN) is reported to modulate phenotypic alterations and varicose vein development, but there is little understanding of its exact functions in CHD-PAH.

Methods and results SYN expression in the pulmonary arterioles of CHD-PAH patients and shunt-induced PAH rat models was evaluated using immunohistochemistry and western blot. Cell counts and Transwell migration assays were used to assess the effect of SYN on the proliferation and migration capability of human pulmonary smooth muscle cells (hPASMCs). Adeno-associated viruses (AAVs) have been used to suppress SYN expression in the pulmonary arterioles of rats. Such rats were further used to construct a shunt-induced PAH animal model to investigate the function of SYN in PAH and pulmonary vascular remodelling. Compared with the normal control group, SYN expression was found to be clearly up-regulated in the remodelled pulmonary arterioles of CHD-PAH and shunt-induced PAH rat models. In addition, SYN suppression increased the expression of hPASMC contractile-phenotype markers and decreased the expression of synthetic phenotype markers, in contrast to the control group. SYN suppression also dramatically attenuated the proliferation and migration capability of hPASMCs. Conversely, SYN overexpression promoted phenotypic switch, proliferation, and migration of hPASMCs, whereas these effects were notably alleviated by the protein kinase B (AKT) inhibitor MK-2206. Furthermore, we confirmed that SYN suppression mitigated PAH and pulmonary vascular remodelling induced by high blood flow in vivo.

Conclusions Our findings indicated that SYN may represent a promising therapeutic target in the treatment of CHD-PAH.

Keywords Congenital heart disease (CHD); Pulmonary heart hypertension (PAH); Pulmonary smooth muscle cells (PASMCs); Synemin (SYN)

Introduction

Pulmonary arterial hypertension (PAH) is a life-threatening vascular disease characterized by elevated pulmonary arterial pressure and increased pulmonary vascular resistance, eventually contributing to right ventricular dysfunction and death.1 Sustained pulmonary vasoconstriction and vascular remodelling are regarded as the most important pathological changes in PAH, and vascular remodelling is mainly caused by pulmonary arterial smooth muscle cells (PASMCs).2,3 It is well documented that PASMCs display plasticity, modulating their phenotype from contractile to proliferative in PAH.4,5 Phenotype-switched PASMCs always exhibit increased hyperproliferation and migration, characterized by decreased expression of contractile-phenotype marker proteins, such as alpha-smooth muscle actin (α-SMA) and cardiac myosin...
heavy chain (SM-MHC), and by increased expression of synthetic-phenotype marker proteins, such as c-MYC, proliferating cell nuclear antigen (PCNA), and others.\(^6,7\) Despite recent advances in the understanding and treatment of PAH which may stabilize or decelerate disease progression, morbidity and mortality rates of this disease continue to rise.\(^8\) PAH is a common post-operative complication in patients with congenital heart disease (CHD).\(^9\) Reportedly, data from a national CHD registry and a cohort of tertiary European CHD centres show that the overall prevalence of PAH in adult CHD patients is 4–28% and that of Eisenmenger’s syndrome is 1–6%.\(^10,11\) However, the precise molecular mechanisms underlying pulmonary vascular remodelling in CHD-PAH, including treatment targets, remain obscure. Identification of the factors involved in pulmonary vascular remodelling may help block or reverse the progression of CHD-PAH.

Synemin (SYN), a unique cytoplasmic intermediate filament protein, has been identified in all types of muscle cells, many non-muscle cells, and certain types of cancer cells.\(^12\) SYN mediates the association of desmin with Z-disks and stabilizes junctional complexes in the sarcolemma.\(^13,14\) Furthermore, it also functions as an A-kinase anchoring protein (AKAP), which implies that it may modulate the activation of certain signaling cascades, such as those controlled by protein kinase B (AKT), type II regulatory (R) subunit \(\alpha\) of protein kinase A (PKA-RII), and extracellular signal-regulated kinases (ERK) 1/2.\(^15,16\) It has been confirmed that SYN positively regulates glioblastoma cell proliferation via persistent activation of AKT.\(^17\) In addition, SYN down-regulation can sharply decrease the migration and proliferation of astrocytoma cells.\(^18\) Interestingly, a previous study indicated that SYN is differentially expressed in vascular smooth muscle cells (VSMCs) isolated from varicose veins, which in turn aggravate VSMC dysfunction and varicose vein progression.\(^19\) However, the involvement of SYN in the modulation of PASMC function and pulmonary vascular remodelling in CHD-PAH has not been studied.

In the present study, the expression levels of SYN in lung tissues of CHD-PAH patients and shunt-induced PAH rat models were investigated. The effect and molecular mechanisms of SYN in phenotypic switch, proliferation, and migration of human PASMCs (hPASMCs) were investigated in vitro. Moreover, the role of SYN in PAH and pulmonary vascular remodelling induced by high blood flow in vivo was explored. These findings may provide promising therapeutic targets for CHD-PAH treatment.

### Materials and methods

#### Lung tissue collection

All clinical lung tissue samples were acquired from patients with CHD-PAH after repair surgery, as previously described.\(^20\) After a one-year follow-up, the patients were divided into two groups: reversible CHD-PAH (mean pulmonary artery pressure [mPAP] < 25 mmHg, \(n = 18\)) and irreversible CHD-PAH (mPAP ≥25 mmHg, \(n = 4\)).\(^21\) The pathological grading of pulmonary arterioles was assessed using Heath-Edwards classification system.\(^22\) Normal lung samples were obtained from patients with bronchial carcinomas. All human studies were approved by the Medical Ethics Committee of Fuwai Hospital. Written informed consent was obtained from all patients.

### Shunt-induced pulmonary heart hypertension model

Male Sprague–Dawley (SD) rats (8 weeks old, 270–300 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and used for the establishment of PAH models. All animal experiments were performed in strict accordance with the Institutional Animal Care and Use Committee (IACUC). The rats were kept in ordinary cages at a standard room temperature of 22 ± 3°C with a 12 h light cycle period. Rats were divided into the following three groups: (1) control (\(n = 6\)), (2) sham (\(n = 6\)), and (3) shunt-induced PAH (\(n = 10\)). In the PAH group, rats underwent subcutaneous injection of monocrotaline (MCT; 60 mg/kg) and abdominal aortocaval shunt (AV) surgery 1 week after injection to mimic shunt-induced PAH.\(^21,23\) Four weeks later, right heart catheterization was performed to measure the right ventricular systolic pressure (RVSP), pulmonary arterial systolic pressure (PASP), and mean pulmonary artery pressure (mPAP) using a Power Lab data acquisition system. Subsequently, lung tissues and hearts from the three groups were isolated for biochemical experiments. The right ventricle (RV) free wall, left ventricle (LV), and septum (LV + S) were separated and weighed. The right heart hypertrophy index (RVHI) was calculated using the equation RV/(LV + S) to assess right heart hypertrophy.

### Transfection with adeno-associated viruses

An adeno-associated virus (AAV) vector encoding SYN was applied to inhibit the expression of SYN in the pulmonary arterioles. After pentobarbital anaesthesia (50 mg/kg intraperitoneally), SD rats (male, 7 weeks, 240–270 g) were administered a one-time intratracheal instillation of AAVs (\(1 \times 10^{12}\) transducing units in 150 \(\mu\)L PBS) or 150 \(\mu\)L PBS. Seven days after gene transfer, some of the rats were randomly sacrificed, and lung tissues were cut into frozen slices for evaluation of transduction efficiency. The intensity of adenoviral-mediated green fluorescent protein (GFP) was used to explore its efficiency using a fluorescent confocal microscope. Next, the remaining AAV-transfected rats were
used to construct shunt-induced PAH animal models after confirmation of transduction success.

**Measurement of pulmonary arterial remodelling**

Paraffin-embedded sections were stained with haematoxylin and eosin (H&E) or elastic Van-Van Gieson (EVG) to observe the structure of the pulmonary arteries with external diameters of 25–200 μm. The ratio of vascular wall area/total vascular area (WA%) and ratio of vascular wall thickness/vascular external diameter (WT%) of the pulmonary meta were calculated.

**Immunohistochemistry**

Immunohistochemistry was performed on 4 μm-thick lung sections of paraffin-embedded tissue. After routine preparation and unmasking of antigens, the sections were incubated with specific primary antibodies (Table S1) followed by incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. The average optical density (AOD) represents the expression of SYN protein in each immunohistochemistry image. Morphological analysis in our study was performed by a blinded investigator.

**Immunofluorescent**

After blocking with normal goat serum, the sections were incubated with primary antibodies at 4°C overnight and then incubated in the dark with Alexa Fluor®-conjugated secondary antibodies (Abcam, USA) and 4′-6-diamidino-2-phenylindole (Sigma Aldrich, USA) for 1 h at room temperature. The stained sections were then observed under a confocal laser scanning microscope.

**Isolation and treatment of pulmonary smooth muscle cells**

PASMCs from the medial layer of the pulmonary arteries were acquired as previously described. The purity of the

![Figure 1](image.png)
PASMCs was confirmed by immunofluorescence staining for α-SMA or platelet endothelial cell adhesion molecule-1 (CD31) under a confocal laser scanning microscope.

**Transfection with lentivirus**

The hPASMCs at passages 2–4 were used in our study. LV-siSYN (hU6-MCS-Ubiquitin-EGFP-ires-puromycin) and LV-SYN (Ubi-MCS-3FLAG-SV40-EGFP-ires-puromycin) were used to modulate the expression of SYN in hPASMCs, and LV-siCON and LV-CON were used as controls. The knockdown or up-regulation efficiency was evaluated by western blot analysis.

**Cell proliferation assay**

Cells were plated in 12-well plates at a density of $7.5 \times 10^4$ cells per well. After 12 h, the number of cells was counted manually using a haemocytometer at ×400 magnification.

**Transwell migration assay**

Cell migration was assessed in 24-well chamber plates (Corning Inc., USA). Briefly, $2 \times 10^5$ cells suspended in 200 μL serum-free SMCM were plated in the upper compartment of the chamber, and 1100 μL SMCM with 2% FBS was added to the lower compartment of the chamber. After 36 h, the cells were fixed with formaldehyde for 90 min, stained with 0.1% crystal violet for 30 min, and washed three times with PBS. The migration of the control cells was set at 100%.

**Western blot analysis**

Proteins were prepared in radioimmunoprecipitation (RIPA) lysis buffer containing both protease and phosphatase inhibitor cocktails (Roche, Canada), and the protein concentration was determined using a Pierce bicinchoninic acid assay (BCA) protein assay kit. Proteins (50 μg) were separated on a 4–12% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel (Thermo Fisher Scientific, USA) by electrophoresis. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with 5%

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Figure 2  SYN was notably increased in remodelled pulmonary arteries of shunt-induced PAH rats. (A) Paraffin sections of lung tissues from control, sham and MCT-AV rat groups were immunohistochemically stained with anti-SYN antibody. Black arrows point SYN-positive cells. Size bars represent 20 μm. (B) Western blot for SYN in lung tissues from sham and MCT-AV rat groups. GAPDH was used as a loading control. Data are shown as the mean ± SEM. n = 5 biologically independent rats. **P < 0.01. (C) PASMCs from control, sham and MCT-AV rat groups were immunofluorescently stained with anti-SYN, anti-α-SMA and anti-CD31 antibodies. Size bars represent 25 μm. Data are shown as the mean ± SEM. n = 5 biologically independent rats. *P < 0.05, **P < 0.01.
powdered milk in TBST for 1 h, the membrane was incubated with the respective primary antibodies (Table S1) overnight at 4°C. This was followed by further incubation with the corresponding horseradish peroxidase (HRP)-labelled secondary antibodies at room temperature for 1 h. Finally, proteins were visualized using enhanced chemiluminescence (ECL; Thermo Fisher Scientific) reagents to acquire images. The relative expression of the target proteins was reported as the protein/GAPDH ratio.

Statistical analysis

Data are representative of three independent experiments. All values are presented as means ± standard error of the mean (SEM) obtained using IBM SPSS Statistics 24. Statistical significance (\( P < 0.05 \)) was determined using Student’s t-test for two groups or one-way ANOVA followed by Bonferroni’s multiple comparison test. Spearman’s rank correlation coefficient was used to describe the relationship between SYN expression and pulmonary vessel pathological grading.

Results

SYN was significantly increased in the remodelled pulmonary arteries of CHD-PAH patients and shunt-induced PAH rat model

Immunohistochemistry results showed that the expression of SYN was distinctly elevated in PAMSCs from remodelled pulmonary arterioles of CHD-PAH patients and in a shunt-induced PAH rat model in comparison with that in the

Figure 3 SYN enhanced phenotypic switch of hPASMCs in vitro. (A) Western blot for phenotypic markers of stably transfected hPASMCs. GAPDH was used as a loading control. (B) The stably transfected hPASMCs were immunofluorescently stained with anti-α-SMA antibody. Size bars represent 25 μm. Data are shown as the mean ± SEM. n = 3 biologically independent group. *: LV-siSYN vs. LV-siCON; #: LV-SYN vs. LV-CON. **P < 0.01, ***P < 0.001; *P < 0.05, **P < 0.01, ***P < 0.001.
normal control group (Figure 1A and Figure 2A). Western blot analysis further confirmed these results (Figure 2B). However, SYN expression was not significantly correlated with the pathologic grading of pulmonary arterioles in patients with CHD-PAH (Figure 1B). Moreover, immunofluorescence results revealed that SYN was enriched in the PASMCs of remodelled pulmonary arterioles of CHD-PAH patients (Figure 1C). SYN expression was markedly up-regulated in PASMCs from shunt-induced PAH rats (Figure 2C). Collectively, our results indicated that SYN overexpression in PASMCs may be involved in pulmonary vascular remodelling in CHD-PAH.

**SYN enhanced phenotypic switch, proliferation and migration capability of hPASMCs in vitro**

To explore the effects of SYN in regulating phenotypic switch, proliferation, and migration capability of hPASMCs, cells were infected with lentivirus expressing shRNA targeting SYN or scrambled control. Transfection efficiency was determined using western blot. As shown in Figure 3A, the expression of SYN was down-regulated or up-regulated by lentivirus expressing shRNA targeting SYN. Interestingly, SYN suppression notably increased the expression of hPASMC contractile-phenotype markers (α-SMA, SM-MHC, and smoothelin (SMTN)) and inhibited the expression of synthetic phenotype markers (c-MYC, PCNA, and tropomyosin-4 (TPM4)) (Figure 3A and 3B). The results of cell counts and Transwell migration assays showed that SYN suppression attenuated the proliferation and migration capability of PASMCs (Figure 4). In contrast, SYN overexpression resulted in the opposite effects (Figures 3 and 4). Taken together, these results indicated that SYN up-regulation enhanced phenotypic switch, proliferation, and migration of hPASMCs, which might contribute to pulmonary arterial remodelling.

**Figure 4** SYN enhanced proliferation and migration capability of hPASMCs. The proliferation and migration capability of hPASMCs in each group was evaluated by cell counting and Transwell migration assays, respectively. Data are shown as the mean ± SEM. n = 3 biologically independent group. **P < 0.01.
SYN enhanced hPASMC phenotypic switch via AKT signalling pathway

Emerging evidence has demonstrated that AKT signalling participates in the modulation of the phenotypic switch, proliferation, and migration of PASMCs in PAH. In addition, Pitre et al. showed that SYN positively promotes glioblastoma cell proliferation by promoting AKT activation. As shown in Figure 5A, SYN suppression inhibited AKT activation in hPASMCs, whereas SYN overexpression resulted in the opposite result. We further explored whether SYN regulated hPASMC dysfunction through AKT. We used a specific AKT inhibitor, MK-2206, to suppress AKT activation. As shown in Figure 5B, MK-2206 markedly alleviated AKT activation. Moreover, SYN overexpression promoted the phenotypic switch of hPAMSCs, whereas this effect was reversed by the AKT inhibitor MK-2206 (Figure 5C). In summary, these results indicated that SYN regulates the phenotypic switch of hPASMC through AKT signalling.

SYN knockdown apparently alleviated shunt-induced PAH and pulmonary vascular remodelling

To explore the effects of SYN on shunt-induced PAH and pulmonary vascular remodelling, rats were intratracheally instilled with AAVs to modulate the expression of SYN in the pulmonary vessels. As shown in Figure 6A and 6B, delivery of AAVs led to more intense green fluorescence in the pulmonary arterioles of rats compared with those in animals treated with PBS. Administration of AAV-siSYN decreased the lung expression of SYN. After 7 days, the rats were used to construct a shunt-induced PAH model. As shown in Figure 6C and 6D, mPAP, RVSP, PASP, mPAP, RVHI, and distal pulmonary artery remodelling (WT% and WA%) were increased in shunt-induced PAH rats; however, these haemodynamic parameters were reversed by SYN knockdown. These results indicated that SYN suppression inhibited the progression of shunt-induced PAH and pulmonary vascular remodelling.

Figure 5 SYN enhanced phenotypic switch of hPASMCs via AKT signalling pathway in vitro. (A) Western blot for p-AKT and AKT of stably transfected hPASMCs, GAPDH was used as a loading control. (B) Western blot for p-AKT and AKT of hPASMCs transfected with LV-con and LV-SYN in the presence or absence of AKT inhibitor MK-2206, GAPDH was used as a loading control. (C) Western blot for phenotypic markers of hPASMCs transfected with LV-con and LV-SYN in the presence or absence of AKT inhibitor MK-2206. Data are shown as the mean ± SEM. n = 3 biologically independent group. *: LV-CON + MK-2206 vs. LV-CON; #: LV-SYN + MK2206 vs. LV-SYN. **P < 0.05, ***P < 0.01, ****P < 0.001; #:P < 0.05, ##P < 0.01.
Discussion

Pulmonary vascular remodelling, the pathological hallmark of CHD-PAH, is attributed to hyperproliferation and hypermigration of PASMCs. Proteomic analysis was used to explore novel targets to better understand the underlying pathological mechanisms and to guide the treatment of CHD-PAH patients. Our results showed that SYN was distinctly up-regulated in lung tissues of CHD-PAH patients compared with the normal control group. Our present experiments showed that the expression of SYN was significantly elevated in PASMCs of remodelled pulmonary arteries in CHD-PAH patients and in the shunt-induced PAH rat model, implying that SYN may participate in the pathological processes of pulmonary vascular remodelling induced by high blood flow. In addition, SYN modulated the phenotypic switch, proliferation, and migration capabilities of hPAMSCs in vitro. Moreover, SYN suppression attenuated PAH and pulmonary vascular remodelling in a shunt-induced PAH rat model.

SYN is a large and unique member of the intermediate filament protein family. It has been reported that SYN is present in VSMCs and its expression is changed under pathological conditions. Reportedly, there is a significant up-regulation of SYN in Müller cells following retinal injury. SYN is rarely expressed in portal tract fibroblasts under normal conditions, but is overexpressed in severe inflammatory diseases associated with portal expansion. Notably, in patients with CHD-PAH, there is an inflammatory profile, especially in Eisenmenger syndrome patients. In contrast, a
previous study found that SYN was notably down-regulated in VSMCs isolated from varicose veins.\textsuperscript{19} It has also been reported that SYN is down-regulated by platelet-derived growth factor beta (PDGF-β) and interferon gamma (IFN-γ), exposure to shear flow stress, and oxidized low-density lipoprotein (oxLDL) loading.\textsuperscript{30} Many studies have shown that PDGF-β levels are distinctively increased during the pathological development of PAH.\textsuperscript{34,35} In our study, SYN was remarkably up-regulated in PAMSCs of remodelled pulmonary tissue of CHD-PAH patients and in a shunt-induced PAH rat model, which might be the result of the combined action of these cytokines. However, the specific mechanisms regulating SYN elevation require further investigation. Considering the key roles of PASMCs in pulmonary vascular remodelling of CHD-PAH, these findings indicate that SYN might be involved in the pathological process of CHD-PAH development.

Recent studies have confirmed that SYN participates in cell growth, differentiation, and other fundamental biological processes. For example, SYN promotes the proliferation of glioblastoma cells.\textsuperscript{17} In addition, SYN contributes to the migratory properties of astrocytoma cells by influencing actin cytoskeleton dynamics.\textsuperscript{18} SYN is involved in focal adhesion dynamics and is essential for cell adhesion and migration.\textsuperscript{36} To further explore the role of SYN in pulmonary vascular remodelling in CHD-PAH, we first investigated whether SYN mediates phenotypic switch, proliferation, and migration of hPASMCs \textit{in vitro}. The results showed that SYN knockdown inhibited hPASMC phenotypic switch, proliferation, and migration, while SYN overexpression resulted in the opposite effects, which implied that SYN aggravated hPASMC dysfunction. Moreover, consistent with previously described findings, our animal experiments demonstrated that SYN knockdown improved PAH and mitigated pulmonary vascular remodelling in a shunt-induced PAH rat model. These findings revealed that SYN plays a crucial role in the pathological development of CHD-PAH. However, SYN knockdown promotes the phenotypic switch of saphenous vein smooth muscle cells from a contractile to a synthetic state and ultimately contributes to the development of varicose veins.\textsuperscript{19} We speculate that this paradox might be due to the various experimental models of pathological conditions and is worthy of further exploration.

The AKT signalling pathway is a classic pathway that modulates PASMC dysfunction. Several studies have reported that the activation of AKT promotes phenotypic switch, proliferation, and migration of PASMCs, thus intensifying pulmonary vascular remodelling in PAH.\textsuperscript{25–27} In addition, it has been demonstrated that SYN enhances the proliferation of glioblastoma cells via activation of the AKT signalling pathway.\textsuperscript{17} Here, we provided direct evidence that SYN overexpression promoted hPASMCs dysfunction, and these effects were reversed by an AKT inhibitor. Therefore, AKT activation may be an important reason for SYN-enhanced hPASMC dysfunction. However, how SYN mediates AKT activation in hPASMC has not yet been investigated, and extensive research will be carried out to study this mechanism in the future.

Conclusions

Therefore, the purpose of the present study was to clarify the mechanism by which SYN mediates PASMC dysfunction and to show that SYN might be a potential target for the treatment of CHD-PAH.

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Conflict of interests

All authors declare they have no actual or potential competing financial interest.

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Consent for publication

All authors have read and agreed towards the submission of the manuscript.

Data availability statement

All raw data generated or used during the study are available from the corresponding author by request.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Antibodies information for immunohistochemistry (IHC), immunofluorescent (IF) and western blot.
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