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ORIGINAL ARTICLE

MiR-138 suppresses airway smooth muscle cell proliferation through the PI3K/AKT signaling pathway by targeting PDK1

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ABSTRACT

Background: Airway smooth muscle cells (ASMCs) play important physiological roles in the lung, and their abnormal proliferation directly contributes to airway remodeling during development of lung diseases such as asthma. MicroRNAs are small yet versatile gene tuners that regulate a variety of cellular processes, including cell growth and proliferation, but little is known about the precise role of microRNAs in the proliferation of ASMCs. **Methods:** In this study, human ASMCs from asthmatic and non-asthmatic donors were used. MicroRNA and mRNA expression were measured by quantitative real-time PCR. Dual-luciferase reporter assays were performed to determine whether microRNA-138 (miR-138) binds directly to 3-phosphoinositide-dependent protein kinase-1 (PDK1) 3' untranslated region (3'-UTR) to alter gene expression. **Results:** The results showed that overexpression of miR-138 reduced proliferation of human ASMCs, whereas inhibition of miR-138 increased proliferation of ASMCs. MiR-138 directly suppressed PDK1 expression by targeting the 3'-UTR of the gene. MiR-138 controls ASMC proliferation through directly inhibiting the phosphoinositide 3-kinase (PI3K) pathway. **Conclusions:** Our study indicated that miR-138 regulation of PI3K signaling in ASMCs by altering the expression of PDK1 can have a profound impact on cell proliferation.

KEYWORDS airway smooth muscle cells (ASMCs), miR-138, 3-phosphoinositide-dependent protein kinase-1 (PDK1), phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway

INTRODUCTION

Asthma is a chronic inflammatory disease of the bronchial airway characterized by chronic airway inflammation, airway hyperresponsiveness, and airway remodeling [1, 2]. A previous study demonstrated that airway remodeling is a characteristic feature of asthma and may be observed from the early stages [3]. The main characteristics of airway remodeling include epithelial detachment, subepithelial fibrosis, and hypertrophy and hyperplasia airway smooth

muscle cells (ASMCs) and goblet cells [3, 4]. Airway smooth muscle (ASM) [5] is likely to be an important contributor to chronic airflow obstruction because of hyperplastic and hypertrophic qualities, leading to increased thickening and narrowing of the airways [6, 7]. ASMCs are structural entities of airways that are believed to confer an abnormally exaggerated bronchoconstriction in asthma, the phenomenon commonly known as airway hyperresponsiveness. A majority of asthma patients show a significant increase in ASM bundles, likely due to increased cell numbers, collectively contributing to airway remodeling [8].

MicroRNAs (miRNAs) are an abundant class of small (18–25 nucleotides), non-coding, single-stranded RNAs found in diverse organisms. They have emerged as important post-transcriptional regulators of gene expression [9, 10]. MiRNAs are

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known to play critical roles in the regulation of gene expression and in disease pathogenesis. Studies have identified several miRNAs (miR-25, miR-10a, and miR-708) that were significantly down-regulated in human ASMCs and have the potential to control airway inflammation and proliferation of ASMCs [11–13].

The phosphoinositide 3-kinases (PI3K) are a family of proteins that catalyze the phosphorylation of the 3-OH position of phosphoinositides and generate lipids that control a wide variety of intracellular signaling pathways. They are classified into 3 families according to their structure and substrate specificity and are thought to have distinct biological roles. Recent studies have suggested that numerous components of the PI3K pathway play crucial roles in the expression and activation of inflammatory mediators, inflammatory cell recruitment, immune cell function, airway remodeling, and corticosteroid insensitivity in chronic inflammatory respiratory disease. Selective PI3K inhibitors have been developed that reduce inflammation and some characteristics of disease in experimental animal models. Targeting specific PI3K isoforms that may be overexpressed or overactive in disease should allow for selective treatment of respiratory diseases. Encouraging data from animal models, primary cells, and clinical studies in other diseases suggest that inhibitors of PI3K/AKT may prove to be useful novel therapies in the treatment of asthma and chronic obstructive pulmonary disease. 3'-phosphoinositide dependent kinase-1 (PDK1) is part of the PI3K/AKT pathway, one of the major cellular pathways involved in cell survival, differentiation, growth, and protein expression [14].

In this study, bioinformatics tools were used to determine potential miRNA response elements in the 3'-untranslated region (UTR) of the human PDK1 gene. Expression of miR-138, which was identified by one of the target prediction algorithms, and its functional role in PDK1 expression were determined in asthmatic ASMCs and non-asthmatic ASMCs. MiR-138 levels were inversely correlated with those of PDK1 in ASMCs. In addition, miR-138 directly regulated the expression of PDK1. PDK1 was shown to be negatively regulated by miR-138 at the post-transcriptional level through a specific target site within the 3'-UTR of the gene. The overexpression of miR-138 may have resulted in the down-regulation of PDK1. Our data indicate that PDK1 is a gene that has been identified as a direct target of miR-138 and is a key component in PI3K/AKT signaling, a well-documented pathway that regulates ASMCs proliferation.

METHODS

Culture and Transfection of ASMCs

Human ASMCs were isolated from the lobar or main bronchus obtained from lung resection donors, using procedures approved by the Division of Thoracic Surgery and the Human Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (all from Gibco, USA). Cells from passages 3–8 were used for the experiments.

MiR-138 mimics and inhibitors (Qiagen, Valencia, CA, USA) were used to overexpress and inhibit miR-138, respectively. Nontargeting scrambled small interfering RNA (siRNA; Qiagen) was used as a control for miR-138 mimic experiments; miRNA inhibitor control (Qiagen) was used as a control for miR-138 inhibitor experiments. Transfections of miR-138 mimics and inhibitors were performed using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Total RNA Isolation and Quantitative Real Time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The purity and concentration of total RNA were determined in an ultraviolet spectrophotometer (Eppendorf, Germany). Quantitative real-time PCR was performed using the Quanti-TectSYBR Green PCR mixture on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative expression of mRNA or miRNA was evaluated by using the $2^{-\Delta\Delta C_t}$ method and all experiments were run in triplicate. The expression level of GAPDH was used as an internal control for mRNAs, and U6 level was regarded as the internal miRNA control.

MTT Assay

Cells cultured under normal conditions were inoculated uniformly into 96-well culture plates at a concentration of 3×10^5 cells/mL. After adherent cell culture, transfections were conducted for miR-138 mimics, nonspecific controls, or miR-138 inhibitors according to the Lipofectamine 2000 transfection manual. The normal control group was also established. Forty-eight hours after transfection, 100 μ L

of MTT (0.5 mg/mL) solution was added to each well. Then, cells were incubated at 37°C for 4 hours and 100 μ L DMSO was added to dissolve the formazan crystals. A microplate reader (Bio-Tek, USA) was used to measure the OD values at 570 nm. Each experimental group contained 10 replicate wells, and the experiment was repeated 3 times.

Colony Formation Assay

Cells were seeded in a 6-well plate (0.5×10^3 cells per well) and cultured for 10 days until the colonies were formed. Colonies were then fixed with 10% formaldehyde for 10 minutes and stained for 5 minutes with 1.0% crystal violet. All experiments were performed in triplicate.

Western Blot Analysis

Cells were lysed in Radio Immunoprecipitation Assay (RIPA) buffer plus protease inhibitors (Roche, IN, USA). Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Logan, UT, USA). Equal amounts of protein were loaded, separated on 10% SDS-PAGE, and then transferred to a PVDF membrane (Millipore, Boston, MA, USA). Nonspecific protein interactions were blocked by incubation with 3% fat-free milk in TBS buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.6) at 4°C for 1 hour. The membranes were incubated with primary antibodies (anti-PDK1 antibody (1/1000; Cell Signaling Technology, USA) or anti-GAPDH antibody (1/2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Unbound antibody was removed by washing in TBST buffer 3 times (10 minutes/wash). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Zhongshan, Beijing, China) at 25°C for 1 hour, and followed by washing with TBST buffer 3 times (10 minutes/wash). The blots were developed with chemiluminescent ECL reagent (Millipore). GAPDH was used as an internal control.

Vector Construction and Luciferase Reporter Assays

The whole 3'-UTR of the PDK1 gene was amplified from genomic DNA and cloned into pGL-3-vector (Promega, San Luis, CA, USA) immediately downstream of the Renilla luciferase gene. Mutation in 3'-UTR of the PDK1 gene was generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA, USA). The whole 3'-UTR of both the PDK1 gene and the mutated gene was identified by sequencing, before massive PCR ampli-

fication. Approximately, 1×10^5 cells per well were seeded into 24-well plates for 24 hours before transfection. Cells were co-transfected with pGL-3 firefly luciferase reporter (50 ng), pRL-TK Renilla luciferase reporter (10 ng), and miR-138 mimic or control (50 nM) using Lipofectamine 2000 (Invitrogen). A luciferase reporter construct containing the miR-138 consensus target sequence served as the positive control and the pRL-TK vector served as the internal control. Cell lysates were prepared using Passive Lysis Buffer (Promega) 48 hours after transfection, and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega). Results were normalized to Renilla luciferase and expressed as relative luciferase activity (Firefly LUC/Renilla LUC). Experiments were independently repeated 3 times.

Statistical Analysis

All computations were carried out in SPSS version 13.0 for Windows. Data were expressed as mean $\pm$ standard deviation (SD). Analysis of variance (ANOVA) was used to determine statistical differences among the groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

The Expression of MiR-138 in ASMCs

MiR-138 was expressed at a much higher level in non-asthmatic ASMCs compared to asthmatic ASMCs (Figure 1A). Expression of miR-138 was significantly higher in the miR-138 mimic transfected group than in the control group ($P < 0.01$; Figure 1B). In the miR-138 inhibitor transfected group, miR-138 expression was significantly lower than in the control group ($P < 0.05$). Therefore, miR-138 was overexpressed in ASMCs by the transfection of miR-138 mimic and its expression was inhibited by the transfection of miR-138 inhibitor.

MiR-138 Suppresses Proliferation of ASMCs

MTT assay of the effect of miR-138 on cell proliferation in human ASMCs transiently transfected either with miR-138 mimic or inhibitor revealed that miR-138 inhibited cell proliferation. Increased expression of miR-138 dramatically suppressed cell proliferation in ASMCs (Figure 2A). Correspondingly, after transfection with miR-138 inhibitors, cell proliferation was significantly accelerated in comparison with the control group. These findings demonstrate that

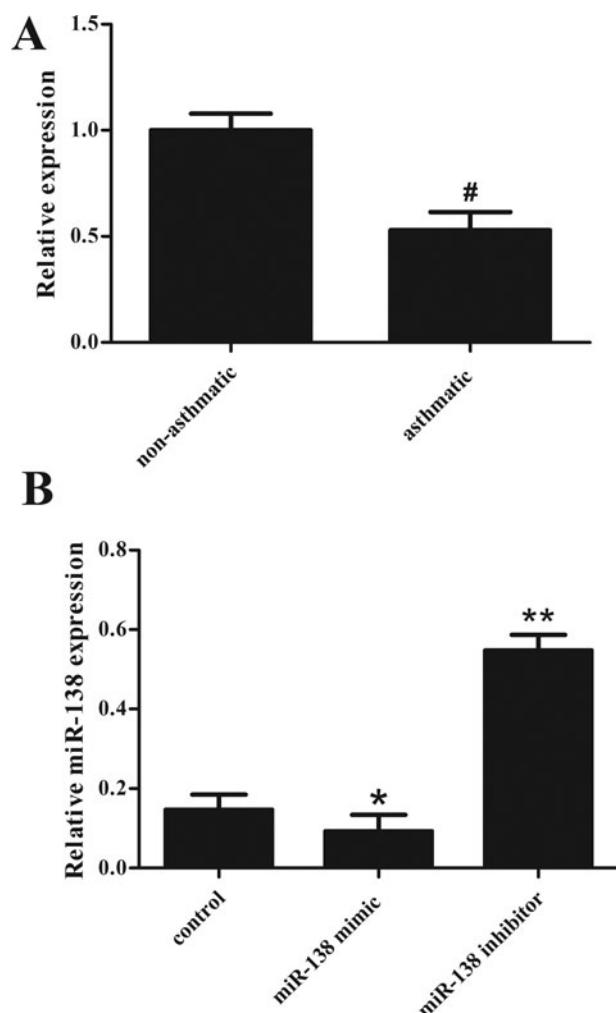


FIGURE 1. Real-time PCR analysis of miR-138 expression in asthmatic ASMCs (A). [#] $P < 0.05$ versus non-asthmatic. The effects of miR-138 mimic or miR-138 inhibitor transfection on the expression of miR-138 in human ASMCs (B). ^{**} $P < 0.01$ and ^{*} $P < 0.05$ versus control group.

miR-138 expression significantly reduced the proliferation of ASMCs. Moreover, the colony formation assay revealed that miR-138 overexpression inhibited proliferation in ASMCs (Figure 2B). Conversely, decreased expression of miR-138 promoted cell proliferation. Together, these results indicated that miR-138 is a negative regulator of human ASMC proliferation.

MiR-138 Directly Binds to 3'-UTR of PDK1

MiRNAs function by suppressing the expression of their target genes. We next determined which genes in the PI3K/AKT pathway are the direct miR-138 targets that mediate the function of the miRNA. By using the TargetScan and PicTar algorithms, we found

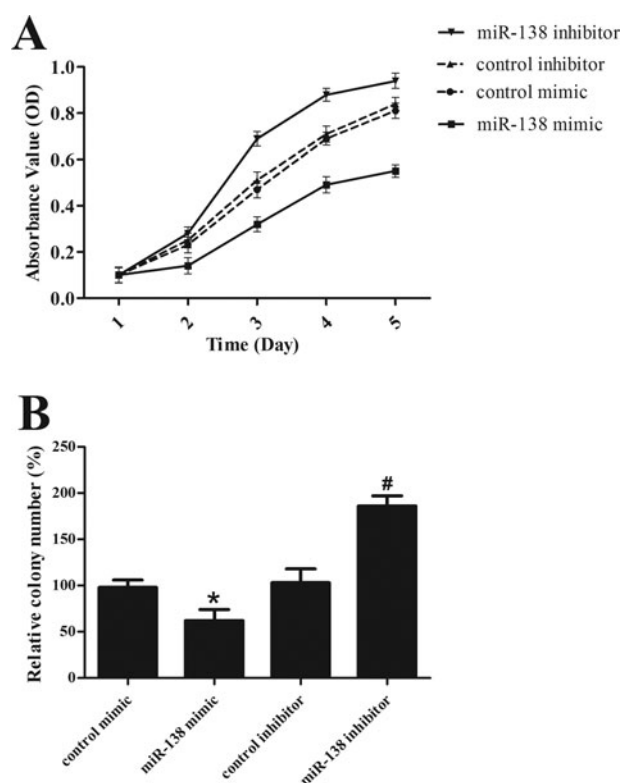


FIGURE 2. MiR-138 inhibits ASMC proliferation. (A) The effect of miR-138 on cell proliferation was evaluated by MTT assay after being transfected with miR-138-mimic or miR-138-inhibitor. Data are mean $\pm$ SD of 3 independent experiments. (B) Colony formation assays were performed to confirm the effect of miR-138 on cell growth. ^{*} $P < 0.05$ versus control mimic, [#] $P < 0.05$ versus control inhibitor.

that PDK1 is a putative target of miR-138 (Figure 3A). To confirm whether miR-138 directly targeted the 3'-UTR of PDK1, we performed luciferase assays using the miR-138 binding sequence present at the 3'-UTR of PDK1 mRNA (WT) or its mutant (Mut) inserted downstream of a luciferase reporter. Luciferase activity in PDK1-WT transfected cells was significantly decreased in miR-138 mimic co-transfected cells compared with that in control mimic co-transfected cells (Figure 3B). In addition, site-directed mutagenesis of the seed region abolished the inhibitory effect of miR-138 mimics and Mut luciferase activity did not change. These results indicate that miR-138 significantly suppressed the activity of WT but not Mut PDK1 3'-UTR in human ASMCs.

MiR-138 Inhibited PDK1 Expression

To further examine the effect of miR-138 on PDK1 expression, we reexamined the effect of miR-138 mimics on PDK1 mRNA along with miR-138 inhibitors. Overexpression of miR-138 significantly

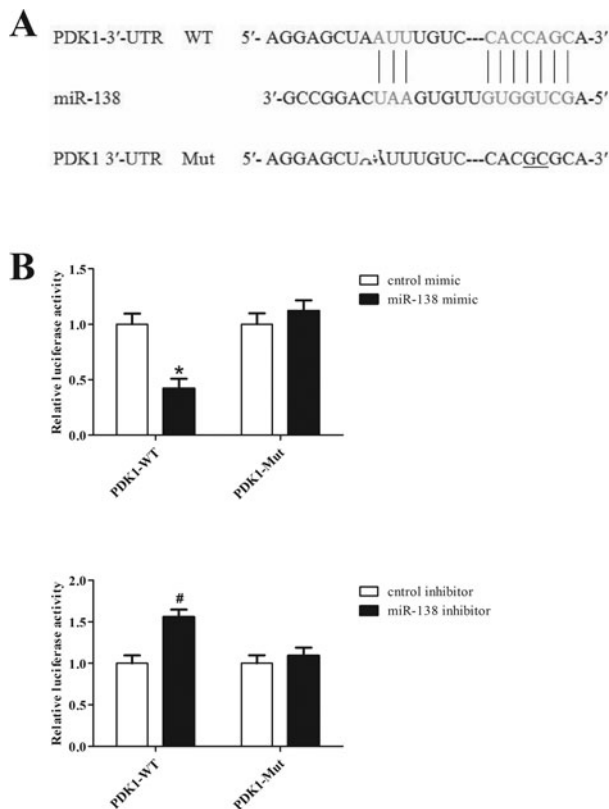


FIGURE 3. MiR-138 directly targets the 3'-UTR of the PDK1 gene. (A) The predicted miR-138 binding site within the PDK1 3'-UTR and a mutated version generated by site directed mutagenesis are shown. (B) A luciferase construct was used to test whether miR-138 directly binds to PDK1. ASMCs were co-transfected with the luciferase construct with either miR-138 mimics or inhibitors and control scrambled siRNA for 48 hours, followed by dual-luciferase assays. The statistical analysis was based on 3 independent experiments. Data represent mean $\pm$ SD. * $P < 0.05$ versus control mimic, # $P < 0.05$ versus control inhibitor.

suppressed the mRNA level of PDK1, while inhibition of miR-138 elevated the mRNA level of PDK1 in human ASMCs (Figure 4A). Furthermore, miR-138 mimics led to significant down-regulation in PDK1 protein expression, whereas miR-138 inhibitors caused a modest increase in PDK1 protein expression (Figure 4B). Taken together, these results reveal that miR-138 could negatively regulate PDK1 expression through a partially complementary binding site in the 3'-UTR of PDK1.

MiR-138 Suppressed the PI3K/AKT Signaling Pathway in ASMCs

PI3K/AKT signaling is a well-defined pathway that is directly associated with cell growth, proliferation, and survival. Given the negative regulation of PI3K/AKT signaling by PDK1, we investigated whether PDK1

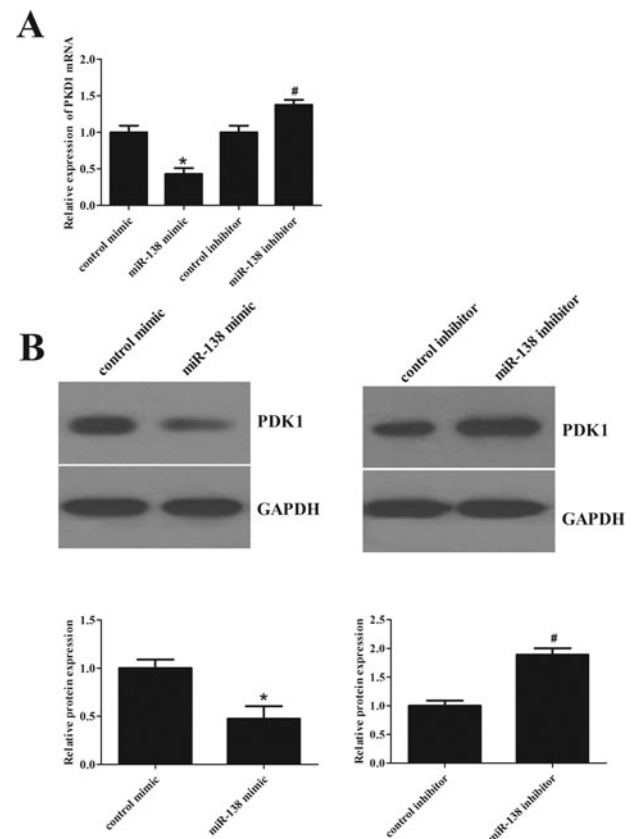


FIGURE 4. Effects of miR-138 on PDK1 expression. (A) Effects of miR-138 mimics and inhibitors on PDK1 mRNA expression. RNA was extracted from ASMCs transfected with scrambled siRNA control, miR-138 mimic, control inhibitor, or miR-138 inhibitor and used for quantitative real time PCR. PDK1 mRNA level was compared between miR-138 and its control-transfected ASMCs (or between miR-138 inhibitor and its control). (B) Effects of miR-138 mimics and inhibitor on PDK1 protein expression. PDK1 protein in the transfected ASMCs was detected using Western blotting. GAPDH was used as a control. Data are mean $\pm$ SD of 3 independent experiments. * $P < 0.05$ versus control mimic, # $P < 0.05$ versus control inhibitor.

inhibition was responsible for the effect of miR-138 on PI3K/AKT signaling. To determine whether miR-138 actually suppresses the PI3K/AKT pathway, we measured levels of phosphorylated AKT, a molecule in the PI3K signaling cascade, as well as levels of PTEN, a phosphatase that terminates PI3K signaling. In cells transfected with the miR-138 mimic, the level of phosphorylated AKT was significantly lower relative to cells transfected with the control mimic (Figure 5A). Consistent with an overall attenuation of PI3K signaling in miR-138 mimic transfected cells, there was a significant increase in PTEN expression compared to expression in cells transfected with the control mimic (Figure 5B). These results suggest that ectopic expression of miR-138

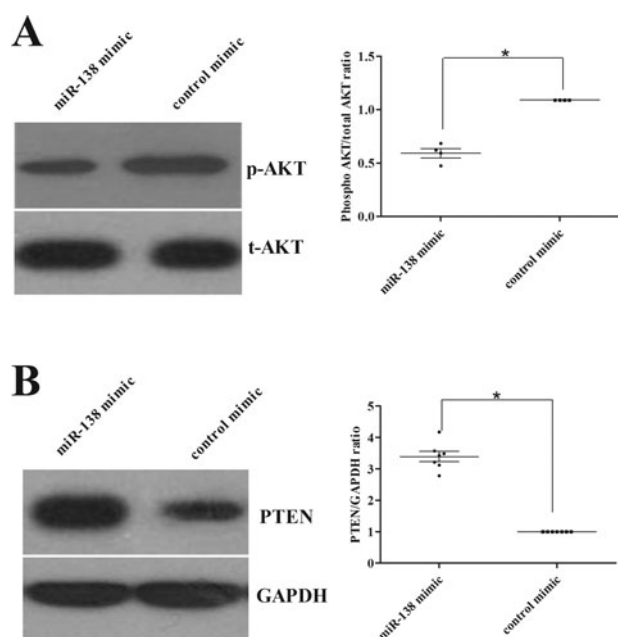


FIGURE 5. Effects of miR-138 on PI3K/AKT pathway. Asthmatic ASMCs were transfected with miR-138 mimic or control mimic to measure total and phosphorylated AKT ($n = 4$) and PTEN expression ($n = 7$) by Western blot analysis. Note significant down-regulation of AKT phosphorylation (A) and up-regulation of PTEN expression (B). Values are means $\pm$ SD. * $P < 0.05$ versus control mimic.

inhibits the PDK1/AKT survival pathway in human ASMCs.

DISCUSSION

Asthma is a complex inflammatory disorder of airways of lungs resulting in airflow obstruction and bronchial hyperresponsiveness to a variety of stimuli and symptoms of wheeze, cough, and breathlessness [15]. The major obstacle in preventing and treating asthma has been our incomplete understanding of its etiology and biological mechanisms. Recent studies have changed our understanding of asthma; it is now known to be a disease in which both inflammatory and structural components are equally involved [16]. Asthma is often associated with structural remodeling of the airways characterized by airway epithelial damage, wall thickening, and subepithelial fibrosis. It is well established that hyperplasia and decreased apoptosis of ASMCs play an important role in the asthmatic airway remodeling.

MiRNAs are known to play critical roles in the regulation of gene expression and in disease pathogenesis [17]. Recent studies have identified several miRNAs whose expression in ASMCs is down-regulated

by inflammatory cytokines [11, 12, 18, 19]. There is also evidence for miRNA regulation of ASM contractility and phenotype [13, 19]. Increased ASM mass due to growth factor-driven hypertrophy and hyperplasia of ASMCs is also a feature of diseases such as asthma [20]. ASMCs proliferation is a major driver of airway narrowing associated with lung diseases such as asthma. Recent reports have identified the specific role of miR-221 and miR-10a in the regulation of proliferation of ASMCs [6, 13]. In the present study, we demonstrated that miR-138 suppresses proliferation of human ASMCs.

The PI3K/AKT pathway is a critical cellular pathway involved in various cell functions such as cell survival, cell differentiation, cell growth, and protein expression [21]. PI3K is the major signaling pathway that regulates ASMC proliferation. Previous studies clearly demonstrated that the PI3K/AKT pathway has a major contribution to cell proliferation in ASMCs obtained from asthmatics [22]. It is well known that PDKs mediate PI3K/AKT signal transduction, and PDK abnormality contributes to the development of many malignancies [23]. PDK1 is a potent regulator in cell growth and a previous study supports its oncogenic role in mammalian cells [24]. PDK1 functions as a molecular kinase belonging to the PI3K signaling pathway and is involved in the regulation of various physiological processes relevant to metabolism, growth, proliferation, and survival. It has recently been reported that the phosphorylated form of AKT, a downstream target of PDK1, has been reported to prevent apoptosis in gastric cancer cells. Our study has shown that miR-138 mRNA levels were inversely correlated with those of PDK1 in ASMCs. The ectopic expression of miR-138 may suppress PDK1 mRNA and protein levels, indicating that miR-138 may also interfere with PI3K/AKT signaling. This regulation stems from direct 3'-UTR binding to the transcript as well as through regulation of signaling involving PI3K/AKT pathways. In addition, we have shown that overexpression of miR-138 increased PTEN expression and an associated decrease in AKT phosphorylation. Zhang *et al.* defined a tumor suppressor function for miR-138 in NSCLC and confirmed that miR-138 could inhibit tumor growth through repression of EZH2 in this cancer [25]. It has been reported that miR-138 appears to be a negative regulator of PASMCA apoptosis by targeting Mst1, and plays an important role in hypoxic pulmonary vascular remodeling (HPVR). Consistent with this study, our results indicated that miR-138 modulates ASMC proliferation by directly targeting PDK1 and the associated PI3K/AKT pathway. Therefore, our findings suggest the possibility that miR-138 is a potential therapeutic target

against PI3K/AKT signaling for preventing ASMC proliferation.

In summary, our identification of miR-138 as a direct regulator of PI3K describes an additional layer of regulation in human ASMC proliferation. We have established that miR-138 is a novel regulator of PI3K/AKT signaling and demonstrated that miR-138 inhibits human ASMC proliferation by suppressing PI3K/AKT signaling through direct targeting of the central PI3K/AKT pathway component, PDK1. Our findings raise the possibility of using miR-138 as a novel therapeutic agent to suppress PI3K/AKT signaling in order to prevent abnormal cell proliferation including that of human ASMCs.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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