

# Rhynchophylline attenuates allergic bronchial asthma by inhibiting transforming growth factor- $\beta$ 1-mediated Smad and mitogen-activated protein kinase signaling transductions *in vivo* and *in vitro*

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**Abstract.** Rhynchophylline (Rhy) is a major active component of *Uncaria rhynchophylla* and exhibits the potential to inhibit the proliferation of airway smooth muscle cells (ASMCs). In the current study, it was hypothesized that Rhy serves a key role in the anti-asthma effect of *Uncaria rhynchophylla* by inhibiting transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)-mediated activation of Smad and mitogen-activated protein kinase (MAPK) signaling. Allergic asthma was induced in mice using ovalbumin (OVA), and the effect of Rhy treatment on inflammatory and allergic responses in the bronchoalveolar lavage fluid (BALF) and serum of mice was determined. Subsequently, the changes in TGF- $\beta$ 1-induced Smad and MAPK signaling following Rhy administration were detected to determine the mechanism associated with this treatment. In addition, TGF- $\beta$ 1 was employed to induce hyperplasia of ASMCs, and the effect of Rhy on proliferation of ASMCs, and Smad and MAPK signaling *in vitro* was also assessed. The administration of Rhy attenuated the recruitment of eosinophils in BALF induced by OVA, which was associated with the suppressed production of immunoglobulin E, interleukin (IL)-13, IL-4 and IL-5. At the molecular level, the administration of Rhy suppressed the expression levels of TGF- $\beta$ 1, Smad4, p-Smad2 and p-Smad3, while it induced the expression of Smad7, indicating the inhibitory effect of Rhy on TGF- $\beta$ 1-mediated Smad and MAPK signaling. Furthermore, Rhy inhibited the proliferation of ASMCs and, similar to the results of the *in vivo* assay, it blocked the pro-hyperplasia signaling transduction *in vitro*. In conclusion, the current study

demonstrated the anti-asthma effect of Rhy, which depended on the inhibition of TGF- $\beta$ 1-mediated Smad and MAPK signaling.

## Introduction

As a chronic airway disease, asthma is characterized by airway inflammation, remodeling and hyper-responsiveness (1). The disorder affects over 300 million people worldwide and can be induced by environmental antigens that increase the secreting activity of lymphocytes and eosinophils (2). Significant heterogeneities exist among asthma patients, which makes the clinical management of asthma challenging currently (3,4). Therefore, a comprehensive understanding of the mechanism driving the onset of asthma is imperative for the development of novel treatment strategies.

In recent years, it is well recognized that 'airway remodeling' is a relatively more chronic feature of asthma (1). Airway remodeling represents structural abnormalities in bronchial walls and is characterized by airway smooth muscle (ASM) hypertrophy and hyperplasia (1). The induced secreting activity of lymphocytes and eosinophils during asthma initiates inflammatory responses and stimulates the accumulation of extracellular matrix in ASM cells (ASMCs) (1,5). The secretion of extracellular matrix in ASMCs has been inferred to be the major driving force of epithelial dysplasia and denudation, and mucus gland hyperplasia associated with asthma (6,7). Other previous studies have also confirmed the increase of ASMC mass following exposure to inflammatory factors (8), implying the complex interplay among inflammation, ASMCs and asthma attacks. Therefore, attenuating hypertrophy and hyperplasia of ASMCs has been proposed to be a promising method for inhibiting airway remodeling and managing asthma (1,9).

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) protein is a crucial regulator for the establishment of body structure and tissue differentiation by influencing cell proliferation, differentiation and migration (10). This agent has been widely employed as an inducer for an *in vitro* hyperplasia model of ASMCs (11-13). TGF- $\beta$ 1 exerts its function in cell hyperplasia via multiple mechanisms, including Smad-dependent and non-Smad-dependent manners (10). In the study by Meng *et al* (14), the authors demonstrated that the disruption of

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Smad4 influences the signaling transduction of TGF- $\beta$ 1/Smad3, which attenuates inflammation and fibrosis in the kidney. As reported by Chen and Khalil (11), the phosphorylation of mitogen-activated protein kinases (MAPKs) by TGF- $\beta$ 1 increases the proliferation of ASMCs. Furthermore, interactions among TGF- $\beta$ 1, Smad and MAPK signaling have also been verified by different studies (10,11). Taken together, it is reasonable to verify the possibility of managing asthma by interrupting the interactions among TGF- $\beta$ 1, Smad and MAPK signaling.

*Uncaria rhynchophylla*, also known as ‘Gou Teng’, is a traditional Chinese herb that has been used in the treatment of cardiovascular and brain disorders for centuries (15-17). The major pharmacologically active components of *Uncaria rhynchophylla* include rhynchophylline (Rhy), isorhynchophylline, hirsutine and corynantheine (15,18), among which Rhy has displayed the potential to inhibit the proliferation of ASMCs (19,20). Given the fact that *Uncaria rhynchophylla* is capable of attenuating asthma as a Chinese medicine formula (21), it is hypothesized that Rhy may serve a key role in the anti-asthma effect of *Uncaria rhynchophylla*. To verify this hypothesis, asthma symptoms were induced in mice using ovalbumin (OVA), and the treatment potential of Rhy was assessed in the present study. Furthermore, the inhibiting effect of Rhy on ASMC hyperplasia induced by TGF- $\beta$ 1 was detected. By focusing on TGF- $\beta$ 1, Smad and MAPK pathways, the current study also attempted to uncover the mechanism associated with the anti-asthma effect of Rhy. The data revealed that Rhy effectively attenuated the symptoms of asthma and inhibited the proliferation of ASMCs by blocking TGF- $\beta$ 1-induced activation of Smad and MAPK signaling.

## Materials and methods

**Chemicals and agents.** OVA (cat. no. A5503) and MTT (cat. no. M-2128) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Enzyme-linked immunosorbent assay (ELISA) kits for the detection of murine immunoglobulin E (IgE; cat. no. EK2751), interleukin 4 (IL-4; cat. no. EK2041/2), IL-5 (cat. no. EK2051) and IL-13 (cat. no. EK2131/2) were purchased from MultiSciences Biotech Co., Ltd. (Hangzhou, China). TGF- $\beta$ 1 (cat. no. RPA124Mu01) was obtained from USCN Business Co., Ltd. (Wuhan, China). Rhy (cat. no. R102720) and the TGF- $\beta$ 1 inhibitor SB431542 (cat. no. S125924) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Radioimmunoprecipitation assay lysis buffer (cat. no. P0013B) and a protein concentration determination kit using the bicinchoninic acid (BCA) method (cat. no. P0009) were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

**Antibodies.** An antibody against TGF- $\beta$ 1 (cat. no. BA0290) was purchased from Boster Biological Technology, Ltd. (Wuhan, China). Antibodies against Smad2 (cat. no. 5339), phosphorylated (p)-Smad2 (Ser465/467; cat. no. 3108), Smad3 (cat. no. 9523), p-Smad3 (Ser423/425; cat. no. 9520), extracellular signal-regulated kinase (ERK; cat. no. 4695) and p-ERK (Thr202/Tyr204; cat. no. 4370) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against Smad4 (cat. no. D120124), Smad7 (cat. no. D160746), p38 (cat. no. D151619) and p-p38 (Thr180/Tyr182; cat. no. D151619)

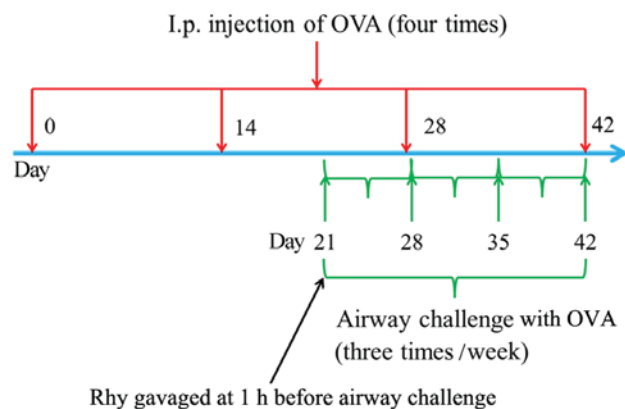


Figure 1. A schematic overview of asthma induction in mice. OVA, ovalbumin; Rhy, rhynchophylline; i.p., intraperitoneal.

were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Antibodies against proliferating cell nuclear antigen (PCNA),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and calponin were purchased from ProteinTech Group, Inc. (Chicago, IL, USA). Horseradish peroxidase (HRP)-conjugated goat-anti rabbit (cat. no. A0208), goat anti-mouse (cat. no. A0216) IgG secondary and Cy3-labeled secondary antibodies (cat. no. A0516) were obtained from Beyotime Institute of Biotechnology. Antibody against internal reference protein  $\beta$ -actin were purchased from Bioss (Beijing, China).

**Allergic asthma induction and Rhy treatment.** All the animal assays were performed following the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of No.1 People's Hospital (Jining, China). A total of 24 female BALB/c mice (8-week-old) were purchased from Changsheng Biotechnology Co., Ltd. (Liaoning, China) and housed in cages at room temperature (20-25°C) with a constant humidity (55±5%) and with food and water available *ad libitum*. The 24 mice were randomly divided into four groups (6 mice in each group), including the blank (untreated mice), sham, asthma and Rhy groups, and were raised for 42 days under the same conditions. To induce allergic asthma, mice in the asthma and Rhy groups were intraperitoneally injected with 20  $\mu$ g OVA (22) for four times, on days 0, 14, 28 and 42 of the model induction, respectively (Fig. 1). Between days 21 and 42 of the induction, mice were subjected to airway challenges with OVA (1%, w/v) using an atomizer for 30 min three times per week (Fig. 1). Mice in the sham group underwent the same procedure as that conducted in the asthma and Rhy groups, but with PBS replacing OVA. In the Rhy treatment group, mice were gavaged with 40 mg/kg Rhy, as previously described (23,24), at 1 h before airway challenge with OVA between days 21 and 42 of the induction (Fig. 1). At 24 h after the last challenge, blood samples and bronchoalveolar lavage fluid (BALF) of mice were collected. Subsequently, mice were sacrificed by pentobarbital sodium overdose (200 mg/kg). Following perfusion of the left ventricle using normal saline, lungs were collected, fixed in 10% neutral buffered formalin and stored at -70°C for subsequent assays.

**Hematoxylin and eosin (H&E) staining.** H&E staining of BALF samples was conducted following previously published protocols (25). Briefly, ~1 ml BALF was recovered through

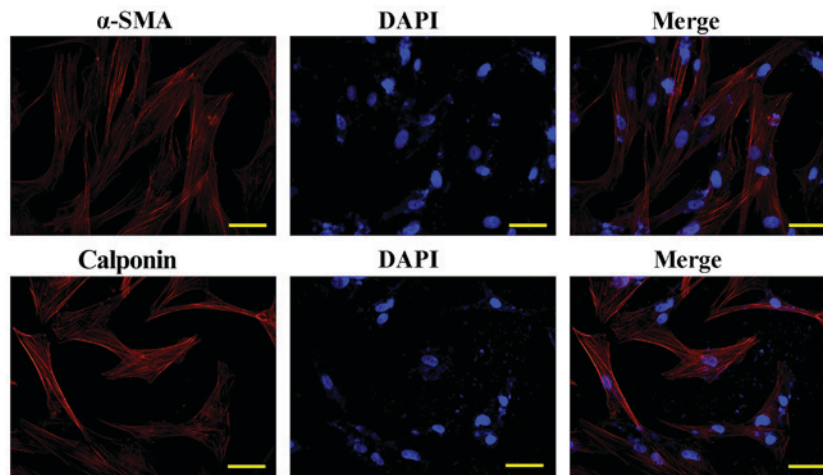


Figure 2. Identification of airway smooth muscle cells by immunofluorescence detection. The positive detection of calponin and  $\alpha$ -SMA indicated typical surface antigen characteristics of ASMCs) Magnification, x400. Scale bar, 200  $\mu$ m.  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; DAPI, 4',6-diamino-2-phenylindole; ASMCs, airway smooth muscle cells.

centrifugation at 1,367  $\times$  g for 15 min at 4°C, and cellular component was obtained from the centrifugal sediment. Next, 0.1 ml sediment was smeared onto a slide and placed into Bouin solution (4% formaldehyde) for perfusion fixation and dehydrated using different concentrations of alcohol. Subsequent to vitrifying in dimethylbenzene, the slide was embedded in paraffin, sectioned and stained with H&E. Images were captured using a microscope (BX53; Olympus Corporation, Tokyo, Japan) at magnification, x200 and the average number of eosinophils per 500 cells was calculated for each group.

**ELISA for determination of IgE, IL-4, IL-5 and IL-13 levels.** The production of IgE in the serum of mice, and the production of IL-4, IL-5, and IL-13 in BALF samples were detected using the corresponding ELISA kits, according to the manufacturer's protocol.

**Western blot analysis.** Cells or tissues were initially incubated with radioimmunoprecipitation assay lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) supplemented with 1% phenylmethane sulfonyl fluoride and placed on ice for 5 min. Next, the mixture was centrifuged at 10,005  $\times$  g for 4 min, and total protein was collected from the supernatant. Protein concentration was then determined using the BCA method. In total, 20  $\mu$ g protein in a 20  $\mu$ l solution was subjected to 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 80 V for 2.5 h and then transferred onto polyvinylidene difluoride membranes at 80 V for 1-2 h. Following rinsing with Tris-buffered saline/Tween-20 (TBST) for 5 min, the membranes were blocked with skimmed milk solution (5%, m/v) for 1 h at room temperature. The membranes were then incubated at 4°C overnight with primary antibodies against TGF $\beta$ 1 (1:300), p-Smad2 (1:1,000), Smad2 (1:1,000), p-Smad3 (1:1,000), Smad3 (1:1,000), Smad4 (1:500), Smad7 (1:500), p-ERK1/2 (1:2,000), ERK1/2 (1:1,000), p-p38 (1:1,000), p38 (1:500), and  $\beta$ -actin (1:500). After washing with TBST for four times, HRP-conjugated IgG secondary antibodies (1:5,000) were added onto the membranes and incubated for 45 min at 37°C. Blots were then developed by incubating the

membranes with Beyo ECL Plus reagent (Beyotime Institute of Biotechnology) for 5 min, and the relative expression levels of proteins were analyzed using the Gel-Pro-Analyzer software (Media Cybernetics, Inc., USA).

**Isolation of ASMCs.** ASMCs were isolated from the healthy female BALB/c 8-week-old mice (which were purchased and housed under the aforementioned conditions) following previously published procedures (26). Briefly, 10 mice were sacrificed using 50 mg/kg pentobarbital sodium, and bronchus tissues were collected. Following washing twice using PBS, the tissues were cut into small sections, and incubated with 0.1% collagenase and 0.1% trypsin in 15-ml tubes at 37°C for 20 min. Cultures were then filtered with a 150- $\mu$ m strainer and centrifuged at 309  $\times$  g for 7 min in Dulbecco's modified Eagle's medium (DMEM). Subsequent to incubation with 0.1% collagenase and 0.1% trypsin and further centrifugation at 309  $\times$  g for 7 min at 37°C, the supernatants were discarded, and precipitates were cultured in DMEM at 37°C in an atmosphere containing 5% CO<sub>2</sub> and 95% air. ASMCs were identified by immunofluorescence detection of calponin and  $\alpha$ -SMA (Fig. 2), and cells of passages 3-5 were employed for subsequent assays.

For *in vitro* assays, cells were divided into four groups as follows: Blank group, which contained ASMCs; TGF- $\beta$ 1 group, in which ASMCs were initially cultured in 0.2% BSA/DMEM serum-free medium to arrest cell growth and then incubated with 5 ng/ml TGF- $\beta$ 1 for 24 h (11); Rhy group, in which ASMCs were initially cultured in 0.2% BSA/DMEM serum-free medium, and then incubated with 5 ng/ml TGF- $\beta$ 1 and 10  $\mu$ M Rhy for 24 h; SB431542 group, in which ASMCs were initially cultured in 0.2% BSA/DMEM serum-free medium, and then incubated with 5 ng/ml TGF- $\beta$ 1 and 10  $\mu$ M SB431542 for 24 h (27). Upon completion of the culture, cells were collected for subsequent assays.

**MTT assay.** The cell viability of ASMCs was detected by an MTT assay. Briefly, the culture medium of ASMCs was replaced by DMEM supplemented with 0.5 mg/ml MTT, and cells were cultured for another 4 h at 37°C. Next, supernatants were aspirated, and 200  $\mu$ l dimethyl sulfoxide was added into

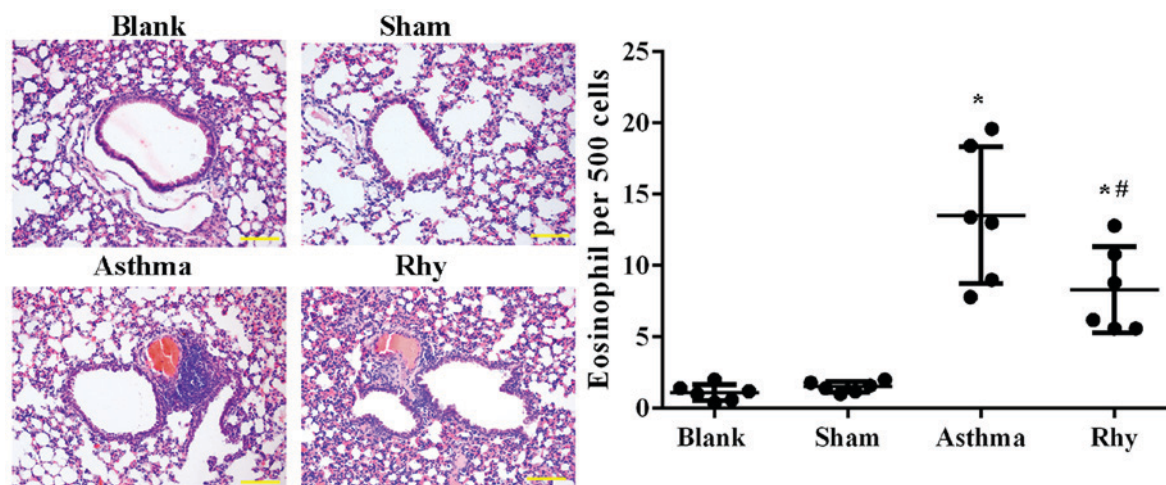


Figure 3. Administration of Rhy attenuated OVA-induced inflammatory cell recruitment in the BALF of mice. Allergic asthma was induced by OVA, BALF samples were collected and smeared onto slides for hematoxylin and eosin staining, and the number of eosinophils was recorded. Six replicates of each assay were performed, and data are presented as the mean  $\pm$  standard deviation. Magnification,  $\times 100$ . Scale bar,  $50 \mu\text{m}$ . \* $P < 0.05$  vs. sham group; # $P < 0.05$  vs. asthma group. Rhy, rhynchophylline; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid.

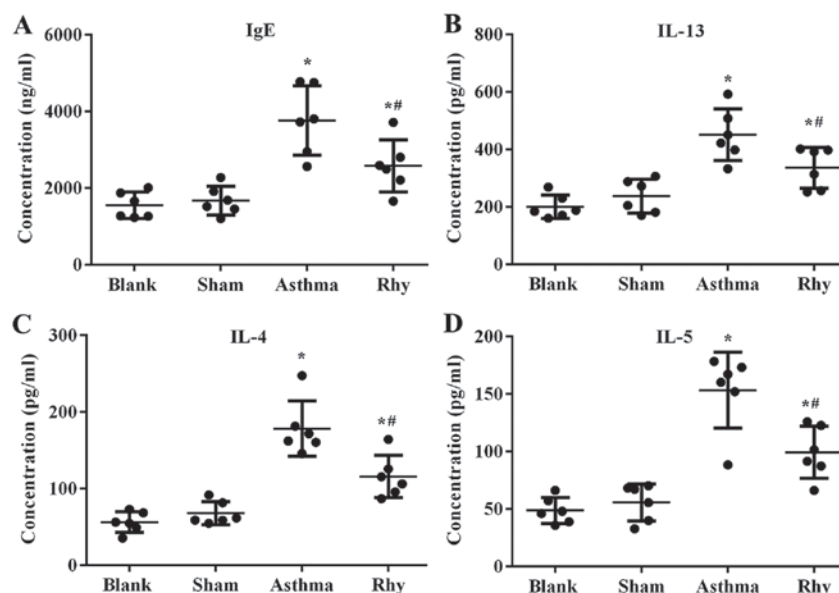


Figure 4. Administration of Rhy decreased the OVA-induced production of IgE, IL-13, IL-4 and IL-5. Allergic asthma was induced by OVA administration in mice. The production of (A) IgE, (B) IL-13, (C) IL-4 and (D) IL-5 was detected by enzyme-linked immunosorbent assay. Six replicates of each assay were performed, and data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. sham group; # $P < 0.05$  vs. asthma group. Rhy, rhynchophylline; OVA, ovalbumin; IgE, immunoglobulin E; IL, interleukin.

each well of a 96-well plate ( $4 \times 10^3$ /well). Cell viability was represented by the optical density value at 570 nm, as detected using a microplate reader (ELX-800; BioTek Instruments, Inc., Winooski, VT, USA).

**Immunofluorescence analysis.** Cells were seeded in 14-well chambers ( $4 \times 10^3$ /well) and allowed to grow into a monolayer. Next, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 30 min. Subsequent to incubation with 10% goat serum for 15 min at room temperature, cells were incubated with primary antibodies against PCNA (1:50),  $\alpha$ -SMA (1:50) and calponin (1:50) at  $4^\circ\text{C}$  overnight. Following three washings using PBS, Cy3-labeled secondary antibody (1:200) was added and incubated for 1 h

at room temperature in the dark. After washing with PBS, cells were stained with 4',6-diamino-2-phenylindole for 5 min. Images were captured with a fluorescent microscope (BX53; Olympus Corporation) at magnification,  $\times 400$ .

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. One-way analysis of variance and post-hoc multiple comparisons were performed using a general linear model. Duncan's test was used for post-hoc multiple comparisons in order to control type I error. A statistically significant difference was considered when the two-tailed P-value was  $< 0.05$ . All the statistical analyses and graph plotting were conducted using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

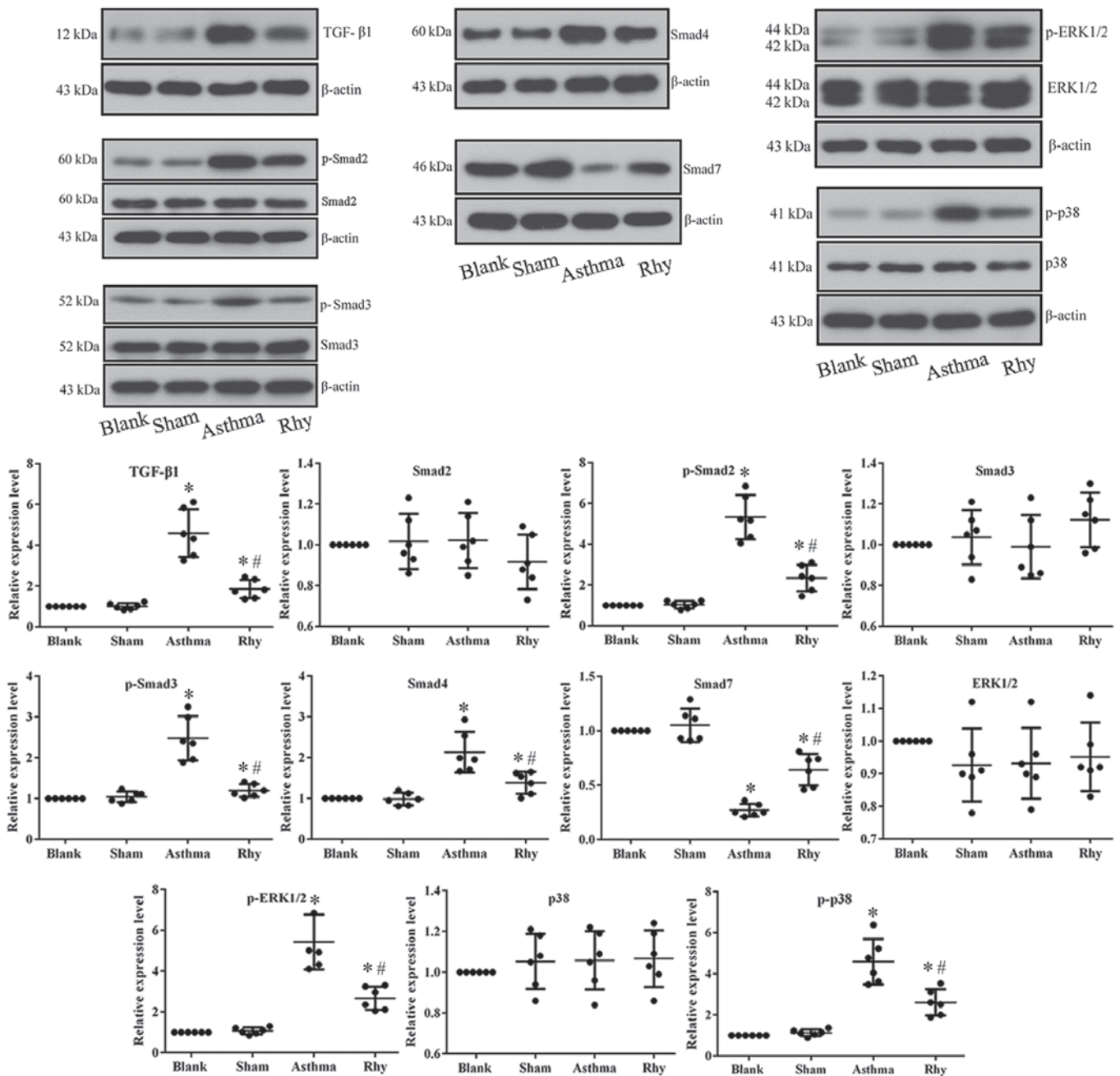


Figure 5. Administration of Rhy exerted an anti-asthma function by suppressing the TGF- $\beta$ 1-mediated Smad and MAPK signaling *in vivo*. Allergic asthma was induced by ovalbumin administration in mice. Subsequently, lung tissues were collected and subjected to western blotting in order to determine the expression levels of molecules involved in TGF- $\beta$ 1-induced Smad and MAPK signaling. Six replicates of each assay were performed, and data are presented as the mean  $\pm$  standard deviation. \* $P$ <0.05 vs. sham group; # $P$ <0.05 vs. asthma group. Rhy, rhyncophylline; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

## Results

*Rhy attenuates the recruitment of inflammatory cells in BALF induced by OVA.* The induction of the asthma model was first evaluated by H&E staining. As shown in Fig. 3, a significantly greater number of eosinophils was recorded in mice in the asthma group as compared with those in the blank and sham groups ( $P$ <0.05). The results confirmed the establishment of the allergic asthma model. Similarly, asthma mice treated with Rhy had a significantly lower number of eosinophils (Fig. 3) compared with that in the asthma group (Fig. 3), evidently

indicating the control of inflammatory cell recruitment by Rhy treatment.

*Rhy suppresses the production of IgE and pro-inflammatory cytokines induced by OVA.* Concomitant with the increase in eosinophil number, the levels of IgE in the serum and pro-inflammatory cytokines IL-13, IL-4 and IL-5 in BALF were found to be induced by OVA administration (Fig. 4), representing the initiation of OVA-induced allergic and inflammatory responses in the lungs. By contrast, mice treated with Rhy displayed lower levels of IgE and pro-inflammatory

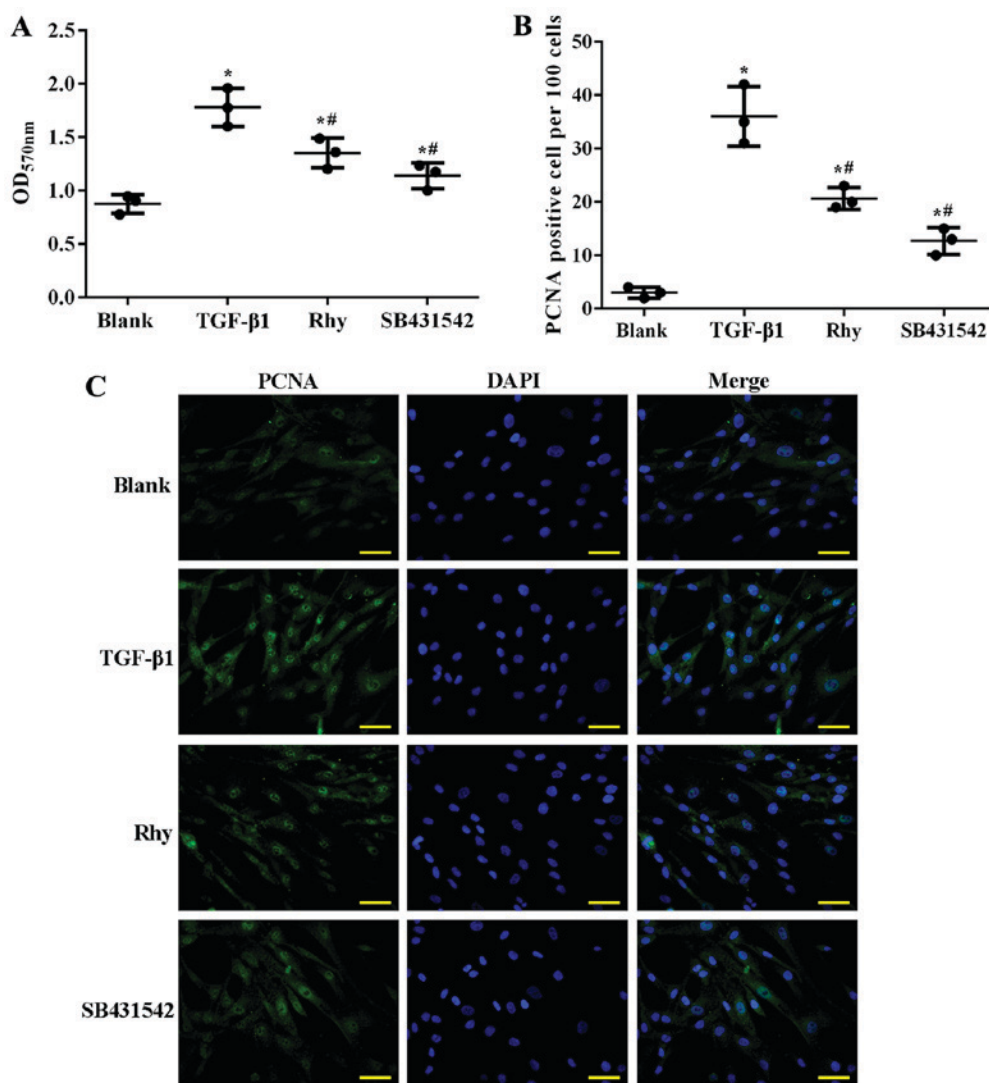


Figure 6. Administration of Rhy attenuated TGF- $\beta$ 1-induced hyperplasia of airway smooth muscle cells. The cells were incubated with 5 ng/ml TGF- $\beta$ 1 alone, or in combination with 10  $\mu$ M Rhy or 10  $\mu$ M SB431542 for 24 h. (A) Cell viability was detected with an MTT assay. (B) PCNA-positive cells and (C) immunofluorescence staining images are shown. Each assay was conducted in triplicate, and data are presented as the mean  $\pm$  standard deviation. Magnification,  $\times$ 400. Scale bar, 200  $\mu$ m. Rhy, rhynchophylline; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; PCNA, proliferating cell nuclear antigen; DAPI, 4',6-diamino-2-phenylindole. \* $P$ <0.05 vs Blank group. \*\* $P$ <0.05 vs. TGF- $\beta$ 1 group.

cytokines compared with those in the asthma group ( $P$ <0.05; Fig. 4). The results revealed the anti-inflammation effect of Rhy during the onset of asthma.

*Rhy inhibits the TGF- $\beta$ 1-induced activation of Smad and MAPK signaling in vivo.* To uncover the mechanism driving the anti-asthma effect of Rhy, the activation of TGF- $\beta$ 1-mediated Smad and MAPK signaling in lung tissues was detected. The data demonstrated that OVA administration in the asthma group significantly induced the expression of TGF- $\beta$ 1, which further initiated Smad signaling by markedly increasing the expression of Smad4, and the phosphorylation of Smad2 and Smad3, while significantly decreasing the expression of Smad7 (Fig. 5). The mechanism is important for the fibrosis process associated with asthma (14). Furthermore, the overproduction of TGF- $\beta$ 1 in the asthma group induced the activation of MAPK pathway (i.e., the increased levels of p-ERK1/2 and p-p38; Fig. 5), which is known to promote hyperplasia of ASMCs in the airway and exacerbate asthma symptoms (10,11). However,

treatment with Rhy reversed the expression patterns of all aforementioned indicators in lung tissues (Fig. 5), inhibiting the pro-fibrosis and pro-hyperplasia signaling transduction induced by OVA.

*Rhy inhibits ASMC proliferation by blocking TGF- $\beta$ 1-mediated Smad and MAPK signaling in vitro.* To verify whether Rhy exerted its anti-asthma effect by suppressing hyperplasia of ASMCs, treatment with Rhy or TGF- $\beta$ 1 inhibitor SB431542 in TGF- $\beta$ 1-treated ASMCs was performed in the current study, and the effect of the administrations on the proliferation of ASMCs and on Smad and MAPK signaling was assessed. Similar to the effect of OVA on mice, TGF- $\beta$ 1 induced proliferation of ASMCs. As detected by MTT assay, significantly higher cell viability (Fig. 6A) and higher production of PCNA (Fig. 6B and C) were observed in the TGF- $\beta$ 1 group as compared with the blank group ( $P$ <0.05). However, when ASMCs were co-incubated with TGF- $\beta$ 1 and Rhy, or TGF- $\beta$ 1 and SB431542, the cell viability and production of PCNA were

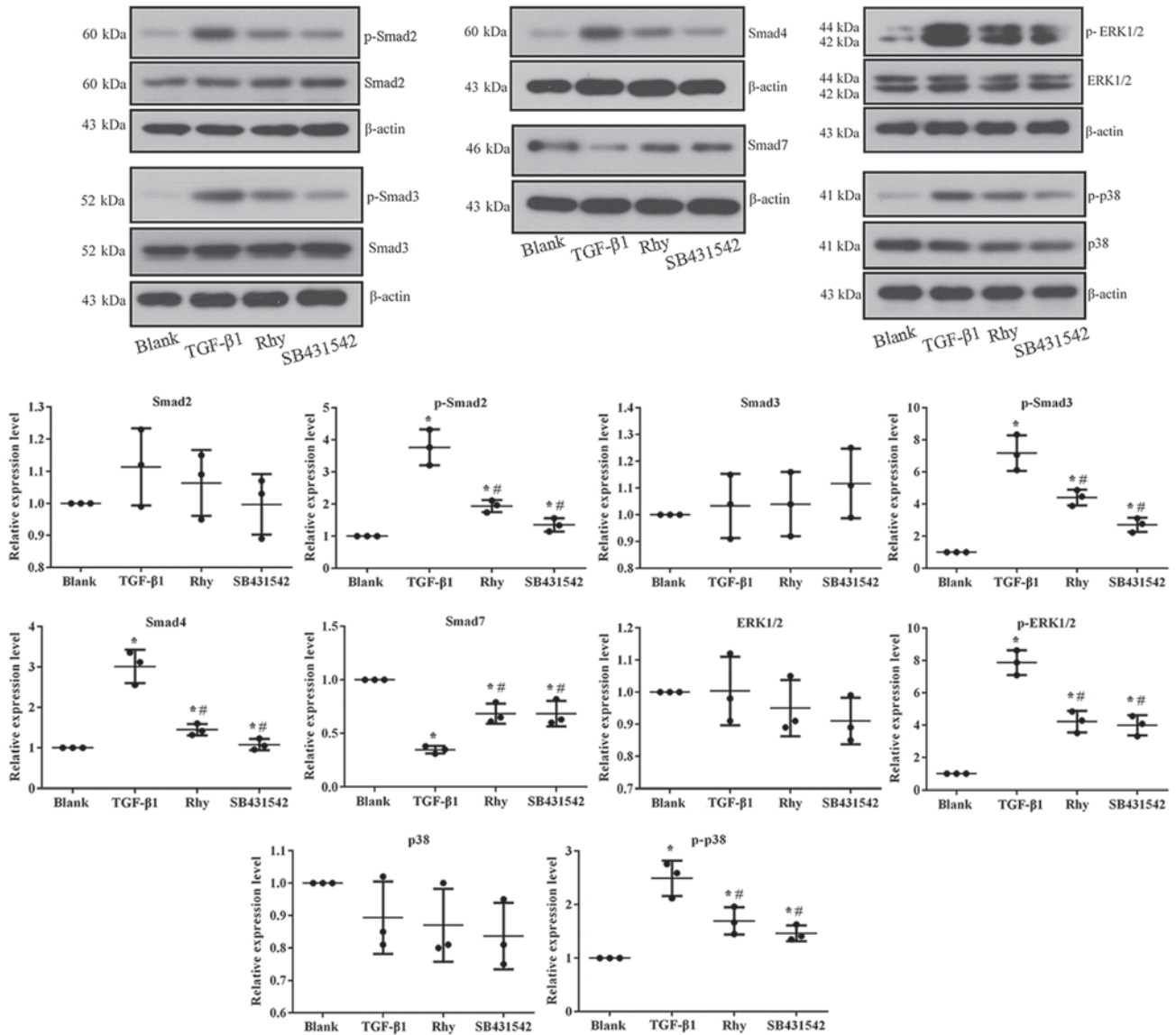


Figure 7. Administration of Rhy suppressed TGF- $\beta$ 1-mediated Smad and MAPK signaling in airway smooth muscle cells. The cells were incubated with 5 ng/ml TGF- $\beta$ 1 alone, or in combination with 10  $\mu$ M Rhy or 10  $\mu$ M SB431542 for 24 h. Western blotting was performed to determine the expression levels of molecules involved in TGF- $\beta$ 1-mediated Smad and MAPK signaling. Each assay was conducted in triplicate, and data are presented as the mean  $\pm$  standard deviation. \* $P$ <0.05 vs. blank group; # $P$ <0.05 vs. TGF- $\beta$ 1 group. Rhy, rhynchophylline; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

significantly inhibited ( $P$ <0.05; Fig. 6A-C), evidently inferring that Rhy inhibited the proliferation of ASMCs in a parallel pattern to that of TGF- $\beta$ 1 inhibitor. At the molecular level, TGF- $\beta$ 1 treatment induced the activation of Smad and MAPK pathways, while treatment with Rhy or SB431542 reversed the expression levels of factors involved in these pathways (Fig. 7), similar to the observations in the mouse model.

## Discussion

Hyperplasia of ASMCs has been reported to induce a variety of pathological symptoms, including atherosclerosis, hypertension and asthma (28,29). Among the factors causing ASMC hyperplasia, TGF- $\beta$ 1 is effective in inducing ASMC proliferation and has been employed as a method to establish asthma *in vitro* models (11-13). Therefore, targeting TGF- $\beta$ 1 has been conceived to be a promising strategy for the treatment of asthma. Rhy, one

of the major pharmacologically active components of *Uncaria rhynchophylla*, was selected in the current study for the management of asthma *in vivo* and *in vitro*. The findings of the current study demonstrated that Rhy was able to attenuate inflammatory and allergic symptoms *in vivo*, and to inhibit hyperplasia of ASMCs *in vitro* by blocking the TGF- $\beta$ 1-mediated Smad and MAPK signaling.

*Uncaria rhynchophylla* is a herb that is widely used in traditional Chinese medicine against hypertension, light headedness, dizziness, convulsion and numbness (20). In the study by Sun *et al* (21), the authors concluded that total alkaloids extracted from *Uncaria rhynchophylla* exhibited an anti-asthma effect in cavy asthma models. However, few studies have followed on these results and conducted a more comprehensive assessment to reveal the pharmacological components involved in the treatment of asthma with this herb. The active components purified from *Uncaria*

*rhynchophylla* include Rhy, isorhynchophylline, hirsutine and corynantheine, among which Rhy has been reported to inhibit the proliferation of ASMCs (19,20). Therefore, it was hypothesized in the current study that the anti-asthma effect of *Uncaria rhynchophylla* total alkaloids may depend on the function of Rhy. Based on the results of *in vivo* assays, it was revealed that the administration of Rhy attenuated the recruitment of inflammatory cells and suppressed the production of IgE, as well as pro-inflammatory cytokines, in asthma mice, representing the effective treatment of Rhy against asthma. Furthermore, incubating TGF- $\beta$ 1-treated ASMCs with Rhy inhibited the proliferation of these cells. Taken together, the results evidently indicated that Rhy was able to attenuate the progression of asthma by inhibiting hyperplasia of ASMCs.

To explore the mechanism driving the effect of Rhy on ASMCs, the activities of TGF- $\beta$ 1-mediated Smad and MAPK pathways were detected. Rhy treatment inhibited the activation of Smad pathway by inducing the expression of Smad7, and markedly suppressing the expression levels of Smad4, p-Smad2 and p-Smad3. The effect was comparable to that observed upon exposure to the TGF- $\beta$ 1 specific inhibitor SB431542, indicating a TGF- $\beta$ 1 inhibition-dependent pattern of Rhy in treating asthma. Two sources for TGF- $\beta$ 1 recruitment exist in airways, including inflammatory cells and residential airway cells (30,31), and this recruitment in turn leads to increased production of collagen I and fibronectin (32). Thus, it is concluded that TGF- $\beta$ 1 serves a determining role in the progression of chronic asthma with repeated episode of injury and inflammation (32). In addition, a previous study demonstrated that overproduction of TGF- $\beta$ 1 enhances proliferation of ASMCs via phosphorylation of MAPKs (11). Activation of MAPK pathway is required for TGF- $\beta$ 1-mediated initiation of Smad signaling, which is central to inflammation and fibrosis processes (14). In addition, co-expression of Smad2 and Smad4 enhances the activation of p38 (11). Interactions between TGF- $\beta$ 1, Smads and MAPKs constitute a positive loop in promoting the proliferation of ASMCs, and further induce pathological symptoms associated with asthma. Therefore, Rhy would interrupt signaling transduction between Smads and MAPKs, and attenuate impairments induced by TGF- $\beta$ 1 overproduction during asthma attacks.

However, shortcomings also exist in the current experimental design. The endotoxin contamination of Rhy cannot be excluded and only a preliminary conclusion that Rhy was able to attenuate allergic bronchial asthma could be provided in the present study. In order to fully explore the medicinal value of Rhy and other active components of *Uncaria rhynchophylla* total alkaloids, further comprehensive investigation is required in the future.

In conclusion, the current study demonstrated the anti-asthma effect of Rhy, which depended on the inhibition of TGF- $\beta$ 1-mediated Smad and MAPK signaling. The findings suggested the clinical application potential of Rhy for managing asthma and other disorders resulting from smooth muscle cell hyperplasia.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

MW and HL collected data and wrote the manuscript. YZ and CL also performed data collection and analyzed the data. GZ designed the experiment, approved the submission and was responsible for the project.

#### Ethics approval and consent to participate

All the animal assays were performed following the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of the No. 1 People's Hospital (Jining, China).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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