Six1 Promotes Epithelial-Mesenchymal Transition in Bronchial Epithelial Cells via the TGFβ1/Smad Signalling Pathway

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Keywords

Airway remodelling · Six1 · Epithelial-mesenchymal transition · TGFβ1/Smad signalling pathway · Asthma

Abstract

Introduction: The homeodomain transcription factor sine oculis homeobox homolog 1 (Six1) plays a crucial role in embryogenesis and is not expressed in normal adult tissue but is expressed in many pathological processes, including airway remodelling in asthma. The current study aimed to reveal the effects of Six1 in regulating the airway remodelling and its possible mechanism. **Methods:** A mouse model of ovalbumin-induced asthma-associated airway wall remodelling and a bronchial epithelial cell (16HBE) model of transforming growth factor β1 (TGFβ1)-induced epithelial-mesenchymal transition (EMT) were used to investigate the role of Six1. Then, 16HBE cells were transformed with Six1 expression vectors and treated with a TGFβ1 pathway inhibitor to determine the role of Six1 in EMT. The effect of Six1 and its possible mechanism were assessed by immunohistochemistry, RT-PCR, and Western blot. **Results:** Six1 expression was elevated in the lungs in an OVA mouse model of allergic asthma and in 16HBE cells treated with TGFβ1. Six1 overex-

pression promoted an EMT-like phenotype with a decreased protein expression of E-cadherin and increased protein expression of α-smooth muscle actin (α-SMA) as well as fibronectin in 16HBE cells; these effects appeared to promote TGFβ1 and phospho-Smad2 (pSmad2) production, which are the main products of the TGFβ1/Smad signalling pathway, which could be reduced by a TGFβ1 inhibitor. **Conclusion:** These data reveal that Six1 and TGFβ1 are potentially a part of an autocrine feedback loop that induces EMT, and these factors can be reduced by blocking the TGFβ1/Smad signalling pathway. As such, these factors may represent a promising novel therapeutic target for airway remodelling in asthma.

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Introduction

Sine oculis homeobox homolog 1 (Six1) is a member of the sine oculis homeobox gene family that is located on the human chromosome 14q23. Accumulating evidence has shown that Six1 plays key roles in embryonic development

Edited by: H.-U. Simon, Bern.

processes, including cell proliferation, differentiation, survival, and migration. However, it is expressed at low levels in normal adult tissues [1]. Its contribution to carcinogenesis has been described in several malignancies and diseases, such as lung cancer and pulmonary fibrosis [2, 3]. In our previous studies, we demonstrated that the expression of Six1 was increased significantly in the bronchoalveolar lavage fluid from children with asthma and lung tissue of a mouse model of asthma induced by OVA compared with the respective normal controls, while downregulation of Six1 inhibited airway inflammation and reversed airway remodelling in a mouse model of asthma [4]. It has been confirmed that Six1 plays a certain role in the pathogenesis of asthma, but the molecular mechanism of Six1 in airway remodelling in asthma is still unclear.

Airway remodelling is one of the main mechanisms involved in the adverse bronchus and lung effects of asthma. It is caused by the frequent repair of damage induced by chronic inflammation in the airway wall and can lead to structural changes. Airway remodelling is one of the factors that decreases the effect of treatments in asthma [5], so finding effective options that have the capacity to reverse the ongoing remodelling process is very important. Studies have found that airway remodelling begins in the early stages of bronchial asthma when epithelial cells show characteristics of mesenchymal cells [6, 7]. It is suggested that epithelial-mesenchymal transition (EMT) may play an important role in the airway remodelling progression.

EMT is the transformation of epithelial cells into mesenchymal cells, and EMT is executed in response to various signalling factors that induce the expression of specific transcription factors. Among these factors, transforming growth factor β 1 (TGF β 1) has been recognized to play a key role in regulating the induction of fibrosis by affecting EMT in various organs, including the airway wall [8, 9]. TGF β 1 mainly activates signalling pathways and transcriptional regulators of the Smad pathway to exert its biological activity [10–12]. Therefore, it is of great significance to explore the mechanisms of TGF β 1/Smad and EMT in fibrosis and airway remodelling, which may lead to an effective treatment for bronchial asthma.

In this article, we evaluated the impact of Six1 expression on fibrosis and EMT phenotypes in bronchial epithelial cells. We identified that the transformation of bronchial epithelial cells into fibroblasts was associated with airway remodelling. These results demonstrate the potential utility of targeting Six1 and the TGF β 1/Smad signalling pathway in the treatment of airway remodelling.

Materials and Methods

OVA-Induced Mouse Model of Asthma

Twenty-four healthy male BALB/c mice (Shanghai Model Organisms Center Inc., Shanghai, China), weighing 18 ± 5 g, were randomly divided into 2 groups: the control group and the asthma group. The mice in the asthma group were sensitized on days 0, 7, and 14 by 20 μ g OVA (Sigma, St. Louis, MO, USA) emulsified in 1 mg of aluminium hydroxide (Sigma, St. Louis, MO, USA) in a total volume of 200 μ L. From the 21st day, the mice were exposed to 1% OVA aerosol for 30 min every other day for 8 weeks. Mice that received PBS at the same volume and frequency were used as controls.

Tissue Samples and Immunohistochemistry

Twenty-four hours after the last challenge, lung tissues were removed from the mice and immediately fixed in 10% buffered formalin. Then, they were embedded in paraffin. Sections (4 μ m) were prepared and then stained with haematoxylin and eosin (HE). The slides were also incubated in 0.01 mol/L citric acid buffer (pH 6.0) for 15 min. To inactivate endogenous peroxidase, the sections were incubated in 3 g/L H₂O₂ for 30 min. Then, they were blocked with 1:10 normal horse serum for 30 min. Slides were incubated with an anti-Six1 (1:300; Affinity, China) primary antibody overnight at 4°C, followed by incubation with a secondary antibody and streptavidin-conjugated horseradish peroxidase for approximately 30 min. Diaminobenzidine chromogen solution was applied as the final staining step. The slides were observed under a microscope.

Cells Cultured and Treated with TGF β 1

The normal bronchial epithelium cell line-16HBE (Chinese Academy of Medical Sciences, Beijing, China) was cultured in 1,640 medium (Gibco, Gaithersburg, MD, USA) containing 10% foetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) in a humidified atmosphere of 5% CO₂ at 37°C. Cells without contamination were selected for the next experiments. Cells ($1-1.5 \times 10^5$) were plated in 6-well plates in media (2.0 mL/well). When the cells reached approximately 60–70% confluency, they were treated with 10 ng/mL TGF β 1 (PeproTech, Rocky Hill, NJ, USA) for 24, 48, and 72 h. After this treatment process, the cells were harvested.

Six1 Overexpression in 16HBE Cells

A Six1 (NM_005982)-GV146 overexpression plasmid in glycerol (Jikai Company, Shanghai, China) was inoculated into LB medium containing kanamycin, and the solution was oscillated at 37°C and 250 rpm for 16 h. The plasmid was extracted according to the instructions of the plasmid extraction kit (TIANGEN Biotech, Beijing, China). The 16HBE cells showing the most growth were divided into Six1 group (receiving the Six1-GV146 plasmid), negative control group (receiving the synthetic nonsense-GV146 plasmid), and blank control group (receiving liposomes). When the cells were grown to approximately 90% confluency, the plasmid was transfected into the cells using Lipofectamine™ 2000 (Thermo Fisher Scientific Co., Waltham, MA, USA). The culture medium was replaced with serum-free medium during transfection. After 6 h, the medium was replaced with serum-containing medium. The cells with the best transfection efficiency after 48 h were chosen.

Table 1. The sequences of the primers

Name	Forward primer	Reverse primer
Six1	AGTTCTCGCCTCACAACCAC	ACACCCCTCGACTTCTCCTT
E-cad	ACACCATCCTCAGCCAAGA	CGTAGGGAACTCTCTCGGT
α -SMA	TGTTGCCCCTGAAGAGCATC	GAGACAGCACCGCCTGGATA
GAPDH	TCATGGGTGTGAACCATGAGAA	GGCATGGACTGTGGTCATGAG

Six1, sine oculis homeobox homolog 1; E-cad, E-cadherin; α -SMA, α -smooth muscle actin.

Inhibition of the TGF β 1 Signalling Pathway

The Six1 plasmid was transfected into 16HBE cells using Lipofectamine™ 2000. After 6 h of transfection, the culture medium was replaced with medium containing serum, and the cells were exposed to a 10 μ mol/L TGF β 1 inhibitor (HY-101275) (MedChemExpress, Monmouth Junction, NJ, USA) for 24 h.

RT-PCR

Total RNA was isolated from each group of cells using RNAiso Plus (Takara, Shiga, Japan). Messenger RNA (mRNA) was reverse-transcribed into complementary DNA (cDNA) by using the PrimeScript™ RT Reagent Kit with gDNA Eraser (RR047A) (Takara, Shiga, Japan). Then, the instructions of the TB Green® Premix Ex Taq™ II kit (RR820A) (Takara, Shiga, Japan) were used to prepare the total reaction mixture. The sequences of the primers are shown in Table 1 (Beijing Ruibo Xingke Biotechnology Co., Beijing, China). GAPDH was used as the internal control. RT-PCR assays were performed on the Light Cycler 480 system. The relative expression of mRNAs was determined using the $2^{-\Delta\Delta CT}$ method.

Western Blot

A 100-1 solution of RIPA:PMSF (Solarbio, Beijing, China) was used to extract total cellular proteins. The BCA Protein Assay Kit (Solarbio, Beijing, China) was used to measure the protein content. A 10% sodium dodecyl sulfate-polyacrylamide gel was prepared and used to separate the proteins. Then, the protein samples were transferred to PVDF membranes and blocked with 5% nonfat milk for 1 h. The PVDF membranes were incubated with primary antibodies against the following targets overnight in a shaking bed at 4°C: E-cadherin (E-cad; 1:1,000; Cell Signalling Technology, Danvers, MA, USA), α -smooth muscle actin (α -SMA; 1:1,000; Cell Signalling Technology, Danvers, MA, USA), phospho-Smad2 (pSmad; 1:1,000; Cell Signalling Technology, Danvers, MA, USA), TGF β 1 (1:1,000; Abcam, Cambridge, MA, USA), Six1 (1:1,000; Affinity, China), fibronectin (FN; 1:1,000; Elabscience, Wuhan, China), GAPDH (1:1,000; Cell Signalling Technology, Danvers, MA, USA), and β -actin (1:1,000; Cell Signalling Technology, Danvers, MA, USA). Membranes were incubated with secondary antibody (1:2,000; Elabscience, Wuhan, China) for approximately 60 min. The proteins were visualized by using electrochemiluminescence (Beyotime, Shanghai, China) and exposed to X-ray. The results were quantified with ImageJ software.

Statistical Analysis

Data are presented as the mean $\pm$ standard deviation ($x \pm s$) as determined by SPSS 25.0 and GraphPad Prism 5.0 statistical soft-

Fig. 1. Tissue sections with HE and Six1 immunohistochemical staining: lung tissue sections obtained from mice 24 h after the last OVA challenge were stained with HE and immunohistochemically stained for Six1 (magnification, $\times 100$). Airway tissues were HE stained: control group (a); asthma group (b). The expression of Six1 in the lung tissue was assessed using immunohistochemical staining. Positive staining is depicted in brown: control group (c); asthma group (d). Six1, sine oculis homeobox homolog 1; HE, haematoxylin and eosin.

ware. Statistical differences among experimental groups were evaluated by one-way ANOVA, followed by Fisher's least significant difference multiple comparison post hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

The Expression of Six1 Was Increased in OVA-Induced Asthmatic Mice

We found that the mice in the control group were lively and active and that their breathing was stable and their hair was smooth, while the mice in the asthma group

Fig. 2. TGFβ1 promotes EMT and increases Six1 in 16HBE cells. **A** Phenotypic changes were observed through an inverted microscope: normal group of 16HBE cells (**a**); 16HBE cells treated with TGFβ1 for 24 h (**b**); 16HBE cells treated with TGFβ1 for 48 h (**c**); and 16HBE cells treated with TGFβ1 for 72 h (magnification, ×40) (**d**). **B** The expression of E-cad and α-SMA mRNA was determined by RT-PCR (* $p < 0.05$, ** $p < 0.01$). **C** The expression of E-cad and α-SMA was measured using the Western blot analysis (* $p < 0.05$,

** $p < 0.01$). **D** The expression of FN mRNA and protein was measured by RT-PCR and Western blot analysis (** $p < 0.01$). **E** The expression of Six1 mRNA and protein was measured by RT-PCR and Western blot analysis (** $p < 0.01$). Six1, sine oculis homeobox homolog 1; E-cad, E-cadherin; EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; mRNA, messenger RNA; FN, fibronectin; TGFβ1, transforming growth factor β1.

Fig. 3. Six1 promotes EMT in 16HBE cells in vitro. **A** Transfection efficiency was observed through an inverted fluorescence microscope after transfection for 24 h. The cells with green fluorescence were successfully transfected. Six1 group: 16HBE cells treated with the Six1-GV146 plasmid (**a**); negative control group: 16HBE cells treated with the synthetic nonsense-GV146 plasmid (**b**); and blank control group: 16HBE cells transfected with liposomes (**c**); images **a'**, **b'**, **c'** were the same views of **a**, **b**, **c** taken under normal white light, respectively (magnification, ×40). **B** The expression of Six1

mRNA and protein was determined by RT-PCR and Western blot (* $p < 0.05$, ** $p < 0.01$). **C** Phenotypic changes after 48 h transfection were evaluated via an inverted microscope: Six1 group (**a**); negative control group (**b**); and blank control group (magnification, ×100) (**c**). **D** The expression of E-cad and α-SMA protein were determined by Western blot (* $p < 0.05$, ** $p < 0.01$). Six1, sine oculis homeobox homolog 1; EMT, epithelial-mesenchymal transition; E-cad, E-cadherin; α-SMA, α-smooth muscle actin.

(For figure see next page.)

A

B

C

D

showed sneezing, shortness of breath, and restlessness. Tissue sections stained with HE were observed under a microscope. In the control group, the bronchial inner wall was smooth with no obvious damage. There was no exfoliation of epithelial cells and no infiltration of inflammatory cells (Fig. 1a). Compared with that of the control group, the airway wall of the asthma group was significantly thickened, and inflammatory cells were significantly increased and more widely distributed in the asthma group. The thickened airway smooth muscle and increased airway mucus in the asthma group can be seen in Figure 1b. These findings confirmed that the mouse model of asthma was established successfully. Next, we examined the expression of Six1, which was significantly higher in the lungs of the asthma group than in those of the control group (Fig. 1c, d).

TGFβ1 Promoted EMT and Increased Six1 in 16HBE Cells

16HBE cells treated with TGFβ1 (10 ng/mL) were observed under an inverted microscope: epithelial cells activated with TGFβ1 transformed into an elongated spindle shape, while cells in the normal group displayed typical cobblestone-like morphology. In addition, the longer the TGFβ1 treatment was administered, the more obvious the morphological changes in 16HBE cells (Fig. 2A).

Compared with the normal group, the asthma group had significantly increased expression of α-SMA mRNA, while the expression of E-cad mRNA was decreased, and the longer the 16HBE cells were treated with TGFβ1, the lower the expression of E-cad and higher the expression of α-SMA mRNA (Fig. 2B). In addition, the Western blot analysis showed the same trend in the expression of α-SMA and the expression of E-cad at the protein level (Fig. 2C). Moreover, the expression of FN mRNA reached a peak at 48 h, and the protein expression was increased in a dose- and time-dependent manner (Fig. 2D). Moreover, as expected, the expression of Six1 mRNA and protein in TGFβ1-16HBE cells were increased compared with that in the normal group, and the longer the cells were treated with TGFβ1, the higher the expression of Six1 in the cells (Fig. 2E).

Six1 Promoted EMT in 16HBE Cells

The transfection efficiency of the Six1 overexpression vector in 16HBE cells was observed through an inverted fluorescence microscope (Fig. 3A). The Six1-GV146 plasmid greatly increased Six1 expression in 16HBE cells at 48 h after transfection (Fig. 3B). These results confirmed that Six1 was successfully transfected into 16HBE cells.

Following the overexpression of Six1 in 16HBE cells, morphological changes were observed by inverted microscopy. The Six1 group showed cells with a fusiform shape and the intercellular gap junctions were enlarged compared with those in the negative control group and blank control group, in which cells maintained the typical cobblestone appearance (Fig. 3C). Protein expression was detected by Western blot: the α-SMA expression in the Six1 overexpression group was significantly higher than that in the control group, while the E-cad expression was lower in the Six1 overexpression group than the control group. There were no significant differences between the negative control group and the blank control group (Fig. 3D).

Six1 Promoted EMT in 16HBE Cells Via the TGFβ1/Smad Signalling Pathway

To determine the validity of our hypothesis that the EMT induced by Six1 occurred through the TGFβ1/Smad signalling pathway, we further measured the protein expression of TGFβ1 and phospho-Smad2 (pSmad2) in Six1-overexpressing 16HBE cells. The expression of TGFβ1 and pSmad2 protein in the Six1-overexpressing 16HBE cells was significantly increased compared with that in the negative control group and the blank control group cells (Fig. 4A). After inhibiting the TGFβ1 signalling pathway for 24 h, the intercellular space between cells in the inhibitor group was significantly smaller than that in the Six1 control group, and the morphology of the cells treated with the inhibitor was closer to the normal cobblestone appearance (Fig. 4B). The Western blot results showed that the expression of TGFβ1 and pSmad2 in cells treated with the inhibitor was considerably lower than that in cells without inhibition (Fig. 4C). The expression of epithelial markers was increased, while that of mesenchymal markers was decreased in the inhibitor group (Fig. 4D).

16HBE Cell Fibrosis Was Closely Related to EMT

When we estimated the FN level, which is a marker of myofibroblasts, in 16HBE cells transfected with the Six1 overexpression plasmid and treated with the TGFβ1 pathway inhibitor, we found that the level of FN was significantly increased compared with that in the negative control group and blank control group (Fig. 5a). The expression of FN in the group that received the TGFβ1 signalling pathway inhibitor was significantly decreased compared with that in the group overexpressing Six1 but not treated with the inhibitor (Fig. 5b).

Fig. 4. Six1 induces EMT in 16HBE cells through the TGF β 1/Smad signalling pathway. **A** Western blot analysis was performed to determine the expression of TGF β 1 and pSmad2 in 16HBE cells transfected with the Six1 overexpression plasmid (** $p < 0.01$) (c). **B** Phenotypic changes in Six1-overexpressing 16HBE cells after inhibition of the TGF β 1 pathway with an inhibitor for 24 h were observed with an inverted microscope (magnification, $\times 100$): Six1 control group (a) and inhibitor group (b). **C** Western blot analysis was used to determine the expression of TGF β 1 and pSmad2 in the Six1 control group and inhibitor group (** $p < 0.01$). **D** Western blot analysis was used to determine the expression of E-cad and α -SMA protein in the Six1 control group and inhibitor group (** $p < 0.01$). Six1, sine oculis homeobox homolog 1; EMT, epithelial-mesenchymal transition; TGF β 1, transforming growth factor β 1; α -SMA, α -smooth muscle actin; E-cad, E-cadherin.

Discussion

The results of the current study together with our prior observations on the expression of Six1 in the bronchoalveolar lavage fluid of children with asthma and lung tissue of a mouse model of asthma [4] show that Six1

plays a necessary role in the process of airway remodelling in asthma. Our studies have demonstrated that Six1 can promote EMT in bronchial epithelial cells via the TGF β 1/Smad signalling pathway, which is critical for fibrosis and remodelling of the airway wall. Since Six1 is expressed at low levels in normal adult tissues, the Six1 or TGF β 1/

Fig. 5. The expression of FN in 16HBE cells is closely related to EMT. **a** Western blot analysis was used to determine the expression of FN in 16HBE cells transfected with the Six1 overexpression plasmid (** $p < 0.01$). **b** Western blot analysis was used to determine the expression of FN in Six1-16HBE cells treated with a TGF β 1 inhibitor (** $p < 0.01$). Six1, sine oculis homeobox homolog 1; EMT, epithelial-mesenchymal transition; TGF β 1, transforming growth factor β 1; FN, fibronectin.

Smad signalling pathway may be a therapeutic target to prevent EMT and fibrosis in asthma.

Bronchial asthma is a long-term recurrent chronic immune inflammatory disease in which the airway epithelium is damaged and repaired repeatedly, and it induces both structural and functional changes in the airway wall [13–15]. Bronchial epithelial cells not only serve as a physical barrier but also play an important part in the process of innate immunity and inflammatory responses [16, 17]. Since the bronchioles are constantly exposed to inhaled pathogens from the environment, dysregulation of airway epithelial cell function related to environmental triggers may contribute to the pathogenesis of asthma. Therefore, asthmatic mice and human bronchial epithelial cells (16HBE) were used to explore the effect of Six1 on bronchial epithelial cells and its possible mechanism in airway remodelling in our experiment. We found substantially thickened lung tissue and extensive fibrosis in OVA-sensitized asthmatic mice, concomitant with increased expression of Six1 in bronchus and lung tissue. We also demonstrated that Six1 mRNA and protein was overexpressed in TGF β 1-stimulated 16HBE cells. These results are consistent with our previous predictions.

Accumulating evidence has confirmed that TGF β 1 is one of the most predominant inducers of EMT and fibro-

sis and is significantly increased in asthma [18–21]. In our study, TGF β 1 was used to establish a model of EMT in 16HBE cells. To explore EMT-related changes, the mRNA and protein expression levels of α -SMA and E-cad were detected. α -SMA and E-cad are important markers of mesenchymal cells and epithelial cells, respectively, and changes in their expression can result in changes in the cell morphology [13]. Numerous studies have confirmed that EMT of epithelial cells is a key mechanism in fibrosis, which is one of the main outcomes during the progression of airway remodelling [22]. In the process of EMT, epithelial cells are transformed into mesenchymal cells, which makes the cells invasive, mobile, and resistant [23, 24]. The epithelial cells change from cells forming a cobblestone-like monolayer to movable fusiform cells with increased intercellular spacing at gap junctions. Following EMT activation, EMT markers of phenotype changes, such as ZO-1 and E-cad are downregulated, while α -SMA and vimentin are increased, which suggest that the cells are undergoing EMT [25, 26]. Cells gain migration and invasion abilities, resulting in molecular remodelling and phenotypic changes. These are essential elements in the development of diseases, including asthma [27, 28]. Our findings are consistent with the findings of these previous experiments. We observed morphological changes in

16HBE cells, which changed from cells forming a cobblestone-like monolayer to motile spindle-shaped cells with increased intracellular spacing at gap junctions. We also demonstrated that the EMT in the bronchial epithelial cells was characterized by the expected decrease in E-cad expression and increase in α -SMA expression as well as the associated increase in FN expression at the protein level.

In this study, we focused on the correlation between Six1 and EMT in TGF β 1-stimulated 16HBE cells. The longer the cells were treated with TGF β 1, the higher the expression of Six1 and FN. It can be inferred that Six1 plays an essential role in the progression of EMT. Six1 is one of the homeobox genes discovered in recent years that plays an important role in embryonic development. It is expressed at low levels in normal adult tissues but is overexpressed in a variety of tumours and diseases. A large number of experiments have confirmed that Six1-induced EMT enhances tumour progression, invasion, and metastasis in several malignancies [29, 30]. In respiratory diseases, Six1 overexpression is closely related to the proliferation and migration of lung cancer cells [31]. To further explore the effect of Six1 in 16HBE cells, the Six1 (NM_005982)-GV146 plasmid was used to overexpress Six1 in 16HBE cells. We found that Six1 overexpression promoted an EMT-like phenotype in 16HBE cells, and the morphological changes and marker expression of cells transfected with Six1 were consistent with those of the EMT model treated with TGF β 1. In experiments analysing how Six1-induced EMT in 16HBE cells, we observed the TGF β 1/Smad signalling pathway and found that TGF β 1 and pSmad2 were increased significantly in Six1-overexpressing cells compared with control cells. After using inhibitors targeting the TGF β 1 signalling pathway, the expression of TGF β 1 and pSmad2 decreased significantly. Most importantly, the EMT and fibrosis in Six1-overexpressing 16HBE cells were also obviously inhibited by administration of the TGF β 1 pathway inhibitor. Our study demonstrated that Six1 expression was induced by TGF β 1 and causes TGF β 1 secretion, activating the TGF β 1 receptor and promoting subsequent Smad2 phosphorylation. The TGF β 1/Smad signalling pathway was found to be critical for EMT in bronchial epithelial cells, and specifically targeting the TGF β 1/Smad pathway attenuated Six1-stimulated EMT and FN expression in 16HBE cells.

In conclusion, our results confirm that Six1 promotes EMT in 16HBE cells via the TGF β 1/Smad signalling pathway. These results may enhance our understanding of the

mechanism of airway remodelling in asthma, further demonstrating the potential application of strategies targeting airway remodelling for treatment.

Acknowledgement

We thank Dr. Chong Wang for his advice.

Statement of Ethics

All experimental procedures were approved by the Medical Ethics Committee of the Affiliated Hospital of Qingdao University (QYFY WZLL 25921).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

National Natural Science Foundation of China, grant number 81700029; Natural Science Foundation of Shandong Province, grant number ZR2017MH017; and Qingdao Scientific and Technologic Development Program, grant number 19-6-32-nsh.

Author Contributions

Wenxin Wang, Zhaochuan Yang, Meixiang Li, and Zhenghai Qu designed the studies; Wenxin Wang, Zhaochuan Yang, Meixiang Li, Zhenhong Wang, and Yanchun Shan performed the experiments; Zhenhong Wang and Yanchun Shan analysed the data; and all authors reviewed the paper.

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