



Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en



Original article

Targeted inhibition of Six1 attenuates allergic airway inflammation and remodeling in asthmatic mice



Zhao-Chuan Yang^a, Ming-Ji Yi^a, Yan-Chun Shan^a, Chong Wang^b, Ni Ran^a, Li-Ying Jin^c,
 Peng Fu^a, Xue-Ying Feng^a, Lei Xu^b, Zheng-Hai Qu^{b,*}

^a Department of Child Health Care, The Affiliated Hospital of Qingdao University, Qingdao 266003, PR China

^b Department of Pediatrics, The Affiliated Hospital of Qingdao University, Qingdao 266003, PR China

^c Cerebrovascular Disease Research Institute, The Affiliated Hospital of Qingdao University, Qingdao 266003, PR China

ARTICLE INFO

Article history:

Received 4 October 2016

Received in revised form 24 October 2016

Accepted 30 October 2016

Keywords:

Asthma
 Six1
 Airway inflammation
 Airway remodeling
 Nuclear factor κB

ABSTRACT

Asthma is an inflammatory disease of the airways, characterized by lung eosinophilia, mucus hypersecretion by goblet cells and airway hyperresponsiveness to inhaled allergens. The purpose of this study was to evaluate the effects of Six1 on airway inflammation and remodeling and the underlying mechanisms in a murine model of chronic asthma. Female BALB/c mice were randomly divided into four groups: phosphate-buffered saline control, ovalbumin (OVA)-induced asthma group, OVA + siNC and OVA + siSix1. In this mice model, Six1 expression level was significantly elevated in OVA-induced asthma of mice. Additionally, downregulation of Six1 dramatically decreased OVA-challenged inflammation, infiltration, and mucus production. Moreover, silencing of Six1 resulted in decreased levels of immunoglobulin E and inflammatory mediators and reduced inflammatory cell accumulation, as well as inhibiting the expression of important mediators including matrix metalloproteinase MMP-2 and MMP-9, which is related to airway remodeling. Further analysis indicated that silencing of Six1 can significantly inhibit NF-κB pathway activation in the lungs. In conclusion, these findings indicated that the downregulation of Six1 effectively inhibited airway inflammation and reversed airway remodeling, which suggest that Six1 represents a promising therapeutic strategy for human allergic asthma.

© 2016 Published by Elsevier Masson SAS.

1. Introduction

Asthma is a common chronic inflammatory disease with an incidence that has markedly increased over the past two decades [1,2]. Asthma is characterized by chronic airway inflammation, with the infiltration of T-lymphocytes, eosinophils, and monocytes/macrophages, and is associated with the increased expression of several inflammatory proteins, including cytokines, enzymes, receptors and adhesion molecules [3,4]. In addition to the presence of inflammatory cells in the airway, the airways demonstrate varying levels of structural changes termed airway remodeling [5]. Currently, no effective therapy is clinically available to treat asthma. Therefore, it is important to explore the mechanisms and seek new strategies for the prevention and treatment of airway inflammation and remodeling.

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 [6,7]. Six1 was initially identified as a TGF-β-inducible gene and has been cloned in mouse and human. Six1 is generated in particular by nonhematopoietic cells such as cells of the mesenchymal lineage by stimuli including TGF-β, IL-1β, TNF-α, IL-6, and LPS. Six1 released from these mesenchymal cells targets immune cells to express proinflammatory cytokines and chemokines [8]. 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 Six1 influencing the bone morphogenic protein (BMP) pathway to inhibit pSmad 1, 5, 8 signaling, as well as Six1 influencing the AKT pathway, the AMP activated protein kinase pathway, the TLR4/CD14 pathway, and Na/K-ATPase membrane potential [9]. Six1 has not previously been reported in asthma or allergy but has been studied in embryogenesis, tumor development, cardiac disease, arthritis, and wound healing [10,11]. The predominant effect of Six1 appears to be proinflammatory, although anti-inflammatory effects of Six1 have also been described [12].

* Corresponding author at: Department of Pediatrics, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao 266003, Shandong Province, PR China.
 E-mail address: quzhenghai@163.com (Z.-H. Qu).

Based on this evidence, we hypothesized that Six1 may be involved in the pathogenesis of airway inflammation and remodeling. The purpose of this study was to investigate the therapeutic effect of Six1 in OVA-induced asthma and the underlying mechanisms.

2. Materials and methods

2.1. Animal experimental protocols

Thirty-six healthy female BABL/c mice, 6–8 weeks old, weighing 18–24 g were randomly divided into four groups: phosphate buffered saline control (PBS), OVA-induced asthma group (OVA), OVA+siNC and OVA+siSix1. The asthmatic models were established by OVA. The mice were sensitized on days 0, 7 and 14 by intraperitoneal injection of 20 mg OVA emulsified in 1 mg of aluminum hydroxide in a total volume of 0.2 mL 7 days after the last sensitization, the mice were exposed to 1% OVA aerosol for up to 30 min every other day for 7 days. The 1% OVA aerosol was generated by a compressed air atomizer driven by filling a perspex cylinder chamber (diameter 50 cm, height 50 cm) with a nebulized solution. The mice in the PBS group were treated with PBS instead of OVA. To assess the effect of Six1 on pulmonary inflammation and fibrosis, mice received a single i.n. instillation of 30 μ L of a lentiviral vector expressing siSix1 and GFP or GFP alone (5×10^8 lentiviral particles/mice) 1 day after intratracheal injection of OVA. All the experiments described below were performed in accordance with the regulations of the Center of Animal Experiments of The Affiliated Hospital of Qingdao University.

2.2. Bronchoalveolar lavage fluid analysis

At 24 h after the last challenge, bronchoalveolar lavage fluid (BAL) was obtained from the mice under anesthesia using 1 mL sterile isotonic saline. Lavage was performed 4 times in each mouse, and the total volume was collected separately. Cells from BAL fluid were suspended in phosphate-buffered saline and counted, and cytopspins were prepared (2000 rpm, 10 min) and stained with Wright-Giemsa. Differential counts of at least 400 cells were carried out in the high-power field of a microscope, and cells were identified based on their morphologic features.

2.3. Tissue samples

Lungs were removed from the mice after killing 24 h after the last challenge. The tissues from the left lung were directly obtained from the surgical suite and immediately fixed in 10% buffered formalin and then embedded in paraffin. Sections (5 μ m) were prepared and stained with hematoxylin and eosin. Additionally, periodic acid–Schiff staining was performed to identify mucus production in epithelial cells, and the number of positive cells per unit length of basement membrane perimeter was determined. Quantitative analysis was performed blinded as described.

2.4. Immunohistochemistry

The expression of Six1 was assessed by semiquantitative immunohistochemistry. After being deparaffinized, the sections were incubated in 0.01 mol/L citric acid buffer (pH 6.0) for 15 min in a microwave for antigen retrieval. After cooling, the sections were incubated in 3 g/L H₂O₂ for 30 min, to inactivate endogenous peroxidase. After blocking by 1:10 normal horse serum for 30 min, the supernatant was discarded. Primary anti-mouse Six1 (1:300 dilution) was added overnight at 4 °C. Then, biotinylated goat anti-rat secondary antibody and streptavidin horseradish peroxidase were added to the slides and incubated for 30 min at room

temperature. Staining was completed by incubation with diaminobenzidine chromogen solution at room temperature. The stained cells were mounted and viewed under light microscopy.

2.5. Enzyme linked immunosorbent assay (ELISA)

At 24 h after the last challenge, BAL was obtained from the mice under anaesthesia using 1 mL sterile isotonic saline. Lavage was performed four times in each mouse and the total volume was collected separately. The lavage fluid sample was immediately centrifuged at 2000 rpm for 10 min at room temperature, and stored at –80 °C until use. The IL-4, IL-13 and IFN- γ levels were then assayed with ELISA kit according to the manufacturer's instructions.

2.6. Western blotting

Total protein was isolated from the right lung using a lysis buffer and quantified using protein quantification reagents from Bio-Rad. Next, 100 μ g of the protein were suspended in 5 $\times$ reducing sample buffer, boiled for 5 min, electrophoresed on 10% SDS-PAGE gels, and then transferred to polyvinylidene difluoride membranes by electroblotting. The membrane was blocked in 1% BSA/0.05% Tween-20/PBS solution overnight at 4 °C, followed by incubation with the primary antibody for 24 h. A horseradish peroxidase-labeled IgG was used as the secondary antibody. The blots were then developed by incubation in a chemiluminescence substrate and exposed to X-ray films.

2.7. Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical comparisons of the data from the various groups were performed using the Student's *t*-test. Differences between groups were considered statistically significant at *P* < 0.05.

3. Results

We have 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. The expression of Six1 in OVA-induced asthma model

We first examined the expression of Six1 in control and OVA-challenged mice lungs, using western blotting. The results as shown in Fig. 1A, Six1 expression was significantly higher in the lungs of OVA-induced asthmatic mice than in those of PBS-treated mice. We further demonstrated the effect of the knockdown of Six1 on the expression of Six1 determined by western blotting, respectively, in each group. The results showed that Six1 silencing effectively downregulate the expression of Six1 (Fig. 1B and C).

3.2. Knockdown of Six1 alleviated airway inflammatory in OVA-induced asthma model

We further determined the effects of Six1 silencing on OVA-specific IgE by measuring the levels of OVA-specific IgE in the serum. As shown in Fig. 2A, OVA-specific IgE was increased in asthmatic mice, and the downregulation of Six1 resulted in a significant reduction in the levels of OVA-specific IgE. To investigate whether the absence of Six1 regulated the level of inflammatory cytokines after OVA challenge, inflammatory cytokine concentrations in supernatants were measured in BALF. As expected, OVA-challenged mice demonstrated increased

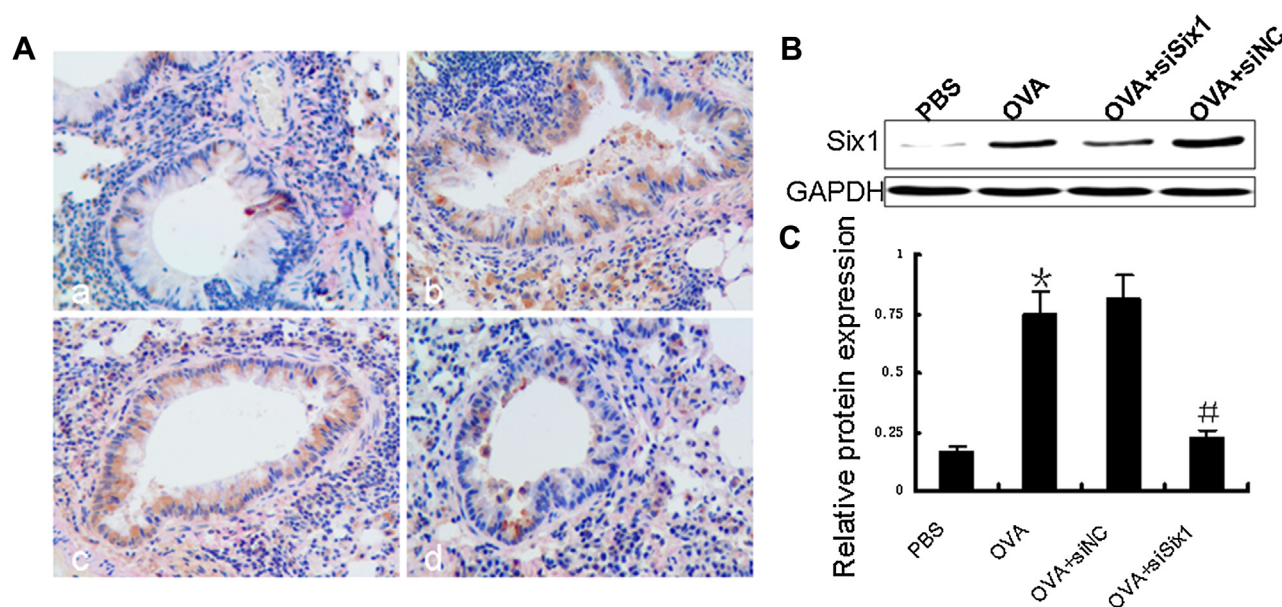


Fig. 1. The expression of Six1 in OVA-induced asthma model. (A) Expression of Six1 in lung tissue was determined by immunohistochemical staining. Positive staining is depicted in brown. Positive staining showed Six1 expression in the epithelium, macrophage leukocytes and in smooth muscle. a, PBS control group; b, chronic asthma group; c, siNC + OVA; d, siSix1 + OVA. (B) Western blotting analysis of Six1 levels in lung tissue. (C) The effect of silencing of Six1 on Six1 protein expression, as determined by western blotting. * $P < 0.05$ vs. PBS, # $P < 0.05$ vs. OVA + siNC.

concentrations of inflammatory cytokines (such as IL-4, IL-13 and IFN- γ) (Fig. 2B). In fact, these inflammatory cytokine concentrations appeared to be higher in OVA-induced mice. Knockdown

of Six1 significantly reduced total cell numbers in bronchoalveolar lavage fluid (BALF). The increase in Eosinophils is generally considered the hall-mark of asthma onset and neutrophils is

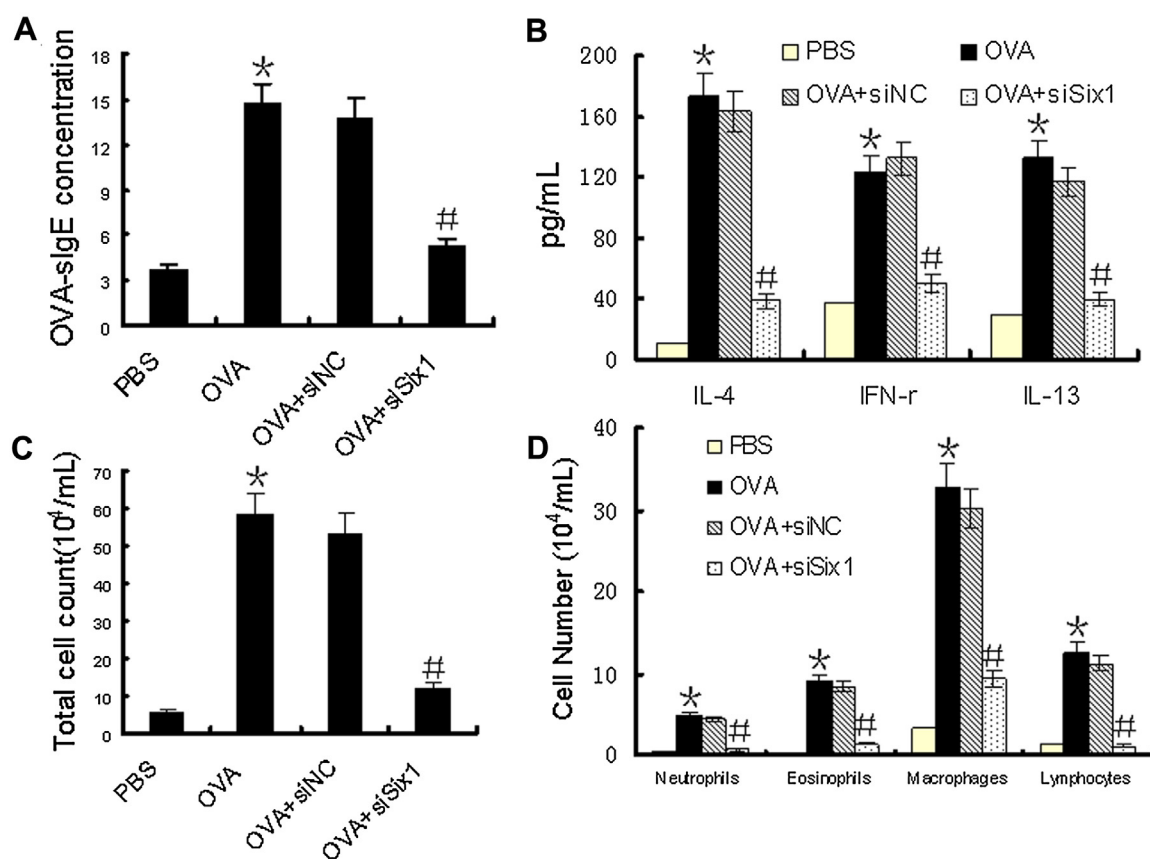


Fig. 2. Knockdown of Six1 reduced OVA-induced airway inflammation. (A) The level of OVA-specific IgE in serum. (B) Effects of Six1 blockade on BALF cytokine concentrations in a murine model of asthma. The concentrations of IL-4, IL-13 and IFN- γ were analyzed using ELISA. (C) Inflammatory cell counts in BALF were obtained from challenged mice after their final OVA challenge. (D) Differential cell counts were performed on a minimum of 400 cells to identify Neutrophils, Eosinophils, Macrophages and Lymphocytes. * $P < 0.05$ vs. PBS, # $P < 0.05$ vs. OVA + siNC.

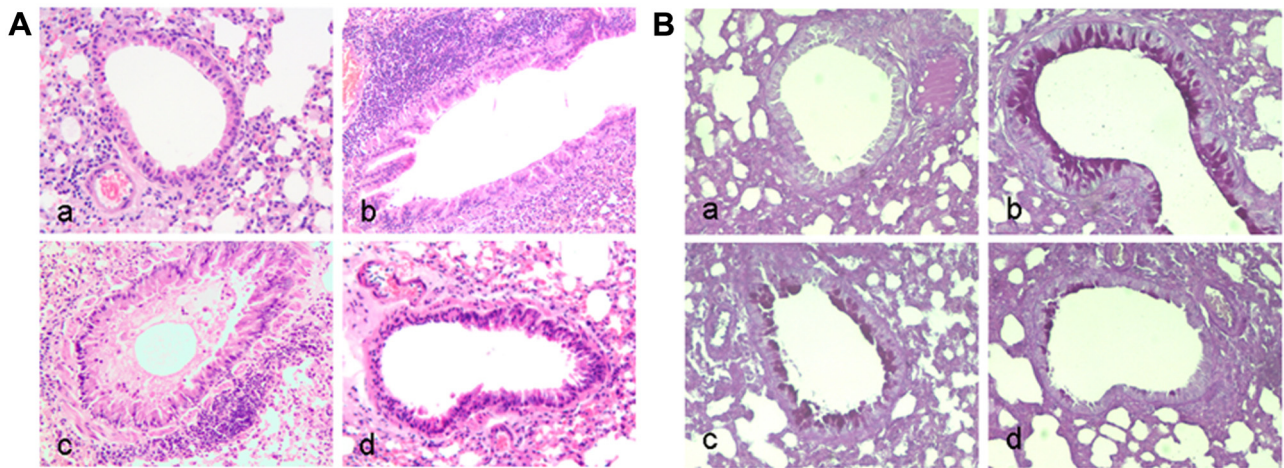


Fig. 3. The effect of Six1 on airway remodeling in an OVA-induced asthma model. (A) Lung tissue sections obtained from mice 24 h after the last OVA challenge were stained with H&E. (B) Periodic acid-Schiff staining for mucus secretion. a, PBS control group; b, chronic asthma group; c, siNC + OVA; d, siSix1 + OVA. all photos were captured at $\times 100$ magnification.

considered the indicator of inflammation. The total number of inflammatory cells, including neutrophils, eosinophils, macrophages and lymphocytes, was increased in the BALF from mice in the OVA-induced asthmatic group compared to PBS control mice. Moreover, Six1 blockade significantly reduced the numbers of neutrophils, eosinophils, macrophages and lymphocytes, in comparison with OVA-challenged mice (Fig. 2C and D). Knockdown of Six1 significantly reduced the level of cytokines. Thus, these results may partly reflect the possibility of promising clinical effects for the Six1 in asthma.

3.3. Knockdown of Six1 relieved airway remodeling in OVA-induced asthma model

Histological analyses showed that OVA-induced inflammation, infiltration, and pathological changes in the lung tissues, and knockdown of Six1 significantly reduced the extent of inflammation and cellular infiltration. In addition, the pathological changes of the lung tissues were milder (Fig. 3A). Furthermore, in the OVA-challenged mice, mucus secretion and goblet cell hyperplasia were clearly observed, as a violet color in the bronchial airways,

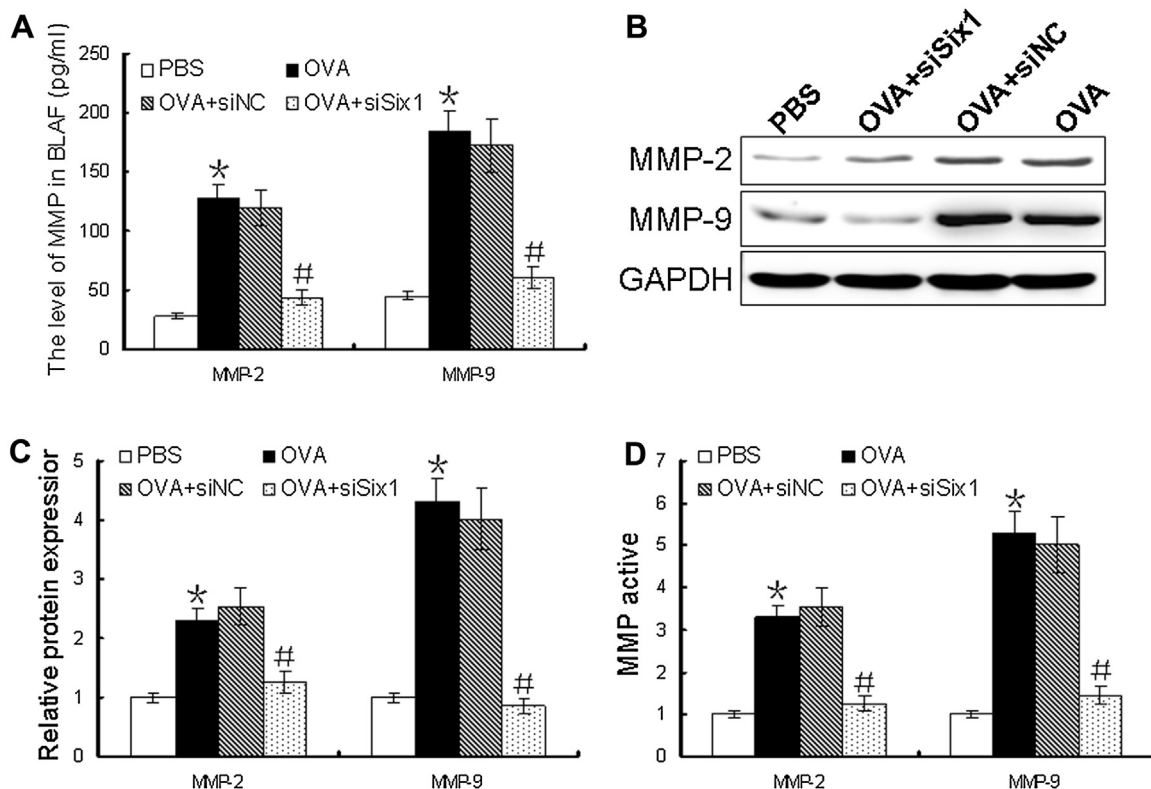


Fig. 4. The effect of Six1 on airway remodeling in an OVA-induced asthma model. (A) Real time PCR analysis of MMP-2 and MMP-9 expression in BALF. (B and C) The protein expression of MMP-2 and MMP-9 in lung tissue, as detected by western blotting. (D) The activities of MMP-2 and MMP-9 were detected by using Biotrak TM activity assay. * $P < 0.05$ vs. PBS, # $P < 0.05$ vs. OVA + siNC.

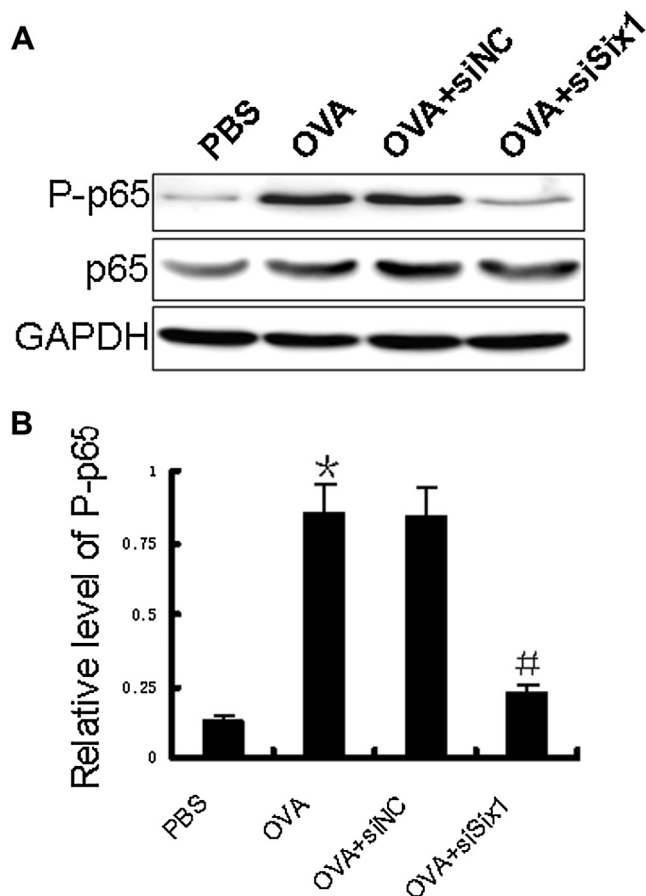


Fig. 5. Silencing of Six1 inhibits NF- κ B signaling pathway. (A) Effect of Six1 on OVA-induced NF- κ B activation. (B) Quantification of band intensity in Fig. A. GAPDH was used as an internal loading standard. * $P < 0.05$ vs. PBS, # $P < 0.05$ vs. OVA + siNC.

compared with the control group. Intervention by Six1 significantly reduced goblet cell hyperplasia (Fig. 3B).

MMPs are a significant pathogenic factor in asthmatic airway remodeling. The level of MMP-2 and MMP-9 (Fig. 4A) were significantly reduced in the BALF from mice in the silencing of Six1 group compared with the mice in the OVA-induced group. We further confirmed the changes in MMP-2 and MMP-9 expression levels by western blot analysis of lung tissue (Fig. 4B and C). In addition, the high MMP-2 and MMP-9 activity detected using Biotrak TM activity assay from the chronic asthmatic mice decreased following the knockdown of Six1 (Fig. 4D). The above results demonstrated that Six1 silencing dramatically impaired airway remodeling in OVA-induced asthma model.

3.4. Silencing of Six1 suppressed NF- κ B pathway activation

It has been reported that OVA-induced airway inflammation was mediated by upregulation of the NF- κ B pathway [13]. To uncover the potential mechanism of inhibitory effects induced by silencing of Six1, the western blotting results showed that p65 was significantly decreased in Six1 silenced group compared to that in control (Fig. 5). Our results showed that silencing of Six1 can significantly inhibit NF- κ B pathway activation in the lungs.

4. Discussion

In the present study, we demonstrated that silencing of Six1 inhibits airway inflammation and remodeling in an OVA-induced mice model of chronic asthma. First, we showed that Six1

expression was significantly higher in chronic asthmatic mice. Moreover, in comparison with OVA-induced asthmatic mice, the knockdown of Six1 exhibited reduced airway inflammation. We also showed that downregulation of Six1 directly affects the expression and secretion of MMP-2 and MMP-9, which are important mediators of airway remodeling. Additionally, we showed that silencing of Six1 can significantly inhibit NF- κ B pathway activation in the lungs. For the first time, our study demonstrated that Six1 may be involved in the pathogenesis of asthma, including airway inflammation and remodeling.

Airway inflammation and remodeling are the two most characterized pathological features of asthma [14,15]. Airway inflammation is a characteristic feature of allergic airway disease, and inflammatory cells that infiltrate the airways are thought to contribute to remodeling by increasing the proliferation and migration of airway SMCs. Resulting from activation of inflammatory cells including eosinophils, neutrophils, macrophages, mast cells, and T lymphocytes, asthma is now generally accepted as a chronic airway inflammatory disorder [16,17]. In this study, we found that the number of these cells was higher in the chronic asthmatic mice than in the PBS-treated control mice, and that the knockdown of Six1 significantly reduced inflammatory cell accumulation. It was believed that the inflammatory mediators and cytokines released by accumulated eosinophils in airway resulted in mucus hypersecretion, increased vascular permeability, bronchial hyper-responsiveness, and smooth muscle contraction [18]. Furthermore, cytokines and growth factors released by inflammatory cells such as neutrophils are also involved in initiation of airway remodeling. A recent study reported that concentrations of Th2 cell-derived cytokines, such as IL-4, IL-5, and IL-13, are elevated in BALF after airway allergen challenge in patients with asthma [19]. The results indicated that silencing of Six1 downregulated the level of inflammatory cytokines. Therefore, we concluded that silencing of Six1, leading to reduce airway inflammation, might exert beneficial effects on the expression of genes associated with lung remodeling.

The pivotal importance of NF- κ B activation in bronchial epithelial cells and immune cells has been demonstrated both in murine models of asthma and in asthma patients [20]. Therefore, NF- κ B has emerged as a promising molecular target for the treatment of asthma. NF- κ B activation within airway epithelium is necessary to fully induce the recruitment of inflammatory cells. NF- κ B activation in airways drives a majority of the Ag-induced inflammation and is a significant contributor to adaptive immune responses and structural remodeling of the airway wall [21]. Its increased activation has been demonstrated in lungs after allergen challenge and in airway epithelial cells and macrophages [22]. Results in this study indicated that OVA significantly increases p65 protein expression in lung tissues, whereas downregulation of Six1 markedly suppresses NF- κ B activity in an OVA-induced model of asthma. These findings suggested that the therapeutic effects of Six1 were correlated with NF- κ B signaling.

In conclusion, this study demonstrated that Six1 plays an important role in the development of OVA-induced chronic airway remodeling in a mice model. Inhibition of Six1 markedly reduces the development of airway inflammation and remodeling that contributes to asthma. The targeted disruption of Six1 may provide a therapeutic rationale for combating obstructive airway diseases such as asthma.

Competing interest

None of the authors have any financial and/or personal relationships with other people or organizations that could inappropriately influence or bias the work.

Funding

This study was supported by the Natural Science Foundation of the Shandong Province (No. ZR2014HP002) and Science and Technology Foundation of the Shandong Province (No. 2010GWZ 20216).

References

- [1] N.S. Lai, T.Y. Tsai, M. Koo, M.C. Lu, Association of rheumatoid arthritis with allergic diseases: a nationwide population-based cohort study, *Allergy Asthma Proc.* 36 (2015) 99–103, doi:http://dx.doi.org/10.2500/aap.2015.36.3871.
- [2] J.C. Cardet, A.A. White, N.A. Barrett, A.M. Feldweg, P.G. Wickner, J. Savage, et al., Alcohol-induced respiratory symptoms are common in patients with aspirin exacerbated respiratory disease, *J. Allergy Clin. Immunol. Pract.* 2 (2014) 208–213, doi:http://dx.doi.org/10.1016/j.jaip.2014.05.003.
- [3] S. Park, M.S. Park, K.H. Jung, J. Song, Y.A. Kim, H.J. Cho, et al., Treatment with pyranopyran-1, 8-dione attenuates airway responses in cockroach allergen sensitized asthma in mice, *PLoS One* 9 (2014) e87558, doi:http://dx.doi.org/10.1371/journal.pone.0087558.
- [4] C. Pilzner, F. Bühlung, T. Reinheckel, C. Chwieralski, A. Rathinasamy, H.D. Lauenstein, et al., Allergic airway inflammation in mice deficient for the antigen-processing protease cathepsin E, *Int. Arch. Allergy Immunol.* 159 (2012) 367–383, doi:http://dx.doi.org/10.1159/000338288.
- [5] B.T. Köksal, O.Y. Ozbek, N. Bayraktar, A.C. Yazici, Evaluation of angiotensin II and 2, vascular endothelial growth factor, and tumor necrosis factor alpha levels in asthmatic children, *Allergy Asthma Proc.* 35 (2014) 482–488, doi:http://dx.doi.org/10.2500/aap.2014.35.3793.
- [6] D. Liu, X.X. Zhang, B.X. Xi, D.Y. Wan, L. Li, J. Zhou, et al., Sine oculis homeobox homolog 1 promotes DNA replication and cell proliferation in cervical cancer, *Int. J. Oncol.* 45 (2014) 1232–1240, doi:http://dx.doi.org/10.3892/ijo.2014.2510.
- [7] D. Liu, L. Li, X.X. Zhang, D.Y. Wan, B.X. Xi, Z. Hu, et al., SIX1 promotes tumor lymphangiogenesis by coordinating TGF β signals that increase expression of VEGF-C, *Cancer Res.* 74 (2014) 5597–5607, doi:http://dx.doi.org/10.1158/0008-5472.CAN-13-3598.
- [8] R. Iwanaga, C.A. Wang, D.S. Micalizzi, J.C. Harrell, P. Jedlicka, C.A. Sartorius, et al., Expression of Six1 in luminal breast cancers predicts poor prognosis and promotes increases in tumor initiating cells by activation of extracellular signal-regulated kinase and transforming growth factor-beta signaling pathways, *Breast Cancer Res.* 14 (2014) R100, doi:http://dx.doi.org/10.1186/bcr3219.
- [9] T. Kawasaki, M. Takahashi, H. Yajima, Y. Mori, K. Kawakami, Six1 is required for mouse dental follicle cell and human periodontal ligament-derived cell proliferation, *Dev. Growth Differ.* 58 (2016) 530–545, doi:http://dx.doi.org/10.1111/dgd.12291.
- [10] E. Li, G. Wang, J. Xiao, J. Ling, Y. Yang, B. Xie, A SIX1 homolog in *Fusarium oxysporum* f. sp. *conglutinans* is required for full virulence on cabbage, *PLoS One* 11 (2016) e0152273, doi:http://dx.doi.org/10.1371/journal.pone.0152273.
- [11] H. Wang, X. Li, H. Liu, L. Sun, R. Zhang, L. Li, et al., Six1 induces protein synthesis signaling expression in duck myoblasts mainly via up-regulation of mTOR, *Genet. Mol. Biol.* 39 (2016) 151–161, doi:http://dx.doi.org/10.1590/1678-4685-GMB-2015-0075.
- [12] L.L. O'Brien, Q. Guo, Y. Lee, T. Tran, J.D. Benazet, P.H. Whitney, et al., Differential regulation of mouse and human nephron progenitors by the Six family of transcriptional regulators, *Development* 143 (2016) 595–608, doi:http://dx.doi.org/10.1242/dev.127175.
- [13] Z.C. Yang, Z.H. Qu, M.J. Yi, C. Wang, N. Ran, N. Xie, et al., Astragalus extract attenuates allergic airway inflammation and inhibits nuclear factor κ B expression in asthmatic mice, *Am. J. Med. Sci.* 346 (2013) 390–395, doi:http://dx.doi.org/10.1097/MAJ.0b013e3182753175.
- [14] F. Liu, N.X. Xuan, S.M. Ying, W. Li, Z.H. Chen, H.H. Shen, Herbal medicines for asthmatic inflammation: from basic researches to clinical applications, *Mediat. Inflamm.* 2016 (2016) 6943135, doi:http://dx.doi.org/10.1155/2016/6943135.
- [15] Y. Zhao, B. Tian, R.G. Sadygov, Y. Zhang, A.R. Brasier, Integrative proteomic analysis reveals reprogramming tumor necrosis factor signaling in epithelial mesenchymal transition, *J. Proteom.* 148 (2016) 126–138, doi:http://dx.doi.org/10.1016/j.jprot.2016.07.014.
- [16] M. Pang, H. Wang, J.Z. Bai, D. Cao, Y. Jiang, C. Zhang, et al., Recombinant rat CC16 protein inhibits LPS-induced MMP-9 expression via NF- κ B pathway in rat tracheal epithelial cells, *Exp. Biol. Med.* 240 (2015) 1266–1278, doi:http://dx.doi.org/10.1177/1535370215570202.
- [17] Z.H. Qu, Z.C. Yang, L. Chen, Z.D. Lv, M.J. Yi, N. Ran, Inhibition airway remodeling and transforming growth factor- β 1/Smad signaling pathway by astragalus extract in asthmatic mice, *Int. J. Mol. Med.* 29 (2012) 564–568, doi:http://dx.doi.org/10.3892/ijmm.2011.868.
- [18] J.W. Lee, H.A. Park, O.K. Kwon, Y.G. Jang, J.Y. Kim, B.K. Choi, et al., Asiatic acid inhibits pulmonary inflammation induced by cigarette smoke, *Int. Immunopharmacol.* 139 (2016) 208–217, doi:http://dx.doi.org/10.1016/j.intimp.2016.07.010.
- [19] J. Zhang, Q. Fu, Z. Ren, Y. Wang, C. Wang, T. Shen, et al., Changes of serum cytokines-related Th1/Th2/Th17 concentration in patients with postmenopausal osteoporosis, *Gynecol. Endocrinol.* 31 (2015) 183–190, doi:http://dx.doi.org/10.3109/09513590.2014.975683.
- [20] K. Mori, T. Fujisawa, H. Kusagaya, K. Yamanaka, D. Hashimoto, N. Enomoto, et al., Synergistic proinflammatory responses by IL-17A and toll-like receptor 3 in human airway epithelial cells, *PLoS One* 10 (2015) e0139491, doi:http://dx.doi.org/10.1371/journal.pone.0139491.
- [21] K. Kurakula, M. Vos, A. Logiantara, J.J. Roelofs, M.A. Nieuwenhuis, G.H. Koppelman, et al., Nuclear receptor $\text{nr}77$ attenuates airway inflammation in mice by suppressing NF- κ B activity in lung epithelial cells, *J. Immunol.* 195 (2015) 1388–1398, doi:http://dx.doi.org/10.4049/jimmunol.1401714.
- [22] M. Contoli, K. Ito, A. Padovani, D. Poletti, B. Marku, M.R. Edwards, et al., Th2 cytokines impair innate immune responses to rhinovirus in respiratory epithelial cells, *Allergy* 70 (2015) 910–920, doi:http://dx.doi.org/10.1111/all.12627.