**MicroRNA-138 promotes proliferation and suppresses mitochondrial depolarization in human pulmonary artery smooth muscle cells through targeting TASK-1**

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**Abstract.** MicroRNA (miR)-138 serves an important role in the proliferation, differentiation and apoptosis of human pulmonary artery smooth muscle cells (HPASMCs), indicating the involvement of miR-138 in the development and progression of pulmonary artery hypertension (PAH). Potassium channel subfamily K member 3 (TASK-1), a two-pore domain K⁺ channel, is expressed in HPASMCs and is associated with hypoxic PAH. However, whether miR-138 mediates PAH through targeting TASK-1 is not known. In the present study, HPASMCs were transfected with miR-138 mimic to establish a PAH model in vitro, and the effects of a miR-138 inhibitor and a TASK-1 inhibitor (A293) were examined. Cell proliferation and mitochondrial membrane potential (MMP) were measured by CCK-8 assay and flow cytometry, respectively. Reverse transcription-quantitative polymerase chain reaction and western blotting were performed to examine the expression of miR-138, TASK-1, Bcl-2, caspase-3 and activation of extracellular signal-regulated kinase 1/2 (ERK1/2). A dual-luciferase reporter assay was also used to analyse the expression level of TASK-1 in HPASMCs. The results of the present study demonstrated that the miR-138 mimic promoted proliferation and MMP level, which was similar to the effect of A293 treatment on HPASMCs. However, the miR-138 inhibitor inhibited the effects induced by miR-138 mimic or A293 treatment, as demonstrated by a decrease in proliferation and MMP level in HPASMCs, accompanied by a decrease of Bcl-2 and an increase of caspase-3 expression levels, as well as ERK1/2 activation. The dual-luciferase reporter assay indicated that TASK-1 expression was negatively regulated by miR-138. The results of the present study suggested that miR-138 promoted proliferation and suppressed mitochondrial depolarization of HPASMCs by targeting TASK-1.

**Introduction**

Pulmonary artery hypertension (PAH) is a disease caused by a variety of elements, and is characterized by a progressive increase in pulmonary circulatory resistance and pressure in the pulmonary artery (PA), which results in mortality owing to right-sided heart failure (1). Although the mechanism of PAH remains unclear, hypoxia is a known trigger factor. Hypoxia-induced PAH and sustained pulmonary vascular contraction have anti-apoptotic effects, which lead to imbalanced pulmonary vascular remodeling (2). Regeneration and extended survival time of PA smooth muscle cells (PASMCs) may lead to hypertrophy, vascular wall thickening, vascular diameter narrowing and increased resistance to blood flow and perfusion (3). Therefore, an imbalance in the proliferation and apoptosis in PASMCs may be associated with the development and progression of pulmonary vascular remodeling and targeting the progression of vascular remodeling may serve as a novel treatment strategy.

MicroRNAs (miRNAs) are small endogenous non-coding molecules ~22 nucleotides in length that act on the 3'-untranslated region (3'-UTR) of target mRNAs and negatively regulate gene expression. Post-transcriptional regulation by miRNAs affects a number of physiological and pathological processes, including cell proliferation and apoptosis, tumor development and various cardiovascular diseases (4-7). In previous studies involving exposure to hypoxia, a variety of miRNAs were revealed to be dysregulated in PAs, including miRNA (miR)-138, miR-204, miR-20a, miR-145 and miR-190, in which miR-138 expression was revealed to be increased and therefore may serve an important role in hypoxic pulmonary vascular remodeling (8-12). It was demonstrated that miR-138 was expressed in the PASMCs and was regulated by hypoxia. Exogenous miR-138...
expression inhibited apoptosis and caspase activation in PASMCs and also inhibited the Bcl-2 expression (9). In hypoxic conditions, overexpression of miR-138 enhanced the expression levels of anti-apoptotic protein Bcl-2 and cleaved caspase-3 (13). Previous studies have also demonstrated that lowering of oxygen from 21 to 2.5% induced the phosphorylation of extracellular signal regulated kinase (ERK)1/2 in PASMCs and also suggested that the ERK1/2 signaling pathway is associated with hypoxia-induced proliferation of PASMCs (14). These results suggest that Bcl-2, caspase-3 and the ERK1/2 signaling pathway may be associated with miR-138-induced hypoxia in PAH.

Potassium channel subfamily K member 3 (TASK-1) is a member of the two-pore domain potassium channel family that has been described in rabbit PASMCs (15) and in rat PAs (16). TASK-1 activity is dependent on external pH and oxygen tension, which suggested that this potassium channel may be a sensor of variations in external pH and hypoxia in PAs. In the present study, miR-138 mimic was used to transfect human PASMCs (HPASMCs) to establish an in vitro hypoxic PAH model. The hypothesis that miR-138 contributes to the regulation of proliferation, membrane potential and apoptosis of HPASMCs by targeting TASK-1 expression was investigated. Therefore, the results may provide an important mechanism for the hypoxic relaxation of PAs.

Materials and methods

Cell culture. Lung tissues were obtained at lung resection from 3 patients (male/female, 2/1; ages 45-68 years) with lung cancer, but without pulmonary vessel disease and arterial hypoxemia, from The First Affiliated Hospital of Bengbu Medical College (Bengbu, China) between May 2010 and February 2015. Tissues were placed on a plate and washed with ice-cold PBS containing 100 mg/ml penicillin G and 50 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). HPASMCs were isolated from distal human PAs (<1 mm external diameter), and the smooth muscle phenotype was confirmed immunohistochemically using an α-smooth muscle actin (α-SMA) antibody (Fig. 1A), as previously described (17,18). HPASMCs were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) and maintained at 37°C in 5% CO₂ until passage three or six. Ethical approval was obtained from The First Affiliated Hospital of Bengbu Medical College Ethics Committee (Bengbu, China) and written informed consent was obtained from all patients included.

Cell treatment. miR-138 mimic (5'-AGCUGGUGUUGU GAAUCAGGCCG-3') was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). To establish the hypoxic PAH model, HPASMCs (3x10⁵ cell/well) were seeded in 6-well plates and transiently transfected with miR-138 mimic (20 µM) (19) for 6 h at 37°C using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. HPASMCs that were not subjected to transfection were used as a control. Following 4-8 h transfection, the miR-138 mimic-transfected HPASMCs (model) were subsequently transfected with an miR-138 inhibitor (20 µM; GE Healthcare Dharmacon, Inc., Lafayette, CO, USA) or control inhibitors (non-targeting miR-138 control inhibitor; 20 µM; GE Healthcare Dharmacon, Inc.) for 6 h at 37°C using Lipofectamine® 2000 as described above. In addition, miR-138 mimic-transfected HPASMCs (model) were also treated with A293 (100 µM; Sanofi S.A., Paris, France) for 4 h at 37°C in the absence or presence of miR-138 inhibitor transfection. Following a 48 h time interval post-transfection, subsequent analyses were performed. A total of 6 experiment groups were used in subsequent analyses: Control group (untreated HPASMCs), model group (miR-138 mimic - transfected HPASMCs), model + control inhibitors group (model + non-targeting miR-138 control inhibitor), model + miR-138 inhibitor group, model + A293 group, and the model + A293 + miR-138 inhibitor group.

Cell counting kit-8 (CCK-8) assay. CCK-8 (Dojindo Molecular Technologies, Rockville, MD, USA) was used to evaluate the effects of miR-138, as previously described (20). HPASMCs were seeded in 96-well plates at 5x10⁴ or 1x10⁵ cells/well in RPMI-1640 at 37°C. CCK-8 solution was added to the wells at 0, 24, 48 or 72 h, according to the manufacturer's instructions. The plates were incubated for 1 h at 37°C in 5% CO₂, incubator conditions and the absorbance was read at 450 nm using a microplate reader (Shanghai Utraro Medical Instrument Co., Ltd., Shanghai, China).

Measurement of mitochondrial membrane potential (MMP). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) probe (Molecular Probes; Thermo Fisher Scientific, Inc.) was used to detect the alterations in MMP, which aggregates in the intact mitochondria of non-apoptotic cells emitting orange-red fluorescence and is widely distributed in apoptotic cells, emitting green fluorescence in the monomeric form at 488 nm (21). Cells (5x10⁵ cells/well) were cultured in 24-well plate overnight at 37°C. Following transfection with the miR-138 mimic or miR-138 inhibitor as aforementioned, HPASMCs were washed with PBS, incubated with JC-1 (100 nM) for 20 min at 37°C and subsequently subjected to flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Following this, the results were subsequently analyzed using CellQuest software (version 5.1; BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from HPASMCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. cDNA was synthesized from RNA with a MMLV RT Reagent kit (Thermo Fisher Scientific, Inc.) for 60 min at 37°C, followed by 95°C for 5 min, as described previously (22). Primers 5'-AGCUGGUGUUGU GAAUCAGGCCG-3' and reverse, 5'-GCA GTG TGC CCA GGC ATA AC CAA AG-3' and reverse, 5'-GCAGTGTCGCCAGGCATA LIU et al. MicroRNA-138 TARGETS TASK-1
AG-3'；Bcl-2 forward, 5'-AGC TGA GCG AGT GTC TCA AG-3' and reverse, 5'-TGT CCA GCC CAT GAT GGT TC-3'; caspase-3 forward, 5'-AAC TGG ACT GTG GCA TTG AG-3' and reverse, 5'-ACA AAG CGA CTG GAT GAA CC-3'; U6 forward, 5'-CTC GCT TCG GCA GCA CA-3' and reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3'; GAPDH forward, 5'-CAC CCA CTC CTC CAC CTT TG-3' and reverse, 5'-CCA CCA CCC TGT TGC TGT AG-3'. Relative quantification of miR-138 expression levels was determined using the 2^(-ΔΔCq) method (22). U6 was used as an internal standard for the normalization of miR-138 expression. GAPDH used to for the normalization of the non-miRNA expression data.

Western blot analysis. Western blot analysis was performed according to standard procedures. Briefly, total protein was isolated from HPASMCs using radio-immunoprecipitation buffer (Amyjet Scientific, Inc., Wuhan, China) for 10 min at 95°C, followed by centrifugation at 400 x g at 25°C for 10 min. The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (cat. no. PICPI23223; Thermo Fisher Scientific, Inc.). A total of 15 μl protein was loaded into each well and separated by 10% SDS-PAGE (Amyjet Scientific, Inc.) and transferred to polyvinylidene difluoride membranes (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The membranes were blocked with...
fat-free milk for 1 h at 25°C and subsequently incubated with primary antibodies against TASK-1 (1:800, cat. no. ab49433; Abcam, Cambridge, MA, USA), Bcl-2 (1:300, cat. no. Sc-492; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), caspase-3 (1:500, cat. no. ab44976; Abcam), phosphorylated (p)-ERK1/2 (1:1,000, cat. no. 4376), ERK1/2 (1:1,000, cat. no. 4695) and GAPDH (1:2,000, cat. no. 5174) (all from Cell Signaling Technology, Inc.) secondary antibodies for 1 h at 37°C. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000, cat. no. A0208), donkey anti-goat IgG (1:1,000, cat. no. A0181) and goat anti-mouse IgG (1:1,000, cat. no. A0216) (all from Beyotime Institute of Biotechnology, Haimen, China) for 2 h at 25°C. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000, cat. no. A0208), donkey anti-goat IgG (1:1,000, cat. no. A0181) and goat anti-rabbit IgG (1:1,000, cat. no. A0216) (all from Beyotime Institute of Biotechnology, Haimen, China) secondary antibodies for 1 h at 37°C. Signals were detected using an enhanced chemiluminescence kit (EMD Millipore, Billerica, MA, USA), and the signal intensity was determined using ImageJ 1.46 software (National Institutes of Health, Bethesda, MD, USA). GAPDH was used to normalize the protein expression data.

Dual-luciferase reporter assays. TASK-1 was predicted to interact with miR-138 by bioinformatics analysis using TargetScan, which is able to predict biological targets of miRNAs by searching for the presence of 8, 7, and 6mer sites that match the seed region of each miRNA (23). The mutant and wild-type 3'-UTR of human TASK-1 were synthesized and inserted downstream of the firefly luciferase gene in the pGL3 reporter vector (Promega Corporation, Madison, WI, USA), yielding pGL3-mut-TASK-1 and wild-type pGL3-TASK-1, respectively. HPASMCs (5x10^3 cell/well) were seeded in 96-well plates, pGL3-mut-TASK-1 (50 ng) or wild-type pGL3-TASK-1 (50 ng) plasmids were co-transfected with miR-138 mimic (5 ng) or miR-138 inhibitor (5 ng) using Lipofectamine® 2000 at 37°C. Following 24 h, cells were lysed and activities of firefly luciferase and Renilla luciferase were examined using the Dual-Luciferase Reporter assay system (Promega Corporation). Firefly luciferase activity was normalized to Renilla luciferase activity.

Statistical analysis. Data are expressed as the mean ± standard deviation. All presented data were representative of a minimum of three independent experiments. Statistical analysis was performed using one-way analysis of variance followed by Tukey’s post hoc test. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-138 mimic promote proliferation and suppress mitochondrial depolarization of HPASMCs. miR-138 mimic was transfected into HPASMCs to establish a PAH model. As revealed in Fig. 1B, transfection of HPASMCs
with the miR-138 mimic (model) significantly increased the expression of miR-138 by 2.61-fold compared with untreated control HPASMCs, whereas model cells treated with the miR-138 inhibitor exhibited a significant decrease in miR-138 expression compared with the model group. However, the miR-138 control inhibitor did not exhibit a marked effect on miR-138 expression compared with the model cells. PAH model HPASMCs were also treated with 100 µM A293, a TASK-1 inhibitor, which significantly enhanced miR-138 expression compared with the control group, and the model + A293 treated HPASMCs with miR-138 inhibitor transfection demonstrated a significant decrease in miR-138 expression compared with the model + A293 only group (P<0.01; Fig. 1B).

The effects of miR-138 on cell proliferation of HPASMCs were examined using a CCK-8 assay. Transfection of HPASMCs with the miR-138 mimic (model) significantly increased cell proliferation by 45.0% at 72 h compared with untransfected control cells, whereas PAH model cells treated with the miR-138 inhibitor exhibited a significant decrease in proliferation compared with the model group (P<0.001; Fig. 1C). However, the miR-138 control inhibitor did not exhibit a significant effect on cell proliferation compared with the model cells. PAH model HPASMCs were also treated with A293 (100 µM), which significantly enhanced cell proliferation by 14.5% at 72 h compared with the model group, and the model + A293 treated HPASMCs with miR-138 inhibitor transfection significantly decreased the proliferation compared with the model + A293 only group (P<0.01; Fig. 1B).

The role of miR-138 on mitochondrial function in HPASMCs was investigated by measuring the MMP by flow cytometry. Treatment with miR-138 mimic (model group) resulted in an increase in MMP levels by 2.70-fold compared with the control (Fig. 1D). Model cells treated with the miR-138 inhibitor decreased the MMP level significantly compared with model and the A293-treated model cells (P<0.01). These results suggested that miR-138 mimic suppress mitochondrial depolarization of HPASMCs.

miR-138 mimic regulate TASK-1 and apoptosis-associated protein expression in HPASMCs. Following treatment with miR-138 mimic, the mRNA expression of TASK-1 was significantly decreased by 58.9% in HPASMCs compared with the control group (P<0.001; Fig. 2A). However, miR-138 inhibitor transfected model cells demonstrated a significantly increased expression of TASK-1 mRNA compared with the model group and the model + A293 + miR-138 inhibitor group. Furthermore, miR-138 mimic (model) significantly increased the mRNA expression level of Bcl-2 and decreased the mRNA expression level of caspase-3, compared with the untreated control (P<0.001; Fig. 2A); however, co-treatment with the miR-138 inhibitor significantly reduced the expression levels of Bcl-2 and significantly increased the expression of caspase-3 mRNA compared with the model group and the model + A293 + miR-138 inhibitor group, respectively. Similar results were also observed by western blot analysis (Fig. 2B and C).

miR-138 mimic activate ERK1/2 signaling in HPASMCs. Following treatment with the miR-138 mimic, the ratio of p-ERK1/2 to ERK1/2 was significantly increased in HPASMCs by 40.1% compared with the control (P<0.01; Fig. 3). However, co-treatment with the miR-138 inhibitor decreased the expression of p-ERK1/2 by 20.3 and 37.5% compared with the model group and the model + A293 + miR-138 inhibitor group, respectively. These data indicated that miR-138 mimic activated ERK1/2 signaling in HPASMCs.

miR-138 targets TASK-1 in HPASMCs. To investigate the regulatory mechanism of miR-138, bioinformatics analysis (TargetScan) was used, which identified that TASK-1 mRNA contained potential miR-138 binding sites (Fig. 4A). To confirm TASK-1 as a miR-138-regulated target in HPASMCs,
wild-type and mutant versions of the TASK-1 3'-UTR were cloned and inserted into a pG3 luciferase reporter vector. The luciferase assay demonstrated that miR-138 mimic transfection significantly suppressed luciferase activity in cells co-transfected with the wild-type TASK-1 3'-UTR compared with cells co-transfected with the mutant TASK-1 3'-UTR (P<0.001; Fig. 4B), and that treatment with the miR-138 inhibitor significantly increased luciferase activity in the presence of TASK-1 3'-UTR compared with cells co-transfected with the mutant TASK-1 3'-UTR (P<0.05; Fig. 4C). These data suggested that TASK-1 may be a direct target of miR-138 in HPASMCs.

Discussion

In the present study, evidence for the role for miR-138 in regulating HPASMCs proliferation, membrane potential and apoptosis in hypoxic PAH, along with the underlying mechanisms has been demonstrated. The importance of miRNAs in pathological processes is being recognized, particularly in cardiovascular disease (24), and a number of miRNAs have been implicated in signal transduction pathways relevant to PAH (25,26). miR-190 regulates hypoxic pulmonary vascular contractility by targeting a voltage-gated potassium channel (27). miR-190 demonstrated negative correlation with the expression of a voltage-dependent K+ channel protein under hypoxia (12). miR-21 in PAH was previously demonstrated to regulate cell proliferation and apoptosis by regulating the expression of proteins that regulate Bcl-2 and Akt signaling pathways (28). These previous data suggested that miRNAs are important in vascular cell fate and are involved in the progression of PAH. The present study focused on miR-138 and demonstrated its effects on proliferation, membrane potential and apoptosis in HPASMCs.

The results of the present study indicated that miR-138 promoted proliferation and attenuated mitochondrial depolarization in HPASMCs. The deregulation of miR-138 is frequently associated with the inhibition of proliferation and migration of smooth muscle cells (29). The signaling pathway underlying the miR-138-associated anti-apoptosis of HPASMCs was also examined in the present study, and it was demonstrated that miR-138 induces ERK1/2 phosphorylation that leads to the cleavage of procaspase-3 and the upregulation of Bcl-2. Caspase activity is known to induce mitochondrial damage during apoptosis (30).

According to bioinformatics-based analysis and luciferase assay, TASK-1 was identified as a direct target of miR-138 in HPASMCs. TASK-1 has been demonstrated previously to serve a role in neuronal apoptosis (31), which further suggested that K+ two-pore channels may exhibit a certain role as modulators of cell proliferation. The mechanism by which K+ channels may alter the proliferative state of cells has been an open question for several years. TASK-1 is expressed in HPASMCs and is hypoxia-sensitive, and controls the resting membrane potential (15), therefore suggesting an important role for TASK-1 K+ channels in the regulation of pulmonary vascular tone.

In conclusion, the results from the present study indicated that miR-138 may serve an important role in the proliferation, membrane potential and apoptosis of HPASMCs. miR-138 overexpression was demonstrated to suppress TASK-1 expression, activate ERK1/2 signaling, and induce of Bcl-2 expression and decrease caspase-3 expression. Therefore, the stabilization of the miR-138 level may be a novel strategy for the clinical treatment of PAH.

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