



TGF- β 1-induced airway smooth muscle cell proliferation involves TRPM7-dependent calcium influx via TGF β R/SMAD3 (Revision)

Ming Chen^{a,1}, Wei Zhang^{b,1}, Jianting Shi^a, Shanping Jiang^{a,*}

^a Department of Respiratory Medicine, Sun Yat-Sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong Province 510120, China

^b Department of Respiratory Medicine, Xili People's Hospital, Shenzhen, Guangdong Province 518055, China

ARTICLE INFO

Keywords:

Asthma
Airway smooth muscle cells
TRPM7
TGF- β 1
TGF β R/SMAD3

ABSTRACT

Background: TRPM7 in mast cells plays a key role in asthma. However, the effect of TRPM7 on TGF- β 1-induced airway smooth muscle cell (ASMC) proliferation remains unclear. Therefore, we designed this study to explore whether TRPM7 is involved in TGF- β 1-induced ASMC proliferation.

Methods: An asthmatic mouse model was established, and the expressions of TGF- β 1 and TRPM7 in mice lungs were detected using enzyme-linked immunosorbent assay (ELISA) and western blotting. In addition, murine ASMCs were cultured and stimulated with TGF- β 1. Possible TGF- β 1 and TRPM7 interactions were examined using RT-PCR and western blotting analyses with ASMCs. The effect of TRPM7 knockdown on ASMC calcium influx was assessed by the fluorescent indicator indo-1. MTT and flow cytometry were applied to evaluate the effects of TRPM7 knockdown on ASMC proliferation and apoptosis.

Results: TGF- β 1 elevated the expression of TRPM7 via TGF β R/SMAD3. Knocking down TRPM7 decreased the intracellular Ca^{2+} concentration and cellular proliferation of ASMCs, although apoptosis remained unaffected.

Conclusions: Our initial findings suggest that TRPM7 inhibition may prevent asthma-induced airway remodeling.

1. Introduction

Asthma is a chronic airway inflammatory of the airways involving different cells and cytokines. The chronic inflammation may lead to structural changes in the airways, called airway remodeling (Kim et al., 2018). In the pathophysiology of asthma, airway smooth muscle is a crucial factor for the progression of airway remodeling (Siddiqui et al., 2018).

The cytokine transforming growth factor β 1 (TGF- β 1) has been reported to play an important role in severe asthma (Ojiaku et al., 2017; Chen et al., 2011). In vitro, TGF- β 1 stimulates the proliferation and migration of smooth muscle cells (Pan et al., 2018). It induces the proliferation of cultured airway smooth muscle cells (ASMCs) (Chen et al., 2017a).

The TGF β receptor (TGF β R) is critical for TGF- β 1 signaling and its biological actions. SMADs are a superfamily of signal transduction molecules that can transmit signals from the cell surface receptors to

the nucleus directly (Chen et al., 2016). TGF- β 1 is shown to modulate intracellular SMAD proteins through transmembrane Ser-Thr kinase receptors. Among the SMAD proteins, SMAD3 plays an essential role in the TGF- β 1/TGF β R/SMAD signaling pathway.

The transient receptor potential melastatin 7 (TRPM7) has ion channel and kinase activities (Faouzi et al., 2017). It permeates Ca^{2+} , Mg^{2+} , and some trace metal ions, suggesting a possible function in calcium ion (Ca^{2+}) influx (Gotru et al., 2018). Investigations have reported a role of TRPM7 in cell survival, apoptosis, proliferation and cell cycle progression (Huang et al., 2017; Yang et al., 2017; Beesetty et al., 2018). We have previously reported the upregulation of TRPM7 in asthmatic mice, in which it is known to regulate Ca^{2+} entry (Ng et al., 2012).

It is reported that Ca^{2+} is an intracellular messenger in the TGF- β 1 activation process (Alevizopoulos et al., 1997; Ishiyama et al., 1996). In NIH3T3 cells, TGF- β 1 stimulates Ca^{2+} entry and elevates the intracellular Ca^{2+} concentration [Ca^{2+}]_i, which contributes to the CTF-1

Abbreviations: ASMCs, airway smooth muscle cells; BALF, bronchoalveolar lavage fluid; ROCC, receptor-operated Ca^{2+} channels; SMAD, drosophila mothers against decapentaplegic protein; SIS3, specific inhibitor of Smad3; SOCC, store-operated Ca^{2+} channels; [Ca^{2+}]_i, intracellular Ca^{2+} concentration; NC, negative control; OVA, ovalbumin; TGF- β 1, transforming growth factor- β 1; TGF β R, transforming growth factor- β 1 receptor; TRPC, transient receptor potential channel; TRPM7, transient receptor potential melastatin 7

* Corresponding author.

E-mail address: shanpingjiang@126.com (S. Jiang).

¹ These authors contributed equally to this work.

<https://doi.org/10.1016/j.molimm.2018.09.015>

Received 4 June 2018; Received in revised form 17 September 2018; Accepted 21 September 2018

0161-5890/© 2018 Elsevier Ltd. All rights reserved.

transcriptional activity induction. TGF- β 1 increases the level of $[Ca^{2+}]_i$, which is largely dependent on Ca^{2+} influx in MIN6 insulinoma cells. However, the effects of TRPM7 and the relationship between TRPM7 and Ca^{2+} influx in TGF- β 1/TGF β R/SMAD3 signaling are still unclear.

We aimed to investigate whether TRPM7 is involved in ASMC proliferation induced by TGF- β 1 and elucidate the possible relationship between TRPM7 and Ca^{2+} influx.

2. Material and methods

2.1. Animals

Adult 6- to 8-week-old BALB/c adult female mice were obtained from the Center of Experiment Animal of Sun Yat-sen University (Certificate of Conformity: Guangdong Experimental Animal Testing by certificate No. SCXK (粵) 2017-0029). We conducted all the experiments were performed as previously described (Chen et al., 2017a) in accordance with the regulations of the Center of Animal Experiments of Sun Yat-sen University. Ethical approval for this investigation was obtained from the Research Ethics Committee, Sun Yat-sen University.

2.2. Reagents

Chicken egg OVA (grade V, A5503) and antibodies against TRPM7 were purchased from Sigma (St Louis, MO), and specific inhibitor of SMAD3 (SIS3) and specific blocker of TGF- β receptor (LY2109761) were purchased from MedChemExpress (New Jersey, USA). Aluminum hydroxide was purchased from the Guangzhou Chemical Reagent Factory (China). CHO-derived recombinant human TGF- β 1 and TGF- β 1 ELISA-kit were purchased from R&D Systems (Minneapolis, MN), and the Annexin V-FITC/PI Apoptosis Detection Kit and Lipofectamine 2000 reagent were obtained from Invitrogen (Carlsbad, CA). ECL reagent kit was purchased from Millipore (Boston, MA, USA) and MTT from Chemicon (Temecula, CA, USA).

2.3. Sensitization and antigen challenge

Fifty female BALB/c mice were randomly divided into five groups, each group comprising 10 mice as described (Chen et al., 2017a).

The control group comprised NS sensitization/challenge mice treated with saline (200 μ l) on days 0 and 14. Seven days after the last sensitization, the mice were exposed to saline aerosol for up to 30 min, three times a week, for 8 weeks.

The treatment groups comprised asthmatic mice: group 0 W (OVA sensitization/0 weeks OVA challenge), group 2 W (OVA sensitization/2 weeks OVA challenge), group 4 W (OVA sensitization/4 weeks OVA challenge), group 8 W (OVA sensitization/8 weeks OVA challenge). The asthmatic mice were sensitized with intraperitoneal injections of 10- μ g OVA emulsified in 1-mg of aluminum hydroxide in a total volume of 200 μ l on days 0 and 14. Seven days after the last sensitization, the mice were exposed to ovalbumin aerosol (2.5% w/v diluted in sterile physiological saline) for up to 30 min, three times a week, for 2 (group 2 W), 4 (group 4 W), 8 (group 8 W) weeks. The aerosol (particle size ranging from 2.0 to 6.0 μ m) was generated by a nebulizer (Ultrasonic nebulizer boy037G6000, Pari, Germany) driven by filling a perspex cylinder chamber (diameter, 50 cm; height, 50 cm) with a nebulized solution.

2.4. Lung histology

Twenty-four hours after the last challenge, the mice were sacrificed by cervical dislocation. Both the lungs from each mouse were removed for histologic examination, as previously described (Chen et al., 2017a). Briefly, the left lung tissues were fixed with 10% neutral buffered formalin, and the specimens were rehydrated and embedded in paraffin. Small (5 μ m) sections of the fixed embedded tissues were cut using a

rotary microtome, and the sliced sections were placed on glass slides, before deparaffinizing and staining them sequentially with hematoxylin and eosin. Semi-quantitative determinations based on the immunohistochemical staining (streptavidin-peroxidase, SP) were used to detect TRPM7 levels in the lung tissue, as described elsewhere (Chen et al., 2017a).

2.5. Measurement of TGF- β 1 level in BALF

Twenty-four hours after the last challenge, bronchoalveolar lavage fluid (BALF) was obtained from the mice under anaesthesia using 1 ml sterile isotonic saline, as previously described (Chen et al., 2011). Lavage was performed four times for each mouse and the total volume was collected. The volume of fluid collected in each mouse ranged from 3.0 to 3.5 ml. The lavage fluid was centrifuged at 3000 rpm at 4°C for 10 min. TGF- β 1 concentrations in the BALF were then measured using an ELISA-kit according to the protocol offered by manufacturer's instructions.

2.6. Cell Culture and treatment

The ASMCs were derived from OVA-sensitized mice. ASMCs were prepared by the explant method from trachea and bronchi of BALB/c mice, as described elsewhere (Chen et al., 2017a). Briefly, tracheas and bronchi were dissected and incubated in Hanks' balanced salt solution (HBSS) with 0.1% collagenase solution (Sigma Chemical Co., St. Louis, MO) at 37°C for 20 min. The tissues were cut into 0.5-mm³ sections, and then placed in a DMEM medium containing 20% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin in a 5% CO₂ incubator at 37°C. ASMCs were identified by the typical "hill and valley" growth patterns and by immunocytochemistry staining for α -smooth muscle actin. ASMCs were then cultured in a DMEM medium supplemented with penicillin, streptomycin (as mentioned above), and 10% FBS. The culture medium was changed every 2 days. Confluent cells at passage numbers 3–4 were used for the experiments.

The cells were treated with NC, TGF- β 1 (0, 0.1, 1, 5 and 10 ng/mL), LY2109761 (5 μ M) + TGF- β 1 (10 ng/mL), SIS3 (10 μ M) + TGF- β 1 (10 ng/mL), and shTRPM7 + TGF- β 1 (10 ng/mL) after serum-free starvation of ASMCs for 12 h.

2.7. SiRNA transfections

ShTRPM7 was transfected into ASMCs as detailed elsewhere (Penner and Fleig, 2007). SiRNA oligonucleotides against TRPM7 genes or scrambled sequences were designed and synthesized by the Shanghai Gena Pharma Corporation (Shanghai, China) and contained the following sequences: TRPM7 siRNA (sense: 5'-GGCACCUCUUUAUCAUU AATT-3'; antisense: 5'-UUAAGAUAAAGGUGCCTT-3'); si-control with scrambled sequence (sense: 5'-UUCUCCGACGUGUCACGUTT-3'; antisense: 5'-CGUGACACGUUCGGAGAATT-3'). ASMCs were cultured (1×10^5 cells) in 6-well plates with antibiotic-free DMEM for 24 h and then subjected to transfection with siRNA using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's protocol. The knockdown efficiency was determined by real-time PCR and western blot analyses. Three independent transfection experiments were completed.

2.8. Real-time reverse transcription PCR

Total RNA was isolated from ASMCs using TRIzol reagent (Invitrogen, USA). cDNA was synthesized from total RNA using the ThermoScript RT-PCR synthesis kit (Fermentas, Canada) according to the manufacturer's instructions. Real-time PCR was performed under standard protocols as detailed elsewhere (Chen et al., 2017a), using β -actin (forward: 5'-GGCTGTATTCCTCCATCG-3'; reverse: 5'-CCAG TTGTAACAATGCCATG-3') and TRPM7 (forward: 5'-TCCTGTGACA

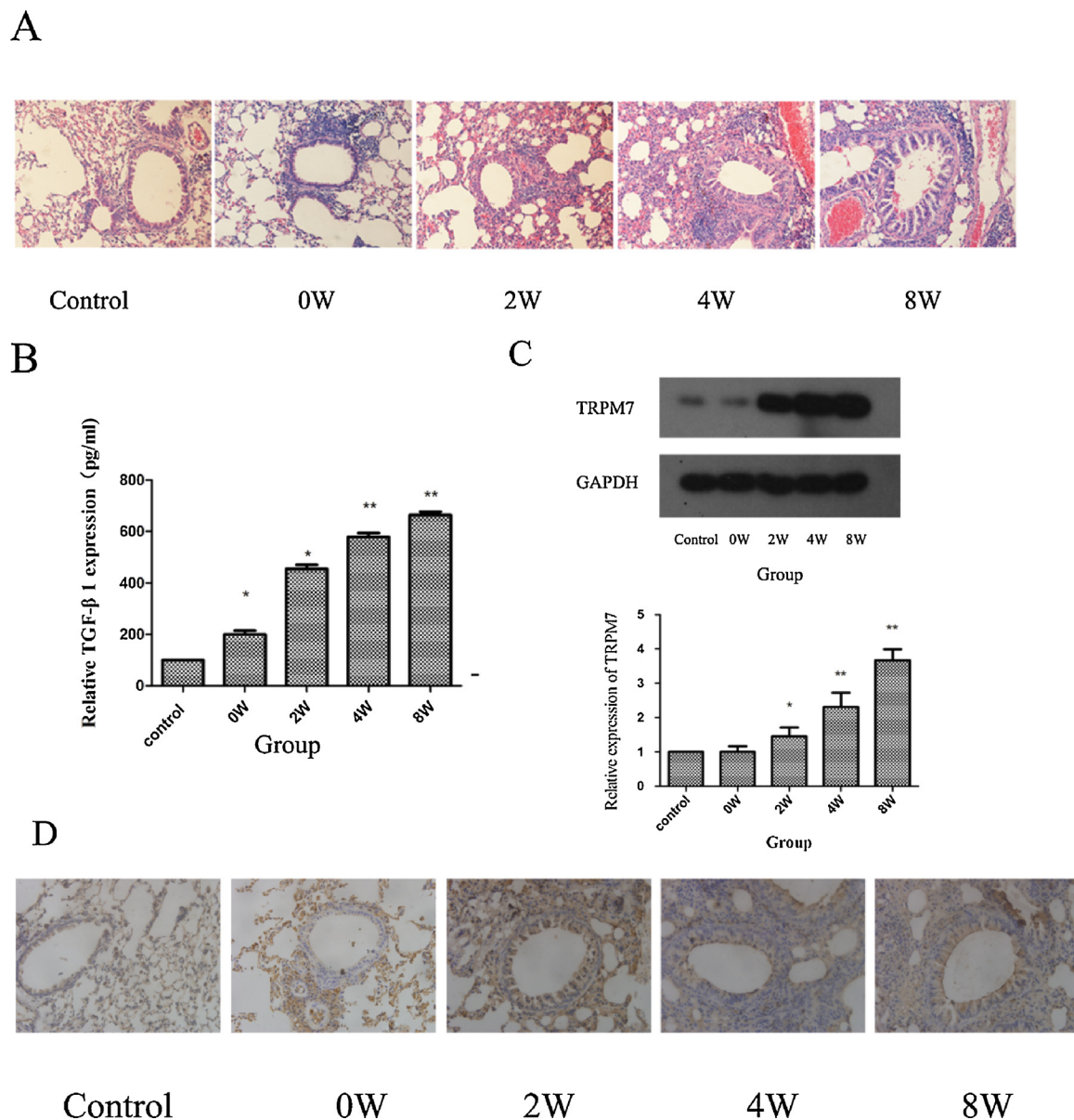


Fig. 1. TGF- β 1 and TRPM7 expression in asthmatic mice. (A) Photomicrographs of HE stain of lung tissue isolated from asthmatic mice at different stages ($\times 200$). Compared with the control group, asthmatic mice showed increased airway remodeling. (B) The time-dependent expression of TGF- β 1 in asthmatic mice was higher than that in control mice ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$, Asthma group versus control group. (C) Increased time-dependent TRPM7 expression in asthmatic mice by time-dependent ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$ versus 0W group. (D) Positive staining showing TRPM7 expression in the epithelium, macrophage, leukocyte and smooth muscle.

TGGTTTGG -3'; reverse: 5'-CAGGCGTAGTCATTCCTCT -3') as primers. Real-time PCR conditions were as follows: 1 cycle at 95°C for 1 min 1 cycle, and 40 cycles of amplification at 95°C for 15 s and at 60°C for 30 s. A melt curve analysis was performed at 95°C for 30 s, 60°C for 30 min, and 95°C for 30 s. Each experiment was repeated at least three times from three independent RNA samples (each sample extracted from at least three lung lobes or wells of ASMCs). The fold-change for mRNA relative to β -actin was determined by the formula: $2^{-\Delta\Delta Ct}$.

2.9. Western blotting

TRPM7 was detected by western blotting as previously described (Chen et al., 2017a). Mice lung tissues and ASMCs were harvested on ice, lysed with RIPA buffer containing 20-mM Tris-HCl (pH 7.5), 150-mM NaCl, 1-mM Na_2EDTA , 1-mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5-mM sodium pyrophosphate, 1-mM beta-

glycerophosphate, 1-mM Na_3VO_4 and 1- $\mu\text{g/mL}$ leupeptin. In addition, proteinase inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets were also added. The BCA method was used to determine the protein concentrations. Equal protein amounts were separated by 10% SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were then blocked for 1 h at room temperature in 5% non-fat dry milk and incubated overnight at 4°C with a special antibody. After washing in TBS-T solution, the membranes were incubated further with horseradish peroxidase-conjugated IgG secondary antibody. The blots were then washed three times in TBS-T, and antibody-bound protein was visualized with an Enhanced Chemiluminescence (ECL) kit.

2.10. Measurement of $[\text{Ca}^{2+}]_i$

Intracellular calcium, $[\text{Ca}^{2+}]_i$, was recorded using a fluorometric

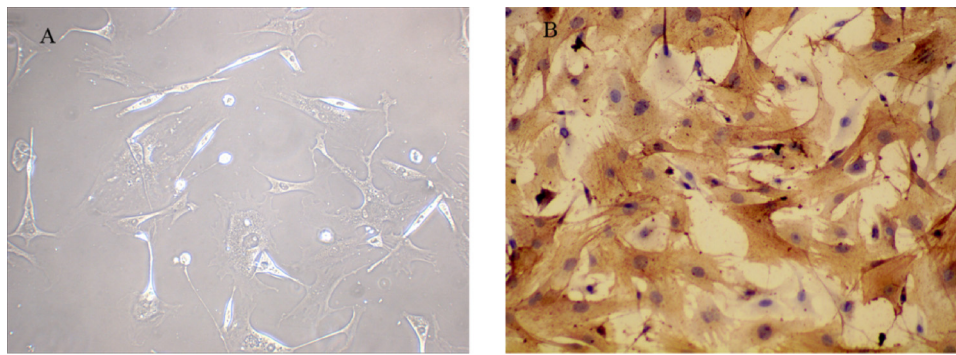


Fig. 2. Culture and identification of ASMCs isolated from mouse. (A) ASMCs displayed the characteristic “hill and valley” appearance (phase contrast microscope $\times 100$). (B) Positive expression of α -actin in ASMCs (immunocytochemical staining $\times 100$).

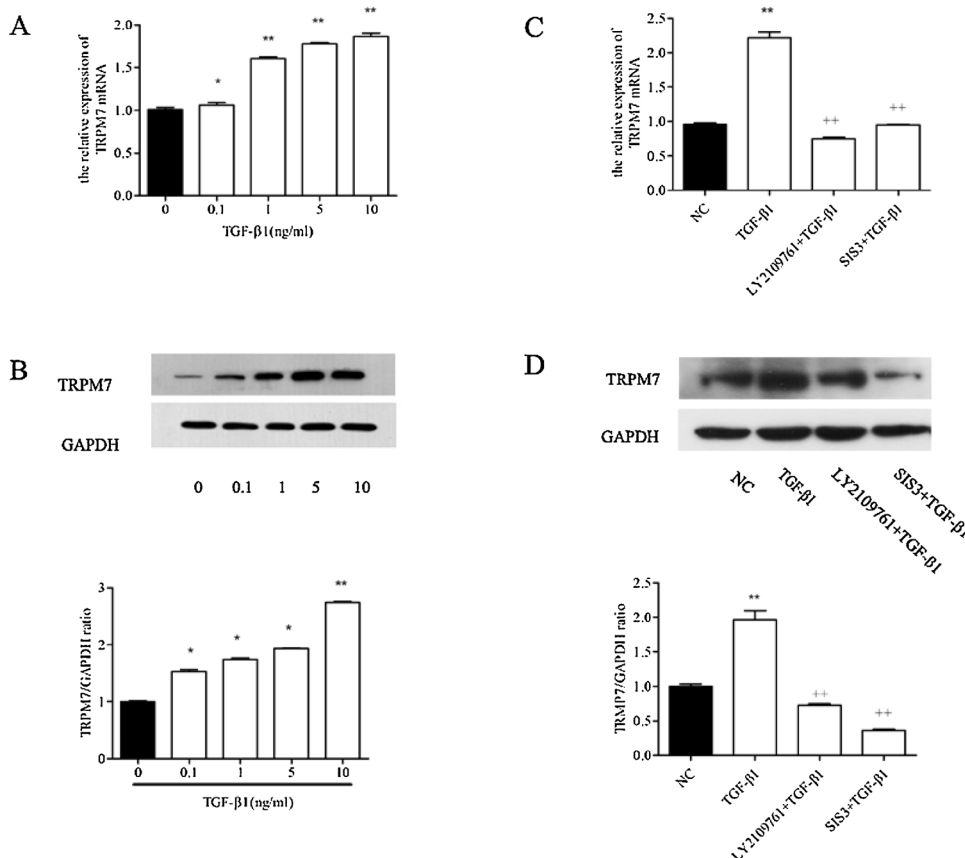


Fig. 3. TGF- β 1 induces the expression of TRPM7 via SMAD signaling in ASMCs. (A and B) Both mRNA and protein TRPM7 levels were elevated in a dose-dependent manner. (C and D) TRPM7 mRNA and protein expressions were effectively suppressed by LY2109761 and SIS3 in ASMCs compared with those in 10 ng/mL TGF- β 1-stimulated cells. * $P < 0.05$, ** $P < 0.01$, versus NC group; + $P < 0.05$, ++ $P < 0.01$, versus TGF- β 1 group. We performed three independent experiments.

ratio technique (indo-1 fluorescence) as described elsewhere (Zhang et al., 2018). ASMCs at 1×10^6 /mL were seeded onto coverslips with different treatments for 24 h. The control group was added the appropriate volume of α -Hank solution. Before detection, the cells were incubated in serum-free culture containing 10-mg/L Fluo3-AM (Dojindo, Kyushu, Japan) and 0.1% F-127 (Dojindo) at 37°C for 30 min. For detection, Ca^{2+} -free extracellular fluid containing 0.1 mmol/L EGTA was added. The slides were fixed to the bottom of the recording dish and then placed in a laser scanning confocal microscope (Leica, Solms, Germany), with an excitation wavelength of 488 nm and a detection wavelength of 515 nm. The Ca^{2+} fluorescence intensity of smooth muscle cells was observed under different conditions at $200 \times$ magnification was observed. The $[\text{Ca}^{2+}]_i$ was calculated based on the Ca^{2+} fluorescence intensity.

2.11. MTT cell proliferation assay

ASMC proliferation was also measured by using MTT assays as described elsewhere (Chen et al., 2015). Cells were incubated with 5 mg/mL MTT (Chemicon, Temecula, CA, USA) in phosphate-buffered saline (PBS) for 4 h at 37°C in 5% CO_2 . Subsequently, 150 μ L of DMSO reagent was added to dissolve the formazan crystals. An ELISA plate reader (MN 3663; Molecular Devices, Sunnyvale, CA, USA) with a reference wave length of 490 nm was used to determine the optical density. Each experiment was performed in triplicate.

2.12. Flow cytometry analysis of cell apoptosis

Different cell samples were observed for cell apoptosis using Flow cytometry as described elsewhere (Chen et al., 2017a). The trypsin-treated cells were centrifuged at 1000 rpm for 5 min. The cell pellets were resuspended with 500 μ L of binding buffer at a concentration of

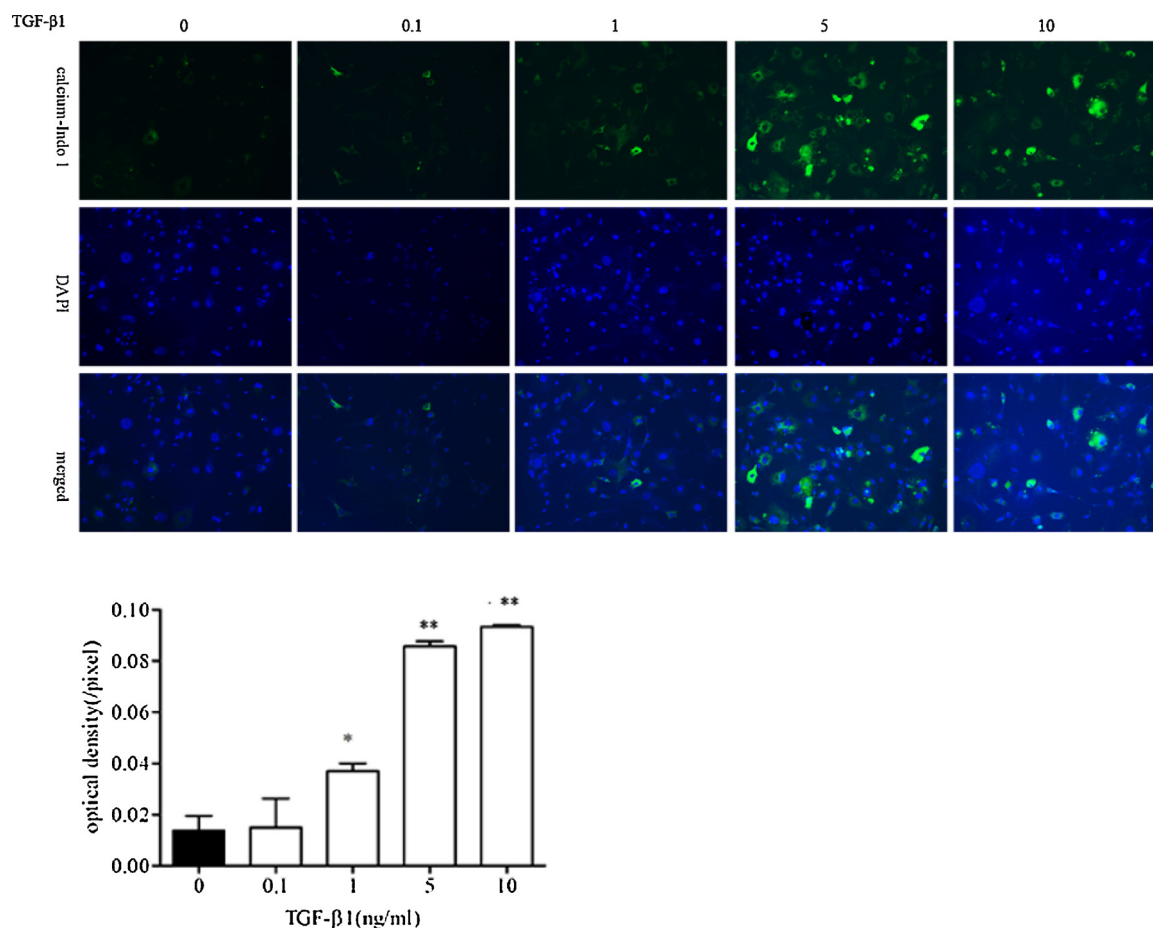


Fig. 4. TGF- β 1 the increased intracellular Ca^{2+} concentration in ASMCs. TGF- β 1 increased $[\text{Ca}^{2+}]_i$ in a dose-dependent manner, but NC did not ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$, versus NC group. Three independent experiments were performed.

10^6 cells/mL, after washing twice with PBS at 1000 rpm for 5 min. Subsequently, 5 mL of FITC-conjugated Annexin V and 5 mL of PI were added to the cells and incubated at room temperature for 15 min in the dark. The samples were analyzed within 1 h after staining. Each experiment was conducted in triplicate.

2.13. Statistical analysis

Data were analyzed using the GraphPad Prism software (version 5, GraphPad Software). Results are expressed as mean $\pm$ SEM. One-way analysis of variance and Dunnett multiple comparison tests were used. P values were two-sided and subject to a significance level of 5%.

3. Result

3.1. TGF- β 1 and TRPM7 expression in asthmatic mice

We successfully established an asthmatic mouse model (Fig. 1A). TGF- β 1 and TRPM7 were expressed in the mice in a time-dependent manner at higher levels than in control mice ($P < 0.05$) (Fig. 1B and C). In Fig. 1D, the positive staining in brown shows TRPM7 expression in the airway smooth muscle, epithelium, and macrophage leukocytes.

3.2. TGF- β 1 induces TRPM7 expression via SMAD signaling in ASMCs

We identified primary ASMC cultures obtained from mice airways by their characteristic “hill and valley” appearance under phase contrast microscope and by positive immunofluorescence staining of α -smooth muscle actin stained brown (Chen et al., 2017a). Both results

confirmed the identity of the cells as smooth muscle cells with identical morphology (Fig. 2).

To study the interaction between TGF- β 1 and TRPM7 in mice ASMCs, we quantified the expressions of TRPM7 mRNA and protein in ASMCs induced by TGF- β 1. In ASMCs treated with 0, 0.1, 1, 5, 10 ng/mL of TGF- β 1, the expressions of TRPM7 mRNA and protein were increased in a dose-dependent manner, as shown in Fig. 3A and B ($P < 0.05$). To explore the rationale for the increase, we used LY2109761 to block the TGF- β receptor. We also used SIS3 to suppress SMAD3 phosphorylation. As shown in Fig. 3C and D, TRPM7 mRNA and protein expressions were effectively inhibited by LY2109761 and by SIS3 in ASMCs compared to the levels in 10-ng/mL TGF- β 1-treated cells ($P < 0.05$).

3.3. TRPM7 knockdown decreases intracellular Ca^{2+} concentration in TGF- β 1 stimulated ASMCs

To uncover the possible relationship between TRPM7 and Ca^{2+} influx, we assessed the intracellular Ca^{2+} concentrations in ASMCs by using fluorescent indicator indo-1. TGF- β 1 increased $[\text{Ca}^{2+}]_i$ in a dose-dependent manner, whereas NC did not, as seen in Fig. 4. LY2109761 and SIS3 significantly decreased $[\text{Ca}^{2+}]_i$ in ASMCs compared to that in TGF- β 1 stimulated cells ($P < 0.05$; Fig. 5). Next, we explored whether knocking down TRPM7 had an impact on the $[\text{Ca}^{2+}]_i$ in ASMCs. ShTRPM7 was transfected into ASMCs. As displayed in Fig. 6, TRPM7 knockdown significantly reduced the $[\text{Ca}^{2+}]_i$ compared to that in NC-stimulated cells significantly ($P < 0.05$) in Fig. 7.

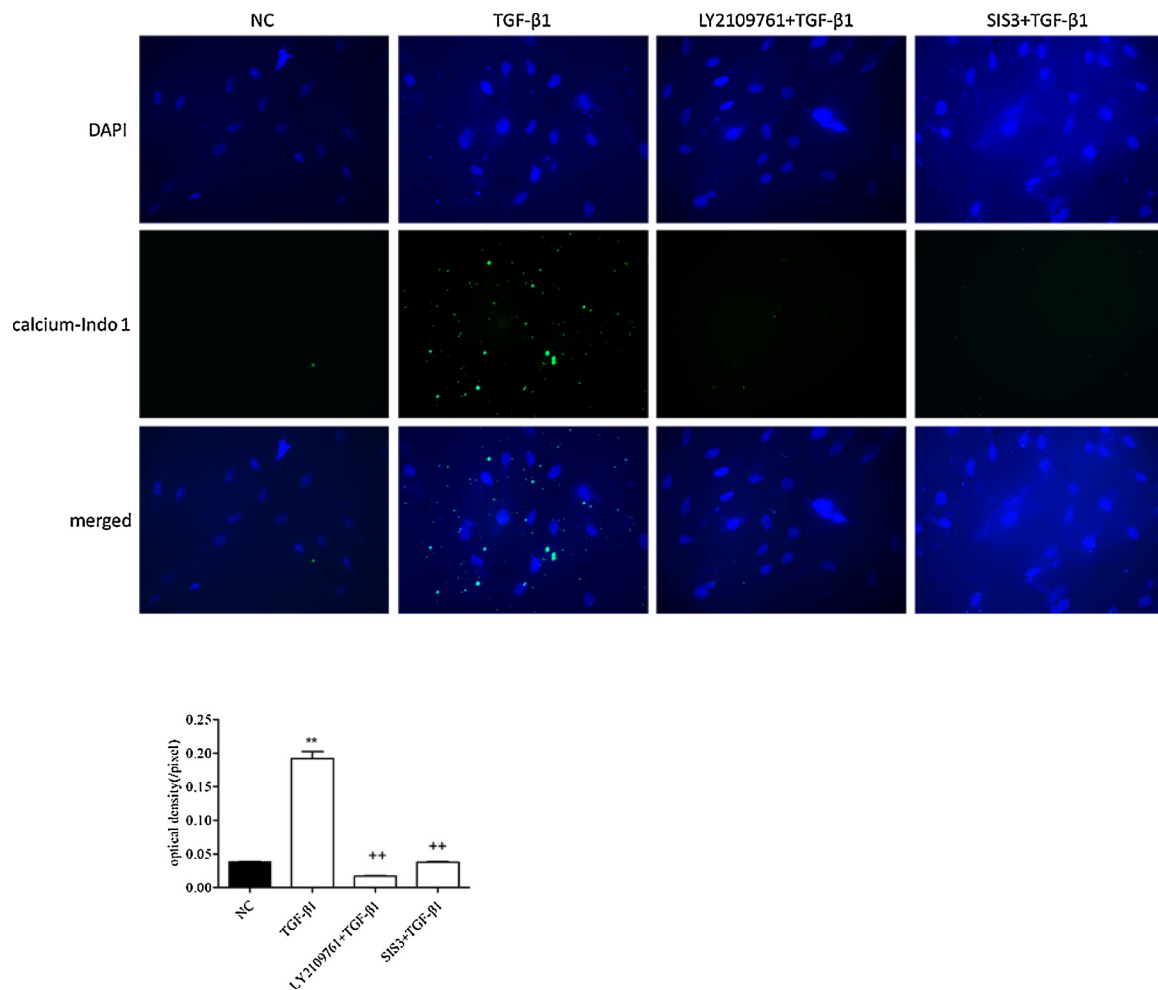


Fig. 5. LY2109761 and SIS3 decrease intracellular Ca^{2+} concentration in TGF- β 1 stimulated ASMCs. LY2109761 and SIS3 decreased $[\text{Ca}^{2+}]_i$ in ASMCs, whereas TGF- β 1 increased it ($P < 0.05$). We found no statistical significance in $[\text{Ca}^{2+}]_i$ when LY2109761 and SIS3 were compared with NC-stimulated cells ($P > 0.05$). * $P < 0.05$, ** $P < 0.01$, versus NC group; + $P < 0.05$, ++ $P < 0.01$, versus TGF- β 1 group. Three independent experiments were performed.

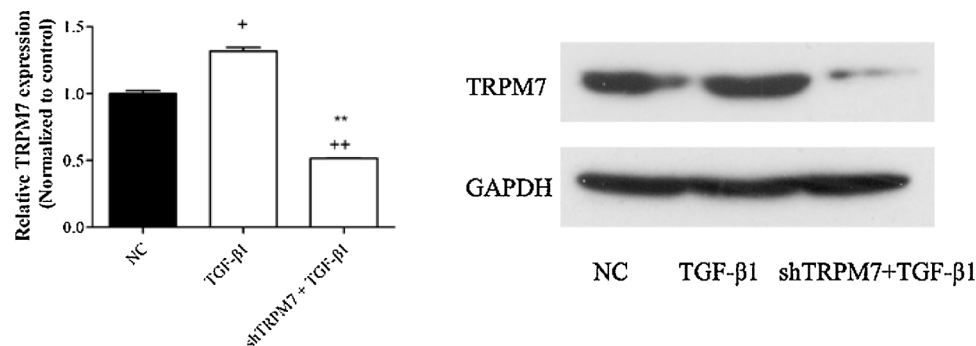


Fig. 6. TRPM7 shRNA knockdown reduced the TRPM7 mRNA and protein expressions in ASMCs. * $P < 0.05$, ** $P < 0.01$, versus NC group; + $P < 0.05$, ++ $P < 0.01$, versus TGF- β 1 group. Three independent experiments were performed.

3.4. Effect of TRPM7 knockdown on TGF- β 1-induced ASMC proliferation and apoptosis

As displayed in Fig. 8A, TGF- β 1 increased, whereas LY2109761 and SIS3 decreased, the ASMC growth rate ($P < 0.05$). TRPM7 knockdown significantly suppressed the growth rate of ASMCs compared with that of TGF- β 1-stimulated cells ($P < 0.05$). Our results indicate that knocking down TRPM7 inhibits ASMC proliferation.

To determine the effect of TRPM7 knockdown on TGF- β 1-induced ASMC apoptosis, we stained the above-mentioned cell samples with

Annexin V-FITC/PI, and applied by flow cytometry. According to results given in Fig. 8B, TGF- β 1 can reduce the apoptosis rate of ASMCs compared to that of the NC group ($P < 0.05$). However, neither TRPM7 knockdown nor SIS3/LY2109761 significantly reduced the apoptosis rate of ASMCs ($P > 0.05$).

4. Discussion

Our study demonstrated that TGF- β 1 increases the expression of TRPM7 in ASMCs through TGF β R/SMAD3 pathway, and that knocking

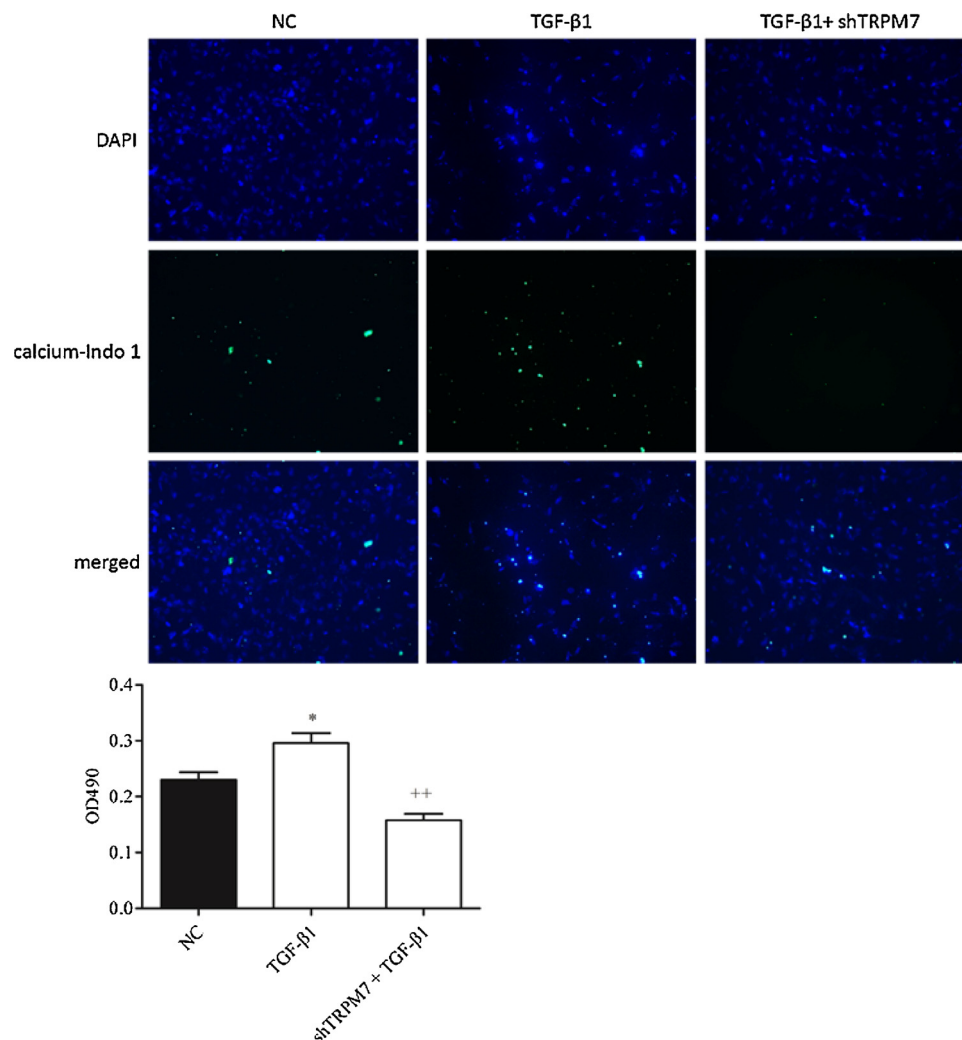


Fig. 7. TRPM7 knockdown decreased intracellular Ca^{2+} concentration in TGF- β 1-stimulated ASMCs. TGF- β 1 increased $[\text{Ca}^{2+}]_i$, whereas NC did not ($P < 0.05$). TRPM7 knockdown significantly reduced $[\text{Ca}^{2+}]_i$ in TGF- β 1-stimulated cells ($P < 0.05$), although no statistical significance was found between those cells and NC-stimulated cells ($P > 0.05$). * $P < 0.05$, ** $P < 0.01$, versus NC group; + $P < 0.05$, ++ $P < 0.01$, versus TGF- β 1 group. Three independent experiments were performed.

down TRPM7 decreases the Ca^{2+} influx and ASMC proliferation without affecting apoptosis. Consequently, TRPM7 may be a potential target for the severe asthma treatment.

TRPM7 is a melastatin-related subfamily of TRP channels (TRPCs) (Park et al., 2014; Kraft and Harteneck, 2005). TRPC genes encode Ca^{2+} channels which are responsible for Ca^{2+} influx (Nilius, 2007; Zhang et al., 2018). TRPM7 is a channel protein permeating diverse metal ions, such as Ca^{2+} and Mg^{2+} , and it possesses an alpha kinase domain in its C-terminal with kinase activity (Penner and Fleig, 2007). Our previous work found that TRPM7 is highly expressed and promotes Ca^{2+} influx in the asthmatic mice (Ng et al., 2012). TRPM7 can be activated by several stimuli, such as low extracellular Mg^{2+} (Yee et al., 2011), PDGF-BB (Fang et al., 2013), epidermal growth factor (EGF) (Gao et al., 2011) and TGF- β 1 (Fang et al., 2014). In this study, we showed that TGF- β 1 induced TRPM7 increased in a dose-dependent manner in ASMCs.

Studies have provided evidence for the role of TGF- β 1 in airway remodeling (Chen et al., 2015). In our study, we have established an asthmatic mice model with airway remodeling that expresses TGF- β 1 and TRPM7 in a time-dependent manner at higher levels than control group. In vitro, LY2109761, a blocker of the TGF- β receptor, abrogated the TRPM7 expression induced by TGF- β 1. Moreover, SIS3 which suppresses SMAD3 phosphorylation, reduced TRPM7 levels. Overall,

Our results suggest that TRPM7 expression may be regulated by the TGF- β 1/TGF β R/SMAD3 signaling pathway at least in part.

Severe asthma is associated with ASMC proliferation, which leads to remodeling and irreversible airway narrowing. ASMC hyperplasia and hypertrophy have been regarded as primary factors leading to irreversible airway obstruction during severe asthma. Intracellular free Ca^{2+} is a second messenger in ASMCs for asthma-related functions, such as contraction, proliferation, apoptosis and cytokine production (Sakai et al., 2017). The Ca^{2+} influx is largely regulated by voltage-independent nonselective cation channels and voltage-dependent Ca^{2+} channels in ASMCs, such as receptor-operated Ca^{2+} channels (ROCC) and store-operated Ca^{2+} channels (SOCC) (Chen and Sanderson, 2017; Boie et al., 2017). Changing the $[\text{Ca}^{2+}]_i$ has a significant effect in the proliferation and metabolism of ASMCs (Chen et al., 2017b). In addition, increased Ca^{2+} entry is a vital cause for ASMC proliferation (Gao et al., 2013). In this study, $[\text{Ca}^{2+}]_i$ increases due to TGF- β 1 stimulation in ASMCs were inhibited by knockdown of TRPM7 with siRNAs. These results are the first to demonstrate that Ca^{2+} influx via TRPM7 stimulates ASMC proliferation, suggesting that the elevated channel activity associated with the disease could contribute to airway remodeling. In addition, we showed that knockdown of TRPM7 attenuates TGF- β 1-induced ASMC proliferation, suggesting that TRPM7 activity is stimulated by TGF- β 1. However, neither TRPM7 knockdown nor SIS3/

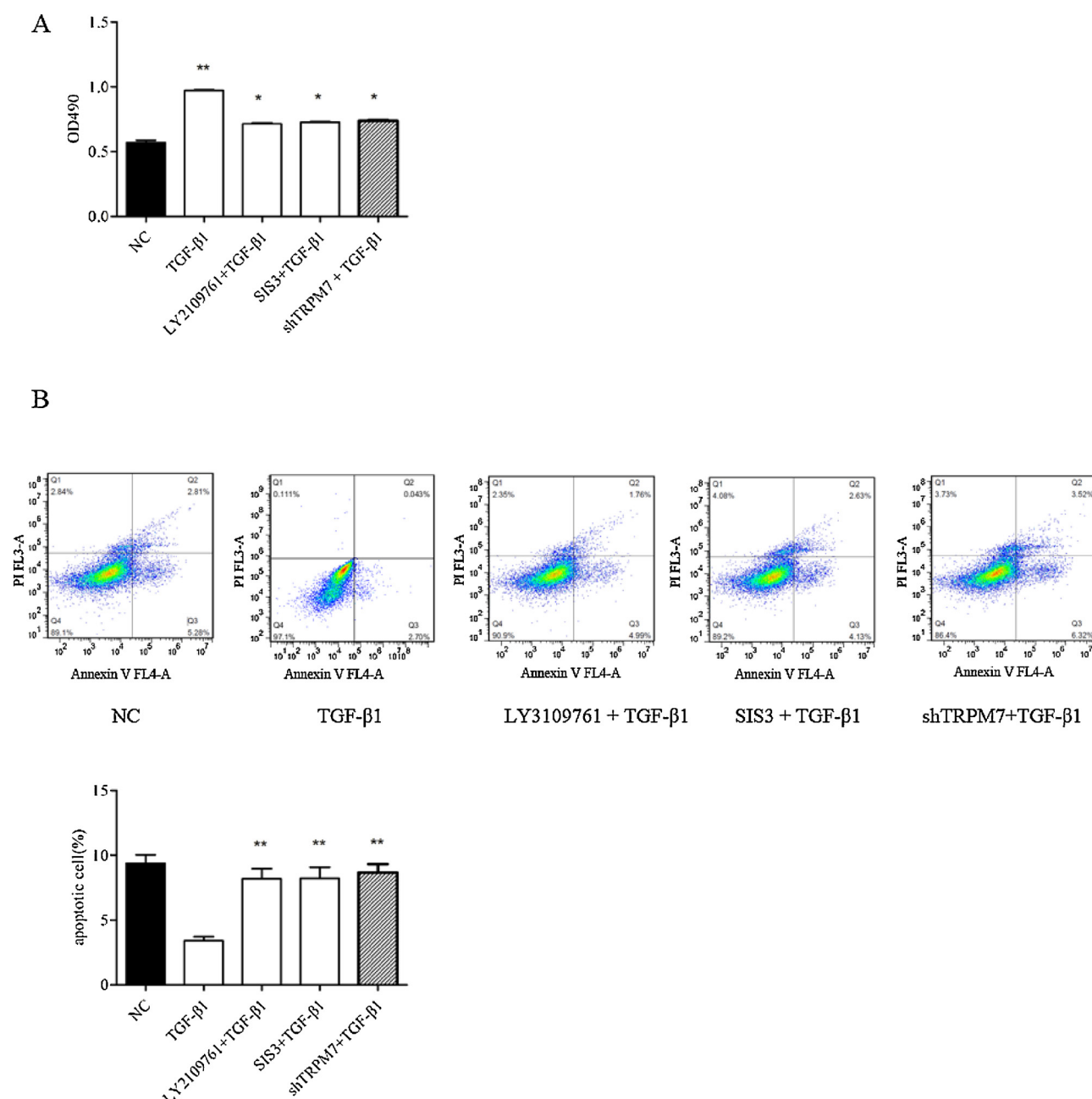


Fig. 8. Effect of TRPM7 knockdown on TGF-β1-induced ASMC proliferation and apoptosis. (A) TGF-β1 increased the growth rate of ASMCs, whereas LY2109761 and SIS3 decreased the growth rates ($P < 0.05$). TRPM7 knockdown significantly decreased the growth rate of ASMCs compared with the wild-type growth rate ($P < 0.05$). (B) TGF-β1 in media reduces the apoptosis rate of ASMCs compared with NC group in media ($P < 0.05$). However neither TRPM7 knockdown nor SIS3/LY2109761 significantly can reduced the apoptosis rate of ASMCs ($P > 0.05$). * $P < 0.05$, ** $P < 0.01$, versus NC group. Three independent experiments were performed.

Mannich reaction was used to functionalize phenolic molecules

LY2109761 reduced the apoptosis rate of ASMCs. Our initial findings support the concept that blocking of TRPM7 could be useful for preventing asthma-induced airway remodeling.

In conclusion, TGF-β1 in ASMCs stimulates TRPM7 expression via a TGFβR/SMAD3 pathway, and TRPM7 knockdown decreases the intracellular Ca^{2+} concentration and cellular proliferation without affecting apoptosis. Therefore, TRPM7 may be a potential target for preventing asthma-induced airway remodeling.

Conflict of interest

The authors have no financial or commercial conflict of interest.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81500018) (81700033) and by the Guangdong Natural Science Foundation (2016A030313332).

References

- Alevizopoulos, A., Dusserre, Y., Rüegg, U., Mermod, N., 1997. Regulation of the transforming growth factor beta-responsive transcription factor CTF-1 by calcineurin and calcium/calmodulin-dependent protein kinase IV. *J. Biol. Chem.* 272 (September (38)), 23597–23605.
- Beesetty, P., Wiczerzak, K.B., Gibson, J.N., Kaitsuka, T., Luu, C.T., Matsushita, M.,

- Kozak, J.A., 2018. Inactivation of TRPM7 kinase in mice results in enlarged spleens, reduced T-cell proliferation and diminished store-operated calcium entry. *Sci. Rep.* 8 (Feb (1)), 3023.
- Boie, S., Chen, J., Sanderson, M.J., Sneyd, J., 2017. The relative contributions of store-operated and voltage-gated Ca^{2+} channels to the control of Ca^{2+} oscillations in airway smooth muscle. *J. Physiol.* 595 (May (10)), 3129–3141.
- Chen, J., Sanderson, M.J., 2017. Store-operated calcium entry is required for sustained contraction and Ca^{2+} oscillations of airway smooth muscle. *J. Physiol.* 595 (May (10)), 3203–3218.
- Chen, Ming, Lv, Zhiqiang, Jiang, Shanping, 2011. The effects of triptolide on airway remodelling and transforming growth factor- β 1/Smad signalling pathway in ovalbumin-sensitized mice. *Immunology* 132 (3), 376–384.
- Chen, M., Lv, Z., Huang, L., Zhang, W., Lin, X., Shi, J., Zhang, W., Liang, R., Jiang, S., 2015. Triptolide inhibits TGF- β 1-induced cell proliferation in rat airway smooth muscle cells by suppressing Smad signaling. *Exp. Cell Res.* 331 (February(2)), 362–368.
- Chen, M., Huang, L., Zhang, W., Shi, J., Lin, X., Lv, Z., Zhang, W., Liang, R., Jiang, S., 2016. MiR-23b controls TGF- β 1 induced airway smooth muscle cell proliferation via TGF β R2/p-Smad3 signals. *Mol. Immunol.* 70 (Feb), 84–93.
- Chen, M., Shi, J., Zhang, W., Huang, L., Lin, X., Lv, Z., Zhang, W., Liang, R., Jiang, S., 2017a. MiR-23b controls TGF- β 1 induced airway smooth muscle cell proliferation via direct targeting of Smad3. *Pulm. Pharmacol. Ther.* 42 (February), 33–42.
- Chen, J., Miller, M., Unno, H., Rosenthal, P., Sanderson, M.J., Broide, D.H., 2017b. Orosomucoid-like 3 (ORMDL3) upregulates airway smooth muscle proliferation, contraction, and Ca^{2+} oscillations in asthma. *J. Allergy Clin. Immunol.* (September (7)) pii: S0091-6749(17)31427-6.
- Fang, L., Zhan, S., Huang, C., Cheng, X., Lv, X., Si, H., Li, J., 2013. TRPM7 channel regulates PDGF-BB-induced proliferation of hepatic stellate cells via PI3K and ERK pathways. *Toxicol. Appl. Pharmacol.* 272 (November (3)), 713–725.
- Fang, L., Huang, C., Meng, X., Wu, B., Ma, T., Liu, X., Zhu, Q., Zhan, S., Li, J., 2014. TGF- β 1-elevated TRPM7 channel regulates collagen expression in hepatic stellate cells via TGF- β 1/Smad pathway. *Toxicol. Appl. Pharmacol.* 280 (October (2)), 335–344.
- Faouzi, M., Kilch, T., Horgen, F.D., Fleig, A., Penner, R., 2017. The TRPM7 channel kinase regulates store-operated calcium entry. *J. Physiol.* 595 (May (10)), 3165–3180.
- Gao, H., Chen, X., Du, X., Guan, B., Liu, Y., Zhang, H., 2011. EGF enhances the migration of cancer cells by up-regulation of TRPM7. *Cell Calcium* 50 (December (6)), 559–568.
- Gao, Y.D., Zheng, J.W., Li, P., Cheng, M., Yang, J., 2013. Store-operated Ca^{2+} entry is involved in transforming growth factor- β 1 facilitated proliferation of rat airway smooth muscle cells. *J. Asthma* 50 (June (5)), 439–448.
- Gotru, S.K., Chen, W., Kraft, P., Becker, I.C., Wolf, K., Stritt, S., Zierler, S., Hermanns, H.M., Rao, D., Perraud, A.L., Schmitz, C., Zahedi, R.P., Noy, P.J., Tomlinson, M.G., Dandekar, T., Matsushita, M., Chubanov, V., Gudermann, T., Stoll, G., Nieswandt, B., Braun, A., 2018. TRPM7 kinase controls calcium responses in arterial thrombosis and stroke in mice. *Arterioscler. Thromb. Vasc. Biol.* 38 (February (2)), 344–352.
- Huang, J., Furuya, H., Faouzi, M., Zhang, Z., Monteilh-Zoller, M., Kawabata, K.G., David Horgen, F., Kawamori, T., Penner, R., Fleig, A., 2017. Erratum to: Inhibition of TRPM7 suppresses cell proliferation of colon adenocarcinoma in vitro and induces hypomagnesemia in vivo without affecting azoxymethane-induced early colon cancer in mice. *Cell Commun. Signal* 15 (September(1)), 31.
- Ishiyama, N., Shibata, H., Kanzaki, M., Shiozaki, S., Miyazaki, J., Kobayashi, I., Kojima, I., 1996. Calcium as a second messenger of the action of transforming growth factor-beta on insulin secretion. *Mol. Cell. Endocrinol.* 117 (March (1)), 1–6.
- Kim, S., Nam Jin, K., Cho, S.H., Hyun Lee, C., Kang, H.R., 2018. Severe asthma phenotypes classified by site of airway involvement and remodeling via chest CT scan. *J. Investig. Allergol. Clin. Immunol.*
- Kraft, R., Harteneck, C., 2005. The mammalian melastatin-related transient receptor potential cation channels: an overview. *Pflugers Arch.* 451 (October (1)), 204–211.
- Ng, N.M., Jiang, S.P., Lv, Z.Q., 2012. Retrovirus-mediated siRNA targeting TRPM7 gene induces apoptosis in RBL-2H3 cells. *Eur. Rev. Med. Pharmacol. Sci.* 16 (September (9)), 1172–1178.
- Nilius, B., 2007. Transient receptor potential (TRP) cation channels: rewarding unique proteins. *Bull. Mem. Acad. R. Med. Belg.* 162 (3-4), 244–253.
- Ojiaku, C.A., Yoo, E.J., Panettieri Jr., R.A., 2017. Transforming growth factor β 1 function in airway remodeling and hyperresponsiveness. The missing link? *Am. J. Respir. Cell Mol. Biol.* 56 (April(4)), 432–442.
- Pan, Y., Liu, L., Li, S., Wang, K., Ke, R., Shi, W., Wang, J., Yan, X., Zhang, Q., Wang, Q., Chai, L., Xie, X., Li, M., 2018. Activation of AMPK inhibits TGF- β 1-induced airway smooth muscle cells proliferation and its potential mechanisms. *Sci. Rep.* 8 (February (1)), 3624.
- Park, H.S., Hong, C., Kim, B.J., So, I., 2014. The pathophysiologic roles of TRPM7 channel. *Korean J. Physiol. Pharmacol.* 18 (February(1)), 15–23.
- Penner, R., Fleig, A., 2007. The Mg^{2+} and Mg^{2+} -nucleotide-regulated channel-kinase TRPM7. *Handb. Exp. Pharmacol.* (179), 313–328.
- Sakai, H., Suto, W., Kai, Y., Chiba, Y., 2017. Mechanisms underlying the pathogenesis of hyper-contraction of bronchial smooth muscle in allergic asthma. *J. Smooth Muscle Res.* 53 (0), 37–47.
- Siddiqui, S., Shikotra, A., Richardson, M., Doran, E., Choy, D., Bell, A., Austin, C.D., Eastham-Anderson, J., Hargadon, B., Arron, J.R., Wardlaw, A., Brightling, C.E., Heaney, L.G., Bradding, P., 2018. Airway pathological heterogeneity in asthma: visualization of disease microclusters using topological data analysis. *J. Allergy Clin. Immunol.* (March) pii: S0091-6749(18)30039-3.
- Yang, M., Fang, J., Liu, Q., Wang, Y., Zhang, Z., 2017. Role of ROS-TRPM7-ERK1/2 axis in high concentration glucose-mediated proliferation and phenotype switching of rat aortic vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 494 (December(3-4)), 526–533.
- Yee, N.S., Zhou, W., Liang, I.C., 2011. Transient receptor potential ion channel Trpm7 regulates exocrine pancreatic epithelial proliferation by Mg^{2+} -sensitive Socs3a signaling in development and cancer. *Dis. Model. Mech.* 4 (March(2)), 240–254.
- Zhang, X., Zhao, Z., Ma, L., Guo, Y., Li, X., Zhao, L., Tian, C., Tang, X., Cheng, D., Chen, Z., Zhang, L., 2018. The effects of transient receptor potential channel (TRPC) on airway smooth muscle cell isolated from asthma model mice. *J. Cell. Biochem.* 1–12.