

MicroRNA-15a-5p induces pulmonary artery smooth muscle cell apoptosis in a pulmonary arterial hypertension model via the VEGF/p38/MMP-2 signaling pathway

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Abstract. The aim of the present study was to investigate the role of microRNA-15a-5p (miR-15a-5p) in pulmonary arterial hypertension (PAH) and elucidate the underlying pro-apoptotic mechanism. Reverse transcription-quantitative PCR analysis and gene microarray hybridization were used to measure the expression of miR-15a-5p in the lung tissues of rats with monocrotaline (MCT)-induced PAH. Flow cytometry and caspase-3/9 activity assays were adopted to measure the apoptosis of pulmonary artery smooth muscle cells (PASMCs). The expression of apoptosis-related proteins was analyzed using western blotting. The results demonstrated that the expression of miR-15a-5p was significantly increased in the lung tissues of rats with MCT-induced PAH. In addition, the overexpression of miR-15a-5p reduced PASMC proliferation, induced apoptosis, promoted the activity of caspase-3/9, induced the protein expression of B-cell lymphoma 2-associated X protein (Bax), decreased the expression of B-cell lymphoma 2 (Bcl-2), increased inflammation, as indicated by the expression of tumor necrosis factor- α (TNF)- α and interleukin (IL)-1 β , IL-6 and IL-18, suppressed the protein expression of vascular endothelial growth factor (VEGF), and promoted the protein expression levels of phosphorylated (p)-p38 mitogen-activated protein kinase (p38) and matrix metalloproteinase (MMP)-2 in the PASMCs of rats with MCT-induced PAH. By contrast,

the downregulation of miR-15a-5p increased cell proliferation, decreased apoptosis, reduced the activity of caspase-3/9 and the protein expression of Bax, increased the expression of Bcl-2, inhibited inflammation (as suggested by the expression of TNF- α , IL-1 β , IL-6 and IL-18), induced the protein expression of VEGF, and suppressed the protein expression of p-p38 and MMP-2 in the PASMCs of rats with MCT-induced PAH. The inhibition of VEGF attenuated the effects of the overexpression of miR-15a-5p on the inhibition of cell proliferation, apoptotic rate, caspase-3/9 activity and protein expression of Bax, and it attenuated the increased inflammation, as indicated by the protein expression of p38 and MMP-2 in the PASMCs. In conclusion, the data of the present study demonstrated that miR-15a-5p induced the apoptosis of PASMCs in an animal model of PAH via the VEGF/p38/MMP-2 signaling pathway. However, further research is required to fully elucidate the role of miR-15a-5p in the development of PAH.

Introduction

Pulmonary hypertension (PH), a progressive disease of various origins, is associated with pulmonary vascular remodeling and results in right heart dysfunction (1). According to the classification of the World Health Organization (2), pulmonary arterial hypertension (PAH) is a major type of PH. The main histopathological manifestations of PAH include vasoconstriction, endothelial cell proliferation and fibrosis, smooth muscle cell proliferation and thrombosis in small pulmonary arteries, which lead to the elevation of pulmonary vascular resistance and, consequently, result in PAH (3,4). Despite the development and application of new types of anti-PAH drugs, the clinical management of PAH patients represents a challenge, and this condition is associated with high mortality rates and markedly poor prognosis (5). Long-term PAH induces vascular remodeling, thus resulting in pulmonary vascular complications, which are a direct cause of death.

Cell apoptosis is a type of cell death process, which is induced by the prestored cell death program and is triggered by factors *in vivo* and *in vitro* (6); it is also referred to as programmed cell death. Apoptosis is characterized by relatively intact cell membrane and organelles, cell shrinkage

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and karyopyknosis in terms of morphology (7). This phenomenon is completely different from the comprehensive cell structural dissolution and destruction that occur in necrosis. The early changes caused by apoptosis occur on the cell membrane surface. The balance between cell proliferation and apoptosis is an important mechanism for maintaining the functional integrity of the organs (8).

MicroRNAs (miRNAs) are a class of small, endogenous, non-coding RNA molecules, 21-22 nucleotides in length. These small miRNAs target one or more mRNAs, and they regulate gene expression through inhibiting target mRNAs or suppressing their translation (9). miRNAs are derived from non-coding RNAs. They transform from pri-miRNAs to pre-miRNAs (9). In addition, they produce functionally mature miRNAs through the Dicer complex. The mature miRNA can bind with the RNA-induced silencing complex (10) and exhibits complementary binding with target mRNAs; therefore, it can inhibit or prevent their translation. Several studies have indicated that miRNAs are involved in almost all pathophysiological processes, including embryonic development, cell differentiation, angiogenesis, tumor formation and damage repair (11).

Vascular endothelial growth factor (VEGF) is the most important factor for pulmonary vascular development (12). VEGF is a cytokine that remains multifunctional during the entire embryonic, fetal and postnatal periods. In addition, VEGF is the most potent and critical regulatory factor of pulmonary vascular growth and maintenance, and it is involved in the development of hyperoxia-induced lung injury in newborns. VEGF mainly acts during the early stage of pulmonary angiogenesis (13); it can promote the formation of an original vascular network. The expression levels of VEGF and its receptors (VEGFRs) are increased in the lung tissue under chronic hypoxic conditions and in lung tissue samples following monocrotaline (MCT)-induced lung injury (14). The upregulated expression of VEGF and VEGFR1-2 has been associated with pulmonary arteriolar endothelial and smooth muscle cell growth, thus contributing to pulmonary angiogenesis (15). MicroRNA-15a-5p (miR-15a-5p) can target VEGF to regulate inflammation, fibrosis, viability and matrix degradation, and it participates in several pathophysiological processes (16,17).

The aim of the present study was to investigate the role of miR-15a-5p in MCT-induced PAH and the underlying pro-apoptotic mechanism by establishing an animal model of PAH. It was also investigated whether the effects of miR-15a-5p on cell apoptosis and modulation of p38 mitogen-activated kinase (MAPK; p38)/matrix metalloproteinase (MMP)-2 levels in pulmonary artery smooth muscle cells (PASMCs) were mediated by targeting VEGF.

Materials and methods

Animal model. Male Wistar rats (8-10 weeks old, 220-250 g) were purchased from the Experimental Animal Center of Capital Medical University (Beijing, China). The rats (n=12) were maintained under standard conditions, including temperature 23±1°C, humidity 55-60% and a 12-h light/dark cycle, and were given access to food and water *ad libitum*. The rats were randomly assigned to the control and PAH groups. PAH

was induced in the rats by subcutaneous injection of 60 mg/kg MCT (Sigma-Aldrich; EMD Millipore) under anesthesia with 35 mg/kg pentobarbital sodium by intraperitoneal injection. After 21 days, lung tissues were collected and immediately frozen at -70°C for enzyme analysis.

Hematoxylin and eosin staining. Lung tissues were washed with PBS and fixed with 4% paraformaldehyde for 24 h at room temperature. Lung tissues were then cut into 5- μ m sections and stained with hematoxylin and eosin for 15 min at room temperature. Lung tissues were observed using confocal microscopy (magnification, x100; Leica SP5, Argon laser; Leica Microsystems, Inc.).

Specimens and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from the lung tissue samples or transfected pulmonary artery smooth muscle cells (PASMCs; see below) using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized from total RNA using the miRNA reverse transcription kit (Takara Biotechnology Co., Ltd.). The expression of miR-15a-5p was measured in triplicate on a Prism 7300 real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) using the following thermocycling conditions: Pre-denaturation at 95°C for 10 min and amplification at 95°C for 30 sec and at 60°C for 30 sec for 40 cycles. The sequences of primer pairs used to screen the transgenic mice were as follows: miR-15a, forward 5'-GTC CTCATCGCATAACCATAACA-3' and reverse 5'-GCTGAA GTAAGGTTGGCAATA-3'; U6 forward 5'-GCTTCGGCA GCACATATACTAAAAT-3' and reverse 5'-CGCTTCACG AATTTGCGTGTCAT-3'. The relative expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (18).

Gene microarray hybridization. Total RNA was hybridized using the G4471A-021828 platform (Agilent Technologies, Inc.) and data were quantified with Feature Extraction Software A.10.7.3.1 (Agilent Technologies, Inc.).

Cell culture. Pulmonary artery tissue samples were incubated in Hanks' solution containing collagenase (1.5 mg/ml, Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 4°C. The adventitia was carefully stripped off using fine forceps, and the endothelium was removed. The remaining smooth muscle was digested with collagenase and elastase for 50 min at 37°C. The PASMCs were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO₂ incubator. After growing to 70-80% confluence, 2x10⁵ cells were transfected with miR-15a-5p (5'-TAAGGCACGCGGTGAATGCC-3') and negative mimics (5'-CCCCCCCCCCCCCCCC-3') using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection for 4 h, a VEGF inhibitor (regorafenib, 4 nM, MedChemExpress) was added to the cells for 44 h at 37°C. Next, the cells (2x10⁵ cells) were transfected with miR-15a-5p and VEGF plasmid for 48 h.

Cell proliferation assays. For the CCK-8 assay, the PASMCs were transfected with miR-15a-5p, and 10 μ l of CCK-8 solution was added to each well for 2 h at 37°C. The absorbance

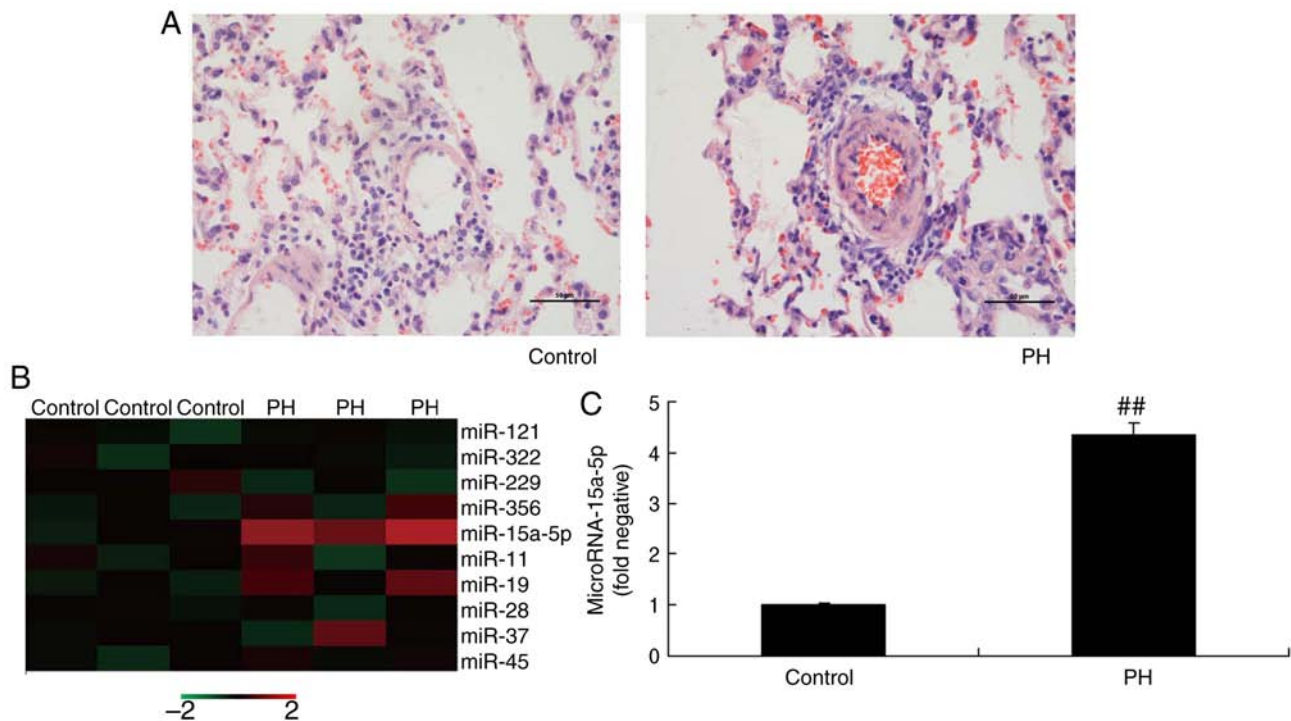


Figure 1. Expression of miR-15a-5p in the lung tissue of rats with monocrotaline-induced pulmonary arterial hypertension. (A) Pulmonary arterial hypertension examined by hematoxylin and eosin staining (original magnification, x100). (B) Expression of miRNAs by gene chip. (C) Expression of miR-15a-5p. $^{##}P < 0.01$ vs. control group. miR, microRNA; control, normal group; PH, pulmonary hypertension.

of each well was measured with a microplate reader (Bio-Tek Instruments, Inc.) set at 570 nM.

Apoptosis assay. At 48 h post-transfection, the PASCs (1×10^6) were harvested, and 5 μ l Annexin V-FITC (Key GEN Biotech Co., Ltd.) and 5 μ l PI solution (KeyGEN Biotech Co., Ltd.) were added and stained for 15 min in the dark. The relative percentage of apoptosis was analyzed by flow cytometry (EPICS XL-MCL; Beckman Coulter, Inc.).

Caspase-3 and caspase-9 activity assays. Caspase-3 and caspase-9 activity was measured by using caspase-3 (cat. no. C1115, Beyotime Institute of Biotechnology) and caspase-9 activity assay kits (cat. no. C1158, Beyotime Institute of Biotechnology). At 48 h post-transfection, the PASCs (1×10^6) were harvested and lysed at 4°C for 30 min using radioimmunoprecipitation assay (RIPA) buffer (cat. no. P0013K, Beyotime Institute of Biotechnology). Total protein was quantified using a bicinchoninic acid (BCA) assay, followed by incubation with 10 μ l Ac-DEVD-pNA and 10 μ l Ac-LEHD-pNA at 37°C for 2 h. The absorbance of each well was measured with a microplate reader (Bio-Tek Instruments, Inc.) set at 405 nM.

Western blot analysis. The PASCs were lysed at 4°C for 30 min using RIPA buffer and total protein was quantified using a BCA assay. Total protein (50 μ g) was separated by electrophoresis on 8-10% sodium dodecyl sulfate-polyacrylamide gels and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% skimmed milk in 1X Tris-buffered saline with 0.1% Tween-20 (TBST) followed by incubation overnight at 4°C with the following primary antibodies:

Anti-B-cell lymphoma 2 (Bcl-2; cat. no. sc-509; 1:1,000, Santa Cruz Biotechnology, Inc.), anti-Bcl-2-associated X protein (Bax; cat. no. sc-20067; 1:1,000, Santa Cruz Biotechnology, Inc.), anti-VEGF (cat. no. sc-81670; 1:500, Santa Cruz Biotechnology, Inc.), anti-phosphorylated (p)-p38 MAPK (cat. no. sc-17852-R; 1:1,000, Santa Cruz Biotechnology, Inc.), anti-p38 MAPK (cat. no. 8690S; 1:1,000, Cell Signaling Technology, Inc.), anti-MMP-2 (cat. no. sc-53630; 1:1,000, Santa Cruz Biotechnology, Inc.) and anti-GAPDH (cat. no. sc-51631; 1:5,000, Santa Cruz Biotechnology, Inc.). The membranes were washed with TBST and then incubated with horseradish peroxidase-conjugated secondary antibody (cat. no. sc-2004, 1:5,000, Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The membranes were detected by enhanced chemiluminescence (Bio-Rad Laboratories, Inc.) using a ChemiDoc MP system (Bio-Rad Laboratories, Inc.).

ELISA. At 48 h post-transfection, the supernatant was collected following centrifugation at 1,000 x g and 4°C for 10 min and used to measure the levels of tumor necrosis factor (TNF)- α (cat. no. RAB0477), interleukin (IL)-1 β (cat. no. RAB0274), IL-6 (cat. no. RAB0308) and IL-18 (cat. no. RAB0268) using ELISA kits (Sigma-Aldrich; EMD Millipore). The absorbance of each well was measured with a microplate reader (Bio-Tek Instruments, Inc.) set at 450 nM.

Immunofluorescence. At 48 h post-transfection, the PASCs were washed with PBS and fixed with 4% paraformaldehyde for 20 min. The cells were incubated with 5% bovine serum albumin (KeyGEN Biotech Co., Ltd.) and 0.1% Tris-X100 in PBS for 1 h and incubated with VEGF (1:100, Santa Cruz Biotechnology, Inc.) at 4°C overnight. The cells were

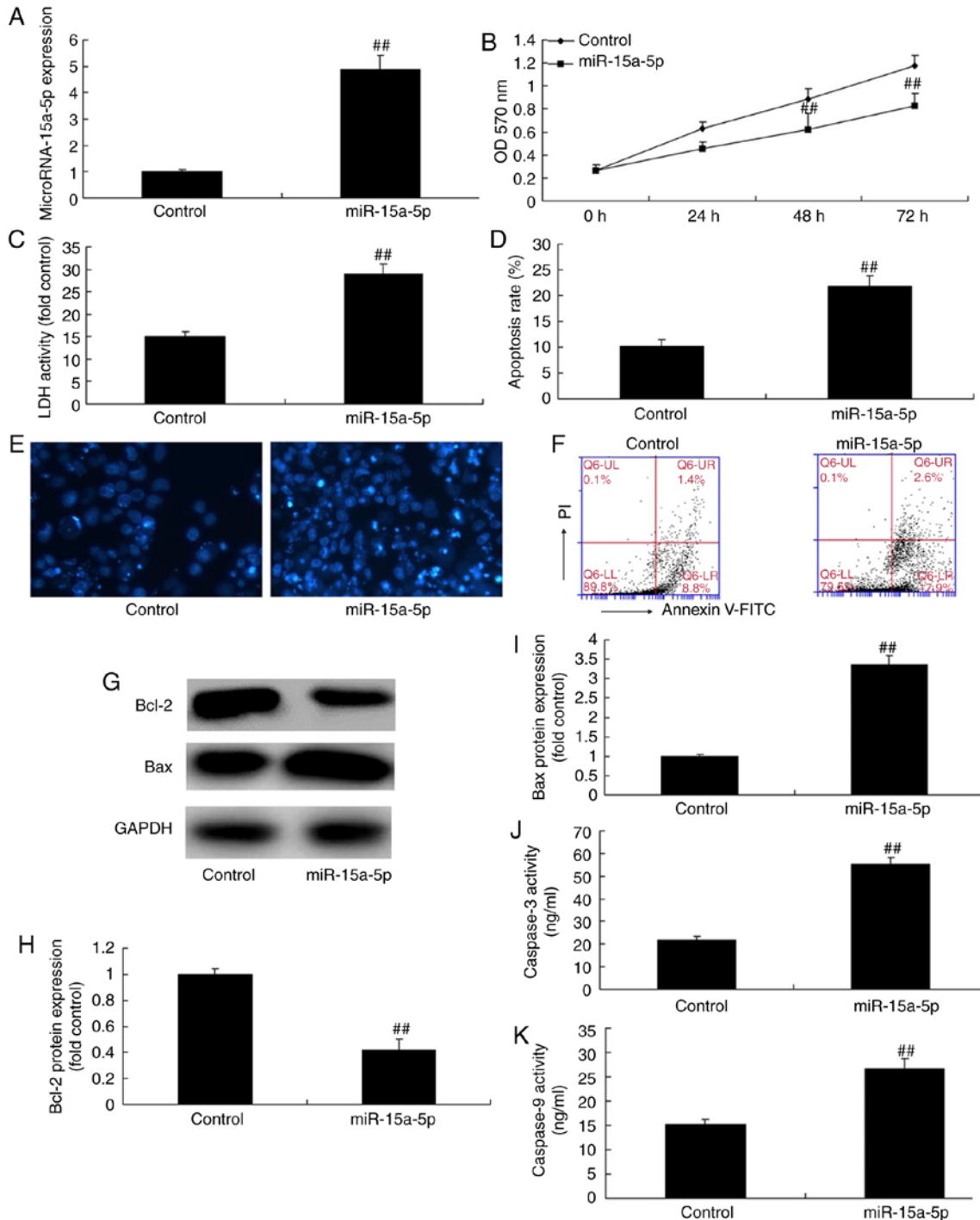


Figure 2. Overexpression of miR-15a-5p inhibited the proliferation and promoted the apoptosis of PAMSCs from rats with monocrotaline-induced pulmonary arterial hypertension. (A) Expression of miR-15a-5p; (B) cell proliferation; (C) LDH levels; (D) apoptosis rate; (E) DAPI assay (original magnification, $\times 100$); (F) detection of cell apoptosis by flow cytometry; (G) western blotting and (H and I) quantification of Bcl-2 and Bax protein expression, respectively; (J and K) caspase-3 and caspase-9 activity, respectively. $^{##}P < 0.01$ vs. control group. miR, microRNA; control, negative control group; miR-15a-5p, overexpression of miR-15a-5p; PAMSCs, pulmonary artery smooth muscle cells; LDH, lactate dehydrogenase; Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; OD, optical density.

then washed with PBS and incubated with goat anti-rabbit IgG-CFL 555 (sc-362272; 1:100, Santa Cruz Biotechnology, Inc.) for 1 h at 37°C and stained with a DAPI assay for 15 min in the dark. The cells were observed using confocal microscopy (Leica SP5, Argon laser; Leica Microsystems, Inc.).

Statistical analysis. The data are expressed as the mean \pm standard deviation using SPSS 17.0 (SPSS, Inc.). Student's t-test and

one-way analysis of variance followed by a Bonferroni-Dunn test were used for statistical analyses. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-15a-5p in rats with MCT-induced PAH. The expression of miR-15a-5p was examined in PAH rats

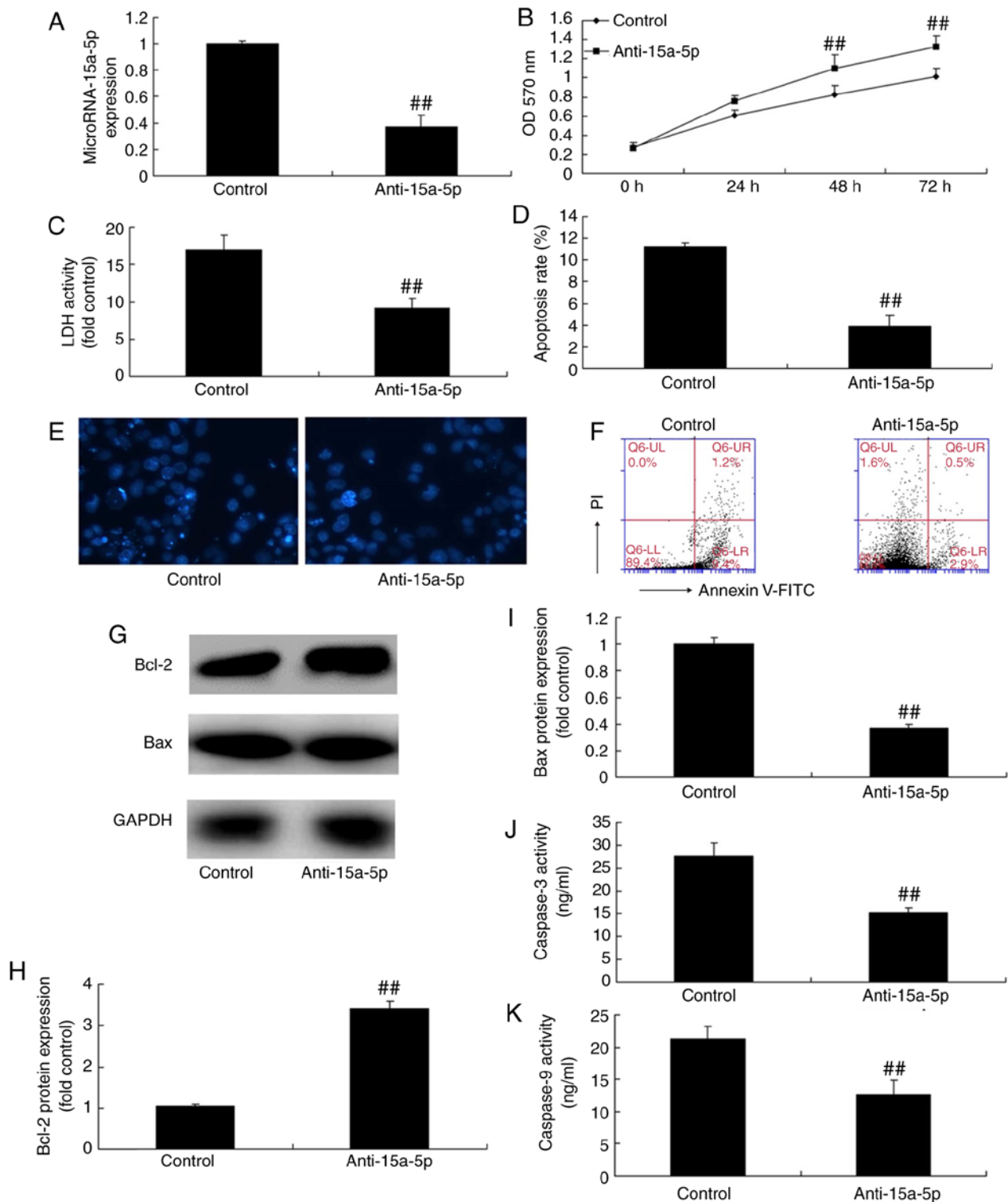


Figure 3. Downregulation of miR-15a-5p increased proliferation and decreased apoptosis of PASMCs from rats with monocrotaline-induced pulmonary arterial hypertension. Cell proliferation and apoptosis in monocrotaline-induced pulmonary hypertension by downregulation of miR-15a-5p. (A) Expression of miR-15a-5p; (B) cell proliferation; (C) LDH levels; (D) apoptosis rate; (E) DAPI assay (original magnification, x100); (F) detection of cell apoptosis by flow cytometry; (G) western blotting and (H and I) quantification of Bcl-2 and Bax protein expression, respectively; (J and K) caspase-3 and caspase-9 activity, respectively. ## $P < 0.01$ vs. control group. miR, microRNA; control, negative control group; anti-15a-5p, suppression of miR-15a-5p; PASMCs, pulmonary artery smooth muscle cells; LDH, lactate dehydrogenase; Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; OD, optical density.

using RT-qPCR analysis and a gene chip microarray. As shown in Fig. 1A, H&E staining revealed that, compared with the control group, vascular lesions in PAH rats were observed in the arteries present in bronchovascular bundles, and they were typically characterized by fibrocellular endothelial prolifera-

tion in the intimal layer and medial muscular hypertrophy. The expression of several miRNAs was downregulated in PAH rats, compared with that in the control group (Fig. 1B). However, the expression of miR-15a-5p was significantly increased in PAH rats (Fig. 1C). Taken together, these results indicate that

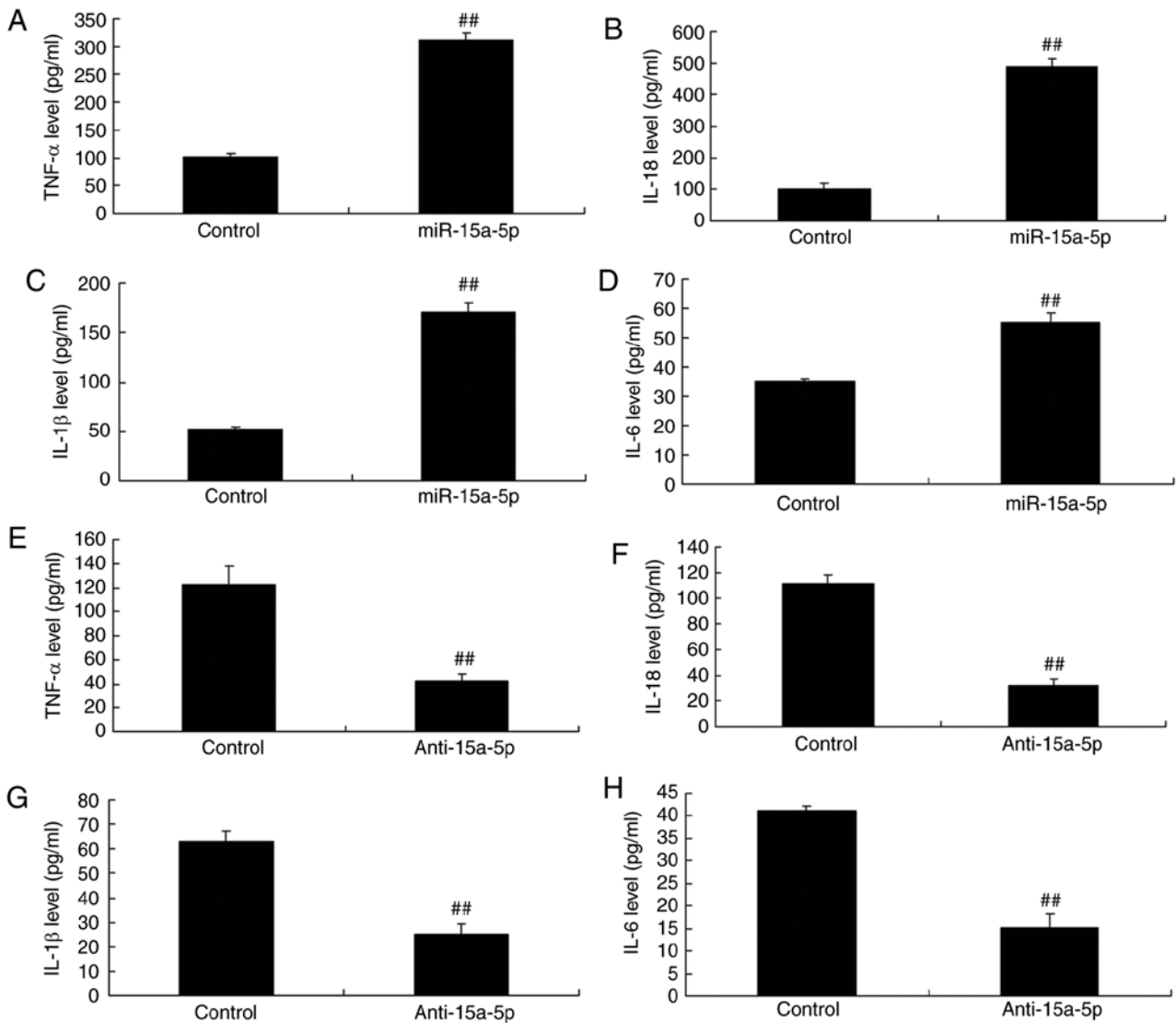


Figure 4. Changes of Inflammation in PSMCs from rats with monocrotaline-induced pulmonary arterial hypertension by miR-15a-5p. Levels of (A) TNF- α , (B) IL-18, (C) IL-1 β and (D) IL-6 with overexpression of miR-15a-5p. Levels of (E) TNF- α , (F) IL-18, (G) IL-1 β and (H) IL-6 with downregulation of miR-15a-5p. ##P<0.01 vs. control group. miR, microRNA; control, negative control group; miR-15a-5p, overexpression of miR-15a-5p; anti-15a-5p, suppression of miA-15a-5p; PSMCs, pulmonary artery smooth muscle cells; TNF, tumor necrosis factor; IL, interleukin.

the expression of miR-15a-5p is upregulated in PAH, and it may represent an important regulatory factor.

Overexpression of miR-15a-5p inhibits the proliferation and promotes the apoptosis of PSMCs in rats with MCT-induced PAH. The expression of miR-15a-5p was upregulated in the PSMCs by using miR-15a-5p mimics. As shown in Fig. 2A, the miR-15a-5p mimics increased the expression of miR-15a-5p in the cells, compared with that in the cells in the negative control group. As shown in Fig. 2B-K, the overexpression of miR-15a-5p led to significantly reduced cell proliferation and significantly increased the levels of lactate dehydrogenase (LDH) and the apoptosis of PSMCs. The overexpression of miR-15a-5p significantly increased the activity of caspase-3/9 and the protein expression of Bax, and it decreased the expression of Bcl-2 in the PSMCs of rats with MCT-induced PAH.

Downregulation of miR-15a-5p promotes the proliferation and inhibits the apoptosis of PSMCs in rats with MCT-induced

PAH. To examine the mechanism of the apoptosis induced by anti-miR-15a-5p, the PSMCs were transfected with anti-miR-15a-5p mimics. As shown in Fig. 3A, the expression of miR-15a-5p was downregulated in the cells compared with that in cells of the negative control group. As expected, cell proliferation was increased, whereas the level of LDH and apoptosis rate were suppressed in the PSMCs following transfection (Fig. 3B-F). In addition, the reduced expression of miR-15a-5p led to significant inhibition of caspase-3/9 activity and protein expression of Bax, and it promoted the expression of Bcl-2 in the PSMCs of rats with MCT-induced PAH (Fig. 3G-K).

Effects of miR-15a-5p on inflammation in the PSMCs of rats with MCT-induced PAH. Subsequently, the potential role of miR-15a-5p in inflammation in PSMCs was examined. As shown in Fig. 4A-D, the levels of TNF- α , IL-1 β , IL-18 and IL-6 were significantly increased in the PSMCs overexpressing miR-15a-5p. The role of miR-15a-5p in inflammation in the

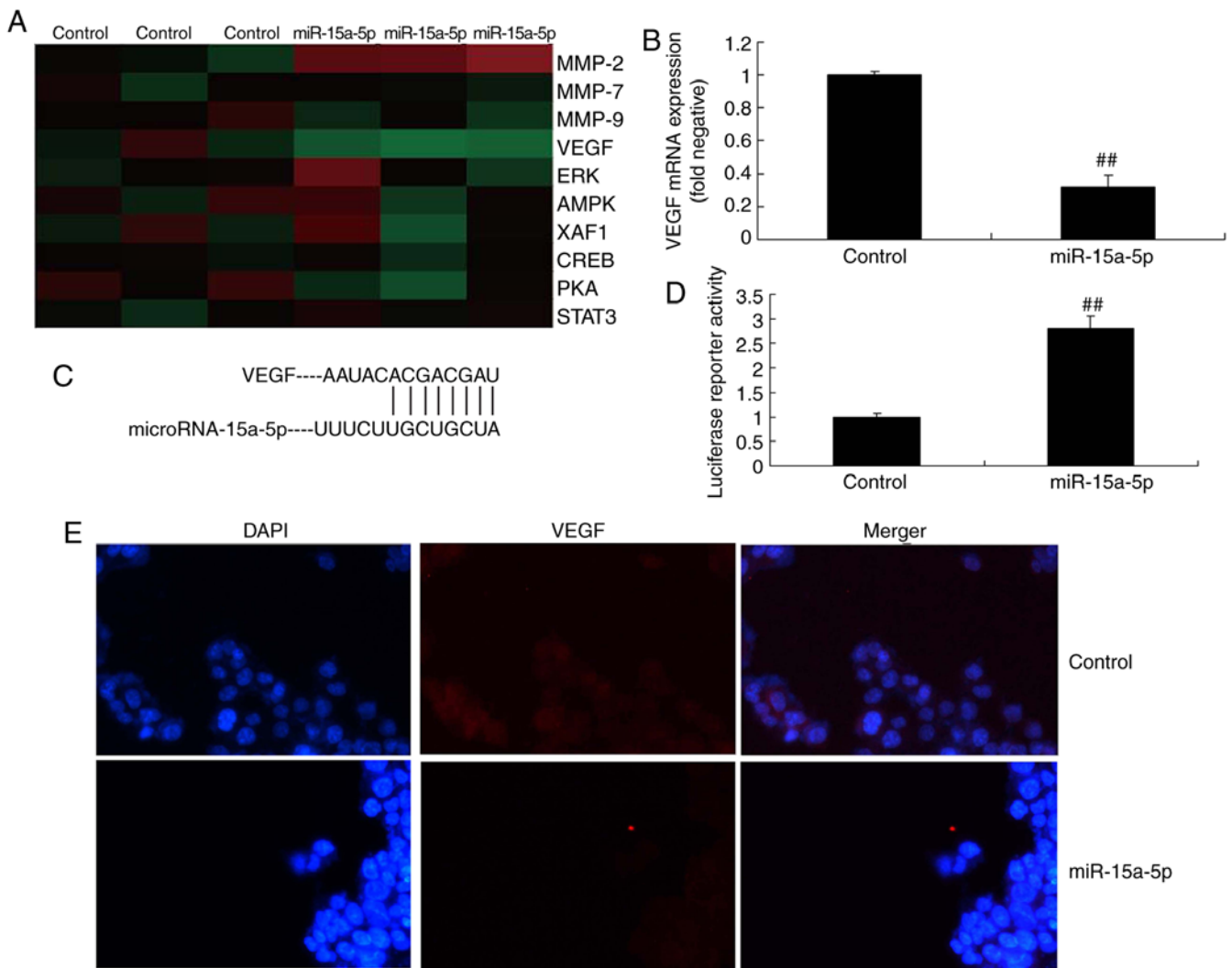


Figure 5. miR-15a-5p targeted the VEGF signaling pathway in PSMCs from rats with monocrotaline-induced pulmonary arterial hypertension. (A) Signaling pathway gene chip analysis. (B) mRNA expression of VEGF. (C) miR-15a-5p targets VEGFA. (D) Immunofluorescence results and (E) images showing the protein expression of VEGF (original magnification, x100). ^{##}P<0.01 vs. control group. miR, microRNA; Control, negative control group; miR-15a-5p, overexpression of miR-15a-5p; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase; AMPK, AMP-activated protein kinase; XAF1, X-linked inhibitor of apoptosis protein-associated factor 1; CREB, cAMP response element-binding protein; PKA, protein kinase A; STAT3, signal transducer and activator of transcription 3; PSMCs, pulmonary artery smooth muscle cells.

PASMCs was further examined. As shown in Fig. 4E-H, the levels of TNF- α , IL-1 β , IL-18 and IL-6 in the PSMCs were lower when the miR-15a-5p expression was downregulated, as compared with those in cells of the negative control group.

miR-15a-5p targets the VEGF/p38/MMP-2 signaling pathway.

A gene chip was used to examine the changes in regulatory proteins, and it revealed that the protein expression of VEGF differed in the PSMCs of rats with MCT-induced PAH with varied expression of miR-15a-5p (Fig. 5A). The RT-qPCR analysis demonstrated that overexpression of miR-15a-5p suppressed the mRNA expression of VEGF compared with that in the negative control group (Fig. 5B). Subsequently, it was inferred that miR-15a-5p targeted VEGF (Fig. 5C). Immunofluorescence demonstrated that the overexpression of miR-15a-5p suppressed the protein expression of VEGF (Fig. 5D and E). Western blotting was then performed to analyze the protein expression, and it demonstrated that the overexpression of miR-15a-5p significantly suppressed the

protein expression of VEGF and enhanced the protein expression of p-p38 and MMP-2 in the PSMCs (Fig. 6A-D). By contrast, following suppression of miR-15a-5p, the protein expression of VEGF was enhanced and the protein expression of p-p38 and MMP-2 was significantly suppressed in the PSMCs (Fig. 6E-H). Taken together, these results indicate that miR-15a-5p may exert a protective effect against MCT-induced PAH via the VEGF/p38/MMP-2 signaling pathway.

Inhibition of VEGF attenuates the effects of anti-miR-15a-5p on the PSMCs of rats with MCT-induced PAH.

As expected, western blot analysis demonstrated that the inhibition of VEGF by 4 nM regorafenib for 48 h suppressed the protein expression of VEGF and Bcl-2 and induced the protein expression of p-p38, MMP-2 and Bax in PSMCs, which were downregulated by miR-15a-5p inhibition (Fig. 7A-F). The molecular mechanism by which VEGF reverses the effects of miR-15a-5p inhibition on MCT-induced PAH was then investigated. As shown in Fig. 8A-E, the inhibition of VEGF attenuated the effects of

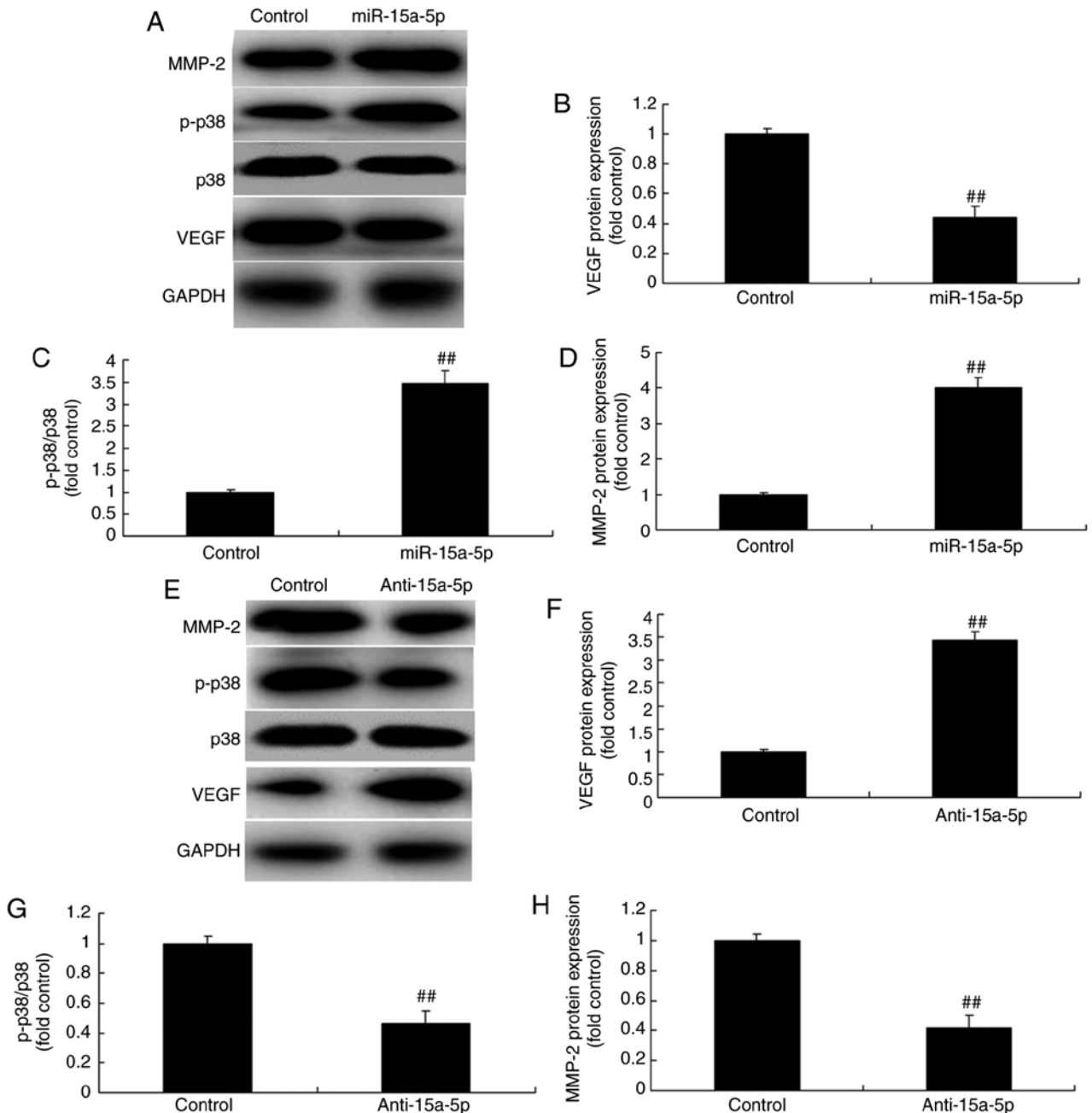


Figure 6. miR-15a-5p regulated the VEGF/p38/MMP-2 signaling pathway in PASMCs from rats with monocrotaline-induced pulmonary arterial hypertension. (A) Protein expression of VEGF, p-p38, p38 and MMP-2 was detected using western blot analysis. Quantification of (B) VEGF, (C) p-p38 and (D) MMP-2 protein expression with overexpression of miRNA-15a-5p. (E) Protein expression of VEGF, p-p38, p38 and MMP-2 detected using western blot analysis. Quantification of (F) VEGF, (G) p-p38 and (H) MMP-2 protein expression with downregulation of miR-15a-5p. ^{##} $P < 0.01$ vs. control group. miR, microRNA; control, negative control group; miR-15a-5p, miR-15a-5p overexpression; anti-15a-5p, downregulation of miR-15a-5p; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; PASMCs, pulmonary artery smooth muscle cells; p-p38, phosphorylated p38.

anti-miR-15a-5p on cell proliferation and increased the apoptotic rate and activity of LDH in the PASMCs. In addition, the inhibition of VEGF significantly increased the activity of caspase-3/9 in MCT-induced PAH, which was decreased by inhibition of miR-15a-5p (Fig. 8F and G). Furthermore, the inhibition of VEGF significantly increased the levels of TNF- α , IL-1 β , IL-18, and IL-6 in MCT-induced PAH, which were inhibited by miR-15a-5p downregulation (Fig. 8H-K).

Upregulation of VEGF attenuates the effects of miR-15a-5p on PASMCs in rats with MCT-induced PAH. To determine

the effect of VEGF on the function of miR-15a-5p in PAH, a VEGF plasmid was used. This plasmid reduced the protein expression levels of p-p38, MMP-2, and Bax, and increased the protein expression level of Bcl-2 in PASMCs overexpressing miR-15a-5p, compared with that in the miR-15a-5p overexpression group without the plasmid (Fig. 9A-F). As shown in Fig. 10A-G, the upregulation of VEGF increased cell proliferation, reduced LDH activity and apoptotic rate, and inhibited the activity of caspase-3/9 in PASMCs with miR-15a-5p overexpression, compared with cells with miR-15a-5p overexpression alone (Fig. 10F and G). Therefore,

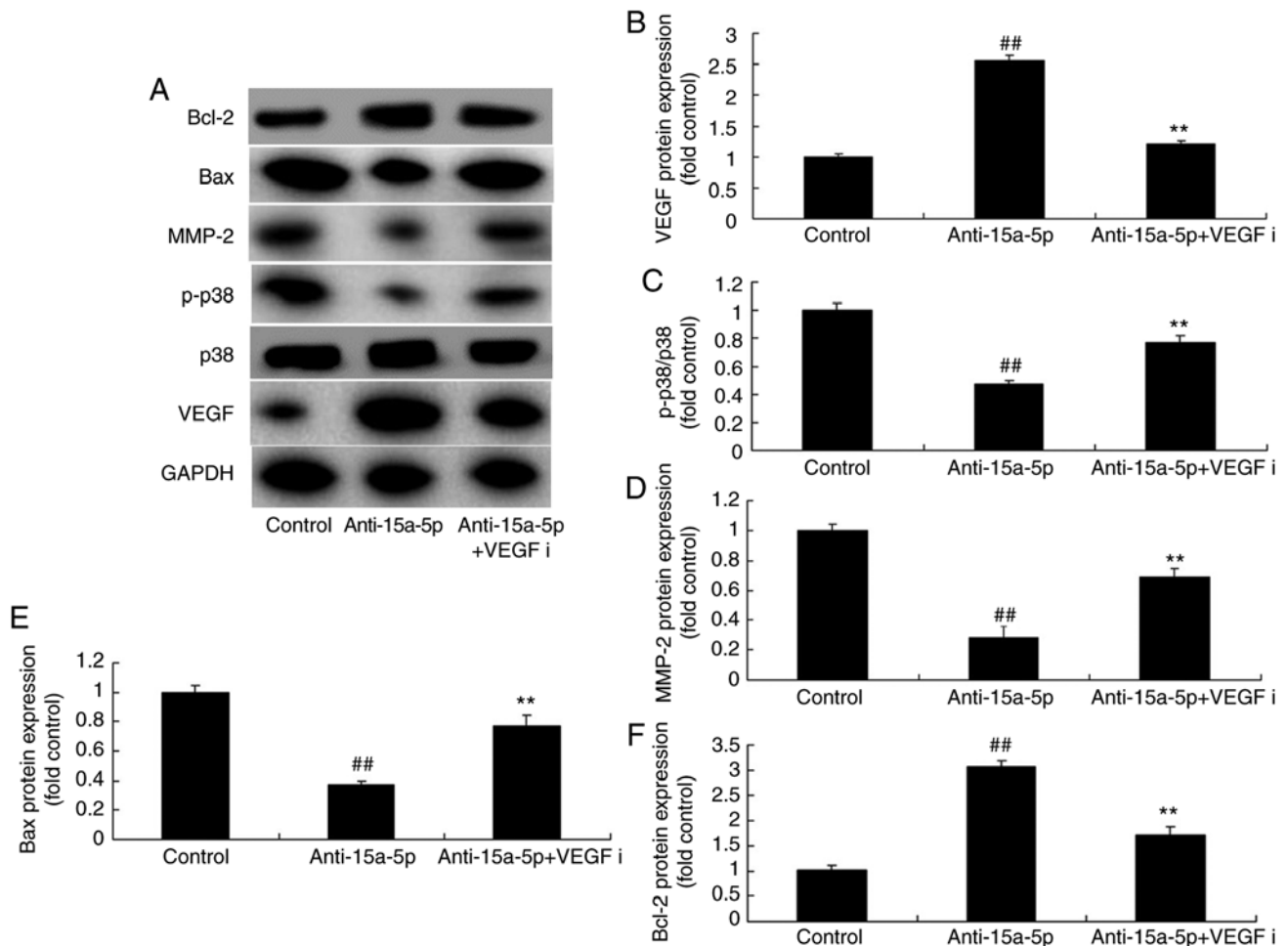


Figure 7. Inhibition of VEGF affected the VEGF/p38/MMP-2 signaling pathway in PASMCs from rats with monocrotaline-induced pulmonary arterial hypertension by attenuating the effects of miR-15a-5p inhibition. (A) Expression of VEGF, p-p38, MMP-2, Bax and Bcl-2 proteins detected using western blot analysis. Quantification of (B) VEGF, (C) p-p38, (D) MMP-2, (E) Bax and (F) Bcl-2 protein expression. ^{##}P<0.01 vs. control group, ^{**}P<0.01 vs. anti-15a-5p group. VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; PASMCs, pulmonary artery smooth muscle cells; p-p38, phosphorylated p38; Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; control, negative control group; miR, microRNA; anti-15a-5p, downregulation of miR-15a-5p; VEGFi, VEGF inhibitor.

VEGF upregulation significantly suppressed the levels of TNF- α , IL-1 β , IL-18 and IL-6 in the PASMCs overexpressing miR-15a-5p (Fig. 10H-K).

Discussion

Pulmonary vascular development includes vascular genesis and formation (19). Vascular genesis marks the process of differentiation of stromal cells to form blood vessels (19). By contrast, vascular formation is the process during which existing vessels form new vessels through germination (3). It has been suggested that multiple factors regulate pulmonary vascular development (3), and VEGF is one of the most important factors. It has been reported that the regulatory effects of premature birth, partial oxygen pressure, inflammatory cytokines, and other signal systems on the expression of normal growth factors may affect lung and pulmonary vascular growth. However, most studies to date have mainly focused on VEGF. VEGF is a multi-functional cytokine that regulates cell proliferation, migration, survival and differentiation in several physiological and pathological processes (20). VEGF is involved in endothelial PAS domain-containing protein 1

mutation-induced congenital heart disease (21). In addition, it is well known that chronic hypoxia increases VEGF expression in lung tissues and that VEGF is likely to be a modulator of chronic hypoxia-induced pulmonary vascular remodeling (14). It has also been reported that VEGF is increased in rats with hypoxia and MCT-induced PH and vascular remodeling (14). Serum VEGF levels are associated with the presence of PAH in systemic sclerosis (22). Being an angiogenic biomarker, the plasma concentration of VEGF is higher in PH patients (23). Moreover, the VEGF receptor inhibitor Su5416 may induce endothelial cell apoptosis, but Su5416 combined with chronic hypoxia induced severe PH in a rat model (24,25). The results of the present study demonstrated that the expression of miR-15a-5p in lung tissue was increased in the PAH group compared with that in the control group. In addition, the overexpression of miR-15a-5p inhibited the proliferation and promoted the apoptosis of PASMCs in rats with MCT-induced PAH through the suppression of VEGF expression. Ye *et al* reported that miR-15a reduced inflammation in the retinal endothelial cell barrier by reducing transforming growth factor- β 3/VEGF signaling (26). However, the results were obtained from one

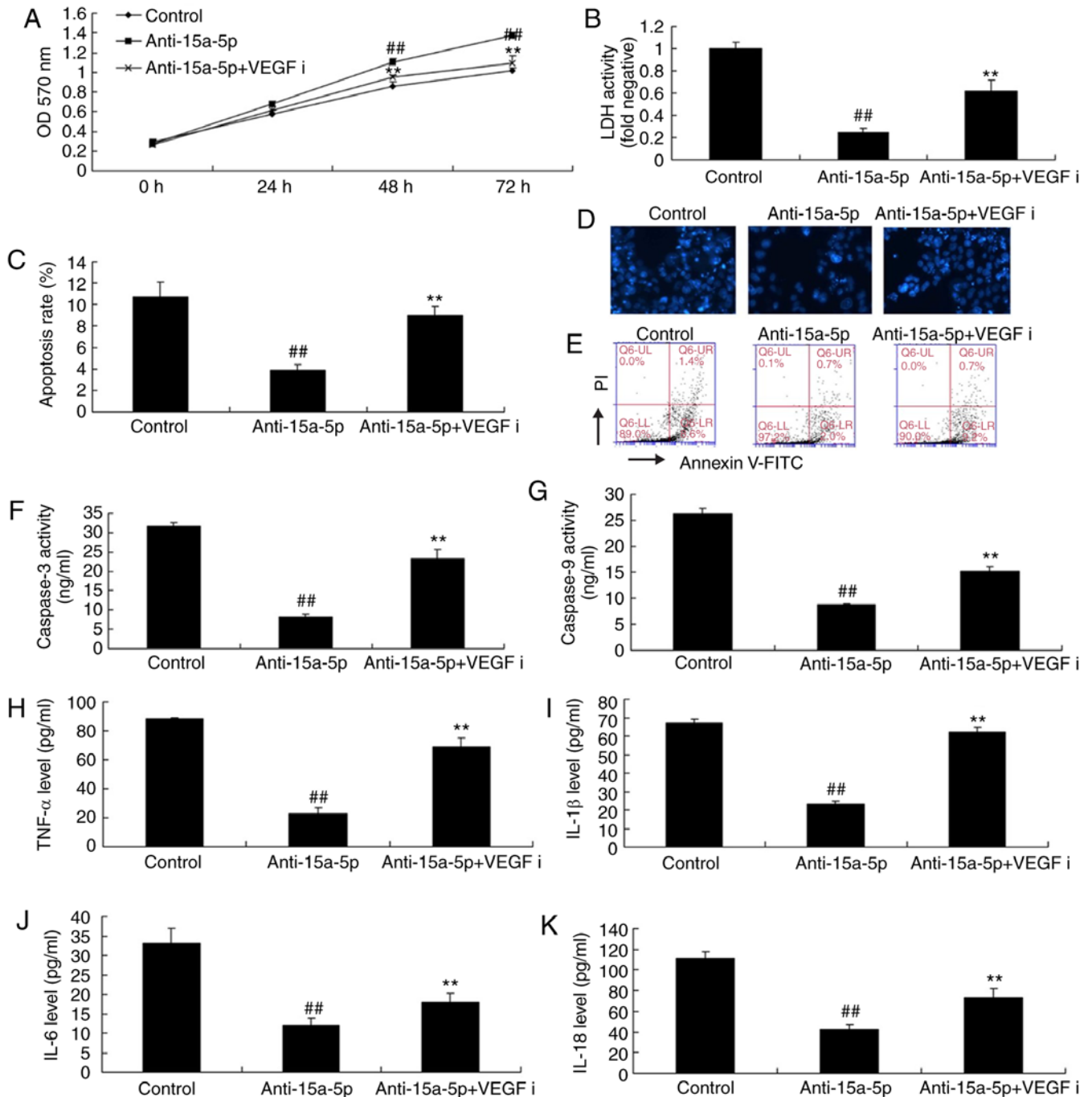


Figure 8. Inhibition of VEGF reduced the effects of anti-miR-15a-5p on PAMSCs from rats with monocrotaline-induced pulmonary arterial hypertension. (A) Cell proliferation; (B) LDH levels; (C) apoptosis rate; (D) DAPI assay (original magnification, $\times 100$); (E) cell apoptosis determined by flow cytometry; activity of (F) caspase-3 and (G) caspase-9. Levels of (H) TNF- α , (I) IL-1 β , (J) IL-6 and (K) IL-18. $^{##}P < 0.01$ vs. control group, $^{**}P < 0.01$ vs. anti-15a-5p group. miR, microRNA; VEGF, vascular endothelial growth factor; PAMSCs, pulmonary artery smooth muscle cells; LDH, lactate dehydrogenase; control, negative control group; anti-15a-5p, downregulation of miR-15a-5p; VEGFi, VEGF inhibitor group.

primary cell type only, which is a limitation of the present study.

Vascular smooth muscle cells (VSMCs) are an important component of the tunica media. Smooth muscle cell proliferation and apoptosis resistance are the major reasons for tunica media thickening in vascular modeling (27) and are important factors that induce vascular remodeling. Inhibiting SMC proliferation and promoting apoptosis are the focus of investigation on vascular remodeling (8). It has been demonstrated that multiple bioactive substances can regulate the function

of VSMCs (8). These include nitric oxide, angiotensin II, platelet-derived growth factor and VEGF. The present study indicated that miR-15a-5p induced apoptosis and decreased proliferation of PAMSCs by inhibiting VEGF expression. Upregulation of VEGF attenuated the effects of miR-15a-5p on PAMSCs. Inflammation contributes to exaggerated contractility and proliferation of vascular cells (28). In the present study, it was observed that overexpression of miR-15a-5p significantly promoted cell apoptosis and was associated with the increase in TNF- α , IL-1 β , IL-18, and IL-6 expression in PAMSCs.

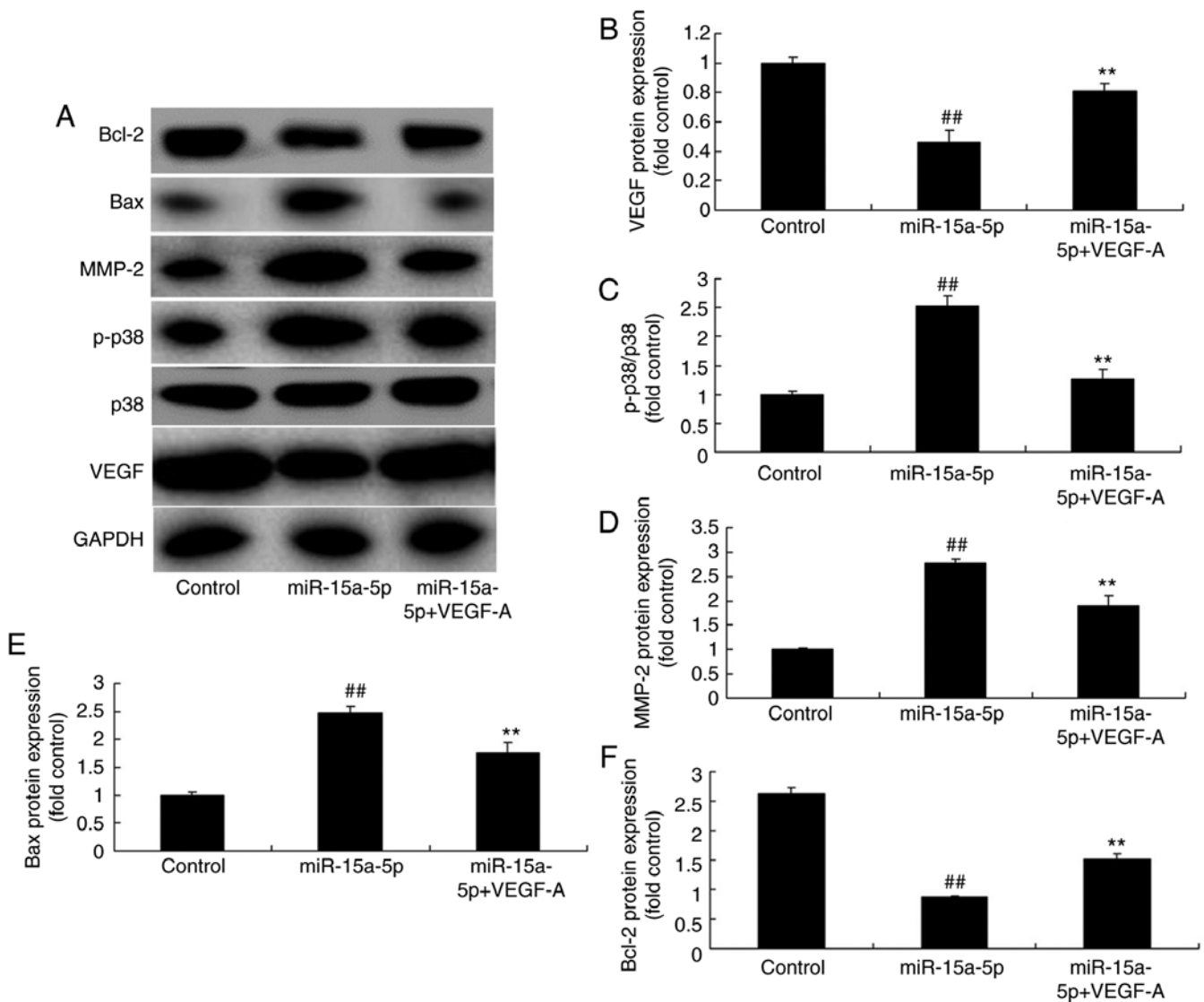


Figure 9. Upregulation of VEGF affected the VEGF/p38/MMP-2 signaling pathway in PSMCs from rats with monocrotaline-induced pulmonary arterial hypertension by the effects of miR-15a-5p. (A) Results of the protein expression of VEGF, p38, MMP-2, Bax and Bcl-2 measured using western blot analysis. Protein expression of (B) VEGF, (C) p-p38, (D) MMP-2, (E) Bax and (F) Bcl-2. ^{##}P<0.01 vs. control, ^{**}P<0.01 vs. miR-15a-5p group. Control, negative control group; miR, microRNA; miR-15a-5p, miR-15a-5p overexpression group; miR-15a-5p-VEGF-A, miRNA-15a-5p and VEGF plasmid group; VEGF, vascular endothelial growth factor; p-p38, phosphorylated-p38; MMP, matrix metalloproteinase; PSMCs, pulmonary artery smooth muscle cells; Bax, B cell lymphoma-2-associated X protein; Bcl-2, B-cell lymphoma 2.

However, a number of studies have reported that miR-15a-5p inhibited inflammation and alleviated several pathological processes. For example, Liu *et al* reported that miR-15a-5p alleviated the atherosclerotic inflammatory response and arterial injury in diabetic atherosclerotic rats (29). In addition, it was reported that miR-15a deficiency facilitated inflammation by directing M1 macrophage polarization in the tumor (30). These conclusions were not consistent with the findings of the present study. This may be attributed to PASMCM apoptosis. Apoptotic cells may become pro-inflammatory through the release of pro-inflammatory cytokines and DAMPs (31). The present study only focused on the inflammation in PASMCMs, but the role of miR-15a-5p in inflammation in MCT-induced PAH *in vivo* remains to be elucidated.

MAPKs are a family of serine-threonine kinases that can be activated by various stimuli. MAPK is most closely associated with the regulation of cell proliferation and it

is a type of biological signal transduction protein kinase in eukaryotic cells. MAPK is a common pathway for information transduction between extracellular signals and cell nuclei. This pathway uses the conserved three-level kinase cascade reaction for signal transduction. The MAPK pathway is activated by diverse extracellular and intracellular stimuli, including growth factors, cytokines and transcription factors; therefore, it can mediate numerous intracellular biological effects (32). Ferrari *et al* reported that VEGF signaling regulated TGF- β 1-induced endothelial cell apoptosis by shifting p38 from a pro-survival to a pro-apoptotic isoform (33). In the present study, miR-15a-5p increased p-p38 levels by targeting VEGF and promoted PASMCM apoptosis in the MCT-induced PAH model. Shi *et al* demonstrated that miR-15a-5p regulates MAPK in human adipocyte differentiation and obesity (34).

Extracellular matrix (ECM) remodeling is important in pulmonary vascular remodeling. The ECM mainly includes

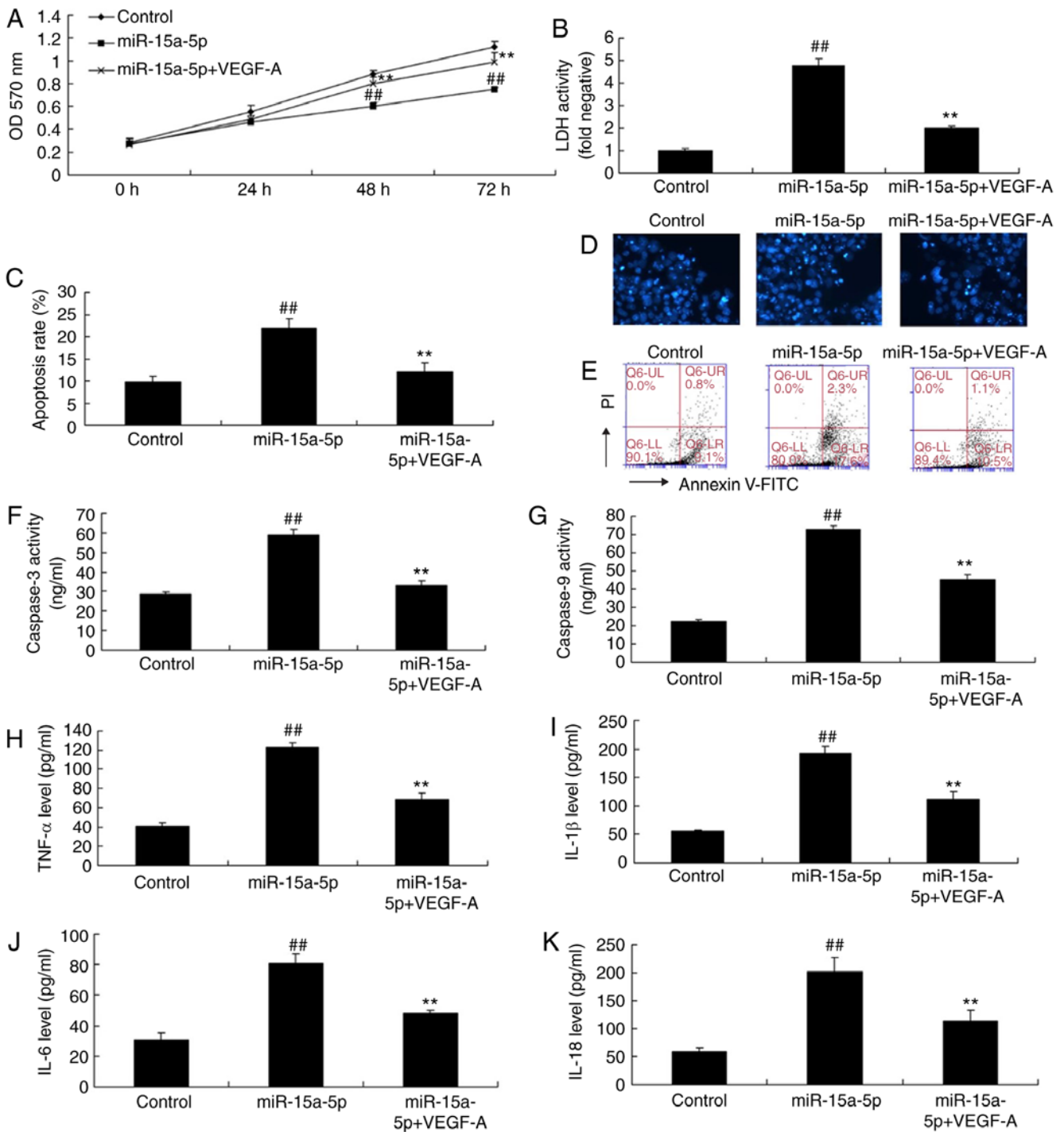


Figure 10. Upregulation of VEGF reduced the effects of miR-15a-5p on the PAMSCs of rats with monocrotaline-induced pulmonary arterial hypertension. (A) Cell proliferation, (B) LDH levels, (C) apoptosis rate, (D) DAPI assay (original magnification, x100), (E) cell apoptosis analysis by flow cytometry, activity of (F) caspase-3 and (G) caspase-9, levels of (H) TNF- α , (I) IL-18, (J) IL-1 β and (K) IL-6. $^{##}P < 0.01$ vs. control; $^{**}P < 0.01$ vs. miR-15a-5p group. Control, negative control group; miR, microRNA; miR-15a-5p, miR-15a-5p overexpression group; miR-15a-5p-VEGF-A, miR-15a-5p and VEGF plasmid group; VEGF, vascular endothelial growth factor; PAMSCs, pulmonary artery smooth muscle cells; LDH, lactate dehydrogenase; TNF, tumor necrosis factor; IL, interleukin; OD, optical density.

collagen, elastic fibers, proteoglycans and glycoproteins (35), among which collagen and elastic fibers are the most abundant structural components in the ECM. These substances form the complicated structural network of the vascular walls (35). ECM exists in a dynamic balance between continuous production and degradation under physiological conditions. MMPs are a group of zinc ion-dependent proteolytic enzymes. In

addition, ECM fragments degraded by MMP-9 exert chemotactic effects on inflammatory cells. Elastic fiber-derived chemokines in the respiratory tract of long-term smokers can induce aggregation of monocytes/macrophages (36), thereby aggravating the pulmonary inflammatory response and leading to destruction of the alveolar wall. In addition to the ECM degradation, MMPs also exhibit pro- and anti-apoptotic

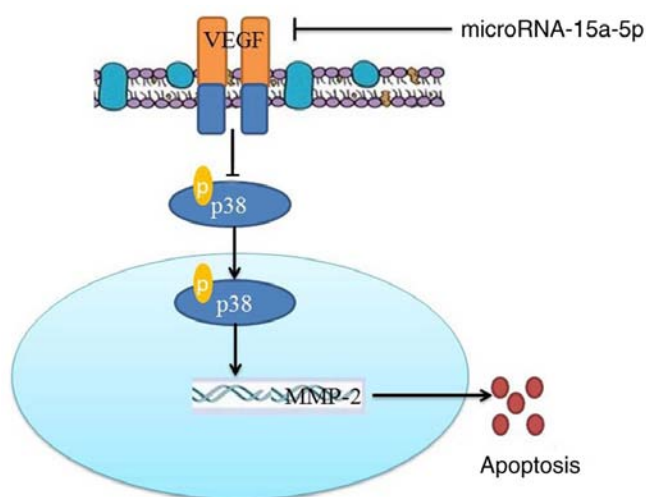


Figure 11. MicroRNA-15a-5p induces pulmonary artery smooth muscle cell apoptosis in monocrotaline-induced pulmonary arterial hypertension via the VEGF/p38/MMP-2 signaling pathway. VEGF, vascular endothelial growth factor; p-p38, phosphorylated-p38; MMP, matrix metalloproteinase.

activity and affect cell apoptosis (37). Their anti-apoptotic action includes cleavage of the Fas ligand and activation of the serine/threonine kinase AKT, also referred to as protein kinase B. The pro-apoptotic activity of MMPs is usually associated with changes in ECM composition. MMPs cause apoptosis by cleaving adhesion molecules. MMP-3 induces apoptosis when overexpressed in epithelial cells, possibly by digestion of laminin (38). A pentanoic acid derivative targeting MMP-2 was shown to induce apoptosis in a chronic myeloid leukemia cell line (39). Yu *et al* reported that microRNA-2861 targeted MMP-2 to regulate the proliferation and apoptosis of ectopic endometrial cells in women with endometriosis (40). The results of the present study demonstrated that the overexpression of miR-15a-5p promoted the protein expression of MMP-2 and increased PASM cell apoptosis in the MCT-induced PAH model.

The Bcl-2 gene family is closely associated with mitochondrial regulation of cell apoptosis. Bcl-2 and Bax are the main members of the Bcl-2 family, and Bcl-2 inhibits whereas Bax promotes cell apoptosis (41). It has been reported that Bcl-2 is located in the mitochondrial permeability transition pore (PT pore) in the outer mitochondrial membrane. The pro-apoptotic Bcl-2 gene family can promote the opening of the PT pore when exogenous or endogenous stimulating factors act on the mitochondrion; it releases cytochrome *c*, activates the caspase cascade reaction, and binds with apoptotic protease-activating factor 1 (Apaf-1). It also initiates the Apaf-1-caspase-9-caspase-3 cell apoptin cascade reaction and induces cell apoptosis (41). In the present study, the overexpression of miR-15a-5p reduced cell proliferation, induced apoptosis, and promoted the activity of caspase-3/9 and the protein expression of Bax in the PSMCs of rats with MCT-induced PAH. Chen *et al* reported that miR-15a-5p suppressed cell survival and metastasis and induced apoptosis in chronic myeloid leukemia (42).

In conclusion, the present study demonstrated that miR-15a-5p induced apoptosis of PSMCs in rats with MCT-induced PAH through targeting the VEGF/p38/MMP-2

signaling pathway (Fig. 11). The results indicated that miR-15a-5p may be involved in the pathogenesis of PAH, and it may be a potential therapeutic target for PAH.

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Availability of data and materials

The analysed datasets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

GZ designed the experiments; WZ, YL, XX, SW, YL and MS performed the experiments. WZ and GZ analysed the data; GZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Beijing Anzhen Hospital, Capital Medical University (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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