

MiR-204-5p Inhibits Transforming Growth Factor- β 1-Induced Proliferation and Extracellular Matrix Production of Airway Smooth Muscle Cells by Regulating Six1 in Asthma

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Keywords

Asthma · Airway remodeling · Proliferation · MiR-204-5p · Six1

Abstract

Background: Transforming growth factor- β 1 (TGF- β 1)-induced proliferation of airway smooth muscle cells plays critical roles in the development of airway remodeling. Six1 (sine oculis homeobox homolog 1) has been demonstrated to be involved in airway inflammation and remodeling in asthmatic mice. **Objectives:** The aim of this work was to investigate the potential role of miR-204-5p in the proliferation and extracellular matrix (ECM) production of airway smooth muscle cells in asthma. **Methods:** Real-time PCR was used to measure the expression of miR-204-5p in asthmatic airway smooth muscle cells. Cell viability and apoptosis were detected to evaluate the effect of miR-204-5p on airway smooth muscle cells. Dual-luciferase reporter experiments were applied to identify the target genes of miR-204-5p. **Results:** MiR-204-5p was downregulated notably in asthmatic airway smooth muscle cells as well as cells stimulated with TGF- β 1. Overexpression of miR-204-5p markedly suppressed the TGF- β 1-induced proliferation of airway smooth muscle

cells and the deposition of ECM, whereas the inhibition of miR-204-5p significantly enhanced the proliferation of airway smooth muscle cells and upregulated the level of fibronectin and collagen III. Furthermore, subsequent analyses demonstrated that Six1 was a direct target of miR-204-5p, and Western blot further indicated that miR-204-5p negatively regulated the expression of Six1. Most importantly, the restoration of Six1 expression reversed the inhibitory effect of miR-204-5p on TGF- β 1-induced proliferation and ECM production. **Conclusions:** MiR-204-5p inhibits TGF- β 1-induced proliferation and ECM production of airway smooth muscle cells by regulating Six1, identifying a potential therapeutic target for preventing airway remodeling in asthma.

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Introduction

Asthma is a common chronic respiratory disease worldwide, which is characterized by airway hyper-responsiveness, airway inflammation, and airway remodeling [1, 2]. Airway remodeling is a multifaceted process

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that encompasses several structural changes in the airway wall, including basement membrane thickening, extracellular matrix (ECM) deposition, and airway smooth muscle cell hypertrophy and hyperplasia [3, 4]. Abnormal airway smooth muscle cell growth may contribute to airway remodeling, leading to increased airway narrowing and obstruction in asthmatic patients [5]. Transforming growth factor- β 1 (TGF- β 1), a key mediator of fibrotic responses, is increased in asthma and drives airway remodeling by inducing the expression of ECM proteins. Furthermore, TGF- β 1 secretion is related to increased ECM protein production, leading to enhanced proliferation [6]. Therefore, the inhibition of airway smooth muscle cell proliferation will be beneficial for airway remodeling.

MicroRNAs (miRNAs) are a kind of abundant, endogenous, small non-coding RNA molecule. They can regulate gene expression through binding to complementary sequences in the 3'-untranslated regions (3'-UTR) of specific target mRNAs, which leads to mRNA degradation or translational termination [7, 8]. miRNAs have been reported to have potential therapeutic effects in airway smooth muscle cells on underlying processes in asthma, and our previous studies found that miRNA-448-5p inhibits pulmonary fibrosis in asthmatic mice [9–11]. MiR-204-5p has been reported to be involved in tumor progression by regulating numerous oncogenic activities, including proliferation, metastasis, chemotherapy resistance, and autophagy [12–14]. Chen et al. [15] reported that the miR-204 downregulation could be a vital facilitator for tubular-cell ECM production induced by TGF- β 1 in renal fibrosis in a mouse model. A recent study found that miR-204-5p inhibits the differentiation of myoblast by regulating estrogen-related receptor- γ (ERR γ) and myocyte enhancer factor 2C (MEF2C) in airway inflammation [16]. However, the biological role of miR-204-5p in airway remodeling is still unclear.

Six1 (sine oculis homeobox homolog 1) is encoded by the Six1 gene, which has been identified as a TGF- β 1-inducible gene and plays important roles in the development of organs [17–19]. Six1 was shown to be overexpressed in various types of cancer and is known to be involved in tumor proliferation by regulating c-myc, cyclin-D1, and cyclin-A1 [20, 21]. Our previous research has revealed that the downregulation of Six1 effectively inhibited the airway inflammation and remodeling in asthma [22]. MiR-204-5p has been reported to suppress tumor proliferation by regulating Six1 in breast cancer and hepatocellular cancer [12, 13]. However, whether miR-204-5p and Six1 exert the same function in asthma has not been demonstrated. In this current study, we il-

lustrated the potential functions of miR-204-5p in airway smooth muscle cell proliferation and the relationship between miR-204-5p and Six1. Our data provided the first evidence that miR-204-5p regulated airway smooth muscle cell proliferation through the miR-204-5p/Six1 pathway, suggesting that miR-204-5p and Six1 could be potential therapeutic targets for preventing airway remodeling in asthma.

Materials and Methods

Human Specimens Collection

Airway smooth muscle cells from primary asthmatic human subjects ($n = 9$) were obtained from patients who underwent bronchial biopsy at the Affiliated Hospital of Qingdao University (China). Airway smooth muscle cells from non-asthmatic human subjects ($n = 9$) were obtained from the main or lobar bronchus of non-asthmatic patients who underwent lung resection surgery. Absence of tumor infiltration in the tissue was defined by pathological diagnosis. Subjects with asthma were recruited based on the criteria of the Chinese guidelines for the prevention and management of bronchial asthma (Primary Health Care Version). The procedure of primary airway smooth muscle cells extraction was as described previously [23]. All the patients who enrolled in the investigation provided written informed consent. This study was approved by the Human Ethics Committee of the Affiliated Hospital of Qingdao University.

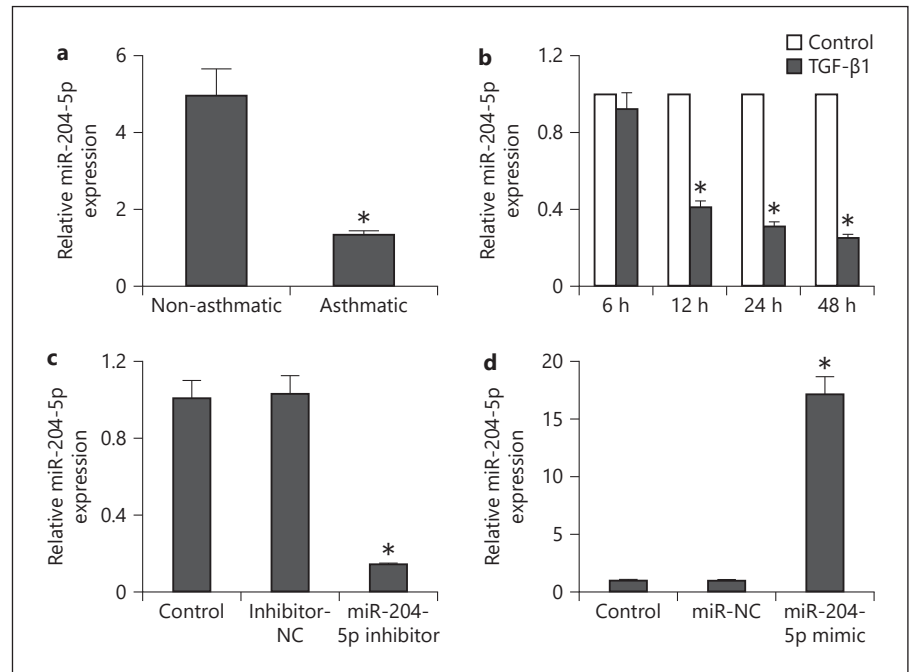
Cell Culture and Treatment

The human airway smooth muscle cell line was obtained from the Cancer Research Institute of Beijing, China. The cells were cultivated in T75 tissue culture flasks in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μ g/mL streptomycin, 2 mM L-glutamine, and 20 mM hydroxyethyl piperazine ethanesulfonic acid and in a humidified incubator containing 5% CO₂ at 37 °C. The miR-204-5p mimic, mimic negative control, inhibitor, and inhibitor negative control were purchased from RiboBio (Guangzhou, China). Cells were transfected with miR-204-5p mimic (100 μ M) or inhibitor (100 μ M) using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocols. The full-length Six1 cDNA was purchased from GeneCopeia Inc. (Maryland, MD, USA), and was subcloned into the eukaryotic expression vector pcDNA3.1 (+). The vector pcDNA3.1 (+) was used as a negative control. After infection with lentiviruses for 24 h, the cells were treated with TGF- β 1 (10 ng/mL; R&D Systems, Minneapolis, MN, USA) and then harvested.

Western Blot

In brief, 50 μ g of the protein was separated by (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA, USA). Subsequently, the membrane was blocked in 1% BSA/0.05% Tween/PBS solution overnight at 4 °C, followed by incubation with the primary antibody, i.e., rabbit monoclonal antibodies to either human fibronectin (ab32419), collagen III (ab184993), Bax (ab32503), Bcl-2 (ab32124), phosphorylated-Smad 3 (ab52903), or Smad 3

Fig. 1. MiR-204-5p was decreased in asthmatic airway smooth muscle cells and TGF- β 1-stimulated airway smooth muscle cells. The level of miR-204-5p in asthmatic airway smooth muscle cells (**a**) and TGF- β 1-stimulated airway smooth muscle cells (**b**) was analyzed by real-time PCR and normalized to U6. After transfection with miR-204-5p inhibitor (**c**) or mimic (**d**), the expression of miR-204-5p in airway smooth muscle cells was measured by real-time PCR and normalized to U6. * $p < 0.05$ versus TGF- β 1-stimulated, miR-NC-transfected airway smooth muscle cells.



(ab40854; Abcam, Cambridge, MA, USA) for 15 h. Horseradish peroxidase-conjugated secondary anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) was adopted to develop signals with an ECL system (GE Healthcare Life Sciences, Little Chalfont, UK).

RNA Isolation and Real-Time PCR Analysis

miRNA was isolated using a Qiagen miRNeasy kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's protocol. Then, reverse transcription of RNA to cDNA was performed (TaKaRa, Kumanoto, Japan). The level of the miRNA and internal control (U6) were measured using quantitative real-time PCR (Bio-Rad, Hercules, CA, USA). The primer sequence of miR-204-5p was as follows: 5'-TTCCCTTTG TCATCCTATGCCT-3'. The primer sequence of U6 was as follows: 5'-CTCGCT TCGGCAG-CACA-3'. U6 and miRNA common primers were also purchased from TaKaRa. The $2^{-\Delta\Delta Ct}$ method was utilized to evaluate relative expression levels.

Cell Proliferation Assay

Cell Counting Kit-8 assay (CCK-8, Dojindo, Kumamoto, Japan) was used to evaluate the proliferation effect of miR-204-5p on airway smooth muscle cells. The cells were plated into 96-well plates in triplicate at a density of 2×10^4 cells/well and then cultured for approximately 12 h at 37°C. Next, 10 μ L of CCK-8 was added, and the plates were incubated in the dark at 37°C for 2 h. The proportion of viable cells was evaluated by measuring the optical density at 490 nm.

Flow Cytometry

Airway smooth muscle cells were resuspended in PBS at a concentration of 2×10^6 /mL and fixed in 2 mL of methanol for 20 min at 4°C. After the fixation of the cells, the mixture was incubated in 1 mL of propidium iodide solution for 20 min at 37°C. Finally,

airway smooth muscle cells were resuspended in 1 mL of PBS and analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA).

Acridine Orange/Ethidium Bromide Staining

Acridine orange/ethidium bromide (AO/EB) staining identifies alive, early apoptotic, and late apoptotic cells. Briefly, airway smooth muscle cells were washed with PBS and immediately treated with AO (150 μ g/mL) for 10 min and EB (150 μ g/mL) for 10 min at room temperature. The cells were then examined under a fluorescence microscope (Leica, Germany).

Dual-Luciferase Reporter Assay

The target gene 3'-UTR was cloned into multiple cloning sites downstream of the pGL6-miR (Beyotime, Wuhan, China) luciferase gene expression vector. The Six1 3'-UTR-WT reporter plasmid was constructed containing the miR-204-5p binding site, and the Six1 3'-UTR-Mut reporter plasmid was constructed containing the mutation site to which miR-204-5p cannot bind. The HEK-293T cells were seeded into 24-well plates per well. In 20 h, following with miRNA mimics (500 ng), pGL6-miR Six1 3'-UTR WT/MT (500 ng), pRL-SV40 (10 ng) were co-transfected into each well by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the cell lysis was collected, and luciferase and Renilla activity was measured by the Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was performed with SPSS 17.0 software (SPSS, San Rafael, CA, USA). All the data in this study are presented as the mean $\pm$ SD from least three independent experiments. Statistical comparisons of the data from the various groups were performed using the Student *t* test. *p* values < 0.05 were considered statistically significant.

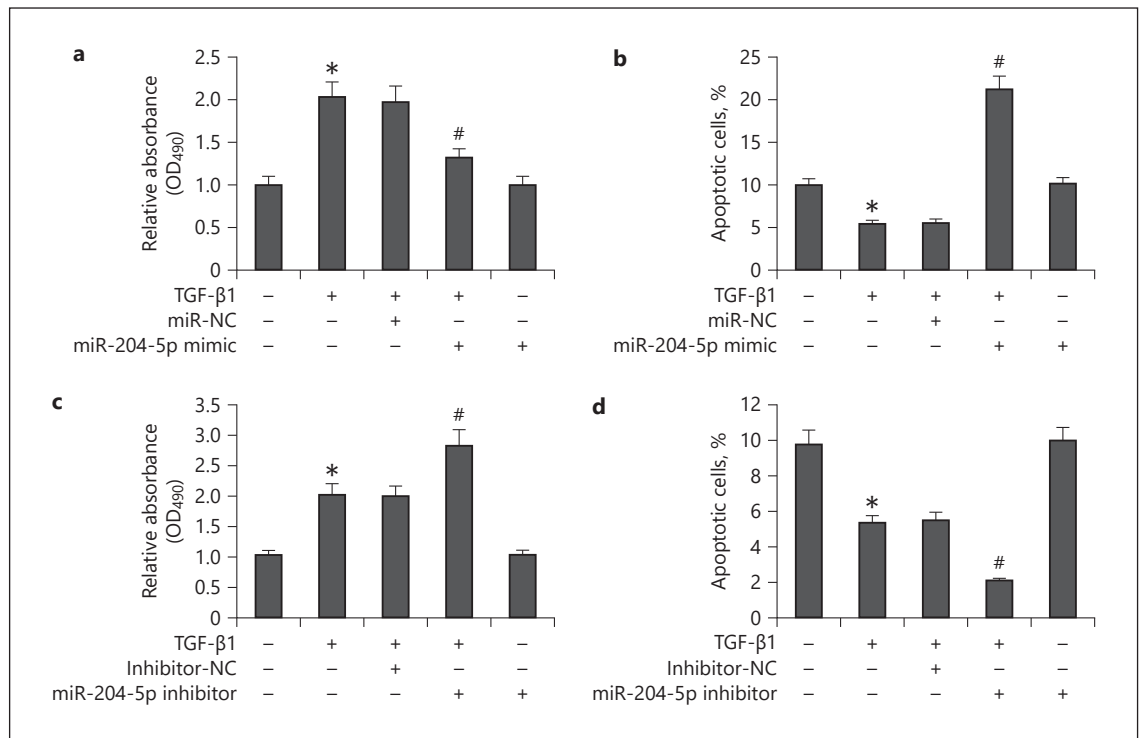


Fig. 2. MiR-204-5p inhibited the proliferation and promoted apoptosis of airway smooth muscle cells. Airway smooth muscle cells stimulated with TGF-β1 (10 ng/mL) after transfection with miR-204-5p mimic or inhibitor. **a, c** The proliferation of airway smooth muscle cells was determined by CCK-8 assay. **b, d** The apoptosis of airway smooth muscle cells was detected by flow cytometry. * $p < 0.05$ versus negative controls; # $p < 0.05$ versus miR-NC, inhibitor-NC, or vehicle + TGF-β1.

Results

MiR-204-5p Was Decreased in Asthmatic Airway Smooth Muscle Cells

We first analyzed the level of miR-204-5p in airway smooth muscle cells from asthmatic and non-asthmatic humans by real-time PCR. As shown in Figure 1a, miR-204-5p expression was lower in airway smooth muscle cells from asthmatic humans than from non-asthmatic humans ($p < 0.05$). We also found that TGF-β1 significantly downregulated the level of miR-204-5p in airway smooth muscle cells (Fig. 1b). After transfection for 48 h, the level of miR-204-5p was significantly increased in the miR-204-5p mimic group ($p < 0.05$) and decreased in the miR-204-5p inhibitor group ($p < 0.05$; Fig. 1c, d).

MiR-204-5p Suppressed the Proliferation of Airway Smooth Muscle Cells and Promoted Cell Apoptosis

We analyzed the role of miR-204-5p on cell proliferation and apoptosis by using flow cytometry and AO/EB

staining. As shown in Figures 2 and 3a, miR-204-5p significantly suppressed the proliferation of airway smooth muscle cells, whereas the miR-204-5p inhibitor suppressed cell apoptosis in airway smooth muscle cells ($p < 0.05$). Bax, the proapoptotic regulator, was markedly increased in the cells overexpressed with miR-204-5p in comparison with TGF-β1-stimulated cells, while the antiapoptotic regulator Bcl-2 decreased in airway smooth muscle cells ($p < 0.05$; Fig. 3b).

MiR-204-5p Inhibited ECM Production in Airway Smooth Muscle Cells

Airway smooth muscle cells play an important role in airway remodeling by synthesizing ECM proteins that could affect airway remodeling. We next sought to determine the functional role of miR-204-5p in TGF-β1-induced expression of fibronectin and collagen III. The data showed that stimulation with TGF-β1 induced fibronectin and collagen III production in airway smooth muscle cells ($p < 0.05$). The results also demonstrated that overexpression of

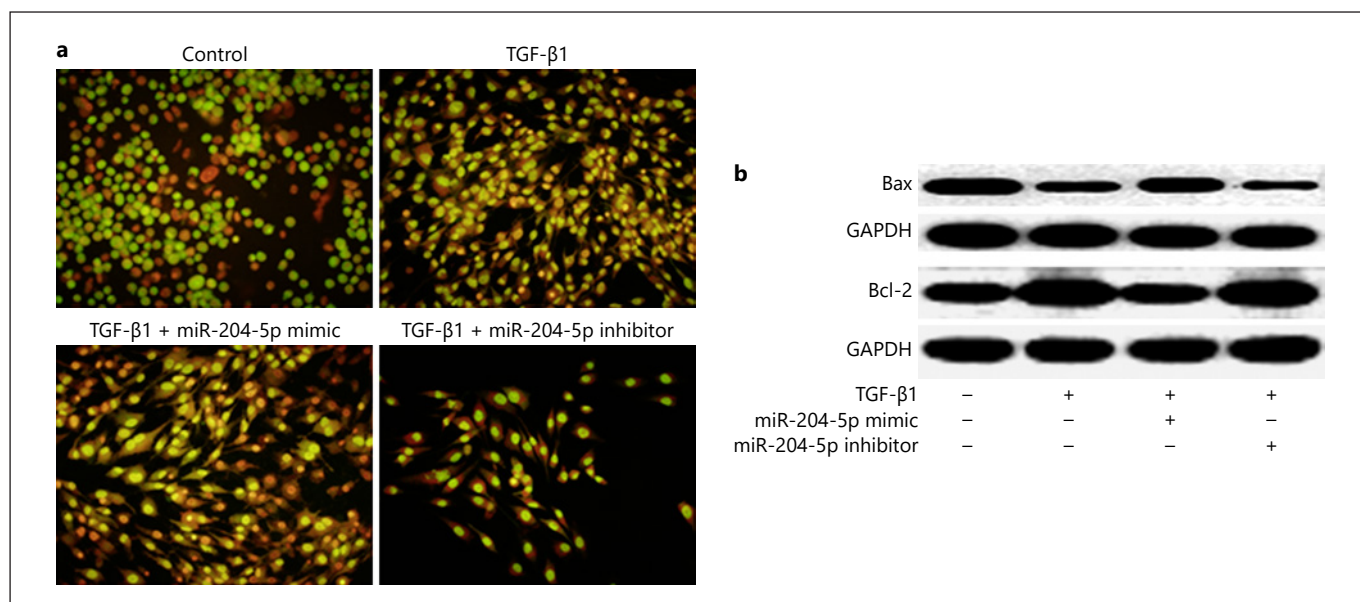


Fig. 3. In situ detection of apoptosis. Airway smooth muscle cells stimulated with TGF- β 1 (10 ng/mL) after being transfected with miR-204-5p mimic or inhibitor for 24 h. **a** The apoptosis of airway

smooth muscle cells was observed by AO/EB staining. Original magnification $\times 100$. **b** The level of Bax and Bcl-2 expression in airway smooth muscle cells was measured by Western blot.

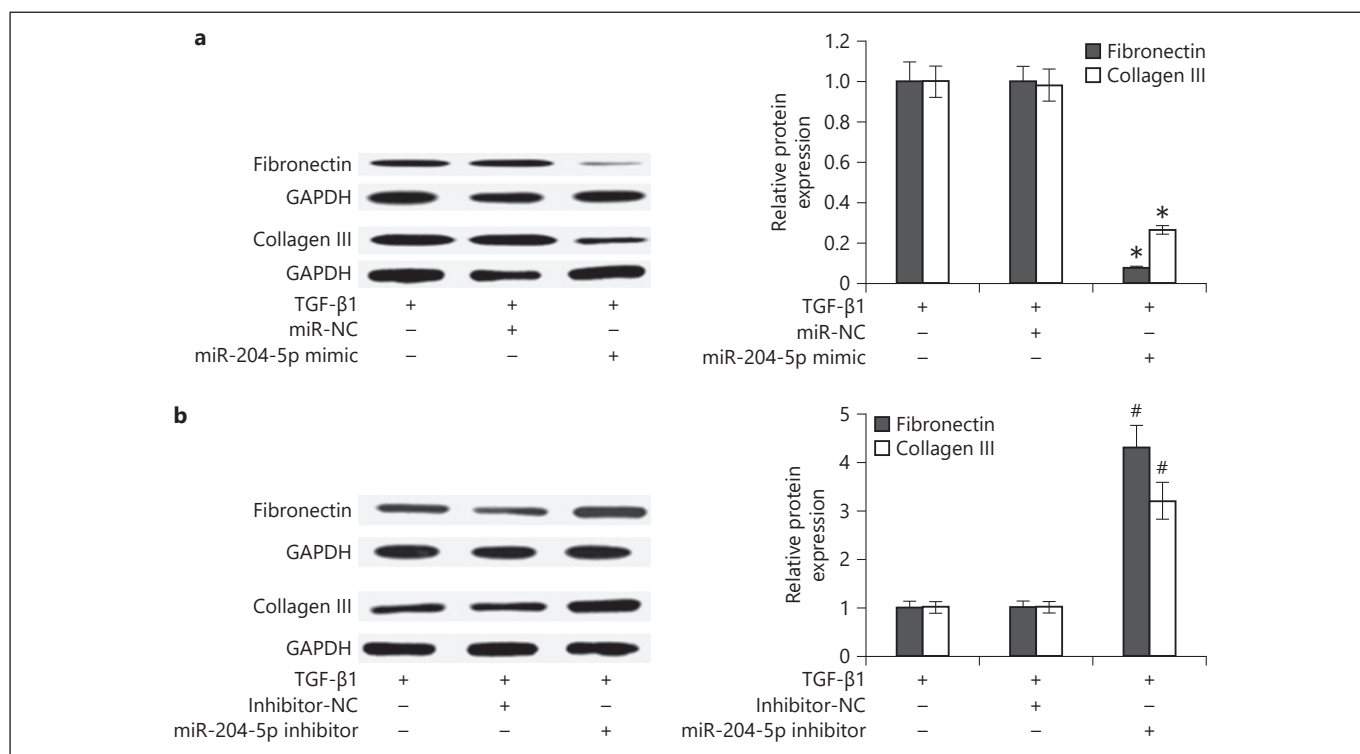


Fig. 4. MiR-204-5p suppressed ECM deposition in airway smooth muscle cells. Airway smooth muscle cells were treated with TGF- β 1 (10 ng/mL) for 24 h after transfection with miR-204-5p mimic (**a**) or inhibitor (**b**), and the level of fibronectin and collagen III in airway smooth muscle cells was measured by Western blot. The data

represent the mean $\pm$ SD. Experiments were repeated in triplicate. * $p < 0.05$ versus TGF- β 1, TGF- β 1 + miR-NC-transfected airway smooth muscle cells; # $p < 0.05$ versus TGF- β 1, TGF- β 1 + inhibitor-NC-transfected airway smooth muscle cells.

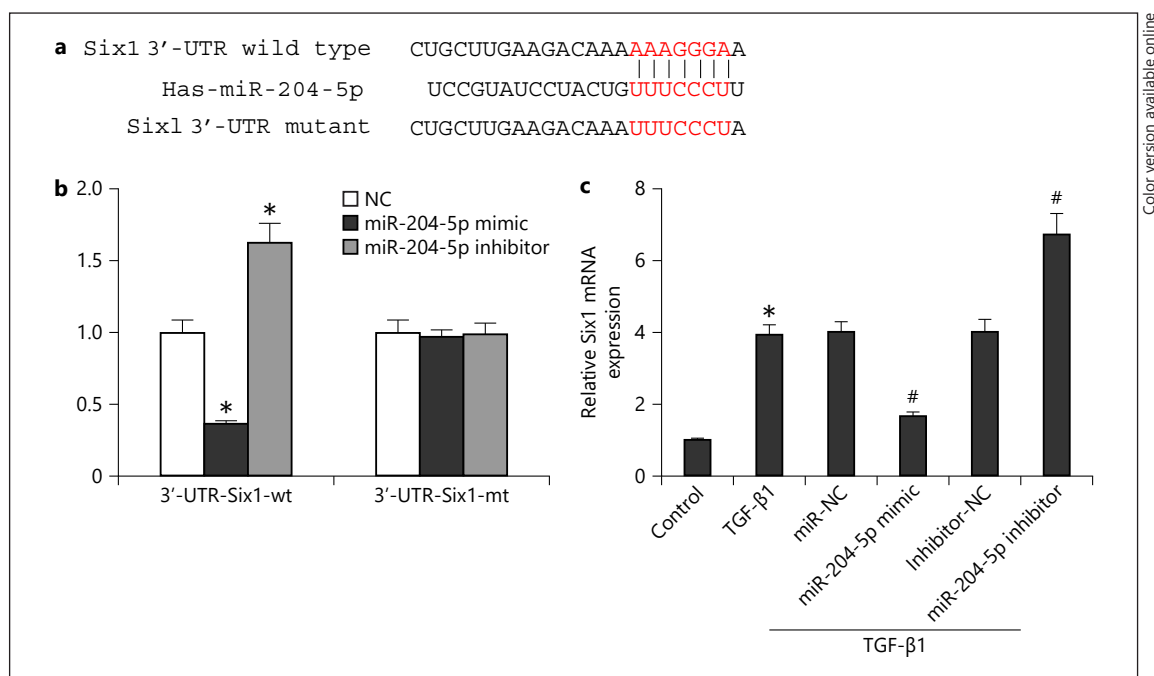


Fig. 5. Six1 was a target gene of miR-204-5p. **a** Schematic of the predicted miR-204-5p target sequences binding site and the mutant site in the Six1 3'-UTR. **b** Dual-luciferase reporter assay was performed using airway smooth muscle cells. Cells were co-transfected with miR-204-5p mimics or an miR-204-5p inhibitor along with a reporter vector containing either a wild-type or mutant Six1

3'-UTR. **c** Airway smooth muscle cells were transfected with miR-204-5p mimic or an miR-204-5p inhibitor for 24 h, then treated with TGF-β1 (10 ng/mL) for 24 h. The level of Six1 was detected by real-time PCR. * $p < 0.05$ versus control cells; # $p < 0.05$ versus TGF-β1-stimulated, scramble control-transfected airway smooth muscle cells.

miR-204-5p significantly decreased the expression of fibronectin and collagen III, whereas the inhibition of miR-204-5p apparently promoted the expression of ECM in airway smooth muscle cells ($p < 0.05$; Fig. 4).

Six1 Is a Direct Target of MiR-204-5p

TargetScan 6.2 (<http://www.targetscan.org/index.html>) identified Six1 as a potential target gene of miR-204-5p. The Six1 3'-UTR contains a conserved binding site for miR-204-5p (Fig. 5a). To verify whether miR-204-5p directly binds to the 3'-UTR of Six1, we performed a dual-luciferase reporter assay. The results showed that miR-204-5p overexpression significantly inhibited the luciferase activity of the reporter vector containing the WT Six1 3'-UTR. In contrast, miR-204-5p inhibition markedly promoted the luciferase activity of the reporter vector containing the WT Six1 3'-UTR. However, these effects were significantly reduced for the reporter vector containing the MT Six1 3'-UTR (Fig. 5b), indicating that miR-204-5p directly targets the 3'-UTR of Six1. To investigate whether miR-204-5p directly targets the Six1 gene, we transfected TGF-β1-stimulated airway smooth mus-

cle cells with a miR-204-5p mimic. As shown in Figure 5c, transfection of the miR-204-5p mimic remarkably reduced Six1 mRNA expression, while transfection of the miR-204-5p inhibitor increased Six1 expression ($p < 0.05$). Taken together, these results suggest that Six1 is a direct target gene of miR-204-5p.

Overexpression of Six1 Partially Reversed the Effects of miR-204-5p on TGF-β1-Stimulated Cell Proliferation and ECM Production

The level of Six1 was significantly increased after transfection of airway smooth muscle cells with the pcDNA-Six1 and miR-204-5p mimic compared with cells transfected with pcDNA3.1 vector and miR-204-5p mimic (Fig. 6). Overexpression of Six1 markedly reversed the effect of the upregulation of miR-204-5p on the proliferation of airway smooth muscle cells. Flow cytometry showed that Six1 overexpression ameliorated apoptosis induced by the miR-204-5p. In addition, restoring Six1 reversed the inhibitory effect of miR-204-5p on the TGF-β1-stimulated ECM deposition in airway smooth muscle cells ($p < 0.05$; Fig. 7).

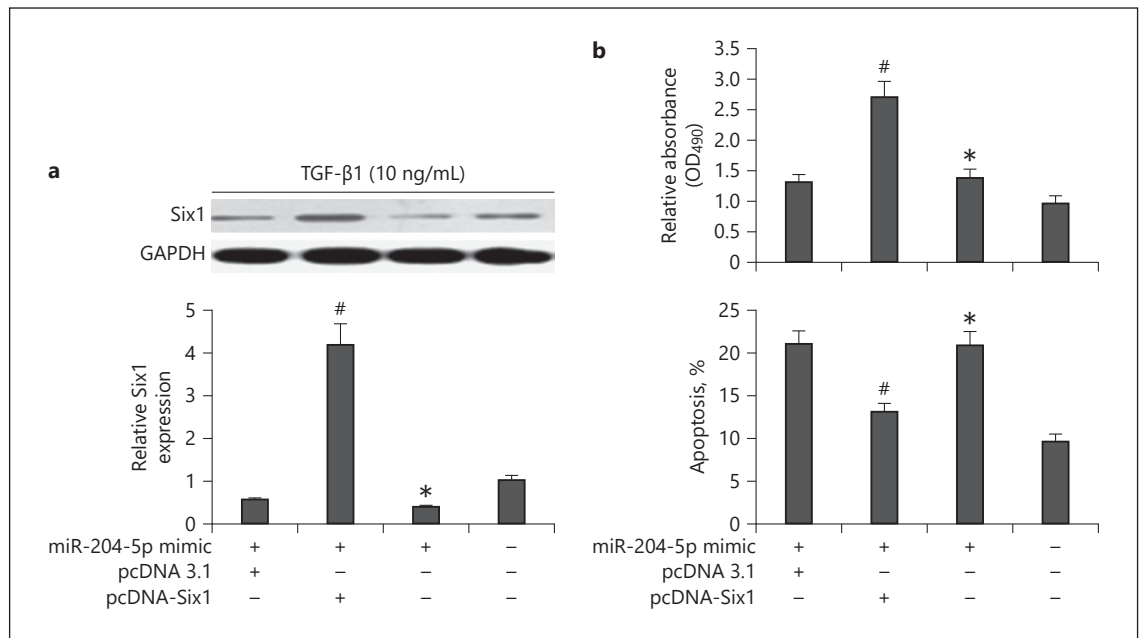


Fig. 6. Restoration of Six1 expression reverses the inhibitory effects of miR-204-5p on TGF-β1-induced cell proliferation and apoptosis. Airway smooth muscle cells were treated with TGF-β1 (10 ng/mL) for 24 h after being transfected with miR-204-5p mimic or pcDNA-Six1. **a** The expression of Six1 was measured by Western blot. **b** The proliferation (upper) and apoptosis (lower) of airway smooth muscle cells were determined by CCK-8 assay and flow cytometry. * $p < 0.05$ versus control cells; # $p < 0.05$ versus miR-204-5p mimic-transfected airway smooth muscle cells.

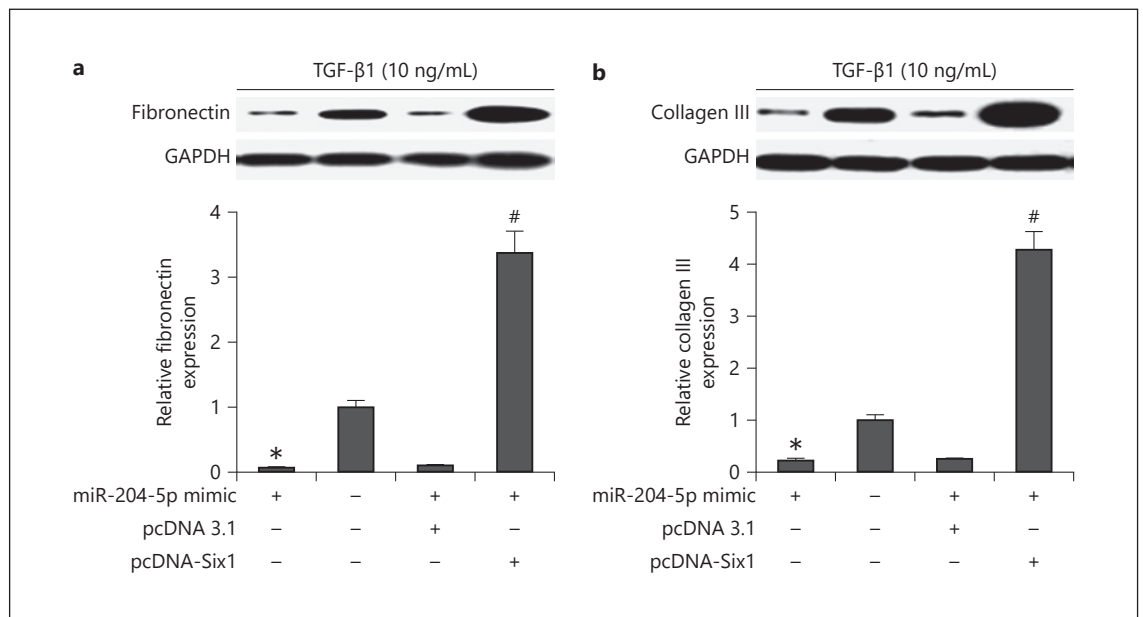


Fig. 7. Restoration of Six1 expression reverses the inhibitory effects of miR-204-5p on TGF-β1-induced ECM production. Airway smooth muscle cells were treated with TGF-β1 (10 ng/mL) for 24 h after being transfected with miR-204-5p mimic or pcDNA-Six1. The level of fibronectin (**a**) and collagen III (**b**) expression was measured by Western blot. * $p < 0.05$ versus control cells; # $p < 0.05$ versus miR-204-5p mimic-transfected airway smooth muscle cells.

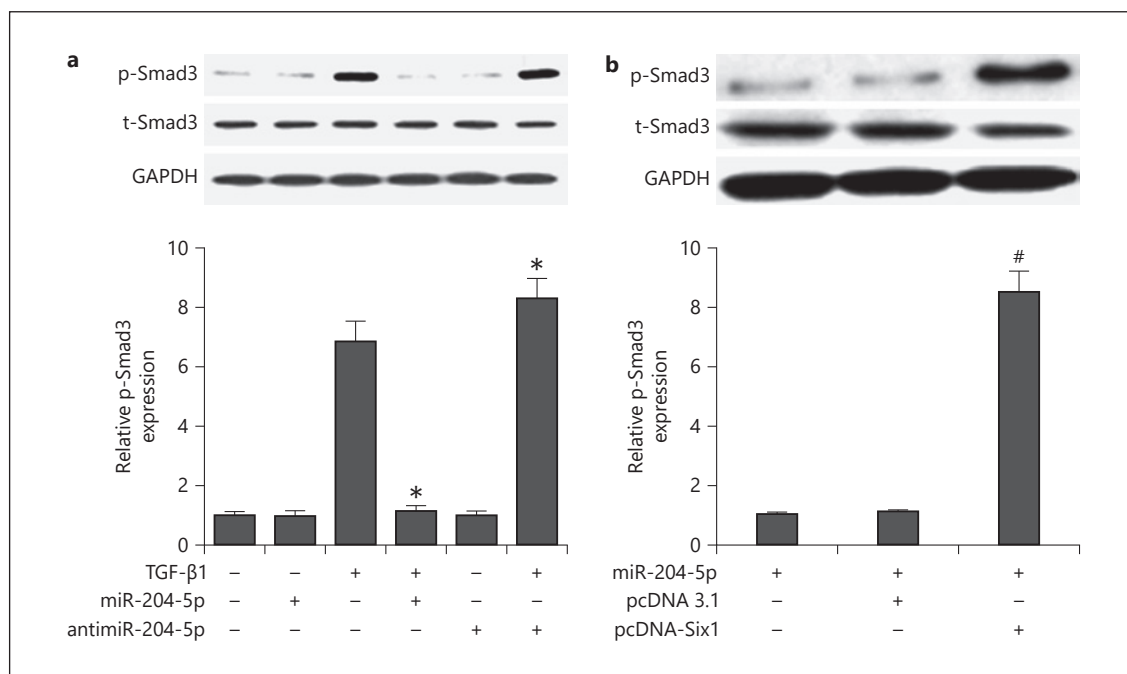


Fig. 8. MiR-204-5p negatively regulated the TGF/Smad3 signaling pathway in airway smooth muscle cells. **a** The level of Smad3 in airway smooth muscle cells transfected with the miR-204-5p mimic or inhibitor was measured by Western blot. **b** Airway smooth muscle cells were co-transfected with miR-204-5p mimic together with pcDNA-Six1, and the expression of phosphorylated Smad3 was evaluated by Western blot. * $p < 0.05$ versus TGF-β1-treated airway smooth muscle cells; # $p < 0.05$ versus miR-204-5p mimic-transfected airway smooth muscle cells.

MiR-204-5p Negatively Regulated the TGF/Smad3 Signaling Pathway in Airway Smooth Muscle Cells

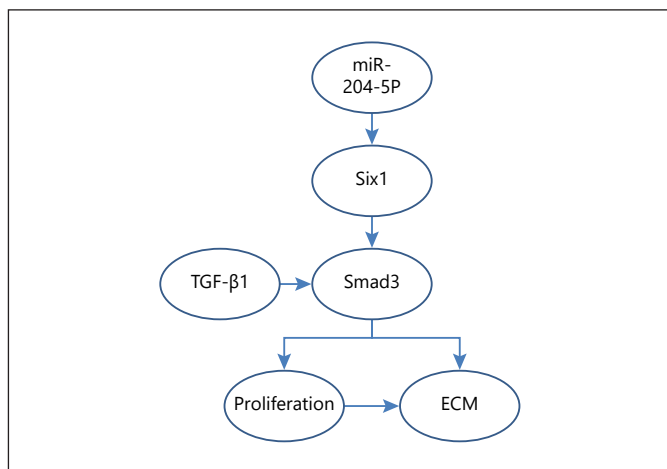
Previous reports have shown that the TGF/Smad pathway can regulate multiple cellular functions, including cell growth, apoptosis, or death. In this study, we found that miR-204-5p did not affect the level of phosphorylated Smad3 in the absence of TGF-β1. Meanwhile, the introduction of miR-204-5p significantly decreased the TGF-β1-induced phosphorylated Smad3 expression, while knockdown of miR-204-5p increased the expression of phosphorylated Smad3 ($p < 0.05$; Fig. 8a). We also found that additional overexpression of Six1 restored the inhibitory effect of miR-204-5p on the Smad3 phosphorylation (Fig. 8b).

Discussion

It is well known that the proliferation and ECM production of airway smooth muscle cells play an important role in the development of airway remodeling [24]. Previous studies have suggested that multiple factors, such as

TGF-β1 and platelet-derived growth factor, can regulate the proliferation of airway smooth muscle cells, contributing to airway remodeling [25]. Therefore, the inhibition of airway smooth muscle cell proliferation will be beneficial for relieving airway remodeling in asthma.

Accumulating evidence has shown that targeting specific miRNAs effectively prevents the proliferation of airway smooth muscle cells [26, 27]. MiRNA-based therapy may be a promising approach for relieving asthma. Previous studies have suggested that miR-204-5p can regulate multiple cellular functions including proliferation, autophagy, and metastasis [10, 11]. In posterior capsule opacification, miR-204-5p regulates epithelial-mesenchymal transition by regulating the Smad4 gene [28]. More importantly, a recent study found that miR-204-5p inhibits the differentiation of myoblasts by regulating ERRγ and MEF2C [16]. These data indicated an interesting connection between the miR-204-5p and its potential roles in asthma. In our study, we investigated a marked downregulation of miR-204-5p in asthmatic airway smooth muscle cells and TGF-β1-induced airway smooth muscle cells. Furthermore, another major discovery of



Color version available online

Fig. 9. The schematic represents miR-204-5p-regulated airway smooth muscle cell proliferation and ECM production via targeting Six1 in asthma.

our study was that miR-204-5p markedly inhibited the proliferation and ECM production of airway smooth muscle cells (Fig. 1–4). Our results indicated for the first time that miR-204-5p may be a negative regulator of airway smooth muscle cell proliferation and airway remodeling in asthma.

To explore the biological functions of miR-204-5p in regulating ECM production and airway smooth muscle cell proliferation, we performed a bioinformatics analysis using TargetScan 6.2 and predicted that Six1 was a target gene of miR-204-5p. Our previous studies suggested that the downregulation of Six1 effectively reversed airway remodeling and inhibited airway inflammation in asthma [22]. Six1 function was identified as a TGF- β 1-inducible gene, and plays a central role in regulating the differentiation of mesenchymal precursors. Here, we found that miR-204-5p directly targeted Six1 to affect the proliferation and ECM production of airway smooth muscle cells and to modulate the TGF- β 1-stimulated expression of phosphorylated Smad3. Moreover, knockdown of miR-204-5p aggravated TGF- β 1-stimulated increased Six1 expression, suggesting an inverse regulatory interaction between miR-204-5p and Six1 in asthma. Most importantly, our current study further confirmed that restoration of Six1 expression reversed the inhibitory effects of miR-204-5p on TGF- β 1-induced proliferation and ECM production in asthma (Fig. 5–8). Overall, these findings revealed that miR-204-5p inhibits the proliferation and ECM production of airway smooth muscle cells by downregulating Six1.

Taken together, our results confirmed that miR-204-5p is significantly decreased in asthmatic airway smooth muscle cells. Additionally, miR-204-5p suppresses the proliferation and ECM production of airway smooth muscle cells by directly regulating Six1 (Fig. 9). Thus, miR-204-5p might function as an inhibitor of airway remodeling by regulating Six1, which may be a potential strategy for preventing airway remodeling in asthma.

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Statement of Ethics

This study was reviewed and approved by the Human Ethics Committee of the Affiliated Hospital of Qingdao University.

Disclosure Statement

The authors declare that no competing interests exist.

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Author Contributions

Z.Y., Z.Q., and X.L. designed the studies; Z.Y., M.Y., Z.L., Y.W., and X.L. performed the experiments; N.R. and Y.S. analyzed the data. All authors reviewed the paper.

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