

Original Paper

MicroRNA-200a Affects the Proliferation of Airway Smooth Muscle Cells and Airway Remodeling by Targeting FOXC1 via the PI3K/AKT Signaling Pathway in Ovalbumin-Induced Asthmatic Mice

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Key Words

MicroRNA-200a • FOXC1 • PI3K/AKT signaling pathway • Asthma • Airway smooth muscle cell • Airway remodeling

Abstract

Background/Aims: The etiology of asthma, which is a complicated disorder with various symptoms, including wheezing, coughing, and airflow obstruction, remains poorly understood. In addition, the effects of microRNAs (miRs) have not been explored. This study explored the effect of microRNA-200a (miR-200a) on airway smooth muscle cells (ASMCs) and airway remodeling in asthmatic mice. Furthermore, we speculated that miR-200a achieves its effect by targeting FOXC1 via the PI3K/AKT signaling pathway based on differentially expressed gene screening, target miR predictions and a bioinformatics analysis. **Methods:** Eighty mice were assigned to a saline group or an ovalbumin (OVA) group, and the OVA group was transfected with a series of inhibitors, activators, and siRNAs to test the established mouse model. Airway reactivity and the ratio of eosinophils (EOSs) to leukocytes were detected. An ELISA was adopted to measure the levels of interleukin (IL)-4, IL-6, IL-8, tumor necrosis factor (TNF)- α , and immunoglobulin E (IgE). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blotting were used to determine the expression of FOXC1, PI3K, AKT, NF- κ B, cyclin D1, TGF- β 1 and p-AKT in ASMCs. Finally, CCK-8 assays were performed to detect cell proliferation and flow cytometry to detect apoptosis and cell cycle entry. **Results:** The bioinformatics analysis indicated that miR-200a mediated the PI3K/AKT signaling pathway by targeting FOXC1. In addition, mouse models of asthma were established. An elevated expression of miR-200a, a decreased mRNA and protein expression of FOXC1, PI3K, AKT, NF- κ B, cyclin D1 and TGF- β 1, a decreased expression of p-AKT, suppressed cell proliferation, accelerated apoptosis, and an increased number of cells at the G0/G1 phase were observed

following the upregulation of miR-200a and downregulation of FOXC1. **Conclusion:** The overexpression of miR-200a may downregulate FOXC1, thereby inhibiting the activation of the PI3K/AKT signaling pathway and ultimately suppressing ASMC proliferation and airway remodeling in asthmatic mice. This evidence supports the potential that miR-200a represents a new approach to treating asthma.

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Introduction

Asthma is a complicated disorder, and the main symptoms include wheezing, coughing, dyspnea, chest tightness, variable airflow, airway chronic inflammation, airway wall remodeling, airflow obstruction, and airway hyperresponsiveness [1, 2]. Due to airway remodeling resulting from glandular hyperplasia, epithelial damage, smooth muscle cell hyperplasia, and airway wall fibrosis, patients with asthma seem to exhibit a more rapid onset of lung dysfunction than healthy individuals [3]. In particular, airway eosinophil (EOS) accumulation is considered a signal of airway inflammation in asthma [4]. The disease is clinically divided into atopic and nonatopic asthma or controlled and uncontrolled asthma on the basis of the level of response to therapy [5]. Inflammation has various potential triggers, including reactions to lung infection [6], and the prevention of symptoms by decreasing airway hyperreactivity and inflammation has been a vital goal in asthma treatment [7]. Recently, with the development of gene treatment, disease progression (deregulation of genes) during the earlier stage has gradually replaced the immune basis of asthma as the focus of research investigating the treatment of asthma [8]. However, knowledge regarding the specific effects of microRNAs (miRs), especially on immunologically driven and inflammatory disorders, remains limited among the scientific community [9].

miRs are short noncoding RNAs ~22 nucleotides long that are involved in cellular processes at the posttranscriptional level by targeting the 3' untranslated region of mRNA, causing a reduction in the translation of proteins or the degradation of mRNAs [10]. miRs have been reported to have potential therapeutic effects in airway epithelial cells on underlying processes in asthma [11]. miR-200a is a member of the miR-200 family, which comprises the following two clusters: 1) miR-200c and miR-141 and 2) miR-200b, miR-200a and miR-429 [12]. Some studies have demonstrated that miR-200a plays an important role in the progression of human cancers, such as hepatocellular carcinoma, breast cancer and non-small cell lung cancer [13-15]. Forkhead box C1 (FOXC1) belongs to the FOX transcription factor family, which has recently been suggested as an emerging marker of and therapeutic target for human tumors [16]. Previously, FOXC1 has also been reported to participate in the activity of several cancers, such as breast cancer, hepatocellular cancer, and lung cancer [17]. The phosphoinositide 3-kinase (PI3K)/AKT signaling pathway could be indirectly regulated by miR-107 to suppress the metastasis of non-small cell lung cancer [18]. In addition, this pathway has been reported to play a regulatory role in allergic airway inflammation in asthma [19]. Furthermore, network analyses, such as the Gene Expression Omnibus (GEO) database, represent an emerging strategy to discuss the complex heterogeneous disorder of asthma at the system level [20]. We speculated that miR-200a may play a role in asthma by targeting FOXC1 through the PI3K/AKT signaling pathway.

Materials and Methods

Ethics statement

This experiment was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. In addition, all procedures performed in this experiment complied with the approved guidelines and were carried out in a way that minimized pain in the mice.

Table 1. Gene chip profile of asthma

Accession	Platform	Organism	Tissue	Sample
GSE27876	GPL6480	Homo sapiens	peripheral blood	five subjects with severe asthma and five normal subjects
GSE6858	GPL1261	Mus musculus	whole lung	mouse group with ovalbumin-induced asthma and PBS-treated control group; 4 replicates in each group

Bioinformatics analysis

We searched for asthma-related gene expression chips in the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). The GSE27876 [21] and GSE6858 [22] asthma-related chips were used to screen for differentially expressed genes. The gene chip profile is shown in Table 1. The affy package of the R language [23] was applied to perform the background correction and standardized preprocessing of the chip expression data, and the limma package of the R language [24] was used to screen for differentially expressed asthma-related genes. The threshold used in the differential gene screening was a p -value < 0.05 and $|\text{LogFoldChange}| > 2$, and a heat map of the differentially expressed genes was generated. DigSee (<http://210.107.182.61/geneSearch/>), which is a disease gene search engine [25], was employed to retrieve asthma-related genes using "Asthma" as a key word. Based on the protein-related information provided by the String database (<https://string-db.org/>) [26], the interaction between asthma-related differentially expressed genes and disease genes was analyzed and visualized online by Cytoscape 3.6.0 software [27]. TargetScan (http://www.targetscan.org/vert_71/), miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>) and microRNA (<http://34.236.212.39/microrna/getGeneForm.do>) were used to predict the target miRs of the differentially expressed genes. Custom Venn diagrams were calculated and generated using the Venn online analysis tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) to compare the differentially expressed genes or miR predictions.

Experimental animals

In total, 80 specific-pathogen-free (SPF) BABL/c female mice with a mean body weight of 21 ± 2 g and 4~8 weeks old were obtained from the Laboratory Animal Center of Zhengzhou University. The mice were fed standard granular feed in the Laboratory of Barrier Environmental Animals, the First Affiliated Hospital of Zhengzhou University and maintained at a moderate temperature of $22 \pm 2^\circ\text{C}$, with a relative humidity of $60 \pm 2\%$ and free access to water and food.

Model establishment and grouping

Eighty mice were randomly assigned to the saline (10 mice) or ovalbumin (OVA) (70 mice) groups. The mice in the OVA group were used to establish a model of asthma [28]. The mice in the OVA group were intraperitoneally injected with 0.2 mL of a solution containing aluminum hydroxide (2 mg) and OVA (20 g) on the 1st, 8th and 15th d. On the 21st d, the mice were anesthetized with an injection of 50 mg/kg of pentobarbital sodium, followed by intranasal infusion of budesonide solution. After 2 h, the mice were stimulated with 0.2% OVA (50 μL per mouse) until the 52nd d. The mice in the saline group were injected with normal saline on the 1st, 8th and 15th d. On the 21st d, the mice were administered normal saline at the same frequency as the mice in the asthma group. The appearance of irritability, ecphysis, salivary secretion, incontinence and abdominal cramps in the mice in the OVA group indicated the successful establishment of the asthma model. Airway reactivity and the proportion of EOS were measured. The mice were sacrificed after hemolysis, and their right lung tissues were preserved at -80°C .

Sixty mice successfully modeling asthma were randomly assigned to the following 6 groups: blank, negative control (NC), miR-200a mimic, miR-200a inhibitor, siRNA-FOXC1, and miR-200a mimic + siRNA-FOXC1 groups ($n = 10$ for each group). After being anesthetized with the inhalation of OVA solution, the mice in the miR-200a mimic group were intranasally instilled with a lentivirus miR-200a mimic (5×10^7 TU/mL) (200 μL per mouse) (purchased from Guangzhou RiboBio Biotechnology Co., Ltd., Guangzhou, Guangdong, China); the mice in the miR-200a inhibitor group received the lentivirus miR-200a inhibitor; the mice in the NC group received the NC lentivirus (lentivirus including the lentivirus vector of the miR-200a mimic, miR-

200a inhibitor, NC and siRNA-FOXC1); the mice in the siRNA-FOXC1 group received si-FOXC1; and the mice in the miR-200a mimic + siRNA-FOXC1 group received the lentivirus miR-200a mimic and siRNA-FOXC1. All instilled volumes were the same in each group. The NC sequence was constructed by Shanghai Genechem Co., Ltd. (Shanghai, China). The lentivirus vector was an LV-has plasmid. The blank group was untreated [29].

Dual luciferase reporter gene assay

A dual luciferase reporter gene assay was used to determine whether FOXC1 was a direct target gene of miR-200a. Artificially synthesized FOXC1 3'UTR was introduced into a pMIR-reporter (Huayueyang Biotechnology Co., Ltd., Beijing, China) using the endonuclease sites SpeI and Hind III. The mutation site of a complementary sequence of the seed sequence was designed in FOXC1 wild-type (WT), and the target fragment was inserted into the pMIR-reporter reporter plasmid with T4 DNA ligase after restriction enzyme digestion. The luciferase reporter WT and mutant (MUT) plasmids with the correct sequences were co-transfected with miR-200a into HEK-293T cells (Shanghai Beinuo Biotechnology Co., Ltd., Shanghai, China). After a 48-h transfection, the cells were collected and lysed, and their luciferase activity was detected by a firefly luciferase assay kit.

Tissue processing and grouping

The tissue experiments were grouped into the control, blank, NC, miR-200a mimic, miR-200a inhibitor, siRNA-FOXC1, and miR-200a mimic + siRNA-FOXC1 groups with 10 mice per group. The total RNA was extracted from the cells, and the product obtained from the amplification of the NC, miR-200a mimic, miR-200a inhibitor and siRNA-FOXC1 primers was digested by EcoRI and XhoI, followed by electrophoresis and gel extraction. The extracted fragments were connected by T4DNA ligase. The bacterium coli was further transformed, and clones were selected, followed by plasmid extraction and enzyme digestion. The mice in the NC group were treated with pentobarbital sodium on the 26th d after the model was successfully established and transnasally dripped with the NC plasmid and Lipofectamine 2000 mixture (50 µL) at a volume ratio of 1:2. Then, the mice were twirled to distribute the plasmid evenly in the airway. The mice in the miR-200a mimic, miR-200a inhibitor, siRNA-FOXC1, and miR-200a mimic + siRNA-FOXC1 groups were transnasally dripped with their corresponding plasmids in the same manner as the mice in the NC group.

Determination of airway reactivity

Forty-eight hours after grouping, the mice were anesthetized by pentobarbital sodium. Using a trachea cannula connected to a breathing machine and an atomizing device, the airway reactivity of the mice was measured on the 1st, 7th and 14th d of atomization stimulation using a Buxco lung function apparatus. Methacholine (Mch) was used as an activator, and the values of different inspiratory resistance (RI) caused by a doubled Mch dose (within 0.39 - 200 g/L) were measured until the original RI value doubled. Then, the Mch dose, namely, PC_{100'}, was converted into logarithms and analyzed statistically. Higher PC₁₀₀ values indicate lower airway reactivity [30].

Detection of proportion of EOS

Forty-eight hours after the transnasal dripping, the mice were anesthetized by 50 mg/kg of pentobarbital sodium via a peritoneal injection. A trocar was inserted into the airway, and the thoracic cavity was exposed. The right lung was connected to the trocar and washed with 0.3 mL of cold fetal bovine serum (FBS) to obtain bronchoalveolar lavage fluid (BALF). Then, the BALF was centrifuged at 1500 r/min for 10 min to remove the supernatant, and the cell deposition was resuspended for the cell smearing and staining. The ratio of EOSs to total leukocytes was counted under an optical microscope.

Hematoxylin-eosin (HE) staining

Lung tissues from the mice in each group were fixed in 4% paraformaldehyde for 24 h; successively dehydrated with 80%, 90%, and 100% ethanol and n-butyl alcohol; wax-dipped in a wax box at 60°C; and sectioned after embedding. The 5-µm serial sections were extended at 45°C, dried at 60°C for 1 h and dewaxed by xylene. After hydration, routine HE staining was carried out. First, the prepared sections were routinely dewaxed and hydrated with gradient ethanol, stained with hematoxylin for 2 min (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and washed with tap water for 10 s, followed by color separation

with hydrochloric acid ethanol for 10 s. Then, the sections were washed with distilled water for 1 min and stained with eosin for 1 min. The sections were washed again with distilled water for 10 s, dehydrated with gradient ethanol, cleared in xylene, and fixed in neutral balata. Finally, the morphological variations in the lung tissues were observed under an optical microscope (XP-330, Bingyu Optical Instrument Co., Ltd., Shanghai, China).

Masson's staining

A part of the lung tissues from each group was fixed in 4% paraformaldehyde for 24 h, successively dehydrated with 80%, 90%, and 100% ethanol and n-butyl alcohol, wax-dipped in a wax box at 60°C, and sectioned after embedding. The sections were dewaxed for hydration, stained with Regaud's hematoxylin dye solution for 5 - 10 min, and washed with distilled water. Then, the sections were stained with Masson's ponceau fuchsin acid liquid for 5 - 10 min, immersed in a 2% acetic acid aqueous solution, and differentiated by a 1% aqueous molybdate solution for 3-5 min. Next, the sections were stained with aniline blue and light green anionic dye for 5 min and briefly immersed in a 0.2% acetic acid aqueous solution, followed by dehydration with gradient ethanol, clearing with xylene, and neutral balata fixation. Each section was observed under a microscope (400×), and each mouse was identified by a 5 - 7 complete bronchial cross; finally, the collagen deposition area was quantitatively analyzed using NIH ImageJ image analysis software.

Immunohistochemistry

A part of lung tissues from each group was fixed in 4% paraformaldehyde for 24 h, successively dehydrated with 80%, 90%, and 100% ethanol and n-butyl alcohol, wax-dipped in wax box at 60°C, and sectioned after embedding. The 5-μm serial sections were extended at 45°C, dried at 60°C for 1 h, and dewaxed by xylene. The sections were dehydrated by gradient ethanol, soaked in 3% H₂O₂ for 10 min, and washed with distilled water. Then, the sections were repaired by a high-pressure method for 90 s, cooled at room temperature, and washed with PBS 3 times for 3 min each time. Then, the sections were supplemented with 100 μL of a 5% bovine serum albumin (BSA) block solution and incubated at 37°C for 30 min. Subsequently, the sections were supplemented with 100 μL of the diluted primary antibodies rabbit-anti-FOXC1 (1: 200, ab115201, Abcam, Inc., MA, USA) and transforming growth factor-β1 (TGF-β1, 1: 250, ab64715, Abcam, Inc., MA, USA) and incubated at 4°C overnight. Then, the sections were washed 3 times (3 min per wash), supplemented with a diluted biotin-labeled goat anti-rabbit secondary antibody solution (1: 100, HY90046, Hengyuan Biotechnology Co., Ltd., Shanghai, China), and incubated at 37°C for 30 min. Next, the sections were washed with PBS, supplemented with streptomycin avidin peroxidase chain (Zhongshan Biotechnology Co., Ltd., Beijing, China) and incubated at 37°C for 30 min. The sections were washed with PBS again 3 times (3 min per wash) and developed by diaminobenzidine (DAB, Bioss Biotech, Beijing, China) at room temperature. Then, the sections were soaked in hematoxylin for 5 min and washed with tap water. Subsequently, the sections were immersed and washed with 1% hydrochloric acid ethanol for 4 s, followed by washing with running water for 20 min to return to blue. The standard for protein-positive cells is that normal positive cells are brownish yellow [31]. Positive protein expression was assessed in five randomly selected high-power fields (400×, 100 cells/field) per section. Then, the percentage of positive cells was calculated for scoring as follows tumor positive cells/tumor cells > 10% was regarded as positive (+); otherwise, the result was regarded as negative (-). The experiment was repeated three times. Three small and medium-sized bronchi with complete cross sections per mouse were selected under a light microscope (400×), and Image-Pro Plus 6 medical image analysis software was adopted to determine the perimeter of the basement membrane (Pbm), total bronchial wall area (WAt), airway smooth muscle area (WAm), and ASMC (N). The results are presented as pre-units of the length of the basal membrane area and shown as Pbm, WAt, WAm and N.

Enzyme-linked immunosorbent assay (ELISA)

Serum was extracted from the mice in each group for ELISA. In strict accordance with the instructions of the ELISA kit (eBioscience, Thermo Fisher Scientific, Inc., Waltham, MA, USA), the ELISA kit was kept at room temperature for 20 min, and the detergent was prepared. After the standards were dissolved, 100 μL were added into a reaction plate to generate the standard curve. Then, the samples (100 μL) were added to the reaction plate, incubated at 37°C for 90 min, washed and supplemented with 100 μL of biotinylated antibody working fluid, and incubated at 37°C for 60 min. Then, the samples were washed, supplemented

with 100 μ L of fresh enzyme-binding agent (in the dark) working fluid and incubated at 37°C for 30 min. The plate was washed 3 times, supplemented with 100 μ L of substrate, incubated while avoiding light at 37°C for 15 min, and immediately supplemented with stop buffer to stop the reaction. Within 3 min, the optical density (OD) value was measured by a multimode microplate reader (Synergy 2, BioTek Instruments, Inc., VT, USA) at 450 nm. A standard curve was generated according to the OD value, and the contents of interleukin-4 (IL-4, hj-C14128, Shanghai Hongju Industrial Co., Ltd., Shanghai, China), IL-8 (hj-C14126, Shanghai Hongju Industrial Co., Ltd., Shanghai, China), tumor necrosis factor- α (TNF- α , hj-C14134, Shanghai Hongju Industrial Co., Ltd., Shanghai, China) and immunoglobulin E (IgE) (ab117718, Abcam, Inc., Cambridge, MA, USA) were measured.

Culture of ASMCs

Primary ASMCs from mice in each group were isolated and cultured. Trachea, other bronchi and lung tissues were rinsed with D-Hanks solution for 3~5 min and transferred to a culture dish of germ-free rubber tile after the blood was washed away. The complete trachea and bronchi were isolated, and the adventitia and connective tissues were stripped to render the trachea and bronchi completely transparent. Then, the abovementioned tissues were cut into blocks (approximately 2×2 mm²) and placed in a culture flask for adherent culture, and Dulbecco-modified Eagle medium (DMEM) containing 20% FBS was added to wet the blocks. Next, the flask was inverted and incubated in a 5% CO₂ incubator at 37°C for 2 h. After the blocks were tightly adherent, the flask was turned again, and the blocks were soaked in the culture medium, which was changed every 4~5 d until the cell confluency reached 80%. Then, the cells were detached by trypsin and transferred to a new flask and allowed to stand for 30 min. The differential adhesion method was adopted to purify the cells, and the adherent rate of the mixed desmocytes was higher than that of the ASMCs. After the impure cells were adhered in advance, the remaining cells were transferred to another flask and supplemented with DMEM containing 10% FBS. The cells were repeatedly purified and subcultured to reach 100% purity. ASMCs at 3~8 generations were used in the subsequent experiments.

Primary ASMC identification

ASMCs (1×10^5 cells/mL) were inoculated into a 6-well plate with a prepared germ-free cover glass. Each well was supplemented with 1 mL of serum-free DMEM and placed in an incubator (37°C, 5% CO₂) for incubation for 4 h and immunofluorescent labeling. Subsequently, the cells were fixed in 4% paraformaldehyde (1 mL/well) for 20 min. The paraformaldehyde was absorbed, and the cells were washed with PBS 3 times. Each well was sealed with 200 μ L of PBS containing 10% goat serum and incubated at room temperature for 30 min; then, the sealing solution was extracted. The cells were supplemented with mice α -Actin antibody (1: 400) and incubated at 4°C overnight. On the morning of the following day, the primary antibody was absorbed, and the cells were washed with PBS three times. Then, the cells were incubated with a secondary goat-anti-mouse antibody (1: 100) for 15 min and washed with PBS three times. Subsequently, the cells were incubated with horseradish peroxidase labeled streptavidin for 15 min and washed with PBS again three times. Then, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, 5 μ g/mL) for 2 min and sealed by glycerophosphate buffer. The cellular immunofluorescence was observed under a fluorescence microscope (excitation wavelength: 495 nm; absorption wavelength: 520 nm) and photographed.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Liquid nitrogen was added to the lung tissues, and the lung tissues were ground to a fine powder. The TRIzol method (15596-018, Invitrogen, New York, CA, USA) was used to extract the total RNA from the cells, and the concentration and purity of the RNA were detected. According to the PrimeScript™ RT Reagent Kit (RR037A, Takara, Dalian, China), the sample RNA was reverse transcribed to cDNA in a total volume of 25 μ L. The reaction conditions were as follows: 37°C for 15 min (reverse transcription) and 85°C for 5 s (reverse transcriptase inactivation). The cDNA was diluted with 65 μ L of diethyl pyrocarbonate (DEPC) and fully mixed. According to the instructions of the SYBR® Premix Ex Taq™ II kit, cDNA was selected for RT-qPCR. The reaction system (50 μ L) included 25 μ L of SYBR® Premix Ex Taq™ II (2 $\times$), 2 μ L of PCR forward primers, 2 μ L of PCR reverse primers, 10 μ M PCR buffer, 1 μ L of ROX Reference Dye (50 $\times$), 4 μ L of DNA template and 16 μ L of ddH₂O. RT-qPCR was carried out using an ABI PRISM® 7300 system (ABI, USA). The reaction condition included pre-denaturation at 95°C for 4 min, 40 cycles of denaturation at 94°C for 30 s,

annealing at 58°C for 30 s and extension at 72°C for 1 min. U6 served as an internal reference for miR-200a, and β -actin served as an internal reference for the other primers. All primers were designed and synthesized by Wuhan Boster Biological Technology Corporation (Wuhan, Hubei, China), and the sequences of the primers are shown in Table 2. The $2^{-\Delta\Delta Ct}$ method refers to the ratio of the target genes in the OVA group to that in the saline group, and the formulae were as follows: $\Delta\Delta Ct = \Delta Ct_{OVA \text{ group}} - \Delta Ct_{saline \text{ group}}$ and $\Delta Ct = Ct_{target \text{ genes}} - Ct_{\beta\text{-actin}}$ [32]. Ct refers to the number of amplified cycles required for the

real-time fluorescence intensity of the reaction to reach the set threshold, with amplification increasing logarithmically. The experiment was repeated three times independently (this was also applied for the cell experiments).

Western blot analysis

A portion of the lung tissues from the control and asthma groups was placed in liquid nitrogen. The lung tissues were ground to a fine powder and supplemented with 1 mL of tissue lysate, including 50 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 5 μ g/mL aprotinin and 2 mmol/L phenylmethylsulfonylfluoride (PMSF). Then, the tissues were homogenized in an ice-bath, lysed with protein lysate at 4°C for 30 min, shaken once every 10 min, and centrifuged at 12000 r/min at 4°C for 20 min after discarding the lipid layer. The supernatant was removed to detect the protein concentration in each sample using a bicinchoninic acid (BCA) kit (Yesen Biotech. Co., Ltd., Shanghai, China), and the loading quantity of protein was adjusted to 30 μ g/lane using deionized water. The 10% SDS separation gel and 4% spacer gel were prepared. The samples and sample loading buffer were mixed, boiled for 5 min, and equivalently added to the lanes after ice-bath immersion and centrifugation using a micropipette for electrophoretic separation. After the separation, the proteins on the gel were transferred onto a nitrocellulose filter (NC filter) that was sealed at 4°C overnight by 5% dried skimmed milk. The membranes were incubated with diluted primary rabbit polyclonal FOXC1 (ab115201, Abcam, Cambridge, MA, USA) (1: 250 – 1: 1000), PI3K (ab151549, Abcam, Cambridge, MA, USA) (1: 1000), AKT (ab8805, Abcam, Cambridge, MA, USA) (1: 500), nuclear factor-kappa B (NF- κ B, ab32536, Abcam, Cambridge, MA, USA) (1: 50000 – 1: 100000), cyclin D1 (ab134175, Abcam, Cambridge, MA, USA) (1: 10000 – 1: 50000), TGF- β (ab25121, Abcam, Cambridge, MA, USA) (1: 2000) and p-AKT (ab38449, Abcam, Cambridge, MA, USA) (1: 500 – 1: 1000) antibodies overnight and washed 3 times with PBS at room temperature (5 min per wash). Then, the membrane was treated with horseradish peroxidase labeled by a goat-anti-rabbit IgG secondary antibody (1: 1000, Wuhan Boster Biological Technology Co., Ltd., Hubei, China), shaken and incubated at 37°C for 1 h. Subsequently, the membrane was washed with PBS 3 times at room temperature (5 min per wash). The membrane was soaked in enhanced chemiluminescence (ECL) solution (Pierce, Waltham, MA, USA) at room temperature for 1 min. Then, the fluid was absorbed, and the membrane was covered with a preservative film. The results were observed by X-ray exposure, developed and fixed in a dark room. With β -actin as an internal reference, the ratio of the gray value of the target band to that of the internal reference band was used as the relative expression level of the proteins (this was also suitable for the cell experiments).

Table 2. Primer sequences of miR-200a, FOXC1, PI3K, AKT, NF- κ B, U6, and β -actin

Genes	Sequences (5'-3')
miR-200a	Forward: AAAGTACGGCCGCGCCAGTTATTAACAGTGTCTTTCTTTT Reverse: CTAGAAAAAGAAAAACACTGTTTAATAACTGGCGCGCTAGTTT
FOXC1	Forward: GGCAAGAATTGATCCGGTA Reverse: TGGATGGCCATGGTGATGAGC
PI3K	Forward: ACACCCGCGTTTGGACTATGG Reverse: GGCTACAGTAGTGGGCTTGG
AKT	Forward: ATGAACGACGTAGCCATTGTG Reverse: TTGTAGCCAATAAAGGTGCCAT
NF- κ B	Forward: CAGCCCGCACACGCCATGTTTCAG Reverse: CATGGAGTCCAGCCGT
Cyclin D1	Forward: GCGTACCCTGACACCAATCTC Reverse: ACTTGAAGTAAGATACGGAGGGC
TGF- β 1	Forward: CCACCTGCAAGACCATCGAC Reverse: CTGGCGAGCCTAGTTTGGAC
U6	Forward: GCCCGCCCTCCGCCGCCGCC Reverse: ATATGGAACGCTTACGAATT
β -actin	Forward: AGAAGATCTGGCACCACACC Reverse: GAACCAGGAGTTAAGAACACG

Cell counting kit-8 (CCK-8) assay

Cells obtained from each group were digested by trypsin 48 h after transfection, and the density was adjusted to 2×10^4 cells/mL. The cell suspension was inoculated into a 96-well plate (100 μ L suspension in each well), and cells from each group were placed in 5 duplicate wells. The plate was incubated in a 37°C incubator. Three wells were randomly selected 12, 24, 36, 48 and 72 h after incubation. Each well was supplemented with 10 μ L of CCK-8 and incubated for 2 h continuously. Finally, the OD value was measured at a wavelength of 450 nm by a microplate reader (Multiskan FC, Thermo Fisher, New York, NY, USA), and a cell growth curve was generated. The experiment was repeated 3 times.

Flow cytometry

Propidium iodide (PI) single staining was used to detect the cell cycle. The cell processing and collection were carried out using the same methods mentioned above. The cells were fixed in 70% cold ethanol overnight at 4°C, centrifuged at 800 g to remove the supernatant, washed two times with PBS containing 1% FBS, resuspended in 400 μ L of binding buffer, supplemented with 50 μ L of RNA (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) and incubated at 37°C for 30 min. Next, the cells were supplemented with 50 μ L of 50 mg/L PI (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) and incubated while avoiding light at room temperature for 30 min. Finally, the cell cycle entry was detected by flow cytometry.

Annexin V-fluorescein isothiocyanate (FITC)/PI double staining was used to detect cell apoptosis. CFPAC-1 cells were inoculated into a 6-well plate (2×10^5 cells/well). The blank, NC, and transfection groups were set up for transfection (100 nmol/L); then, 72 h after transfection, the medium was removed, and the cells were washed with precooled PBS, detached by trypsin, and collected into a 15 mL centrifuge tube for centrifugation at 800 g after removing the supernatant. Next, the deposition was washed twice with PBS. Using the Annexin V-FITC Apoptosis Detection Kit I (556547, BD Biosciences, Franklin Lakes, NJ, USA), the cells were resuspended in 500 μ L of binding buffer, supplemented with 5 μ L of FITC and 5 μ L of PI, mixed and incubated while avoiding light for 15 min. Finally, cell apoptosis was analyzed by flow cytometry.

Statistical analysis

The statistical analyses were performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The measurement data are expressed as the mean $\pm$ standard deviation, and a *t*-test was used for comparisons between two groups. One-way ANOVA analysis was adopted for comparisons among multiple groups. *p* < 0.05 was considered statistically significant.

Results

Bioinformatics analysis of miR-200a in asthma

Using the GSE27876 and GSE6858 chips, 3,271 and 686 differentially expressed asthma-related genes were identified, respectively, using a *p*-value < 0.05 and |LogFoldChange| > 2 as the threshold for the screening. Sorted by |LogFoldChange|, the first 700 genes in GSE27876 and GSE6858 were selected for comparison. A Venn diagram was generated (Fig. 1A) and showed that there were 16 differentially expressed genes at the intersection. In addition, asthma-related genes were retrieved from DigSee, and the top 20 genes were selected as disease genes. Along with the 16 differentially expressed genes, these genes were used for a gene interaction analysis to map the gene interaction network (Fig. 1B). In this network, the differentially expressed genes MUC4 and FOXC1 had strong interactions with the disease genes. MUC4, which is a gene encoding mucus proteins, is associated with respiratory diseases [33, 34]. FOXC1 is a member of the forkhead family of transcription factors, and few studies have focused on its role in asthma. In addition, a heat map of the top 20 differentially expressed genes in the GSE6865 chip was plotted (Fig. 1C). The expression of FOXC1 in mice with asthma was significantly higher than that in the mice in the control group using both the GSE6865 and GSE27876 chips (Fig. 1D). TargetScan, miRWalk, and microRNA were applied to predict miRs capable of regulating FOXC1. There were 36 miRs with Pct values using TargetScan and 34 miRs with Pct values using microRNA, and the first 100 miRs predicted using miRWalk were compared with those predicted using TargetScan and microRNA. The

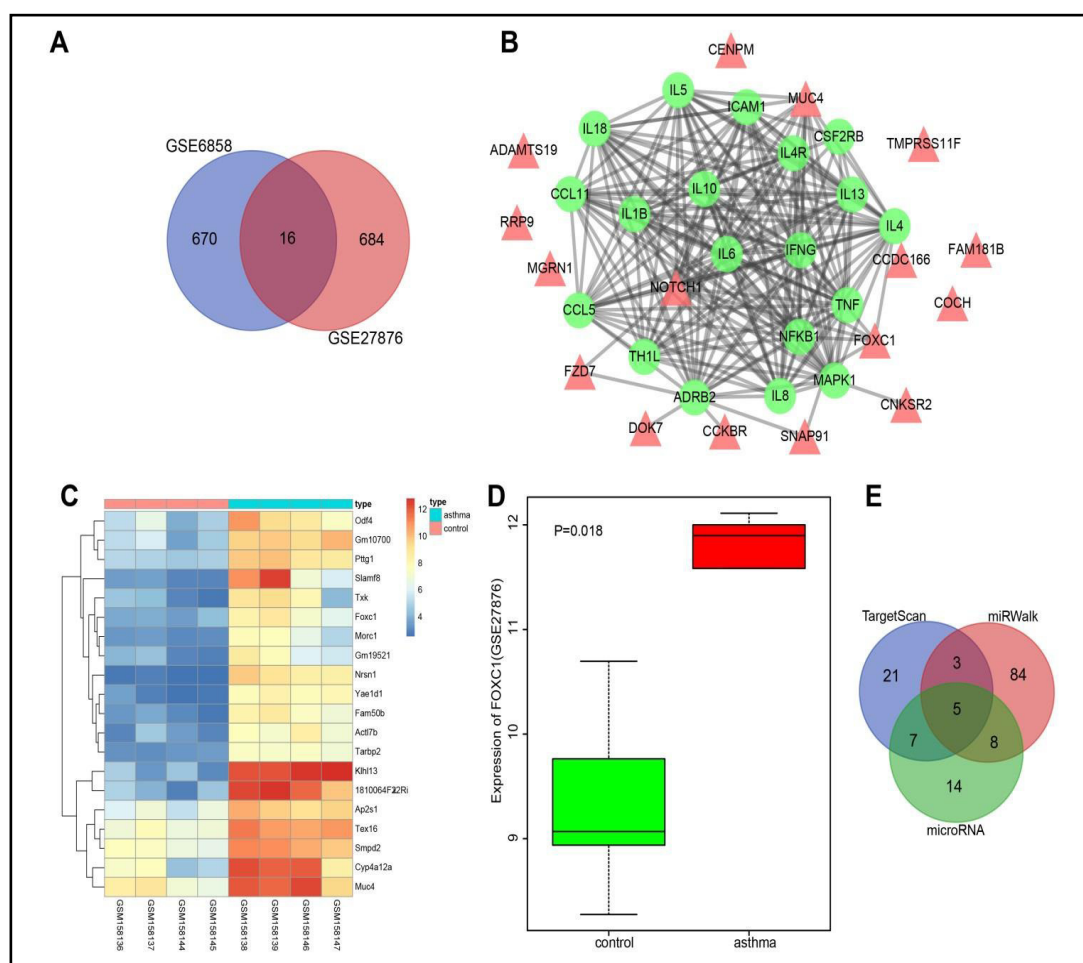


Fig. 1. Bioinformatics analysis of the role of molecules in asthma. Note: A, 16 intersections of miRs in the comparison of the first 700 differentially expressed genes using the GSE27876 and GSE6858 gene chips; B, interaction network of asthma-related differentially expressed genes and disease genes; red represents differentially expressed genes, and green represents disease genes; C, heat maps of the first 20 differentially expressed genes in the GSE6865 chips; sample number is presented by the abscissa, and the differentially expressed genes are presented by the ordinate. The histogram shown on the upper right provides the color scale, and each rectangle corresponds to an expression value of the sample. Red indicates a high expression level, and blue represents a low expression level; D, change in the expression levels of FOXC1 in the GSE27876 chip; E, comparison of miRs predicted by TargetScan, miRWalk, and microRNA. There are 5 intersections of miRs, miRs, microRNAs.

results illustrated that there were 5 intersections of miRs as follows: mmu-miR-200a-3p, mmu-miR-141-3p, mmu-miR-138-5p, mmu-miR-204-5p, and mmu-miR-137-3p (Fig. 1E); thus, these 5 miRs may regulate FOXC1. Studies have shown that miR-138 [35], miR-204 [36] and miR-137 [37] can regulate FOXC1. Few studies have investigated the association among miR-200a, miR-141 and FOXC1. The inhibition of the PI3K/AKT signaling pathway has positive effects on asthma relief [38-40]. miR-200a can inhibit the PI3K/AKT signaling pathway [41, 42], while miR-141 activates the PI3K/AKT signaling pathway [43]. Therefore, in our study, we focused on the role of miR-200a in asthma and speculated that miR-200a could target FOXC1-mediated PI3K/AKT signaling pathways.

Table 3. Successful mouse model of asthma

Groups	Sample sizes (n)	Number of mice successfully modeled (n)	Rate of successful model establishment (%)
Saline	10	10	100
OVA	70	60	85.7

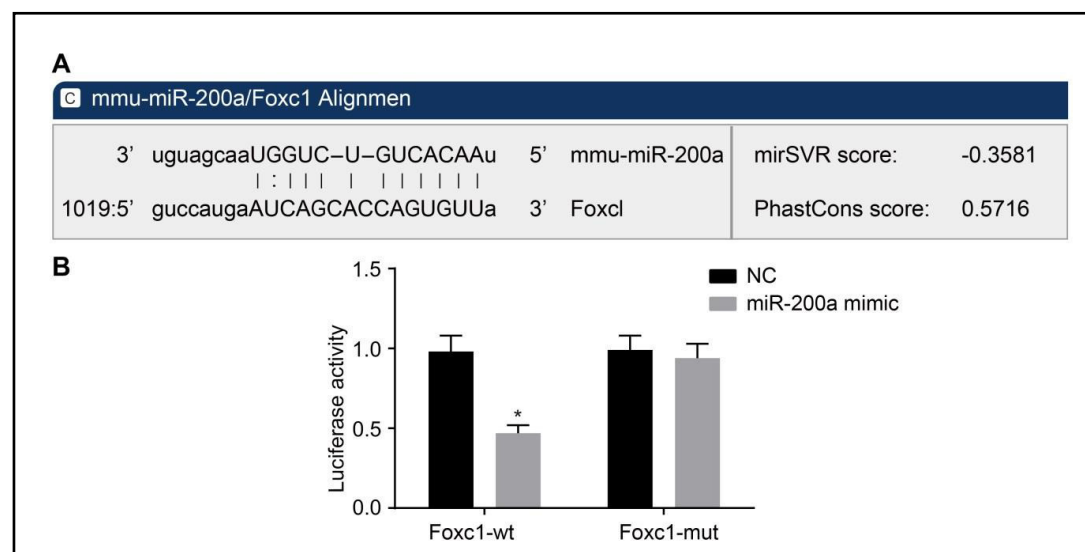


Fig. 2. FOXC1 is a target gene of miR-200a as determined by a dual luciferase reporter gene assay. Note: A, the predicted binding sites of miR-200a in the FOXC1 3'UTR; B, dual luciferase reporter gene assay; *, $p < 0.05$ compared with the NC group; n = 3; miR-200a, microRNA-200a; NC, negative control.

Mouse model of asthma was successfully established

The mice in the saline group behaved normally and had a regular respiratory rhythm after saline nebulization. The selected mice in the saline group were all normal. During the model establishment process, the mice in the OVA group showed signs and symptoms, such as dysphoria, polypnea, nose scratching, cough, slight tremor of the limbs, humpback and reduced mobility, and, during the later stage, mild cyanosis and nodding respiration were observed, indicating that OVA successfully established asthma in the mice. After nebulization, ten mice in the OVA group did not appear to have dysphoria, polypnea, ptialism, gatism and abdominal spasm. The rate of successful model establishment was 85.7% (Table 3).

FOXC1 is a target gene of miR-200a

The analysis using online software revealed specific binding areas between the FOXC1 sequence and the miR-200a sequence, and FOXC1 was potentially a target gene of miR-200a (Fig. 2A). A dual luciferase reporter gene assay was applied to determine whether FOXC1 was a target gene (Fig. 2B). The result showed that compared with the NC group, the luciferase activity of Wt-miR-200a/FOXC1 in the miR-200a mimic group was decreased ($p < 0.05$), while the luciferase activity of the mutant 3'UTR showed no significant difference ($p > 0.05$), indicating that miR-200a could specifically bind FOXC1.

MiR-200a targets the expression of the FOXC1 gene

After interfering with miR-200a and the Foxc1 gene, the mRNA expression of miR-200a and FOXC1 was measured by RT-qPCR (Fig. 3A) and a western blot analysis (Fig. 3B-C). Compared with the control group, the other groups had significantly decreased miR-200a expression and increased FOXC1 expression (all $p < 0.05$). There was no significant difference in the expression of miR-200a and FOXC1 between the blank and NC groups ($p > 0.05$). Compared with the blank group, the miR-200a mimic group displayed significantly higher miR-200a expression and significantly lower FOXC1 expression; the miR-200a inhibitor

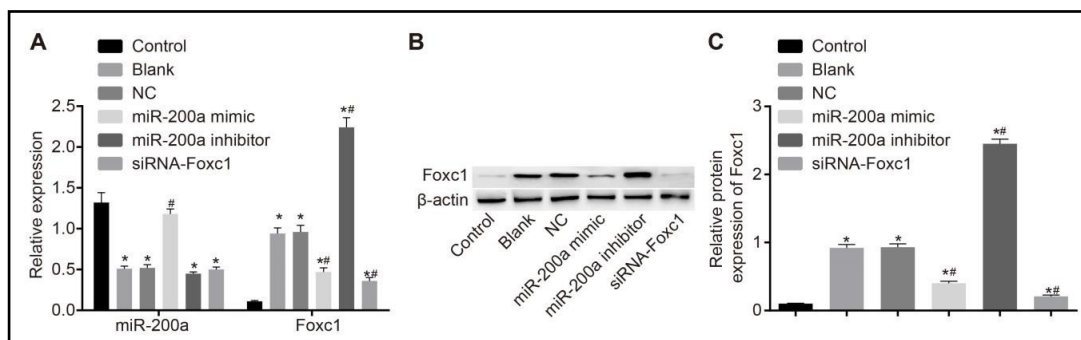


Fig. 3. miR-200a targets the expression of FOXC1 as determined by RT-qPCR and western blot analysis. Note: A, mRNA expression of FOXC1 was downregulated in the miR-200a mimic group but upregulated in the miR-200a inhibitor group; B and C, protein expression of FOXC1 was inhibited in the miR-200a mimic group but elevated in the miR-200a inhibitor group.

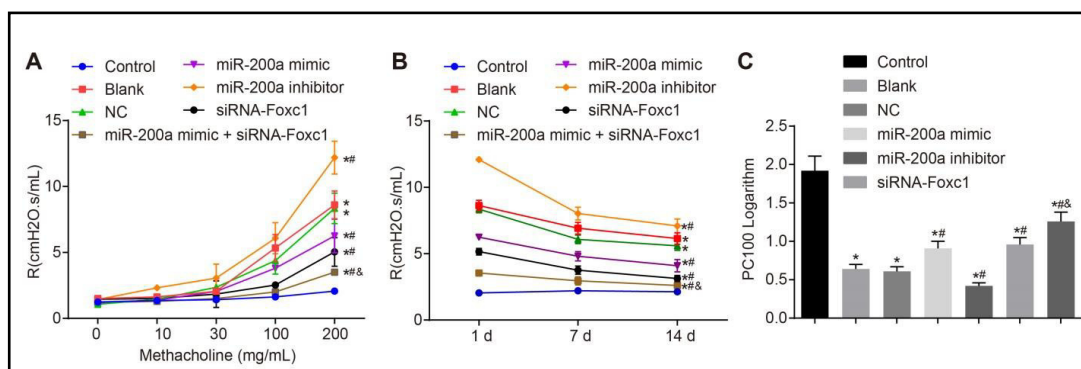


Fig. 4. miR-200a attenuates airway reactivity in mice with asthma as determined by the airway resistance response. Note: A, Methacholine dose-response curve of airway resistance on the 1st day; B, airway resistance response in mice treated with 200 mg/mL of Methacholine during different time periods; C, airway resistance response in mice with asthma in each group; *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; n = 10; NC, negative control; miR-200a, microRNA-200a.

group demonstrated the opposite trend. The siRNA-FOXC1 group showed no significant difference in miR-200a expression, but FOXC1 expression was significantly suppressed ($p < 0.05$).

High levels of miR-200a and silencing FOXC1 repress airway reactivity in mice with asthma

The results of the airway resistance measurements in the mice from each group are shown in Fig. 4A. When the Mch dose was above 30 mg/mL, the airway resistance in the mice in the other groups was significantly increased compared with that in the control group (all $p < 0.05$). Compared with the blank group, the NC group showed no significant difference in airway resistance, and the miR-200a mimic, siRNA-FOXC1, and miR-200a mimic + siRNA-FOXC1 groups exhibited a remarkable decrease, while the miR-200a inhibitor group exhibited an increase (all $p < 0.05$). Compared with the miR-200a mimic and siRNA-FOXC1 groups, the miR-200a mimic + siRNA-FOXC1 group had reduced airway resistance ($p < 0.05$). When the dose of Methacholine was 200 mg/mL, the results of the airway resistance measurements in each group are shown in Fig. 4B. Except for the control group, the other groups had significantly higher airway resistance on the 1st day than on the 7th and 14th days (all $p < 0.05$). Compared with the control group, the other groups showed significantly elevated airway resistance (all $p < 0.05$); there was no significant difference in airway resistance between the NC and blank groups ($p > 0.05$). Compared with the blank group, the miR-200a

mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups illustrated significantly inhibited airway resistance, whereas the miR-200a inhibitor group demonstrated promoted airway resistance ($p < 0.05$). The miR-200a mimic + siRNA-FOXC1 group displayed significantly lower airway resistance than the miR-200a mimic and siRNA-FOXC1 groups ($p < 0.05$).

The results of the airway reactivity measurements in the mice from each group are shown in Fig. 4C. Compared with the control group, the PC_{100} logarithms in the mice in the other groups obviously decreased, suggesting that the airway reactivity in the mice in the other groups significantly increased ($p < 0.05$). Compared with the blank group, no significant difference in airway reactivity was found in the NC group; however, decreased airway reactivity was revealed in the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups, while elevated airway reactivity was found in the miR-200a inhibitor group (all $p < 0.05$). Compared with the miR-200a mimic and siRNA-FOXC1 groups, the airway reactivity was significantly decreased in the miR-200a mimic + siRNA-FOXC1 group ($p < 0.05$). These results indicate that airway reactivity is inhibited at higher levels of miR-200a and following the silencing of FOXC1.

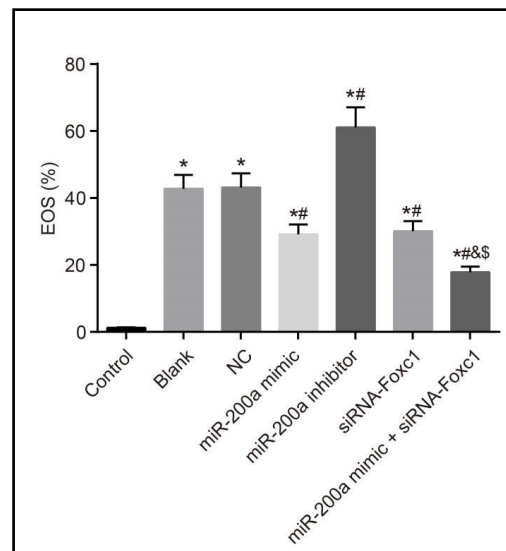


Fig. 5. miR-200a decreases the proportion of EOSs in BALF from mice with asthma as determined by the airway resistance response. Note: *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; $n = 10$; NC, negative control; miR-200a, microRNA-200a; EOSs, eosinophils; BALF, bronchoalveolar lavage fluid.

High levels of miR-200a and silencing FOXC1 limit the proportion of EOSs in mice with asthma

The results of the EOS proportion measurements in each group are shown in Fig. 5. Compared with the control group, the other groups had increased proportions of EOSs ($p < 0.05$). Compared with the blank group, the NC group had no significant difference in the proportion of EOS, but the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups showed significantly decreased proportions of EOSs, and the miR-200a inhibitor group showed an increased proportion of EOSs ($p < 0.05$). Compared with the miR-200a mimic and siRNA-FOXC1 groups, the proportion of EOSs was significantly decreased in the miR-200a mimic + siRNA-FOXC1 group ($p < 0.05$). These results reveal that upregulated miR-200a or downregulated FOXC1 can restrain the proportion of EOSs in asthma.

High levels of miR-200a and silencing FOXC1 protect against damage to the alveolar epithelium and collagen deposition in mice with asthma

The histopathological changes are shown in Fig. 6. The tissue sections from the mice in the control group presented complete alveolar wall structures, orderly arrangement of alveolar epithelial cells, normal thickness of alveolar walls and discontinuous smooth muscle layers. The tissues from the mice in the blank and NC groups showed impaired alveolar wall structures, incassated alveolar walls, incassated and continuous smooth muscle layers, proliferated bronchiolar smooth muscle cells, a large amount of eosinophil infiltration around the bronchi and arteries, increased folds of bronchial mucous membranes and exfoliated mucous membranes. The arrangement of the alveolar epithelium was relatively organized, with mild alveolar damage in the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups. The tissues from the mice in the miR-200a inhibitor group

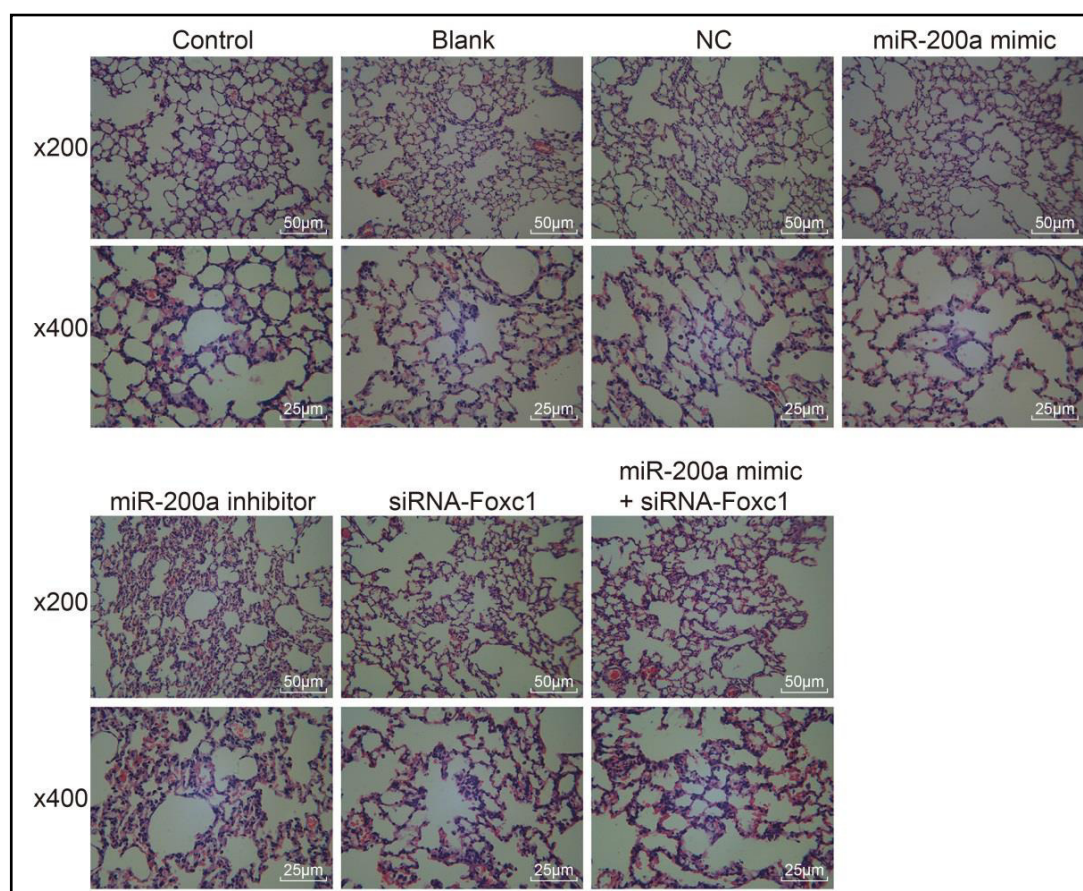


Fig. 6. Histopathological observation maps of mice in the saline group and OVA group as detected by HE staining. Note: the first line shows the original magnification of 200 $\times$, and the second line shows the original magnification of 400 $\times$; NC, negative control; miR-200a, microRNA-200a; HE, hematoxylin-eosin; OVA, ovalbumin.

had seriously impaired alveolar wall structures. In addition, the Masson's staining results in each group are shown in Fig. 7. The mice in the control group had normal lung tissues. In the blank, NC and siRNA-FOXC1 groups, the lung tissues were dim in color, and the lungs were swollen with a greater volume, widened alveolar septum and more collagen deposition. In the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups, the lungs were slightly swollen and impaired. The collagen deposition area in the mice in each group was quantitatively analyzed by online software. The results showed that compared with the control group, the other groups had an elevated collagen deposition area ($p < 0.05$). Compared with the blank group, the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups showed a decreased collagen deposition area ($p < 0.05$), the miR-200a inhibitor group showed an increased collagen deposition area ($p < 0.05$), and the NC group showed no significant difference ($p > 0.05$). Compared with the miR-200a mimic and siRNA-FOXC1 groups, the miR-200a mimic + siRNA-FOXC1 group showed a smaller collagen deposition area ($p < 0.05$). Altogether, the upregulation of miR-200a and knockdown of FOXC1 could prevent alveolar cell damage and collagen deposition in asthma.

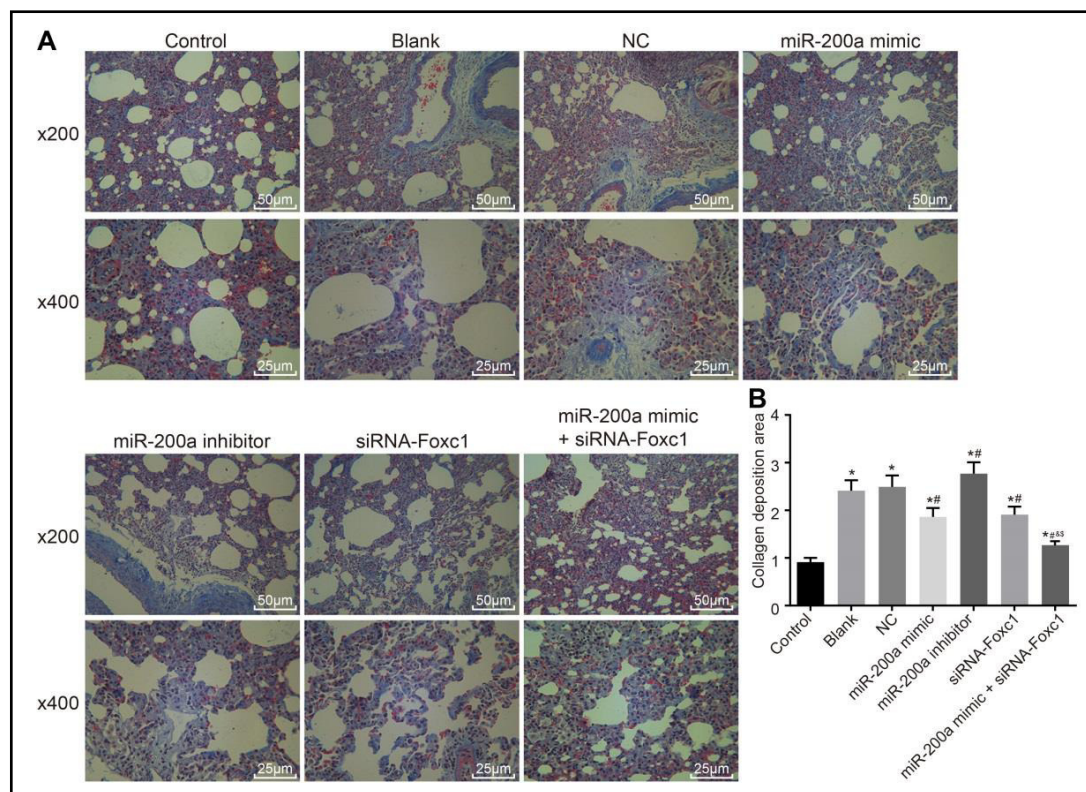


Fig. 7. miR-200a and silencing FOXC1 prevent collagen deposition in lung tissues from mice with asthma as detected by Masson's staining (400 $\times$). Note: A, lung tissues from mice analyzed by Masson's staining; B, comparison of the collagen deposition area; the first line shows the original magnification of 200 $\times$, and the second line shows the original magnification of 400 $\times$; *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; $n = 10$; NC, negative control; miR-200a, microRNA-200a.

High levels of miR-200a and silencing FOXC1 regulate the expression of FOXC1 and TGF- β 1 and bronchus-related factors

The immunohistochemistry results are shown in Fig. 8. Compared with the control group, the other groups had an elevated positive expression rate of FOXC1 and TGF- β 1 ($p < 0.05$). Compared with the blank group, the positive expression rate of FOXC1 and TGF- β 1 was decreased in the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups ($p < 0.05$), increased in the miR-200a inhibitor group ($p < 0.05$) and unchanged in the NC group ($p > 0.05$). Compared with the miR-200a mimic and siRNA-FOXC1 groups, the positive expression rate of FOXC1 and TGF- β 1 in the miR-200a mimic + siRNA-FOXC1 group was obviously decreased ($p < 0.05$).

Moreover, the values of Pbm, Wat, WAm, and N in the mice in each group are shown in Table 4. Compared with the NC group, the values of Pbm, Wat, WAm, and N were increased in the other groups ($p < 0.05$). Compared with the blank group, the values of Pbm, Wat, WAm, and N in the NC group showed no statistical significance ($p > 0.05$); the values of Pbm, Wat, WAm, and N in the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups were decreased ($p < 0.05$); and the values of Pbm, Wat, WAm, and N in the miR-200a inhibitor group were elevated ($p < 0.05$). Compared with the miR-200a mimic and siRNA-FOXC1 groups, the values of Pbm, Wat, WAm, and N in the miR-200a mimic + siRNA-FOXC1 group were decreased ($p < 0.05$). Altogether, miR-200a decreased the positive expression rate of FOXC1 and TGF- β 1 and the values of Pbm, Wat, WAm and N, thus regulating the expression of FOXC1 and TGF- β 1 and bronchus-related factors by targeting FOXC1.

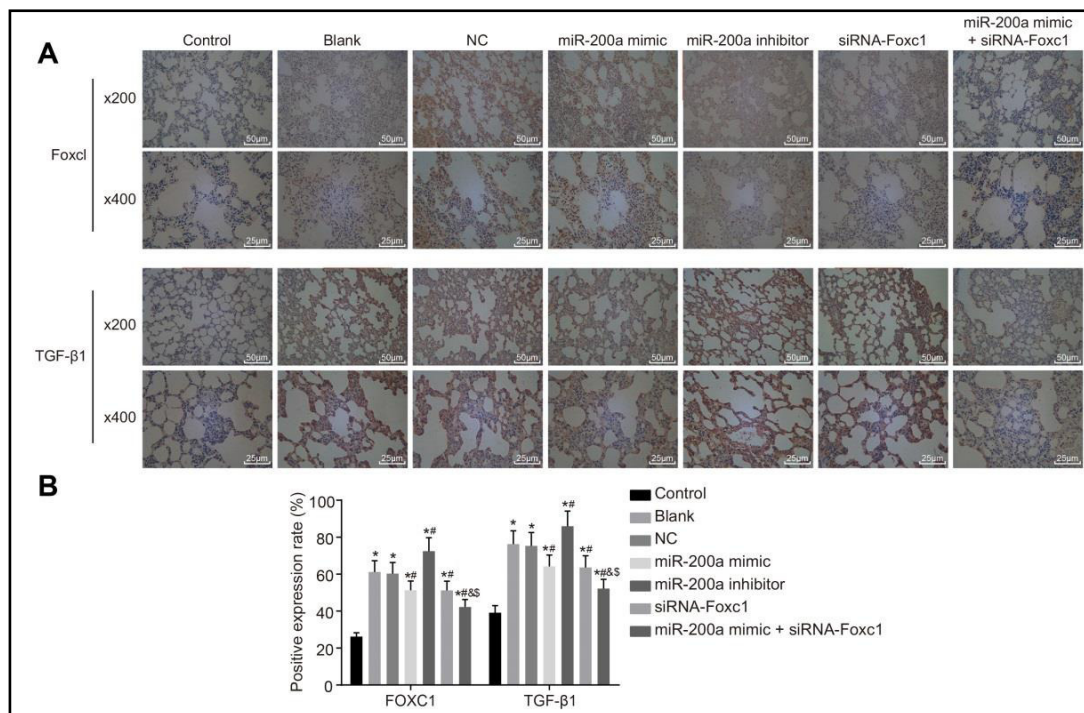


Fig. 8. miR-200a regulates the expression levels of FOXC1 and TGF- β 1 in mice with asthma as detected by immunohistochemistry. Note: A, lung tissues observed by immunohistochemistry (the first line shows the original magnification of 200 $\times$, and the second line shows the original magnification of 400 $\times$); B, comparison of the positive expression rate of FOXC1 and TGF- β 1 in lung tissues; *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; $n = 10$; NC, negative control; miR-200a, microRNA-200a.

Table 4. miR-200a and silencing FOXC1 regulate the activity of bronchus-related factors in mice with asthma. $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; $n = 10$; miR-200a, microRNA-200a; NC, negative control; FOXC1, forkhead box C1; Pbm, basement membrane; WAt, total bronchial wall area; WAm, airway smooth muscle area; ASMC, arterial smooth muscle cell

Groups	Pbm (μ m)	WAt (μ m)	WAm (μ m)	ASMC nucleus (N)
Control	2617.12 $\pm$ 21.01	51.48 $\pm$ 5.09	11.18 $\pm$ 1.09	2.28 $\pm$ 0.21
Blank	2764.25 $\pm$ 22.56*	84.11 $\pm$ 7.81*	29.98 $\pm$ 3.01*	20.22 $\pm$ 1.89*
NC	2779.71 $\pm$ 28.88*	83.91 $\pm$ 8.01*	30.24 $\pm$ 3.02*	21.18 $\pm$ 2.08*
miR-200a mimic	2701.22 $\pm$ 21.24*#	73.28 $\pm$ 7.11*#	23.04 $\pm$ 2.11*#	14.28 $\pm$ 1.19*#
miR-200a inhibitor	2837.35 $\pm$ 23.26*#	96.14 $\pm$ 9.42*#	40.25 $\pm$ 3.99*#	27.26 $\pm$ 2.44*#
siRNA-Foxc1	2704.56 $\pm$ 21.41*#	72.14 $\pm$ 7.16*#	22.98 $\pm$ 2.17*#	14.99 $\pm$ 1.16*#
miR-200a mimic + siRNA-Foxc1	2666.19 $\pm$ 20.17*#&	62.04 $\pm$ 6.21*#&	16.22 $\pm$ 1.42*#&	8.24 $\pm$ 0.71*#&

High levels of miR-200a and silencing FOXC1 inhibit the activity of inflammatory cytokines

The levels of IL-4, IL-6, IL-8, TNF- α and IgE in the mice in each group are shown in Table 5. Compared with the control group, the other groups had increased levels of IL-4, IL-6, IL-8, TNF- α and IgE ($p < 0.05$). Compared with the blank group, the NC group showed no significant difference in the levels of IL-4, IL-6, IL-8, TNF- α and IgE ($p > 0.05$); the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups had decreased levels of IL-4, IL-6, IL-8, TNF- α and IgE ($p < 0.05$); and the miR-200a inhibitor group had increased levels of IL-4, IL-6, IL-8, TNF- α and IgE ($p < 0.05$). Compared with the miR-200a mimic and siRNA-FOXC1 groups, the miR-200a mimic + siRNA-FOXC1 group had decreased levels of IL-4, IL-6, IL-8, TNF- α and IgE ($p < 0.05$). These results illustrated that miR-200a suppressed the levels of IL-4, IL-6, IL-8, TNF- α and IgE by targeting FOXC1.

Table 5. miR-200a and silencing FOXC1 inactivate the activity of inflammatory cytokines in mice with asthma as detected by an ELISA. Note: *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; n = 10; miR-200a, microRNA-200a; ELISA, enzyme-linked immunosorbent assay; NC, negative control; IL, Interleukin; TNF- α , tumor necrosis factor α ; IgE, immunoglobulin E

Groups	IL-4 (ng/mL)	IL-6 (pg/mL)	IL-8 (ng/mL)	TNF- α (pg/mL)	IgE (μ g/mL)
Control	0.25 $\pm$ 0.02	95.27 $\pm$ 9.42	46.28 $\pm$ 4.66	111.09 $\pm$ 11.07	0.24 $\pm$ 0.02
Blank	0.51 $\pm$ 0.05*	488.11 $\pm$ 41.02*	71.72 $\pm$ 6.24*	188.26 $\pm$ 15.28*	0.68 $\pm$ 0.06*
NC	0.55 $\pm$ 0.05*	481.28 $\pm$ 41.65*	70.93 $\pm$ 6.64*	189.67 $\pm$ 15.98*	0.71 $\pm$ 0.07*
miR-200a mimic	0.41 $\pm$ 0.04*#	296.16 $\pm$ 27.16*#	63.01 $\pm$ 5.45*#	162.08 $\pm$ 15.74*#	0.55 $\pm$ 0.05*#
miR-200a inhibitor	0.74 $\pm$ 0.07*#	535.1 $\pm$ 51.12*#	81.28 $\pm$ 6.92*#	213.11 $\pm$ 20.07*#	0.89 $\pm$ 0.09*#
siRNA-Foxc1	0.40 $\pm$ 0.04*#	291.11 $\pm$ 25.88*#	62.17 $\pm$ 5.22*#	160.37 $\pm$ 15.26*#	0.58 $\pm$ 0.05*#
miR-200a mimic + siRNA-Foxc1	0.33 $\pm$	142.26 $\pm$	54.18 $\pm$	138.26 $\pm$	0.44 $\pm$
Foxc1	0.03*#&	11.08*#&	5.09*#&	13.02*#&	0.04*#&

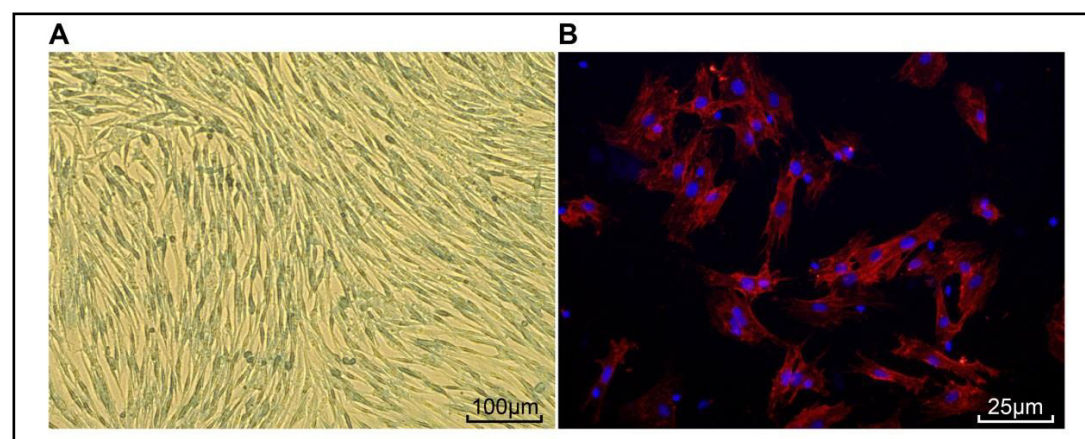


Fig. 9. Immunofluorescence of ASMCs from mice with asthma. Note: A, morphology of ASMCs (100 $\times$); B, ASMCs observed under a fluorescence microscope (400 $\times$); ASMCs, arterial smooth muscle cells.

Cultured cells were identified as ASMCs by immunohistochemistry staining of α -actin

Primary ASMCs were observed by microscopy. Four hours after culture, the cells exhibited a macrospindle, abundant cytoplasm and centered nuclei, and the cells at a higher density exhibited a partially overlapping, characteristic “peak valley” shape (Fig. 9A). Immunohistochemistry staining of α -actin was adopted to detect α -actin in ASMCs by SP immunohistochemistry. The results of the DAB staining were positive if the cytoplasm was brownish yellow or brownish red and the nuclei were blue. Under fine fluorescence microscopy, all nuclei of cells were positively stained in all groups, and the cytoplasm showed strong positive staining, indicating that the cultured cells were ASMCs (Fig. 9B).

miR-200a inactivates the PI3K/AKT signaling pathway by targeting FOXC1 in asthma

miR-200a expression and the mRNA and protein expression of related genes after transfection are shown in Fig. 10. Compared with the control group, the other groups had increased mRNA and protein expression of PI3K, AKT, NF- κ B, cyclin D1 and TGF- β 1 and higher expression of p-AKT ($p < 0.05$). Compared with the blank group, the NC groups showed no significant difference ($p > 0.05$), and the miR-200a mimic and miR-200a mimic + siRNA-FOXC1 groups showed decreased mRNA and protein expression of PI3K, AKT, NF- κ B, cyclin D1 and TGF- β 1 and decreased p-AKT expression ($p < 0.05$). Compared with the blank group, the miR-200a inhibitor group had significantly elevated mRNA and protein expression of PI3K, AKT, NF- κ B, cyclin D1 and TGF- β 1 and elevated expression of p-AKT ($p < 0.05$). Compared with the blank group, the siRNA-FOXC1 group showed decreased mRNA and protein expression of PI3K, AKT, NF- κ B, cyclin D1 and TGF- β 1 and decreased expression of p-AKT ($p < 0.05$). Compared with the miR-200a mimic group, the miR-200a mimic + siRNA-

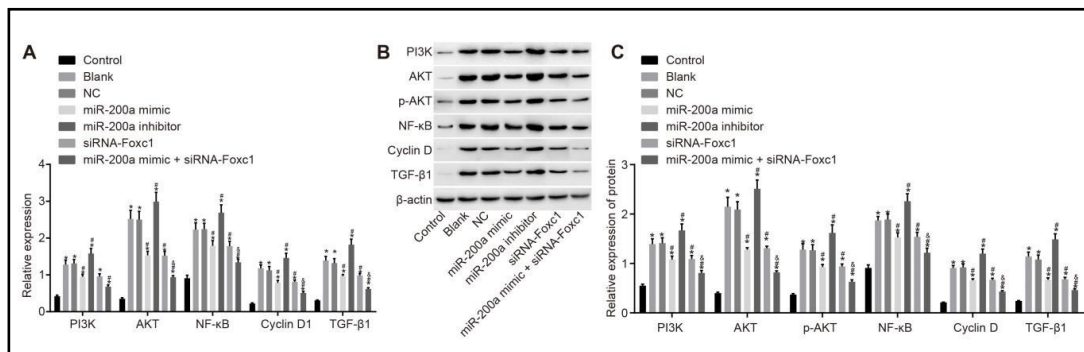


Fig. 10. miR-200a negatively regulates the PI3K/AKT signaling pathway. Note: A, mRNA expression of PI3K, NF-κB, cyclin D1, AKT, p-AKT and TGF-β1; B, protein bands of PI3K, NF-κB, cyclin D1, AKT, p-AKT and TGF-β1; C, protein expression of PI3K, NF-κB, cyclin D1, AKT, p-AKT and TGF-β1; *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; $n = 3$; NC, negative control; miR-200a, microRNA-200a; NF-κB, nuclear factor-kappa B; TGF-β1, transforming growth factor-β1; PI3K, phosphoinositide 3-kinase.

FOXC1 group showed decreased mRNA and protein expression of PI3K, AKT, NF-κB, cyclin D1 and TGF-β1 and decreased expression of p-AKT ($p < 0.05$). These results showed that a high level of miR-200a or the silencing of FOXC1 lowers the mRNA and protein expression of PI3K, AKT, NF-κB, cyclin D1, and TGF-β1, thus regulating the PI3K/AKT signaling pathway by targeting FOXC1 in mice with asthma.

High levels of miR-200a and silencing FOXC1 suppress cell proliferation and facilitate cell apoptosis in asthma

The results of the proliferation in each group after transfection are shown in Fig. 11. Compared with the control group, the other groups showed accelerated cell proliferation ($p < 0.05$). Compared with the blank group, the NC group showed no significant differences in cell proliferation ($p > 0.05$), but the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups showed suppressed cell proliferation, while the miR-200a inhibitor group showed strengthened cell proliferation ($p < 0.05$). Compared with the miR-200a mimic and siRNA-FOXC1 groups, cell proliferation was suppressed in the miR-200a mimic + siRNA-FOXC1 group ($p < 0.05$). In addition, the cell cycle in each group was observed as shown in Fig. 12. Compared with the control group, the number of cells at the G0/G1 phase was decreased in the other groups ($p < 0.05$). Compared with the blank group, the NC group showed no significant differences in the cell cycle ($p > 0.05$), but the miR-200a mimic, siRNA-FOXC1 and miR-200a mimic + siRNA-FOXC1 groups showed an increased number of cells at the G0/G1 phase and a decreased number of cells at the S phase, while the miR-200a inhibitor group showed a decreased number of cells at the G0/G1 phase and an increased number of cells at the S phase ($p < 0.05$). Compared with the miR-200a mimic and siRNA-FOXC1 groups, the number of cells at the G0/G1 phase increased and the number

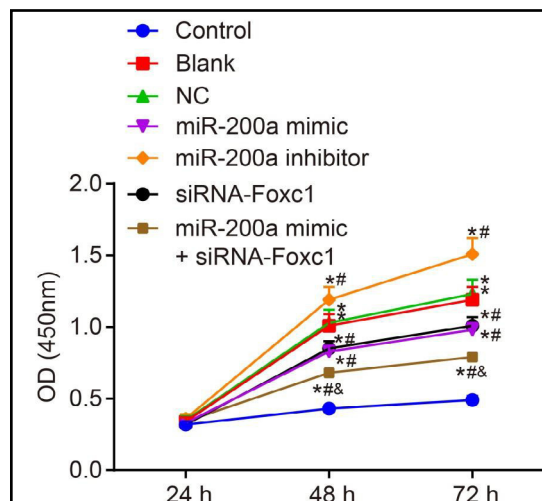


Fig. 11. miR-200a and silencing FOXC1 inhibit cell proliferation in mice with asthma as detected by a cell counting kit-8 assay. Note: *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; $n = 3$; NC, negative control; miR-200a, microRNA-200a.

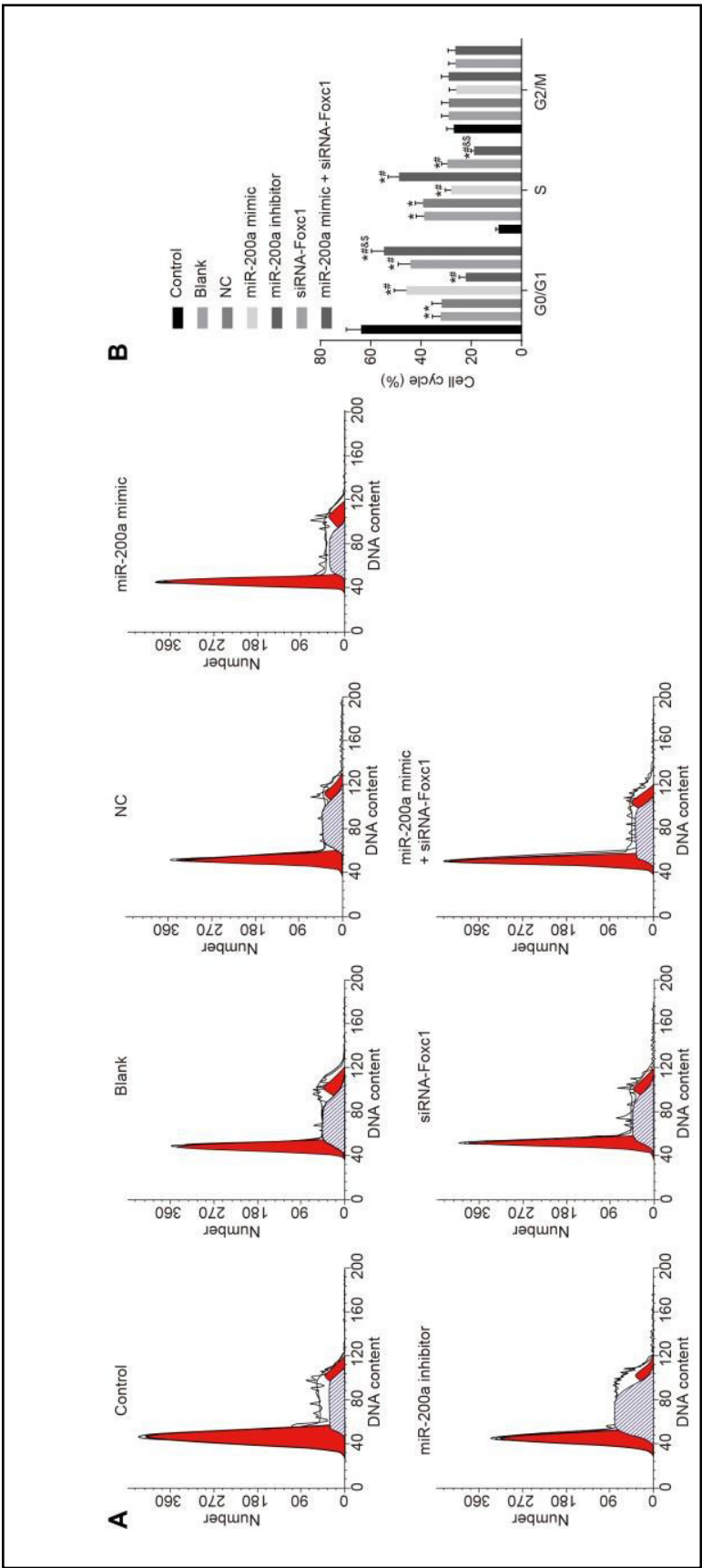


Fig. 12. miR-200a and silencing FOXC1 regulate the cell cycle in mice with asthma as detected by flow cytometry. Note: A, distribution of the cell cycle in each group; B, comparison of the cell cycle in each group; *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; n = 3; NC, negative control; miR-200a, microRNA-200a.

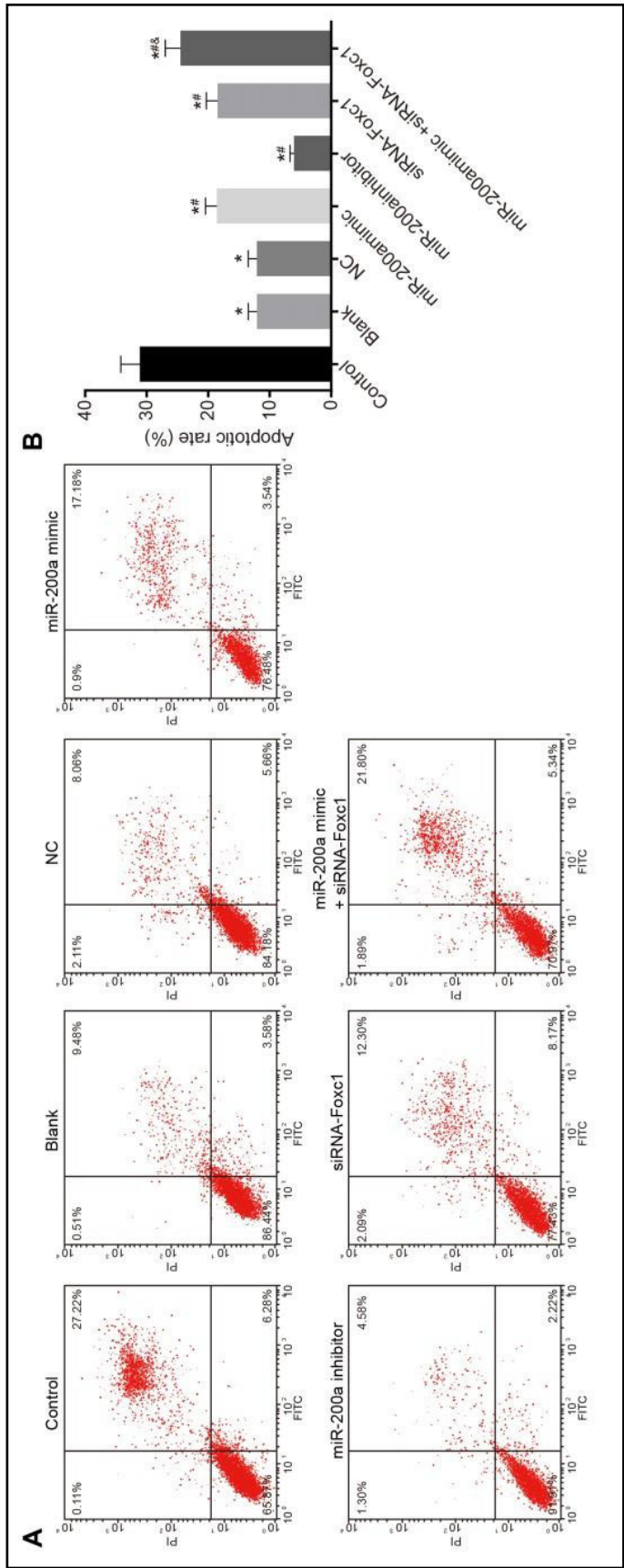


Fig. 13. miR-200a and silencing FOXC1 promote cell apoptosis in mice with asthma as detected by flow cytometry. Note: A, flow chart of cell apoptosis in each group; B, comparison of cell apoptosis in each group; *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the blank group; &, $p < 0.05$ compared with the miR-200a mimic group; $n = 3$; NC, negative control; miR-200a, microRNA-200a.

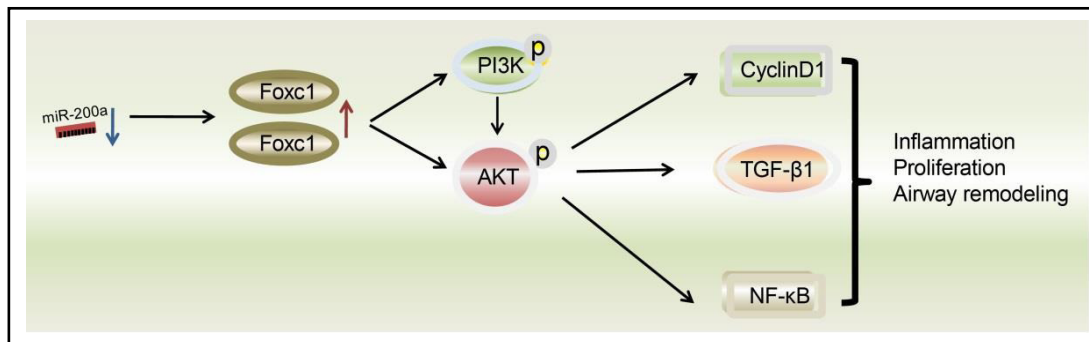


Fig. 14. Mechanical map. Our study suggested that the overexpression of miR-200a may downregulate FOXC1, thereby inhibiting the activation of the PI3K/AKT signaling pathway and ultimately suppressing ASM proliferation and airway remodeling in asthmatic mice.

of cells at the S phase decreased in the miR-200a mimic + siRNA-FOXC1 group ($p < 0.05$). Moreover, cell apoptosis in each group is shown in Fig. 13. Compared with the control group, the other groups had a decreased apoptosis rate ($p < 0.05$). Compared with the blank group, the NC and miR-200a inhibitor + siRNA-FOXC1 groups had no significant difference in the apoptosis rate ($p > 0.05$), but the miR-200a mimic and siRNA-FOXC1 groups had an elevated apoptosis rate, while the miR-200a inhibitor group had a decreased apoptosis rate ($p < 0.05$). Altogether, high levels of miR-200a or the silencing of FOXC1 could inhibit cell proliferation and promote cell apoptosis, thus regulating the cell cycle in asthma.

Discussion

Due to smoking, pollution, widely used antibiotics and infectious stimuli produced by unsanitary practices, allergy-related diseases, such as allergic asthma, have proliferated, especially among children [44]. miRs have been identified to be involved in bronchial epithelial changes associated with allergic asthma and suggested as a new strategy for the treatment of asthma [45]. In this study, we explored whether and how miR-200a affects OVA-induced allergic mice by targeting FOXC1 via the PI3K/AKT signaling pathway.

First, compared with the control group, the other groups had increased positive protein expression rates of FOXC1 and TGF- β 1 in lung tissues. FOXC1, which is a novel hypoxia-induced transcription factor, is upregulated in hypoxic areas of lung cancer tissues and plays a key role in the tumor microenvironment and promotes lung cancer progression [17]. TGF- β 1 is a key mediator of fibrosis and induces the differentiation of fibroblasts into myofibroblasts, which are the effector cells in asthma whose expression is increased in asthma [46]. Compared with the blank group, the positive expression rates of FOXC1 and TGF- β 1 in lung tissues were decreased in the miR-200a mimic groups. Furthermore, miR-200a specifically bound FOXC1. miR-200a have been reported to play an inhibitory role in fibrosis by suppressing the TGF- β 1-mediated endothelial-mesenchymal transition [47]. The TGF β -induced epithelial-to-mesenchymal transition in tongue squamous cell carcinoma could be inhibited by miR-639 via the suppression of the expression of FOXC1, and miR-200b has also been reported to inhibit the TGF β -induced epithelial-to-mesenchymal transition [48]. Based on the above information, we may conclude that miR-200a negatively targets FOXC1.

Second, compared with the blank group, the miR-200a mimic group showed a lower mRNA and protein expression of FOXC1, PI3K, AKT, NF- κ B, cyclin D1 and TGF- β 1. FOXC1 has been shown to activate the PI3K/AKT signaling pathway [49]. The PI3K enzyme acts on membrane PI and activates Akt to phosphorylate several downstream proteins, such as NF- κ B and GSK3- β [50]. Through the PI3K/AKT signaling pathway, FOXC1 is able to promote the epithelial-to-mesenchymal transition in cervical carcinoma [49]. Cyclin D1 is an important

initiator and activator of the cell cycle, and its overexpression has been found in various human cancers, including breast cancer [51]. The inactivation of the PI3K/AKT signaling pathway has been reported to induce the expression of p16INK4a to suppress the expression of cyclin D1 [52]. Compared with the blank group, the miR-200a mimic group showed a lower proliferation rate and a higher apoptosis rate. NF- κ B, which is a molecule downstream of the PI3K/AKT signaling pathway, has been reported by Li *et al.* to have a proliferative effect on endothelial progenitor cells [53]. Cyclin D1 participates in the G1 phase progression of the cell cycle in proliferating cells via the activation of cyclin dependent kinase 2 (CDK2), CDK4, or CDK6 [54]. Through the downregulation of IKBKB, miR-200b is also able to inhibit the activation of NF- κ B, which suppresses breast cancer cell growth and migration [55]. In addition, the inactivation of NF- κ B through the pentaerythritol tetranitrate-Akt-IKK β axis results in lower cyclin D1 expression, suppressing renal cancer cell proliferation [56]. Therefore, miR-200a could inhibit the expression of FOXC1 and inactivate the PI3K/AKT signaling pathway and its downstream genes NF- κ B and cyclin D1 to inhibit cell proliferation.

Third, compared with the blank group, the miR-200a mimic group had decreased levels of IL-4, IL-6, IL-8, TNF- α and IgE. In addition, miR-200a could decrease airway reactivity, EOS accumulation and the collagen deposition area. Lung inflammation in asthma results from the activation of innate immune cells stimulated by an allergen, followed by aggravated inflammation caused by Th2 (including IL-4 and IL-6) and the synthesis of IgE antibody, which induces the secretion of inflammatory mediators [57]. IL-8 is a proinflammatory cytokine that has been reported to be a potent chemoattractant of neutrophils during airway inflammation [58]. TNF- α has been shown to induce airway hyperreactivity, while anti-TNF- α antibodies are able to suppress allergen-induced airway hyperreactivity in mice [59]. EOSs may be activated and recruited by Th2 cytokines (IL-4), inducing airway hyperreactivity [60, 61]. miRs negatively target PI3K/AKT signaling, limiting IL-4-mediated murine macrophage proliferation [62]. IL-6 has been reported to promote the airway remodeling process, which could be enhanced by the PI3K/AKT signaling pathway [63]. Additionally, the activation of AKT has been demonstrated to upregulate the expression of IL-6 protein, while LY294002, which is an AKT inhibitor, and AKT siRNA suppressed SIRT1-regulated IL-6 expression in an asthmatic mouse model and 16HBE cells [64]. In addition, IL-8 expression has been shown to be regulated by the PI3K/AKT signaling pathway [65]. The PI3K/AKT signaling pathway also plays a key role in the IgE-mediated maturation and activation of mast cells [66, 67]. The suppression of the PI3K/AKT signaling pathway has been reported to lead to the inhibition of TNF- α -induced inflammation [68]. The PI3K/AKT signaling pathway has been reported to be indirectly suppressed by miR-200c and miR-200b [69, 70]; thus, we may assume that miR-200a decreases the levels of IL-4, IL-6, IL-8, TNF- α and IgE by inhibiting the PI3K/AKT signaling pathway to inhibit lung inflammation.

Conclusion

In summary, miR-200a could inhibit ASMC proliferation and airway remodeling in a mouse model of allergic asthma by downregulating the expression of FOXC1, which regulates the PI3K/AKT signaling pathway (Fig. 14). The causes of asthma are various; however, we studied only OVA as a model of allergen-induced asthma, and other types of allergen-induced asthma should be further investigated.

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Disclosure Statement

The authors declare no potential conflicts of interest.

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