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MicroRNA-126 affects cell apoptosis, proliferation, cell cycle and modulates VEGF/TGF-β levels in pulmonary artery endothelial cells

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Abstract. – OBJECTIVE: In the clinic, therapeutic options for pulmonary arterial hypertension are limited; therefore, investigating the therapeutic strategies and novel therapies is critical for pulmonary arterial hypertension (PAH) treatment. This study aimed to evaluate the role of miRNA-126 (miR-126) and its associated signaling pathways and specific mechanisms for the pathogenesis of PAH.

MATERIALS AND METHODS: The pulmonary artery endothelial cells (PAECs) were isolated and identified. The miR-126 mimic and miR-126 inhibitor were synthesized. LV-3-miR-126 mimic viral vector and LV-3-miR-126 inhibitor vector were established and infected into pulmonary artery endothelial cells. Expression of sprouty-related EVH1 domain-containing protein 1 (SPRED1), phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) and miR-126 were detected using Real-time PCR (RT-PCR). Cell apoptosis (Annexin V-PE/7-AAD) and proliferation (PKH26) were examined by using FACScan flow cytometry. Vascular endothelial growth factor (VEGF), transforming growth factor β1 (TGF-β1) and TGF-β3 levels were evaluated using enzyme-linked immunosorbent assay (ELISA) kits.

RESULTS: miR-126 inhibited the endothelial cells related to SPRED1 and PIK3R2 expression. Over-expression of miR-126 significantly inhibited the PAECs apoptosis compared to PAECs and blank LV-3 vector group ($p<0.05$). miR-126 significantly triggered the PAECs proliferation compared to PAECs and blank LV-3 vector group ($p<0.05$). In functional analysis, miR-126 mimic significantly increased the cells amounts of S phases compared to PAECs and blank LV-3 vector group ($p<0.05$). Pre-infection with miR-126 mimic significantly enhanced the levels of VEGF, TGF-β1, and TGF-β3 compared to PAECs and blank LV-3 vector group ($p<0.05$).

CONCLUSIONS: miR-126 could affect cell apoptosis, proliferation, cell cycle, and modulate VEGF/TGF-β levels.

Key Words

Pulmonary arterial hypertension, MiRNA-126, Pulmonary artery endothelial cells, VEGF.

Introduction

Pulmonary arterial hypertension (PAH)[1] is a kind of severe disease, which is clinically assigned as the mean pulmonary arterial pressure (PAP) more than 25 mmHg at rest, as assessed by the right-side heart catheterization[2,3]. PAH always leads to decreased exercise tolerance and causes poor prognosis when the disease is untreated[4,5]. Clinically, the PAH always causes heart failure or other cardiovascular diseases[6]. PAH is also characterized by the proliferative and obstructive remodeling of the pulmonary vessel wall[7], medial thickening of large pulmonary muscular arteries[8] and is associated with the pathogenesis process of the chronic obstructive pulmonary diseases[9]. The previous studies[5,10] showed that the treatment options for the PAH are limited and un-effective in clinic; therefore, investigating the therapeutic strategies novel therapies is critical for PAH therapy. Recently, the aim of long-term treatment is to cause the regression of vascular remodeling, decrease the pulmonary vasoconstriction, reduce the proliferation, and prevent the inflammation and thrombosis[11]. MicroRNAs (miRNAs) are a series of small, non-coding, endogenous single-strand RNAs with a length of 20 to 25 nucleotides[12]. MiRNAs act as the gene regulators by triggering the messenger RNA (mRNA) degradation and translational repression[13]. miRNAs usually regulate various biological functions or processes, including cell proliferation, cell apoptosis, cell differentiation, neoplastic transformation and metabolism[14]. Researchers[15,16] reported that the abnormal expression of miRNAs is related to many diseases' pathogenesis, including cancers, autoimmune diseases, and inflammatory diseases. Previous scholars[17,18] proved that miRNAs play critical roles in the vascular system disorders, such as ocular neovascularization and tumor angiogenesis, and also play an important role in vascular development. Among the miRNAs, miRNA-126 plays

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an anti-atherosclerotic role in some diseases by activating the epithelial cells[19], triggering the growth of epithelial cells, and improving the status of the epithelial cells. Actually, the miR-126 is considered as the primary miRNA distributed in the endothelial cells, and which is closely correlated with the angiogenesis[20]. Till now, there were few studies focused on the expression and the specific role of miRNA-126 in PAH. Therefore, this work aimed to evaluate the role of miRNA-126 and its possible signaling pathways and specific mechanisms in the pathogenesis of PAH. Finally, we are expecting to provide the experimental findings for the clinical research on the therapy of PAH.

Materials and Methods

Isolation and Culture of Pulmonary Artery Endothelial Cells

The rat pulmonary arteries were obtained from Sprague Dawley (SD) rats (with the weight of 200 to 220 g), which were purchased from Experimental Animal Center of Third Military Medical University (Chongqing, China). The pulmonary arteries were obtained surgically for the rats and characterized as previously described[21,22]. Then, the pulmonary artery endothelial cells (PAECs) were harvested using 0.25% trypsin (Beyotime Biotech, Co. Int, Beijing, China) digestion at 37°C for 30 min. Next, the isolated PAECs were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA), and supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 μg/ml of streptomycin and 100 U/ml of penicillin (Gibco, Grand Island, NY, USA), 10 U/ml of heparin sulfate (Fisher Scientific, Waltham, MA, USA) at 37°C and 5% CO_2. The isolated PAECs always exhibit the typical cobblestone morphology and positively express the factor VIII according to the immunofluorescence. Identification for the PAECs was confirmed by factor VIII antigen and the acetylated low-density lipoprotein uptake[23]. Throughout the total course of this study, the PAECs have maintained the levels of VIII. This study was approved by the Ethics Committee of Daping Hospital, Third Military Medical University (Chongqing, China).

Plasmid Construction

The pG-LV3 lentiviral vector (LV3, GenePhama Co. Ltd, Shanghai, China) was employed to establish the pG-LV3-miR-126 mimic plasmid and pG-LV3-miR-126 inhibitor plasmid. Meanwhile, the negative sham oligonucleotides for the miR-126 were also synthesized by the GenePhama Co. Ltd. (Shanghai, China). The oligonucleotide sequences of the miR-126 mimic and inhibitor were listed as follows: miR-126 mimic sequences: 5'-TCGTACCG TGAGTAATAATGCG TGGTCA-3' and miR-126 inhibitor sequence: 5'-CGCATTATTACTCACG-GTACG A-3', respectively. Later, we synthesized the miR-126 related DNA double-chain artificially by using the sequences as the template sequences. After that, the synthesized double-chain sequences were sub-cloned into the pG-LV3 plasmid to form the LV-3-miR-126 mimic, LV3-miR-126 inhibitor and LV-3-miR-126 sham plasmid.

Lentivirus Packaging

The 293T cells were cultured by using the above methods. One day before the transfection, the 293T cells were seeded into a 10-cm dish. The LV-3-miR-126 mimic, LV3-miR-126 inhibitor, LV-3-miR-126 sham, the blank vector LV-3 plasmid, and the packing plasmids, including PG-p1-VSVG, PG-P2-REV, PG-P3-RRE, were transfected by using the RNAi-mate (GenePhama Co., Ltd, Shanghai, China), according to the instruction of the manufacturer. 72 h after the transfection, the supernatant was harvested by centrifugation at 2200×g at 4°C for 5 min. Then, the harvested supernatant was passed through a 0.45 μm syringe filter, and the supernatant was cleared by centrifugation again at 20000×g 4°C for 60 min. The titer of the isolated virus was evaluated by examining the expression of the GFP according to the manufacturer's instructions. The packaged lentiviruses were designated of the LV-3-miR-126 mimic, LV3-miR-126 inhibitor, and LV3 plasmid. Finally, the sequences of the above vectors were confirmed by using the sequence analysis method. Briefly, the synthesized lentivirus vectors were transformed to the *Escherichia Coli* DH5α competent cells. Then, the DH5α cells were inoculated to the agarose culture medium to screen the positive clone. Positive clones were identified by using enzyme digestion method. Finally, the sequences of the enzyme digestive vectors were examined by using the Sanger sequencing method according to the previous study[24].

PAECs Infection with LV-3-miR-126 Mimic and LV3-miR-126 Inhibitor Plasmid

The PAECs were infected with the LV-3-miR-126 mimic, LV3-miR-126 inhibitor and LV3 vector, at a multiplicity of infection ratio of 15[25], supplemented with the 5 μg/ml polybrene (Ge-

Table I. Primers for the RT-PCR assay.

Gene		Primers	Length (bp)
SPRED1	Forward Reverse	GGACACTTCCCGTTCCCTG CTGGCTCACTTGGCTTTGC	148
PIK3R2	Forward Reverse	ACCTGACTTCCCTGTGCTGC ACCTGACTTCCCTGTGCTGC	105
miR-126	Forward Reverse	GGTCGTACCGTGAGTA GAGCAGGCTGGAGAA	156
β-actin	Forward Reverse	TGACGTGGACATCCGCAAAG CTGGAAGGTGGACAGCGAGG	205

nePhama Co. Ltd, Shanghai, China). Then, the infection efficiency of the viral vectors was detected by microscopic analysis for the GFP fluorescence.

RNA Extraction, Reverse Transcription and Real-Time PCR

The total RNA of the PAECs was extracted by using the TRIzol kit and method (Beyotime Biotech, Co. Int, Shanghai, China) according to the manufacturer's instructions. Then, the extracted total RNA was reversely transcribed by using reverse transcription kit (Western Biotech. Chongqing, China) to synthesize the complementary DNA (cDNA), which would be used in the following Real-time PCR (RT-PCR) assay. PCR primers for sprouty-related, EVH1 domain-containing protein 1 (SPRED1), phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) and miR-126 were synthesized by Western Biotech. Inc. Co. (Chongqing, China) and the respective sequences were listed in Table I. The SYBR Green I Real-time PCR kit (Western Biotech. Chongqing, China) was used for amplification as described, according to the manufacturer's instructions. The amplification conditions for the RT-PCR were listed as the followings: 4 min at 94°C, followed by 35 cycles of 20 s at 94°C, 30 s at 60°C and 30 s at 72°C, and terminated at 10 min at 72°C. All the reactions were performed in triplicate with the final volume of 50 μl. The reactions were performed on a Real-time PCR instrument (Eppendorf, Hamburg, Germany). The $2^{-\Delta Ct}$ ($2^{-[(Ct\ of\ gene) - (Ct\ of\ U6)]}$) method was employed for the RT-PCR products analysis.

Apoptosis Assay

The evaluation of the apoptosis was performed basing on the manufacturer's instruments of the Annexin V-PE/7-AAD apoptosis detection kit (BD Biosciences, Bedford, MA, USA). Forty-eight hours post the viral plasmids transfection, the PAECs were harvested and suspended in 450 μl Annexin V binding buffer. Then, the Annexin V-PE (1 μl) and 7-AAD (5 μl) were added to the PAECs, and the PAECs were incubated for 5 min in the dark. The PAECs samples must be analyzed by the FACScan flow cytometry (BD Biosciences, Bedford, MA, USA) within 1 h post-staining. According to the classical method for flow cytometry, the staining cells were divided into necrotic cells, apoptotic cells (including early apoptotic and late apoptotic cells), and viable cells by calculating with the Cell Quest software (BD Biosciences, Franklin Lakes, NJ, USA). Finally, the apoptotic cell percentages for every group were calculated and compared. All the assays were performed at least in triplicate.

PKH26 Staining for PAECs Proliferation

PKH26 is a kind of lipophilic dye and emission of red fluorescence, and which can also be irreversibly combined with the cell membranes[26]. Meanwhile, the PKH26 can conduct fluorescence labeling for many kinds of cell lines. Briefly, 2×10^7 cells were trypsinized and counted through the trypan blue (Gibco, Rockville, MD, USA) by using the haemocytometer for the cell viability and the total cell number before staining. PAECs cells in each group were stained with the PKH26 dye (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 2×10^{-6} M/ml for 5 min and blocked with 1% BSA. Then, the PAECs were washed twice, plated and passaged to obtain to induce the PKH-26 dilution in two weeks. The fluorescence-activated PAECs sorting was conducted on the single cell suspensions by using the FACScan flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) 2 weeks post-labeling of PKH26. The PAECs were monitored with the light microscope (Model: CK40; Olympus, Tokyo, Japan) and the live imaging fluorescence microscope (Olympus, Tokyo, Japan). Subsequently, the proliferation index was calculated according to the previous study described[27].

Enzyme-Linked Immunosorbent Assay (ELISA)

The PAECs were cultured and digested by the 0.25% trypsin, and the cell suspension was diluted with PBS at the final concentration of 10^5 cells/ml. The above PAECs were frozen and thawing repeatedly to induce the cell lysis. Then, the cells were centrifuged at 200 r/min for 20 min, and supernatants were harvested for the ELISA assay. The supernatant was analyzed for the vascular endothelial growth factor (VEGF), transforming growth factor β1 (TGF-β1) and TGF-β3 concentration by using rat VEGF-A ELISA kit, rat TGF-β1 ELISA kit, and rat TGF-β3 ELISA kit (Sangon Biotech, Shanghai, China), respectively. The concentrations of the VEGF, TGF-β1, and TGF-β3 were examined by the color intensity of solutions by using ELISA microplate reader (Bio-Tek, Winooski, VT, USA) at the 450 nm and 570 nm, respectively. The optical imperfections were also corrected by reading the color intensity at 570 nm and subtracting from the reading at 450 nm. Finally, the VEGF, TGF-β1, and TGF-β3 concentration were calculated by comparing the associated readings with the pre-drawn standard curve, using the known concentrations. All the assays were performed at least in triplicate.

Immunohistochemistry

The PAECs were cultured on the cover-slip (Nunc, Rochester, NY, USA), and fixed with the 4% paraformaldehyde (Sangon Biotech, Shanghai, China) for 15 min. After washing with PBS for 3 times (5 min per time), the endogenous peroxidase was inactivated by treating with the 3% hydrogen peroxide for 5 min at the room temperature. Then, the cells were blocked with 5% BSA for 20 min and washed for 3 times again. The PAECs were incubated with rabbit anti-factor VIII polyclonal antibody (1:500 in PBS) (Catalogue No. ab203590; Abcam, Cambridge, MA, USA) at 4°C overnight. Then, the PAECs were washed for three times with PBS and incubated with the goat anti-rabbit peroxidase-conjugated IgG (1:500 in PBS) (Catalogue No. ab6721, Abcam, Cambridge, MA, USA) at room temperature for 1 h. Finally, the PAECs were washed for three times, immersed in the alkaline phosphatase labeled diaminobenzidine (ZSGB Bio. Inc. Co., Beijing, China), and rinsed in the distilled water. The images of stained PAECs were observed and acquired by using an inverted fluorescence microscope (Model: IX51; Olympus, Tokyo, Japan).

Statistical Analysis

All the data in this study were analyzed using SPSS software 19.0 (SPSS Inc., Armonk, NY, USA) and described as mean ± SD from at least three independent experiments. The Student's *t*-test was used for comparison between two groups. Tukey's post-hoc test which validated the analysis of variance (ANOVA) was employed for comparing the data among groups. *p*-value less than 0.05 was considered as a significant difference.

Results

Isolation and Identification for the PAECs

PAECs were isolated from the rat pulmonary arteries. They grew with increasing cell intensity following the culture days (Figure 1A). To confirm that the isolated cells are the PAECs, the marker molecule, factor VIII, was examined by using the immunohistochemistry assay. The results indicated that the cells positively expressed the factor VIII, but a negative control can't express factor VIII (Figure 1B). Meanwhile, the acetylated low-density lipoprotein uptake was also examined to confirm the isolated PAECs. The results showed that the ligand uptake in the PAECs was significantly higher compared to the negative cells (Figure 1C, $p<0.05$). Therefore, according to the observation, the factor VIII, and the acetylated low-density lipoprotein uptake findings, we observed that the isolated cells are the PAECs, which would be used in the following experiments.

LV-3-miR-126 Mimic and LV3-miR-126 Inhibitor were Successfully Established

In this study, the lentivirus was packed, the LV-3-miR-126 mimic and LV3-miR-126 inhibitor viral vectors were successfully constructed and confirmed by using the sequence analysis method. Meanwhile, the immunofluorescence assay results showed that the LV-3-miR-126 mimic and LV3-miR-126 inhibitor viral vector were expressed in PAECs with higher infection efficiency (Figure 1D).

MiR-126 Inhibits the Endothelial Cells Related SPRED1 and PIK3R2 Expression

The results indicated that the miR-126 expression was significantly increased in LV-3-miR-126 mimic group, significantly decreased in LV3-miR-126 inhibitor group compared to the blank PAECs and LV3 vector group (Figure 2A, $p<0.05$). Moreover, the SPRED1 (Figure 2B) and PIK3R2 (Figure 2C) levels were decreased

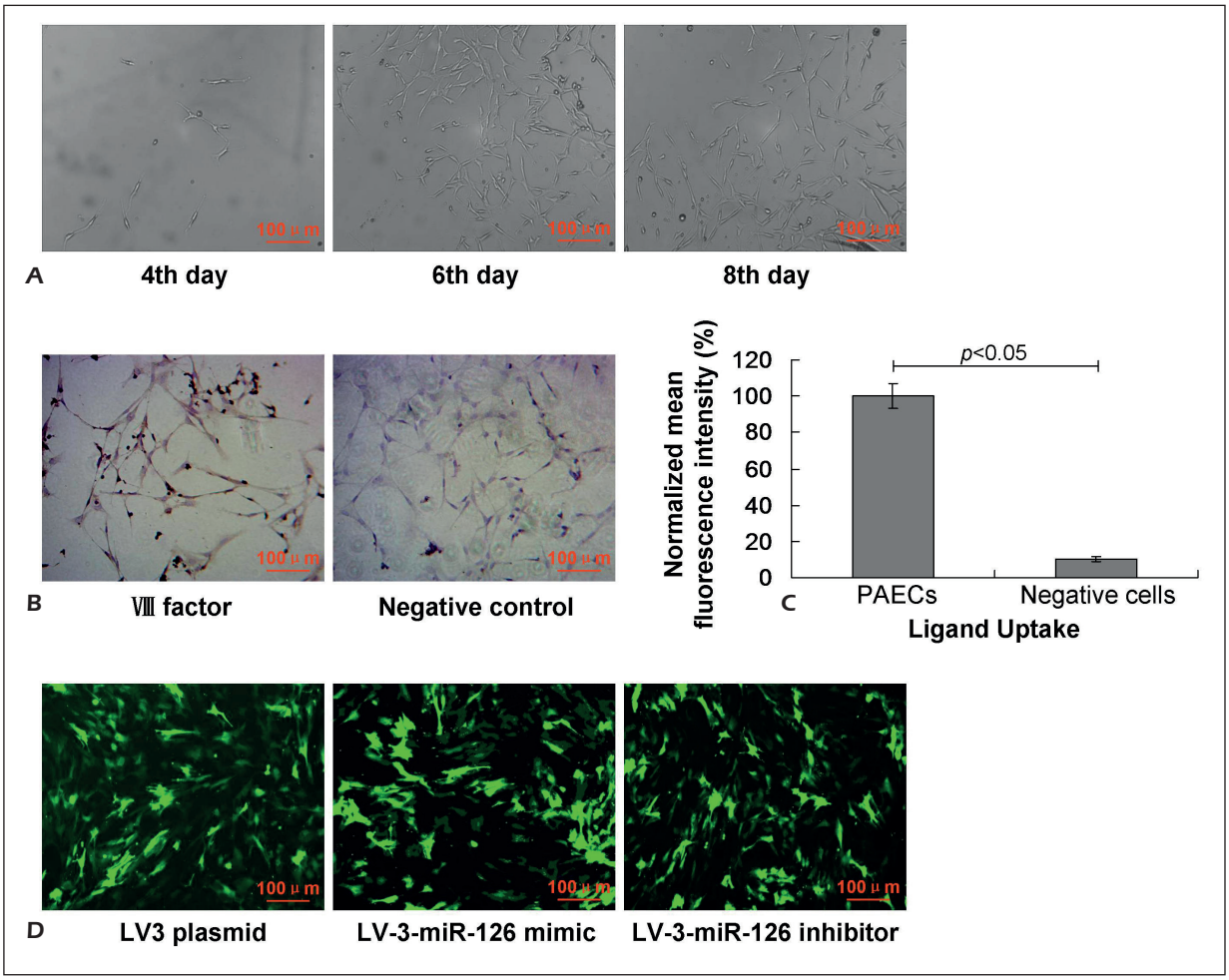

Figure 1. Identification for the isolated pulmonary artery endothelial cells and evaluation for the viral vector infection. **A**, Isolated cells observed under light microscopy. **B**, Identification for the isolated pulmonary artery endothelial cells by examining the factor VIII. The cells incubated with rabbit anti-factor VIII antibody. **C**, Identification for the isolated PAECs by examining the acetylated low-density lipoprotein uptake levels. **D**, Observation for viral vectors infection efficiency. The scale bars have been added in the images.

in LV-3-miR-126 mimic group and significantly increased in LV3-miR-126 inhibitor group, compared to the blank PAECs and LV3 vector group ($p<0.05$).

MiR-126 Inhibits the PAECs Apoptosis

To explore the effects of miR-126 on the cell growth of PAECs, the apoptosis was evaluated in the miR-126 mimic and miR-126 treated PAECs. The results showed that the over-expression of miR-126 could significantly decrease the apoptosis rate compared to the blank PAECs and LV3 vector group (Figure 3, $p<0.05$). Moreover, when the PAECs were treated with miR-126 inhibitor, the cell apoptosis rate was significantly increased compared to the blank PAECs and LV3 vector group (Figure 3, $p<0.05$).

MiR-126 Triggers PAECs Proliferation

According to the above findings for the miR-126 which caused apoptosis, the cell proliferation of PAECs was also evaluated. The results indicated that the over-expression of miR-126 significantly increased cell proliferation compared to the blank PAECs and LV3 vector group (Figure 4, $p<0.05$). Furthermore, the miR-126 inhibition significantly decreased the cell proliferation compared to the blank PAECs and LV3 vector group (Figure 4, $p<0.05$).

MiR-126 Regulates Cell Cycle in PAECs

In order to investigate the special role of miR-126 in the PAECs, the PAECs were infected with the LV-3-miR-126 mimic, miR-126 inhibitor or the blank LV3 viral vectors, and the DNA profiles

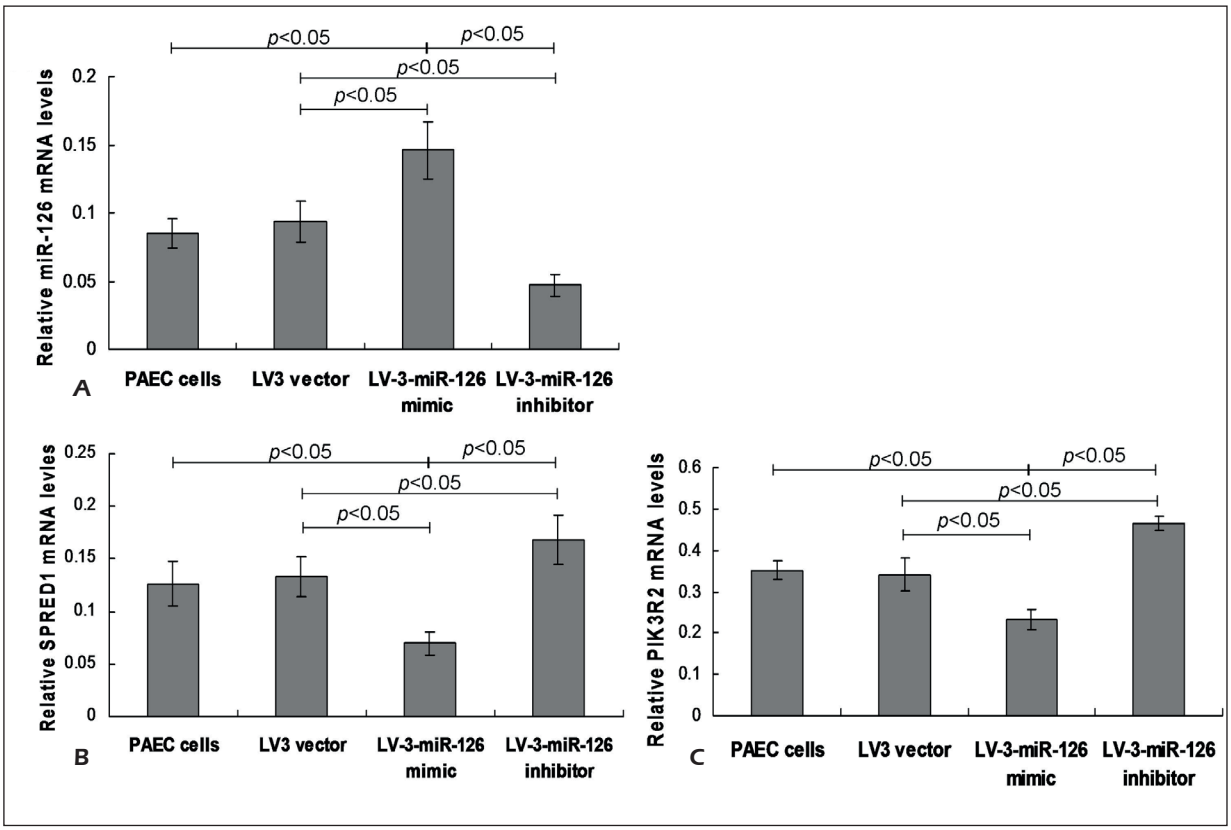

Figure 2. RT-PCR assay evaluation for miR-126, SPRED1 and PIK3R2 mRNA expression. **A**, Statistical analysis for miR-126 mRNA expression. **B**, Statistical analysis for SPRED1 mRNA expression. **C**, Statistical analysis for PIK3R2 mRNA expression. Data were presented as the mean ± SD with at least three independent experiments. $p<0.05$ represents the significant differences between the groups, which was illustrated in the images.

of S, G1 and G2 phase were examined by using the flow cytometry. According to the Figure 5, the cells in S phase were significantly increased and the cells in G1 phase were significantly decreased in the miR-126 mimic group compared to the blank PAECs and LV3 vector group (Figure 5, $p<0.05$). Meanwhile, the inhibition of the miR-126 slightly decreased the cells in the S phase, but without statistical significance (Figure 5, $p>0.05$). This finding suggests the positive regulation of miR-126 in the S phase in PAECs.

Changes of VEGF and TGF-β levels in PAECs

The effects of miR-126 on the VEGF and TGF-β expression in PAECs cells pre-infection with miR-126 mimic, miR-126 inhibitor, and the blank LV3 vector were evaluated by using the ELISA. Pre-infection with miR-126 mimic increased VEGF expression, however, pre-infection with miR-126 inhibitor decreased VEGF expression in PAECs (Figure 6A). Moreover, the pre-infection with miR-126 mimic also increased TGF-β1 (Figure 6B) and TGF-β3 (Figure 6C) expression, and pre-infection with miR-126 inhibitor decreased the TGF-β1 and TGF-β3 expression in PAECs. These results suggest that miR-126 may play a negative regulative role of VEGF and TGF-β in PAECs.

Discussion

Previously Oguz et al[7] illustrated that PAH has multi-factorial pathogenesis involving various biochemical pathway in the PAECs. Therefore, we speculated that improving the PAECs proliferation or remodeling the pulmonary vessel is the potential therapeutic method to PAH. Therefore, we investigated the effects of the miR-126 on the apoptosis and the proliferation of PAECs, and we explored the further mechanism for these effects. PAH causes the pulmonary vessel hypoxia, ischemia, and apoptosis or damage of the PAECs, and

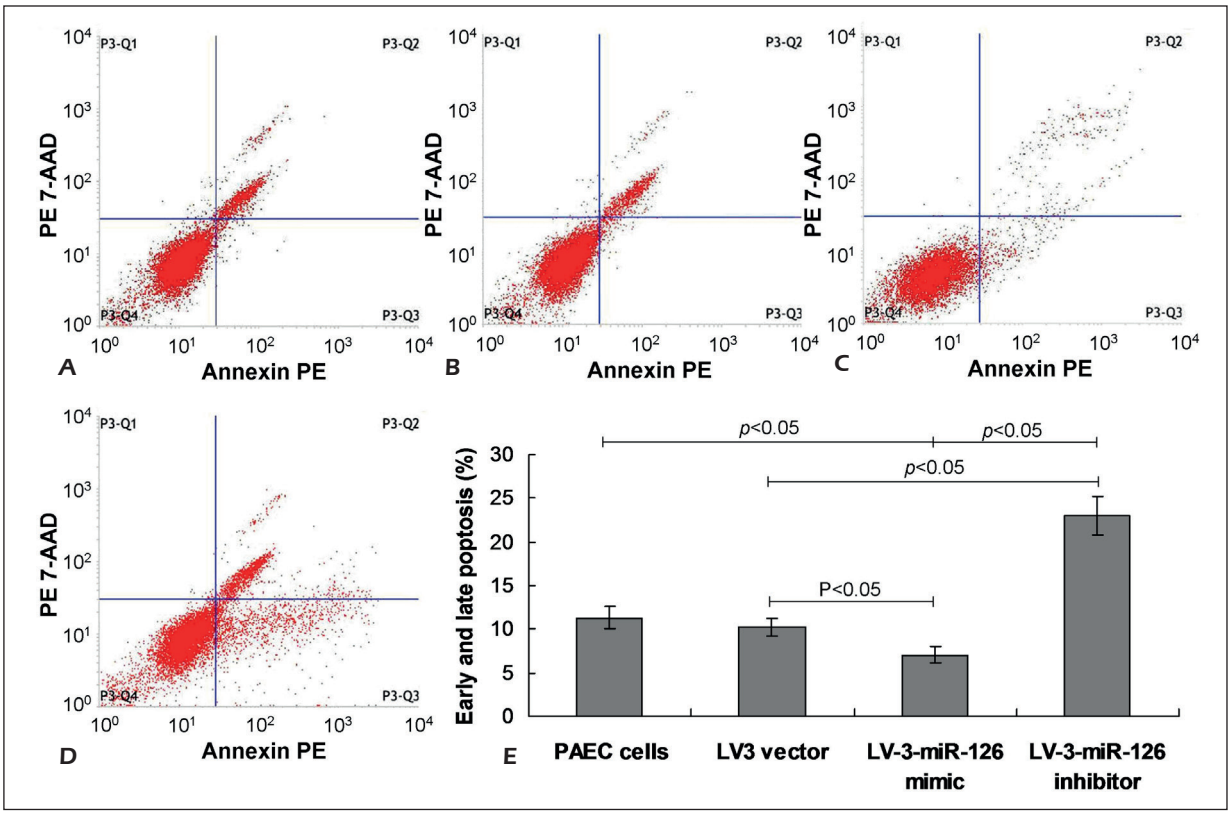

Figure 3. Evaluation for the apoptosis by using FACScan flow cytometry. **A**, Flow cytometry image for PAECs. **B**, Flow cytometry image for blank LV-3 vector. **C**, Flow cytometry image for LV-3-miR-126 mimic infected cells. **D**, Flow cytometry image for LV-3-miR-126 inhibitor infected cells. **E**, Statistical analysis for the cell apoptosis in each group. All the cells were stained with Annexin V-PE/7-AAD apoptosis detection kit and examined by FACScan flow cytometry. $p<0.05$ represents the significant differences between the groups, which was illustrated in the images.

further triggers a series of functional and structural changes in the pulmonary artery endothelial cells. Recently, the miR-126 has been proved to be an important molecule in the development and angiogenesis of the vascular system[28,29]. However, the function of miR-126 remains controversial in the angiogenesis, according to the previous reports[20,28,30] focusing on the role of miR-126 in the endothelial cells. Wang et al[20] found that the deletion of the miR-126 genes triggers the defects of endothelial cell proliferation and angiogenesis and damage the vascular integrity. However, Bai et al[31] proved the beneficial function of miR-126 in the pathological vascularization. Ye et al[31] found that the miR-126 may halt the hypoxia-induced neovascularization. In this study, our results indicated that the pre-infection of the miR-126 mimic viral vector could inhibit the PAECs apoptosis and enhance the PAECs proliferation. However, Zhou et al[12] reported that the miR-126 always triggers the tumor cell apoptosis and decreases the tumor cell proliferation, whose conclusion is un-consistent with our result. We speculated that miR-126 may play its role in multi-aspects in different diseases or different cell lines. According to the literature recordings, when the cells undergo the toxicity, stimuli, stress or tumorigenesis, miR-126 mainly triggers the apoptosis. When the cells undergo physical injury and mechanical damage, the miR-126 may trigger the compensatory mechanism and trigger cell proliferation. Therefore, the role of miR-126 in various diseases also needs to be fully clarified in the following studies. In the cell cycle and functional analysis, the results indicated that the up-regulation of the miR-126 triggered the enhanced cells amounts in S phase and induced the activation of the cell cycle, which illustrates an angiogenesis activator role for the miR-126 in PAECs. However, the miR-126 inhibitor can't trigger significant changes in the various phases of the cell cycle. The most possible speculation is that miR-126 level is relatively lower in normal PAECs, and therefore the effects of miR-126 inhibitor are

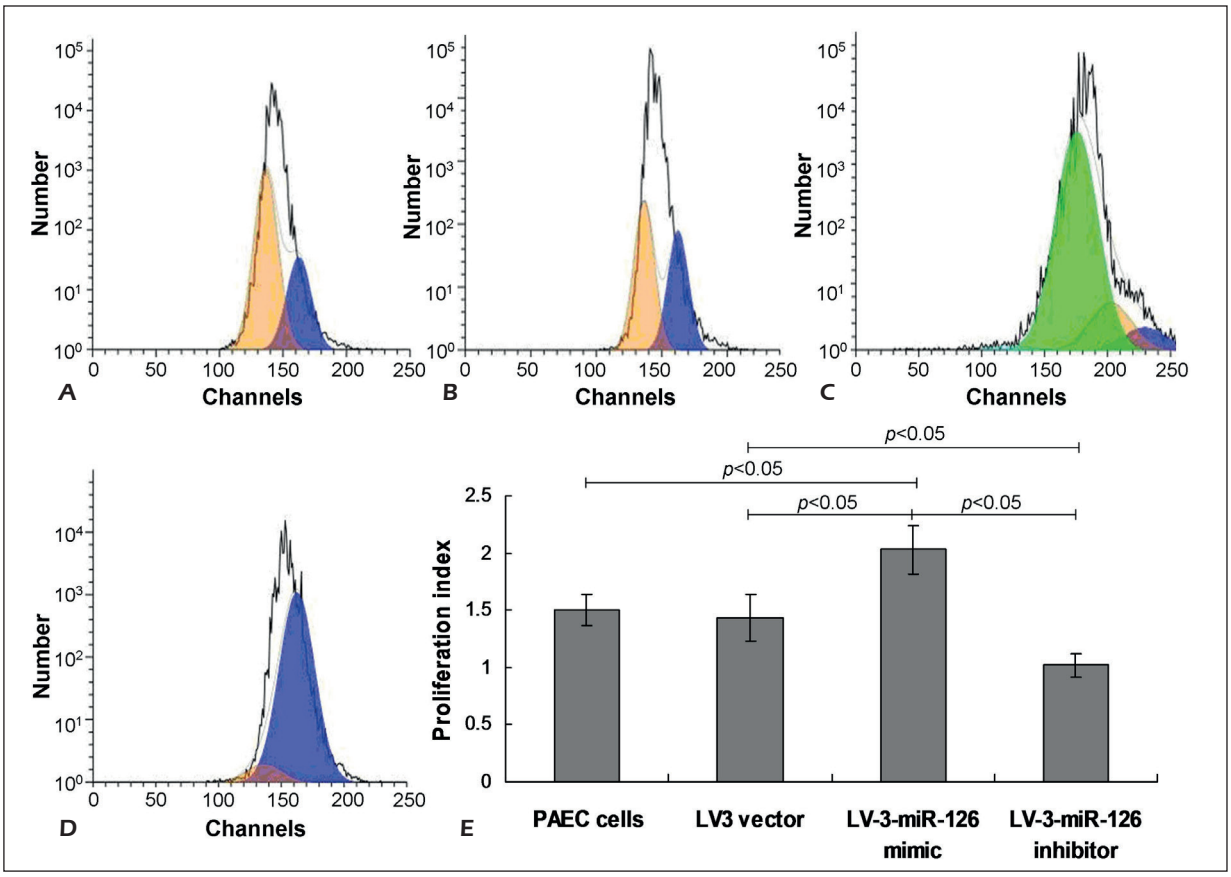

Figure 4. Observation for the cell proliferation by using FACScan flow cytometry. **A**, Flow cytometry image for PAECs. **B**, Flow cytometry image for blank LV-3 vector. **C**, Flow cytometry image for LV-3-miR-126 mimic infected cells. **D**, Flow cytometry image for LV-3-miR-126 inhibitor infected cells. **E**, Statistical analysis for the cell apoptosis in each group. All the cells were stained with PKH26 and examined by FACScan flow cytometry. Data were presented as the mean ± SD with at least three independent experiments. $p<0.05$ represents the significant differences between the groups, which was illustrated in the images.

limited. However, over-expression of miR-126 mimic could play its role effectively. Previous scholars[7,32] showed that many biomarkers, such as the growth factors, the adhesion molecules, and the brain natriuretic peptide, have been proposed for the development, diagnosis, and prognosis of PAH. Vascular Endothelial Growth Factor A (VEGF-A) is a member of the VEGF protein family, which always acts as a mediator for the pulmonary angiogenesis in both pulmonary hypertensive disorder and healthy individuals[33,34]. Therefore, we speculated that VEGF-A may be an important biomarker for the pathogenesis of PAH. In this work, miR-126 triggers significantly and sustains the increase of the VEGF, which is closely related to the neovascularization. To demonstrate the effects of miR-126 mimic, VEGF expression was detected after being infected with miR-126 inhibitor viral vector. MiR-126 inhibitor significantly inhibited the VEGF expression, which together with miR-126 results suggest that the miR-126 induced the up-regulation of VEGF expression. Previous studies[35-37] reported that the levels and activity of transforming growth factor beta (TGF-β) significantly increased in the PAH patients and animal models. TGF-β1 is a kind of multi-functional growth factor, which could regulate a broad range of biological processes, including cell differentiation, cell proliferation, migration, survival, etc.[38]. TGF-β3 plays an important role in cell differentiation and embryogenesis, and also mediates the inflammatory response, cell proliferation, tissue remodeling and formation[39]. Therefore, we also examined the levels of TGF-β1 and TGF-β3 in the miR-126 mimic or miR-126 inhibitor-treated PAECs. The results showed that both of the TGF-β1 and TGF-β3 levels were significantly increased when treated with

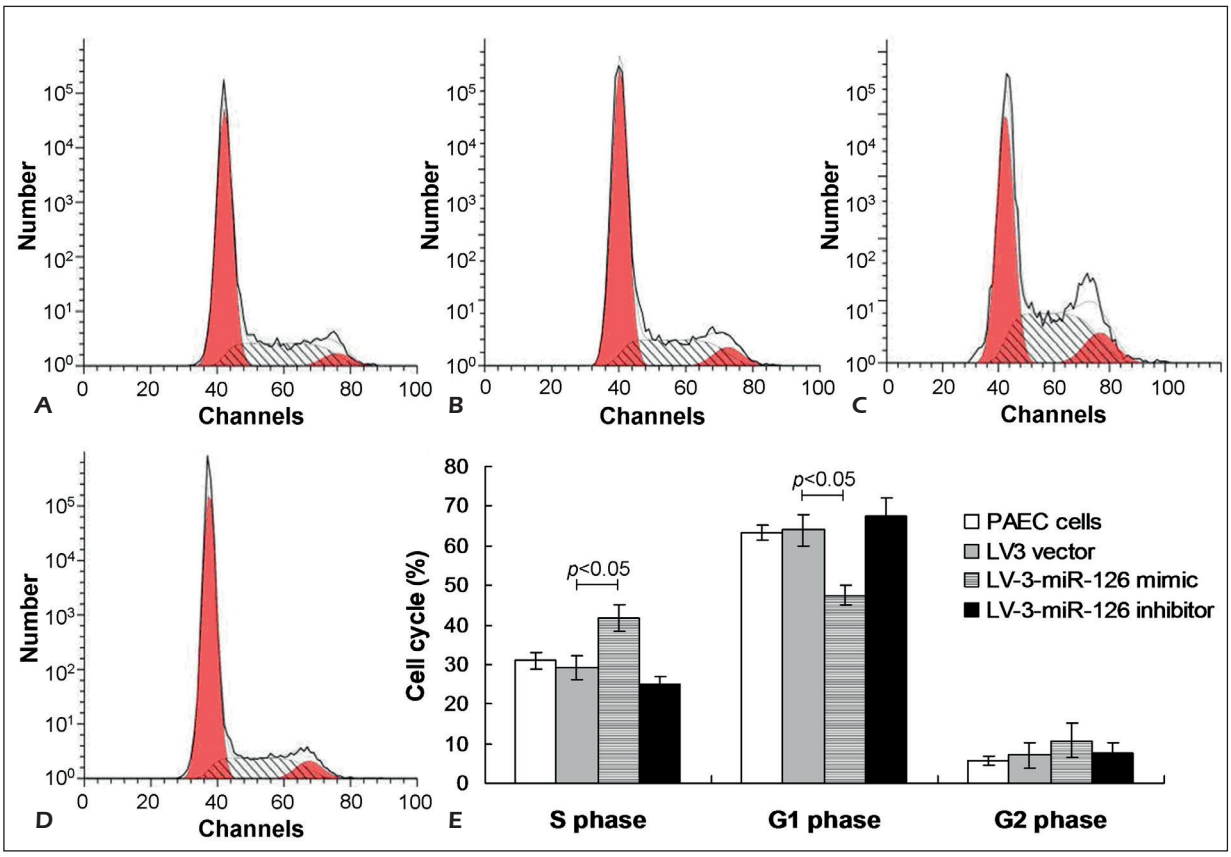

Figure 5. Up-regulation and down-regulation of miR-126 change the cells amounts in S phase in PAECs. The PAECs were treated with miR-126 mimic, miR-126 inhibitor or LV-3 blank vector. The cells were stained and analyzed for the cell cycle. **A**, Representative FACS plots for the cell cycle phases of PAECs cells. **B**, Representative FACS plots for the blank LV-3 vector cells. **C**, Representative FACS plots for the miR-126 mimic treated cells. **D**, Representative FACS plots for the miR-126 inhibitor-treated cells. **E**, Cell cycle analysis for the above FACS plots in each group. Data were presented as the mean ± SD with at least three independent experiments. $p<0.05$ represents the significant differences between the groups, which was illustrated in the images.

miR-126 mimic and decreased when treated with miR-126 inhibitor. Taken together, the results of VEGF and TGF-β suggest that miR-126 may be a promising therapeutic target for HAP therapy in clinic. However, there must be a few biomarkers or mechanism for the miR-126 regulated VEGF and TGF-β, which would be investigated in the following study. In this study, we also found that the SPRED1 and PIK3R2 levels were decreased in LV-3-miR-126 mimic group compared to other groups. The sprouty-related Ena/VASP homology 1 domain-containing protein 1 (SPRED1) is a regulator of growth factor and cytokine-induced ERK activation[40]. Meanwhile, the phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2) could also regulate cell growth by triggering the PIK/Akt signaling pathway[41]. Wang et al[20] reported that the miR-126 could activate the PAECs triggering the SPRED1 and PIK3R2. Therefore, in this research, we investigated the levels of SPRED1 and PIK3R2. However, no more experiments were performed, and no more details and data were provided in the present study. Although we received some interesting and significant findings, there were also a few limitations. Firstly, the morphological analysis was not performed for investigating the expression levels of miR-126, which would make the results more convincing. Secondary, roles of SPRED1 and PIK3R2 were not clarified. Thirdly, the stress conditions were not emphasized in this study, which may also involve the effects of miR-126. Fourthly, we have not validated the changes of miR-126 in the human specimen or animal models. Fifthly, the association between the VEGF over-expression and the development of PAH was not investigated in this report. Sixthly, the isolated PAECs cells did not illustrate a typical cobblestone morphology,

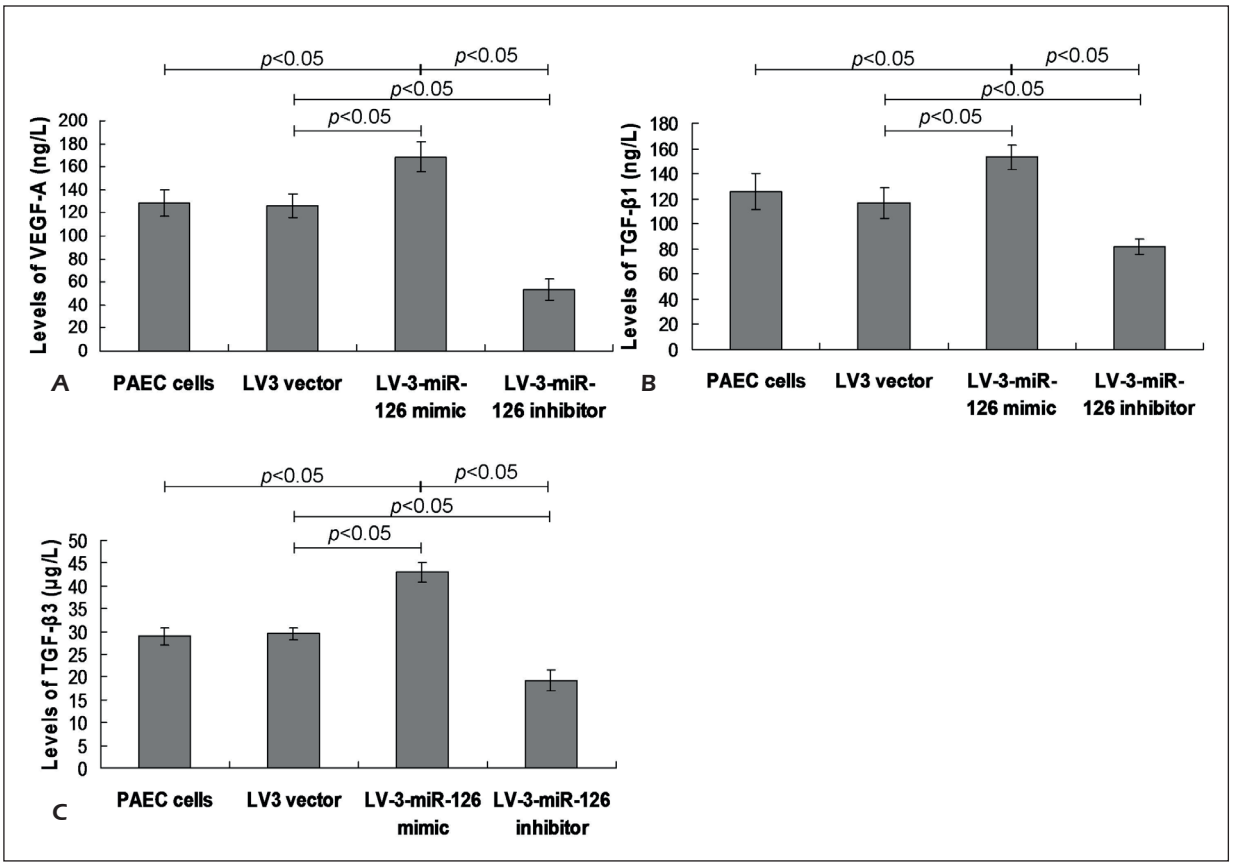

Figure 6. Effects of miR-126 on the VEGF and TGF-β expression in PAECs treated with miR-126 mimic and miR-126 inhibitor. **A**, Examination for the VEGF expression. **B**, Examination for the TGF-β1 expression. **C**, Examination for the TGF-β3 expression. The VEGF, TGF-β1 and TGF-β3 were detected by using the ELISA kit, respectively. Data were presented as the mean ± SD with at least three independent experiments. $p<0.05$ represents the significant differences between the groups, which was illustrated in the images.

but a spindle-like morphology, which may be caused by the bad culture condition. In a further paper, we would improve the culture condition for the PAECs.

Conclusions

We proved that miR-126 affects cell apoptosis, proliferation, cell cycle and levels of VEGF/TGF-β. The present study is an original and first study that focuses on the expression and the specific role of miRNA-126 in PAH.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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