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To cite this article: Jianting Shi, Ming Chen, Lihua Ouyang, Linjie Huang, Xiaoling Lin, Wei Zhang, Ruiyun Liang, Zhiqiang Lv, Shanying Liu & Shanping Jiang (2017) Airway smooth muscle cells from ovalbumin-sensitized mice show increased proliferative response to TGF β 1 due to upregulation of Smad3 and TGF β RII, Journal of Asthma, 54:5, 467-475, DOI: [10.1080/02770903.2016.1225760](https://doi.org/10.1080/02770903.2016.1225760)

To link to this article: <http://dx.doi.org/10.1080/02770903.2016.1225760>



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Accepted author version posted online: 01 Dec 2016.
Published online: 01 Dec 2016.



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MECHANISMS

Airway smooth muscle cells from ovalbumin-sensitized mice show increased proliferative response to TGF β 1 due to upregulation of Smad3 and TGF β RII

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ABSTRACT

Objective: This study aimed to elucidate the role of Transforming growth factor (TGF)- β 1 signaling in the proliferation of airway smooth muscle cells (ASMCs). **Background:** TGF- β 1 is an important cytokine in airway remodeling in asthma. However, results of studies focusing on the effect of TGF β 1 on proliferation of ASMCs are controversial. **Methods:** An allergic model that mimics airway remodeling in chronic asthma was established and primary ASMCs were cultured. Cell proliferation was detected by viable cell counting and Cell Counting Kit (CCK)-8 analysis. Expression and phosphorylation of Smad3, type 1 TGF β receptor (TGF β RI), type 2 TGF β receptor (TGF β RII), extracellular signal-regulated kinase (ERK)-1/2, p38 mitogen-activated protein kinase (MAPK), C-Jun N-terminal kinase (JNK) and AKT were detected by western blot. siRNAs were used to knock down Smad3 and TGF β RII. **Results:** Smad3 and TGF β RII were up-regulated in primary ASMCs isolated from ovalbumin (OVA)-sensitized mice as compared with ASMCs isolated from unsensitized control mice, which persisted for at least four passages. TGF β 1 stimulated proliferation of ASMCs isolated from OVA-sensitized mice, which was inhibited by specific siRNA targeting Smad3 or TGF β RII. However ASMCs from control mice showed no proliferative response to TGF β 1. TGF β 1-induced proliferation of ASMCs from OVA-sensitized mice was markedly attenuated by PD-98059, a specific ERK1/2 inhibitor. TGF β 1 induced ERK1/2 phosphorylation within 15 minute, which was partially blocked by specific inhibitor of Smad3 (SIS3). **Conclusions:** ASMCs isolated from OVA-sensitized mice showed hyper-proliferation upon TGF β 1 stimulation. This might have been associated with up-regulated Smad3 and TGF β RII and mediated by ERK1/2 downstream to Smad3.

ARTICLE HISTORY

Received 21 April 2016
Revised 7 August 2016
Accepted 14 August 2016

KEYWORDS

Airway smooth muscle cell;
asthma; ERK1/2; Smad3;
TGF β 1

Introduction

Asthma is a chronic inflammatory disorder of the airways. Multiple cells and cellular components are involved in its pathophysiological processes. The frequent occurrence of injury and repair initiated by chronic inflammation could lead to structural changes in airways, such as airway wall thickening, subepithelial fibrosis, increased smooth muscle mass, angiogenesis, and increased mucous glands, which are collectively called airway remodeling [1–3]. The proliferation of airway smooth muscle cells (ASMCs) plays a crucial role in these processes. Enhanced ASMC hyperplasia increases the airway smooth muscle mass and contributes to subepithelial fibrosis and narrowing of the airways [4].

Transforming growth factor (TGF)- β 1 is one of the main factors involved in asthma airway remodeling. It is secreted by several types of cells, such as macrophages,

epithelial cells, fibroblasts, and eosinophils [5]. Elevated levels of TGF β 1 are detected in patients with asthma [3]. It has been proved that TGF β 1 participates in most of the cellular biological processes, leading to airway remodeling, including epithelial changes, subepithelial fibrosis, airway smooth muscle remodeling, and microvascular congestion [3,6].

However, the mechanisms underlying the effect of TGF β 1 on ASMC proliferation are complex, and controversial results have been reported by different investigators. Cohen et al. [7] found that TGF β 1 inhibited mitogen-induced human ASMC proliferation. Shin et al. [8] showed that TGF β 1 had no significant effect on ASMC proliferation. Chen et al. [9] indicated that TGF β 1 stimulated bovine ASMC proliferation through p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)1/2. Chung's group found that TGF β 1 enhanced the proliferative effect of

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fetal bovine serum (FBS), but did not stimulate human ASMC proliferation by itself [4].

The most plausible explanation for the controversial effects of TGF β 1 on ASMCs is the difference in cellular signaling pathways. TGF β 1 signaling can be transduced through numerous downstream signaling pathways, among which, the Smad family has been the most thoroughly investigated. TGF β 1 binds to a complex of transmembrane receptor serine/threonine kinases composed of type 1 TGF β receptor (TGF β RI) and type 2 TGF β receptor (TGF β RII), and induces transphosphorylation of TGF β RI by TGF β RII kinases. Consequently, activated TGF β RI phosphorylates selected Smads (Smad2 and Smad3) at their C-terminal serine residues, and these receptor-activated Smads combine with Smad4 to form activated complexes. Activated Smad complexes translocate into the nucleus, where they regulate the transcription of target genes by binding to DNA at specific Smad-binding elements [10]. In addition to the Smad family signaling pathway, TGF β 1 signaling is also transduced through other pathways, such as ERK1/2, C-Jun N-terminal kinase (JNK), p38 MAPK, and phosphatidylinositol 3-kinase (PI3K) [11–13].

In this study, we established an allergic mouse model that mimicked asthma and compared the effect of TGF β 1 on proliferation of ASMCs isolated from ovalbumin (OVA)-sensitized and control mice. The purpose of this study was to elucidate the role of TGF β 1 and its downstream signaling pathways in the proliferation of ASMCs and asthma airway remodeling.

Materials and methods

Animals

Forty female adult BALB/c mice were obtained and maintained in a pathogen-free environment in the Laboratory Animal Centre of Sun Yat-sen University (Guangzhou, China). All experiments were performed according to the institutional regulations. Ethical approval for this study was obtained from the Animal Research Ethics Committee of Sun Yat-sen University. Mice were housed in a temperature-controlled room with 12-h dark/light cycles. Food and water were allowed *ad libitum*.

Reagents

Chicken egg OVA (grade V, A5503) and specific inhibitor of Smad3 (SIS3) were purchased from Sigma (St Louis, MO); aluminum hydroxide was purchased from Guangzhou Chemical Reagent Factory (China). Chinese hamster ovary (CHO)-derived recombinant human TGF β 1 was purchased from R&D Systems (Minneapolis, MN). Antibodies against total and phosphorylated Smad3 (Ser423/425), p44/42 MAPK (Thr202/Tyr204),

p38-MAPK (Thr180/Tyr182), JNK (Thr183/Tyr185), and AKT (Ser473) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against TGF β RI and TGF β RII were from Millipore (Boston, MA). Selective inhibitors of PI3K (LY-294002), ERK (PD-98059), JNK (SP-600125), and p38 MAPK (SB-203580) were from Selleck (Houston, TX). Annexin V-FITC/PI Apoptosis Detection Kit was from Invitrogen (Carlsbad, CA). Cell culture medium, FBS, and Lipofectamine 2000 reagent were obtained from Gibco/Life Technologies (Grand Island, NY).

Sensitization and antigen challenge

An allergy model that mimicked asthma was established in mice, as described previously by Temelkovski et al. [14] with slight modification. In the OVA group, mice were sensitized and challenged with OVA. They were sensitized by an intraperitoneal injection of 10- μ g OVA emulsified with 1-mg aluminum hydroxide, in a total volume of 200 μ l on days 0 and 14. Mice were exposed to OVA aerosol (2.5% w/v diluted in sterile physiological saline) 7 days after the second sensitization, and then exposed to the aerosol for up to 30 minutes, three times per week for 8 weeks. The 2.0–6.0 μ m aerosol was generated by a nebulizer (Ultrasonic nebulizer boy037G6000, Pari, Starnberg, Germany). In the control group, mice were treated synchronously with physiological saline.

Lung histology

Twenty-four hours after the last challenge, all mice were killed by cervical dislocation under anesthesia. The lungs were removed, fixed with 10% neutral buffered formalin, dehydrated, and embedded in paraffin. The tissues were cut into 5- μ m sections, deparaffinized, and stained with hematoxylin and eosin (H&E) to assess airway remodeling. Images were captured with a digital camera. At least 10 bronchioles with an inner diameter of 100–200 μ m were analyzed on each slide.

Culture of ASMCs

ASMCs were prepared by the explant method from mouse trachea. After killing the mice by cervical dislocation, the tracheas were freed from lung tissue, connective tissue, bronchial vessels, and adherent fat, and the endothelial layer of the intima was removed. The remaining tissues were cut into 1 $\times$ 1 $\times$ 1 mm³ cubes and explanted into culture flasks. Primary cells were then cultured in Dulbecco's Modified Eagle's medium (DMEM)/F12 medium with 20% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified environment with 5% CO₂ at 37°C. Cells were subcultured by continuous passage. ASMCs were identified by the typical "hill and valley" growth

pattern and immunocytochemical staining of α -smooth muscle actin (α -SMA) as previously described [15]. Cells at confluence by the third or fourth passage were used for further experiments.

Western blotting

Whole cell lysates from cultured ASMCs were extracted with RIPA (Radio-immunoprecipitation assay) buffer (Cell Signaling Technology). Transmembrane proteins were extracted with Transmembrane Protein Extraction Kit (Millipore). Proteinase inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets were also added. Protein concentrations were measured using the bicinchoninic acid method. Equal amounts of protein (20 μ g) were separated by 10–12% SDS-PAGE, transferred to PVDF (Polyvinylidene fluoride) membranes, blocked in 5% non-fat milk at room temperature for 1 hour, and incubated with the corresponding antibodies at 4°C overnight. After washing five times in Tris-buffered saline+Tween (TBS-T) solution, membranes were incubated with horseradish-peroxidase-conjugated IgG secondary antibodies, and the antibody-bound proteins were quantified by an enhanced chemiluminescence kit from Millipore.

Cell counting

Cells were seeded at 2×10^4 cells/well in six-well plates, pretreated with specific inhibitors or siRNAs followed by TGF- β 1 treatment for 24, 48 and 72 hours, respectively, and counted on hemocytometers.

CCK8 assay

The proliferation of ASMCs was also measured by CCK-8 reagents (Dojindo, Japan). Cells were seeded in 96-well plates at 5×10^3 cells/well and grown in DMEM/F12 medium with 10% FBS for 24 hours. Ten microliters of CCK8 solution was added to 100- μ l culture medium and incubated at 37°C for 4 hours. The optical density (OD) was determined by a microplate reader (MN 3663; Molecular Devices, Sunnyvale, CA) with a reference wavelength of 450 nm.

siRNA transfection

siRNA for Smad3 (siRNA ID no. 156946), TGF β RII (siRNA ID no. 64894), and control (siRNA-1 AM4611) were purchased from Ambion (Austin, TX). Cells were plated in six-well culture plates at 1×10^4 cells/well overnight. The culture medium was replaced with 2-ml Opti-MEM I medium (Gibco, Invitrogen, China). ASMCs were transfected with 200-nM siSmad3, siTGF β RII, or control siRNA by Lipofectamine 2000 reagent.

Flow cytometry analysis of apoptosis

Cells were trypsinized, resuspended in PBS (Phosphate buffer saline), and centrifuged at 150 g for 5 minutes. After washing twice with PBS, 5-ml FITC-conjugated Annexin V and 5-ml PI were added to the suspension, and cells were incubated for 15 minutes in the dark at room temperature. The samples were analyzed by a flow cytometer within 30 minutes after staining (FACScalibur; BD Biosciences, Heidelberg, Germany).

Statistical analysis

Experiments in this study were performed at least three times. Data were analyzed by GraphPad Prism version 5 (GraphPad Software, San Diego, CA). Results were expressed as mean $\pm$ SEM. One-way analysis of variance and Dunnett's multiple comparison tests were adopted. Two-side *P* values were subject to a significance level of 5%.

Results

Establishment of mouse allergic model

Successful establishment of an allergic model that mimicked asthma was verified by the signs and lung histology after challenge. Mice sensitized to OVA demonstrated cough, tachypnea or cyanosis, and typical changes in lung histology, including inflammatory cell infiltration and airway remodeling as shown in Figure 1.

Smad3 and TGF β RII were upregulated in ASMCs from OVA-sensitized mice

Primary ASMCs obtained from tissue explants of OVA-sensitized and control mice were identified by their characteristic "hill and valley" appearance with positive staining of α -SMA under phase-contrast fluorescence microscopy (Figure S1). No apparent morphological difference was detected between ASMCs from OVA-sensitized and control mice (Figure 2a). Expression of Smad3 and TGF β RII was significantly upregulated in ASMCs from OVA-sensitized mice as compared with ASMCs from control mice, and these changes were sustained for at least four passages (Figure 2b). There were no apparent changes in the expression of TGF β RI.

TGF β 1 stimulated proliferation of ASMCs from OVA-sensitized mice

TGF β 1 stimulated proliferation of ASMCs from OVA-sensitized mice in a dose-dependent manner and peaked at 10 ng/ml (Figure 2c). No proliferative response was

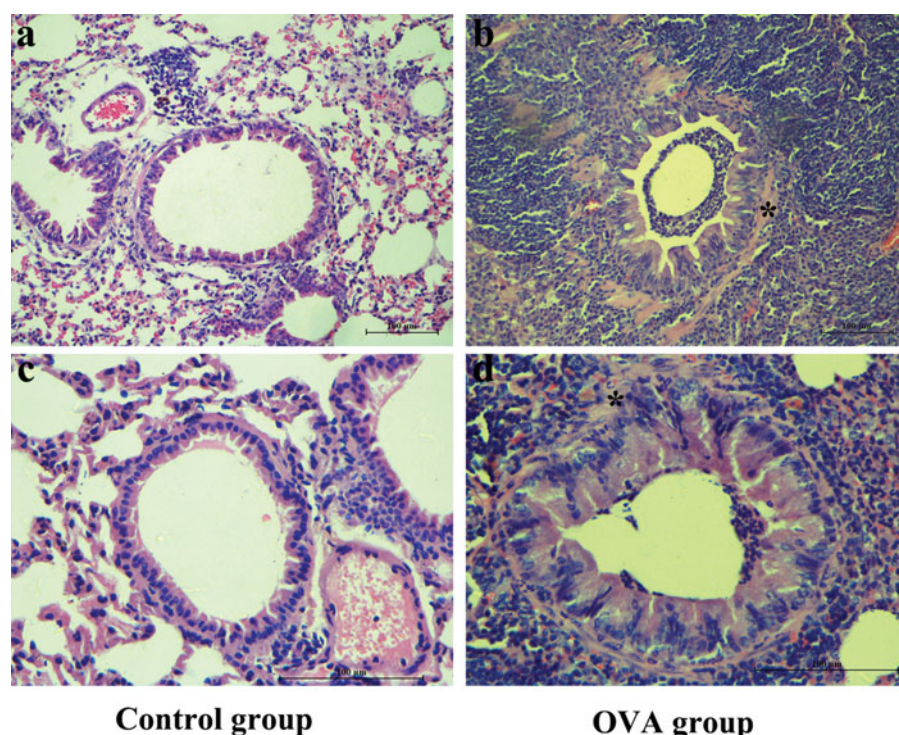


Figure 1. Histology of lung tissue (H&E stain). Twenty-four hours after the last challenge, lungs were removed from the mice after death, fixed, and stained. Representative photomicrographs of control group at (a) 20 $\times$ and (c) 40 $\times$ magnification and OVA group at (b) 20 $\times$ and (d) 40 $\times$ magnification. There was notable inflammatory cell infiltration, airway wall thickening and increased smooth muscle in OVA-sensitized mice. *Proliferation of smooth muscle cells.

observed in ASMCs from control mice upon stimulation with 1–20 ng/ml TGF β 1. No difference in apoptosis rates was detected between the two types of ASMCs or in the presence or absence of TGF β 1 (Figure 2d).

RNAi of Smad3 or TGF β RII attenuated proliferative effect of TGF β 1 in ASMCs from OVA-sensitized mice

Gene knockdown efficacy of siRNA was confirmed by Western blot analysis of corresponding target proteins (Figure 3a). Knockdown of Smad3 or TGF β RII was associated with reduced proliferation of ASMCs from OVA-sensitized mice in response to TGF β 1, and the inhibitory effect was more profound with siSmad3 than with siTGF β RII (Figure 3b). Knockdown of Smad3 or TGF β RII did not affect the apoptosis rate in ASMCs from OVA-sensitized mice (Figure 3c).

TGF β 1 induced ASMCs proliferation through Smad3/ERK1/2

Phosphorylation of ERK1/2 was significantly increased at 15 minutes after TGF β 1 stimulation in ASMCs (Airway smooth muscle cell) isolated from OVA-sensitized mice, but not in ASMCs from control mice (Figure 4a). No significant difference in the expression or phosphorylation

of p38MAPK, JNK, or AKT was detected between these groups. Phosphorylation of ERK1/2 was totally inhibited by the specific inhibitor of ERK1/2, PD-98059, at 30 μ M, and partially inhibited by SIS3, a specific inhibitor of Smad3, at 10 μ M (Figure 4b). This suggests that ERK1/2 is a signaling molecule downstream of Smad3. Specific inhibitors of PI3K (10 μ M LY-294002), ERK (30 μ M PD-98059), JNK (50 μ M SP-600125), and p38 MAPK (10 μ M SB-203580) were added to the culture medium of ASMCs from OVA-sensitized mice 30 minutes before TGF β 1 stimulation to explore further the involvement of these pathways in TGF β 1 signaling. TGF β 1-induced proliferation of ASMCs from OVA-sensitized mice was only inhibited with PD-98059 treatment (Figure 4c). Apoptosis of ASMCs was not affected by PD-98059 (Figure 4d). These results suggest collectively that ERK1/2 is the signaling molecule involved in the proliferative effect of TGF β 1 in ASMCs from OVA-sensitized mice, acting downstream of Smad3, while p38MAPK, JNK, and PI3K/AKT may not participate in the process.

Discussion

We showed that endogenous Smad3 and TGF β RII expression was increased in ASMCs isolated from OVA-sensitized mice compared with ASMCs from control

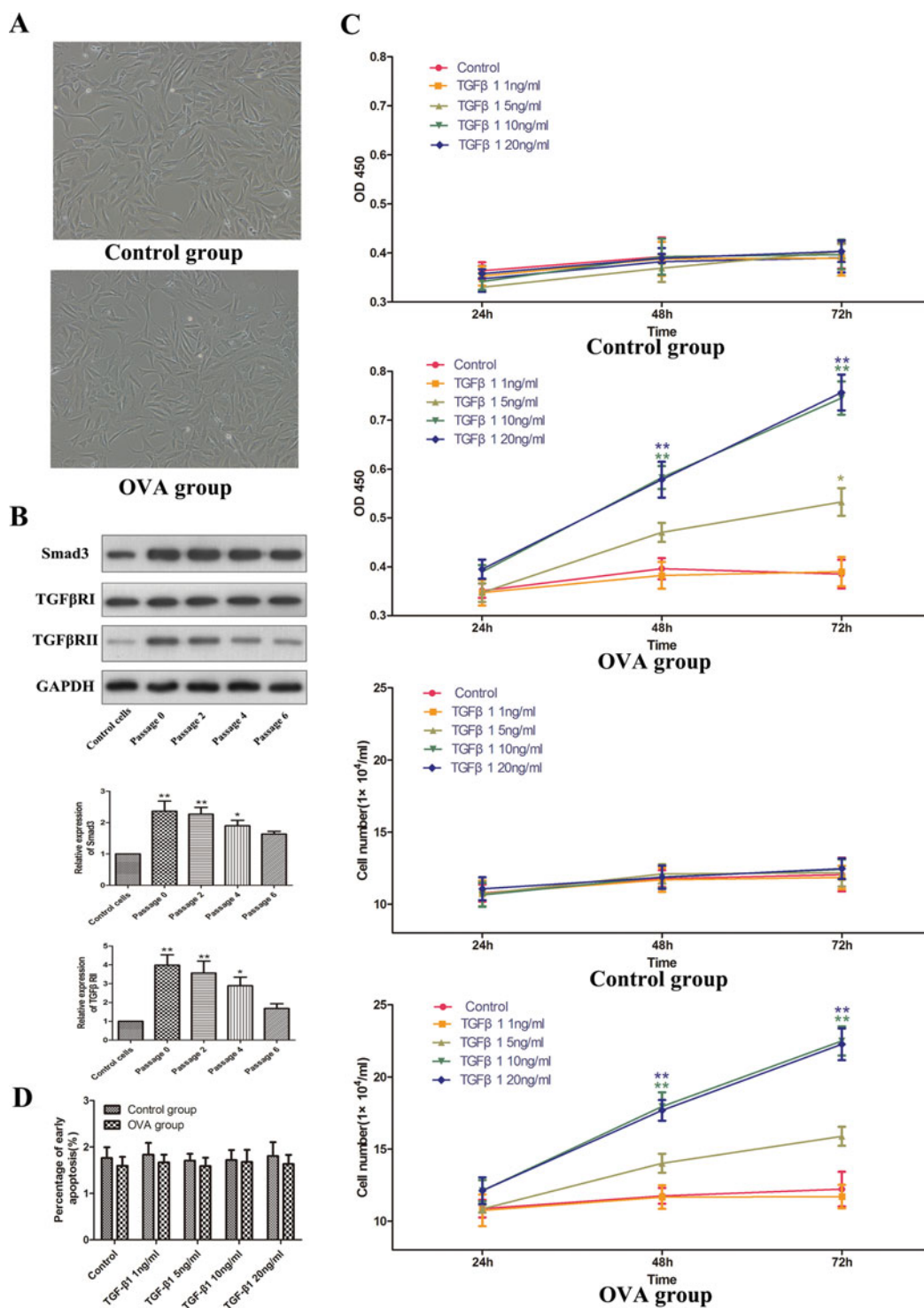


Figure 2. Culture of ASMCs and different response to TGF β 1 stimulation between ASMCs from OVA-sensitized and control mice. (a) ASMCs displayed the characteristic “hill and valley” appearance (phase contrast, 100 $\times$). There was no apparent morphological difference between these two types of cells. (b) Western blot analysis showed markedly elevated expression of Smad3 and TGF β RII but not TGF β RI in ASMCs from OVA-sensitized mice, which was sustained for at least four passages. Control cells: primary cultured ASMCs from healthy mice; Passage 0: primary cultured ASMCs from OVA-sensitized mice; Passages 2–6: sub-cultured ASMCs from OVA-sensitized mice. * $p < 0.05$, ** $p < 0.01$ versus control cells. (c) Growth curve of the two types of ASMCs stimulated by different concentrations of TGF β 1, using CCK-8 analysis and cell counting, respectively. These two methods reached similar results. TGF β 1 stimulated ASMC proliferation from OVA-sensitized mice dose dependently and reached maximal efficacy at 10 ng/ml. ASMCs from healthy mice did not respond to stimulation with 1–20 ng/ml TGF β 1. Control: ASMCs cultured without TGF β 1 stimulation. * $p < 0.05$, ** $p < 0.01$ versus controls at the same time point. (d) Results of flow cytometry analysis of apoptosis. Y axis shows the percentage of cells in lower-left quadrant of flow cytometry chart, which represents early apoptosis. There was no difference in apoptosis rate among these four groups ($p > 0.05$). All experiments were repeated three times.

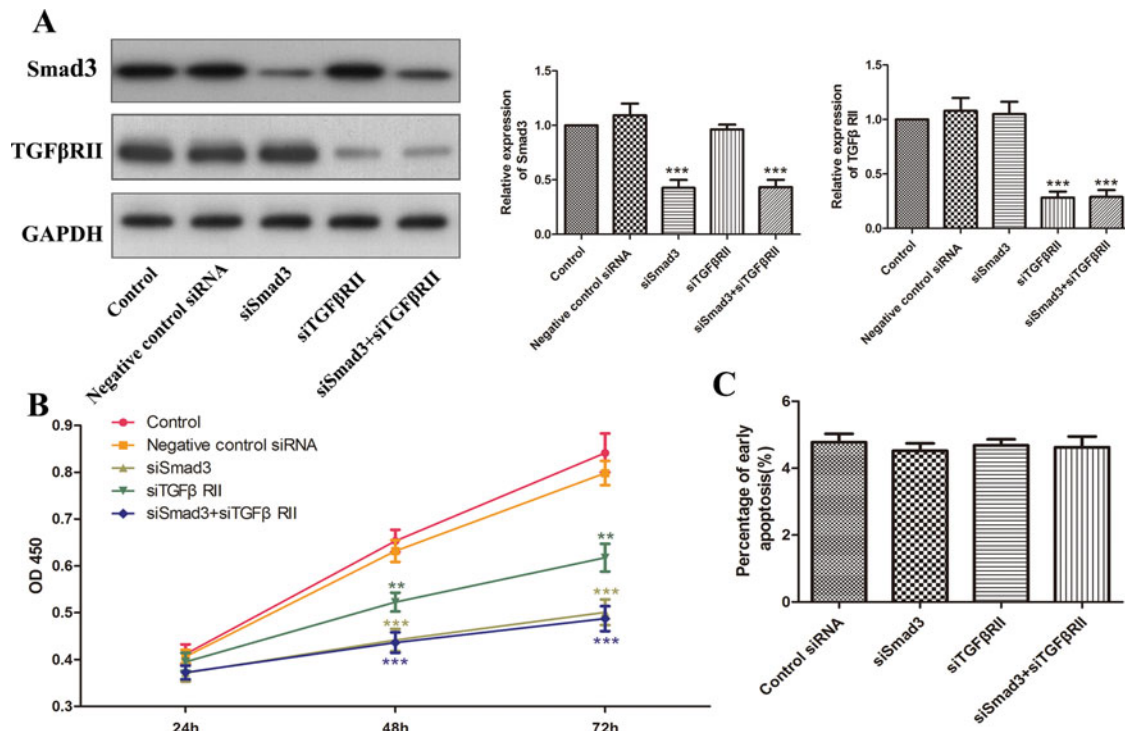


Figure 3. Downregulation of Smad3 or TGFβRII inhibited TGFβ1-induced proliferation of ASMCs from OVA-sensitized mice. (a) Results of Western blot analysis of Smad3 and TGFβRII in ASMCs from OVA-sensitized mice treated with or without siRNA. siRNA of Smad3 and TGFβRII induced silencing of the corresponding gene in 48 hours. *** $p < 0.001$ versus negative control siRNA group. (b) Growth curve of ASMCs from sensitized mice using CCK-8 analysis. TGFβ1-induced proliferation of ASMCs was significantly inhibited by siRNA of Smad3, TGFβRII, or both Smad3 and TGFβRII. ** $p < 0.01$, *** $p < 0.001$ vs negative control siRNA group. (c) Results of flow cytometry analysis of apoptosis. There was no difference in apoptosis rate among these four groups. All experiments were repeated three times.

mice. Downregulation of Smad3 or TGFβRII inhibited TGFβ1-induced proliferation of ASMCs from OVA-sensitized mice. TGFβ1-induced proliferation of ASMCs might be mediated by Smad3/ERK1/2.

The proliferation of ASMCs is one of the most important mechanisms in the airway remodeling of asthma. Increased proliferation of ASMCs isolated from patients with asthma was first found by Johnson et al. [16] *in vitro*. However, increased proliferation of ASMCs was not detected both *in vivo* and *in vitro* in the study of Ward et al. [17]. This discrepancy was not clarified until the study of Hassan et al. [18]. They obtained bronchoscopic biopsies from patients with moderate or severe asthma and healthy controls, and quantified the proliferation of ASMCs by measuring proliferating cell nuclear antigen (PCNA) *in vivo*. They found that the muscle area was 3.4-fold greater, and the fraction of PCNA⁺ nuclei in muscle was fivefold greater in patients with severe asthma compared with healthy individuals. This confirmed the role of ASMC proliferation in airway remodeling in asthma.

TGFβ1 is a crucial mediator in tissue remodeling in asthma and is believed to play an important role in most of the cellular biological processes, leading to airway remodeling [5,19]. However, the mechanisms by which TGFβ1 exerts these effects have not been

clarified. Although TGFβ1 is a profibrotic factor involved in airway remodeling, data concerning its role in cell growth and migration remain controversial. Our results reveal that the response of ASMCs to TGFβ1 stimulation depends on the expression level of Smad3 and TGFβRII. In the basal state, there is less Smad3 expression in ASMCs. The proliferative effect mediated through Smad3-dependent pathways and the anti-proliferative effect mediated through Smad3-independent pathways reach balanced state. ASMCs isolated from healthy mice do not respond to TGFβ1 stimulation. However, expression of Smad3 and TGFβRII increases during chronic inflammation after OVA challenge. In the presence of upregulated Smad3 and TGFβRII, ASMCs undergo a transition, so that their response to TGFβ1 is to undergo strong proliferation. Therefore, up-regulation of Smad3 and TGFβRII, which might be induced by inflammation, appears to be crucial in the proliferative effect of TGFβ1 on ASMCs from OVA-sensitized mice.

In our study, upregulation of Smad3 and TGFβRII in ASMCs induced *in vivo* was sustained for at least four passages during culture *in vitro*. A similar phenomenon was also observed by Chang et al. [20]. Their study revealed that abnormal proliferative response and relative corticosteroid insensitivity could last for up to five cell culture

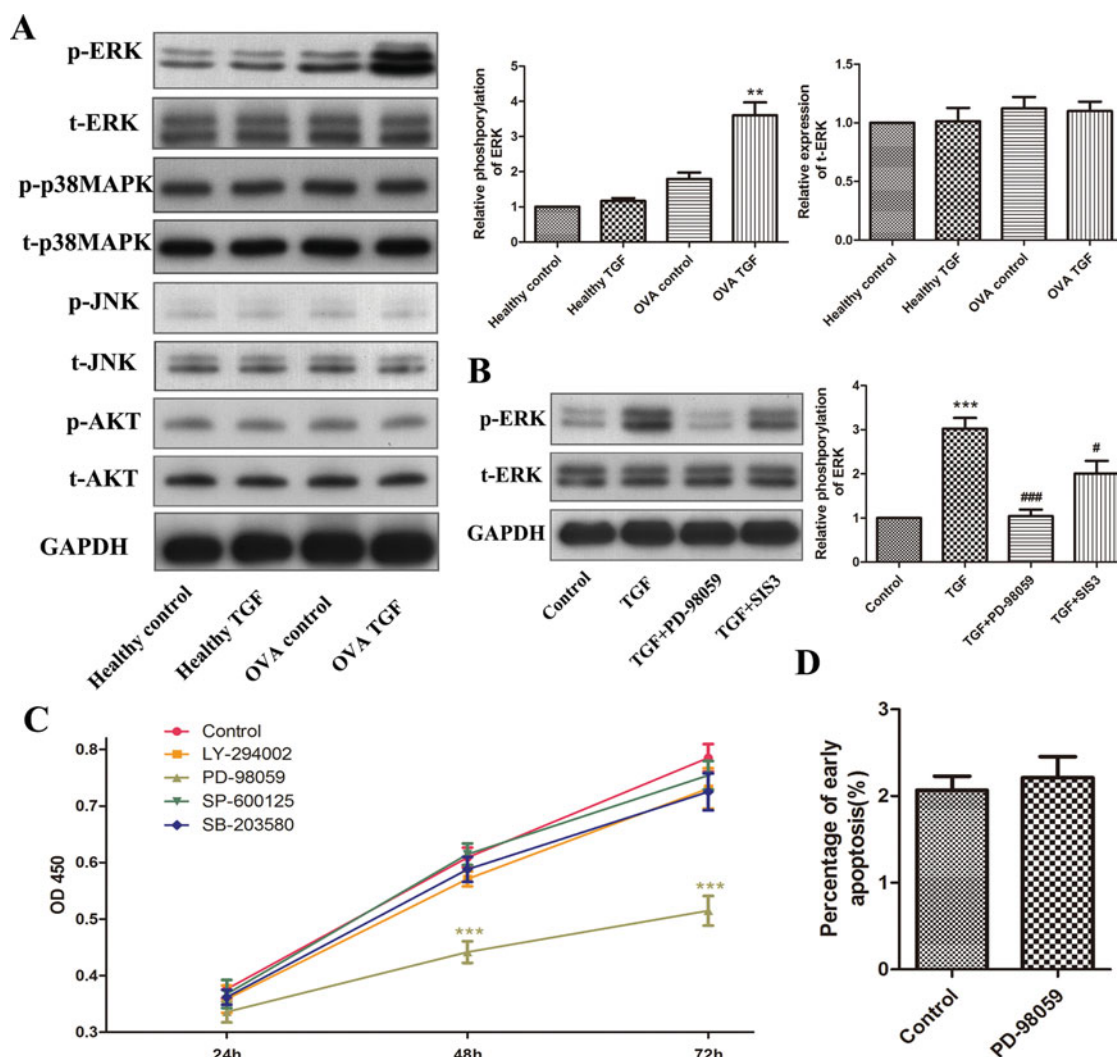


Figure 4. Proliferative effect of TGF β 1 in ASMCs from OVA-sensitized mice was transduced through the Smad3/ERK1/2 pathway. (a) Western blot analysis of phosphorylation of ERK1/2. Phosphorylation of ERK1/2 was significantly enhanced 15 minutes after TGF β 1 stimulation in ASMCs from OVA-sensitized but not healthy mice (** $p < 0.01$ vs. OVA control group). There was no significant difference in expression of total ERK1/2 between these two types of ASMCs ($p > 0.05$). There was no significant difference in expression or phosphorylation of p38MAPK, JNK, or AKT among these groups ($p > 0.05$). (b) Phosphorylation of ERK1/2 was totally inhibited by specific ERK1/2 inhibitor (PD-98059, 30 μ M) and partially inhibited by SIS3 (10 μ M) (*** $p < 0.001$ vs. control group, ### $p < 0.001$, # $p < 0.05$ vs. TGF group). (c) CCK-8 analysis showed the effect of selective inhibitors of PI3K (10 μ M LY-294002), ERK1/2 (30 μ M PD-98059), JNK (50 μ M SP-600125), and p38 MAPK (10 μ M SB-203580) on TGF β 1-induced proliferation of ASMCs from OVA-sensitized mice (*** $p < 0.001$ vs control group). (d) Flow cytometry analysis of apoptosis. Selective inhibition of ERK1/2 did not affect the apoptosis rate of ASMCs from OVA-sensitized mice.

passages, possibly due to altered or rewired intracellular signaling pathways or epigenetic mechanisms [4,21].

Consistent with our former findings, the proliferative effect of TGF β 1 on ASMCs from OVA-sensitized mice is mediated by Smads and ERK1/2 [15,22]. Surprisingly, in contrast to our results, Xie et al. [23] obtained ASMCs by laser capture microdissection of bronchial biopsies from patients with asthma and individuals without asthma, and found that TGF β 1 acted in an autocrine fashion to induce ASMC hyperplasia independent of the Smad pathway. This discrepancy may be due to different experimental settings. For example, Xie et al. [23] transduced

an adenovirus vector expressing Smad7 into ASMCs and found no involvement of Smads in TGF β 1-induced proliferation. However, Smad7 inhibits both Smad2 and Smad3. Although Smad2 and Smad3 are closely related, they are believed to have some important functional differences. TGF β 1 signaling through Smad2 has been shown to inhibit vascular smooth muscle cell (VSMC) proliferation *in vitro* [24]. In our study, the inhibitory effect of siTGF β RII was less than that of siSmad3, which supports this hypothesis. Besides, in the study of Xie et al. [23], ASMCs were obtained from patients with asthma, in whom the etiology of asthma may differ from that in our

OVA-induced allergic asthma. Various forms of asthma may elicit different effects on the airway and trigger different cellular responses [25].

The role of ERK1/2 in airway remodeling, especially ASMC proliferation, has been well studied. For example, some investigators have shown that inhibition of MAPK-1 and 2, which are upstream of ERK1/2, with the specific MEK inhibitor U-0126 or PD-98059, blocks proliferation of human and bovine ASMCs, indicating that ERK1/2 is required for proliferation [9,26]. Similarly, Xie et al. [23] revealed that mitogenic activity of TGF β 1 on ASMCs was inhibited by PD-98059 [23]. Other results have also shown that PD-98059 inhibits phosphorylation of ERK1/2 and consequently attenuates mitogen-induced cyclin D1 protein expression and DNA synthesis in human ASMCs [27].

We showed that TGF β 1-induced proliferation of ASMCs is mediated by ERK1/2 downstream of Smad3. It has been demonstrated that TGF β 1 can activate ERK1/2 through Smad-dependent pathways in several types of cells. For instance, Zhang et al. [28] found that TGF β 1 can activate ERK1/2 in pancreatic acinar cells through a pathway involving Smad4. Similar results have also been found in other cell types [29,30]. Smads are traditionally thought to work through shuttling into the nucleus and regulating related genes. However, in our study, activation of ERK1/2 was observed 15 minutes after TGF β 1 stimulation. Similarly, another study also found that stimulation of VSMCs with TGF β 1 resulted in phosphorylation of ERK1/2 as early as 15 minutes, which was mediated by Smad3, suggesting that Smad3 immediately and directly interacts with ERK [31]. Recently, this hypothesis was confirmed by immunoprecipitation studies revealing direct protein–protein interaction between Smad3 and ERK1/2 in VSMCs [30].

We acknowledge that the status of ASMCs may be altered by culture in flasks and may not reflect exactly the pathophysiological changes in asthma. In our study, the upregulation of Smad3 and TGF β RII faded over time *in vitro*. In this regard, proliferation of ASMCs and expression of Smad3 and TGF β RII should be analyzed *in situ* to confirm our findings in the future.

In conclusion, our findings demonstrate that in the presence of increased expression of Smad3 and TGF β RII, TGF β 1 stimulates ASMCs proliferation through activation of ERK1/2. Airway remodeling in asthma might be related to TGF β 1 secretion and altered expression of signaling molecules in ASMCs.

Acknowledgments

The authors thank coworker of Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation for technical support. We also thank Animal Experiments

Center of Sun Yat-sen University for the experimentation on animals.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

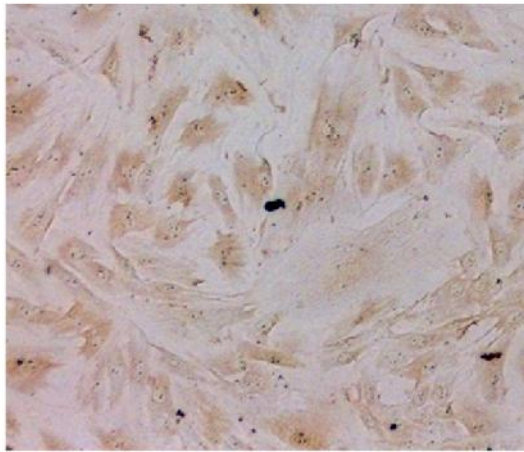
Funding

This work was supported by the National Natural Science Foundation of China (81370120, 81500018) and by the Guangdong Natural Science Foundation (S2013010014803).

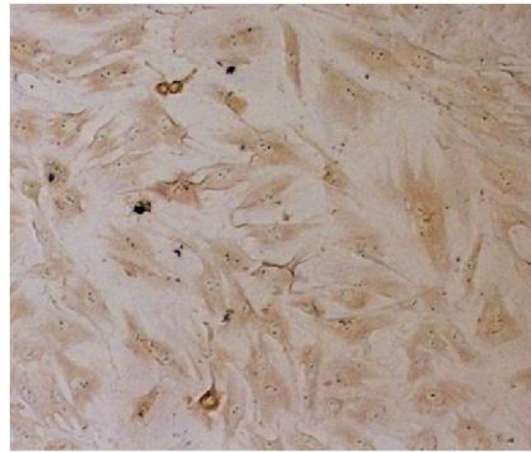
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Control Group



Asthma Group

Figure S1. Culture and identification of airway smooth muscle cells (ASMCs) isolated from control and sensitized mice. Positive expression of α -smooth muscle actin in ASMCs is shown (immunocytochemical staining $\times 200$).