

The Regulatory Role of miR-107–Cdk6-Rb Pathway in Airway Smooth Muscle Cells in Asthma

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Purpose: Airway remodeling is a significant pathological change of asthma. This study aimed to detect differentially expressed microRNAs in the serum of asthma patients and airway smooth muscle cells (ASMCs) of asthmatic mice, exploring their role in the airway remodeling of asthma.

Methods: The differentially expressed microRNAs in the serum of mild and moderate-severe asthma patients compared to healthy subjects were revealed using the “limma” package. Gene Ontology (GO) analysis was used to annotate the functions of microRNA target genes. The relative expressions of miR-107 (miR-107-3p in mice sharing the same sequence) in the primary airway smooth muscle cells (ASMCs) of the asthma mice model were tested by RT-qPCR. Cyclin-dependent kinases 6 (Cdk6), a target gene of miR-107, was predicted by algorithms and validated by dual-luciferase reporter assay and Western blot. The roles of miR-107, Cdk6, and protein Retinoblastoma (Rb) in ASMCs were examined by transwell assay and EDU KIT in vitro.

Results: The expression of miR-107 was down-regulated in both mild and moderate-severe asthma patients. Intriguingly, the level of miR-107 was also decreased in ASMCs of the asthma mice model. Up-regulating miR-107 suppressed ASMCs' proliferation by targeting Cdk6 and the phosphorylation level of Rb. Increasing the expression of Cdk6 or suppressing Rb activity abrogated the proliferation inhibition effect of ASMCs induced by miR-107. In addition, miR-107 also inhibits ASMC migration by targeting Cdk6.

Conclusion: The expression of miR-107 is down-regulated in serums of asthma patients and ASMCs of asthmatic mice. It plays a critical role in regulating the proliferation and migration of ASMCs via targeting Cdk6.

Keywords: asthma, microRNA microarray, airway smooth muscle cells, miR-107, Cdk6

Introduction

Asthma is one of the most common chronic respiratory diseases, with nearly 300 million asthma patients worldwide.¹

Asthma patients suffer from respiratory symptoms such as wheezing, chest tightness, shortness of breath, cough, and variable expiratory airflow limitation. Airflow limitation later can become persistent as a result of airway remodeling.² Airway remodeling is also the cause of lung function decline, correlated with asthma severity.^{3–5} Severe asthma-related airway changes are characterized by diffuse airway narrowing and focal bronchial stenoses.⁵ Also, airway remodeling is responsible for glucocorticoid resistance in asthma patients.⁶ Therefore, although asthma has long been viewed as a chronic inflammatory disease, much evidence shows that airway remodeling equally attributes to its process and deterioration.

Typical airway remodeling includes epithelial damage, increased airway smooth muscle (ASM) mass, subepithelial collagen and proteoglycan deposition, mucus gland hyperplasia, and angiogenesis.⁷ Among alterations of airway

remodeling, increased ASM mass because of hypertrophy and hyperplasia of ASMCs represent a prominent feature.⁸ However, the mechanisms underlying the increase of ASM mass remain poorly understood.

MicroRNA regulates the target gene expression via binding to the 3' untranslated region (3'-UTR) of mRNA, inducing mRNA degradation or suppressing their translation.⁹ In recent years, studies have shown that microRNA plays a vital role in airway remodeling partly by regulating the functions of ASMCs. miR-15b-5p inhibits the migration, proliferation, and extracellular matrix production of ASMCs by regulating yes-associated protein 1(YAP1) expression.¹⁰ miR-21 promotes ASMCs migration and proliferation in asthma via targeting PTEN.¹¹ miR-145 inhibitor resulted in decreased migration and proliferation of ASMCs in a dose-dependent manner.¹² For the whole view about the role of microRNA in asthma, Sharma, R and his colleague had made an informative review.¹³ Nevertheless, our knowledge of the functions and targets of microRNAs in airway remodeling is incomplete.

In this study, we detected the differently expressed microRNAs through analysis of the microarray dataset GSE222894, showing that miR-107 is significantly down-regulated in the serum of mild asthma patients and moderate-severe asthma patients. We tested the relative expression and the role of miR-107 in primary ASMCs from the asthma mice model. We found evidence that miR-107 is critical in regulating the proliferation and migration of ASMCs by targeting Cdk6.

Materials and Methods

Acquisition and Procession of Microarray Dataset

The microRNA expression profiles in mild asthma patients, moderate-severe asthma patients, and healthy subjects were acquired from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) via the “GEOquery” package (version 2.58.0) in R software.¹⁴ The differentially expressed microRNAs between asthma patients (mild and moderate-severe asthma patients) and healthy subjects were identified by using the “limma” package (version 3.46.0)¹⁵ in R software. The target genes of co-downregulated microRNAs were predicted by two databases (targetscan7.1 and miRDB V6). We utilized GO analysis to investigate the target genes enriched biological process (BP), molecular function (MF), and cellular component (CC) with p-value < 0.05 were considered statistically significant.

Animals Experimental Protocol

All mice experiments were approved by PKU-HKUST Medical Center Ethics Committee and followed with Regulations of the People's Republic of China for the Administration of laboratory animals. Ethics approval number is SPHMC2019-190. Female BALB/c mice of 6 weeks of age (Guangdong Medical Laboratory Animal Center, China) were kept in a specific pathogen-free environment. The mice were randomly divided into the asthma group (OVA) and the control group (PBS). The experimental protocol of the chronic asthma mouse model is conducted as described previously.¹⁶ Briefly, mice were intraperitoneally injected with 20μg OVA adsorbed with aluminum hydroxide on days 0, 14, 28, and 42 at the sensitization phase. The mice were challenged with aerosolized OVA for 30 min with an aerosol of 1% OVA at 21 days, three times per week until day 51. Mice in the control group were treated with PBS correspondingly. All animal experiments were conducted within 24h after the last challenge.

The levels of specific Ig E in the serum of mice were tested according to the manufacturer's instructions (BioLegend, California, USA). Respiratory system resistance of mice model tested by Non-invasive pulmonary function tester (Buxco, North Carolina, USA) as published previously.¹⁷

Lung Histology

The right middle lungs of the mice were fixed with 4% formaldehyde and embedded in paraffin. Lung sections were cut and conducted to hematoxylin and eosin (H&E), immunohistochemistry and Immunofluorescence as described previously.^{18,19} The primary antibody used in this part was anti-α-SMA (Abcam, Cambridge, UK).

Isolation of ASMCs

We isolated and cultured ASMCs from the mice model as previously described methods.²⁰ Briefly, lungs were excised and cut in cross-sections of 2–3 mm. The cross-sections were cultured in Dulbecco's Modified Eagle Media with 20% fetal bovine serum, 1% penicillin, and 1% penicillin-streptomycin for 4 days. Then the concentration of serum was reduced to 10%. ASMCs exhibited a typical “hill-and-valley” growth pattern, and immunofluorescence techniques for α -actin revealed that >95% of the cells were ASMCs in culture. Cells in passages 3–6 were used for experiments.

To test the effect of miR-107 on p-Rb expression, ASMCs in this part were cultured with supplemented PDGF-BB (20 ng/mL). ASMCs used in function tests (EDU kit, transwell test, and Western blot) were driven from the asthma mice model group.

Dual-Luciferase Reporter Assay

The sequence of 3'UTR of Cdk6 and the corresponding mutant sequence were inserted into the 3'UTR of psiCHECK-2 plasmid (a gift from the University of Science and Technology of China). In six-well plates, 293T cells (a gift from the University of Science and Technology of China) were cultured to around 70% confluence. Then, the cell co-transfected with luciferase reporter vectors harboring wild type or mutant 3'-UTR Cdk6 site and mimic miR-107 or negative control (NC). We measured the luciferase activity after 30h co-transfection.

Western Blot

Western blot was performed as reported previously.²¹ Briefly, the ASMCs were lysed with the RIPA buffer, adding phosphatase and protease inhibitors (Beyotime, Shanghai, China). The concentration of the proteins was tested with the Bicinchoninic Acid Kit (Thermo Scientific, Rockford, USA). The primary antibodies used in this part were anti-Cdk6 Ab, anti-pRb (Cell Signaling, Inc. Boston, USA), and anti- β -actin Ab (Abcam, Cambridge, UK). The relative level of proteins was measured with the Image J Software (National Institutes of Health, Bethesda, USA).

RT-qPCR

Total RNA was extracted from the ASMCs using the TRIzol RNA Isolation Reagents (sigma, St. Louis, MO, USA). The first-strand cDNA was converted using a reverse transcription system (Promega, Wisconsin, USA) according to the manufacturer's protocols. RT-qPCR analysis was performed using iTaq TM Universal SYBR Green Supermix (Bio-Rad, California, USA). Primers are shown in [Table S1](#). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (for mRNAs) and U6 (for microRNAs) were used for normalization, respectively.

Proliferation and Migration Assays

The percentage of ASMCs undergoing proliferation in culture was determined by EDU (5-ethynyl-2'-deoxyuridine) KIT (RIBOBIO, Guangzhou, China) according to the manufacturer's instructions. EDU is a thymidine analog that can get incorporated into DNA during cell proliferation. Following the incorporation of EDU, a fluorescent molecule was added to react with EDU for fluorescent visualization of proliferating cells.

Transwell chamber (Corning, New York, USA) with 8- μ m pores was used to test the ability of cell migration. After ASMCs transfected with mimic miR-107, inhibitor miR-107, siCdk6, or their negative control (NC) for 48h, we suspended the cells in serum-free DMEM-F12 and added them to the upper chamber at 4×10^4 cells/well, in the lower chamber added with DMEM-F12 with 10% FBS. After 12h of incubation, the residual cells in the upper chamber were gently moved out, and the cells migrating through the membrane to the lower insert surface were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet.

Transfection

Before transfection, the logarithmic growth phase cells were harvested, trypsinized, and inoculated in a 6-well plate at 12×10^4 cells/well. After the cells were cultured for 24 h, cell transfection was performed. Mimic miR-107, inhibitor miR-107, siCdk6, siRb, and their corresponding negative controls (RIBOBIO, Guangzhou, China) were transfected into separated wells according to our experimental designs and instructions of ViaFect Transfection Reagent (Promega,

Wisconsin, USA). The transfection of lentiviral vector Cdk6 and its negative control was performed according to the instructions (GENECHEM, Shanghai, China).

Statistics

All statistical analyses were performed with GraphPad Prism 6.01 software. Significant differences ($P < 0.05$) between the two groups were identified by Student's t-tests.

Results

Detecting and Functional Annotation of Differentially Expressed microRNA

microRNA microarray dataset (GSE222894) was obtained from the GEO database, which includes 5 mild asthma patients, 5 moderate-severe asthma patients, and 5 healthy subjects. We analyzed this dataset by the “limma” package to detect differentially expressed microRNAs in the serum between asthma patients and healthy subjects under the conditions of $P < 0.01$ and $FDR > 3$. We identified 20 significantly co-downregulated microRNAs in the serum of mild and moderate-severe asthma patients compared with healthy subjects. These downregulated microRNAs include miR-223, miR-26b, miR-20b, let-7, miR-15b, miR-107 and so on. Detailed information is listed in [Table S2](#).

The target genes of these microRNAs were predicted by two databases (targetscan7.1 and miRDB V6). Detailed information is listed in [Table S3](#). As the Venn diagram shows in [Figure 1A](#), there are 3114 genes predicted to be regulated by the co-downregulated microRNAs. The microRNAs and their target genes' relationship were represented in the network ([Figure 1B](#)). The GO analysis was used to determine the underlying biological mechanisms. We identified that the GO terms of targeted genes enriched in protein binding, catalytic activity, acting on a protein, intracellular part, positive regulation of the cellular process, and cellular protein modification process ([Figure 1C](#)).

Chronic Asthma Mice Models Show Airway Remodeling Phenotype

To identify the roles of these co-downregulated microRNAs in airway remodeling, we established a mouse model of chronic asthma ([Figure 2A](#)).²² OVA-treated mice exhibited higher airway responsiveness when challenged with Acetylcholine, and they had a higher OVA-specific Ig E antibody in the serum ([Figure 2B and C](#)). OVA treatment also induced the immersion of inflammation cells ([Figure 2D](#)) and increased ASM mass marked by α -SMA ([Figure 2E](#)).

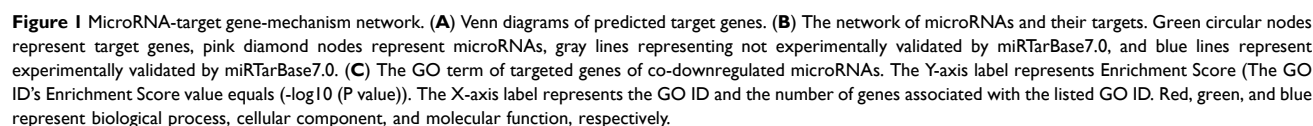
Up-Regulation of miR-107 Inhibits the Proliferation and Migration of Primary ASMCs

We cultured primary ASMCs from the mice model. Our results show that miR-107 is also significantly down-regulated in primary ASMCs from the asthma mice model ([Figure 3A](#)). We have re-tested the expression of miR-107 in serum of asthma patients and healthy volunteers by RT-qPCR. Our data (not published) shows that miR-107 is remarkably reduced in the serum of asthma patients.

Since the expression of miR-107 is down-regulated, we up-regulated the level of miR-107 through mimic miR-107 transfection to identify its biological functions in ASMCs of the asthmatic mice. The transfection efficiency of mimic and inhibitor miR-107 was confirmed by RT-qPCR ([Figure S1](#)). The percentage of ASMCs undergoing proliferation was determined by EDU KIT. The up-regulation of miR-107 suppressed the proliferation rate of ASMCs ([Figure 3B and C](#)). In addition, overexpression of miR-107 significantly inhibited ASMCs migration through transwell cell migration assay ([Figure 3D and E](#)). The hyperplasia and migration of ASMCs are key factors for increased ASM mass. Thus, our data indicate that miR-107 plays a crucial role in regulating ASM mass.

Cdk6 is a Target of miR-107

To illustrate the molecular mechanism underlying the regulation of miR-107 in ASMCs' proliferation and migration, we used bioinformatic algorithms (in miRDB, miRWalk, DIANA, and TargetScan) to predict the potential targets of miR-107. As shown in [Figure 4A](#), bioinformatics analysis identified that the 3'-UTR of Cdk6 is a putative binding site for miR-107, which is evolutionarily conserved. microRNA regulates gene expression by acting on the 3'-UTR of the mRNA. When mimic miR-107 was co-transfected with luciferase reporter vector harboring 3'-UTR site of Cdk6, the



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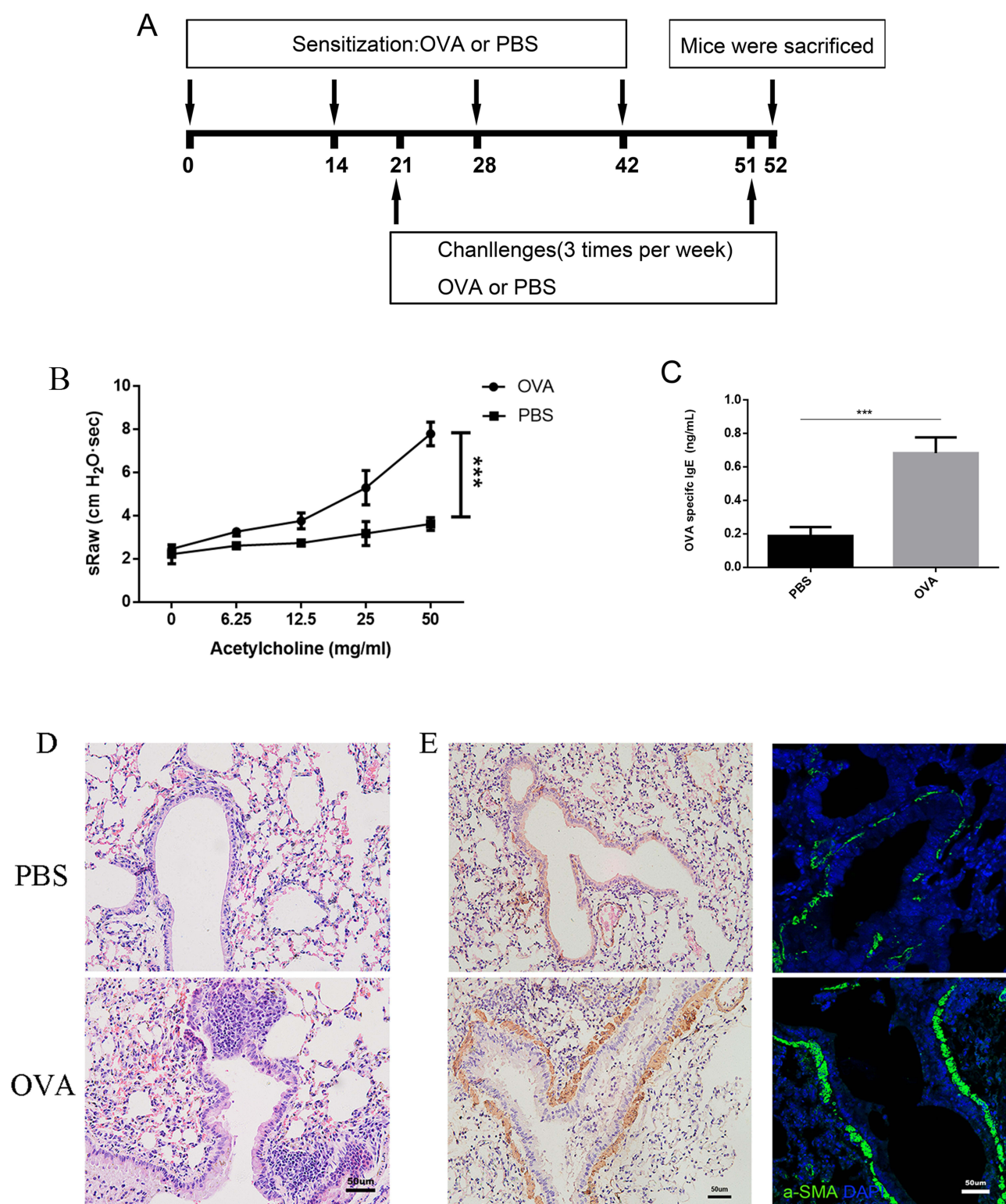


Figure 2 The tissue remodeling in response to OVA treatment in mice. **(A)** Timeline of the experimental protocol. **(B)** Respiratory system resistance test of mice model (***, $P<0.001$). **(C)** the levels of OVA-specific Ig E in the serum of mice model tested by ELISA, (***, $P<0.001$). **(D)** Representative photomicrographs of lung sections stained with H&E were present in OVA-induced asthmatic mice (down) and PBS-treated control mice (up), scale bar=50µm. **(E)** Representative photomicrographs of lung sections stained with IHC (left), and IHF (right). Marked smooth muscle layers by α -SMA were present in OVA mice (down) and PBS mice (up) (blue: DAPI; green: α -SMA), scale bar=50µm.

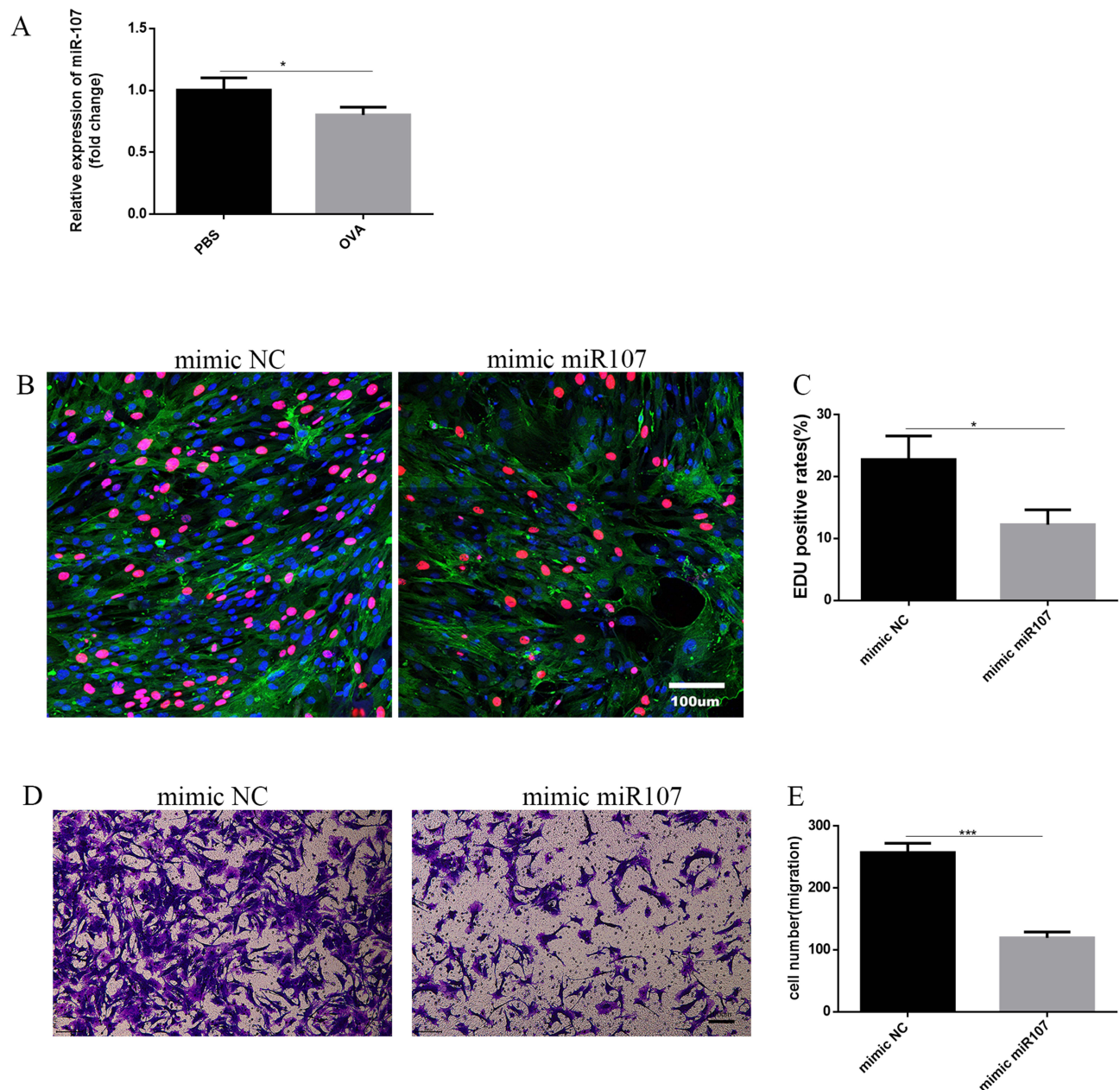


Figure 3 The effects of miR107 on the proliferation and migration of ASMCs. **(A)** the expressions of miR107 in primary ASMCs were compared between PBS-treated control mice and OVA-treated asthmatic mice by RT-qPCR (*, $P < 0.05$). **(B and C)** EDU staining (red) showing quantitative data **(C)** and representative images **(B)** left for negative control ASMCs, right for mimic miR107-treated ASMCs, nuclei stained with DAPI, and α -SMA in green, $P < 0.05$. **(D and E)** transwell cell migration assay showing quantitative data **(E)** and representative images **(D)**; left for negative control ASMCs, right for mimic miR107-treated ASMCs, (*, $P < 0.05$).

expression in primary ASMCs. Transfection of inhibitor miR-107 up-regulated Cdk6 protein expression while it was down-regulated by the overexpression of miR-107 through mimic miR-107 (Figure 4C and D). In addition, when ASMCs were cultured with PDGF-BB, an established stimulator that induces ASMCs proliferation and migration,²³ the phosphorylation level of Rb, a Cdk6 downstream target, was also down-regulated by miR-107 overexpression (Figure 4E and F). These results confirmed that miR-107 targets Cdk6.

Up-Regulation of Cdk6 Reverses miR-107-Mediated Proliferation Inhibition of ASMCs

To investigate whether Cdk6 was involved in the effects of miR-107 on ASMCs proliferation, first, we tested the role of Cdk6 in ASMCs. We transfected ASMCs with RNAi siCdk6 to down-regulate Cdk6 expression; Transfection efficiency was tested by Western blot (Figure 5C). Decreasing the protein level of Cdk6 reduced the proliferating ASMCs

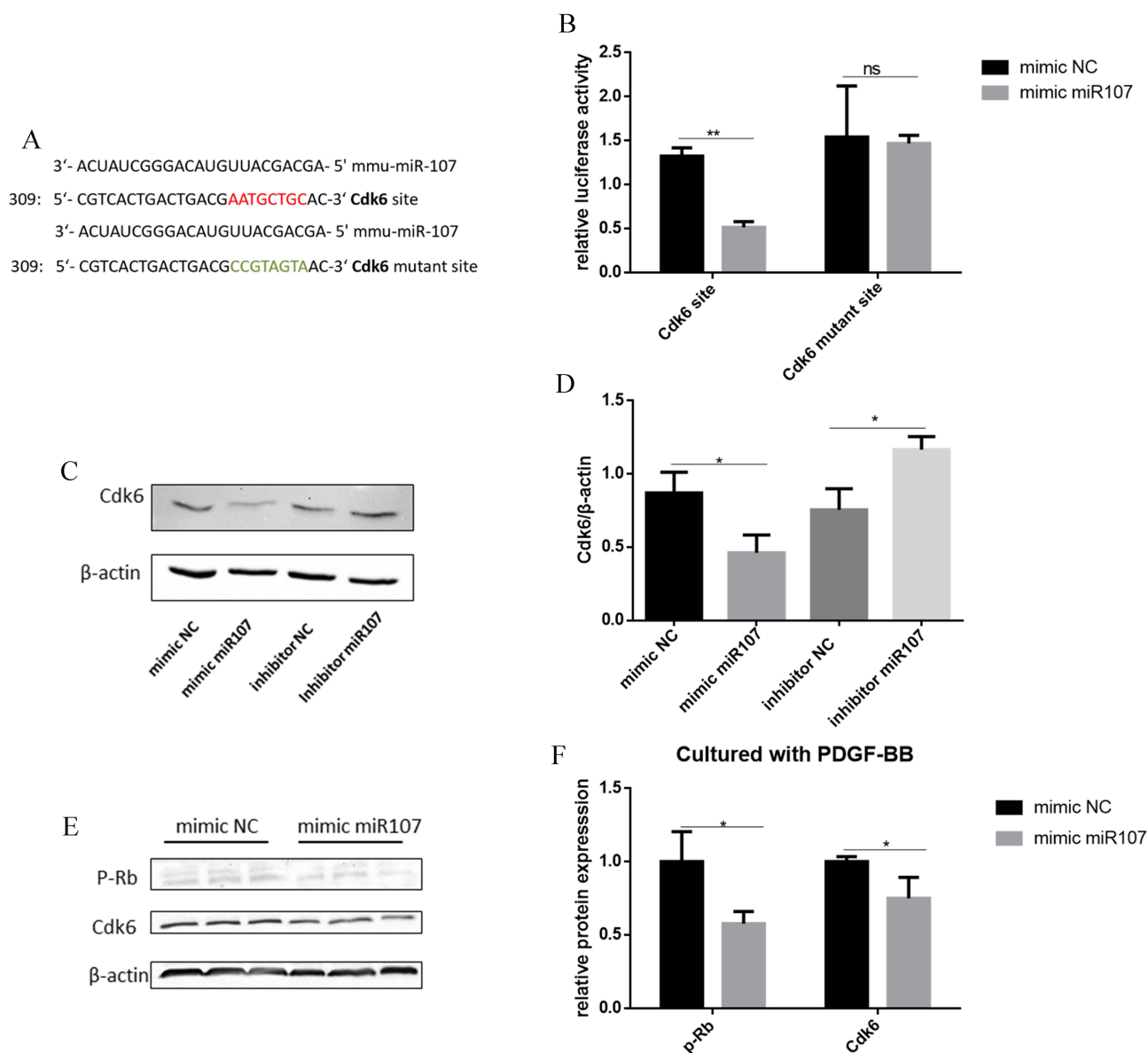


Figure 4 Interaction between miR107 and Cdk6 as well as Rb. **(A)** predicted binding site between miR107 and Cdk6 3'UTR / mutant Cdk6 3'UTR. (Red: Cdk6 site; green: mutant Cdk6 site). **(B)** Dual luciferase assay of miR107 on Cdk6 (**, $P < 0.01$; ns, $P > 0.05$). **(C and D)**, Effects of miR107 on Cdk6 protein expression were analyzed in ASMCs by Western blot **(C)** and statistical analysis **(D)**, all group $P < 0.05$. **(E and F)** Effects of miR107 on Cdk6 and p-Rb expression were analyzed in ASMCs cultured with PDGF-BB by Western blot **(E)** and the statistical analysis **(F)**, (*, $P < 0.05$).

noticeably (Figure 5A and B). We then executed rescue experiments by dividing primary ASMCs into three co-transfection groups (mimic NC & vector NC, mimic miR-107 and vector NC, and mimic miR-107 and vector Cdk6). Western blot shows that the protein level of Cdk6 down-regulated by mimic miR-107 was significantly rescued by vector Cdk6 transfection in ASMCs (Figure 5F). Our data also showed that recovering the level of Cdk6 protein substantially abolished the inhibitory effect of miR-107 on ASMCs proliferation (Figure 5D and E). Therefore, these data declared that the inhibiting effect of miR-107 on ASMCs proliferation was through the regulation of Cdk6.

Rb is Involved in miR-107-Mediated Inhibition of ASMCs Proliferation

Western blot results also showed that the of miR-107 suppressed the phosphorylation level of Rb in primary ASMCS cells (Figure 4E). Rb arrests the cell cycle by binding with transcription factor E2F. Cell cycle-dependent phosphorylation of Rb by Cdk6/4-cyclinD complex inhibits its activity and thus releases E2F allowing cell cycle progression.

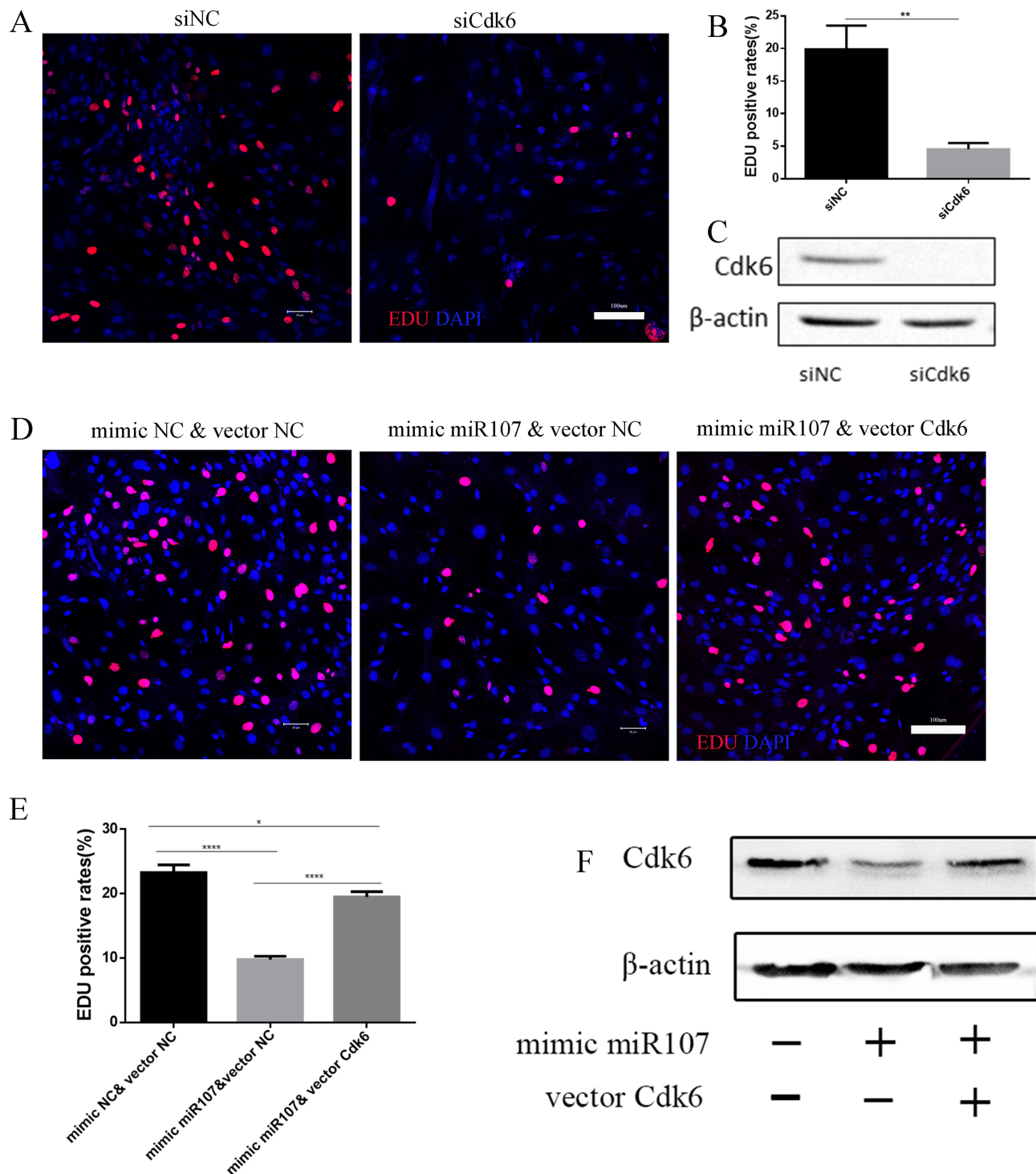


Figure 5 Up-regulation of Cdk6 reverses the effects of miR107 on the proliferation of ASMCs. (**A** and **B**) EDU staining (red) showing quantitative data (**B**) and representative images (**A**); left for negative control ASMCs, right for siCdk6-treated ASMCs), and nuclei stained with DAPI (**, $P < 0.01$). **C**, the transfection efficiency of siCdk6 in ASMCs tested by Western blot. (**D** and **E**) EDU staining (red) showing quantitative data (**E**) and representative images (**D**); left for mimic NC & vector NC co-transfection group, middle for mimic 107 & vector NC co-transfection group and right for mimic 107 & vector Cdk6 co-transfection group), and nuclei stained with DAPI (***, $P < 0.001$; *, $P < 0.05$); (**F**) the transfection efficiency of vector Cdk6 tested by Western blot.

Consistent with this, we use RNAi siRb to down-regulate the expression of Rb and thus its overall binding ability. The transfection efficiency was confirmed by RT-qPCR (Figure 6A). Lower expression of Rb robustly increased the proliferating ASMCs (Figure 6B and C) and rescued the proliferation inhibition of ASMCs caused by mimic miR-107 (Figure 6D and E).

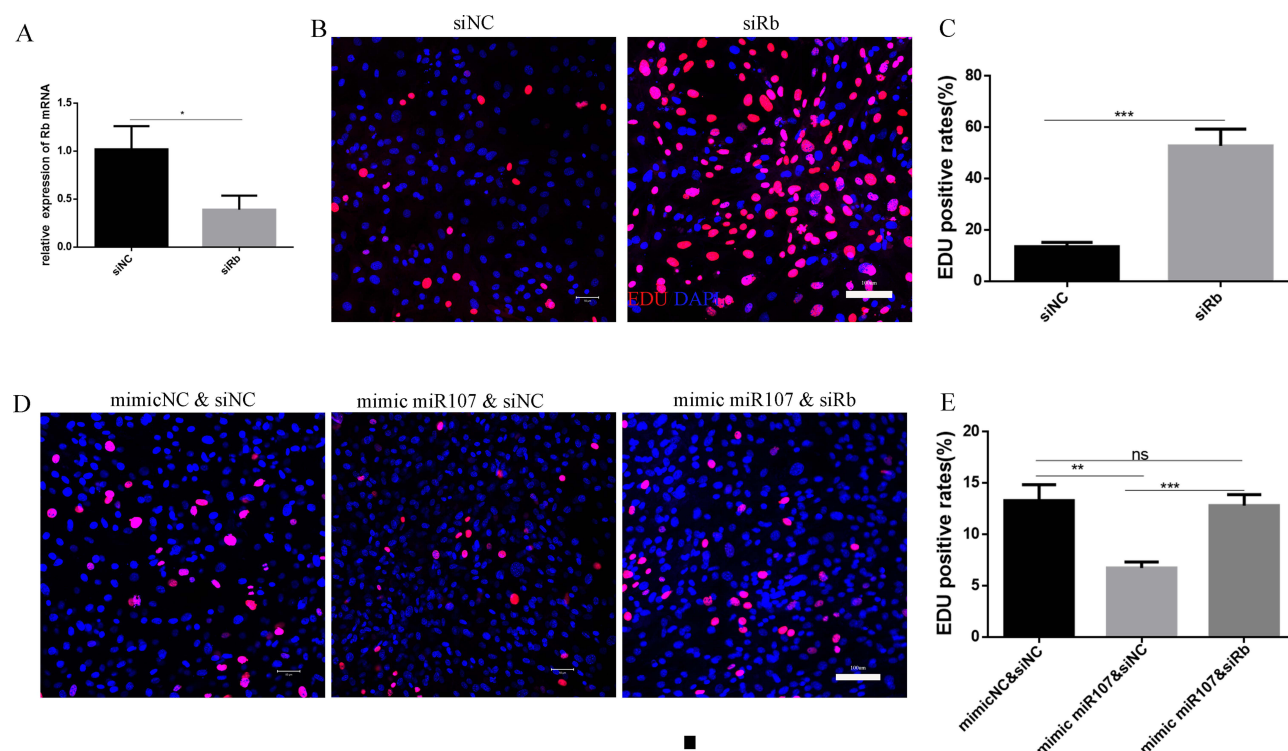


Figure 6 Rb was involved in the effect of miR107 on the proliferation of ASMCs. (A) the transfection efficiency of RNAi siRb in ASMCs tested by RT-qPCR (*, $P < 0.05$). (B and C) EDU staining (red) showing quantitative data (C) and representative images (B); left for negative control ASMCs, right for siRb-treated ASMCs, and nuclei stained with DAPI, (***, $P < 0.001$). (D and E) EDU staining (red) showing quantitative data (E) and representative images (D); left for mimic NC & siNC co-transfection group, middle for mimic miR107 & siNC co-transfection group and right for mimic 107 & siRb co-transfection group, and nuclei stained with DAPI, (***, $P < 0.001$; **, $P < 0.01$; ns, $P > 0.05$).

Cdk6 is Involved in miR-107-Mediated Migration Inhibition of ASMCs

We also tested the role of Cdk6 in the migration of ASMCs. Our results show that decreasing the level of Cdk6 reduced the number of migrated cells (Figure 7A and B) and Cdk6 overexpression partly abrogated the suppressive effect of miR-107 on ASMCs migration (Figure 7C and D). Therefore, these data declared that the effect of miR-107 on ASMCs migration was partially through regulating Cdk6.

Discussion

Pathological changes of asthma include cellular inflammation and tissue remodeling, in which smooth muscle hypertrophy and hyperplasia are significant causes.²⁴ However, the mechanisms underlying such ASM remodeling remain to be elucidated.

In the present study, we focused on microRNAs and their target genes-related signaling pathways to enhance our understanding of the mechanisms underlying asthma. By analyzing the microRNA microarray dataset and RT-qPCR test, we identified that miR-107 was downregulated in the serum of asthma patients and primary ASMCs of the chronic asthma mice model.

miR-107 is a highly evolutionally conserved microRNA belonging to the miR-15/107 group, in which all mature family members share the common sequence AGCAGC in their 5'-end.²⁵ Previous studies shown that miR-107 expression is reduced in lung tumors compared to normal lungs,²⁶ and evidence has also shown that miR-107 can suppress cell proliferation in two LC cell lines and induces G1 cell-cycle arrest by downregulating CCNE1 and CDK6.²⁷ In addition, up-regulation of miR-107 suppresses glioma cell growth through direct targeting of SALL4, CDK6, and NOTCH.²⁸ These reports are consistent with our finding that miR-107 inhibited ASMCs proliferation by targeting Cdk6-pRb. Thus, our data extend the importance of miR107-Cdk6 in asthma other than cancer. miR107 is a pro-metastatic microRNA in Colorectal cancer (CRC) by negatively regulating two metastasis suppressors: DAPK and KLF4,²⁹ while in

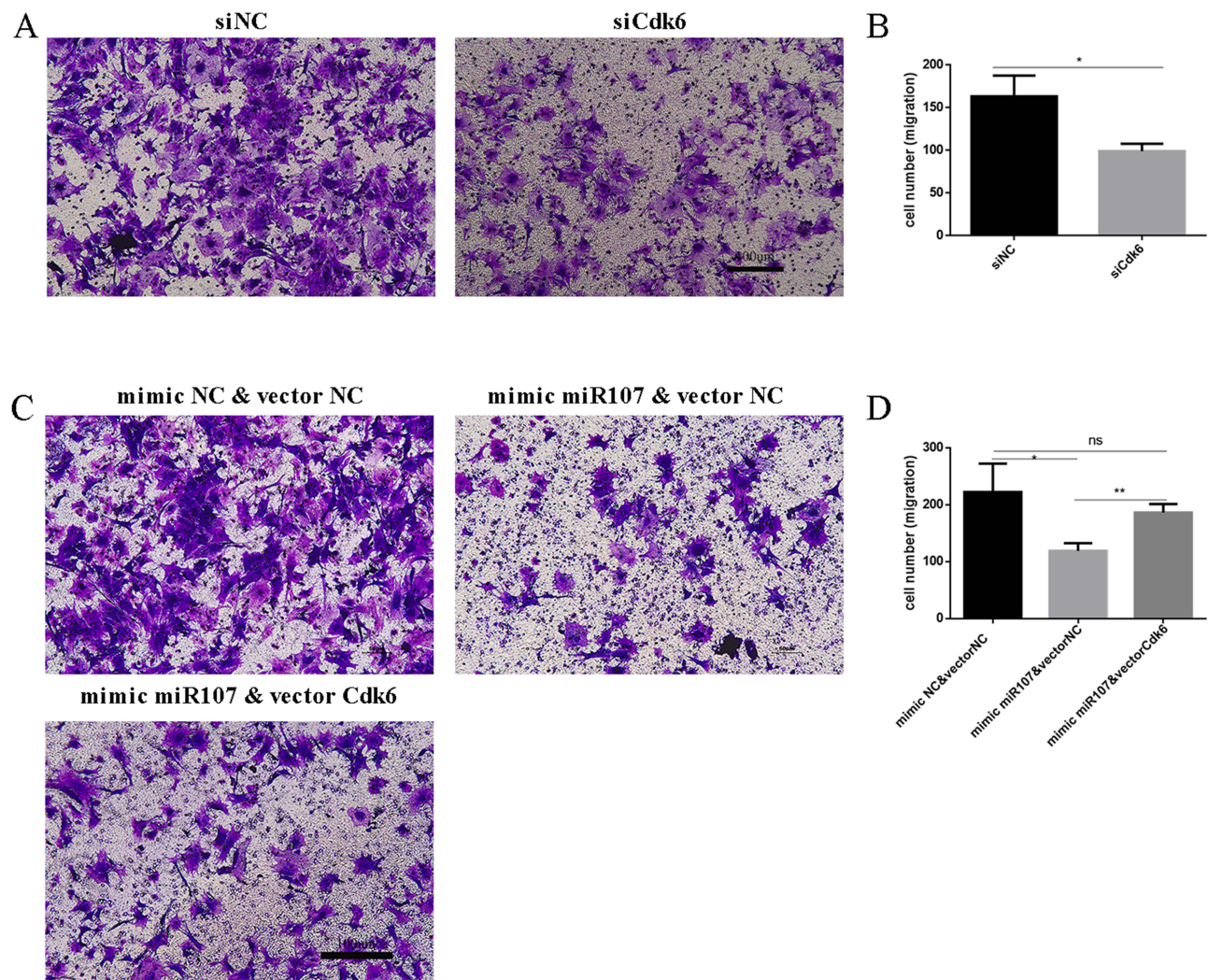


Figure 7 Up-regulation of Cdk6 partially reversed the effect of miR107 on the migration of ASMCs. (A and B) transwell cell migration assay showing quantitative data (B) and representative images ((A); left for negative control ASMCs, right for siCdk6-treated ASMCs), (*, $P < 0.05$). (D and C) transwell cell migration assay showing quantitative data (D) and representative images ((C); up-left for mimic NC & vector NC co-transfection group, up-right for mimic 107 & vector NC co-transfection group and down-left for mimic 107 & vector Cdk6 co-transfection group), (**, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).

our study, we show that miR107 functions as an anti-metastatic microRNA in the migration of ASMCs. The biological function of miR107 vary significantly in different physiological and pathological processes, probably as a result of being targeted to different pathways or genes.³⁰

Several down-regulated serum microRNAs in our analysis of the microarray dataset have been reported to play significant roles in airway remodeling by regulating ASMCs. For example, miR-20b-5p inhibits the proliferation of ASMCs by targeting STAT3.³¹ miR-223 reduces the production of the extracellular matrix by ASMCs via regulating IGF-1R in the PI3K/Akt pathway.³² Upregulation of miR-15b-5p results in the inhibition of TNF- α -induced migration, proliferation, and extracellular matrix deposition of ASMCs by targeting YAP1.¹⁰ Therefore, miR-20b-5p, miR-223, miR-15b-5p, and miR-107 may provide clinical significance to function as serum markers indicating the hyperplasia of ASMCs in asthma patients.

A limitation of this study is that we did not examine the role of miR-107 in asthma airway remodeling in vivo because of the experimental condition limitation. Notwithstanding this limitation, the study suggests that the miR-107-Cdk6 pathway plays a crucial role in regulating the proliferation and migration of ASMCs, which cause the abnormal increase of ASM mass. An implication of this is the possibility that these results may provide a promising area of investigation in the detection, prevention and therapy of airway remodeling.

Abbreviations

GO, Gene Ontology; ASMCs, primary airway smooth muscle cells; EDU, (5-ethynyl-2'-deoxyuridine). Cdk6, Cyclin-dependent kinases 6; Rb, Retinoblastoma; YAP1, yes-associated protein 1; PTEN, Phosphatase and tensin homolog.

Ethics Approval and Informed Consent

The microRNA array test of serum was conducted in accordance with the Declaration of Helsinki, approved by the Medical Ethics Committee of Shenzhen People's Hospital, and all patients provided written informed consent.

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Disclosure

The authors report no conflicts of interest in this work.

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