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Protein tyrosine phosphatase SHP2 regulates TGF- β 1 production in airway epithelia and asthmatic airway remodeling in mice

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Abstract

Background—Transforming growth factor (TGF)- β 1 produced in airway epithelia has been suggested as a contributor to the airway remodeling observed in asthma patients. The protein tyrosine phosphatase SHP2 is a demonstrable modulator of TGF- β 1 production and thus a potential regulator of airway remodeling.

Objectives—To define the signal event by which SHP2 regulates asthmatic responses in airway epithelial cells by using a mouse model of experimental OVA-induced airway remodeling.

Methods—The airways of *Shp2^{flox/flox}* mice were infected with recombinant adenovirus vectors expressing a *Cre* recombinase–green fluorescence protein (GFP) fusion protein as part of allergen provocation studies using mice sensitized with ovalbumin (OVA) and repeatedly challenged with OVA. Several endpoint pathologies were assessed, including airway hyper-responsiveness (AHR), lung inflammatory score, peribronchial collagen deposition, and α -smooth muscle actin (SMA) hyperplasia. *In vitro* studies using airway epithelial cells (BEAS-2B) were used to investigate the

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Conflict of Interests

All authors have reviewed and approved the manuscript for publication and have no conflicts of interest to declare regarding the publication of this manuscript.

Authors Contributions

Hua-Hao Shen defined the research theme, designed many of the experiments, contributed to the assessments and interpretation of experimental results, and wrote an initial draft of this manuscript. Yue-Hai Ke contributed to the design of many of the experiments and contributed to the assessments and interpretation of experimental results presented. Xue-Jun Qin and Gen-Sheng Zhang contributed to the design of many of the experiments, contributed to the assessments and interpretation of experimental results, and also contributed to the writing of the initial draft of this manuscript. Xue Zhang, Zhang-Wei Qiu, Ping-Li Wang, Yan-Wei Li, Wen Li, and Qiang-Ming Xie contributed to the design of the dispersal and colonization experiments as well as the collection of data and their analyses and/or interpretation. James J Lee provided guidance and advice for the design of experiments and provided a critical review of the manuscript at various stages in the submission process.

role of SHP2 in the regulation of pulmonary remodeling events, including the expression of collagen, α -SMA, and TGF- β 1.

Results—Chronic OVA challenges in wild-type mice resulted in airway remodeling and lung dysfunction (e.g., increased inflammatory scores, collagen deposition (fibrosis), smooth muscle hyperplasia, and a significant increase in AHR). These endpoint pathology metrics were each significantly attenuated by conditional *shp2* gene knockdown in airway epithelia. *In vitro* studies using BEAS-2B cells also demonstrated that the level of TGF- β 1 production by these cells correlated with the extent of *shp2* gene expression.

Conclusions—SHP2 activities in airway epithelial cells appear to modulate TGF- β 1 production and, in turn, regulate allergic airway remodeling following allergen provocation.

Clinical Implications—Our findings identify SHP2 as a previously underappreciated contributor to the airway remodeling and lung dysfunction associated with allergen challenge. As such, SHP2 represents a potentially novel therapeutic target for the treatment of asthmatics.

Keywords

airway epithelia; asthma; mice; protein tyrosine phosphatase SHP2; remodeling

Asthma remains a common chronic inflammatory disease whose incidence has markedly increased over the past two decades. In addition, the chronic inflammation associated with a given patient is often linked with the remodeling of airway structure that may impair lung function [1, 2]. These changes include peribronchial fibrosis, fibroblast proliferation and conversion to myofibroblasts, and smooth muscle hypertrophy [3]. Although these features are well recognized, the mechanisms leading to remodeling and the effect of therapy on preventing or reversing these changes are largely unknown. A variety of cells, cytokines, chemokines, and growth factors that are released from inflammatory and structural cells in the airway have been implicated in promoting airway inflammation and, in turn, remodeling events. Furthermore, the cross-talk between epithelium and the underlying mesenchyme appears to be critical for driving remodeling responses in asthma. Findings from long-term patient studies, which have been ongoing for more than a decade, have indicated that transforming growth factor- β 1 (TGF- β 1) may be an important mediator of allergic inflammation and airway remodeling. TGF- β 1 is produced by various cell types in the lung, such as airway epithelial cells and leukocytes comprising the pulmonary pro-inflammatory cell infiltrate [4, 5], and more recent studies suggested that this pulmonary TGF- β 1 contributes to subepithelial fibrosis and airway smooth muscle remodeling linked with many asthma patients [6, 7].

An important positive regulator of many growth factor signaling pathways, including TGF- β , is the protein tyrosine phosphatase SHP2. SHP2 is a cytoplasmic phosphotyrosine phosphatase with two SH2 domains (N-SHP2 and C-SHP2) that is widely expressed in the airway epithelium [8]. Studies of allergic respiratory inflammation have also identified SHP2 as both a positive and negative regulator of key pulmonary immune signaling pathways. These SHP2 functions include fine-tuning of cellular response to cytokines [9–12], effects on inhibitory receptor signaling [13–15], and mediating T-cell development and function [16–18].

In the present study, the role of SHP2 expression in airway epithelial cells was defined further using a mouse model of chronic allergen exposure that is accompanied by characteristic airway remodeling events. These studies avoid the early embryonic lethality associated with homozygous *shp2* knockout mice [19, 20] and instead exploit an inducible *shp2* knockout strategy to specifically delete the expression in the mouse airway epithelia.

Airway epithelial cell *shp2* expression was linked with the airway remodeling events following chronic allergen exposure. Subsequent cell culture studies with BEAS-2B airway epithelial cells showed that the *shp2*-mediated airway remodeling occurring in mice may be mediated by the regulation of airway epithelial TGF- β 1 production. These data suggest that *shp2* may be a key regulator of airway remodeling events and thus a previously underappreciated therapeutic target.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 mice were purchased from the Laboratory Animal Center of Zhejiang University (Hangzhou, China). *Shp2^{flox/flox}* mice in the C57BL/6 background were a generous gift from Dr. Gen-Sheng Feng (University of California at San Diego, USA) [21]. Genotypes were determined by PCR analysis using tail genomic DNA and *shp2^{flox}* primers (Table 1). All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Zhejiang University Medical Laboratory Animal Care and Use Committee.

Chronic OVA exposure

Male and female mice at 6–8 weeks of age (weighing 18–22 g) were sensitized (on protocol days 0 and 14) by two intraperitoneal (*i.p.*) injections (total volume = 100 μ l) of 20 μ g OVA (grade V; Sigma-Aldrich, St Louis, MO, USA) emulsified in 2 mg of Imject[®] alum adjuvant (Pierce, Rockford, IL, USA) dissolved in 0.1 M phosphate-buffered (pH 7.4) saline (PBS). Seven days following the last sensitization, mice were challenged (45 min) with an OVA aerosol generated by ultrasonic nebulization of a 1.5% (w/v) OVA solution in PBS; this challenge phase of the protocol was carried out three times a week (every other day) for eight consecutive weeks. Negative controls were sensitized and challenged with PBS.

Adenovirus preparation

Adenovirus expressing either *Cre* or a *Cre*–green fluorescent protein fusion protein (Ad-*Cre*-GFP) and adenovirus expressing only GFP (Ad-GFP) were purchased from Vector Biolabs, Philadelphia, PA, USA. Viral stocks (2.5×10^7 PFU/ml) were prepared (as per the manufacturer's instructions) by infecting 293T cells.

Shp2 conditional deletion in airway epithelia

Ad-*Cre*-GFP was delivered intratracheally to each mouse (100 μ l, 2.5×10^6 PFU) for 3 days prior to the first OVA challenge to elicit cell-specific deletion of the SHP2 enzyme in airway epithelium. Another group of mice was administered Ad-GFP and used as control animals. The adenoviral administration was repeated 4 weeks later. The efficiency of *shp2* deletion was determined (Appendix S1).

Assessments of allergen-mediated inflammation and airway remodeling events

The details of the measurement of airway inflammatory metrics and specific remodeling events, including the recover and processing of tissues for *in vitro* experimentation, are described in the Appendix S1.

Statistical analyses

Data analysis was performed using the SPSS statistical software package version 17.0 (SPSS Inc., Chicago, IL, USA). Results were plotted using SPSS SIGMAPLOT (version 10.0). Statistical analysis for comparisons between groups was performed using pairwise Student's *t*-tests.

Data are expressed as the mean $\pm$ SEM. Differences between mean values were considered significant when $P < 0.05$.

Chronic OVA challenge leads to increased expression of *shp2* in airway epithelia

As an initial approach toward investigating the possible role of SHP2 in allergic airway remodeling, we assessed the levels of *shp2* mRNA and protein expression in airway epithelial cells of mice subjected to chronic OVA challenge. These studies revealed that *shp2* mRNA and protein levels each increased significantly (relative to unchallenged control mice) in response to chronic OVA challenge (Fig. 1).

Generation of conditional *shp2* knockout in mouse airway epithelia

Shp2^{flox/flox} mice were administered Ad-GFP-*Cre* (via tracheal instillation) to induce *Cre* recombinase expression (i.e., *shp2* gene inactivation among infected airway epithelial cells); these animals are designated as iASKO mice. Assessments of genomic DNA from the airway epithelial cells of iASKO mice demonstrate the nearly complete deletion of the 'floxed' exon of *shp2* (i.e., exon 4) in these mice (Fig. 2A–B). Western blot analysis demonstrated that the deletion of exon 4 was also accompanied with a near-complete loss of SHP2 protein expression in the airway epithelium of iASKO mice (Fig. 2C). Immunohistochemical staining also demonstrated that Ad-GFP-*Cre* infection of the lungs of mice significantly decreased the level of SHP2 expression in the airway epithelium (Fig. 2D–E).

The specific targeting of *shp2* in airway epithelial cells demonstrates a link with the pulmonary pathologies associated with chronic allergen challenge

The overlay of a strategy targeting SHP2 expression in airway epithelial cells with an OVA sensitization/chronic OVA challenge protocol (Fig. 3A) demonstrated the unique importance of airway epithelial cells in the pulmonary changes associated with chronic allergen provocation. Specifically, endpoint assessments of pulmonary inflammatory metrics demonstrated that the loss of SHP2 in airway epithelial cells resulted in a concomitant decreases in allergen-induced inflammation. For example, airway hyper-responsiveness (AHR) observed in iASKO mice was significantly decreased, reaching levels similar to those detected in PBS-treated control animals (Fig. 3B). Moreover, the loss of SHP2 from airway epithelial cells resulted in a smaller bronchoalveolar lavage (BAL) fluid pro-inflammatory cell infiltrate; in particular, BAL eosinophil numbers in iASKO mice showed a significant decrease relative to the eosinophilia observed in OVA-treated control mice (Fig. 3C). The lower pro-inflammatory cell infiltrates of iASKO mice were accompanied by a corresponding decrease in the histopathology observed in these animals (Fig. 3D) that is quantitatively reflected in the peribronchial inflammatory scores of these mice (Fig. 3E).

The specific loss of *shp2* in airway epithelial cells attenuates the remodeling events associated with chronic allergen provocation

The assessments of remodeling metrics linked with chronic OVA challenges [airway smooth muscle hyperplasia and extracellular matrix deposition of collagen (i.e., pulmonary fibrosis)] showed that airway epithelial expression of SHP2 was a contributor to the histopathological changes linked with allergen challenge. Airway smooth muscle remodeling (i.e., smooth muscle thickness and total pulmonary α -smooth muscle actin (SMA) protein expression) was assessed in response to chronic OVA challenge by immunohistochemistry (Fig. 4A–B). iASKO mice chronically challenged with OVA exhibited significantly less α -SMA staining (per μ m of small bronchi basement membrane) and lower total α -SMA levels relative to OVA-treated control animals. However, the baseline levels of airway α -SMA protein

staining and total lung α -SMA protein expression in the airway epithelium of non-OVA-challenged mice were similar in both the control and iASKO mice (Fig. 4A–B).

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Assessments of Masson's trichrome–stained lung tissue showed the *shp2*-dependent effects on peribronchial fibrosis (Fig. 4C–E). Specifically, in both control and iASKO mice, the collagen staining areas of tissue sections (per μ m length of the small bronchi basement membrane) and the total levels of lung collagen at baseline level (i.e., non-OVA-challenged mice) were similar to levels detected in OVA-challenged animals. In contrast, significantly, the groups chronically challenged with OVA displayed a significant increase in both Trichrome staining (Fig. 4D) and total lung collagen levels (Fig. 4E). However, despite the repetitive challenge of iASKO mice with OVA for the same duration as control mice, they exhibited significantly less peribronchial Masson's trichrome staining (Fig. 4D) and total lung collagen (Fig. 4E) relative to control mice.

***Shp2* knockout in airway epithelium decreased TGF- β 1 production**

Our demonstration of a link between airway epithelial cell SHP2 expression and airway remodeling events suggested that this expression was mechanistically linked with the increased levels of TGF- β observed in animals following chronic OVA provocation. Assessments of TGF- β levels in the BAL of these animal groups showed that repetitive OVA challenges of OVA-treated control and iASKO mice induced a significant increase in the amount of TGF- β 1 in BAL relative to saline-challenged control mice. However, similar to the changes observed in pulmonary fibrosis, this increase in TGF- β was significantly lower in the iASKO mice (Fig. 4F). In addition, the area of TGF- β 1-positive staining in airway epithelium (per μ m length of the small bronchi basement membrane) was significantly less in the iASKO mice relative to control animals (Fig. 4G).

Induced overexpression of *shp2* in the airway epithelium modulates the conversion of fibroblasts to myofibroblasts and, in turn, induces collagen production by stimulating TGF- β 1 production

An *in vitro* cell model system using BEAS-2B airway epithelial cells was employed as an additional demonstration of *shp2*-dependent regulation of TGF- β 1 production. Expression levels of *shp2* were manipulated downward in BEAS-2B cells by using siRNA knockdown (*Shp2* KD) and upward through the transfection of a *shp2* overexpression plasmid. These studies showed that BEAS-2B cells endogenously produced SHP2 protein, which decreased after transfection with *shp2* siRNA plasmid (Fig. 5A). In addition, transfection with a *shp2* overexpression cassette significantly increased the levels of SHP2 over endogenous BEAS-2B levels (Fig. 5A). These induced changes in SHP2 expression levels correlated with concomitant changes in TGF- β 1 levels in these cells. That is, TGF- β 1 mRNA levels were significantly decreased in cells with knockdown *shp2* and were significantly increased in *shp2*-overexpressing cells (Fig. 5B). Moreover, these mRNA changes also reflected similar changes observed in TGF- β 1 protein levels found in the culture supernatants from these cells (Fig. 5C).

A direct link between remodeling events and the manipulation of *shp2* gene expression in BEAS-2B cells was achieved through co-culture studies with mouse primary lung fibroblasts (Fig. 5D–H). Specifically, when conditioned medium from *Shp2* KD/BEAS-2B cells (i.e., reduced *shp2* gene expression) was added to mouse primary lung fibroblasts, the production of both collagen (Fig. 5D–E) and α -SMA (Fig. 5F–H) was significantly reduced. In contrast, conditioned medium from *shp2*-overexpressing BEAS-2B cells caused increased production of collagen (Fig. 5D–E) and α -SMA (Fig. 5F–H) in mouse primary lung fibroblasts. More importantly, in each of these cases of *shp2*-overexpressing cell culture supernatants, the addition of anti-TGF- β 1-neutralizing antibody eliminated the induced

increases in collagen (Fig. 5D–E) and α -SMA (Fig. 5F–H) in mouse primary lung fibroblasts; similar results were observed in studies of co-culturing BEAS-2B cells with the human fibroblast cell line HFL1 (data not shown).

Discussion

The bronchial epithelium acts as the barrier to the external environment and plays a vital role in protecting the internal milieu of the lung. Damage to the epithelium is now recognized as a key factor driving airway remodeling. As such, many types of challenges to the epithelium, including pathogens, allergens, and cigarette smoke, can elicit the production of factors that mediate airway inflammation and remodeling [22]. In the present study, our findings revealed that *shp2* is a potential contributor to pathways in allergic respiratory inflammation that lead to airway remodeling in mice. Specifically, we found that *shp2* mRNA and protein levels in the airway epithelium were increased significantly in mice in concert with induced remodeling events following allergen provocation. This link was further examined using a conditional genetic approach to dissect the role of *shp2* in the regulation of OVA-induced asthmatic airway remodeling because homozygous deletion of mouse *shp2* is known to result in early embryonic lethality [19, 20]. We selectively inactivated the *shp2* gene in adult mouse airway epithelia using a *Cre-loxP* strategy by tracheal instillation of Ad-*Cre* into *shp2^{lox/lox}* mice. These studies showed that mice with *shp2* conditional knockout in the airway epithelia exhibited reduced levels of pro-inflammatory cell infiltrates in both the lung and airway lumen and reduced levels of AHR. Moreover, the loss of *shp2* from the airway epithelium led to lower lung inflammation scores with less expression of α -SMA and collagen in lung following OVA challenges. These results suggested that SHP2 signaling in airway epithelia was necessary for the airway remodeling events linked with repetitive allergen challenge in this model of respiratory inflammation.

Our data revealed that the mechanistic role of *shp2*-mediated activities in airway epithelial cells appears to be the induction of TGF- β 1 production from airway epithelium. In the airways, TGF- β is found in various cell types, including epithelial cells and inflammatory cells that colonize the space beneath the basement membrane [23]. There is speculation about the different functions of TGF- β at its different locations. In particular, Kumar and coworkers suggested that the concentration of antigen and the amount of antigen exposure are key elements that determine which cells will preferentially produce TGF- β 1 in allergic asthma. They concluded that whereas eosinophils are the main TGF- β 1-producing cells in acute models of allergic asthma challenged with high doses of antigen, the epithelium is the main source of this cytokine in sensitized animals chronically challenged with low doses of antigen [4]. In this study, we found that TGF- β 1 protein expression in mouse airway epithelial cells was increased after chronic exposure to an allergen and that this increased expression correlated with SHP2 signaling in airway epithelia. Significantly, we showed *in vivo* that knockdown of *shp2* gene expression in airway epithelia was able to reduce the chronic OVA-induced increase in TGF- β 1 production. In addition, our *in vitro* studies demonstrated that the levels of TGF- β 1 mRNA in BEAS-2B cells and the levels of soluble TGF- β 1 protein in culture supernatants parallel SHP2 protein levels. In addition, supernatants from BEAS-2B cells overexpressing SHP2 were able to promote fibroblasts' expressions of α -SMA, collagen type I, and collagen type III. More importantly, this effect could be blocked with an antibody against TGF- β 1, suggesting that TGF- β 1 produced by epithelia contributes to collagen production by lung fibroblasts, as well as fibroblast conversion into myofibroblasts. It remains unclear which signal transduction pathway is involved in the SHP2-mediated production of TGF- β 1 revealed in our study. Liu and colleagues have suggested that SHP2 is a positive transducer of growth factor and cytokine signaling [24], while studies led by Cha and Quintanar-Audelo indicated that SHP2 is an

essential mediator to regulate ERK pathways [25]. We are currently exploring the ERK signaling pathway as a potential contributor to SHP2-mediated allergic airway remodeling.

In conclusion, we have provided evidence demonstrating that protein tyrosine phosphatase SHP2 regulates the TGF- β 1 production in airway epithelial cells and may be involved in asthma pathogenesis and airway remodeling. Our findings identify SHP2 as a new member of the factors suggested to regulate the remodeling events linked with repetitive allergen provocation. As such, we suggest that SHP2 may be a previously underappreciated therapeutic target for intervention; future studies will validate both this potential mechanism and the efficacy of potential therapeutic approaches targeting SHP2.

"Online Materials"

Materials and Methods

The efficiency of Shp2 deletion—To determine the efficiency of *shp2* deletion in the airway epithelia, the tracheal epithelial cells were isolated from eight mice. Briefly, mice were anesthetized and the tracheas were excised and washed in pre-warmed phosphate buffered saline (PBS). Eight tracheae were incubated at 4°C overnight in 10 mL of Dulbecco's modified Eagle's medium (DMEM) containing 1.4 mg/mL of Pronase XIV (Sigma-Aldrich, USA). The cell suspension was collected and centrifuged at 200×g for 10 min. The cell pellets were washed twice with PBS and harvested for genotypes by PCR and SHP2 protein expression by Western blotting. In addition, SHP2 protein expression in airway epithelia was also confirmed by immunohistochemistry using the paraffin-embedded lung sections.

Assessment of airway hyperresponsiveness (AHR)—The measurement of AHR was described previously (1). Twenty hours after the last OVA challenge, the unrestrained conscious mice were put into the chambers and nebulized with PBS followed by increasing doses of MCh (6–100 mg/mL) by using whole-body plethysmography (Buxco Research Systems, Wilmington, NC, USA). At each dose, the values of enhanced pause (Penh), reflecting the degree of AHR, were calculated.

Bronchoalveolar lavage and cytologic analysis—Two days after the final challenge, mice were anesthetized and sacrificed by cardiac puncture bleeding. Lungs were lavaged with 0.8 mL PBS, and approximately 0.6–0.7 mL of bronchoalveolar lavage fluid (BALF) was recovered. All BALF samples were kept on ice until processing. After determining the total cell number on a hemacytometer, BALF samples were spun at 2000 rpm for 5 min at 4°C, after which the supernatants were collected and stored at –80°C for TGF- β 1 analysis. Cell pellets were resuspended in 100 μ L PBS, cytopinned (Thermo Shandon Inc., USA), and stained with Wright-Giemsa to facilitate differential cell counting. All differential counts were performed in a blinded manner and in a randomized order at the end of the study. A total of 300 to 500 cells per cytospin were differentiated using this strategy.

Lung inflammation scores—Immediately upon sacrifice, the lungs were inflated with an intratracheal injection of 10% formalin solution, in order to preserve pulmonary architecture. Then, the lung tissues were embedded in paraffin according to standard pathology protocols. The paraffin-embedded sections were stained with hematoxylin and eosin (H&E) to evaluate immune cell infiltration. A standard quantitative scoring system was used to grade the extent of lung infiltrates (2). In brief, the degree of peribronchial inflammation was evaluated on a scale of 0 to 4: a value of 0 was assigned when no inflammation was detectable; a value of 1 indicated occasional cuffing with inflammatory cells; a value of 2 indicated that most bronchi were surrounded by a ring of inflammatory

cells that were one cell layer deep; a value of 3 was assigned when most bronchi were surrounded by a ring of inflammatory cells that were two to four cells deep; and a value of 4 was assigned when most bronchi were surrounded by a thick layer of inflammatory cells (more than four cells deep).

Measurement of airway smooth muscle thickness—To determine the airway smooth muscle thickness, the α -smooth muscle actin (α -SMA) protein was identified in paraffin-embedded sections of the lung tissue by using immunohistochemical staining with an α -SMA antibody (Abcam, England). The thickness of detected α -SMA was visualized by using a light microscope (BX51; Olympus, Japan) and quantified by the Image Pro 6.1 software (Media Cybernetics). Results were expressed as area of α -SMA staining per micrometer length of bronchioles' basement membrane of 150–200 μ m in internal diameter. At least 10 bronchioles were counted on each slide and the data used for statistic analysis.

Quantitation of peribronchial fibrosis and collagen—Peribronchial fibrosis was detected by Masson trichrome staining. The area of peribronchial Masson trichrome staining (blue color) in paraffin-embedded lung was visualized and quantified using a light microscope and Image Pro 6.1 software, as described above. Results were expressed as the area of Masson trichrome staining per micrometer length of bronchioles' basement membranes that were 150–200 μ m in internal diameter. At least 10 bronchioles were counted on each slide for statistic analysis.

Collagen lung content was measured by quantifying soluble collagen with the Sircol Collagen Assay Kit (Biocolor, Chile), according to the manufacturer's protocol (3). In brief, 100 mg of lung tissues were homogenized and total acid pepsin-soluble collagens were extracted overnight at room temperature using 0.1 mg/mL of pepsin in 10 mL of 0.5 M acetic acid. Each sample was extracted in duplicate. A 1 mL aliquot of Sircol dye reagent was added to 100 μ L of each extraction, centrifuged, and the pellets re-suspended in 1 mL of alkali reagent. Finally, absorbance was read at 555 nm (Thermo Scientific Microplate Reader, Finland).

TGF- β 1 analysis—TGF- β 1 proteins expressed in airway epithelia were detected in the paraffin-embedded sections of the lung tissue by using immunohistochemical staining with anti-TGF- β 1 antibody (Biovision Inc., USA). Soluble TGF- β 1 released into BALF or cultured cells supernatants were assessed by sandwich enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's protocol (R & D Systems Inc., USA). The sensitivity of detection was 7 pg/mL in BALF.

Shp2 overexpression and knockdown in BEAS-2B cells—BEAS-2B cells were cultured in DMEM/F12 medium containing 10% fetal calf serum. BEAS-2B cells were transfected with the expression vector for *shp2*, shRNA against *shp2*, or empty vector (control) (gifts from Dr. Gen-Sheng Feng at the University of California at San Diego, USA) using Lipofectamine Plus (Invitrogen, USA), according to the manufacturer's instructions. Supernatants were collected at 48 hours after transfection and stored at -80°C until further analysis. Cells were harvested for Western blotting or real-time reverse transcription (RT)-PCR, which were performed within three days. Experiments were repeated four times.

Culture of mouse primary lung fibroblasts—Fibroblasts were isolated from the lungs of C57BL/6 mice (4). Lung tissue was dissected from the airways, minced into 2 mm pieces, and placed in tissue culture flasks with a minimal volume of DMEM supplemented with 10% FBS (v/v), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were incubated in a humidified incubator at 37°C under a 5% CO_2 atmosphere. After 20 min, an

appropriate volume of medium was added to the flask, and the cells were maintained until fibroblasts began to migrate out from the tissue. Identification of fibroblasts was based on the morphology and presence of vimentin staining (1:200; Sigma-Aldrich). Cells between generation 2 and 5 were used for experiments and cultured in BEAS-2B-conditioned medium.

α -SMA expression in primary lung fibroblasts determined by Western blotting, RT-PCR, and immunofluorescence—For Western blotting, primary mouse lung fibroblasts were washed with ice-cold PBS, then lysed on ice in 100 μ L of lysis buffer (50 mM Tris, 150 mM NaCl, 20 mM NaF, 1 mM NaVO₃, 1 mM EDTA, 1 μ M PMSF, glycerol, 1% v/v Triton X-100, 0.1% w/v sodium dodecyl sulfate, and protease inhibitor cocktail (Roche, Switzerland). The lysates (20 μ g) were loaded onto a 10% SDS-PAGE gel and electrotransferred onto a nitrocellulose membrane. Blots were incubated with an anti- α -SMA antibody (1:800; Abcam). Immunoblot signals were detected with IRDye[®]680-labeled goat-anti-mouse secondary antibody (1:5000; LI-COR Bioscience, USA) and visualized using the Odyssey Infrared Imaging System (LI-COR Bioscience). A single band was detected at 45–54 kDa. The membranes were reprobed with an anti-GAPDH antibody (1:1500; Sigma-Aldrich) and immunoblot signals were detected with IRDye[®]800-labeled goat-anti-rabbit secondary antibody (LI-COR Bioscience) to verify equal loading of protein in each lane. Densitometric analysis was performed using Quantity One 1-D Analysis Software version 4.62 (Bio-Rad Laboratories Inc, USA). Data were expressed as mean $\pm$ SEM.

For RT-PCR, cells were washed in PBS and then lysed in TriZol reagent (Invitrogen), and RNA was extracted according to the manufacturer's instructions. cDNA was prepared by reverse transcription of RNA using random hexamer primers and Superscript II (Invitrogen). Real-time PCR was performed for 50 cycles on a Step One[™] Real-time PCR System (Applied Biosystems, USA). Each PCR reaction used the following thermal cycling conditions: 37°C for 10 min, 95°C for 5 min, followed by 50 cycles of 95°C for 15 s, 60°C for 20 s, and 68°C for 20 s. Primers are shown in Table 1. For each sample, Δ Ct was used to calculate the differences between target Ct values and the normalizer (housekeeping) gene: Δ Ct = [(target) – Ct (normalizer)]. The comparative $\Delta\Delta$ Ct was used to calculate the differences between each sample's Δ Ct value and the baseline's Δ Ct. The comparative expression level was obtained by transforming the logarithmic values to absolute values using $2^{-\Delta\Delta\text{Ct}}$.

For immunofluorescence, mouse primary lung fibroblasts were seeded onto glass coverslips, fixed in freshly made 4% paraformaldehyde in PBS (pH 7.4) for 20 min, and washed three times in PBS. After permeabilizing with 0.1% Triton X-100 in PBS for 15 min at room temperature and washing three times with PBS, the fibroblasts were blocked in 10% normal goat serum (NGS; Sigma-Aldrich) in PBS for 60 minutes. After, the fibroblasts were incubated with anti- α -SMA antibody (1:100; Abcam) at 4°C overnight in a sealed humid container. After washing three times with PBS, the fibroblasts were incubated with goat anti-mouse IgG conjugated to FITC (1:100; Sigma-Aldrich) at room temperature for 40 min in a sealed humid container in the dark. After washing three times with PBS, the fibroblasts were mounted with Vectashield mounting media (Vector Labs, USA), sealed with nail polish, and viewed under an Olympus BX51 imaging fluorescence microscope.

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Figure 1.

Chronic allergen provocation of C57BL/6 mice elicits *shp2* gene expression in airway epithelial cells. *shp2* mRNA (RT-PCR) and SHP2 protein (Western blot) expression in airway epithelia was compared to saline-treated control lung tissue. Individual lanes in each panel represent different mice examined.

Figure 2.

Generation of iASKO mice and a strategy of airway-specific ablation of *shp2*. (A) Schematic map of the generation of iASKO mice. Exon 4 of the *shp2* gene was targeted by transient *Cre* expression in *shp2*^{fllox/fllox} mice mediated by *Cre* recombinase delivered to airway epithelial cells via an Ad-*Cre*-GFP adenoviral vector (i.e., iASKO mice). (B) Successful deletion of exon 4 in *shp2* (i.e., the generation of an inactive *shp2* allele) was confirmed by allele-specific PCR of genomic DNA isolated from airway epithelial cells of iASKO mice and mice treated with Ad-GFP as control. (C) Western blotting analysis showed that SHP2 protein was absent in airway epithelial cells of *shp2*^{fllox/fllox} mice that also expressed *Cre* recombinase. (D) Immunohistochemistry demonstrated the SHP2 protein expression (brown staining cells) in airway epithelia of *shp2*^{fllox/fllox} mice without *Cre* recombinase (control). (E) Immunohistochemistry demonstrated that SHP2 protein was reduced in airway epithelial cells in iASKO mice. Scale bar: 30 μm.

Figure 3.

The loss of *shp2* in airway epithelial cells leads to a reduction in histopathologies and lung dysfunction following repetitive allergen provocation. (A) The experimental scheme used to establish chronic ovalbumin (OVA)-induced allergic airway remodeling. (W), time scale is weeks; S, OVA/alum sensitization; C, OVA challenge (three times a week); Ad, administration of adenovirus; X, terminal endpoint of study. (B) Airway resistance as characterized by enhanced pause (Penh) following nebulization with increasing doses of methacholine. Results are expressed as mean $\pm$ SEM ($n = 5$ mice/group, in two separate experiments). * $P < 0.05$, OVA-sensitized control group vs OVA-sensitized iASKO. ** $P < 0.05$, OVA-sensitized iASKO vs with PBS-sensitized iASKO. (C) BALF differentials from mice are expressed as mean $\pm$ SEM ($n = 5-6$ mice/group, in two independent experiments). * $P < 0.05$. Eos, eosinophils; LMs, lymphomononuclear cells; Macs, macrophages; Neuts,

neutrophils. (D) Representative photomicrographs (H&E-stained sections) characteristic of the pulmonary histopathology occurring in the lungs of mice from each group. (E) Total lung inflammation was defined as the average of the peribronchial inflammation scores expressed as mean $\pm$ SEM ($n = 5-6$ mice/group, in two separate experiments). Scale bar: 100 μm .

Figure 4.

Shp2 deletion in airway epithelial cells prevents airway smooth muscle hyperplasia and peribronchiolar collagen deposition. All data shown were obtained with groups of $n = 5-6$ mice/group, in two separate experiments. (A) Representative photomicrographs demonstrating α -smooth muscle actin (α -SMA) expression (arrows) in lung tissue detected by immunohistochemistry. Scale bar: 100 μ m. (B) Plot of mean $\pm$ SEM measurements of the area covered by α -SMA staining per μ m length of small bronchi basement membrane. (C) Representative photomicrographs demonstrating peribronchial collagen deposition (arrows) detected by Masson's trichrome staining (blue color). Scale bar: 100 μ m. (D) Image analysis assessing both the intensity and extend of Masson's trichrome staining expressed as mean $\pm$

SEM, respectively. (E and F) Plots of total lung collagen determined by the Collagen Assay kit and TGF- β 1 concentration in BALF measured by ELISA, respectively. Individual values and means (solid lines) for each group are shown. (G) Plot of mean $\pm$ SEM measurements of the area of TGF- β 1 staining in airway epithelia per μ m length of small bronchi basement membrane.

Figure 5.

TGF- β 1 production in BEAS-2B cells and, in turn, production of collagen I, collagen III, and α -smooth muscle actin (α -SMA) in mouse primary lung fibroblasts occur as a function of shp2 gene expression. All control groups were transfected with empty expression vector. (A) Knockdown (KD) and overexpression of SHP2 in BEAS-2B cells were demonstrated by Western blot analysis of cell lysates. (B) TGF- β 1 transcript levels in unstimulated BEAS-2B cells following transfection of *shp2* knockdown vs overexpression cassettes as determined by real-time RT-PCR. Data shown are mean $\pm$ SEM of four independent experiments. (C) TGF- β 1 levels in supernatants of BEAS-2B cells following transfection of *shp2* knockdown

vs overexpression cassettes, as quantified by ELISA. Individual values and means (solid lines) are shown for each group. (D) Collagen I and collagen III gene transcripts induced in mouse primary lung fibroblasts upon stimulation by BEAS-2B supernatants following transfection of *shp2* knockdown vs overexpression cassettes, as measured by real-time RT-PCR. Results are expressed as mean $\pm$ SEM of four independent experiments. (E) Total collagen content (Sircol collagen assay) in primary lung fibroblast culture media following co-culture with BEAS-2B cells transfected with *shp2* knockdown vs overexpression cassettes. Individual values and means (solid lines) are shown for each group. (f–h) Measurements of α -SMA produced upon stimulation by culture supernatants from BEAS-2B cells transfected with *shp2* knockdown vs overexpression cassettes as determined by real-time RT-PCR (F), immunofluorescence staining (G), and Western blot analysis (H). Data are expressed as mean $\pm$ SEM. * indicates statistically significant differences of $P < 0.05$ among the comparisons noted.

Table 1

Gene-specific primers used in this study for RT-PCR

Gene	Forward	Reverse
Mouse <i>shp2^{flox}</i> ^a	5'-ACGTCATGATCCGCTGTCAG-3'	5'-ATGGGAGGGACAGTGCAGTG-3'
Mouse <i>shp2^{flox}</i> ^b	5'-CAGTTGCAACTTTCTTACCTC-3'	5'-GCAGGAGACTGCAGCTCAGTGATG-3'
Human <i>TGF-β1</i>	5'-GTGGAAACCCACAACGAA AT-3'	5'-CACGTGCTGCTCCACTTTTA-3'
Mouse <i>collagen I</i>	5'-TGTGTGCGATGACGTGCAAT-3'	5'-CCCTCGACTCCTACATCTTC-3'
Mouse <i>collagen III</i>	5'-GGTTTCTTCTCACCTTCTTC-3'	5'-ATCCTGAGTCACAGACACATA-3'
Mouse α -SMA	5'-CTGACGCTGAAGTATCCGAT-3'	5'-TCTCAAACATAATCTGGGTC-3'
Mouse β -actin ^c	5'-AGAGGGAAATCGTGCGTGAC-3'	5'-CAATAGTGATGACCTGGCCGT -3'
Human <i>GAPDH</i>	5'-TGGTCACCCAGGGCTGCTTTTAA-3'	5'-CTGGAAGATGGTGATGGGAT-3'
Mouse <i>Eotaxin</i>	5'-AGAGGCTGAGATCCAAGCAG-3'	5'-ACAGATCTCTTTGCCCAACCT-3'
Mouse <i>IL-4</i>	5'-ACGGCACAGAGCTATTGATG-3'	5'-ATGGTGGCTCAGTACTACGA-3'
Mouse <i>IL-5</i>	5'-ATGACTGTGCCTCTGTGCCTGGAGC-3'	5'-CTGTTTTCTCGGAGTAACTGGGG-3'

^a *shp2^{flox/flox}* genotyping.^b *shp2* knockout efficiency.^c Real-time RT-PCR.