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Downregulation of microRNA-637 Increases Risk of Hypoxia-Induced Pulmonary Hypertension by Modulating Expression of Cyclin Dependent Kinase 6 (CDK6) in Pulmonary Smooth Muscle Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The objective of this study was to investigate the molecular mechanism by which miR-637 interferes with the expression of CDK6, which contributes to the development of pulmonary hypertension (PH) with chronic obstructive pulmonary disease (COPD).

Material/Methods: We used an online miRNA database to identify CDK6 as a virtual target of miR-637, and validated the hypothesis using luciferase assay. Furthermore, we transfected SMCs with miR-637 mimics and inhibitor, and expression of CDK6 was determined using Western blot and real-time PCR.

Results: In this study, we identified CDK6 as a target of miR-637 in smooth muscle cells (SMCs), and determined the expression of miR-637 in SMCs from PH patients with COPD and normal controls. We also identified the exact miR-637 binding site in the 3'UTR of CDK6 by using a luciferase reporter system. The mRNA and protein expression levels of CDK6 in SMCs from PH patients with COPD were clearly upregulated compared with the normal controls. Cells exposed to hypoxia also showed notably increased CKD6 mRNA and protein expression levels, and when treated with miR-637 or CDK6 siRNA, this increase in CKD6 expression was clearly attenuated. Additionally, cell viability and cell cycle analysis showed that hypoxia markedly increased viability of SMCs by causing an accumulation in S phase, which was relieved by the introduction of miR-637 or CDK6 siRNA.

Conclusions: Our study proved that the CDK6 gene is a target of miR-637, and demonstrated the regulatory association between miR-637 and CDK6, suggesting a possible therapeutic target for PH, especially in patients with COPD.

MeSH Keywords: Cell Cycle Checkpoints • MicroRNAs • Persistent Fetal Circulation Syndrome

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Background

Pulmonary hypertension (PH) is a lethal disease characterized by vascular remodeling and inappropriate vasoconstriction, leading to elevated pulmonary vascular resistance and eventually to decreased exercise tolerance and increased heart failure [1,2]. Understanding of the molecular pathophysiology of PH has been advancing rapidly but is still largely unclear. Hypoxia is a well-known risk factor of PH, and hypoxia may cause PH by promoting the development of pulmonary vascular remodeling and, thereby, pulmonary vasoconstriction [3]. The alternation of cell cycle progression, as well as proliferation of pulmonary artery smooth muscle cells (PASMCs), may underlie the pathogenesis of pulmonary vascular remodeling [4]. However, the exact molecular mechanism underlying the abnormally enhanced PASMC proliferation remains to be explored.

MicroRNAs (miRNAs) are a class of small non-coding RNAs (21 to 23 nucleotides in length), that have been reported to participate in many biological processes, such as differentiation, cell death, apoptosis, proliferation, and metabolism [5,6]. Recently, many cardiovascular diseases, such as PH, have been found to result from dysregulation of miRNAs expression or differential expression of miRNAs [7,8]. For example, the expression of bone morphogenetic protein receptor type II (BMPR2) is negatively regulated by miR-17 [9], and the dysregulation of its expression has been reported to play a critical role in the control of pulmonary artery blood pressure, vascular remodeling, and proliferation of PASMCs [10]. Additionally, miR-21 is believed to be crucial in regulating migration of smooth muscle cells of pulmonary vessels (VSMC). The abnormal migratory ability of VSMCs caused by dysregulation of miR-21 might result from hypoxia [10].

As a well-established high-throughput technique, microarray analysis is able to identify altered expression profiles of miRNAs at a large scale. Researchers designed a longitudinal, crossover experiment with rats whose PH was caused by monocrotaline or chronic hypoxia, and identified the differential miRNA expression profiles and dysregulated expression of specific miRNAs that might be responsible for the development of PH [11]. In the same study, numerous miRNAs were identified to be differentially expressed, including miR-637, which belongs to a cluster of miRNAs named “hypoxamiRs”, defined as those miRNAs whose expression changes in response to hypoxia. The present study was focused on miR-637 as well as its direct target gene, CDK6. Bioinformatic analysis and luciferase assay were used to validate the regulatory association between miR-637 and CDK6. The roles of the miRNA and CDK6 in the control of PASMC proliferation were also tested in human lung samples and cultured cells.

Material and Methods

Subjects

The study was approved by the Research Ethics Committee at Jilin University, and we obtained written informed consent from each subject recruited in this study. We recruited 21 COPD patients with PH and 18 normal control subjects. We isolated PASMCs from control subjects and COPD patients with PH. The tissue samples were collected from those patients who received surgical intervention for the treatment of lung cancer. We carefully separated the PASMCs from the thin layer of media. All the cells were spindle-shaped, and almost all cells collected were positive for α-smooth muscle actin, a specific marker for smooth muscle cells (data not shown). Tissues were cut into small pieces and incubated in fresh Hank’s balanced salt solution (HBSS) containing 1.0 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) and 2.5 mg/mL collagenase (Worthington Biochemical, Lakewood, NJ) at 37°C for 30 min. Then, we centrifuged the tissues at 300 g for 5 min, and re-suspended them in Medium 231 (Gibco, Grand Island, NE) containing 200 IU/mL streptomycin, 200 ug/mL of penicillin and smooth muscle growth supplement, plated them in dishes, and incubated them in a humidified atmosphere with 5% CO_2 at 37°C.

Transfection of oligonucleotides

The PASMCs were treated with miR-637 mimics, CDK6 siRNA, and the scramble control, which were designed and synthesized by GenePharma (Shanghai, China) using X-treme Gene siRNA Transfection Reagent (Roche Applied Science, Mannheim, Germany). The detailed miRNA and siRNA sequences were: miR-637 (5’-ACUGGGGCUUUCGGGCUCUGCGU-3’) and CDK6 siRNA (5’-GCAAAGACCUACUUCUGAAUU-3’), scramble control (5’-UUCUCCGAACGUGUCACGUTT-3’). The PASMCs cultured in 6-well plates (~50% confluence) were treated with serum deprivation for 24 h. Approximately 2 g of siRNA and 10 l of X-treme Gene siRNA Serum-free DMEM medium were used to dilute Transfection Reagent for 5 min, then we mixed them together at room temperature for another 20 min before cells were added. Cells were incubated for 24 h under normal growth conditions. Then, they replaced by DMEM (5% FBS) and cultured for another 24 h.

Real-time PCR analysis

The total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, and the isolated RNA was reversely transcribed into cDNA with the prime script RT reagent kit. We performed PCR with SYBR® Premix Ex TaqTMII (TaKaRa biotechnology) on a thermocycler ABI Prism 7300 (Applied Biosystems) for 40 cycles. The $2^{-\Delta\Delta CT}$ method was

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used to determine the change in the expression of mRNA or miRNA relative to control samples. U6 and GAPDH were used as an internal control for miR-637 and CDK6, respectively.

Cell cycle analysis

The proportion of G0/G1, S and G2/M phases of PASMCs was detected by flow cytometry. Briefly, the cells were pre-treated with hypoxia with or without other indicated agents and then harvested by trypsinization. We used 70% ethanol for fixing cells at 4°C. We centrifuged the fixed cells at 300 g for 5 min and then re-suspended them in 500 l of staining buffer before detection. A total of 10 ul of RNaseA was added and mixed; then, 25 ul of PI (propidium iodide) was added, and we incubated the suspension for 30 min in a 37°C water bath. Finally, we filtered the cells once through 400-mesh sieves before flow cytometry was performed.

Hypoxia treatment of PASMCs

Hypoxia exposure was done by connecting to a chamber equilibrated with a water-saturated gas mixture of 1% O2, 5% CO2, and 94% N2 at 37°C for 24 h.

Luciferase reporter assays

We searched the online microRNA database (*www.mirdb.org*) and identified CDK6 as a directly regulated target of miR-637 with 2 potential binding sites located in 1510–1516 bp and 7382–7399 bp in the 3'UTR of CDK6. We amplified full-length CDK6 3'UTR and cloned into psiCHECK-2 vectors (named WT). We used the GeneTailor Site-Directed Mutagenesis System (Invitrogen, Guangzhou, China; named MUT) to introduce mutants to replace the virtual binding sites of miR-637 in the 3'UTR. For reporter assays, we cotransfected mt or wt vector and psiCHECK-2 vector as control vector into PASMCs, which overexpresses miR-637 (transfection with miR-637 mimics), and incubated them in 48-well plates. Luciferase assay was performed 48 h after transfection. We used the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI) to perform luciferase assays in accordance with the protocol and we used Renilla luciferase for normalization.

Western blot analysis

PASMCs were lysed with lysis buffer, and the lysate was loaded onto a SDS-PAGE gel (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and then blotted them onto a nitrocellulose membrane (Millipore, Billerica, MA). We blocked the membrane in phosphate-buffered saline with 5% nonfat dry milk and incubated it for 1 h. Subsequently, the membrane was incubated with primary antibody of anti-β-actin (1:1,0000; Sigma-Aldrich) and primary antibody of CDK6 (1:1,000; Cell Signaling Technology, Beverly, MA) overnight at 4°C. HRP-labeled secondary antibody (1:1,0000; Cell Signaling Technology, Beverly, MA) was used to treat the primary antibody-labeled membranes for 1 h. An ECL kit (Amersham ECL detection system, GE Healthcare) was used to detect the signals in the blots. The density of the β-actin band was used to normalize the value of the relative density of the target protein band.

Statistical analysis

Data are presented as means ± standard deviation (SD). Student's unpaired t-test was used to evaluate the difference between the groups. P value <0.05 was considered to be statistically significant.

Results

Expression of miR-637 is depressed in PH tissues in COPD

To investigate the role of miR-637 in the pathogenesis of hypoxia-induced PH, we collected 21 PASMCs from PH patients with COPD and 18 normal control samples. We used qRT-PCR to examine the expression level of miR-637 in each sample. As shown in Figure 1, we found that the expressions of miR-637 in PASMCs collected from PH patients with COPD were markedly lower than that of normal controls.

CDK6 is a potential target of miR-637

Since expression of miR-637 is suppressed in PASMCs from PH patients with COPD, we assumed miR-637 is involved in the development of PH via interfering with the expression of its target genes. As shown in Figure 2, to identify the potential target of miR-637, we therefore used bioinformatics tools and identified 2 possible miR-637 binding sites in the 3'UTR of CDK6: segments 1510-1516 and segments 7382–7388. The 2 matched regions strongly supported the CDK6 gene as a potential target gene of miR-637.

1510–1516 bp in the 3'UTR of CKK6 is the target binding site for miR-637

To verify the CDK6 gene as the direct gene of miR-637, we examined the exact binding region for miR-637 in the 3'UTR of CDK6 gene. Luciferase reporter constructs were established with wild-type or mutants inserted (Figure 2). As shown in Figure 3, the luciferase activity of the wild-type CDK6 group was clearly lower than in the negative controls. However, the Mutant1 CDK6 group showed significantly higher luciferase activity (which is, on the other hand, quite comparable with the negative controls) than in the wild-type CDK6 group, while the Mutant2 CDK6 group merely showed different luciferase

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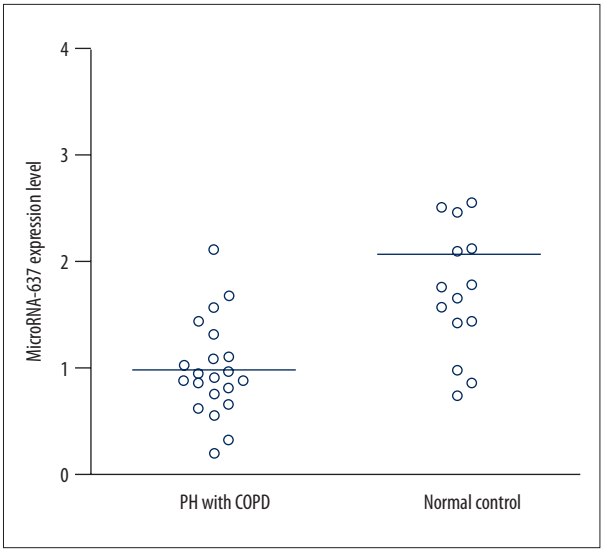

Figure 1. Expression of miR-637 was reduced in PASMCs collected from PH patients with COPD compared with normal controls.

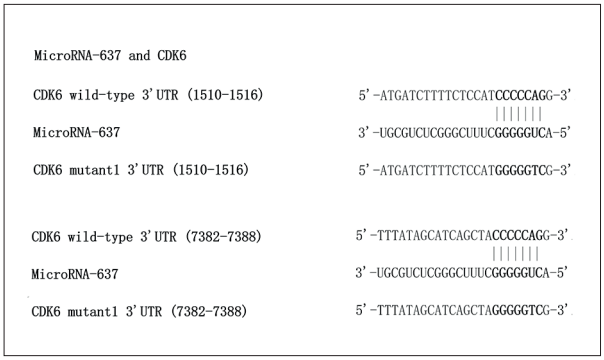

Figure 2. CDK6 was identified as a target of miR-637 with 2 possible binding regions, which accordingly generated Mutant1 CDK6 and Mutant2 CDK6 where the possible binding site sequences were replaced with their complementary sequences.

activity compared with the wild-type CDK6 group. The findings indicate that 1510–1516 bp in the 3'UTR of CKK6 is the target binding site for miR-637.

Negatively related association between miR-637 and CDK6

We also conducted qRT-PCR and Western blot analysis to investigate the regulatory association between miR-637 and CDK6 in PASMCs collected from PH patients with COPD and from normal controls. As shown in Figure 4A, mRNA expression of CDK6 showed an evident decrease among the normal controls compared with the cases, as was also the case with the protein expression of CDK6 (Figure 4B). Considering the former conclusion that expression of miR-637 was higher in normal controls, we concluded that miR-637 would inhibit the expression of CDK6 at the mRNA and protein levels, indicating the negatively-related association between miR-637 and CDK6.

MiR-637 inhibits the mRNA and protein expression of CDK6

To validate the effect of miR-637 upon the predicted target CDK6 gene, we exposed PASMCs to hypoxia, which would help to elucidate the pathologic phenomenon caused by COPD. We also transfected hypoxia-exposed cells with miR-637 mimics (which aggrandizes the effect of miR-637) and CDK6 siRNA (which interferes with the expression of CDK6 mRNA and protein), respectively. As the results in Figure 5A show, cells exposed to hypoxia had increased protein expression of CDK6 compared with the controls, while cells treated with miR-637

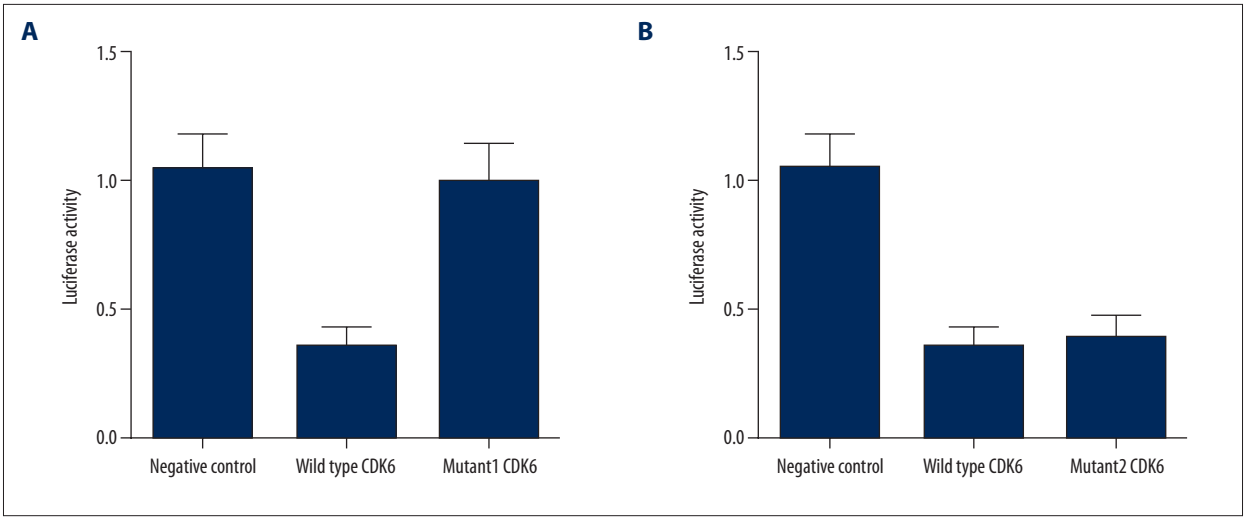

Figure 3. (**A**) Luciferase activity of cells with wild-type CDK6, Mutant1 CDK6 as compared with the negative controls; (**B**) Luciferase activity of cells with wild-type CDK6, Mutant2 CDK6 as compared with the negative controls.

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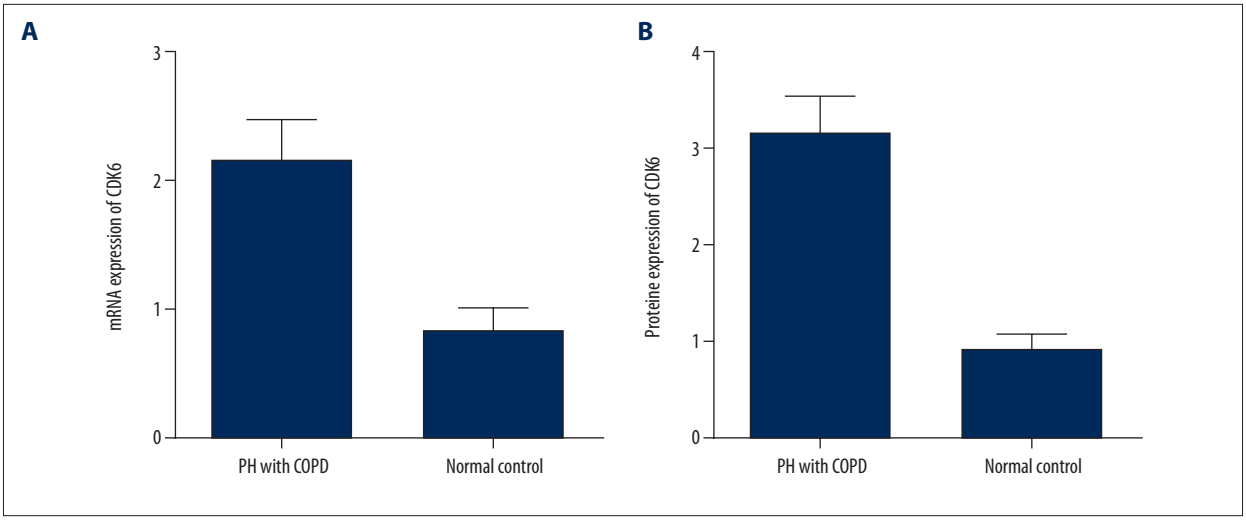

Figure 4. (**A**) CDK6 mRNA expression level in PASMCs from PH patients with COPD was significantly lower compared with normal controls; (**B**) CDK6 protein expression level in PH cells with COPD was significantly lower compared with normal controls.

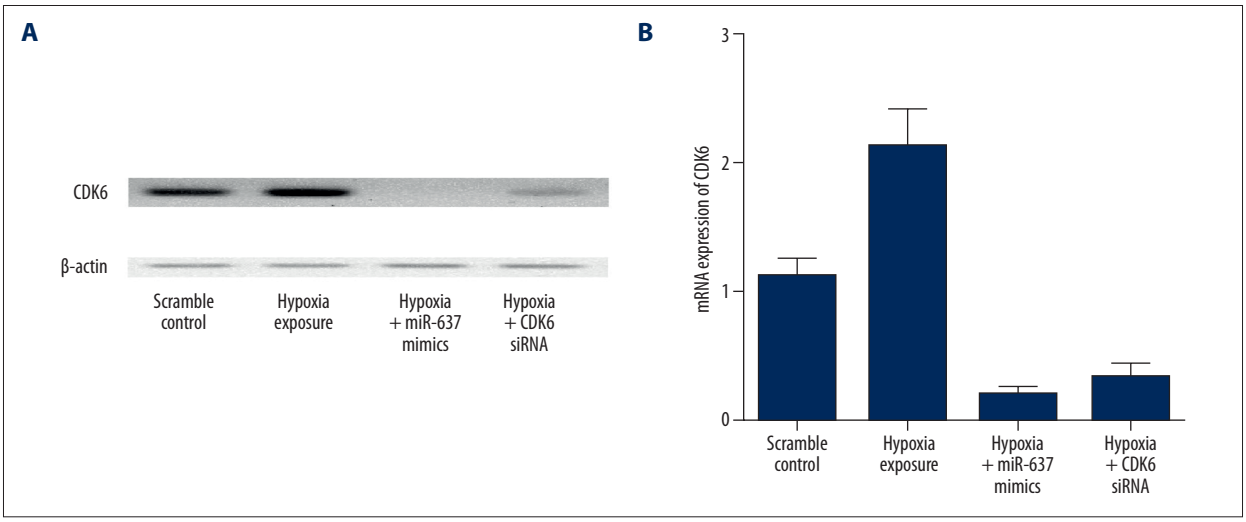

Figure 5. (**A**) CDK6 protein expression levels in cells treated with hypoxia exposure, miR-637 mimics with hypoxia exposure, CDK6 siRNA with hypoxia exposure as compared with the scramble controls; (**B**) CDK6 mRNA expression levels in cells treated with hypoxia exposure, miR-637 mimics with hypoxia exposure, CDK6 siRNA with hypoxia exposure as compared with the scramble controls.

mimics and CDK6 siRNA showed a substantially lower expression of CDK6 compared with the control. Similar results were observed regarding the mRNA expression determined by the qRT-PCR analysis, as shown in Figure 5B. The above findings all support the hypothesis that miR-637 actually downregulated the expression of CDK6.

Influence of miR-637 upon viability and cell cycle status of PASMCs

CDK6 protein is known to be an important regulator of tumor suppression (negatively correlated) and cell cycle progression, especially cell cycle G1 phase progression and G1/S transition. Therefore, cell viability and cell cycle status of PASMCs will also work as indicators to evaluate the effect of miR-637 upon the CDK6 gene. As shown in Figure 6, PASMCs treated with hypoxia exposure showed higher cell viability and more cells in G2/M phase compared with the scramble controls. Hypoxia-exposed PASMCs treated with miR-637 mimics or CDK6 siRNA showed comparably low cell viability and many more cells in S phase compared with the scramble controls. Consequently, the viability and cell cycle fractions of PASMCs all proved the negative effect of miR-637 on CDK6.

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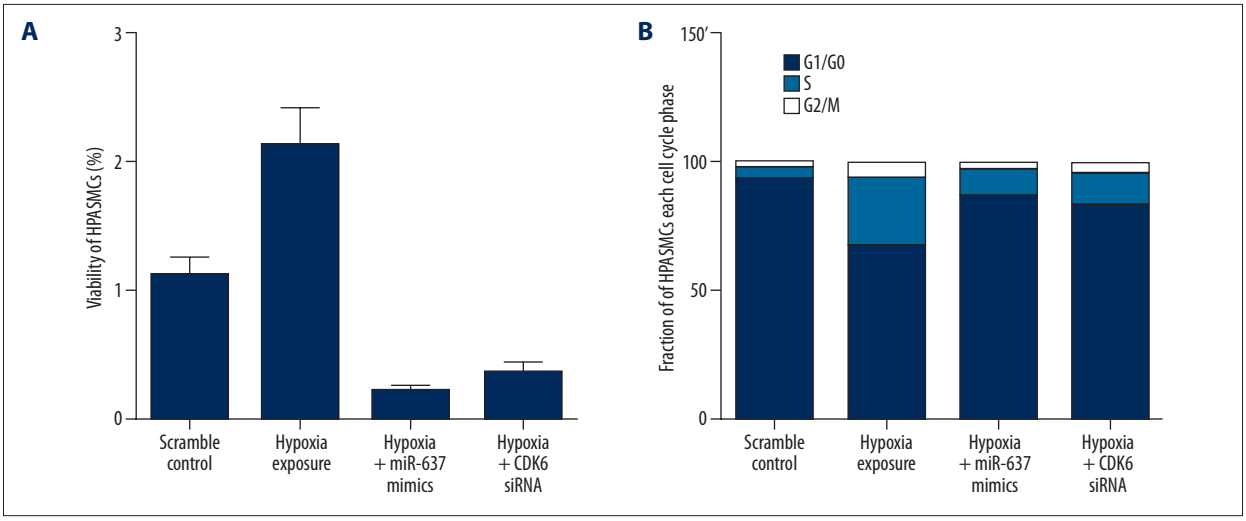

Figure 6. (**A**) Percentage of cell viability in groups of PASMCs treated with hypoxia exposure, miR-637 mimics with hypoxia exposure, CDK6 siRNA with hypoxia exposure as compared with the scramble controls; (**B**). Fraction of cell cycle phase in groups of PASMCs treated with hypoxia exposure, miR-637 mimics with hypoxia exposure, CDK6 siRNA with hypoxia exposure as compared with the scramble controls.

Discussion

In this study we demonstrated that the downregulation of miR-637, which was induced by hypoxia, led to promotion of proliferation in HPASMC via releasing physiological inhibition of CDK6, a regulator of cell cycle progression. Furthermore, we found that the dysregulation of CDK6 caused by aberrant expression of miR-637 leads to abnormally enhanced proliferation of PASMC, which contributes to remodeling of vessels in PH induced by hypoxia.

As a primate-specific miRNA, a high expression of miR-637 has been detected in livers in accordance with the miRbase database. However, there are no adequate reports about the role of miR-637 in any field, not even in the liver. It has been found that the expression of miR-637 is significantly decreased in glioma tissues [12]. Tumor grading was found to be negatively correlated with miR-637 expression [12]. Underexpression of miR-637 increased activity of Akt1, which participates in cell proliferation and survival, and contributes to glioma progression [13,14]. Studies have shown that the AKT pathway regulated by miRNA is critical in glioma progression [15,16]. Another study found that the STAT3 signaling pathway was activated by the downregulation of miR-637, leading to proliferation of breast cancer cells [17]. In our experiment, miR-637 was identified as a putative proliferation suppressor in PASMCs, and we provided a basis for further investigating the potential use of miR-637 in treatment of PH. Although it was discovered several years ago, the functions of miR-637 in control of proliferation of human cells remain obscure. Our findings revealed decreased expression of miR-637 in the PASMCs collected from PH patients with COPD as well as the PASMCs exposed to hypoxia for the first time, which were reversely correlated with cell proliferation. To discover the role of miR-637 in the control of PASMC proliferation, the effect of miR-637 on cell growth was examined in PASMCs, and we found that PASMCs treated with hypoxia exposure showed an increase in cell viability and accumulation in S phase compared with the scramble controls. Treatment with miR-637 mimics or CDK6 siRNA mostly restored the hypoxia-induced proliferation of PASMCs and S phase accumulation, indicating that miR-637 was a growth suppressor in PASMCs exposed to hypoxia, and hypoxia induced abnormally enhanced proliferation of PASMCs, at least partially via downregulation of miR-637.

We also found that miR-637 directly targets the 3-UTR of CDK6 by binding 1510–1516 bp in the 3'UTR of the gene, and that miR-637 overexpresses when CDK6 is knocked down.

Cell division protein kinase 6 (CDK6) is an enzyme encoded by the CDK6 gene [18]. It is regulated by cyclins, specifically by Cyclin D proteins and Cyclin-dependent kinase inhibitor proteins [19]. The protein encoded by this gene is a member of the cyclin-dependent kinase (CDK) family, and CDK family members are highly similar to the gene products of *Saccharomyces cerevisiae* cdc28 [20], and *Schizosaccharomyces pombe* cdc2, and are known to be important regulators of cell cycle progression in the point of regulation named R or restriction point [21,22]. Sequential activation of cyclin-dependent kinases (CDKs) and cyclins regulates cell cycle progression. Cyclin D1 combines with CDK6 to form the Cyclin D1/CDK6 complex, which is critical in regulating cell cycle G1 phase. As a binding partner of Cyclin D1 and part of the activated Cyclin D1/CDK6 complex,

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CDK6 can induce target gene expression, which is essential for entry into S phase [23,24]. In this study we found that cells exposed to hypoxia showed an increased protein expression of CDK6 compared with the controls, while cells treated with miR-637 mimics and CDK6 siRNA showed a substantially lower expression of CDK6 compared with the controls. Similar results were observed regarding the mRNA expression determined by the qRT-PCR analysis. Consistently, we found that hypoxia exposure upregulated the expression of CDK6, causing an increase in the fraction of cells in S phase, as well as an abnormally enhanced proliferation of PASMCs. This effect of CDK6 on the cell cycle status and growth of the cells could be eliminated by downregulation of CDK6 by transfecting the PASMCs with miR-637 and CDK6 siRNA, confirming the role of upregulation of CDK6 and downregulation of miR-637 in the pathogenesis of the development of PH induced by hypoxia.

Conclusions

We confirmed that the expression of hypoxia induced downregulation of miR-637 as well as its negative regulation of the CDK6 pathway in PASMC. We have shown that miR-637 directly targeted CDK6 in PASMC. We demonstrated the functional connection of miR-637 and CDK6 in the process of cell proliferation. We hypothesize that it is essential for cell survival to maintain a balanced level of miR-637, and that miR-637 plays an important role in the control of homeostasis of PASMC proliferation. *In vivo* studies are needed to see whether knockout or overexpression of miR-637 is involved in the development of hypoxia-induced PAH and vascular remodeling.

Conflict of interest

The authors claim no conflicts of interest.

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