

Original Article

Astragalus extract inhibits TGF- β 1-induced EMT of bronchial epithelial cells and airway remodeling in asthmatic mice

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Abstract: Astragalus membranaceus from traditional Chinese herbal medicines previously showed that it possesses a strong anti-inflammatory activity. The purpose of this study was to elucidate the effect of astragalus on allergen-induced airway remodeling and investigate its possible molecular mechanisms. In the present study, we first investigated the role of astragalus extract in the development of airway remodeling in a mouse asthma model. And then we demonstrated that bronchial epithelial cells transforming into myofibroblasts responded to TGF- β 1 and contribute to peribronchial fibrosis in asthma. Furthermore, our study demonstrated that astragalus extract inhibits TGF- β 1-induced EMT of bronchial epithelial cells by inhibiting Snail activity in vitro. Taken together, our current study demonstrated a potential therapeutic value of astragalus extract in the treatment of asthma and it may act by inhibiting the TGF- β 1-induced EMT of bronchial epithelial cells. Our results support the utility of astragalus extract as a herbal medicine for asthma treatment and may have application in the development of anti-inflammatory and anti-asthmatic drugs.

Keywords: Astragalus plant, transforming growth factor- β 1, epithelial-mesenchymal transition, airway remodeling, asthma

Introduction

Asthma is a common chronic inflammatory disease with an incidence that has markedly increased over the past two decades. In addition, the chronic inflammation apparent in a given patient is often associated with the remodeling of the airway structure, which may impair lung function [1, 2]. These changes include peribronchial fibrosis, fibroblast proliferation and conversion to myofibroblasts, and smooth muscle hypertrophy [3]. Myofibroblasts have been suggested to be pivotal factors in the pathogenesis of peribronchial fibrosis. Our previous study has demonstrated that Epithelial-mesenchymal transition (EMT) of bronchial epithelial cells was an important source of myofibroblast in asthmatic mice [4].

EMT is an orchestrated series of events, in which differentiated epithelial cells undergo a phenotypic transition to mesenchymal cells, often fibroblasts and myofibroblasts [5, 6].

During EMT, the epithelial cells lose intracellular junctions, leading to dissociation from the surrounding cells, acquire mesenchymal-like characteristics and become able to migrate away from the original location [7]. This important process was initially recognized during embryonic development and has more recently been identified in tumor progression and organ fibrosis [8]. To date, studies have suggested that kidney proximal tubule epithelial cells undergo EMT to induce interstitial fibrosis in progressive renal disease [9]. In the fibrotic kidney, ~36% of new fibroblasts arise from tubular epithelial cells [10]. EMT may be triggered by different signalling molecules, such as transforming growth factor- β 1 (TGF- β 1), epidermal growth factor, fibroblast growth factor, hepatocyte growth factor and bone morphogenetic proteins [11].

Astragalus membranaceus, is a traditional Chinese herbal medicine used for the treatment of the common cold, diarrhea, fatigue

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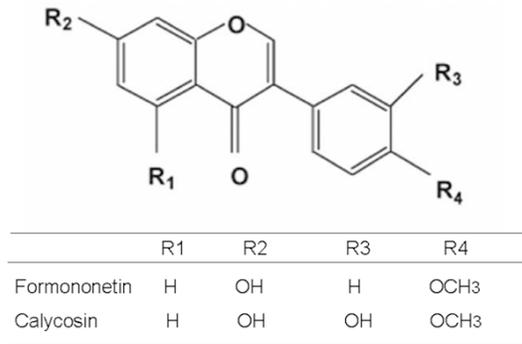


Figure 1. Chemical structures of formononetin, calycosin.

anorexia and cardiac diseases [12, 13]. It has also been used as an immunomodulating agent in treating immunodeficiency diseases and to alleviate the adverse effects of chemotherapeutic drugs. Research studies have been performed to investigate the usefulness of astragalus extract in the treatment of asthma, which can efficiently relieve symptoms and reduce the frequency of asthma attacks [14, 15]. In addition, we have report that astragalus extract inhibits airway remodeling in a mouse asthma model and regulates the TGF- β 1/Smad signaling pathway in ovalbumin-sensitized mice [16]. However, little is known about the underlying mechanisms that regulate this activity.

Materials and methods

Reagents

Astragalus extract (formononetin and calycosin) were obtained from Haerbin Shengtai Botanical Development Co., Ltd. China; their chemical structures are shown in **Figure 1**. Chicken egg ovalbumin (OVA) was purchased from Sigma (USA); TRIzol was purchased from Gibco-BRL (USA); the PCR kit was obtained from Promega (USA); Snail and fibronectin antibodies, as well as secondary antibodies was purchased from Santa Cruz Biotechnology, Inc. (USA). Other laboratory reagents were obtained from Sigma.

Sensitization and antigen challenge

36 healthy female BABL/c mice, weighing 18-24 g were randomly divided into 3 groups, with 12 mice in each group: normal control group (A), asthma group (B), and astragalus extract group (C). The asthmatic model was established by OVA. The mice were sensitized

on Days 0, 7 and 14 by intraperitoneal injection of 20 μ g OVA emulsified in 1 mg of aluminum hydroxide in a total volume of 0.2 ml in groups B and C. Seven days after the last sensitization, the mice were exposed to 1% OVA aerosol for up to 30 min every other day for 8 weeks. The 1% OVA aerosol was generated by a compressed air atomizer driven by filling a Perspex cylinder chamber (diameter 50 cm, height 50 cm) with a nebulized solution. Saline was used in group A instead of OVA. At the same time, mice in group C were treated with 0.4 ml (0.2 g/ml) astragalus extract by gavage before stimulation. All the experiments described below were performed in accordance with the regulations of the Centre of Animal Experiments of Qingdao University.

Tissue samples

Lungs were removed from the mice after sacrificing 24 h after the last challenge. The tissues from the left lung were directly obtained from the surgical suite and immediately fixed in 10% buffered formalin and then embedded in paraffin. Sections (5 μ m) were prepared and stained with hematoxylin and eosin (H&E). Additionally, Periodic acid-Schiff (PAS) staining was performed to identify mucus production in epithelial cells and the number of positive cells per unit length of basement membrane perimeter was determined. The thickness of the submesothelial extracellular matrix was determined after the tissue sections were H&E stained. The average of 10 independent measurements was calculated for each section and then the data were summarized.

Cell line and culture

The 16HBE human bronchial epithelial cells used in this study were obtained from the Cancer Research Institute of Beijing, China. The cells were cultivated in T75 tissue culture flasks in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine and 20 mM hydroxyethyl piperazine ethanesulfonic acid, and incubated in a humidified incubator containing 5% CO₂ at 37°C.

Semi-quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from cells using the TRIzol reagent according to the manufacturer's

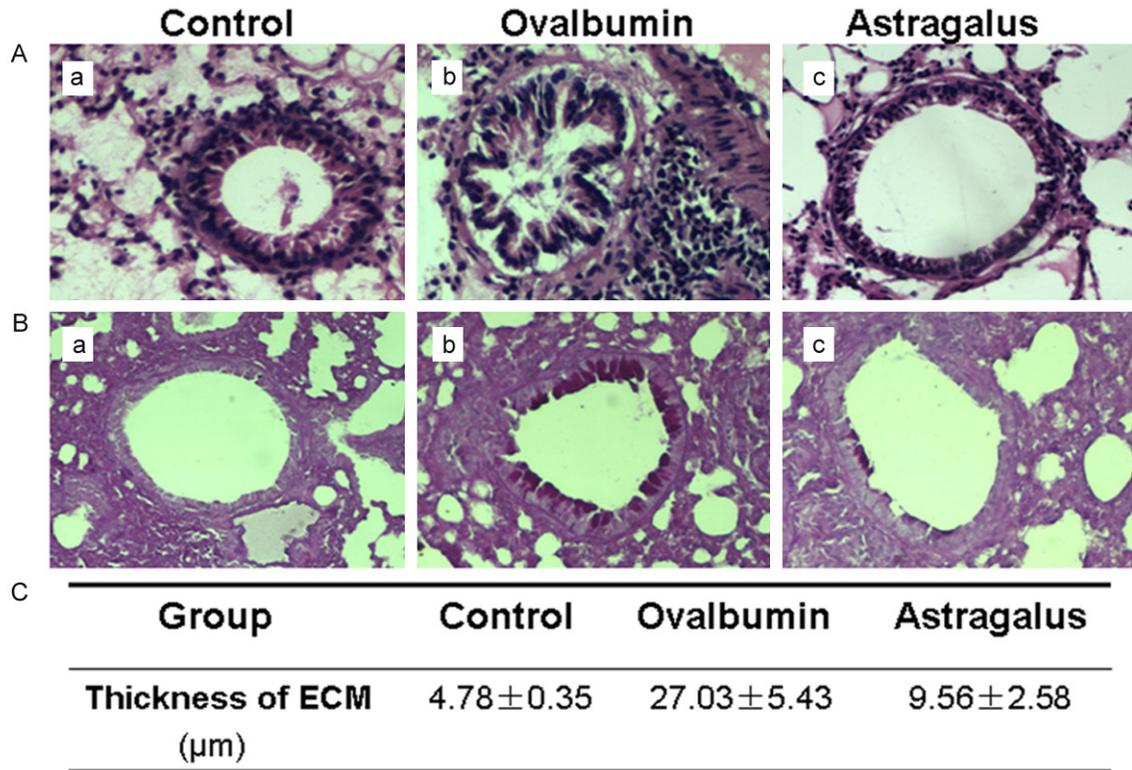


Figure 2. Astragalus extract suppressed OVA-induced lung inflammation and goblet cell hyperplasia. Lung tissue sections obtained from mice 24 hours after the last OVA challenge were stained with H&E (A) and PAS (B). Control group (a), asthmatic group (b) and astragalus extract-treated group (c). All photographs were captured at 100× magnification. (C) Morphometric parameters of airway tissues in three groups. Data are expressed as the mean ± standard error of the mean of at least 3 separate experiments.

Table 1. Primers used for semi-quantitative RT-PCR

Primer	Sequence
Snail-F	5'-CAAGGAATACCTCAGCCTGG-3'
Snail-R	5'-ATTCACATCCAGCACATCCA-3'
E-cadherin-F	5'-TGGGTATTCTCCCATCAG-3'
E-cadherin-R	5'-TTTGTGAGGGAGCTCAGGAT-3'
Vimentin-F	5'-CGCTTCGCCAACTACATC-3'
Vimentin-R	5'-GGTCAGGCTTGGAACATC-3'
β-actin-F	5'-CACCAACTGGGACGACAT-3'
β-actin-R	5'-ACAG CCTGGATAGCAACG-3'

instructions. One microgram of the total cellular RNA was then reverse-transcribed into cDNA for PCR amplification using a kit from Promega. The primer sequences used for PCR have been listed in **Table 1**. Amplification consisted of an initial 5 min incubation at 95°C and then 30 cycles of amplification using 30 s of denaturation at 95°C, 30 s at 56°C, and 60 s at 72°C. The final extension was set for 10 min at 72°C. All data were expressed as the relative differ-

ences between control and treated cells after normalization to β-actin expression.

Western blotting

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

Small interfering RNA (siRNA) treatment

Bronchial epithelial cells were grown to 70% confluence on the culture dishes and the tran-

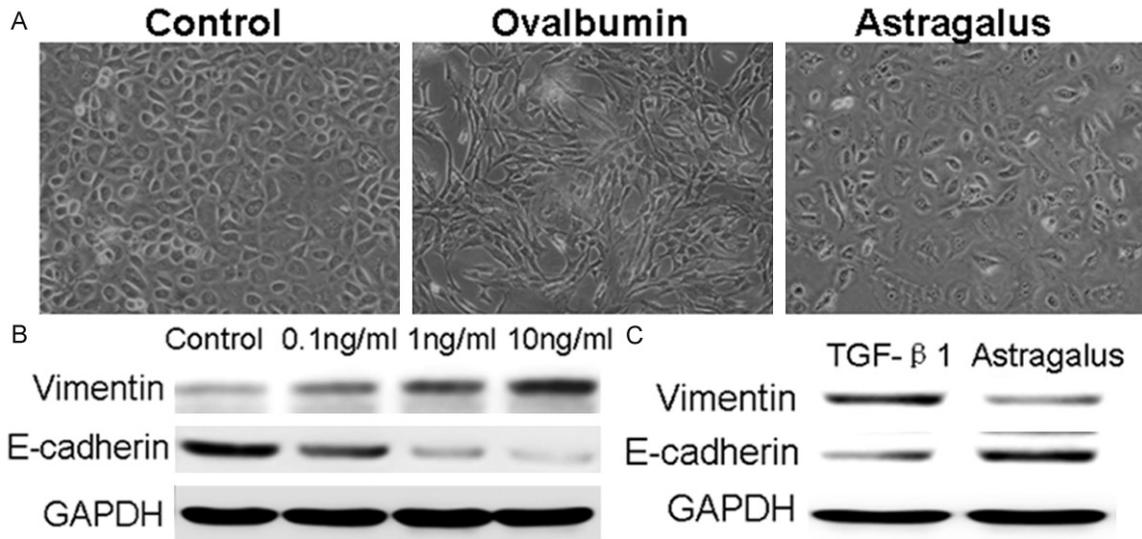


Figure 3. Astragalus extract reverses the epithelial-mesenchymal transition (EMT) of bronchial epithelial cells. Bronchial epithelial cells were incubated in the presence of TGF- β 1 or astragalus extract. A. The phenotypic changes were evaluated using phase contrast microscopy (magnification, $\times 40$). B, C. The expression of E-cadherin and vimentin was measured using western blot analysis.

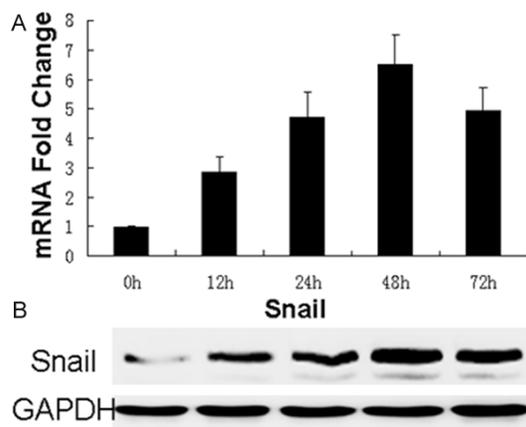


Figure 4. TGF- β 1 increases Snail expression in cultured bronchial epithelial cells. Serum-starved bronchial epithelial cells were incubated with TGF- β 1 for up to 72 h. A. Total RNA was extracted and expression of Snail was measured using semi-quantitative reverse transcription-polymerase chain reaction analysis. B. The expression of Snail protein was measured using western blot analysis. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

sient transfection was performed with specific stealth siRNA against Snail or control siRNA using Lipofectamine 2000, in accordance with the manufacturer's instructions. The target sequences for Snail were 5'-GCGAGCTGCAG-GACTCTAA-3' (No. 1), and 5'-GCGAGT GGTTCT-TCTG CGCTA-3' (No. 2); the scrambled control sequence was 5'-CACATGTTCCGATCTCG GC-3'. Following 6 h incubation with the RNA-complex,

the medium was replaced and 2 ml fresh medium containing 10% FBS was added. The cells were treated and harvested at the indicated times subsequent to the transfection.

Phase contrast microscopy

The phenotypic changes of the bronchial epithelial cells were assessed using phase contrast microscopy. The cultured bronchial epithelial cells were either treated with recombinant TGF- β 1 or left untreated (control) and the morphological changes were then visualized using phase contrast microscopy. The images were collected using a Nikon inverted microscope (Nikon Corp., Tokyo, Japan).

Statistical analysis

For statistical analysis, we used the X^2 test and Fisher's exact test for categorical variables, and the Student's t test or one-way ANOVA test for continuous variables. Relative mRNA expression levels were calculated from quantified data. Data are expressed as mean \pm SD. For statistical analysis SPSS version 18.0 was used throughout, and p values < 0.05 were considered significant.

Results

We have developed a mouse model of airway remodeling through repetitive OVA challenge.

Astragalus inhibits EMT of bronchial epithelial cells

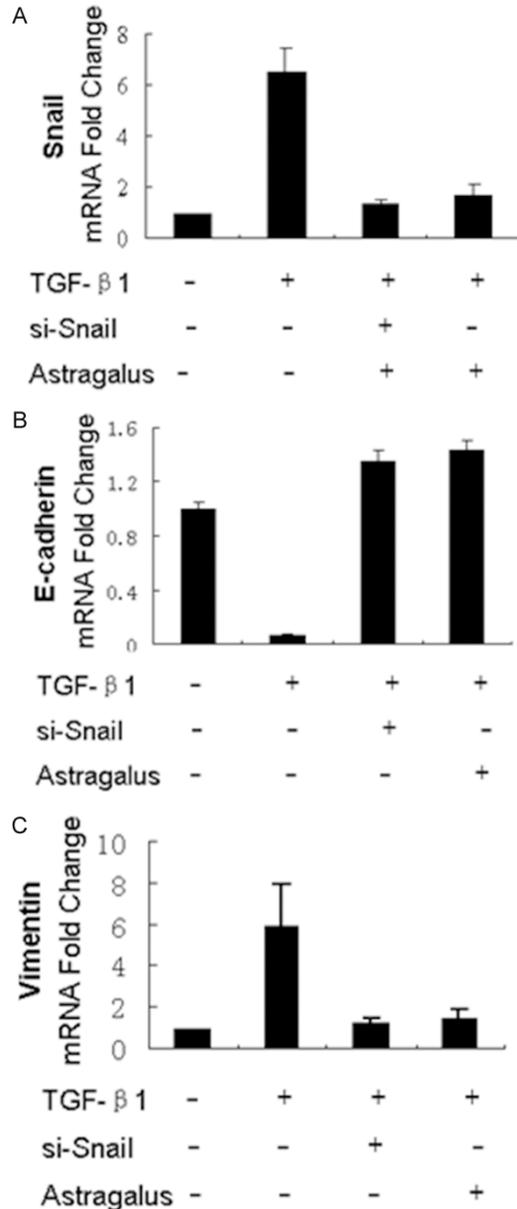


Figure 5. Effects of astragalus extract on TGF- β 1-induced Snail activation in human bronchial epithelial cells. Bronchial epithelial cells transfected with Snail-shRNA or control-shRNA plasmid were starved for 24 h in serum free media and treated with astragalus extract for 48 h. The expression of Snail (A), E-cadherin (B) and vimentin (C) were analyzed by real-time PCR. Values represent fold change as compared to control cells. Data shown as mean \pm SD of two independent experiments each performed in triplicate.

Mice were subjected to OVA challenge three times a week for 8 weeks and developed significant eosinophilic inflammation and airway

remodeling similar to that observed in human chronic asthma.

Astragalus extract inhibited OVA-induced collagen deposition in asthma airway remodeling

We first stained and examined the histology of the airway wall in the four groups of mouse lung tissue. There was a little collagen deposition in the airway wall surrounding the normal mice, and the deposition increased significantly with an extensive distribution in the airway wall surrounding the asthma model mice. Compared with the model group, collagen deposition in the mice treated with astragalus extract was found to be significantly decreased ($P < 0.05$). Compared with control animals, mucus overproduction was clearly observed as a violet color in bronchial airways in OVA-induced mice. In contrast, the extent of mucus staining was markedly diminished in OVA-induced mice treated with astragalus extract (**Figure 2**).

Astragalus extract blocks TGF- β 1-induced mesenchymal transformation in bronchial epithelial cells

Bronchial epithelial cells were activated with recombinant TGF- β 1 and the morphological changes were observed. The epithelial cells cultured in serum-free medium (control) showed a typical polygonal and cobblestone-monolayer morphology. When compared with the control cells, the TGF- β 1-activated epithelial cells exhibited an elongated, spindle-shaped morphology, characteristic of fibroblasts. These morphological changes were associated with the loss of epithelial characteristics, such as E-cadherin, and with the acquisition of certain mesenchymal characteristics, including an increase in vimentin expression. To further elucidate the effect of astragalus extract in TGF- β 1-mediated EMT in bronchial epithelial cells, cells were treated by astragalus extract in vitro. As shown in **Figure 3**, the cellular morphology of TGF- β 1-treated bronchial epithelial cells was reverted from a spindle shape to a more cuboidal/cobblestone shape after astragalus extract treated. We then evaluated the expression of E-cadherin and vimentin in total cell lysates by Western blot analysis. We noted a remarkably reduced expression of vimentin, and most importantly a significant restoration of the junctional protein E-cadherin (**Figure 3**).

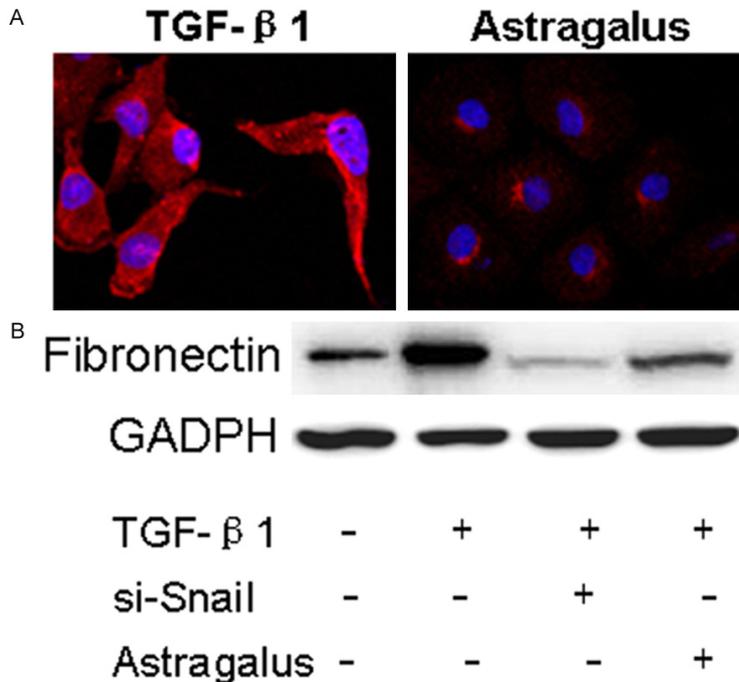


Figure 6. Effects of astragalus extract on fibronectin expression in bronchial epithelial cells. Serum-starved bronchial epithelial cells were incubated with TGF-β1, small interfering RNA (siRNA)-Snail and astragalus extract. A. Confocal immunofluorescence of fibronectin expression in bronchial epithelial cells. B. Expression of fibronectin in bronchial epithelial cells measured using western blot analysis. All photos were obtained at 100× magnification.

Astragalus extract decreases fibronectin expression in bronchial epithelial cells

We next determined whether astragalus extract can affect extracellular matrix production (such as fibronectin) in bronchial epithelial cells. We cultivated and treated them with astragalus extract and then performed western blot analysis. Our data showed that astragalus extract deregulated expression of fibronectin protein, as compared to the control group. Furthermore, we use immunofluorescence microscopy to confirm this protein data. TGF-β1-treated bronchial epithelial cells exhibited a fibrillar pattern of fibronectin-specific proteins after 48 h treatment that was markedly absent in the astragalus extract group (**Figure 6**).

Discussion

Astragalus extract inhibits TGF-β1-induced snail activation in human bronchial epithelial cells

As a result of the correlation between Snail and EMT, we investigated whether the expression of Snail was upregulated by TGF-β1 in bronchial epithelial cells. Compared with the untreated cells, TGF-β1 increased Snail mRNA expression 12 h subsequent to treatment, with the highest level of expression being reached 48 h subsequent to treatment. The inducing effects of TGF-β1 on Snail protein levels were further demonstrated using western blot analysis (**Figure 4**). Astragalus extract markedly inhibited the TGF-β1-induced activation of Snail expression in normal human bronchial epithelial cells. Furthermore, astragalus extract noticeably blocked TGF-β1-induced upregulation of vimentin and decrease of E-cadherin mRNA expression in bronchial epithelial cells. At the same time, down-regulation of Snail resulted in the decrease of the mesenchymal markers vimentin and the increase of the epithelial markers E-cadherin (**Figure 5**).

In the current study, we first investigated the role of astragalus extract in the development of airway remodeling in a mouse asthma model. And then we demonstrated that bronchial epithelial cells transforming into myofibroblasts responded to TGF-β1 and contribute to peribronchial fibrosis in asthma. Furthermore, our study demonstrated that astragalus extract inhibits TGF-β1-induced EMT of bronchial epithelial cells by inhibiting Snail activity in asthmatic mice. Our results support the utility of astragalus extract as a herbal medicine for asthma treatment and may have application in the development of anti-inflammatory and anti-asthmatic drugs.

Airway remodeling is one of the pathophysiological characteristics of asthma, and its main pathological changes include subepithelial fibrosis formation and increased collagen deposition on the airway wall [17]. Our study demonstrated the therapeutic effect of astragalus extract on airway remodeling in allergic airways disease. Astragalus membranaceus extract includes formononetin and calycosin, which

have been identified as the major components responsible for the immunosuppressive and anti-inflammatory effects of this herb [18]. The astragalus extract inhibits several pro-inflammatory cytokines and adhesion molecules that are important mediators of some autoimmune diseases, such as rheumatoid arthritis and asthma, and has been shown to be safe and clinically beneficial in these diseases [19]. In the present study, we observed that the astragalus extract reduced collagen deposition and airway wall thickening involving the reticular basement membrane, smooth muscle layer and epithelial hyperplasia in the mouse model.

TGF- β 1 is a profibrotic cytokine that has been indicated to be an important factor promoting the structural changes of airway remodeling in asthma [20]. Snail, a zinc-finger transcription factor, has been characterized as a key EMT regulator [21]. It has been shown that Snail binds to specific DNA sequences, known as E-boxes, in the promoter of the E-cadherin gene and represses the transcription of E-cadherin [22]. Therefore, the downregulation of the cell-cell adhesion protein E-cadherin has been considered to be characteristic of EMT. Knockout mice deficient in Snail die at gastrulation due to a failure to undergo a complete EMT process, which leads to the formation of an abnormal mesodermal layer that maintains E-cadherin expression. In certain epithelial tumor cell lines, Snail-regulated EMT promotes cell motility and invasion [23]. An inverse correlation between E-cadherin and Snail expression has been observed in cultured epithelial lines established from breast cancer, pancreatic carcinoma and colon cancer. The silencing of Snail by stable RNA interference in epithelial cells was shown to attenuate the complete EMT, which was associated with the upregulation of E-cadherin, the downregulation of mesenchymal markers and the inhibition of invasion [24]. In the present study, our data demonstrated that bronchial epithelial cells underwent a transition from an epithelial to a mesenchymal phenotype following activation with TGF- β 1, which was characterized by a decrease in E-cadherin expression and an increase in vimentin and α -SMA expression. Furthermore, astragalus extract blocks TGF- β 1-induced mesenchymal transformation in bronchial epithelial cells by inhibiting Snail activity in asthmatic mice.

Airway remodeling is one of the pathophysiological characteristics of asthma, and its main pathological changes include subepithelial fibrosis formation and increased collagen deposition on the airway wall [25]. In order to confirm the effect of astragalus extract on peribronchial fibrosis, we showed that astragalus extract decreased fibronectin mRNA production by bronchial epithelial cells; Snail siRNA transfection suppressed fibronectin protein expression in TGF- β 1-treated bronchial epithelial cells. These data further indicated that astragalus extract improved asthma airway remodeling by inhibiting TGF- β 1-induced mesenchymal transformation in bronchial epithelial cells.

In conclusion, our study demonstrated that the astragalus extract inhibited asthma airway wall remodeling through mechanisms involving a block TGF- β 1-induced mesenchymal transformation in bronchial epithelial cells as well as modulation of inhibit Snail signaling in the lung. It suggests the possibility of further developing astragalus extract as a candidate for the systemic therapy of asthma airway remodeling.

Acknowledgements

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Disclosure of conflict of interest

None.

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