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MiR-19a modulates hypoxia-mediated cell proliferation and migration via repressing PTEN in human pulmonary arterial smooth muscle

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ABSTRACT

Aim: The dysfunction of human pulmonary arterial smooth muscle cells (HPASMCs) has been suggested to participate in the pathophysiology of pulmonary arterial hypertension (PAH). This study determined miR-19a expression in hypoxia-induced HPASMCs and explored the mechanistic actions of miR-19a in hypoxia-induced HPASMC proliferation and migration.

Methods: QRT-PCR and western blot assays respectively determined the mRNA and protein expression of miR-19a, phosphatase and tensin homolog (PTEN) and hypoxia-inducible factor-1 alpha (HIF-1α). *In vitro* functional assays determined HPASMC proliferation and migration, respectively. Luciferase reporter assay determined interaction between miR-19a and PTEN. The knockdown effects of miR-19a on PAH were confirmed in *in vivo* mice model.

Results: Hypoxia treatment time-dependently up-regulated miR-19a expression and enhanced cell proliferation in HPASMCs. MiR-19a overexpression increased cell proliferation and migration of HPASMCs, while repression of miR-19a reduced cell proliferative and migratory potentials of hypoxia-treated HPASMCs. Bioinformatics analysis and luciferase reporter assay showed that PTEN 3' untranslated region was targeted by miR-19a, and miR-19a repressed the mRNA and protein expression of PTEN in HPASMCs. Further rescue studies revealed that miR-19a regulated proliferative and migratory potentials of hypoxia-treated HPASMCs via suppressing PTEN expression. In addition, HIF-1α was identified as one of the mediators for the hypoxia-induced aberrant expression levels of miR-19a and PTEN. MiR-19a overexpression enhanced PI3K/AKT signaling, which was attenuated by enforced expression of PTEN in HPASMCs. More importantly, knockdown of miR-19 attenuated the chronic hypoxia-induced PAH in *in vivo* mice model.

Conclusion: This study presented a novel mechanistic action of miR-19a-mediated cell proliferation and migration of HPASMCs.

1. Introduction

Pulmonary arterial hypertension (PAH) is featured by pulmonary arterial vasoconstriction and is a devastating disease that severely threatens the human life, and the risk of developing PAH is increased with aging [1]. Neointima formation in small pulmonary arteries and the hyper-proliferation of pulmonary vascular cells have been suggested to involve in the pathophysiology of PAH [2,3]. PAH progression can eventually lead to the heart failure of left ventricle, which largely contributed to PAH-related deaths [4]. The human pulmonary arterial smooth muscle cells (HPASMCs) hyper-proliferation has been regarded as one of important features in the pulmonary vascular remodelling, and dysfunction of HPASMCs has been found to participate in the pathogenesis of PAH [5]. The common treatments for PAH are mainly targeting on the prostacyclin, endothelin and the nitric oxide pathways [6]. However, these therapies are still far from satisfaction in the

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clinical applications. Therefore, it is necessary for us to explore novel signaling pathways related to the pathophysiology of PAH in order to develop more effective therapies.

MicroRNAs (miRNAs) belong to the non-coding RNA family and has 21–23 nucleotides in length. MiRNAs have been found to exert its actions in suppressing targeted gene expression via forming competitively bindings with the 3′ untranslated region (3′UTR) of targeted genes [7]. Owing to this specific action, miRNAs regulate diverse biological functions including cell differentiation, cell proliferation, cell apoptosis and cell metabolism [8]. Data from the clinical studies have revealed that differentially expressed miRNAs were detected in the peripheral blood and lung tissues from PAH patients [9]. MiR-424 was up-regulated in the peripheral blood and served as a new marker for the progression of PAH [10]. In addition, the circulating miR-206 was identified as an important biomarker in the PAH patients with left heart diseases [11]. Mechanistically, miRNAs have also been shown to regulate the cellular functions of PASMCs, which may be linked to the pathophysiology of PAH. MiR-429 and miR-424-5p were shown to inhibited cell proliferation by down-regulating calcium sensing receptor in HPASMCs [12]. Liu et al., showed that miR-17-5p mediated hypoxia-induced proliferation of HPASMCs via regulating p21 and phosphatase and tensin homolog (PTEN) [13]. Recently, evidence from the clinical investigations showed that enhanced miR-19a expression was found in the plasma from PAH patients [9]. MiR-19a can target PTEN to regulate high mobility group protein B1-inudced proliferation and migration of human airway smooth muscle cells [14]. Moreover, PTEN/PI3K/AKT signaling pathway has been demonstrated to modulate hypoxia-induced pulmonary hypertension [15]. Based on the above evidence, we proposed that miR-19a/PTEN may involve in the pathophysiology of PAH.

In the present study, we detected the aberrant expression of miR-19a in the HPASMCs under hypoxia conditions, and further *in vitro* functional assays were employed to examine the role of miR-19a in the regulating cellular functions of HPASMCs under hypoxia conditions. Furthermore, mechanistic studies were performed to reveal the downstream signaling pathways in the HPASMCs. The present study may provide new evidence to reveal the key functions of miR-19a in the pathophysiology of PAH.

2. Materials and methods

2.1. Cell lines, cell culture and treatment with hypoxia

Human pulmonary artery smooth muscle cells (HPASMCs) were purchased from Sigma-Aldrich (St. Louis, USA). The HPASMCs were kept in the smooth muscle cell growth medium (Sigma-Aldrich) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, USA) and were maintained in a humidified incubator with 5% CO_2 at 37 °C. Cells at passage 4–6 were used for the *in vitro* experimental assays. For the hypoxia treatment, HPASMCs were kept in a gastight molecular incubator chamber (Thermo Fisher Scientific) supplied with 92% N_2, 5% CO_2 and 3% O_2, and after the HPAMSCs were induced with hypoxia for 24, 48 and 72 h, correspondingly, the cells were collected for further experimental assays.

2.2. Oligonucleotides (miRNAs, siRNAs and plasmids) and HPASMCs transfections

The miRNAs for miR-19a overexpression (miR-19a mimics), respective negative control (mimics NC), miR-19a knockdown (miR-19a inhibitors) and respective negative control (inhibitors NC) were designed and synthesized by Ribobio (Guangzhou, China). The plasmids that overexpress PTEN were constructed by cloning the PTEN cDNA into the pcDNA3.1 plasmid (pcDNA3.1-PTEN), and pcDNA3.1 was served as the negative control (GenePharma, Shanghai, China). For the knockdown of hypoxia-inducible factor-1α (HIF-1α), the HIF-1α siRNA and the respective scrambled siRNA were designed and synthetized by

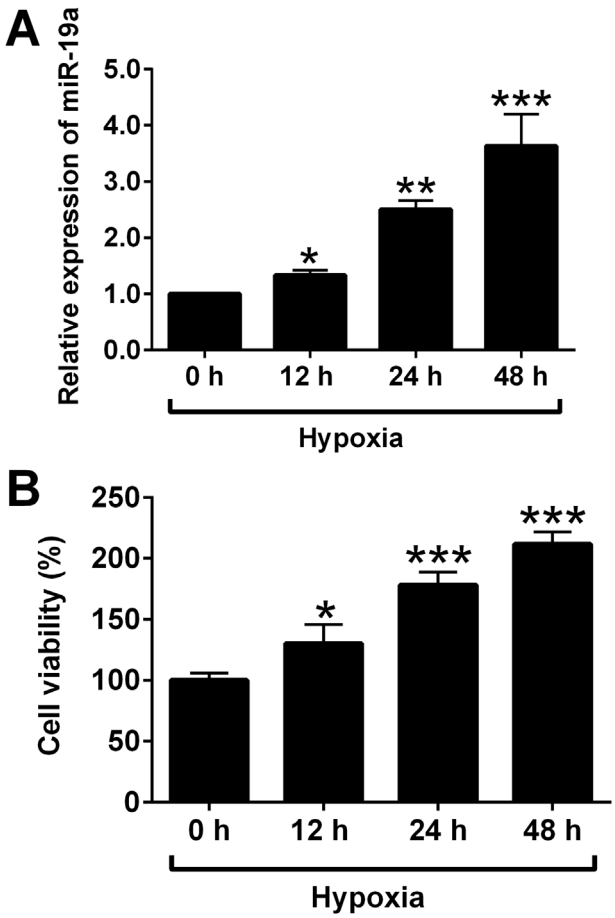

Fig. 1. Hypoxia promoted cell proliferation and upregulation of miR-19a in HPASMCs. (A) The expression of miR-19a in HPASMCs after exposure to hypoxia for 12, 24 and 48 h was determined by qRT-PCR assay. (B) The cell viability of HPASMCs after exposure to hypoxia for 12, 24 and 48 h was evaluated by CCK-8 assay. Data are expressed as mean ± standard deviation (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control groups.

Ribobio. For the cell transfections, HPASMCs were transfected with miRNAs, plasmids or siRNAs by using the Lipofectamine 2000 reagent by following the manufacturer's protocol (Invitrogen, Carlsbad, USA). At 24 post-transfection, the transfected HPASMCs were collected for further experimental assays.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA from treated HPASMCs was isolated using TRIzol reagent (Takara, Dalian, China). For the quantification of miR-19a, One Step Prime script miRNA cDNA Synthesis Kit (Qiagen, Valencia, USA) was used to synthesize cDNA, and RT-PCR was performed on an ABI7900 system (Applied Biosystem, Foster City, USA) by using the miRNA-specific TaqMan MiRNA Assay kit (Applied Biosystems). For the quantification of proliferating cell nuclear antigen (PCNA), PTEN and HIF-1α mRNA, PrimeScript RT reagent Kit (Takara, Dalian, China) was used to synthesize the cDNA, and RT-PCR was performed on an ABI7900 system (Applied Biosystem) using the SYBR Green Premix Ex Taq II kit (Takara). The PCR conditions were as follow: 1 cycle of 95 °C for 30 s, and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. MiR-19a was normalized by using snRNA U6 and the expression levels of other genes were normalized by GAPDH. The folds changes of relevant genes were calculated using comparative Ct method. The sequences of the primers were as follow: miR-19a, forward, 5′-TGGTGTGTGCAAATCTATGCA-3′, and reverse, 5′- CAGTGCGTGTCGTTGGAGT-3'; U6, forward, 5′-CTCG

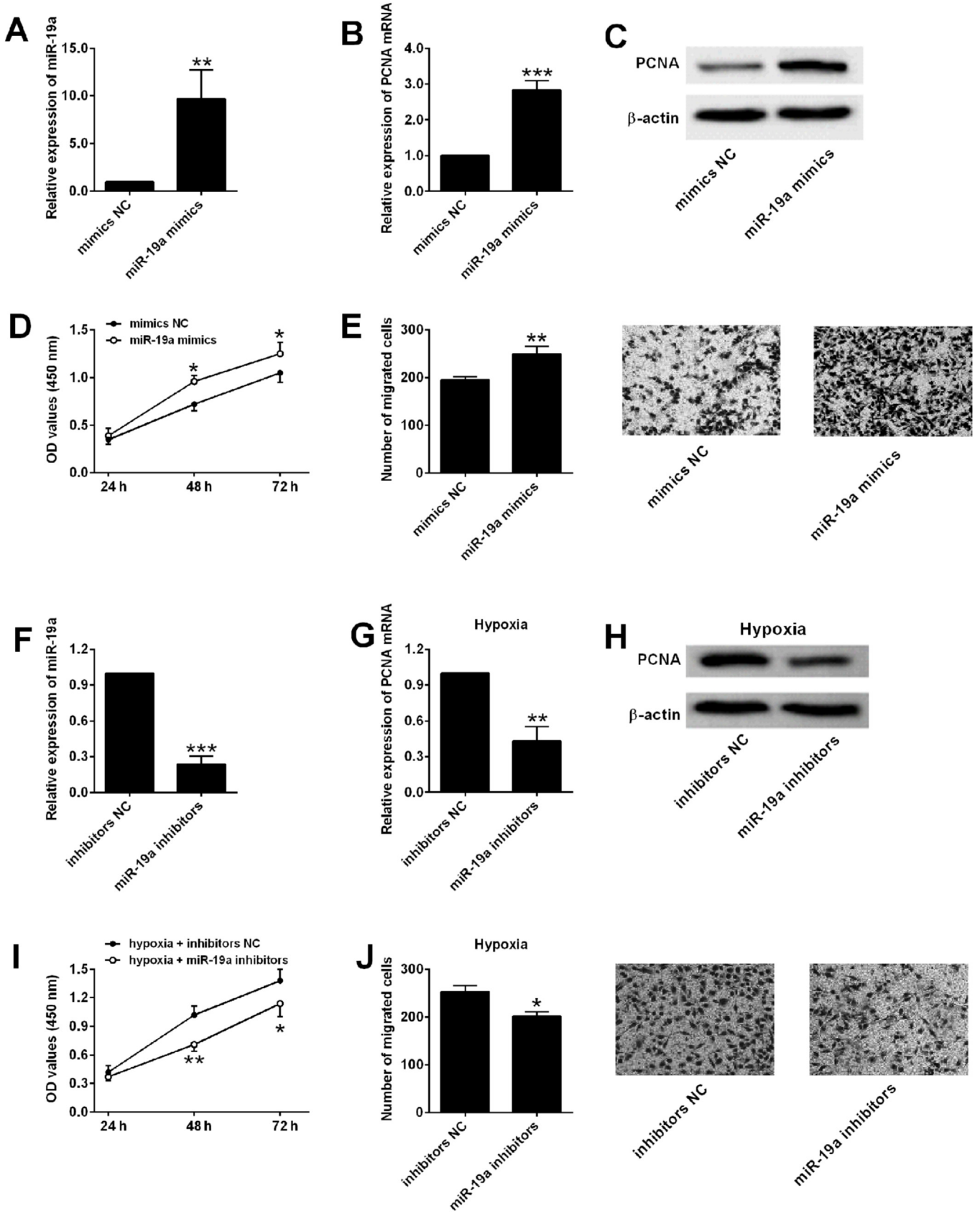

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Fig. 2. The effects of miR-19a on the cell proliferation and migration in HPASMCs after exposure to hypoxia. HPASMCs were transfected with mimics NC or miR-19a mimics, and at 24 h after transfection, (A) the expression of miR-19a in HPASMCs was determined by qRT-PCR; (B&C) the expression of PCNA mRNA and protein in HPASMCs was determined by qRT-PCR and western blot assays, respectively. (D) Cell viability of the HPASMCs was detected by CCK-8 assay at 24, 48 and 72 h post-transfection with mimics NC or miR-19a mimics. (E) Cell migration of the HPASMCs was measured by transwell migration assay at 24 h post-transfection with mimics NC or miR-19a mimics. (F) HPASMCs were transfected with inhibitors NC or miR-19a inhibitors, and at 24 h after transfection, the expression of miR-19a was determined by qRT-PCR. (G&H) The expression of PCNA mRNA and protein in hypoxia-treated HPASMCs was determined by qRT-PCR and western blot assays, respectively, at 24 h post-transfection with inhibitors NC or miR-19a inhibitors. (I) Cell viability of hypoxia-treated HPASMCs was detected by CCK-8 assay at 24, 48 and 72 h post-transfection with inhibitors NC or miR-19a inhibitors. (J) Cell migration of hypoxia-treated HPASMCs was detected by transwell migration assay at 24 h post-transfection with inhibitors NC or miR-19a inhibitors. Data are expressed as mean ± standard deviation (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control groups.

CTTCGGCAGCACATATACT-3′ and reverse, 5′-ACGCTTCACGAATTTGC GTGTC-3'; PTEN, forward, 5′-AGGGACGAACTGGTGTAATGA-3′ and reverse, 5′-CTGGTCCTTACTTCCCCATAGAA-3'; GAPDH, forward, 5′-CGAGCCACATCGCTCAGACA-3′ and reverse, 5′-GTGGTGAAGACGCCA GTGGA-3'.

2.4. Cell counting Kit-8 (CCK-8) assay

CCK-8 assay was carried out to determine the cell viability of the HPASMCs. Briefly, HPASMCs were cultured in a 96-well plate with 1×10^4 cells/well, and after different treatments for indicated time periods, the HPASMCs were treated with CCK-8 solution (10 μl) at room temperature for 2 h. The cell viability was assessed by using a microplate reader to measure the optical density at the wavelength of 450 nm.

2.5. Transwell migration assay

The Transwell migration assay was carried to evaluated cell migration of HPASMCs. The treated HPASMCs were seeded into the non-coated transwell inserts (8 μm pore size, Corning, Cambridge, USA) on the upper chamber containing the culture medium without FBS, and full medium (containing 10% FBS) as a chemoattractant was filled into the lower chamber. After further incubation for 24 h, a sterilized cotton swab was used to clean the non-migrated cells, and the cells that migrated into the lower membrane was fixed with 70% ethanol and stained with 0.5% gentian violet for 30 min at room temperature. The number of migrated cells was counted under a light microscope by randomly selecting 5 fields.

2.6. Western blot assay

Protein extraction from treated HPASMCs was performed by using the RIPA buffer (Bio-Rad, Hercules, USA) supplemented with protease inhibitors (Sigma-Aldrich). The concentrations of the extracted protein were measured by the BCA method (Bio-Rad). Equal amount of proteins (40 μg) were separated by gel electrophoresis (10% SDS-PAGE) and transferred to the polyvinylidene fluoride membranes. The membranes were then incubated with non-fat milk (5% in TBST) at room temperature for 1 h immediately followed by rinsing with TBST for 3 × 5 min. The washed membranes were then probed by corresponding antibodies against PNCA, PTEN, HIF-1α, phosphorylated PI3K (p-PI3K), total PI3K (t-PI3K), phosphorylated AKT (p-AKT), total AKT (t-AKT) and β-actin (Abcam, Cambridge, USA) at 4 °C overnight. Following overnight incubation, the membranes were washed with TBST for 3 × 5 min before incubating with horseradish peroxidase-conjugated antibodies at room temperature for 2 h. The blotting bands were detected by using the ECL detection system (Bio-Rad).

2.7. Reporter plasmids construction and dual-luciferase reporter assay

To construct the 3′TUR reporter plasmids, the 3′UTR of PTEN (wild type or mutant) were subcloned into the pGL3 reporter plasmid (Promega, Madison, USA). Briefly, the HPASMCs were co-transfected with Renilla luciferase pRL-TK vector (Promega), pGL3 3′UTR-reporter vector, and corresponding miRNAs by using Lipofectamine 2000 reagent (Invitrogen). At 48 h after co-transfection, HPASMCs were collected for the determination of firefly and renilla luciferase activities using the Dual-luciferase reporter Assay system (Promega). Renilla luciferase activity was used as internal control for the firefly luciferase activity.

2.8. In vivo chronic hypoxic animal model

All the animal experimental procedures were approved by the Animal Ethics Committee of the Third People's Hospital of Hainan. For the establishment of *in vivo* chromic hypoxia animal model, male C57BL/6 mice (8 weeks old) were exposed to hypoxia for 21 days in an indigenously designed transparent Plexiglas chamber flushed with nitrogen-balanced 10% oxygen at 0.5-3.0 L/min. The CO_2 from the chamber was removed daily by absorption with sodalime (Sigma). For the normoxic control group, mice were placed in the same chamber open to room air. The assessment of PAH was performed by measuring right ventricular systolic pressures and the ratio of right to left ventricle plus septum weight [RV/((LV + S)], systolic arterial pressure (SAP) and heart rate (derived from SAP) at 21 d as described [16,17]. For miRNA treatment, mice exposed to hypoxia at day 7 and 14 were intravenously injected with inhibitors (30 nmol) NC or miR-19a inhibitors (30 nmol). At the end of the experiments, the mice was anaesthetized with intraperitoneal injection of pentobarbitone (40 mg/kg), the serum was isolated from the blood via tail vein incision; for the collection of the whole lung, diaphragm and suprahepatic vena cava were dissected and lung ligaments sectioned, and the lungs were gently turned upside down with two cotton swabs to perform posterior dissection, after that, the whole lung was excised. The collected samples were processed for qRT-PCR assay.

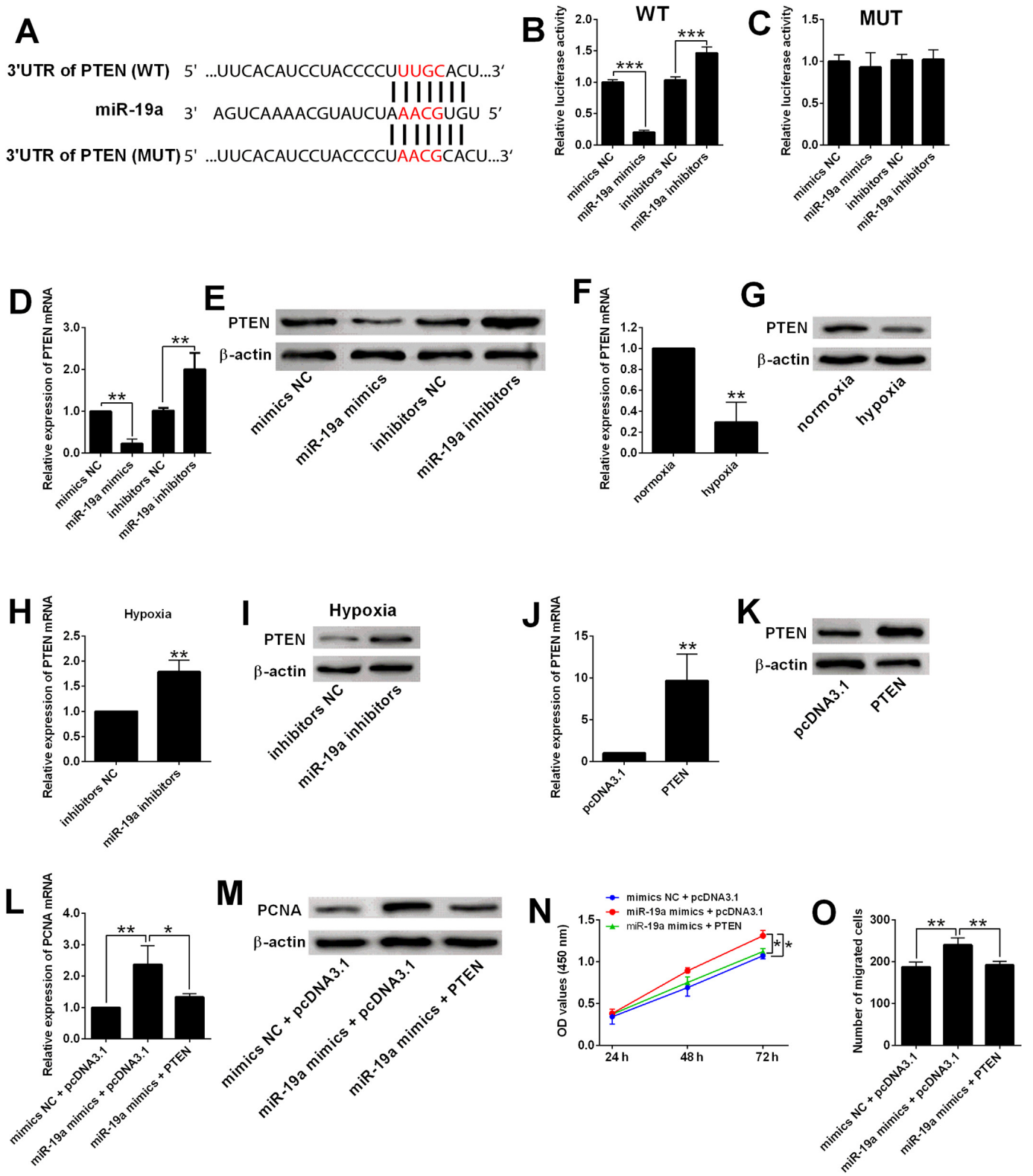
2.9. Statistical analysis

All the data analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, USA). All the data are presented as mean ± standard deviation. Significant differences between treatment groups were analysed using one-way analysis of variance or Student's unpaired t-test, as appropriate. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Hypoxia up-regulated miR-19a and promoted cell proliferation in HPASMCs

MiR-19a expression in the HPASMCs after exposing to the hypoxia (3% O_2) for different durations (12 h, 24 h and 48 h) was first evaluated by qRT-PCR assay, and exposure of HPASMCs to hypoxia for 12, 24 and 48 h significantly increased miR-19a expression when compared to control group (no exposure to hypoxia), and hypoxia time-dependently upregulated the miR-19a expression (Fig. 1A). Consistently, the CCK-8 assay results revealed that hypoxia time-dependently potentiated the cell viability of HPASMCs (Fig. 1B).



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3.2. The cell proliferative and migratory potentials of HPASMCs were enhanced with miR-19a overexpression

Transient overexpression of miR-19a was carried out by transfecting HPASMCs with miR-19a mimics, and transfection by miR-19a mimics markedly increased the miR-19a expression in HPASMCs compared to mimics NC group (Fig. 2A). The increase in the PCNA mRNA and protein expression levels was detected in HPASMCs with miR-19a overexpression (Fig. 2B and C). In addition, the CCK-8 assay showed that miR-19a mimics-transfected HPASMCs showed an enhanced cell viability compared to the NC group (Fig. 2D). The cell migration of HPASMCs after being transfected by miR-19a mimics or mimics NC was

Fig. 3. MiR-19a directly targets the 3′UTR of PTEN. (A) The predicted binding sites between miR-19a and 3′UTR of PTEN. (B&C) The dual luciferase reporter assay was used to determine the relative luciferase activity of the report vectors containing the wild type (WT) or mutant (MUT) 3′UTR of PTEN in the HPASMCs cells transfected with respective miRNAs. (D and E) The expression of PTEN mRNA and protein in HPASMCs was determined by qRT-PCR and western blot assays, respectively, at 24 h post-transfection with respective miRNAs. (F and G) The expression of PTEN mRNA and protein in HPASMCs exposing to normoxia or hypoxia for 48 h was determined by qRT-PCR and western blot assays, respectively. (H&I) The expression of PTEN mRNA and protein in hypoxia-treated HPASMCs was determined by qRT-PCR and western blot assays, respectively, at 24 h post-transfection with inhibitors NC or miR-19a inhibitors. (J&K) The expression of PTEN mRNA and protein was determined by qRT-PCR and western blot assays, respectively, at 24 h post-transfection with pcDNA3.1 or pcDNA3.1-PTEN. (L&M) The expression of PCNA mRNA and protein in HPASMCs was determined by qRT-PCR and western blot assays, respectively, at 24 h post-transfection with mimics NC + pcDNA3.1, miR-19a mimics + pcDNA3.1, or miR-19a mimics + pcDNA3.1-PTEN. (N&O) The cell viability and cell migration of HPASMCs were determined by CCK-8 and transwell migration assays, respectively, after being transfected mimics NC + pcDNA3.1, miR-19a mimics + pcDNA3.1, or miR-19a mimics + pcDNA3.1-PTEN. Data are expressed as mean ± standard deviation (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control groups.

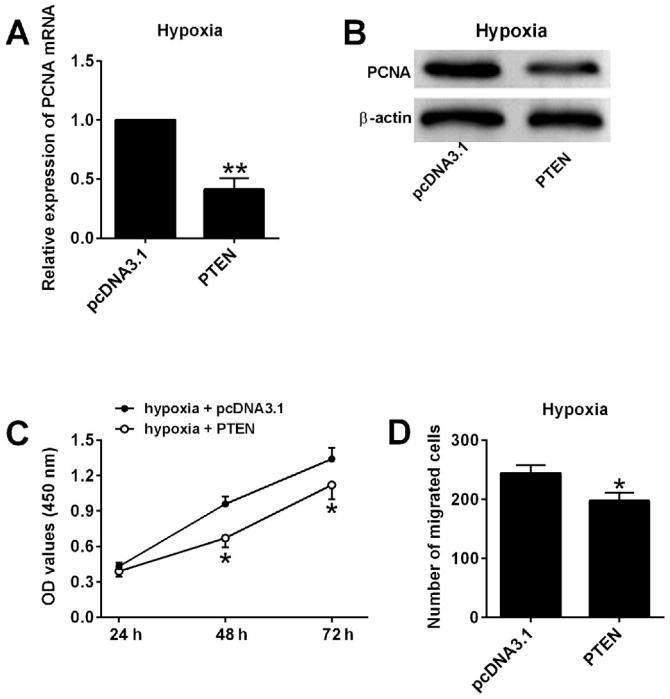

Fig. 4. The effects of PTEN on the cell proliferation and migration in HPASMCs after exposure to hypoxia. (A&B) The expression of PCNA mRNA and protein in hypoxia-treated HPASMCs was determined by qRT-PCR and western blot assays, respectively, at 24 h post-transfection with pcDNA3.1 or pcDNA3.1-PTEN. (C) Cell viability of the hypoxia-treated HPASMCs was detected by CCK-8 assay at 24, 48 and 72 h post-transfection with pcDNA3.1 or pcDNA3.1-PTEN. (D) Cell migration of the hypoxia-treated HPASMCs was measured by transwell migration assay at 24 h post-transfection with pcDNA3.1 or pcDNA3.1-PTEN. Data are expressed as mean ± standard deviation (n = 3). *P < 0.05 and **P < 0.01 compared to control groups.

evaluated by transwell migration assay, and the results demonstrated that miR-19a overexpression caused an increase in the number of migrated cells compared to the mimics NC group (Fig. 2E).

3.3. The cell proliferative and migratory potentials of hypoxia-treated HPASMCs was suppressed with miR-19a knockdown

Knockdown of miR-19a was carried out by transfecting HPASMCs with miR-19a inhibitors, and transfection by miR-19a inhibitors significantly down-regulated miR-19a expression in HPASMCs when compared to inhibitors NC group (Fig. 2F). In the HPASMCs with hypoxia treatment for 48 h, knockdown of miR-19a significantly suppressed the PCNA mRNA and protein expression levels in hypoxia-treated HPASMCs compared to inhibitors NC group (Fig. 2G and H). CCK-8 assay showed that miR-19a knockdown had suppressed the cell viability of the hypoxia-treated HPASMCs (Fig. 2I). Further results from transwell migration assay revealed that miR-19a knockdown inhibited the cell migration of HPASMCs exposing to hypoxia for 48 h (Fig. 2J).

3.4. MiR-19a suppressed the PTEN expression via targeting the 3′UTR of PTEN

To determine the mechanistic actions of miR-19a in regulating the cellular functions of HPASMCs, the TargetScan online tool was carried out, and the predicting results revealed a list of targets that could be potentially targeted by miR-19a (see supplementary data, Table 1), and we further selected PTEN for detailed investigation due to its well-documented role in the regulation of diverse cellular functions. To validate the interactions between PTEN 3′UTR and miR-19a, we constructed the luciferase report vectors with wild type PTEN 3′UTR or mutant PTEN 3′UTR (Fig. 3A). The luciferase activity of the wild type report vector was suppressed with miR-19a overexpression in HPASMCs and was enhanced in HPASMCs with miR-19a knockdown (Fig. 3B). On the other hand, miR-19a failed to affect the luciferase activity of the mutant report vector (Fig. 3C). Further *in vitro* assays showed that the PTEN mRNA and protein expression levels were inhibited in HPASMCs after being transfected miR-19a mimics and were increased in HPASMCs with miR-19a knockdown (Fig. 3D and E). PTEN expression was also examined in HPASMCs with 48 h hypoxia treatment or under normoxia conditions, and HPASMCs exposing to hypoxia had the decreased mRNA and protein expression levels of PTEN comparing to normoxia treatment (Fig. 3F and G). Consistently, knockdown of miR-19a markedly induced an up-regulation of PTEN in the hypoxia-treated HPASMCs compared to inhibitors NC group (Fig. 3H and I). To validate the functional interaction between PTEN 3′UTR and miR-19a, rescue experiments were performed, and the overexpression of PTEN was observed in HPASMCs after being transfected with PTEN-overexpressing constructs (pcDNA3.1-PTEN; Fig. 3J and K). Overexpression of PTEN attenuated the enhanced effects of miR-19a overexpression on the expression of PCNA mRNA and protein in HPASMCs (Fig. 3L and M). The CCK-8 and transwell migration assays revealed that PTEN overexpression partially reversed the increased cell viability and migratory potentials induced by miR-19a overexpression in HPASMCs (Fig. 3N and O).

3.5. The cell proliferation and migration in HPASMCs after exposure to hypoxia were inhibited with PTEN overexpression

We further determined the actions of PTEN in the cellular functions of HPASMCs after being treated with hypoxia for 48 h. Overexpression of PTEN significantly down-regulated the expression levels of PCNA in both mRNA and protein levels compared to the pcDNA3.1 transfection group (Fig. 4A and B). In addition, PTEN overexpression exerted inhibitory effects on the cell viability and cell migration of hypoxia-treated HPASMCs comparing to the control group (Fig. 4C and D). Furthermore, the western blot results showed that miR-19a overexpression enhanced the PI3K/AKT signaling, which was attenuated by enforced expression of PTEN in HPASMCs (Supplementary Fig. S1).

3.6. The HIF-1α mediated effects of hypoxia on the miR-19a and PTEN expression levels in HPASMCs

As HIF-1α is an important mediator in cells under hypoxia

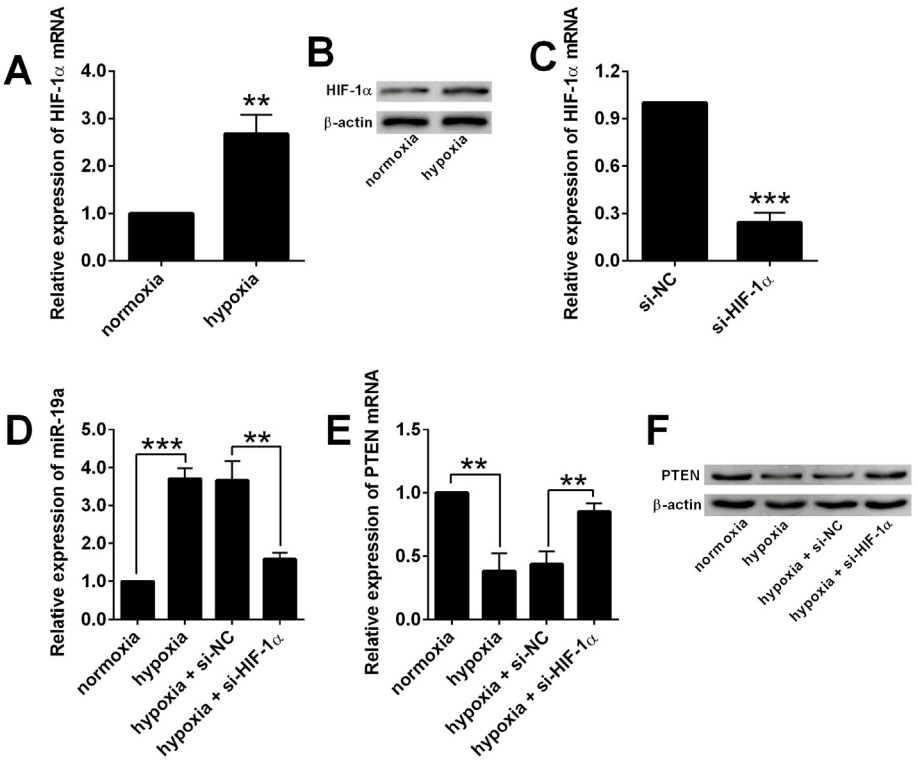

Fig. 5. The HIF-1α mediated the effects of hypoxia on the expression of miR-19a and PTEN in HPASMCs. (A&B) The expression of HIF-1α mRNA and protein in HPASMCs exposing to normoxia or hypoxia for 48 h was determined by qRT-PCR and western blot assays, respectively. (C) The expression of HIF-1α mRNA in HPASMCs was determined by qRT-PCR assay at 24 h post-transfection with si-NC or si-HIF-1α. (D&E) The expression of miR-19a and the expression of PTEN mRNA and protein in HPASMCs received different treatments were determined by qRT-PCR and western blot assays, respectively. Data are expressed as mean ± standard deviation (n = 3). **P < 0.01 and ***P < 0.001 compared to control groups.

conditions, the HIF-1α expression was determined in HPASMCs after being exposed to hypoxia for 48 h. As expected, hypoxia exposure caused a significant upregulation of HIF-1α in HPASMCs comparing to that in normoxia-treated HPASMCs (Fig. 5A and B). The down-regulation of HIF-1α was performed by transfecting HPASMCs with HIF-1α siRNA (Fig. 5C). Knockdown of HIF-1α partially reversed the increased expression of miR-19a, PTEN mRNA and protein induced by hypoxia in HPASMCs (Fig. 5D–F).

3.7. Knockdown of miR-19a attenuated the chronic hypoxia-induced PAH in in vivo chronic hypoxia mice model

The knockdown effects of miR-19a in hypoxia-induced PAH were further evaluated in the *in vivo* animal model. Chronic hypoxia treatment for 21 d induced an increase in the miR-19a expression and a decrease in the PTEN mRNA expression determined from both serum and lung tissues, and the effects were attenuated by the pre-treatment with miR-19a inhibitors (Fig. 6A–D). As expected, chronic hypoxia caused an increase in RVSP and RV/(LV + S) (Fig. 6E–F), suggesting the establishment of PAH model. The miR-19a inhibitors pre-treatment decreased RVSP and RV/(LV + S) in the hypoxia-treated mice (Fig. 6E and F). There is no significant difference in SAP and heart rate between different treatment groups (Fig. 6G and H).

4. Discussion

Hyper-proliferation of HPASMCs has been shown to be a key contributor in the pathophysiology of PAH [18]. In the present study, we found that miR-19a was markedly up-regulated and cell proliferation was enhanced in the hypoxia-induced HPASMCs. Functional *in vitro* assays demonstrated that cell proliferative and migratory potentials of HPASMCs were enhanced with miR-19a overexpression, on other hand, knockdown of miR-19a exerted suppressive effects on hypoxia-treated HPASMCs. Further mechanistic studies revealed that miR-19a regulated proliferation and migration of hypoxia-treated HPASMCs via suppressing PTEN expression. In addition, HIF-1α was identified as one of the mediators for the hypoxia-induced changes in miR-19a expression and PTEN. This study presented a novel mechanistic action of miR-19a-mediated cell proliferation and migration of HPASMCs.

Hypoxia is one of key factors that contribute to PAH, and studies have shown that treatments with chronic hypoxia lead to PAH via inducing hyper-proliferation of HPASMCs and causing vascular remodelling [19]. In this study, we found that hypoxia caused miR-19a upregulation and concurrently increased cell proliferation in HPASMCs, suggesting miR-19a's involvement in the hyper-proliferation of hypoxia-treated HPASMCs. A large body evidence has showed the enhanced effects of miR-19a on the cell proliferation, particularly in cancer cells. MiR-19a was found to potentiate cell proliferation and migration in colorectal cancer cells via targeting T-cell intracellular antigen 1 [20]. In addition, miR-19a also exerted enhanced effects on other types of cancer cells such as ovarian cancer, liver cancer, gastric cancer and pancreatic cancer cells [4,21–24]. In the cardiomyocytes, miR-19a had protective effects on the hypoxia/reoxygenation-induced apoptosis [25]. In our study, we determined the cell viability by using CCK-8 assay, and the expression of PCNA was measured to indicate the proliferative ability of HPASMCs, as PCNA has been shown to be an important marker of cell proliferation [26]. In addition, transwell migration detected the cell migration of HPASMCs. Consistent with previous findings, miR-19a overexpression promoted cell proliferative and migratory potentials of HPASMCs; on the other hand, knockdown of miR-19a attenuated the enhanced effects of hypoxia treatment on the HPASMCs proliferation. All in all, our findings suggested that the effects of hypoxia on the HPASMCs proliferation may involve miR-19a upregulation.

In order to address the mechanistic actions of miR-19a on the cell proliferation and migration of HPASMCs, we performed the bioinformatics prediction and found that PTEN was one of potential genes that targeted by miR-19a, where the interaction between miR-19a and 3′TUR of PTEN was confirmed by luciferase reporter assay in this study. PTEN is well-documented for its tumor suppressive functions and plays pivotal roles in diverse cellular functions [27]. Up to date, PTEN has been studied for its key functions in PAH. Ravi et al., showed that peroxynitrite-mediated inactivation of PTEN was a key mediator of lung microvascular remodelling associated with PAH [28]. Inactivation of

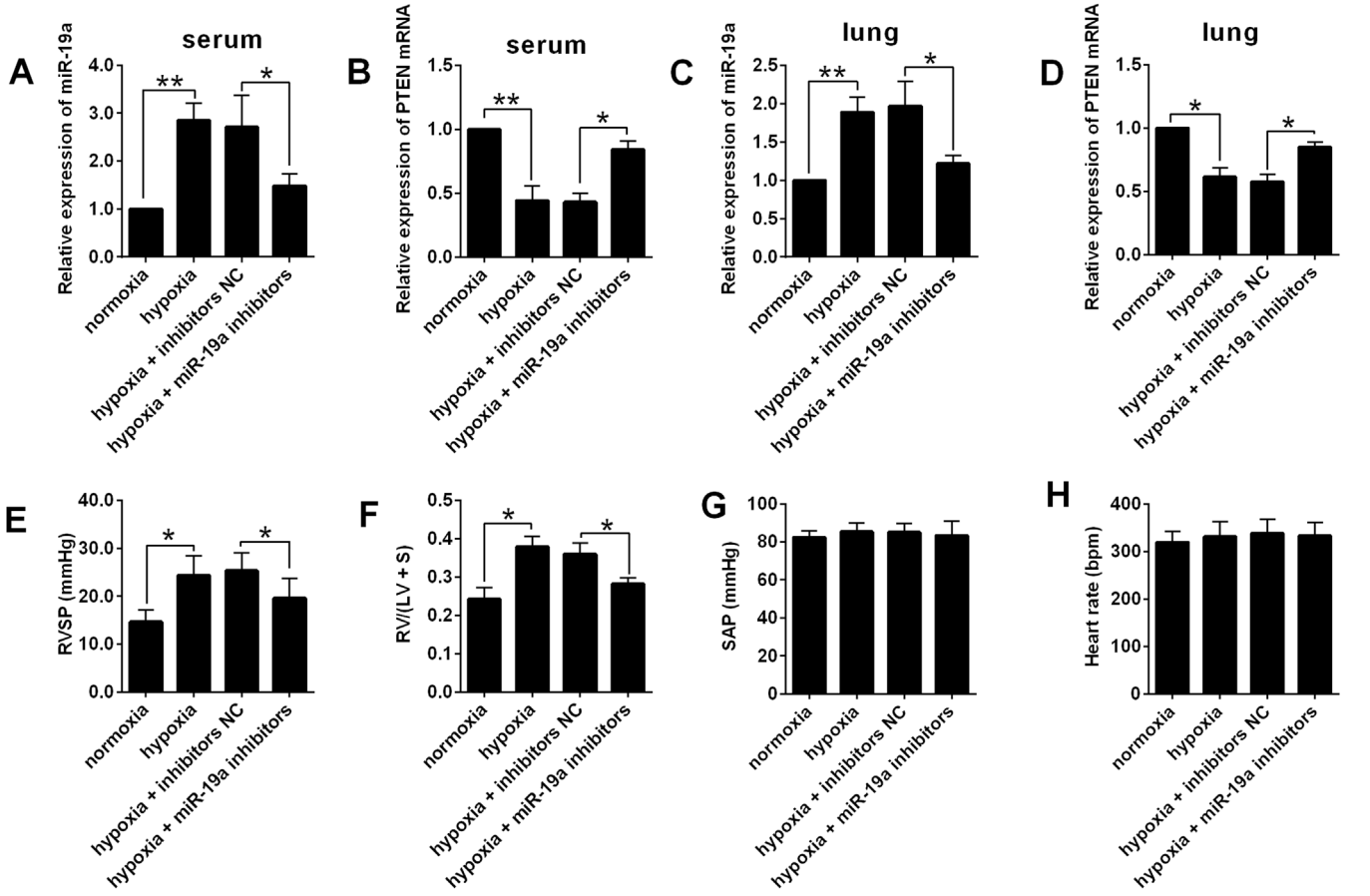

Fig. 6. Knockdown of miR-19a attenuated the chronic hypoxia-induced PAH in *in vivo* chronic hypoxia mice model. Chronic hypoxia-induced PAH in a mice model was established by 3 weeks of hypoxic treatment. For the miRNA treatment, mice were mice exposed to hypoxia at day 7 and 14 were intravenously injected with inhibitors (30 nmol) NC or miR-19a inhibitors (30 nmol). (A) The miR-19a and (B) PTEN mRNA expression levels in the mice serum were detected by qRT-PCR. (C) The miR-19a and (D) PTEN mRNA expression levels in the mice lung tissues were evaluated by qRT-PCR. (E) Right ventricular systolic pressure (RVSP), (F) right ventricular hypertrophy [RV/(LV + S)], (G) SAP and (H) heart rate from different treatment groups were shown. N = 8. *P < 0.05 and **P < 0.05 compared to control groups.

PTEN in PASMCs was found to cause severe pulmonary hypertension by synergizing with hypoxia [29]. A further study demonstrated that PTEN signaling pathway plays a key role in attenuating pathogenic derangements in PAH [30]. Moreover, recent studies showed that miR-19a could target PTEN to regulate the high mobility group protein B1-induced proliferation and migration of human airway smooth muscle cells [14]. In the present study, PTEN was markedly repressed by miR-19a overexpression in HPASMCs, and PTEN overexpression partially restored the enhanced effects on cell proliferation and migration caused by miR-19a overexpression and hypoxia in HPASMCs. Taken together, the hypoxia-induced enhanced proliferation and migration of HPASMCs may involve in the miR-19a/PTEN axis. More importantly, miR-19a was up-regulated and PTEN was down-regulated in the serum and lung tissues from chronic hypoxia-treated mice, and knockdown of miR-19a attenuated PAH induced by chronic hypoxia treatment in the mice, suggesting that targeting of miR-19a may be a promising target for PAH. Studies have demonstrated that miR-19a could target the toll-like receptor 2 (TLR2) to regulate the inflammatory response in rheumatoid fibroblast-like synoviocytes [31], and whether miR-19a can target TLR2 to regulate HPASMC proliferation via inflammatory modulation still requires further investigation. Moreover, the downstream signaling mediators of PTEN include PI3K and AKT, and PTEN/PI3K/AKT signaling pathway has been demonstrated to modulate hypoxia-induced pulmonary hypertension [15]. MiR-19a also regulated the hypoxia/reoxygenation-induced apoptosis in cardiomyocytes PTEN/PI3K/AKT signaling pathway [25]. In this study, western blot results showed that miR-19a overexpression increased p-PI3K and p-AKT protein levels, which was attenuated by the enforced PTEN expression. Thus, miR-19a may regulate the HPASMC proliferation and migration via PTEN/PI3K/AKT signaling, which may be a future direction of our study.

HIF-1α plays an important role in regulating the response to hypoxia by regulating proteins involved in essential biological processes (Wang et al., 2018a). HIF-1α induced upregulation of miR-9 contributed to the hyper-proliferative phenotype in HPASMCs during hypoxia [32]. HIF-1α was also found to regulate endothelin expression via miR-543 in PASMCs, which may consequently contribute to the progression of PAH [33]. Gou et al., revealed that miR-210 overexpression in HPASMCs was a HIF-1α-dependent and miR-210 has an anti-apoptotic action on HPASMCs under hypoxic conditions [34]. In the present study, HIF-1α was induced by hypoxia in HPASMCs, and inhibition of HIF-1α down-regulated the miR-19a expression and increased the PTEN mRNA expression in HPASMCs during hypoxia. Taken together, hypoxia-induced changes in the miR-19a/PTEN axis may be mediated via HIF-1α.

In this study, there are several limitations. The PAH animal model was assessed by hemodynamics in the mice, while the methodology was controversial [35,36]. Electrocardiography (ECG) has been commonly used in the clinic to assess PAH [37], however, the application of ECG in the evaluation of experimental PAH in mice was rare, and future studies may employ electrocardiography evaluation for PAH in the mice. Thus, we should be cautious when interpreting the *in vivo* findings. Our study was mainly focused on the *in vitro* signaling pathways,

whether the pathway is activated in the *in vivo* animal studies remains to be determined in the future studies. Hypoxia can affect cell proliferation and migration in other smooth muscle cells such as venous smooth muscle cells [38], bladder smoot muscle cells [39] and aortic vascular smooth muscle cells [40]; whether hypoxia-mediated these effects via a similar mechanism in these smooth muscle cells is worth of investigation. The targets of miR-19a as predicted by TargetScan tool were not limited to PTEN (see supplementary data, Table 1). Among the predicted targets, insulin-like growth factor-binding protein 3, serum-glucocorticoid regulated kinase 1 and ras homolog family member B have been demonstrated to involve in the pathophysiology of PAH [41–44], and further studies are needed to examine these potential targets.

5. Conclusion

In conclusion, our results showed that miR-19a expression was markedly enhanced in HPASMCs during hypoxia, and knockdown of miR-19a suppressed the cell proliferative and migratory potentials in hypoxia-induced PASMCs. Further mechanistic studies revealed that the hypoxia-induced PASMCs hyper-proliferation and enhanced migration may involve the HIF-1α/miR-19a/PTEN/PI3K/AKT pathway. The current investigations provided evidence for the novel role of miR-19a in modulating cell proliferation and migration of HPASMCs during hypoxia.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116928.

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