Abstract. Epithelial-mesenchymal transition (EMT) provides a valuable source of fibroblasts that produce extracellular matrix in airway walls. The Sonic hedgehog (SHH) signaling pathway plays an essential role in regulating tissue turnover and homeostasis. SHH is strikingly upregulated in the bronchial epithelia during asthma. Snail1 is a major target of SHH signaling, which regulates EMT and fibroblast motility. The present study was designed to ascertain whether the combination of house dust mite (HdM) and transforming growth factor β1 (TGF-β1) could induce EMT via the SHH signaling pathway in human bronchial epithelial cells (HBecs). HBec cultures were treated with HdM/TGF-β1 for different periods of time. The involvement of SHH signaling and EMT biomarkers was evaluated by quantitative real-time PCR, western blotting and immunofluorescence staining. Small-interfering RNA (siRNA) for glioma-associated antigen-1 (Gli1) or cycloporine was used to inhibit SHH signaling in HBecs. HBecs stimulated by HdM/TGF-β1 exhibited morphological features of EMT. e-cadherin (an epithelial marker) was decreased after a 72-h exposure to HdM/TGF-β1 compared to that in the control cells, and the expression of type I collagen and FSP1 (mesenchymal markers) was increased. HdM/TGF-β1 activated the SHH signaling pathway in HBecs, which led to Gli1 nuclear translocation and the transcriptional activation of Snail1 expression. Moreover, gene silencing or the pharmacological inhibition of Gli1 ameliorated EMT. In summary, these findings suggest that HdM/TGF-β1 may induce EMT in HBECs via an SHH signaling mechanism. Inhibition of SHH signaling may be a novel therapeutic method for preventing airway remodeling in asthma.

Introduction

Asthma is a complex type of allergic inflammation that principally involves the conducting airways and is often related to airway remodeling (1). The importance of tissue remodeling, which involves extracellular matrix (ECM) deposition and fibroblast proliferation instead of eosinophilic infiltration in the airway walls, is an early and persistent component of asthma that has been emphasized (2). Fibroblasts may play a key role in peribronchial fibrosis. Nevertheless, the origin of these fibroblasts and the exact pathogenesis of airway remodeling in asthma remain unclear.

The destruction of epithelial integrity is an important event in airway remodeling and airway hyperresponsiveness (3). In recent years, studies have suggested that epithelial-mesenchymal transition (EMT) provides a direct source of fibroblasts (4–6). EMT is a biological process in which epithelial cells undergo multiple biochemical changes to acquire a mesenchymal cell phenotype. House dust mite (HDM) is the major indoor allergen and is associated with allergic response in asthma patients. A previous study demonstrated that HDM combined with transforming growth factor β1 (TGF-β1) can induce marked EMT characteristics (7). Chronic HDM exposure leads to TGF-β expression in the airway epithelium and the induction of EMT (8). These results suggest that EMT may play a unique role in the airway remodeling of asthma. However, the exact molecular mechanism of EMT in allergic asthma remains unclear.

Sonic hedgehog (SHH) signaling is an evolutionarily conserved pathway involved in a variety of biological processes during normal embryonic development and adult tissue homeostasis (9). SHH signaling molecules include a receptor Patched (Ptc) and a signal transducer Smoothened (Smo). Once Hedgehog binds to Ptc, the inhibition of Smo...
is relieved, leading to the translocation of full-length Gli proteins into the nucleus and the activation of the expression of hedgehog target genes (10). The Sonic hedgehog pathway is significantly upregulated in the airway epithelium of children with asthma (11). Snail1 is a major target of the SHH signaling pathway, which regulates EMT and fibroblast motility (12). Shh is expressed in the lung and regulates epithelial-mesenchymal crosstalk (13). A genome-wide association study linked Hedgehog interacting protein (HHIP) and Ptc1 mutations to lung function decline and different asthma phenotypes (14). HHIP acts in a negative feedback loop to attenuate Hedgehog signaling by affecting Hedgehog proteins. However, the mechanism of SHH signaling that contributes to the pathophysiology of asthma is still unclear.

To the best of our knowledge, there has been no systematic study of the possible molecular mechanisms of HDM that may be involved in EMT in human bronchial epithelial cells (HBECs). A previous study demonstrated that HDM alone could not induce cell morphological and phenotypic changes (HBECs). The confluent HBECs were seeded in 24-well plates at a density of 1x10^3 cells/ml in 96-well plates and were transfected with siRNA for Gli1 (5'-AACUCCAAGGGAUAUCAGGA-3') and a negative control. Gene expression was calculated using the 2^ΔΔCt method (16).

**Materials and methods**

**Cell culture.** An HBEC cell line (16HBECs; American Type Culture Collection/ATCC) was cultured in 2 ml of DMEM (Sigma-Aldrich; Merck KGaA) containing 10% foetal bovine serum (FBS) and penicillin (100 U/ml). Prior to treatment, cells were seeded at a density of 5-6x10^5 cells per well in serum (FBS) and penicillin (100 U/ml). Prior to treatment, cells were seeded at a density of 5-6x10^5 cells per well in 6-well plates. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

**Cell viability assay.** To determine a suitable concentration for cyclophamine treatment, a Counting Kit-8 assay (CCK-8) was used to monitor cellular viability. In brief, 16HBECs were seeded at a density 1x10^5 cells/ml in 96-well plates and were treated with cyclophamine at different concentrations (0, 5, 10, 20 and 40 µM) for 72 h. At the end of the treatment, CCK-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well of the plates, and then the plates were incubated at 37°C in an incubator for 4 h. Finally, the absorbance values at 450 nm were measured using a microplate reader (FLX800'TBID, Bio-Tek Instruments, USA). The cell viability was analyzed in triplicate.

**Western blot analysis.** The cells were lysed on ice using RIPA buffer containing protease inhibitors. The cytoplasmic extract (CE) buffer was composed of 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris pH 8.0, 1 mM sodium orthovanadate and 5 mM NaF. The nuclear and cytoplasmic proteins were extracted according to the manufacturer's protocol, and proteins (30 µg/lane) were separated by 10% SDS-PAGE, subsequently electro-transferred onto PVDF membranes. The detection of blotted proteins was performed with anti-E-cadherin (1:500; cat. no. sc-8426), anti-type I collagen (1:1,000; cat. no. sc-59772), anti-Gli1 (1:1,000; cat. no. sc-20687), anti-Snail1 (1:1,000; cat. no. sc-271977), anti-GAPDH (1:1,000; cat. no. sc-47724) (Santa Cruz Biotechnology, Inc.) and anti-FSP1 (1:1,000; cat. no. ab124805; Abcam) antibodies. Primary antibodies were added to the membrane in 5% nonfat dry milk at 4°C overnight and were then incubated with a horseradish peroxidase-linked anti-rabbit or anti-mouse secondary antibody (1:2,000; cat. no. sc-2004, sc-2005; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Immunodetection was performed by chemiluminescence (ECL, Millipore, Billerica, MA, USA). GAPDH was used as an internal control. The gray levels of the blots were measured using ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA). The experiment was repeated three times.

Immunofluorescence staining. Immunofluorescence staining was performed as described previously (15). HDM and TGF-β1 were added to the wells. The cells were incubated with anti-E-cadherin (1:50; cat. no. sc-8426), anti-Gli1 (dilution 1:100; cat. no. sc-20687; Santa Cruz Biotechnology, Inc.), and anti-FSP1 (1:150; cat. no. ab124805; Abcam) antibodies at 4°C overnight, and were then incubated with a horseradish peroxidase-linked anti-rabbit or anti-mouse secondary antibody (1:2,000; cat. no. sc-2004, sc-2005; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Cells were also stained with DAPI. The slides were visualized with a Zeiss Axio Imager 2 microscope (Carl Zeiss AG).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated and reverse transcribed into cDNA using a ReverTra Ace qPCR RT kit (Toyobo Biotechnology, Tokyo, Japan). Gene expression was determined by SYBR Green Real-Time PCR Master Mix (Toyobo Biotechnology). The primer pairs were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. The primer sequences used were as follows: SHH (sense 5'-AAGGATTAGAGGAATCT-3' and antisense 5'-CCCTGTGGTTCTGATGTTGC-3'); Gli1 (sense 5'-GGAAGATGACTGCGCAATGC-3' and antisense 5'-TGGGGGTCTGATGTGGT-3'); Snail1 (sense 5'-TCTTTTCATTCTTTCTCAT-3' and antisense 5'-GCTGGATGTGATGCTTG-3'); GAPDH (sense 5'-GCC TTTTCGTCCCCACTGTC-3' and antisense 5'-GGCTTGG TGTCAGGCTTCT-3'). GAPDH was used as an internal control. Gene expression was calculated using the 2^ΔΔCt method (16).

Blockade of SHH signaling. Small-interfering RNA (siRNA) for Gli1 or cyclophamine was used to inhibit SHH signaling. Cyclophamine is an isolated alkaloid that shows strong potential to bind to SMO and inhibit the SHH signaling pathway (10). Cells (16HBECs) were seeded in 24-well plates at a density of 3x10^4 cells/well and were transfected with siRNA for Gli1 (5'-AACUCCAAGGGAUAUCAGGA-3') and a negative
control siRNA (5'-AACGUACCGGAAUCAACAGA-3') purchased from Sangon Biotech (Shanghai, China), which were used in a previous study (17). siRNA was introduced into cells using Lipofectamine LIXV reagent (Invitrogen; Thermo Fisher Scientific, inc.). Twenty-four hours later, the cells were stimulated with HD/M/TGF-β1. After 72 h, cells were harvested for the detection of downstream target genes in the SHH signaling pathway. All of the siRNA experiments were performed in triplicate.

Cyclopamine (Sigma-Aldrich; Merck KGaA) was dissolved in dimethyl sulfoxide (DMSO) (at 100 mmol/l stock solution) (18). The cells were transfected in 24-well plates at a density of 3x10^4 cells/well. After 24 h, the 16HBECs were stimulated with HD/M/TGF-β1 and were then treated with cyclopamine for 72 h. Control wells were treated with only 0.1% (v/v) DMSO. The total amount of DMSO in the medium never exceeded 1% (v/v). Finally, the cells were harvested for further experiments.

**Statistical analysis.** All data are expressed as the mean ± the standard error of the mean (SEM). Group differences were analyzed by a Student's t-test or one-way ANOVA with Tukey post hoc analysis. Statistical significance was set at P<0.05.
HDM combined with TGF-β1 induces EMT in HBECs. The HDM and TGF-β1 concentrations were chosen according to previous studies (7,19). First, we examined whether HDM/TGF-β1 could induce a morphological change that was characteristic of EMT. Cells (16HBECs) in culture exhibited a cobblestone morphology. However, after stimulation by HDM/TGF-β1, the cells acquired an elongated and mesenchymal-like morphology with a loss of cell-cell contact (Fig. 1A).

After 72 h of exposure to HDM/TGF-β1, western blot analysis demonstrated that the protein level of E-cadherin as decreased; however, the expression levels of mesenchymal markers type I collagen and FSP1 were markedly upregulated compared to that in the controls (Fig. 1C and D). As shown by immunofluorescence staining, the expression of E-cadherin was decreased in the 16HBECs after 72 h of treatment with HDM/TGF-β1, and the expression of FSP1 increased. Magnification, x400. HDM, house dust mite; TGF-β1, transforming growth factor β1; EMT, epithelial-mesenchymal transition; HBECs, human bronchial epithelial cells.

Results

HDM combined with TGF-β1 induces EMT in HBECs. The HDM and TGF-β1 concentrations were chosen according to previous studies (7,19). First, we examined whether HDM/TGF-β1 could induce a morphological change that was characteristic of EMT. Cells (16HBECs) in culture exhibited a cobblestone morphology. However, after stimulation by HDM/TGF-β1, the cells acquired an elongated and mesenchymal-like morphology with a loss of cell-cell contact (Fig. 1A).
HDM combined with TGF-β1 induces an increase in the expression of SHH signaling in HBECs. As shown by western blotting, the cells exposed to HDM/TGF-β1 for 24 h exhibited slightly increased Shh secretion compared to that in the control cells, and this difference gradually increased when cells were exposed for 72 h (Fig. 2A and B). qPCR data demonstrated a significant increase in Shh mRNA expression after 48 h of HDM/TGF-β1 treatment (Fig. 2C). These findings demonstrated that the gene expression and protein levels of Shh were increased. Since Shh has repeatedly been shown to activate SHH-Gli1 signaling in vitro (20,21), we explored whether HDM/TGF-β1 could induce an accumulation of Gli1. Indeed, exposure to HDM/TGF-β1 for 24 h resulted in an accumulation of the total Gli1 levels compared to the control (Fig. 2C). Snail1 is a well-known target of SHH signaling that regulates EMT and fibroblast motility (12). The expression of Snail1 was significantly increased in the 16HBECs exposed to HDM/TGF-β1 for 48 h compared with its expression in the control cells (Fig. 2A-C).

Blockade of SHH signaling inhibits the nuclear translocation of Gli1. The nuclear translocation of Gli1 is a key inducer of Snail1 protein expression (22). The nuclear localization and accumulation of Gli1 are necessary for its transcriptional activity. By immunofluorescence staining, we found that Gli1 protein expression was increased in cells that were treated with HDM/TGF-β1 for 72 h compared to that in untreated cells and was mainly localized in the nucleus of the HDM/TGF-β1-stimulated 16HBECs (Fig. 3A and B).

Using a CCK-8 assay, cyclopamine treatment was shown to lead to the inhibition of 16HBEC survival in a dose-dependent manner (Fig. 1B). In the present study, cyclopamine (10 µM) was used to specifically block the SHH signaling pathway. After silencing of Gli1 using siRNA or cyclopamine, the nuclear translocation and protein expression of Gli1 were significantly decreased (Figs. 3A and B, 4A-C).

Inhibition of SHH signaling attenuates HDM/TGF-β1-induced EMT in 16HBECs. To examine whether SHH signaling mediates HDM/TGF-β1-induced EMT, siRNA was used to silence Gli1. Gli1 siRNA was transfected into 16HBECs and reduced Gli1 protein expression in 16HBECs that were treated or untreated by HDM/TGF-β1. Compared to transfection with a negative control siRNA, transfection with Gli1-specific siRNA significantly decreased the EMT process, as demonstrated by the decreased expression of type I collagen and FSP1 and the increased expression of E-cadherin (Fig. 4A and B). Cultured 16HBECs were treated with cyclopamine following HDM/TGF-β1 stimulation. As shown in Fig. 4A and C, this inhibitor of SHH signaling effectively antagonized the EMT process in vitro.
Figure 3. Blockade of SHH signaling in 16HBECS inhibits the HDM/TGF-β1-induced nuclear translocation of Gli1. The nuclear translocation of Gli1 was assessed by immunofluorescence staining. Cells were stained with an anti-Gli1 antibody (red) and DAPI (blue). Gli1 protein expression was increased in the treated cells compared to its expression in the control cells, and Gli1 was primarily localized to the nuclei of 16HBECS stimulated by HDM/TGF-β1 for 72 h. After the silencing of Gli1 using siRNA (A) or cyclopamine (B) the nuclear translocation of Gli1 was significantly decreased. A negative control siRNA (NC siRNA) with no homology to known genes was used as a non-targeting control siRNA. Scale bar, 10 µm. SHH, Sonic hedgehog; HBECs, human bronchial epithelial cells; HDM, house dust mite; TGF-β1, transforming growth factor β1; Gli1, glioma-associated antigen-1; HBECs, human bronchial epithelial cells.
Discussion

The bronchial epithelium is an initial barrier and may be more susceptible to inhaled noxious agents (7). Airway remodeling is a marked feature of asthma that aims to maintain epithelial integrity. The present study revealed that in human bronchial epithelial cells (HBecs), exposure to HDM/TGF-β1-induced epithelial-mesenchymal transition (EMT), which was identified by the upregulation of mesenchymal markers (FSP1 and type I collagen) and the loss of the adherens junction protein E-cadherin. We believe these findings provide evidence for the upregulation of the Sonic hedgehog (SHH) signaling pathway in house dust mite (HDM)/transforming growth factor β1 (TGF-β1)-mediated EMT in HBecs. In our study, HBecs stimulated by HDM/TGF-β1 exhibited morphological features of EMT; by immunofluorescence staining, the expression of an epithelial marker was decreased, while expression of mesenchymal markers was increased compared to those in the controls. The exposure of HBecs to HDM/TGF-β1 induced EMT. These findings are in line with a previous study that was performed (Gli1). Moreover, the genetic knockout or pharmacological antagonism of Gli1 ameliorated EMT. In general, these findings suggest that HDM/TGF-β1 may trigger the induction of EMT in HBecs via an SHH signaling mechanism. The inhibition of SHH signaling may be a new therapeutic method for preventing airway remodeling in asthma.

Figure 4. Blockade of SHH signaling reduces HDM/TGF-β1-mediated EMT in 16HBecs. Cells (16HBecs) were stimulated with HDM/TGF-β1 in the absence or presence of a Gli1 siRNA or cyclopamine for 72 h, as indicated. Western blotting (A) and quantification (B) showed that the transfection of cells with a Gli1-specific siRNA inhibited the expression of SHH signaling proteins (Gli1 and Snail1) and suppressed HDM/TGF-β1-induced EMT changes (increased expression of E-cadherin and decreased expression of collagen I and FSP1). No antagonistic effect was detected in 16HBecs transfected with a control siRNA. The western blotting (A) and quantification (C) demonstrated that cyclopamine inhibited SHH signaling and attenuated HDM/TGF-β1-induced EMT. *P<0.05 (n=3). SHH, Sonic hedgehog; HDM, house dust mite; TGF-β1, transforming growth factor β1; EMT, epithelial-mesenchymal transition; HBECs, human bronchial epithelial cells; Gli1, glioma-associated antigen-1.
in an HDM-challenged mouse model (24). This study showed that EMT may contribute to asthma, but the role of EMT in asthma needs to be further studied in the future.

The Hedgehog signaling pathway plays an important role during vertebrate embryonic development and tumorigenesis. Patched (Ptc) was found to impede Smoothened (Smo) activity when the Hedgehog ligand is lacking and then prevents the activation of Gli (14). In the present study, we first detected the gene and protein expression of the core molecular components of the SHH signaling pathway in HBECS stimulated by allergens. By western blot analysis, we found that Shh and Gli1 secretion was significantly increased in 16HBECS exposed to HDM/TGF-β1 for 48 h compared to their secretion in control cells, and real-time PCR revealed the significant upregulation of SHH and Gli1 mRNA expression. Our results suggested that the gene expression and protein levels of Shh/Gli1 were upregulated in response to HDM/TGF-β1. By immunofluorescence staining, we found that Gli1 protein expression was increased, and Gli1 was localized primarily to the nucleus of 16HBECS treated with HDM/TGF-β1. The nuclear localization and accumulation of Gli1 is essential to its transcriptional activity. The efficiency of Gli1 siRNA or cyclopamine in the inhibition of Gli1 expression was reported in a previous study (18). After silencing of Gli1 using siRNA or cyclopamine, the nuclear translocation and protein expression of Gli1 were significantly decreased. These findings demonstrated that SHH signaling may play a role in asthma. Our findings are consistent with two previous studies that showed that SHH was significantly increased in the bronchial epithelium in asthma patients (11, 25). A recent study reported that Gli1 protein expression was strongly associated with EMT biomarkers in lung carcinomas (26).

In the present study, the expression of the Snail1 protein was significantly increased in 16HBECS exposed to HDM/TGF-β1. Some studies have emphasized the important roles of the transcription factor Snail1 during the EMT process (27). To examine the role of Shh/Gli1 in HDM/TGF-β1-induced Snail1 activation and the EMT process, we treated HBECS with Gli1 siRNA or cyclopamine. Following the blockade of SHH signaling, 16HBECS stimulated by HDM/TGF-β1 had lower cytoplasmic Snail1 expression as well as reduced EMT biomarker alterations than untreated cells. Based on these data, we proposed that the combination of HDM and TGF-β1 activated Shh/Gli1, which upregulated the nuclear translocation of Gli1 and in turn increased Snail1 expression, finally resulting in EMT.

If SHH signaling is important for EMT induction, the inhibition of SHH signaling may have antiobiotic effects. The use of cyclopamine, a well-characterized Smo inhibitor, is limited in vivo due to its short half-life and off-target effects occur at high doses (28). Ding et al (18) demonstrated that cyclopamine could prevent fibroblast proliferation and matrix synthesis in renal tubulointerstitial fibrosis. The present study showed that cyclopamine antagonized the EMT process and reduced ECM production in vitro. However, the antibiotic effects of cyclopamine in asthma warrant further study.

In summary, these findings demonstrate that the combination of HDM and TGF-β1 may induce EMT in HBECS. The effect was mainly triggered by the SHH signaling pathway, and the inhibition of SHH signaling abolished SHH-induced EMT and ECM synthesis. Therefore, SHH signaling may play a key role in EMT and airway remodeling in asthma, and pharmacological antagonism of SHH signaling may have a therapeutic effect in asthma. In the future, the role of the in vivo blockade of Shh/Gli1 signaling during EMT requires further study.

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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
YZ and WH designed the research study. YZ, WH, LZ and YM performed the experiments, WS and YM analyzed the data and prepared the figures. YZ drafted the manuscript. YZ and YM edited and revised manuscript. YZ and WH interpreted the results of the experiments. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

 Ethics approval and consent to participate
Not applicable.

 Patient consent for publication
Not applicable.

 Competing interests
The authors declare that they have no competing interests.

 References


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