

ORIGINAL RESEARCH ARTICLE

MiR-448-5p inhibits TGF- β 1-induced epithelial-mesenchymal transition and pulmonary fibrosis by targeting Six1 in asthma

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Funding information

National Natural Science Foundation of China, Grant/Award Number: 81700029; Natural Science Foundation of Shandong Province, Grant/Award Numbers: ZR2017PH032, ZR2017MH017; Higher Educational Science and Technology Program of Shandong Province, Grant/Award Number: J17B092; Qingdao Scientific and Technologic Development Program, Grant/Award Number: 16-6-2-15-nsh

Abstract

MicroRNAs (miRNAs) are small yet versatile gene tuners that regulate a variety of cellular processes, including cell growth and proliferation. The aim of this study was to explore how miR-448-5p affects airway remodeling and transforming growth factor- β 1 (TGF- β 1)-stimulated epithelial-mesenchymal transition (EMT) by targeting Sine oculis homeobox homolog 1 (Six1) in asthma. Asthmatic mice models with airway remodeling were induced with ovalbumin solution. MiRNA expression was evaluated using quantitative real-time polymerase chain reaction. Transfection studies of bronchial epithelial cells were performed to determine the target genes. A luciferase reporter assay system was applied to identify whether Six1 is a target gene of miR-448-5p. In the current study, we found that miR-448-5p was dramatically decreased in lung tissues of asthmatic mice and TGF- β 1-stimulated bronchial epithelial cells. In addition, the decreased level of miR-448-5p was closely associated with the increased expression of Six1. Overexpression of miR-448-5p decreased Six1 expression and, in turn, suppressed TGF- β 1-mediated EMT and fibrosis. Next, we predicted that Six1 was a potential target gene of miR-448-5p and demonstrated that miR-448-5p could directly target Six1. An siRNA targeting Six1 was sufficient to suppress TGF- β 1-induced EMT and fibrosis in 16HBE cells. Furthermore, the overexpression of Six1 partially reversed the protective effect of miR-448-5p on TGF- β 1-mediated EMT and fibrosis in bronchial epithelial cells. Taken together, the miR-448-5p/TGF- β 1/Six1 link may play roles in the progression of EMT and pulmonary fibrosis in asthma.

KEYWORDS

airway remodeling, asthma, epithelial-mesenchymal transition (EMT), microRNA-448-5p, Sine oculis homeobox homolog 1 (Six1)

1 | INTRODUCTION

The morbidity and mortality of asthma have increased worldwide, and asthma has become a severe global public health problem (Thomsen et al., 2018). The frequent occurrence of injury and repair initiated by chronic inflammation could lead to structural changes in the airway, collectively termed airway remodeling. Airway remodeling is characterized by airway wall thickening, subepithelial fibrosis,

increased smooth muscle mass, angiogenesis, and increased mucous glands (Breton, Heydet, Starrs, Veldre, & Ghildyal, 2018; Sharma et al., 2017). Our previous studies found that myofibroblasts derived from bronchial epithelial cells via epithelial-mesenchymal transition (EMT) may contribute to pulmonary fibrosis (Yang et al., 2013). Transforming growth factor- β 1 (TGF- β 1) is considered a master switch for the induction of fibrosis by a process of EMT in various organs, including the airway (Charbonneau et al., 2016).

MicroRNAs (miRNAs) are small, short, noncoding RNAs that inhibit gene expression through binding to the 3'-untranslated regions (3'-UTR) of target gene mRNAs (Aquino-Jarquin, 2017). Accumulating evidence has shown that miRNAs play key roles in diverse biological processes, including cell proliferation, differentiation, and apoptosis (Gai et al., 2018). MiR-448-5p is considered an EMT-associated miRNA. Downregulation of miR-448-5p may amplify the positive feedback loop between NF- κ B and miR-448-5p, leading to the induction of EMT and eventually accelerating cancer formation (Li et al., 2011). Recently, a number of studies have also shown that miRNAs play important roles in mouse models of asthma (Chiba & Misawa, 2010; Singh et al., 2017). However, the underlying role of miR-448-5p in the development of asthma has not been explored.

Sine oculis homeobox homolog 1 (Six1), which belongs to the sine oculis homeobox gene family, plays extensive roles in regulating the development of many tissues and organs (Du et al., 2017). Our previous studies indicated that the downregulation of Six1 effectively inhibited airway inflammation and reversed airway remodeling in asthmatic mice (Yang et al., 2016). Six1 was initially identified as a TGF- β -inducible gene and has been cloned in mice and humans. The mechanism by which Six1 influences signaling events in target cells has been the focus of several studies that have identified at least five potential Six1 signaling pathways, including influences of Six1 on the bone morphogenic protein pathway to inhibit phosphorylated Smad 1, 3, 8 signaling, on the AKT pathway, on the AMP-activated protein kinase pathway, on the TLR4/CD14 pathway, and on the Na/K-ATPase membrane potential (Nishimura et al., 2017; Xu, Pirisi, & Creek, 2015).

In this study, we provide experimental evidence that miR-448-5p is essential for EMT and pulmonary fibrosis in asthmatic mice. Moreover, we reveal that a novel posttranscriptional regulatory mechanism of Six1 expression is mediated by miR-448-5p.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

Human bronchial epithelial cells, 16HBE, were obtained from the Cancer Research Institute of Beijing, China. The cells were cultivated in T75 tissue culture flasks in Roswell Park Memorial Institute medium-1640 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. Human bronchial epithelial cells were transfected with miR-448-5p mimic or inhibitor RiboBio, Guangzhou, China by using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA) according to the manufacturer's protocols. After infection with lentiviruses for 24 hr, the cells were treated with or without TGF- β 1 and then harvested.

2.2 | Animal experimental protocols

Female BALB/c mice weighing 18–24 g were randomly divided into normal control and asthma groups; each group had 12 mice. The mouse

model of asthma was established using ovalbumin (OVA). The mice were sensitized on Days 0, 7, and 14 by intraperitoneal injection of 20 μ g of OVA emulsified in 1 mg of aluminum hydroxide in a total volume of 0.2 ml. Seven days after the final sensitization, the mice were exposed to 1% OVA aerosol for $\leq$ 30 min every other day for 8 weeks. The 1% OVA aerosol was generated using a compressed air atomizer driven by filling a Perspex cylinder chamber (diameter 50 cm, height 50 cm) with a nebulized solution. Saline was used in the control group instead of OVA. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee of the Affiliated Hospital of Qingdao University.

2.3 | Vector construct

Full-length Six1 cDNA was purchased from GeneCopoeia and subcloned into the pcDNA3.1(+) vector. The 3'-UTR of Six1 (pMIR-Six1) or the 3'-UTR with mutated binding sites for miR-448-5p (pMIR-Six1-mut) was synthesized by Invitrogen (China) and inserted into pMIR-REPORT, which expresses the firefly luciferase plasmid (Promega).

2.4 | Immunohistochemistry

The expression of Six1 was assessed using semiquantitative immunohistochemistry. After deparaffinization, the sections were incubated in 0.01 mol/L citric acid buffer (pH 6.0) for 15 min in a microwave for antigen retrieval. The sections were then cooled and incubated in 3 g/L H₂O₂ for 30 min to inactivate endogenous peroxidases before blocking with 1:10 normal horse serum for 30 min. The supernatant was then discarded, and primary anti-mouse Six 1 (1:500) was added overnight at 4°C, followed by biotinylated goat anti-rat secondary antibody and streptavidin-horseradish peroxidase. The stained cells were then mounted and viewed using light microscopy.

2.5 | Western blot

Total cellular protein was extracted using a lysis buffer and quantified using protein quantification reagents from Bio-Rad (Hercules, CA). Then, 100 μ g of the protein was suspended in 5 $\times$ reducing sample buffer, boiled for 5 min, electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and transferred to polyvinylidene difluoride membranes using electroblotting. The membranes were subsequently blocked in 1% bovine serum albumin (BSA)/0.05% Tween-20/phosphate-buffered saline solution overnight at 4°C, followed by incubation with the primary antibody for 24 hr. Horseradish peroxidase-labeled immunoglobulin G was used as the secondary antibody. The blots were developed by incubation with a chemiluminescent substrate and exposure to X-ray film.

2.6 | Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA (1 μ g) was reverse-transcribed into cDNA for PCR amplification using a kit from Sigma. Amplification consisted of an initial 5-min incubation at 95°C, followed by 30 cycles of 30 s of

denaturation at 95°C, amplification for 30 s at 56°C, and extension for 60 s at 72°C. The final extension was 10 min at 72°C. Data were expressed as the relative differences between the control and treated cells after normalization to β -actin expression. The primers are listed in Table 1. To determine the expression levels of miRNAs, hydrolysis probe miRNA assays (Applied Biosystems) were performed, and U6 snRNA was used for normalization. The relative expression of the mRNA or miRNA was measured using the $2^{-\Delta\Delta C_t}$ method, where C_t is the fractional cycle number at which the fluorescence of each sample passes a fixed threshold.

2.7 | Luciferase assay

The full-length Six1 3'-UTR was amplified by PCR and cloned downstream of the firefly luciferase gene in the pGL3 vector (Promega, Madison, WI). The vector was named wild-type (wt) 3'-UTR. The GeneTailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA) was used to perform site-directed mutagenesis of the miR-448-5p binding site in Six1 3'-UTR. The resultant mutant was named mutant (mt) 3'-UTR. The cells were transfected with the reporter plasmids and placed in 96-well plates. Luciferase activity was measured according to the manufacturer's instructions (Promega, Beijing, China) and normalized to β -galactosidase activity.

2.8 | Statistical analysis

Data are expressed as the mean $\pm$ standard deviation. Statistical comparisons of the data from the various groups were performed using Student's *t*-test. The relative expression of an mRNA or miRNA

was measured using the $2^{-\Delta\Delta C_t}$ method, and differences between groups were considered statistically significant at $p < 0.05$.

3 | RESULTS

We developed a mouse model of airway remodeling through repetitive OVA challenge. Mice were subjected to OVA and developed significant eosinophilic inflammation and airway remodeling similar to that observed in human chronic asthma.

3.1 | MiR-448-5p was decreased and Six1 expression was increased in asthmatic mice and TGF- β 1-stimulated 16HBE cells

To explore the potential functional roles of miR-448-5p in the processes of airway remodeling, we estimated its level in a mouse model of airway remodeling induced by repetitive OVA challenge and in control subjects by using the quantitative real-time polymerase chain reaction (qRT-PCR) assays. The miR-448-5p level was significantly reduced in asthmatic mice compared with the control group (Figure 1a). In addition, we detected the level of miR-448-5p in TGF- β 1-stimulated 16HBE cells in vitro. TGF- β 1 dramatically downregulated miR-448-5p levels compared with the control group (Figure 1b). Next, we predicted that Six1 might be a direct target of miR-448-5p by using an online database. To prove this, we assessed the expression of Six1 at the mRNA and protein levels in asthmatic mice and TGF- β 1-stimulated 16HBE cells, respectively. We demonstrated that the expression of Six1 was remarkably increased in asthmatic mice and TGF- β 1-stimulated 16HBE cells (Figure 1c,d, and e).

TABLE 1 Quantitative polymerase chain reaction primers

Primer name	Sequence 5'-3'
MiR-448-5p F	GCGTTGCATATGTAGGAT
MiR-448-5p R	CTGGTGTCTGGAGTCGG
MiR-448-5p RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGATGGGAC
Fibronectin F	CGGTGGCTGTCTCAGTCAAAG
Fibronectin R	AAACCTCGGCTTCTCCATAA
Collagen IV F	GGACTACCTGGAACAAAAGGG
Collagen IV R	GCCAAGTATCTCACCTGGATCA
Vimentin F	GACGCCATCAACACCGAGTT
Vimentin R	CTTTGTCTGGTTAGCTGGT
E-cadherin F	CGAGAGCTACACGTTACGG
E-cadherin R	GGGTGTCTGAGGAAAAATAGG
β -actin F	CATTGCTGACAGGATGCAGA
β -actin R	CTGCTGGAAGGTGGACAGTGA
U6 F	CTCGCTTCGGCAGCACA
U6 R	AACGCTTCACGAATTTGCGT
U6 RT	CGCTTCACGAATTTGCGTGTACAT

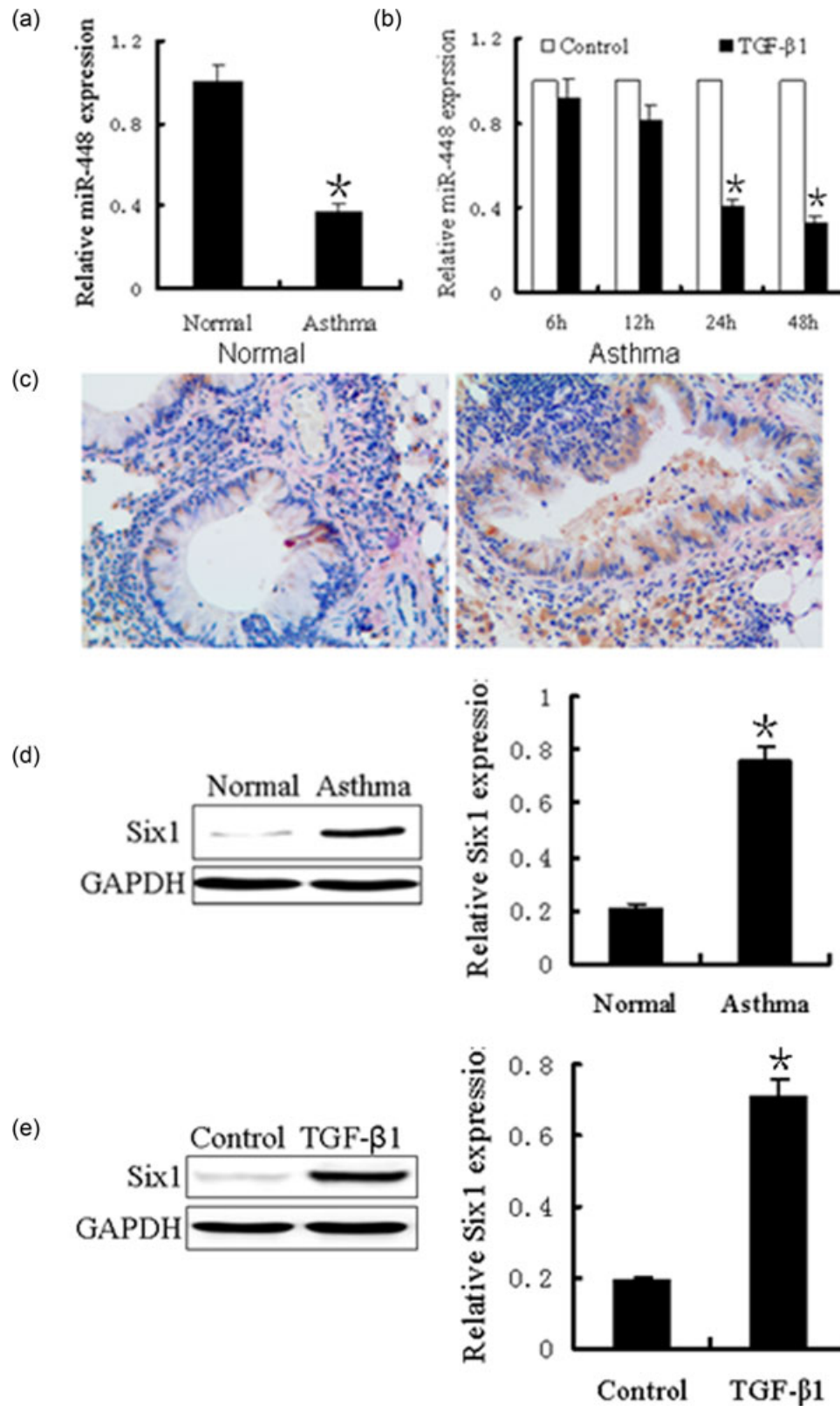


FIGURE 1 The levels of miR-448-5p and Six1 in asthmatic mice and TGF- β 1-induced 16HBE cells. Lung tissue sections were obtained from mice 24 hr after the final OVA challenge. (a) The relative expression of miR-448-5p in normal and asthmatic mice was detected by qRT-PCR. (b) Bronchial epithelial cells were incubated in the presence of TGF- β 1 (10 ng/ml) for 6, 12, 24, or 48 hr. The level of miR-448-5p was analyzed by qRT-PCR and normalized to U6. The expression of Six1 in the lung tissue was assessed using immunohistochemical staining (c) and western blot (d). (e) Bronchial epithelial cells were treated with TGF- β 1 for 24 hr. The level of Six1 was analyzed by western blot. * $p < 0.05$ versus normal tissues or control cells. OVA: ovalbumin; qRT-PCR: quantitative real-time polymerase chain reaction; Six1: Six1; TGF- β 1: transforming growth factor- β 1 [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | Effect of miR-448-5p on TGF- β 1-induced fibrosis in 16HBE cells

To investigate the effect of miR-448-5p on TGF- β 1-stimulated fibrosis in 16HBE cells, we transfected either an miR-448-5p mimic or inhibitor into 16HBE cells and then stimulated them with TGF- β 1. First, we evaluated the miRNA transfection efficiency via the qRT-PCR assay. After transfection for 48 hr, as shown in Figure 2a, the level of miR-448-5p was significantly increased in the miR-448-5p mimic group compared with the miR-NC group. In addition, the miR-448-5p level was

significantly decreased in 16HBE cells transfected with miR-448-5p inhibitor compared with cells treated with inhibitor-NC. To determine the functional role of miR-448-5p in TGF- β 1-stimulated fibrosis of 16HBE cells, we determined fibronectin and collagen IV expression. The introduction of miR-448-5p dramatically suppressed TGF- β 1-stimulated fibronectin and collagen IV expression in 16HBE cells, whereas miR-448-5p knockdown significantly aggravated TGF- β 1-stimulated fibronectin and collagen IV expression. However, miR-448-5p upregulation or downregulation alone did not affect basal gene expression (Figure 2b).

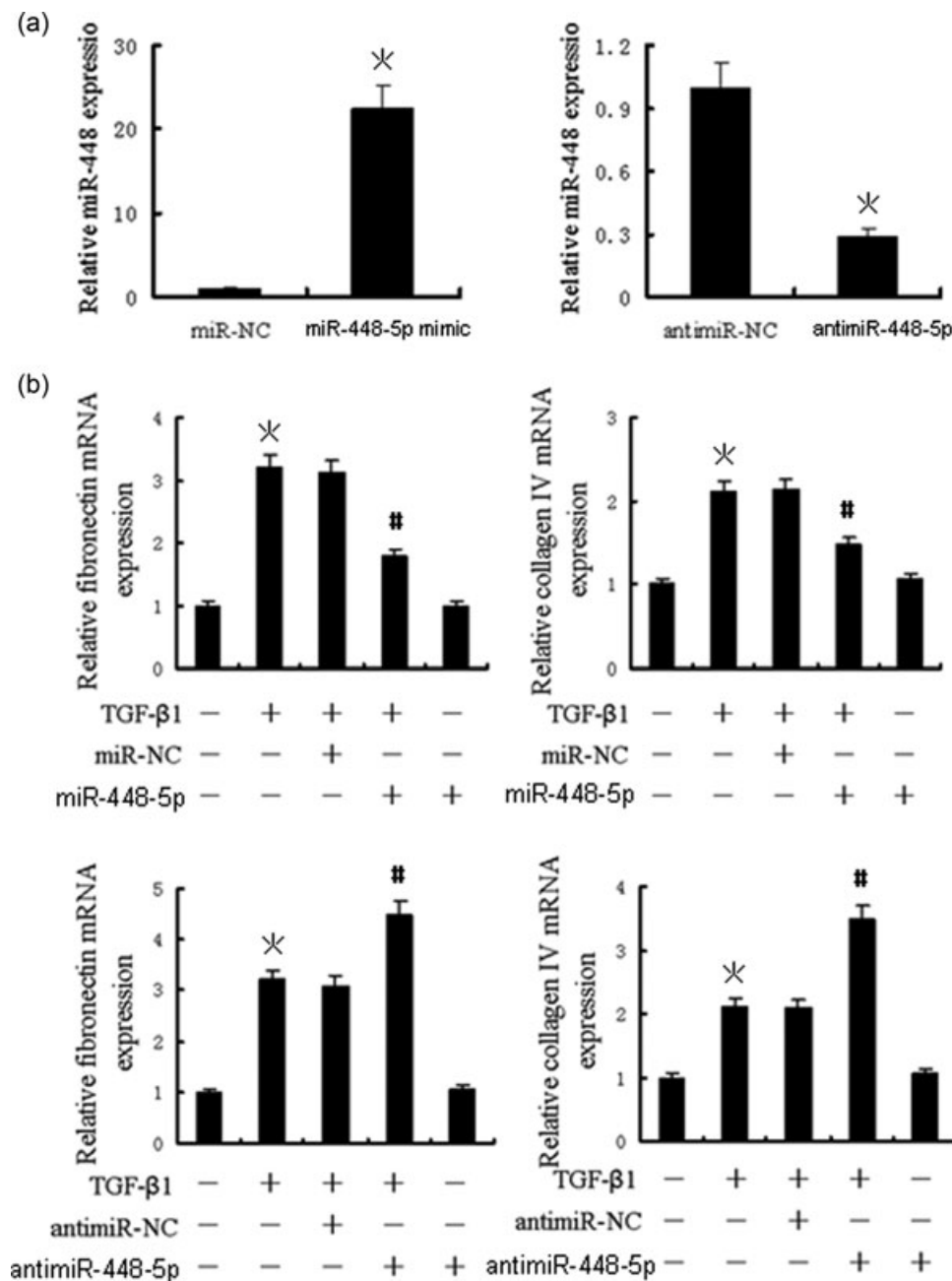


FIGURE 2 Effects of miR-448-5p on TGF- β 1-induced fibrosis. Bronchial epithelial cells were treated with or without TGF- β 1 (10 ng/ml) for 24 hr after transfection with an miR-448-5p mimic or inhibitor. (a) The level of miR-448-5p in bronchial epithelial cells was determined by RT-PCR. Bronchial epithelial cells were treated with TGF- β 1 (10 ng/ml) for 24 hr after transfection with an miR-448-5p mimic or inhibitor. (b) The mRNA expression of fibronectin and collagen IV was determined by qRT-PCR. * p < 0.05 versus control; # p < 0.05 versus miR-NC, inhibitor-NC or vehicle + TGF- β 1. mRNA: messenger RNA; RT-PCR: reverse transcription polymerase chain reaction; TGF- β 1: transforming growth factor- β 1

3.3 | Effect of miR-448-5p on TGF- β 1-stimulated EMT of 16HBE cells

To explore whether the miR-448-5p level was directly linked to EMT in TGF- β 1-stimulated 16HBE cells, we transfected 16HBE cells with an miR-448-5p mimic or inhibitor. Notably, the introduction of miR-448-5p significantly attenuated TGF- β 1-stimulated increased mesenchymal markers such as vimentin and decreased the epithelial marker E-cadherin compared with the TGF- β 1 group (Figure 3). These results indicated that knockdown of miR-448-5p could block the effects of TGF- β 1 on the EMT of 16HBE cells. In addition, our data demonstrated that the introduction or knockdown of miR-448-5p did not have effects on EMT under normal conditions.

3.4 | MiR-448-5p directly targets Six1

The online database TargetScan 6.2 indicated that Six1 is a binding target of miR-448-5p (Figure 4a). To further confirm whether miR-448-5p directly targets Six1, we cloned the Six1 WT 3'-UTR into the

pGL3-luciferase reporter vector and mutated the putative miR-448-5p binding site in the Six1 3'-UTR. We demonstrated that the miR-448-5p mimic or inhibitor significantly suppressed or promoted luciferase activity in 16HBE cells after transfection with Six1 WT 3'-UTR, respectively. Six1 MUT 3'-UTR reversed the functional roles of miR-448-5p (Figure 4b). Next, we performed western blot to detect Six1 expression in 16HBE cells after transfection with the miR-448-5p mimic or inhibitor. The expression of Six1 was dramatically decreased after miR-448-5p overexpression but significantly increased after miR-448-5p knockdown compared with the control group (Figure 4c).

3.5 | Knockdown of Six1 mimicked the effects of overexpression of miR-448-5p

To explore the role of Six1 in 16HBE cells, we transfected 16HBE cells with si-Six1. Silencing Six1 decreased Six1 expression but also significantly attenuated TGF- β 1-stimulated increased expression of vimentin and decreased the expression of E-cadherin (Figure 5a, b).

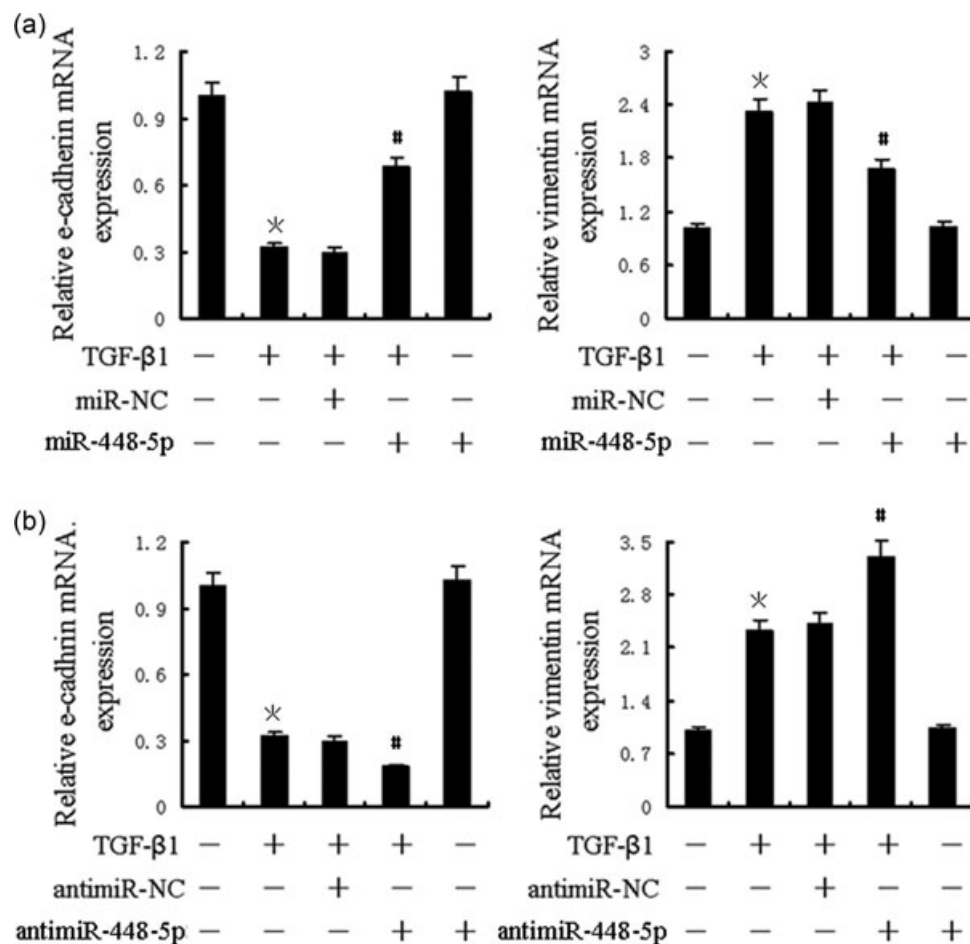


FIGURE 3 Effect of miR-448-5p on TGF- β 1-induced EMT. Bronchial epithelial cells were treated with or without TGF- β 1 (10 ng/ml) for 24 hr after transfection with an miR-448-5p mimic. (a) The expression of vimentin and E-cadherin was determined by qRT-PCR. Bronchial epithelial cells were treated with or without TGF- β 1 (10 ng/ml) for 24 hr after transfection with an miR-448-5p inhibitor. (b) The expression of vimentin and E-cadherin was determined by qRT-PCR. * $p < 0.05$ versus control; # $p < 0.05$ versus miR-NC, inhibitor-NC or vehicle + TGF- β 1. EMT: epithelial-mesenchymal transition; qRT-PCR: quantitative real-time polymerase chain reaction; TGF- β 1: transforming growth factor- β 1

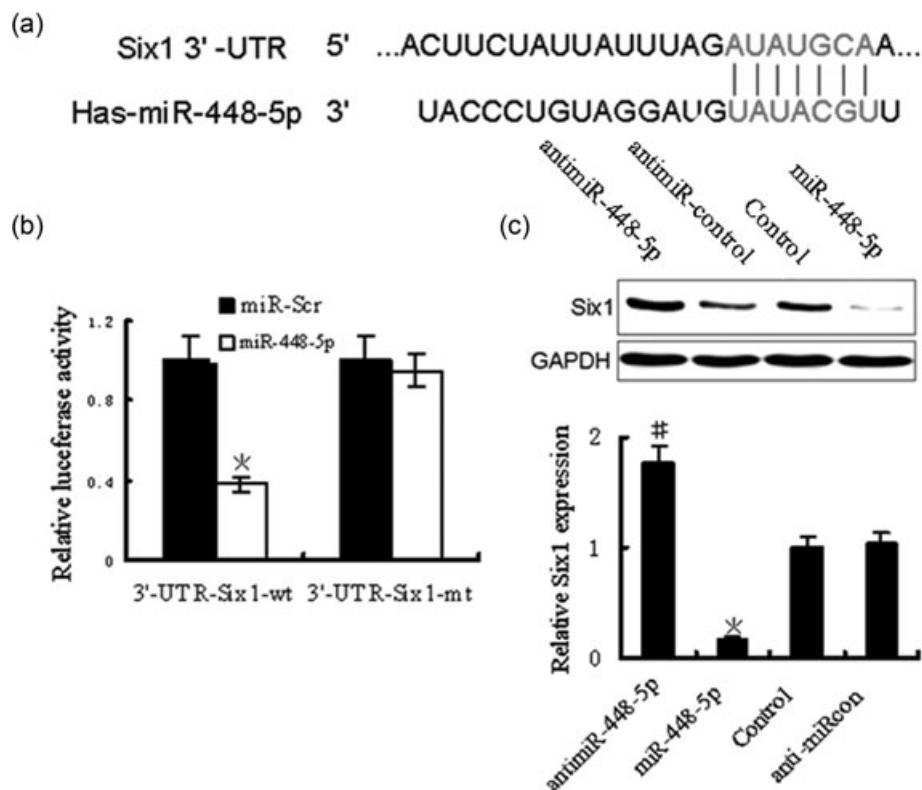


FIGURE 4 Six1 was a direct target of miR-448-5p. (a) Schematic representation of the Six1 3'-UTR showing the putative miRNA target site. (b) Analysis of the relative luciferase activities of Six1-wt, Six1-mut. (c) The expression of Six1 in bronchial epithelial cells transfected with the miR-448-5p mimic, miR-448-5p inhibitors, or anti-miRcontrol was measured by western blot analysis and normalized to GAPDH expression. * $p < 0.05$ versus control; # $p < 0.05$ versus anti-miRcontrol or control. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; miRNA: micro RNA; Six1: Sine oculis homeobox homolog 1; 3'-UTR: 3'-untranslated region

Furthermore, silencing Six1 dramatically reduced TGF- β 1-stimulated expression of fibronectin in 16HBE cells, suggesting that Six1 knockdown suppressed TGF- β 1-stimulated fibrosis (Figure 5c). These results confirmed that Six1 silencing had a very similar phenotype to miR-448-5p overexpression in 16HBE cells.

3.6 | Overexpression of Six1 partially blocked the effects of an miR-448-5p mimic on TGF- β 1-stimulated EMT

To confirm that miR-448-5p inhibited TGF- β 1-stimulated EMT and pulmonary fibrosis of 16HBE cells through a Six1-dependent mechanism, we cotransfected 16HBE cells with the miR-448-5p mimic and pcDNA-Six1. The expression of Six1 was dramatically increased at the mRNA and protein levels after transfection of 16HBE cells with the miR-448-5p mimic and pcDNA-Six1 compared with cells transfected with miR-448-5p mimic and pcDNA3.1 vector (Figure 6a). Upregulation of Six1 promoted TGF- β 1-stimulated EMT in 16HBE cells transfected with the miR-448-5p mimic (Figure 6b). Moreover, the introduction of Six1 blocked the inhibitory effect of miR-448-5p overexpression on the TGF- β 1-stimulated expression of fibronectin and collagen IV in 16HBE cells (Figure 6c).

3.7 | MiR-448-5p negatively regulates the phosphorylated Smad3 signaling pathway

We detected the expression of phosphorylated Smad3, and found that miR-448-5p did not affect the expression of phosphorylated Smad3 in the absence of TGF- β 1. However, the introduction of miR-448-5p significantly decreased the TGF- β 1-induced expression of phosphorylated Smad3 (Figure 7a), whereas knockdown of miR-448-5p increased TGF- β 1-induced expression of phosphorylated Smad3 (Figure 7b). After transfecting 16HBE cells with si-Six1, the expression of phosphorylated Smad3 was determined. The decreased Six1 expression did not affect the expression of phosphorylated Smad3 in 16HBE cells in the absence of TGF- β 1. Interestingly, the TGF- β 1-induced expression of phosphorylated Smad3 was dramatically reduced by Six1 knockdown (Figure 7c). Taken together, these data suggest that miR-448-5p inhibited TGF- β 1-induced fibrogenesis and EMT in 16HBE cells partly by modulation of the phosphorylated Smad3 signaling pathway.

4 | DISCUSSION

Airway remodeling is characterized by airway wall thickening, subepithelial fibrosis, increased smooth muscle mass, angiogenesis,

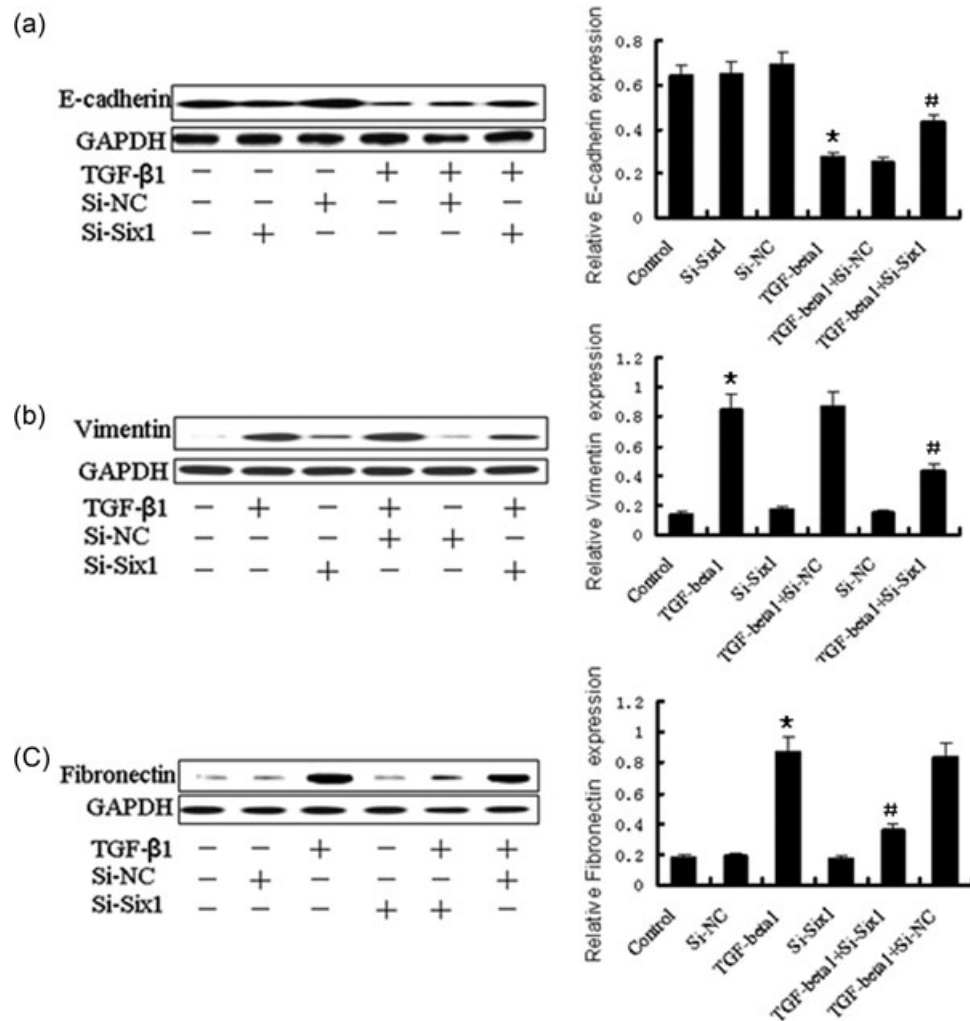


FIGURE 5 Six1 silencing inhibited TGF- β 1-induced EMT and fibrosis in bronchial epithelial cells. Bronchial epithelial cells were treated with TGF- β 1 (10 ng/ml) for 24 hr after transfection with si-Six1 or si-NC. The expression of E-cadherin (a) and vimentin (b) was determined by western blot. To confirm the effect of EMT on pulmonary fibrosis, the expression of fibronectin (c) was determined by western blot. All error bars represent the SE of three experiments. * $p < 0.05$ versus control; # $p < 0.05$ versus TGF- β 1 or vehicle + TGF- β 1. EMT: epithelial-mesenchymal transition; SE: standard error; Six1: Sine oculis homeobox homolog 1; TGF- β 1: transforming growth factor- β 1

and an increase in mucous glands, which may lead to chronic and obstinate asthma with pulmonary function depression (Choi et al., 2018). There is an increasing interest in the functional role of miRNAs in chronic diseases such as asthma (Ge et al., 2016; Plank, Maltby, Mattes, & Foster, 2013). Here, we report that upregulation of miR-448-5p was sufficient to inhibit TGF- β 1-stimulated EMT and fibrosis in bronchial epithelial cells, suggesting that this is a main regulatory event in EMT changes and pulmonary fibrosis. Furthermore, miR-448-5p directly targeted Six1, which was significantly increased by TGF- β 1 stimulation, and our data showed that Six1 knockdown effectively prevented TGF- β 1-stimulated EMT and fibrosis in vitro, indicating a potentially critical role in preventing airway remodeling in asthma.

Increasing reports have confirmed that EMT of the epithelial cells plays a functional role in the development and progression of fibrosis, including asthma (Ma et al., 2018; Wu et al., 2017). Undoubtedly, TGF- β 1 is one of the most predominant inducers of the EMT process

and is dramatically increased in asthma, inducing the EMT process through activation of TGF- β 1 downstream signaling pathways (Shin, Ryu, Oh, & Kang, 2015; Xu et al., 2017). Accumulating evidence has confirmed that specific miRNAs play important roles in the development and progression of airway remodeling. For example, miR-139-5p inhibits proliferation and promotes apoptosis of human airway smooth muscle cells by downregulating the Brg1 gene in asthma (Zhang, Sun, Yu, & Sun, 2017). Cheng et al (2017) reported that miR-145 downregulates mucin 5AC to alleviate airway remodeling and targets epidermal growth factor receptor (EGFR) to inhibit cytokine expression. MiR-448-5p is considered an EMT-associated miRNA (Yang, Wu, Li, Chen, & Shang, 2018; Zhu et al., 2015). In this study, we focused on miR-448-5p as it was markedly reduced in asthmatic mice and significantly downregulated by TGF- β 1 in bronchial epithelial cells. Our findings indicated that miR-448-5p effectively suppressed TGF- β 1-stimulated EMT in 16HBE cells. Moreover, the results revealed that miR-448-5p significantly reduced

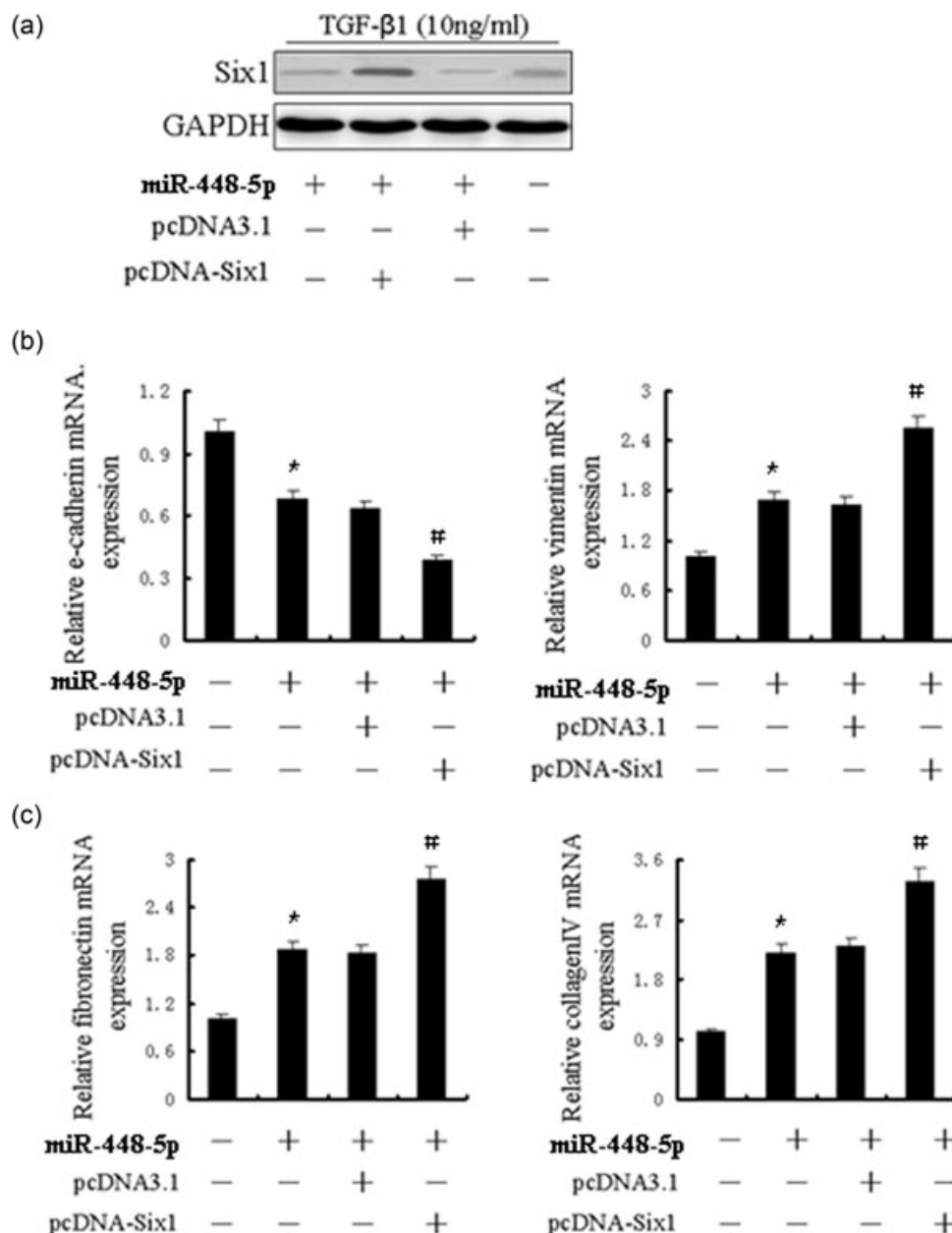


FIGURE 6 Six1 was involved in the effects of miR-448-5p on TGF-β1-induced fibrosis and EMT. Bronchial epithelial cells were transfected with the miR-448-5p mimic and pcDNA-Six1 and then treated with TGF-β1 (10 ng/ml) for 24 hr. (a) The protein expression of Six1 was determined by western blot. (b) The expression of vimentin and E-cadherin was determined by qRT-PCR. (c) The mRNA expression of fibronectin and collagen IV was determined by qRT-PCR. All error bars represent the SE of three experiments. * $p < 0.05$ versus control; # $p < 0.05$ versus TGF-β1 or vehicle + TGF-β1. EMT: epithelial-mesenchymal transition; mRNA: messenger RNA; qRT-PCR: quantitative real-time polymerase chain reaction; SE: standard error; Six1: Sine oculis homeobox homolog 1; TGF-β1: transforming growth factor-β1

TGF-β1-stimulated increased Six1 expression, whereas miR-448-5p knockdown aggravated TGF-β1-stimulated increased Six1 expression, confirming an inverse regulatory relationship between miR-448-5p and Six1 in bronchial epithelial cells. Interestingly, our results indicated that miR-448-5p directly regulated Six1 expression under normal conditions but did not affect EMT and pulmonary fibrosis of bronchial epithelial cells in the absence of TGF-β1. Most importantly, miR-448-5p only modulated TGF-β1-stimulated EMT and subepithelial fibrosis by regulating the expression of phosphorylated Smad3, which was a critical signal for TGF-β1-induced EMT but did not affect

EMT, subepithelial fibrosis, and the expression of phosphorylated Smad3 in the absence of TGF-β1. Finally, we have confirmed for the first time that miR-448-5p is sufficient to attenuate TGF-β1-stimulated effects on EMT and pulmonary fibrosis via the repression of its downstream target, Six1, in 16HBE cells. The above results support and highlight the potential beneficial effects of miR-448-5p on regulating pulmonary fibrosis in asthma.

Our study also demonstrated that specifically targeting Six1 could attenuate TGF-β1-stimulated EMT and pulmonary fibrosis in 16HBE cells. TGF-β1 treatment evokes increased calcium entry in

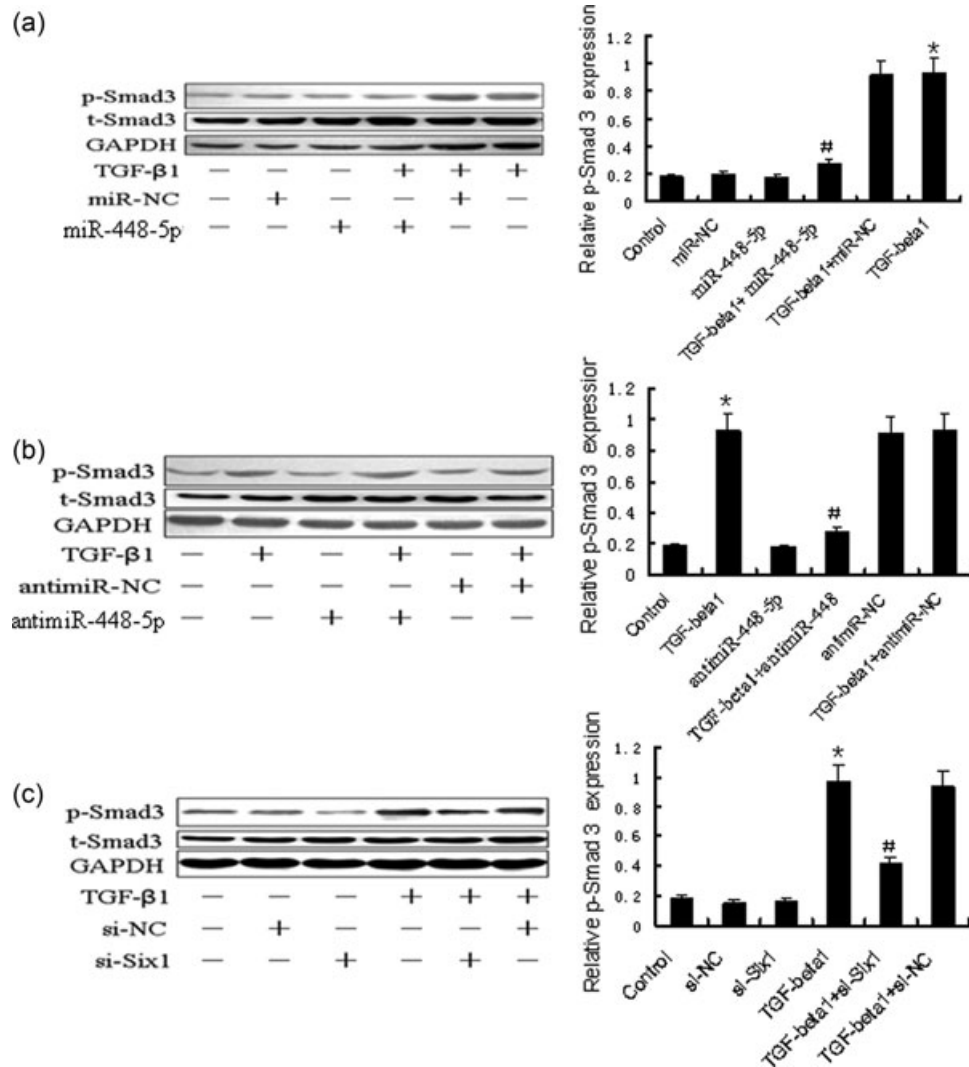


FIGURE 7 MiR-448-5p negatively regulates the phosphorylated Smad3 signaling pathway in 16HBE cells. (a) Western blot analysis of the expression of Smad3 in cells transfected with the miR-448-5p mimic. (b) Western blot analysis of the protein expression levels of Smad3 in cells transfected with the inhibitor. 16HBE cells were treated with TGF- β 1 for 24 hr after transfection with si-Six1 or si-NC. (c) The expression of phosphorylated Smad3 was determined by western blot. All error bars represent the SE of three experiments. * $p < 0.05$ versus control; # $p < 0.05$ versus TGF- β 1 or vehicle + TGF- β 1. Six1: Sine oculis homeobox homolog 1; TGF- β 1: transforming growth factor- β 1

16HBE cells, which was fully blocked when Six1 was knocked down, indicating a potential involvement of Six1 and Six1-mediated calcium influx in TGF- β 1-induced EMT. Consistent with previous studies, we observed that Six1 was an important valid target for preventing EMT. Furthermore, our results confirmed that miR-448-5p directly targeted Six1 to affect the TGF- β 1-stimulated expression of phosphorylated Smad3 and to modulate EMT and pulmonary fibrosis in 16HBE cells. Collectively, our results confirmed an inverse regulatory relationship between Six1 and miR-448-5p in bronchial epithelial cells.

In conclusion, our data demonstrated that miR-448-5p over-expression prevented TGF- β 1-stimulated EMT in asthmatic mice by targeting Six1 expression. The miR-448-5p/TGF- β 1/Six1 link may play roles in the progression of EMT and pulmonary fibrosis in 16HBE cells and may represent novel targets for strategies for preventing airway remodeling in asthma.

ACKNOWLEDGMENTS

This study was supported by National Natural Science Foundation of China (No. 81700029), Natural Science Foundation of Shandong Province (Nos. ZR2017PH032, ZR2017MH017), Higher Educational Science and Technology Program of Shandong Province (No. J17B092), and Qingdao Scientific and Technologic Development Program (No. 16-6-2-15-nsh).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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REFERENCES

- Aquino-Jarquín, G. (2017). Emerging role of CRISPR/Cas9 technology for microRNAs editing in cancer research. *Cancer Research*, 77(24), 6812–6817.
- Breton, J. D., Heydet, D., Starrs, L. M., Veldre, T., & Ghildyal, R. (2018). Molecular changes during TGF β -mediated lung fibroblast-myofibroblast differentiation: Implication for glucocorticoid resistance. *Physiological Reports*, 6(7), e13669.
- Charbonneau, M., Lavoie, R. R., Lauzier, A., Harper, K., McDonald, P. P., & Dubois, C. M. (2016). Platelet-derived growth factor receptor activation promotes the prodestructive invadosome-forming phenotype of synoviocytes from patients with rheumatoid arthritis. *Journal of Immunology*, 196(8), 3264–3275.
- Cheng, Z., Dai, L. L., Wang, X., Jia, L. Q., Jing, X. G., Li, P. F., ... An, L. (2017). MicroRNA-145 downregulates mucin 5AC to alleviate airway remodeling and targets EGFR to inhibit cytokine expression. *Oncotarget*, 8(28), 46312–46325.
- Chiba, Y., & Misawa, M. (2010). MicroRNAs and their therapeutic potential for human diseases: MiR-133a and bronchial smooth muscle hyperresponsiveness in asthma. *Journal of Pharmacological Sciences*, 114(3), 264–268.
- Choi, J. Y., Lee, H. Y., Hur, J., Kim, K. H., Kang, J. Y., Rhee, C. K., & Lee, S. Y. (2018). TRPV1 blocking alleviates airway inflammation and remodeling in a chronic asthma Murine model. *Allergy, Asthma & Immunology Research*, 10(3), 216–224.
- Du, P., Zhao, J., Wang, J., Liu, Y., Ren, H., Patel, R., ... Huang, G. (2017). Sine oculis homeobox homolog 1 regulates mitochondrial apoptosis pathway via caspase-7 in gastric cancer cells. *Journal of Cancer*, 8(4), 636–645.
- Gai, Y. P., Zhao, H. N., Zhao, Y. N., Zhu, B. S., Yuan, S. S., Li, S., ... Ji, X. L. (2018). MiRNA-seq-based profiles of miRNAs in mulberry phloem sap provide insight into the pathogenic mechanisms of mulberry yellow dwarf disease. *Scientific Reports*, 8(1), 812.
- Ge, L., Habel, D. M., Hansbro, P. M., Kim, R. Y., Gharib, S. A., Edelman, J. D., ... Chen, P. (2016). miR-323a-3p regulates lung fibrosis by targeting multiple profibrotic pathways. *JCI Insight*, 1(20), e90301.
- Li, Q. Q., Chen, Z. Q., Cao, X. X., Xu, J. D., Xu, J. W., Chen, Y. Y., ... Xu, Z. D. (2011). Involvement of NF- κ B/miR-448 regulatory feedback loop in chemotherapy-induced epithelial-mesenchymal transition of breast cancer cells. *Cell Death and Differentiation*, 18(1), 16–25.
- Ma, J., Zhang, L., Hao, J., Li, N., Tang, J., & Hao, L. (2018). Upregulation of microRNA-93 inhibits TGF- β 1-induced EMT and renal fibrosis by downregulation of Orai1. *Journal of Pharmacological Sciences*, 136, 218–227.
- Nishimura, T., Tamaoki, M., Komatsuzaki, R., Oue, N., Taniguchi, H., Komatsu, M., ... Sasaki, H. (2017). Six1 maintains tumor basal cells via transforming growth factor- β pathway and associates with poor prognosis in esophageal cancer. *Cancer Science*, 108(2), 216–225.
- Plank, M., Maltby, S., Mattes, J., & Foster, P. S. (2013). Targeting translational control as a novel way to treat inflammatory disease: The emerging role of microRNAs. *Clinical and Experimental Allergy*, 43(9), 981–999.
- Sharma, P., Yi, R., Nayak, A. P., Wang, N., Tang, F., Knight, M. J., ... Deshpande, D. A. (2017). Bitter taste receptor agonists mitigate features of allergic asthma in mice. *Scientific Reports*, 7, 46166.
- Shin, H. S., Ryu, E. S., Oh, E. S., & Kang, D. H. (2015). Endoplasmic reticulum stress as a novel target to ameliorate epithelial-to-mesenchymal transition and apoptosis of human peritoneal mesothelial cells. *Laboratory Investigation*, 95(10), 1157–1173.
- Singh, P. B., Pua, H. H., Happ, H. C., Schneider, C., Von moltke, J., Locksley, R. M., ... Ansel, K. M. (2017). MicroRNA regulation of type 2 innate lymphoid cell homeostasis and function in allergic inflammation. *Journal of Experimental Medicine*, 212, 3627–3643.
- Thomsen, A. M. L., Ehrenstein, V., Riis, A. H., Toft, G., Mikkelsen, E. M., & Olsen, J. (2018). The potential impact of paternal age on risk of asthma in childhood: A study within the Danish national birth cohort. *Respiratory Medicine*, 137, 30–34.
- Wu, C. H., Hsiao, Y. M., Yeh, K. T., Tsou, T. C., Chen, C. Y., Wu, M. F., & Ko, J. L. (2017). Upregulation of microRNA-4417 and its target genes contribute to nickel chloride-promoted lung epithelial cell fibrogenesis and tumorigenesis. *Scientific Reports*, 7(1), 15320.
- Xu, H., Pirisi, L., & Creek, K. E. (2015). Six1 overexpression at early stages of HPV16-mediated transformation of human keratinocytes promotes differentiation resistance and EMT. *Virology*, 474, 144–153.
- Xu, H., Chen, Y., Chen, Q., Xu, H., Wang, Y., Yu, J., ... Xu, B. (2017). DNMT1 regulates IL-6- and TGF- β 1-induced epithelial mesenchymal transition in prostate epithelial cells. *European Journal of Histochemistry*, 61(2), 2775.
- Yang, H., Wu, D., Li, H., Chen, N., & Shang, Y. (2018). Downregulation of microRNA-448 inhibits IL-1 β -induced cartilage degradation in human chondrocytes via upregulation of matrilin-3. *Cellular and Molecular Biology Letters*, 23, 7.
- Yang, Z. C., Yi, M. J., Ran, N., Wang, C., Fu, P., Feng, X. Y., ... Qu, Z. H. (2013). Transforming growth factor- β 1 induces bronchial epithelial cells to mesenchymal transition by activating the Snail pathway and promotes airway remodeling in asthma. *Molecular Medicine Reports*, 8(6), 1663–1668.
- Yang, Z. C., Yi, M. J., Shan, Y. C., Wang, C., Ran, N., Jin, L. Y., ... Qu, Z. H. (2016). Targeted inhibition of Six1 attenuates allergic airway inflammation and remodeling in asthmatic mice. *Biomedicine and Pharmacotherapy*, 84, 1820–1825.
- Zhang, H., Sun, Z., Yu, L., & Sun, J. (2017). MiR-139-5p inhibits proliferation and promoted apoptosis of human airway smooth muscle cells by downregulating the Brg1 gene. *Respiratory Physiology & Neurobiology*, 246, 9–16.
- Zhu, H., Zhou, X., Ma, C., Chang, H., Li, H., Liu, F., & Lu, J. (2015). Low expression of miR-448 induces EMT and promotes invasion by regulating ROCK2 in hepatocellular carcinoma. *Cellular Physiology and Biochemistry*, 36(2), 487–498.

How to cite this article: Yang Z-C, Qu Z-H, Yi M-J, et al. MiR-448-5p inhibits TGF- β 1-induced epithelial-mesenchymal transition and pulmonary fibrosis by targeting Six1 in asthma. *J Cell Physiol*. 2018;1–11. <https://doi.org/10.1002/jcp.27540>