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shRNA for Thymic Stromal Lymphopoietin: A Novel Therapeutic Approach for Pulmonary Fibrosis

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Abstract

Thymic stromal lymphopoietin (TSLP) was recently identified as a master switch for Th2 responses. This study discusses the role of TSLP in pulmonary fibrosis. We show that TGF-β1 (a Fibrogenic Growth Factor) up regulates TSLP proteins in human lung fibroblasts (HFL-1) on a dose- and time-course-dependent basis. Additionally, TSLP increases fibronectin expression on a dose- (1 ng/ml to 100 ng/ml) and time-course-dependent basis concomitantly with the upregulation of pSmad2/3 and Smad4, which is the essential downstream signal regulator for TGF-β. Silencing TSLP by TSLP shRNA dramatically reverses TGF-β1-induced cellular fibrosis concomitantly with the suppression of type I TGF-β receptors and pSmad2/3. Parallel results are observed in vivo. Bleomycin-treated C57BL/6 mice show intense staining for TSLP in fibrotic lung tissue by immunohistochemistry. More importantly, Sirius red and H&E staining from bleomycin-treated mice demonstrate that transfection with TSLP shRNA (by intranasal instillation) dramatically decreases both infiltration of inflammatory cells and deposition of collagen compared to the control. Moreover, a whole-body plethysmography test showed that TSLP shRNA transgenic mice significantly attenuate the increase in airway respiratory resistance induced by bleomycin. Thus, it may be possible to use TSLP shRNA as a novel therapeutic approach for treating pulmonary fibrosis by down-regulating TGF-β signal proteins.

Keywords: TSLP; TGF-β1; Smad; Receptors; Lung fibrosis

Abbreviations: ELISA: Enzyme-Linked Immunosorbent Assay; FBS: Fetal Bovine Serum; FN: Fibronectin; HFL-1: Normal Human Lung Fibroblast Cells; H&E stain: Hematoxylin and Eosin stain; IPF: Idiopathic Pulmonary Fibrosis; PF: Pulmonary Fibrosis; shRNA: Short Hairpin RNA; TGF-β1: Transforming Growth Factor-Beta1; TGF-β RI: Type I TGF-β Receptor; TSLP: Thymic Stromal Lymphopoietin

Introduction

Pulmonary Fibrosis (PF) is the end stage of a wide range of inflammatory lung conditions [1]. It can result from a variety of causes, including lung injury, environmental particle and toxin inhalation, chemotherapy, and systemic autoimmune diseases [2]. Idiopathic Pulmonary Fibrosis (IPF) is the most common type of pulmonary fibrosis. The prognosis for IPF patients is poor and current therapies are ineffective in preventing the onset of respiratory failure. Novel therapeutic approaches for treating pulmonary fibrosis by using molecular targeting of specific signaling pathways activated during fibrotic processes have been proposed for years; however, few successful strategies have been proposed thus far [1,3].

Thymic Stromal Lymphopoietin (TSLP) is a novel IL-7-like cytokine, originally cloned from a murine thymic stromal cell line [4]. It was first identified in conditioned medium supernatants of the mouse thymic stromal cell line Z210R.1 [5]. TSLP has been shown to be highly involved in the pathogenesis of inflammatory diseases [6]. It is produced by epithelial cells, skin keratinocytes, stromal cells, smooth muscle cells, mast cells, and lung fibroblasts [7]. Recent studies have established that high levels of TSLP are associated with airway inflammatory disease in humans and mice [8,9]. Over-expression of TSLP in transgenic mice induces spontaneous airway inflammation and atopic dermatitis [10];

however, little is known about the role of TSLP in regulating fibrosis in pulmonary fibrosis.

TGF-β1 is a multi-functional cytokine that regulates the cell growth, differentiation, and functions of various cell types. It is a "key switch" to induce fibrosis in many tissues, including the lungs. TGF-β1 is upregulated in the lungs of patients with IPF, and expression of active TGF-β1 in rat lungs induces a dramatic fibrotic response. Many effects of TGF-β1 are mediated by signal transducer and transcription factor Smad. Smad2/3 are Receptor-Activated Smad Proteins (R-Smad), which are phosphorylated by TGF-β receptor type I and subsequently form a heteromeric complex with Co-Smad (Smad4) [11]. Such complexes subsequently translocate into the nucleus to regulate the expression of target genes (e.g., fibronectin) [12]. In addition, accumulation of tissue fibrosis, which can lead to abnormalities in organs, occurs during the inflammation process as well. Since TSLP is highly involved in the pathogenesis of inflammatory diseases, it is of great interest to understand whether TGF-β1 synergistically interacts with TSLP

to exert its fibrotic effects in lungs. However, the role of TSLP in the regulation of pulmonary fibrosis has remained unknown up to now.

In this study, we used both in vitro and in vivo models to elucidate the role of TSLP in lung fibrosis. This study demonstrated that TSLP plays a pivotal role in the regulation of pulmonary fibrosis. In addition, TSLP shRNA might act as a novel antagonizing agent against TGF-β1 signaling and fibrosis partly by down-regulating TGF-β RI and pSmad2/3.

Materials and Methods

Cell culture

HFL-1 (CCL-153; American Type Culture Collection, Manassas, VA), a normal human lung fibroblast cell line, was cultured in Ham's F12K (GIBCO,CA,USA) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Hyclone Labs, Sweden, Europe) at 37°C in 5% CO_2. The cells were trypsinized by 0.025% trypsin-EDTA (Hyclone Labs, Sweden, Europe), typically seeded at approximately 70% confluence in complete medium that contained 10% FBS for 24h, and then serum starved for 24 h. For protein assays, HFL-1 was treated with recombinant human TGF-β1 (R&D Systems, Minnesota, USA) and TSLP (R&D Systems, Minnesota, USA) for specific time points.

Animal study

Male C57BL/6 mice, aged six to eight weeks and weighing 18 to 22 g, were obtained from the National Laboratory Animal Center (NLAC, Taipei, Taiwan). The animals were housed in a plastic cage with the Ventilated Micro Isolator System (VMIS) maintained between 21°C and 24°C with a 12h/12h light/dark cycle. The animals received a standard sterile rodent chow diet (altromin 1326, Altromin, Lage, Germany) and distilled water. The experimental protocol adhered to the rules of the Animal Protection Act in Taiwan. A pulmonary fibrosis murine model was performed according to Kolb et al. Briefly, mice were lightly anesthetized with a carbon dioxide/oxygen mix. While anesthetized, intranasal instillation was conducted by placing bleomycin (60 mU/kg, BLEOCIN®, Nippon Kayaku Co., Ltd. Tokyo, Japan) suspended with normal saline in a total volume of 75 μl on the nares as the bleomycin group. C57BL/6 mice were classified into four groups (n=3), including a control group (i.e., sham treatment with 0.2 ml normal saline), a lung fibrosis-inducing group (treated with bleomycin), an shRNA transfection control group (i.e., inoculated with shRNA control vector with transfection reagent), and a lung fibrosis therapeutic group (i.e., inoculated with bleomycin with TSLP shRNA (3 μg/mice, sc-39821-SH, Santa Cruz Biotechnology, CA, USA) in vivo-jetPEI™ transfection reagent (Polyplus-transfection Inc. CA, USA). Intranasal gene transfer procedures were performed as described in the commercial manual (in vivo-jetPEI® DNA & siRNA Delivery kit, Cat # CPT201vK). In brief, after mixing vivo-jetPEI®, 10% glucose, and 3 μg of plasmid DNA, the mixture was incubated for 15 minutes at room temperature, and intranasal instillation was performed afterward. The transfection efficiency for trachea and lung tissues for our routine experiments was steady and mostly around 80-90%. All mice were briefly anesthetized seven days later. Mice were monitored for general health and weighed daily. The study animals were killed 14 days after bleomycin administration. Blood was collected and the lungs were removed for either histological or biochemical analysis.

Airway respiratory resistance by Whole-Body Plethysmography (WBP)

According to Hsieh et al., the airway respiratory resistance was expressed with the enhanced pause (Penh) as a parameter of altered airway function using the MAX-II 1320 Modular Unit (Buxco, Troy, NY, USA) [13]. Briefly, the mice were placed in the plethysmographic measuring chamber. The pressure differences signal was determined by the volume and resultant pressure changes in the chamber during the respiratory cycle of the mouse. Penh is a dimensionless value that represents a function of the proportion of maximal expiratory pressure to maximal inspiratory pressure and the timing of expiration. According to the manufacturer's instructions, Penh was calculated as (Te-Tr)/Tr × (PEP/PIP), where Te is expiratory time (sec.); Tr is relaxation time (sec.) defined as the time of pressure decay to 30% of the total expiratory pressure signal (area under the box pressure signal at expiration); PEP is peak expiratory pressure (ml/sec); and PIP is peak inspiratory pressure (ml/sec). Penh reflects changes in the waveform of the box pressure signal during both inspiration and expiration, and combines these changes with the timing comparison of early and late expiration. Penh was measured for 2.0 min for each experiment.

shRNA transfection *in vitro*

HFL-1 cells were seeded in a six-well tissue culture plate growing to 50-70% confluency in an antibiotic-free medium supplemented with FBS. Serum-free FBS with Fugene6™ Transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN; catalog no. 1814443) was prepared and incubated at room temperature for five minutes. Cells were transfected with TSLP shRNA (Santa Cruz, CA, USA) by pipetting the solution up and down and incubating it at room temperature for 15 minutes according to the transfection manual. After six hours, the cells were collected and cultured in Ham's F12K medium with 10% FBS. The TSLP expression was evaluated using a routine Western blot.

Enzyme-Linked Immunosorbent Assay (ELISA)

To quantify TSLP, TGF-β1, and fibronectin in the supernatant of the cultured HFL-1 cells, conditioned culture medium was collected and centrifuged at 12,000 rpm for five minutes to remove particulates. The clear supernatant was then collected and stored at –80°C until use. Immediately prior to performing the ELISA, the sample was acidified by 1 N HCl followed by the addition of NaOH to equilibrate the pH value. A commercial sandwich ELISA kit was used for the detection of TSLP using Quantikine™ (R&D Systems, Minnesota, USA), fibronectin (Assaypro, Missouri, USA), and TGF-β1 (R&D Systems, Minnesota, USA). The protocols were performed according to the manufacturer's instructions. The absorbance (450 nm) for each sample was analyzed by an ELISA reader. The concentration of each protein was determined by interpolating from a standard curve after the absorbance was assayed.

Western Blotting

Cells were lysed by lysis buffer (10 mM Tris, 1 m MEDTA,1% Triton X-100, 1mM Na3VO4, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM dithiothreitol, and 50 μg/ml phenylmethylsulfonyl fluoride). The crude protein lysate was resolved by 7.5, 10, or 12.5% SDS-PAGE. After protein transfer to a polyvinylidenedifluoride membrane with an electrotransfer unit, the membrane was blocked with 10% (w/v) nonfat milk in Tris-buffered saline for 2 h at 37°C. The blots were probed with a 1:1000 (v/v) dilution of primary antibody. The primary antibodies used were as follows: anti-TSLP (ab-59377), anti-Smad2/3 (sc-8332), anti-pSmad2/3 (sc-11769), anti-Smad4 (sc-7154), and anti-fibronectin (sc-9068). After hybridization at 37°C, the blots were washed and hybridized with 1: 6000 (v/v) dilutions of goat antirabbitIgG, horseradish peroxidase-conjugated secondary antibody (Calbiochem, Darmstadt, Germany), or donkey antigoatIgG-horseradish peroxidase (Santa Cruz, CA, USA).

The signal was generated by adding enhanced chemoluminescent reagent. β-actin was used as an internal control.

Histochemistry and immunohistochemistry

Half of the mice had their lungs inflated for 30 min with 4% paraformaldehyde at 20 cm H_2O pressure and then embedded in paraffin. Multiple 4 μm sections were made of each sample. The sections were then treated twice in Hemo-D for 7 min, twice in 100% ethanol for 5 min, twice in 90% ethanol for 5 min, and finally in distilled H_2O for 5 min. One section of each sample was used for Hematoxylin and Eosin (H&E), Masson's trichrome, or immunohistochemical staining. For immunohistochemistry, the samples were blocked overnight at 4°C in blocking buffer (25 mM Tris pH 7.5, 500 mM NaCl, 1% Tween 20, 10% fish gelatin, and 0.05% NaN_3). The samples were then incubated with 20% glycerol in H_2O for 30 min and washed with blocking buffer. Next, the sections were incubated with 1/100 affinity purified rabbit polyclonal anti-TSLP or 1/2,000 rabbit IgG in blocking buffer for 3 hours at room temperature. The sections were then washed in high-salt buffer (25 mMTris pH 7.5, 500 mM NaCl, and 1% Tween 20) three times for 5 min. A biotinylated anti-rabbit IgG, in blocking buffer (1: 500), was placed on the sections for 30 min at room temperature. The sections were washed once with high-salt buffer then treated for 3–5 min in 3% H_2O_2 in water. The samples were then washed two times for 5 min in high-salt buffer without detergent. Vectastain ABC Elite and diaminobenzidine (Vector Laboratories) kit were used as described in the kit instructions. The sections were then stained for 3 min with 1% methyl green and washed with distilled H_2O. The sections were then dehydrated twice in 90% ethanol for 5 min, twice in 100% ethanol for 7 min, and twice in Hemo-D for 7 min.

Collagen assay

A complete description of this assay can be found in Rojkind [14]. In brief, paraffin-embedded sections were soaked in Hemo-D to remove the paraffin. Samples were then placed in a (1:1) Hemo-D:ethanol mixture, then 100% ethanol, then 50% ethanol, then distilled water followed by 0.5 ml of Sirius red and fast green FCF mixture in 0.1% picric acid. After 30 min, the solution was pulled off, and the samples were washed three times in distilled water. Next, 0.25 ml of NaOH 0.1 N and 0.25 ml of methanol were added for one min. Samples were aspirated and absorbance was measured at 540 and 605 nm using a 96-well plate reader. The amount of noncollagenous protein in milligrams per sample was calculated by measuring the absorbency of the eluate at 605 nm divided by 2.22 (the lung fast green FCF color equivalence). The amount of collagenous protein was calculated by measuring the absorbance of the eluate at 540 nm, subtracting 29% of the absorbance at 605 nm, and then dividing by 36.3 (the Sirius red color equivalence). The total amount of collagen per lung was calculated by multiplying the total protein of the lung with the ratio of collagenous protein to total protein.

Histological scoring

H&E staining results were scored independently by two investigators. A 10x10 square grid on the left ocular was superimposed over a portion of each histological section repeatedly so that all lung tissue was covered once and only once. Using a Nikon light microscope with a 20x ocular and 2x objective lens, we scored each square in the grid as '1' if fibrosis occupied at least 50% of the square and '0' otherwise. The percent of fibrosis for each section was determined by adding the number of squares scored with a 1 and dividing by the number of the squares counted. An average of 549 squares was counted for each section scored.

Statistics

Results were expressed as mean ± SEM. The unpaired Student's t test was used for comparison between two groups. P*<0.05 was considered to be statistically significant. For in vivo experiments, a one-way Analysis of Variance (ANOVA) was used to determine if the means were significantly different (P<0.05). If means were significantly different, a Tukey-Kramer multiple group comparison test was used to compare individual groups. Standard error was indicated for each value by a bar, and significance was listed for each comparison. A 2-test was used to calculate P values for ratios. All values were calculated using GraphPad Prism version 3.00 for Macintosh (GraphPad Software, San Diego, CA).

Results

To elucidate the role of TSLP in the pathogenesis of lung cellular fibrosis, expression of TSLP was assayed. As shown in (Figures 1A and 1B), unconditioned HFL-1 cells show little expression of the TSLP protein. In addition, an exogenous TGF-β1 dose-dependently (0.1, 1, or 10 ng/ml) and significantly increases the expression of TSLP from either extracellular (as determined by ELISA) or intracellular (as determined by Western blot) origin in HFL-1 cells. Thus, TSLP may play an underlying role in TGF-β1-induced biological effects on human lung fibroblasts.
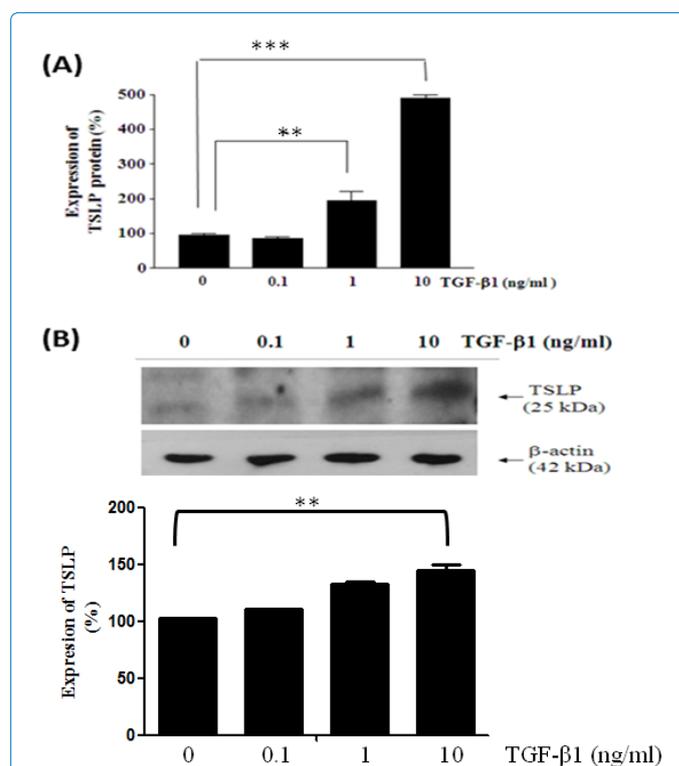
Figure 1: Effects of TGF-β1 on the expression of TSLP in HFL-1 cells. (A) HFL-1 cells were cultured in serum-free medium (0.5% FBS) with TGF-β 1 (0, 0.1, 1, or 10 ng/ml) for 24 h. Supernatants were collected and subjected to ELISA for detection of TSLP. Experiments were duplicated (n=4) and consistent results were observed. HFL-1 cells were treated with TGF-β1 dose-dependently (0, 0.1, 1, or 10 ng/ml, Figure1B) and lyzed. 100 μg of protein lysate was resolved by 12.5% SDS-PAGE. Western blot was performed by polyclonal anti-TSLP followed by the addition of secondary antibodies (1:4000). β-actin was used as an internal control. Experiments were repeated three times and consistent results were observed. P*<0.05, P**<0.01 versus control (0 ng/ml TGF-β1).

Since TSLP has been implicated in the pathogenesis of inflammatory diseases in recent years, it is of further interest to investigate whether TSLP regulates TGF-β1/Smad signaling. According to (Figure 2), TSLP significantly (P<0.01), dose-dependently (0, 1, 10 or 100 ng/ml), and time-course-dependently (from 0 to 24 hours) increases the expression of fibronectin concomitantly with the upregulation of TGF-β type I receptor, Smad2/3, pSmad2/3, collagen and JNK. Thus, TSLP appears essential for the regulation of lung fibrosis since fibrogenic signaling is dramatically induced in lung fibroblast cells following treatment with TSLP.

To investigate the role of TSLP in lung fibroblasts, HFL-1 cells were transiently transfected with TSLP shRNA. As shown in (Figure 3A and 3B), treatment with TSLP shRNA significantly inhibits a TGF-β1 (10 ng/ml)-induced increase in TGF-β RI, pSmad2/3, and fibronectin levels in HFL-1 cells (P<0.01). Thus, we propose that TSLP shRNA may exert fibrosis antagonizing effects by possibly regulating TGF-β RI and its downstream regulator (e.g., pSmad2/3) in lung fibroblast cells. We used C57BL/6 mice treated by bleomycin to mimic pulmonary fibrosis in vivo. Enhanced pause (Penh) is a dimensionless composite parameter that can be used to screen experimental animals for airway hyper-reactivity to obstruction [15-17]. Thus, respiratory resistance examination was performed as shown in (Figure 4). It is evident that the Penh values of the mice treated with bleomycin were significantly higher at the 7- or 21-day time points, indicating the possibility of

infiltration of immune cells and the induction of lung inflammation, which cause airway obstruction. However, mice treated with bleomycin and TSLP shRNA (7 or 21 days) show significantly lower levels of Penh than animals treated with the bleomycin only [18].

To further test the in vivo fibrosis-antagonizing efficacy of TSLP shRNA, bleomycin-treated C57BL/6 mice, a well-documented lung fibrosis model, were used. Mice were handled according to the methods described previously. As shown in (Figure 5), H&E staining of lung tissue from bleomycin-treated mice sacrificed at 7 and 21 days showed increased inflammatory cell infiltration (Figures 5B and 5F). This is a foreseeable effect according to previous literature. Interestingly, a significant decrease in inflammatory cell infiltration was observed in mice treated with bleomycin and inoculated with TSLP shRNA (by intranasal instillation) at both 7 and 21 days (Figures 5D and 5H). As shown in (Figures 6A and 6E), TSLP proteins were nearly un-expressed in lung tissue in controlled mice. However, it is evident that intense staining for TSLP proteins occurs in inflammatory and fibrotic areas in the lung tissue of the mice treated with bleomycin (Figures 6B and 6F). In addition, a significant decrease in the staining of TSLP proteins is observed in mice treated with bleomycin and inoculated with TSLP shRNA at both 7 and 21 days (Figures 6D and 6H).

To further elucidate the fibrosis-regulating role of TSLP shRNA in vivo, lung tissues from bleomycin-treated mice were stained for
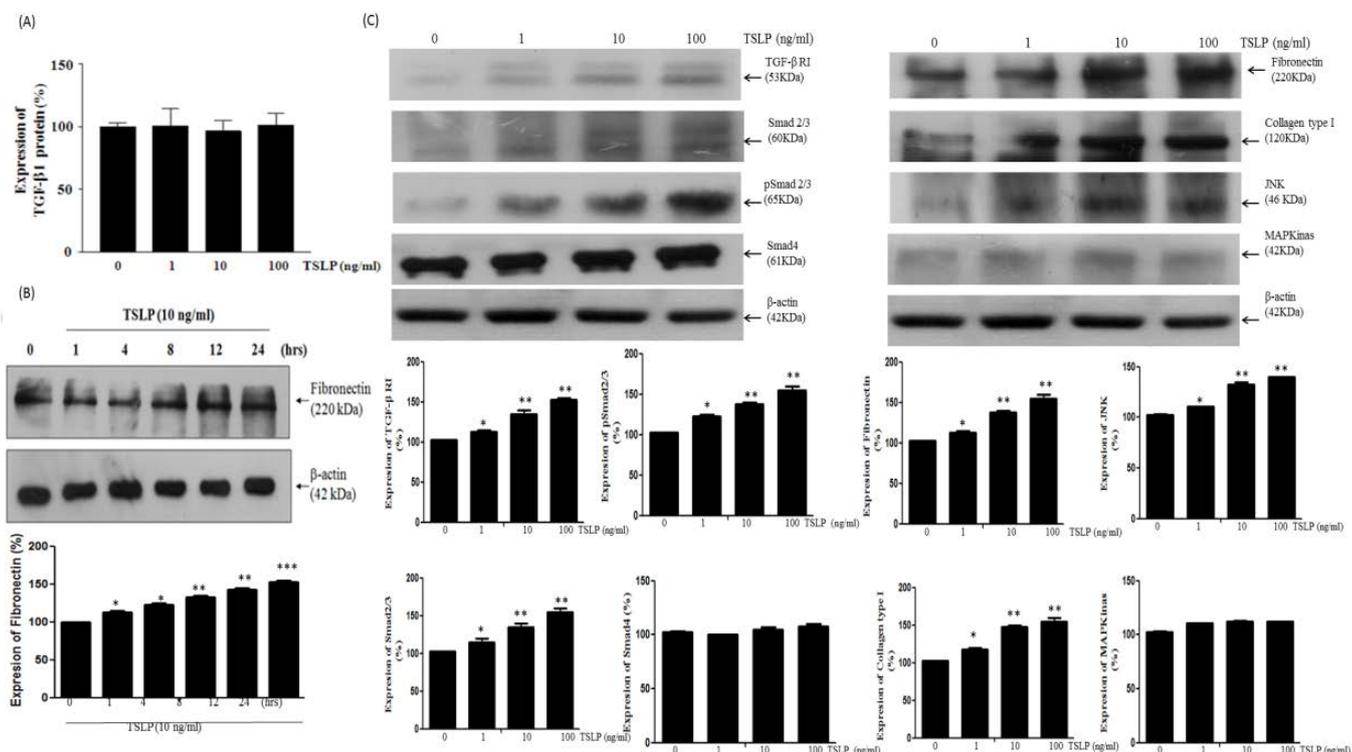

Figure 2: Effects of TSLP on signal transduction and TGF-β1 protein levels in HFL-1 cells. Cells were cultured in 25 cm² flasks. Supernatants from conditioned cells were collected and subjected to ELISA for detection of TGF-β1 and to Western blot for detection of fibronectin. Cells were serum starved (0.5% FBS) and treated dose-dependently (0, 1, 10 or 100 ng/ml for 24 hours, Figure 2A) with TSLP, after which ELISA was used for detection of TGF-β1. Alternatively, cells were serum starved (0.5% FBS) and treated time-course-dependently (0, 1, 4, 8, 12, 24 hours, Figure 2B) with TSLP, after which Western blot was used for detection of fibronectin. Cells were lysed and 100 μg of protein lysate was resolved by 7.5% SDS-PAGE followed by Western blot using antibodies against type I receptor TGF-β, pSmad2/3, Smad2/3, Smad4, fibronectin, collagen, JNK, and MAP Kinase. Experiments were repeated three times and consistent results were observed. Experiments were duplicated (n=4). It is evident that TSLP significantly increases type I receptor TGF-β, pSmad2/3, Smad2/3, fibronectin, collagen, and JNK without affecting the secretion of TGF-β1. P*<0.05, P**<0.01 versus control.
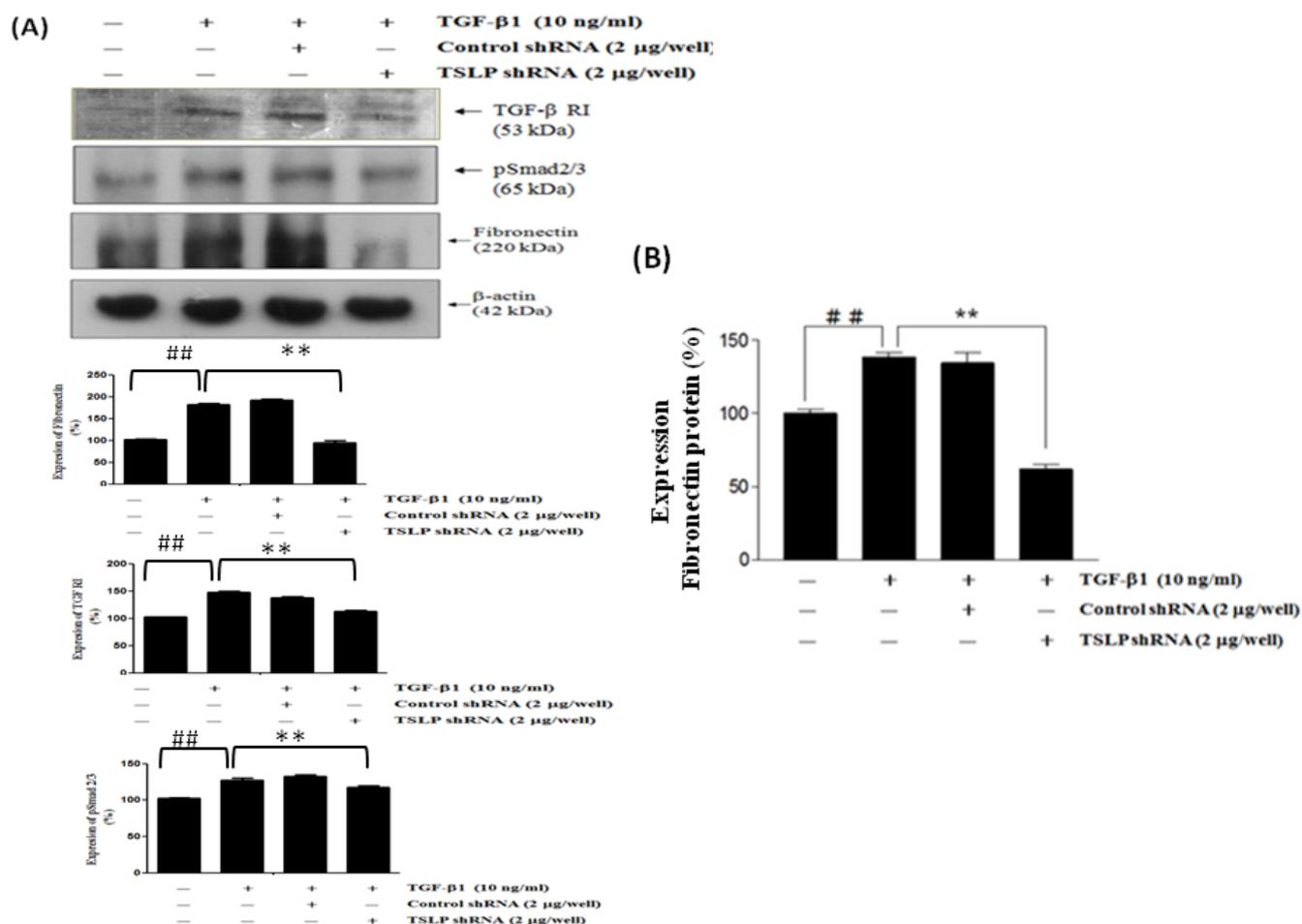
Figure 3: Regulatory effects of TSLP shRNA on the expression of TGF-β RI, pSmad2/3, and fibronectin in HFL-1 cells. (A) Cells were manipulated and transfected as above. The cells were subjected to stimulation of TGF-β1 (10 ng/ml) for 24 hours in serum-free medium. Whole-cell lysates were immunoblotted with anti-TGF-β RI, anti-pSmad2/3, and anti-fibronectin antibodies. (B) Densitometry analysis from Figure 3B shows the group examined by anti-fibronectin. $P^{##}<0.01$ versus control. $P^{**}<0.01$ versus the TGF-β1 control (i.e., the TGF-β1 treated group). The experiment was repeated twice and consistent results were obtained.
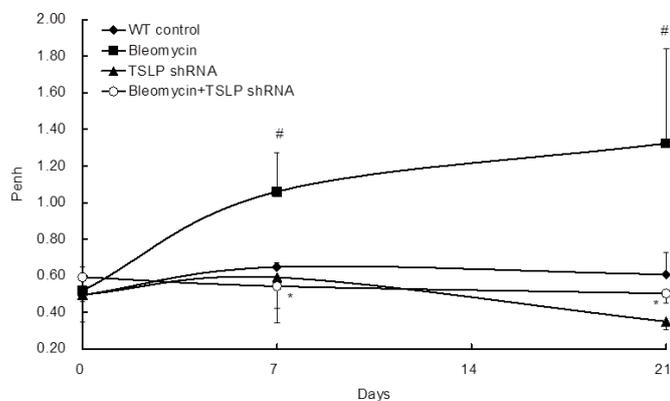
Figure 4: Effects of TSLP shRNA on bleomycin-induced increase in respiratory resistance (Penh). Wild type control group: ◆; Bleomycin group: ■; TSLP shRNA inoculated group: ▲; and bleomycin with TSLP shRNA group: ○ Each point represents mean±SD. #P<0.05, represents significant difference when compared to wild type group. *P<0.05, represents significant difference when compared to bleomycin group.
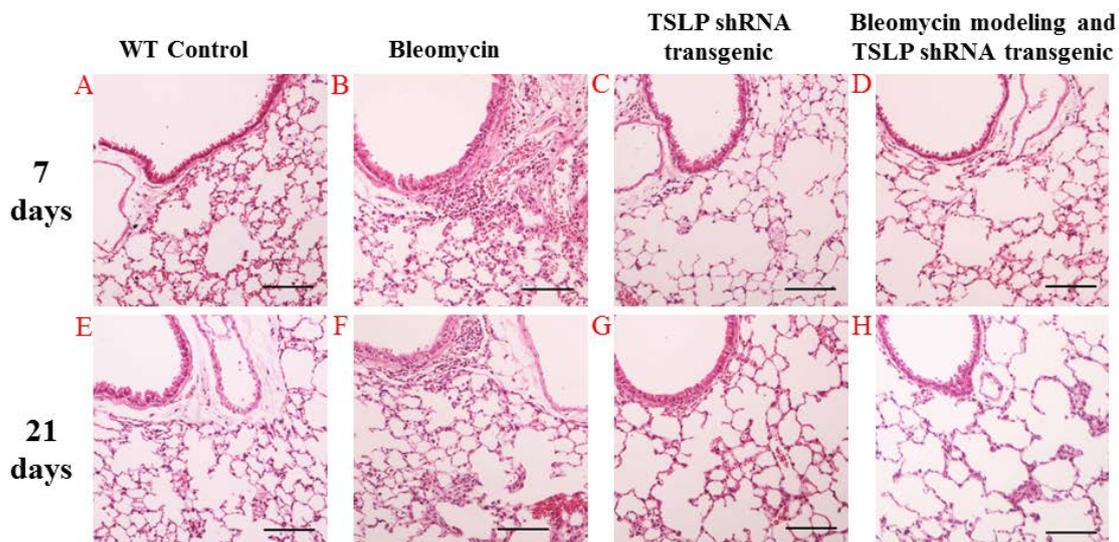
Figure 5: Hematoxylin and Eosin (H&E) staining. A, E: Wild type control mice show no significant histological changes at 7 and 21 days. B, F: Bleomycin-treated mice (i.e., pulmonary fibrosis group) sacrificed at 7 and 21 days show increased inflammatory cell infiltration. C, G: TSLP shRNA transgenic mice (i.e., shRNA control group) show no significant histological changes compared to the control at 7 and 21 days. D, H: Mice treated with bleomycin and TSLP shRNA at 7 and 21 days show a significant decrease in inflammatory cell infiltration compared with bleomycin-treating group. The calibration bar represents 100 μm.
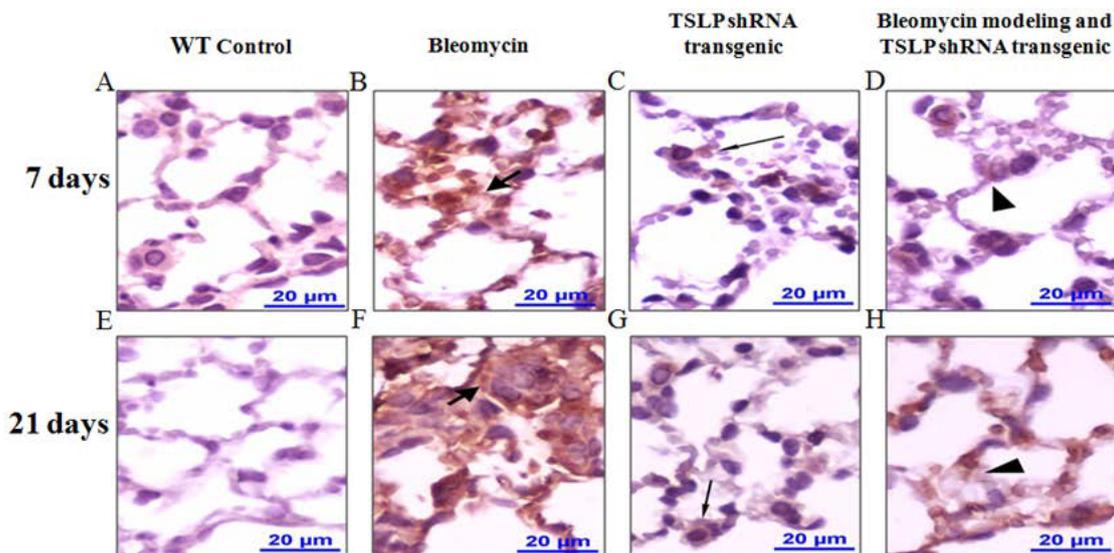

Figure 6: Immunohistochemistry of bleomycin-treated mice shows intense staining for TSLP proteins in the pulmonary tissues. Regardless of treatment with bleomycin or saline, mice pulmonary tissue samples were perfused, fixed, and embedded in paraffin. Immunohistochemistry was performed according to the protocol listed in the Methods section. A, E: Wild-type mouse pulmonary samples (high power) using IgG control show no brown pigment (i.e., no TSLP expression) at either 7 or 21 days. B, F: Bleomycin-treated mouse pulmonary samples show intense staining for TSLP in the foci of the injured pulmonary tissue; these areas appear to be extracellular (thick arrows). C, G: TSLP shRNA transgenic mouse pulmonary samples show mild TSLP staining associated with type II alveolar cells (thin arrows). D, H: After bleomycin modeling, TSLP shRNA transgenic mouse pulmonary samples have smaller foci of the injured pulmonary tissue. TSLP staining appears to be extracellular (arrow heads).

Sirius red for the detection of collagen deposition, which is a major form of extracellular matrix in pulmonary tissue. As shown in (Figure 7), mice were grouped and treated as shown in (Figure 6). Mouse lungs were scored for fibrosis by overlaying a grid onto lung sections stained with Sirius red stain. Each square on the grid was counted as positive if fibrosis occurred in at least 50% of the square. It is evident that there was little collagen deposition under controlled conditions at both 7 and 21 days (Figures 7A and 7E); however, the expression levels of collagen were significantly higher in mice treated with bleomycin at both 7 and 21 days (Figures 7B and 7F). More importantly, TSLP shRNA significantly attenuates the deposition of lung collagen in the mice treated with bleomycin compared to the wild type.

Taken together with previous results, we identified a mechanism for the TSLP-induced cellular fibrosis via up-regulation of receptor type I
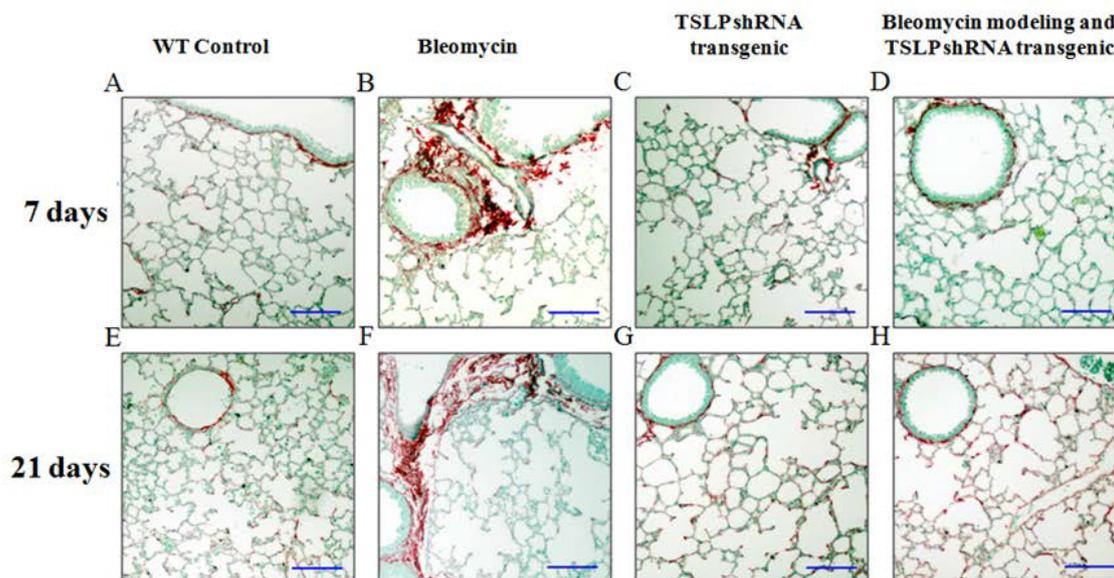
Figure 7: Collagen-rich areas of fibrosis are smaller in TSLP shRNA-inoculated mice in bleomycin modeling. Regardless of treatment with bleomycin or saline, mice pulmonary tissue samples were perfused, fixed, and embedded in paraffin. Sections were stained for collagen with Sirius red (red) and for noncollagenous protein with fast green FCF (green), and then photographed at a magnification of 10. Bleomycin-treated (B, F) animals intensely stained for collagen around large airways and blood vessels; however, both wild-type control (A, E) and TSLP shRNA transgenic control (C, G) animals had foci of fibrosis that stained for collagen. More importantly, pulmonary tissue from a bleomycin-treated mouse inoculated with TSLP shRNA (D, H) and sacrificed at 7 and 21 days shows significantly decreased fibrosis in collagen stain compared to the bleomycin-treated group.
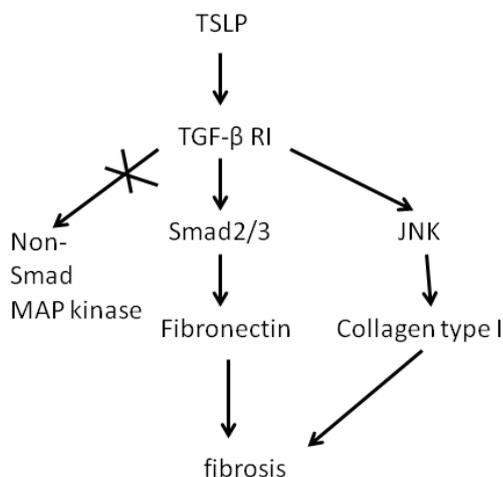
Figure 8: Proposed mechanism for the positive regulation role of TSLP-induced cellular fibrosis via upregulation of receptor type I of TGF-β1. We propose that the fibrosis effects of TSLP are exerted by upregulation of the TGF-β type I receptor, Smad2/3, collagen, and JNK.

of TGF-β1 as shown in (Figure 8). In this study, we found that TSLP induced lung fibrosis by enhancing the JNK pathway. More importantly, we found that TSLP had significantly increased Smads pathway. Thus, it may be possible to use TSLP shRNA as a novel therapeutic approach for treating pulmonary fibrosis by down-regulating TGF-β signal proteins

Discussion

In this study, the pulmonary fibrosis-regulating effect of a novel inflammatory cytokine TSLP is discussed. We demonstrate that TSLP plays a pivotal role in cellular fibrosis in human lung fibroblast cells, possibly by regulating post-receptor signal transducers (e.g.,

Smad2/3) of fibrogenic growth factor, TGF-β. Moreover, TSLP shRNA (3 μg/animal) can significantly silence the expression of TSLP and simultaneously ameliorate bleomycin-induced inflammation (i.e., infiltration of immune cells) and lung fibrosis (i.e., collagen deposition) in C57BL/6 mice. The observation that pulmonary fibrosis may be controlled by regulating inflammatory cytokines is a pivotal one. This hypothesis is supported by many studies showing that TSLP plays an essential role in the pathogenesis of fibrosis [19].

We demonstrate that fibrogenic growth factor TGF-β1 dose-dependently induced an increase in the level of intracellular and extracellular TSLP in HFL-1 cells (Figure 1). Moreover, treatment with

exogenous TSLP from either dose- or dependent significantly induces pulmonary cellular fibrosis (Figure 2). Thus, we propose that TGF-β may exert its fibrosis-inducing effects by inducing the expression of TSLP since TSLP induces Smad pathways and cellular fibrosis in the same manner as TGF-β1. However, we found that exogenous TSLP does not influence the secretion of TGF-β1 (Figure 2A). This observation suggested that TGF-β1 can one-way regulate the expression of TSLP; moreover, there is no positive feedback loop (i.e., autocrine loop) between TGF-β1 and TSLP because TSLP did not affect the expression of TGF-β1. In this study, we examined the involvement of TSLP mechanisms in pulmonary fibrosis, using Western blot assays of fibrosis markers (i.e., fibronctin or collagen). We found out that TSLP has been shown by the upregulation of fibronectin and collagen, one important component of the extracellular matrix, corroborating the findings of Wong et al. In addition, TSLP can significantly enhance the adhesion of eosinophils onto fibronectin.

TSLP transgenic (TSLP tg) mice develop mixed cryoglubulinemia and a systemic inflammatory disease that involves the pulmonary system, the spleen, the liver, and skin [20]. Overexpression of TSLP in the pulmonary systems of mice results in severe allergic airway inflammation. In addition, TSLP is implicated in allergic inflammation. A number of inflammation mediators with the potential to promote TSLP expression are associated with airway inflammation and atopic dermatitis [6,19,21]. TSLP induces a systemic Th2 inflammatory response characterized by increased circulating IgE and IgG1, as well as increased draining lymph node size and cellularity. Thus, some researchers began to notice that there exists a close interaction between the level of TSLP and the extent of tissue fibrosis, since inflammation and tissue fibrosis are common consequences of Th2 responses which are significantly induced by TSLP [22].

Thus, silencing TSLP expression by shRNA appears effective for the amelioration of inflammation in pulmonary tissues. To understand the role of TSLP proteins in bleomycin-induced pulmonary fibrosis further, immunohistochemistry staining was performed. In conjunction with the results shown in (Figure 5 and 6), there was a parallel observation that expression of TSLP proteins appears consistent to the level of pulmonary inflammation. Moreover, silencing the expression of TSLP by using TSLP shRNA enhanced the effectiveness of the treatment of pulmonary inflammation induced by bleomycin. Thus, TSLP shRNA may have significant potential as an agent for the regulation of pulmonary inflammation.

TSLP has been reported to be associated with skin fibrosis [23] and liver fibrosis [24]. Oh et al. demonstrated that the progression of skin fibrosis occurs via TSLP-dependent pathways. Intriguingly, reduction of fibrosis could be achieved by depletion of TSLP [23]. The pulmonary systems of TSLP-transgenic mice showed massive infiltration of leukocytes, goblet cell hyperplasia, and subepithelial fibrosis according to Zhou et al. [25]. These observations are completely consistent and compatible with our findings showing the essential role of TSLP in bleomycin-induced pulmonary fibrosis and the therapeutic potential of TSLP shRNA in treating pulmonary fibrosis. Thus, the current study demonstrates that TSLP may be a central regulator for pulmonary fibrosis.

There are a number of potential applications for targeted, local delivery of shRNA to pulmonary tissues, including the treatment of inflammation, immune conditions, cystic fibrosis, infectious diseases, and cancers [26]. However, many potential shRNA therapies are still at the preclinical trial stage. Despite this, many in vivo animal studies have been performed using shRNA [27-29]. Thus, we propose that

controlling expression of inflammatory cytokines using TSLP shRNA through intranasal instillation might be a potential therapeutic strategy for treating pulmonary fibrosis. This study suggests a promising application for controlling pulmonary inflammation and may be helpful in developing strategies to treat pulmonary fibrosis.

This study proposes that TSLP is an important growth factor with immuno- regulatory significance in the pathogenesis of pulmonary fibrosis. Moreover, the study demonstrates that the silencing of TSLP (i.e., by TSLP shRNA) may have potential for controlling pulmonary inflammation or even pulmonary fibrosis. This study develops a novel therapeutic strategy using growth-factor-based silencing vectors as a tool. The study also promotes the un-expressed of the roles of TSLP in the pathogenesis of pulmonary fibrosis.

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