Shikonin-mediated inhibition of nestin affects hypoxia-induced proliferation of pulmonary artery smooth muscle cells

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Abstract. The imbalance between the proliferation and apoptosis of pulmonary artery smooth muscle cells (PASMCs) is of importance in pulmonary vascular remodeling. Shikonin, a naphthoquinone compound extracted from the Chinese medicinal herb *Lithospermum erythrorhizon*, inhibits the proliferation of rat smooth muscle cells (SMCs). The present study was designed to investigate the effects of shikonin on the proliferation of rat PASMCs and the possible mechanisms involved. Rat PASMCs were cultured under the following five treatment conditions: Normal control; hypoxia for 24 h; hypoxia + 1 μM shikonin for 24 h; hypoxia + 2 μM shikonin for 24 h; and hypoxia + 4 μM shikonin for 24 h. The viability of PASMCs was measured using the Cell Counting Kit-8 assay, the mRNA expression of nestin (NES) in each group was measured by reverse transcription-polymerase chain reaction and the protein expression of NES was measured by western blotting. The proliferation of hypoxic PASMCs transfected with NES-specific small interfering (si)RNA decreased compared with the non-transfected group. These results indicated that hypoxia induced the proliferation of PASMCs through the enhancement of NES expression. The treatment of hypoxic PASMCs with shikonin resulted in a significant downregulation of NES expression and the inhibition of PASMC proliferation.

Introduction

Pulmonary hypertension (PH) is caused by pulmonary vasoconstriction, pulmonary vascular remodeling and *in situ* thrombosis (1). The condition leads to a progressive increase in pulmonary vascular resistance, resulting in the failure of the right side of the heart and, subsequently, the patient succumbs to hypertension sequelae. A previous study indicated that an imbalance between the proliferation and apoptosis of pulmonary artery smooth muscle cells (PASMCs) affects pulmonary vascular remodeling (2). Therefore, investigations into the specific reversal mechanisms for PASMC proliferation are currently an important research topic for the treatment of PH.

Nestin (NES), a member of the intermediate filament family, is expressed in myogenic and neural stem cells, vascular smooth muscle cells, and during the development of their immature descendants (3-5). A number of studies have demonstrated that NES participates in the development of endothelial cells during reparative angiogenesis and tumor vascularization. It is upregulated in the infarcted heart and detected in various types of cancer (6-8). NES has been used extensively as a marker for muscle and neural progenitor cells. The expression of NES decreases in muscle cells following the differentiation of skeletal muscles (4,9), but is resumed in response to injury (10). Similar effects have been observed in the central nervous system (11). The reappearance of NES-positive cells in the neointima of balloon-injured carotid arteries has been demonstrated (5), which implicated NES-positive cells in the progression of vascular remodeling. Furthermore, a previous study of NES-RNA interference in an anti-Thy1 nephritis model reported that the knockdown of NES reduces the proliferation of mesangial cells following injury (12). These results indicated that the re-expression of NES may serve an important role in cell proliferation during the early post-injury phase. However, the expression of NES in PASMCs during the development of, and following injury has not yet been studied.

Shikonin, a natural naphthoquinone dye from the Chinese medicinal herb shikon (*Lithospermum erythrorhizon*), exerts multiple pharmacological activities *in vitro* and *in vivo* (13-15). It has been used as an anti-inflammatory (16) and anti-tumor agent (17) in traditional medicine for a long time. Previous studies have demonstrated that shikonin induces apoptosis in human cancer cells through the upregulation of p73 and the downregulation of E3 ubiquitin-protein ligase UHRF1 (18). Shikonin can also inhibit the proliferation and migration of endothelial (13) and vascular smooth muscle cells (19) *in vitro*. A recent publication reported that shikonin treatment significantly inhibits the morphology of glioma stem cells and NES positive (NES^+), sphere-forming cells and reduces CD133 expression (20). It is known that malignant gliomas have a perivascular location with certain NES^+ cells co-expressing CD133 (21). However, the function and association between

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NES and shikonin in hypoxic PASMCs have not been elucidated. Therefore, the aim of the present study was to test the hypothesis that shikonin-mediated downregulation of NES expression is the mechanistic basis of the inhibition of PASMCs proliferation.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Hyclone; GE Healthcare Life Sciences (Logan, UT, USA). Mouse monoclonal anti-NES antibody (clone 401; cat. no. 556309) was purchased from BD Biosciences (San Jose, CA, USA). Anti-β-actin antibody (cat. no. sc-58673) and anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. sc-51625) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Shikonin was purchased from Shanghai Standard Technology Co., Ltd (Shanghai, China). The Cell Counting Kit 8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The synthesis of small interfering RNAs (siRNAs) was performed by Shanghai GenePharma Co., Ltd (Shanghai, China).

Cell preparation and culture. All experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23) (22). The use of animals was conducted in strict accordance with the Guidance Suggestions for the Care of Laboratory Animals published by the Chinese Ministry of Science and Technology (23). All efforts were made to minimize animal suffering and to reduce the number of animals used in the study. In addition, all animal procedures in the present study were performed in accordance with the guidelines of the Wenzhou Medical University and the National Institutes of Health standards of animal care. The present study was approved by the Animal Ethics Committee of Wenzhou Medical University, including permit number SCXK (Zhejiang 2005-0019 150). A total of 15 10-week-old adult male Sprague-Dawley rats of specific pathogen free grade (weight 200-220 g) were purchased from the Laboratory Animal Center of Beijing (China). The rats (3-5 per cage) were kept in the same room for one week at a controlled temperature (22-24˚C) and relative humidity (50-60%), under a 12-h light-dark cycle. Food and water were accessible. Prior to sacrifice, the rats were anesthetized by intraperitoneal injection of 500 mg/kg 5% chloral hydrate (Merck KGaA, Darmstadt, Germany). Anesthesia was monitored by electroencephalogram and rats were sacrificed by cervical dislocation following deep anesthesia. Rat death was confirmed by checking breathing, heartbeat, pupils and nerve reflex. Subsequently, the rats were immersed in 75% alcohol to disinfect the skin and then the chest was rapidly dissected. Primary cultures of PASMCs were obtained from the intrapulmonary arteries (grades 2-4 arterioles) of Sprague-Dawley rats using the method described by Gunther *et al* (24) with slight modifications. Briefly, the adventitia was stripped off by blunt dissection and the intimal surface was scratched to remove the endothelium. The remaining smooth muscles were placed in DMEM supplemented with 20% FBS for 1-2 days to recover more cells following digestion. The pulmonary arteries were incubated in Hanks' balanced salt solution (Gibco; Thermo Fisher Scientific, Inc.) with 1.5 mg/ml collagenase I (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 90 min. The cells were transferred to a 25 ml culture flask and cultured in DMEM supplemented with 20% FBS and 1% penicillin-streptomycin solution at 37˚C in an atmosphere of 5% CO_2. PASMC morphology was observed using an inverted microscope (TS100-F; Nikon Corporation, Tokyo, Japan) following 24 h of plating. After the PASMCs reached 80-90% confluence, the cells were harvested using trypsin (Promega Corporation, Madison, WI, USA) and passaged. The passaged cells were then recultured in DMEM supplemented with 10% FBS.

Immunofluorescence staining. Cells were seeded at $2x10^4$ cells/ml and fixed using 4% paraformaldehyde for 15 min at room temperature. After washing in PBS for three times, the cells were incubated with 10% H_2O_2 in methanol for 10 min at room temperature to block endoperoxidase activity. Subsequently, the cells were immunolabeled with antibodies to smooth muscle-α actin (α-SMA; 1:100; cat. no. sc-53142; Santa Cruz Biotechnology, Inc.) by incubation overnight in a humid chamber at 4˚C. The sections were incubated with a fluorescein isothiocyanate (FITC)-labeled secondary antibody (1:100; cat. no. F9137; Sigma-Aldrich; Merck KGaA) at 37˚C for 20-30 min in the dark and the nuclei were counterstained by incubation with DAPI (Sigma-Aldrich; Merck KGaA) at 37˚C for 3-5 min. The sections were observed and images were captured using a laser confocal microscope (Leica TCS SP2; Leica Microsystems GmbH, Germany) at different excitation wavelengths (FITC: 450-490 nm and PI: 515-560 nm).

Cell treatments. The PASMCs used for all experiments were between passages 4 and 6. Cells seeded at a density of $1x10^4$ cells/ml were incubated in serum-free DMEM for 48 h to prevent growth. Hypoxia was induced through the incubation of cells in a CO_2-N_2 incubator (Heraeus Holding GmbH, Hanau, Germany) in an atmosphere of 5% O_2/5% CO_2/90% N_2. To study the effects of hypoxia and shikonin on PASMC proliferation and NES expression (mRNA level and protein level), PASMCs were divided into five groups: i) Normal: The cells were cultured in 10% FBS-DMEM in normoxic conditions (21% O_2, 5% CO_2) for 24 h; ii) Hypoxia: The cells were cultured in 10% FBS-DMEM in hypoxic conditions (5% O_2, 5% CO_2) for 24 h; iii) Hypoxia + 1 μM shikonin: The cells were cultured in 10% FBS-DMEM with shikonin (1 μM) for 24 h in hypoxic conditions; iv) Hypoxia + 2 μM shikonin: As for iii), but with 2 μM) and v) Hypoxia + 4 μM shikonin: As for iv), but with 4 μM shikonin. For the small interfering (si)RNA knockdown assay, PASMCs were divided into three groups: i) Blank control: The cells were cultured in 10% FBS-DMEM without NES-siRNA for 48 h; ii) NES-siRNA: The cells were transfected with NES-siRNA for 48 h; iii) Scrambled control: The cells were transfected with a scrambled negative control for 48 h.

siRNA knockdown assay. The specific sets of gene sequences (Shanghai GenePharma, Co., Ltd.) for the siRNA knockdown assay include: NES-siRNA (sense) 5'-GGAGCAGAGAAU

UGUGAAATT-3'; NES-siRNA (antisense) 5'-UUUCACAAU UCUCUGCUCCTT-3'; scrambled negative control (sense) 5'-UUCUCCGAACGUGUCACGUTT-3'; scrambled negative control (antisense) 5'-ACGUGACACGUUCGGAGAATT-3'. Prior to transfection, PASMCs were cultured at $4x10^4$ and $1x10^4$ cells/well in 6-well flat-bottomed plates and 96-well flat-bottomed microplates, respectively, until a confluence of 40-60% was reached. Serum-free DMEM broth was used to dilute 100 pmol NES-siRNA (or scrambled negative control) and 5 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) to 1 ml, respectively. The solutions were stored at 37˚C for 5 min and then mixed and stored at 37˚C for 20 min to form the siRNA/Lipofectamine 2000 mixture. The transfection mixture was added to the culture plates (100 μl per well in the 96-well microplates), incubated for 6 h and then replaced by DMEM medium. The effects of siRNAs were studied by reverse transcription-polymerase chain reaction (RT-PCR), western blotting and CCK-8 assays, which were performed 48 h post-transfection.

RNA extraction and semi-quantitative RT-PCR. Total RNA was isolated from the cells using TRIzol® (Gibco; Thermo Fisher Scientific, Inc.). The RNA concentration was determined by spectrophotometric measurements of the absorbance at a wavelength of 260 nm. RT was performed using the first strand cDNA synthesis kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) with total RNA (1 μg) at 60˚C for 5 min. Subsequently, the sample was placed on ice for at least 1 min. The reaction mixture was heated at 42˚C for 60 min and 70˚C for 5 min to inactivate the reverse transcriptase. The cDNA was subjected to PCR following the addition of 1 μl first strand cDNA reaction mixture to a 25 μl PCR reaction mixture. The specific sets of oligonucleotides for the PCR were: NES (sense) 5'-TGCTGAGTATGTCGTGGAG-3'; NES (antisense) 5'-GTCTTCTGAGTGGCAGTGAT-3'; GAPDH (sense) 5'-CACCTCAAGATGTCCCTTAGTC-3'; GAPDH (antisense) 5'-AAGTAGGGTGGTGAGGGTTG-3'. The PCR conditions comprised 30 cycles of amplification at 95˚C for 30 sec, 65˚C for 30 sec and 72˚C for 1 min, followed by a final extension period at 72˚C for 10 min. PCR amplifications were performed on ABI7500 real-time PCR detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR products (NES 5 μl and GAPDH 2 μl) were subjected to electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide. The optical density (OD) of each band on the gel was measured using the Gelpro 31 system (Wenzhou Aoli Biomedical Instrument Factory, Shanghai, China). The OD values for NES were normalized to the OD values for GAPDH.

Western blot analysis. Total protein was extracted from cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with 1 mM phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology) and protein concentration was quantified by the Bradford method against standard solutions of bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Haimen, China). Equal quantities of protein (50 μg) were separated using 8-12% SDS-PAGE) gel electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The separated proteins were elecrophoretically transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked in Tris-buffered saline with 0.05% Tween 20 (TBST) containing 5% BSA for 90 min at room temperature and then incubated with anti-NES (1:500; BD Biosciences) and anti-β-actin (1:1,000; Santa Cruz Biotechnology, Inc.) antibody at 4˚C overnight. The membranes were washed with TBST buffer and incubated with HRP-labeled goat anti-mouse immunoglobulin G (1:1,000; Santa Cruz Biotechnology, Inc.) at room temperature for 60 min. The HRP intensity was detected using a Pierce™ ECL Western Blotting Substrate kit (Pierce; Thermo Fisher Scientific, Inc.). The Quantity One software (version 4.4.0.36; Bio-Rad Laboratories, Inc.) was used to quantify the density of the bands.

Determination of cell proliferation. PASMCs were cultured at $1x10^4$ cells/well in 96-well flat-bottomed microplates and incubated with 200 μl 10% FBS-DMEM at 37˚C and 5% CO_2 for 2 days (6 replicate wells were used for each group). At the end of the second day, the medium was replaced with 100 μl fresh DMEM without FBS and treated in a similar manner for a further 2 days. CCK-8 reagent (10 ml/well) was added to the wells at the end of the experiment and incubated at 37˚C for 2 h. The absorbance of the solution in each well at 450 nm was then determined using a microplate reader (ELX800; BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. The experimental data are expressed as the mean ± standard deviation of three independent experiments. All data were subjected to normality tests. Comparisons of means among multiple groups were performed using one-way analysis of variance followed by Fisher's least significant difference post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of hypoxia and shikonin on PASMC proliferation. Confluent PASMCs demonstrated typical hill and valley features (Fig. 1A). α-smooth muscle actin is a commonly used marker of myofibroblast formation (25) and can be used to identify PASMCs. Immunofluorescence staining demonstrated that PASMCs were positively stained for α-smooth muscle actin (Fig. 1B). A CCK-8 assay was used to determine whether shikonin inhibited the proliferation of hypoxic PASMCs (Fig. 2). The proliferation of cells in the hypoxia group was significantly increased compared with the normal group ($P<0.05$). Shikonin inhibited hypoxia-stimulated PASMC growth in a dose-dependent manner (Fig. 2). A significant decrease in cell proliferation was also observed in the hypoxia + 4 μM shikonin group ($P<0.05$; Fig. 2).

Effects of hypoxia and shikonin on NES expression. NES expression was significantly increased in the hypoxia group compared with the normal group ($P<0.05$; Fig. 3). Shikonin (4 μM) significantly suppressed the expression of NES in hypoxic PASMCs ($P<0.05$).

RT-PCR was used to quantify the relative levels of NES expression in PASMCs with GAPDH mRNA used as the

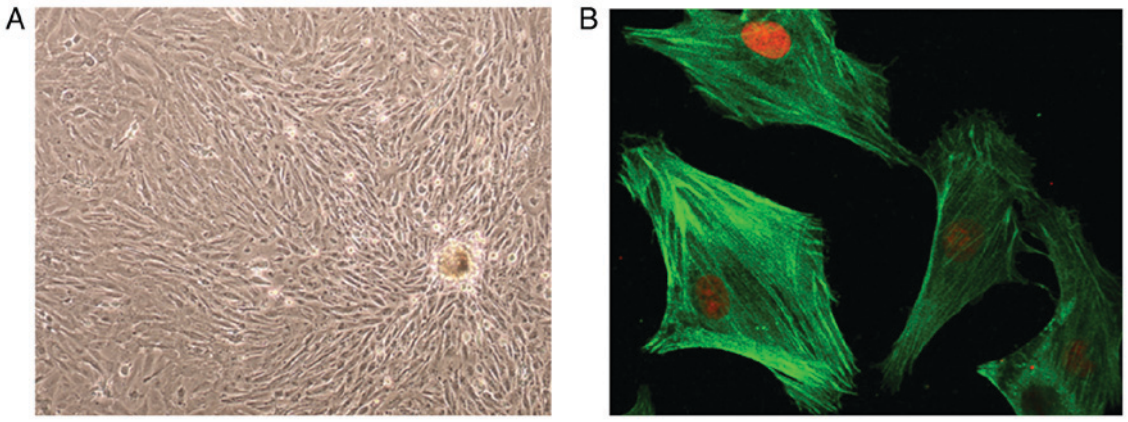

Figure 1. PASMC morphology and growth. (A) Typical 'hill and valley' appearance of PASMCs observed using phase contrast microscopy at a magnification, x400. (B) Positive immunofluorescent identification of α-smooth muscle actin. PASMCs, Pulmonary artery smooth muscle cells.

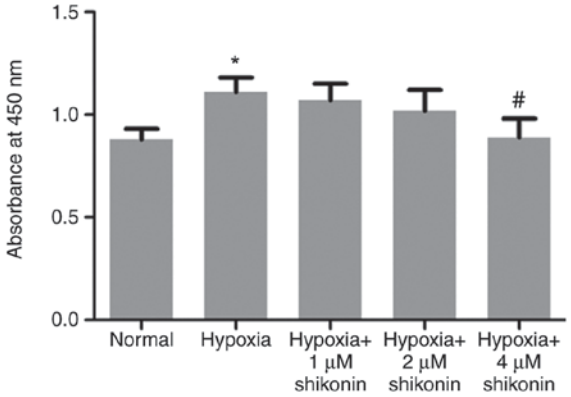

Figure 2. Effect of hypoxia and shikonin on cell proliferation. Pulmonary artery smooth muscle cells were cultured for 24 h in normoxic or hypoxic (5% O_2) conditions in the presence and absence of shikonin. The cell viability was determined using the Cell Counting Kit-8. *P<0.01 vs. the normal and #P<0.01 vs. the hypoxia groups, n=6.

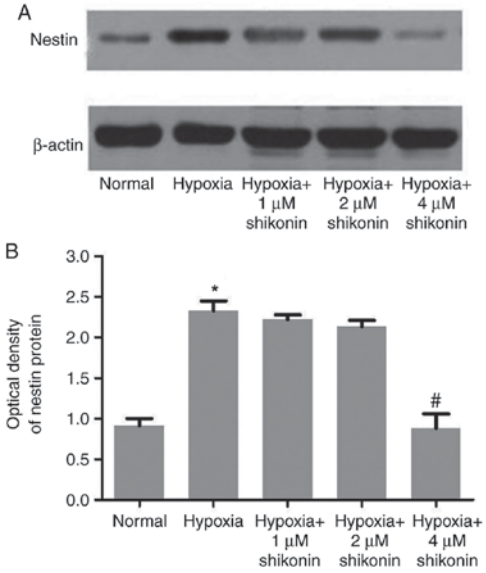

Figure 3. Effects of hypoxia and shikonin on the expression of the NES protein in PASMCs. PASMCs were cultured for 24 h in normoxic or hypoxic (5% O_2) conditions in the presence and absence of shikonin. The protein expression of NES was measured using western blotting. (A) Representative image and (B) quantification of data. *P<0.01 vs. the normal and #P<0.01 vs. the hypoxia groups, n=3. PASMCs, pulmonary artery smooth muscle cell; NES, nestin.

loading control. The expression of NES in the hypoxia group was significantly increased compared with the normal group (P<0.05; Fig. 4). Shikonin (4 μM) significantly inhibited the expression of NES in hypoxic PASMCs at 24 h following treatment (P<0.05).

NES participates in PASMC proliferation. To determine the role of NES in PASMC proliferation, the cells were transfected with scrambled siRNA and Nes siRNA. The expression of NES (P<0.05; Fig. 5) and Nes mRNA (P<0.05; Fig. 6) was significantly suppressed by Nes RNAi. The CCK-8 assay indicated that the proliferation of NES-depleted cells was significantly decreased (P<0.05; Fig. 7).

Discussion

Chronic hypoxia is the most important cause of PH, a chronic obstructive pulmonary disease with a central role in the development of pulmonary heart disease (26). Long-term hypoxia can lead to hypoxic pulmonary vasoconstriction, dysfunction of the pulmonary vascular endothelium and pulmonary vascular remodeling, which culminates in PH. PASMCs can be divided into three phenotypes based on structure and function: The contractile phenotype, the synthetic phenotype and a transitional phenotype between the other two phenotypes (27). Of these three phenotypes, only the synthetic phenotype can be stimulated with mitogens to initiate proliferation (27). In hypoxic conditions, PASMCs transform from the contractile to the synthetic phenotype and migrate from the medial to the intimal layer to increase proliferation. Migration leads to medial thickening of the pulmonary artery and the muscularization of non-muscular vessels. These effects lead to luminal narrowing, increased resistance to blood flow and a weakened buffering effect in the pulmonary vessels, which causes alterations in blood volume and resistance (2). Therefore, these processes serve an important role in pulmonary vascular remodeling. In the present study, rat PASMCs subjected to hypoxic conditions demonstrated an increase in proliferation, which was consistent with the studies described above.

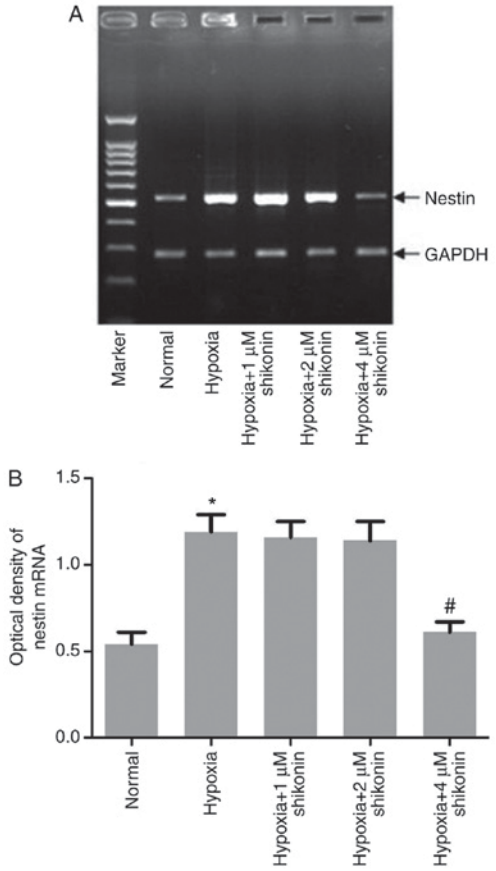

Figure 4. Effects of hypoxia and shikonin on expression of NES mRNA in PASMCs. PASMCs were cultured for 24 h in normoxic or hypoxic (5% O_2) conditions in the presence and absence of shikonin and the mRNA expression of NES was measured using reverse transcription-polymerase chain reaction. (A) Representative image and (B) quantification of data. *P<0.01 vs. the normal and #P<0.01 vs. the hypoxia groups, n=6. PASMCs, pulmonary artery smooth muscle cells; NES, nestin.

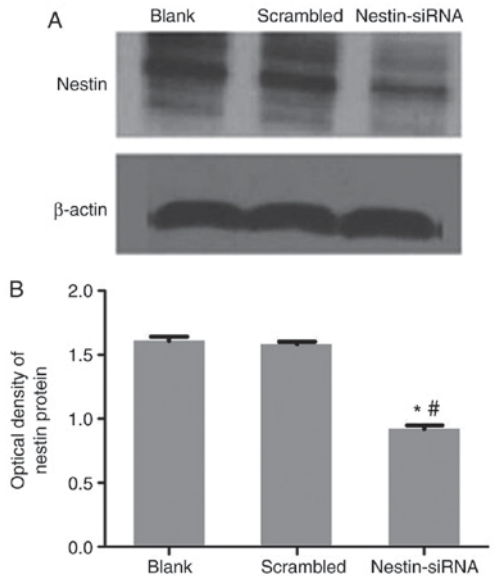

Figure 5. Effect of transfection of scrambled or NES siRNA on expression of NES protein in PASMCs. PASMCs were transfected with NES siRNA or scrambled siRNA for 48 h and the protein expression of NES was measured using western blotting analyses. (A) Representative image and (B) quantification of data. *P<0.01 vs. the blank and #P<0.01 the scrambled groups, n=3. siRNA, short-interfering RNA; PASMCs, pulmonary artery smooth muscle cells; NES, nestin.

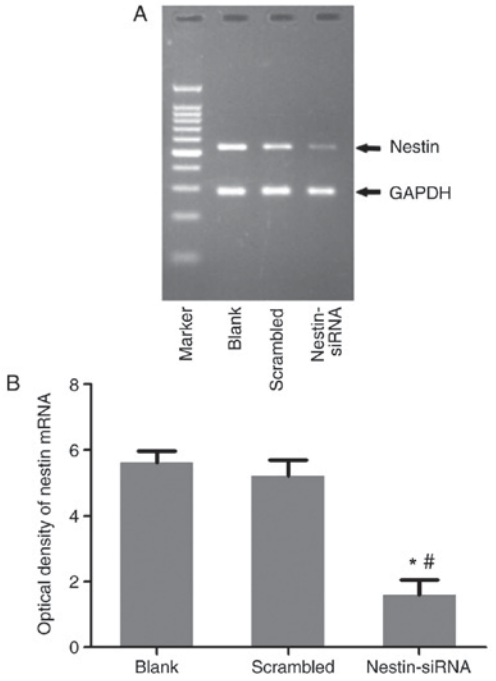

Figure 6. Effect of transfection of scrambled or NES siRNA on mRNA expression of NES in PASMCs. PASMCs were transfected with NES siRNA or scrambled siRNA for 48 h. The mRNA expression of NES was measured using reverse transcription-polymerase chain reaction. (A) Representative image and (B) quantification of data. *P<0.01 vs. the blank and #P<0.01 vs. scrambled groups, n=6. siRNA, short-interfering RNA; PASMCs, pulmonary artery smooth muscle cells; NES, nestin.

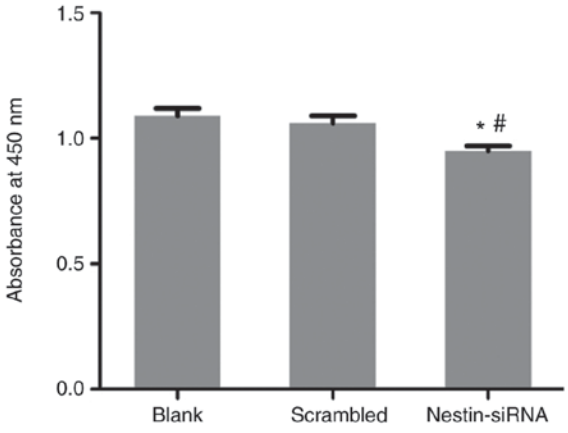

Figure 7. Effect of scrambled or NES siRNA transfection on cell viability. PASMCs were transfected with NES siRNA or scrambled siRNA for 48 h. The cell viability was analyzed using a Cell Counting Kit. *P<0.01 vs. the blank and #P<0.01 vs. the scrambled groups, n=6. PASMCs, pulmonary artery smooth muscle cells; si, small interfering; NES, nestin.

Current investigations on the role of NES in SMCs are limited, with even fewer studies on its function in the lungs: Only a handful of studies have reported the expression of NES in human fetal lung fibroblasts and adult bronchial fibroblasts (28) however increased expression of NES in the stellate SMCs of coronary artery plaques from patients with unstable angina is frequently observed (29). These results indicate the important role of NES in the formation of coronary atherosclerotic plaques. A previous study indicated that serum stimulation restores the expression of NES

in aortic SMCs through triggering the epidermal growth factor (EGF)-mediated extracellular signal-regulated kinase (ERK) pathway (30). The stimulation of the ERK pathway may be responsible for the alterations in the cellular phenotype (5,30,31). The expression of NES in developing arteries has been observed to diminish in mature arteries (5), which indicates that NES expression is time- and region-dependent. The expression of NES is restored during the formation of carotid neointima post-balloon injury and increases gradually with neointimal proliferation (32). Therefore, NES may serve an important role in vascular remodeling. An *in vitro* study identified that hypoxia induces the cellular expression of the NES protein in LLC-PK1 cells, which is accompanied by alterations in cellular morphology (32). In the present study, the increased expression of NES was confirmed in rat PASMCs by RT-PCR and western blotting analyses. Furthermore, the results indicated that hypoxia promoted the upregulation of NES mRNA and protein in PASMCs.

A previous study on ST15A neural progenitor cells reported that NES interferes with cyclin dependent kinase (Cdk)5-dependent apoptosis and exerts a protective effect on these cells (33). It was also reported that RNAi significantly downregulates NES expression in vascular SMCs, which leads to an increase in the H_2O_2-mediated apoptosis of vascular SMCs (34). Therefore, NES has anti-apoptotic effects, which may occur through the activation of the Cdk5-mediated phosphorylation of the B-cell lymphoma 2 (BCL-2)/caspase signaling pathway. Therefore, NES promotes proliferation and inhibits apoptosis. In the present study, the transfection of PASMCs with specific siRNAs resulted in the downregulation of NES expression. The results of the CCK-8 assay indicated that cell proliferation in the RNAi-treated group was significantly decreased compared with the blank control and the scrambled negative transfection control group. Therefore, it was deduced that hypoxia enhanced the proliferation of PASMCs through the upregulation of NES expression.

Shikonin is a naphthoquinone compound extracted from *L. erythrorhizon*. Extensive studies conducted globally have reported the various pharmacological activities of shikonin and its derivatives. Shikonin also exerts significant inhibitory effects on the proliferation and metastasis of multiple types of tumor cells, and can exert pro-apoptotic effects (35-38). A previous study suggested that shikonin can induce cell cycle arrest in the G_0/G_1 phase and thereby inhibit the proliferation of rat vascular SMCs (19). In addition, it was reported that shikonin induces cell-cycle arrest and apoptosis in human epidermoid carcinoma cells in a time- and dose-dependent manner (39). The apoptosis of these cells is partly mediated by the inactivation of nuclear factor (NF)-κB, which occurrs through the modulation of the EGF receptor signaling pathway and the activation of caspases. The study by Zhang *et al* (40) demonstrated that shikonin regulates the activation of the NF-κB and phosphatidyl inositol 3-kinase signaling pathways. Shikonin also modulates the expression of cyclin D1, cyclin E, BCL-2 and BCL2 associated X protein, activates caspase-3 and caspase-9, induces cell cycle arrest, and promotes the apoptosis of VSMCs. In the current study, rat PASMCs cultured in hypoxic conditions treated with shikonin were evaluated by a CCK-8 assay to determine the viability of PASMCs. The results indicated that shikonin inhibited hypoxia-induced PASMC proliferation in a dose-dependent manner. Significant inhibition of cell growth was observed following the addition of 4 μM shikonin and resulted in decreased cell viability compared with the normal group. The effect of the various doses of shikonin were evaluated by RT-PCR and western blotting, and it was determined that shikonin downregulated the hypoxia-induced expression of NES in PASMCs in a dose-dependent manner. However, as it was unclear which signaling pathways were involved in the shikonin-mediated inhibition of NES in hypoxic PASMCs, further studies are required.

In conclusion, significant upregulation of NES occurred in PASMCs cultured in hypoxic conditions *in vitro*. NES exerted protective effects on PASMCs and promoted their proliferation. Furthermore, shikonin inhibited the proliferation of PASMCs through the downregulation of NES expression. Therefore, the present study provided information to suggest a theoretical basis for the use of shikonin in the clinical management of hypoxic PH.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SH and JF conceived the study and designed the experiments. SH and JL performed the experiments. SH and JF wrote the manuscript. JL, LL and YX analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23). The use of animals was conducted in strict accordance with Guidance Suggestions for the Care of Laboratory Animals published by the Chinese Ministry of Science and Technology. All efforts were made to minimize animal suffering and to reduce the number of animals used in the study. In addition, all animal procedures in the present study were performed in accordance with the guidelines of Wenzhou Medical University and the National Institutes of Health standards of animal care. Our study was approved by the Animal Ethics Committee of Wenzhou Medical University, including permit number SCXK (Zhejiang 2005-0019 150).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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