

## REVERSAL OF IL-1 $\beta$ -MEDIATED HUMAN EMBRYONIC PULMONARY FIBROBLAST TRANSDIFFERENTIATION BY TARGETING THE ERK SIGNALING PATHWAY

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**Abstract** - The aim of the present study was to determine whether Interleukin (IL)-1 $\beta$ -mediated human embryonic pulmonary fibroblast transdifferentiation could be reversed by targeting of the ERK signaling pathway. The human embryonic pulmonary fibroblast MRC-5 cell line was used as a model to observe IL-1 $\beta$ -mediated transdifferentiation as well and the inhibitory effects of lentinan (LNT). Cell proliferation was examined by a CCK-8 assay. ERK signaling activity was detected using immunoblotting with phospho-ERK antibody. The expression levels of fibronectin (FN), Col I and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) were assessed by either reverse transcription PCR or the SABC assay. IL-1 $\beta$ -induced-ERK signaling activation in MRC-5 cells was inhibited by pretreatment with the LNT or ERK inhibitor U0126. IL-1 $\beta$ -enhanced cell proliferation and expression of FN, Col I and  $\alpha$ -SMA were also attenuated by the treatment with LNT. Our study revealed that activation of ERK signaling is involved in IL-1 $\beta$ -mediated human embryonic pulmonary fibroblast proliferation, phenotypic switching and collagen secretion. These transdifferentiation events in MRC-5 cells could be reversed with LNT treatment by targeting the ERK signaling pathway

**Key words:** Lentinan, ERK signaling, MRC-5 cell transdifferentiation,  $\alpha$ -SMA

**Abbreviations:** IL – interleukin; LNT – lentinan;  $\alpha$ -SMA – smooth muscle actin; HEPF – human embryonic pulmonary fibroblast; CCK-8 – Cell Counting Kit-8; Col- – collagen type; ECM – extracellular matrix; EMTU – epithelial mesenchymal trophic unit; ERK – external signal regulated kinase; FN – Fibronectin

### INTRODUCTION

Asthma is a chronic airway disorder resulting from bronchial inflammation, which is characterized by bronchial hyperreactivity and airflow obstruction. As the primary pathophysiological characteristic of asthma, airway remodeling is defined based on histological descriptions, including bronchial wall thickening, subepithelial fibrosis, collagen basement membrane deposition, muscle cells hyperplasia or hypertrophy, goblet cell hyperplasia, mucus gland metaplasia or neovascularization formation (Giro-

det et al., 2011; Holgate et al., 2009). The pathological consequences of airway remodeling in asthma may include airway hyperreactivity, continuous airflow obstruction and irreversible pulmonary dysfunction (Elias et al., 1999). Alterations in structural cell function are one of the characteristic aspects of airway remodeling in asthma. During airway remodeling, fibroblast-myofibroblast transdifferentiation induced by chronic airflow inflammation may play an important role in extracellular matrix (ECM) deposition of the basement membrane, resulting in subepithelial reticular layer thickening, luminal

stenosis and airway remodeling (Homer and Elias, 2005).

IL-1 $\beta$  is a widely recognized multifunctional cellular inflammatory factor and plays central roles in initiating an inflammation reaction (Dinarello, 2011). As reported, bronchial lavage fluid collected from asthmatic patients has a high concentration of IL-1 $\beta$  that may underlie an etiology of asthma (Hori-ba et al., 2011). Although functions of IL-1 $\beta$  are extensively studied in acute inflammation, its roles in chronic continuous airway inflammation have not been well defined.

Lentinan (LNT), a purified  $\beta$ -glucan isolated from the fruit body of shiitake (*Lentinula edodes*) has a variety of bioactivities, such as activation of the immune system, inhibition of proliferation and migration of tumor cells, and modulation of fat and carbohydrate metabolism (Rop et al., 2009). Furthermore, a previous study revealed that LNT could inhibit IL-1 $\beta$ -induced fibroblast transdifferentiation (Jin et al., 2011). To gain a better understanding of the molecular mechanisms underlying cell signaling in airway remodeling, we used MRC-5 cells as a model to clarify the roles of IL-1 $\beta$  in human embryonic pulmonary fibroblast transdifferentiation. Considering that IL-1 $\beta$  is an important extracellular external signal regulated kinase (ERK) modulator (Jiang et al., 2004), we also determined whether IL-1 $\beta$ -mediated human embryonic pulmonary fibroblast transdifferentiation could be reversed by targeting the ERK signaling pathway.

## MATERIALS AND METHODS

### *Cell culture and drug treatment*

The human embryonic pulmonary fibroblast MRC-5 cell line was obtained from the American Type Culture Collection (fourth generation). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplied with 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 mg/ml streptomycin with a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. During the 7<sup>th</sup>-15<sup>th</sup> pas-

sages, cells at 80% confluence were starved of serum for 24 h. To observe the effect of IL-1 $\beta$  on ERK activation, starved cells were stimulated with IL-1 $\beta$  (R&D, USA) at a concentration of 10 ng/mL for 1 min, 15 min, 30 min, 1 h, 2 h and 4 h, respectively. To further verify the dose-dependent effect of IL-1 $\beta$ , starved cells were treated with IL-1 $\beta$  at doses of 0.1 ng/ml, 1 ng/ml, 5 ng/ml and 10 ng/ml for 1 h, respectively. Moreover, starved cells were divided into four groups to investigate the effect of LNT on IL-1 $\beta$ -induced proliferation and other related functions. The groups included: (a) control group, (b) IL-1 $\beta$  group (10 ng/mL IL-1 $\beta$ ), (c) LNT (Nanjing Kanghai Pharmacy) group (pretreatment with 4  $\mu$ g/mL LNT for 1h, followed by 10 ng/mL IL-1 $\beta$  treatment); (d) U0126 (CST, USA) group (10  $\mu$ M U0126 pretreatment with 10  $\mu$ M U0126 for 1h, followed by 10 ng/mL IL-1 $\beta$  treatment). Cell lysates or mRNA extracts were prepared for Western immunoblot or RT-PCR at the indicated times (48 h following IL-1 $\beta$  treatment for the expression level of protein or mRNA of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), Fibronectin (FN), and Col I).

### *Western immunoblotting*

Following treatment with drugs, total proteins were extracted and the protein level was measured using a bicinchoninic acid (BCA) protein assay. Equal amounts of protein extracts were then separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and blocked with 5% non-fat dried milk in phosphate buffered saline (PBS) for 1 h. The membranes were incubated with anti-ERK1/2 (1:1000, CST, USA) and anti-p-ERK1/2 (1:1000, CST, USA) overnight at 4°C. After washing with TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20), the membranes were incubated with a horseradish peroxidase (HRP)-labeled secondary antibody (1:300) at RT for 2 h. Signals were visualized using an enhanced chemiluminescence detection kit. Densitometric values were semiquantified using Gel-Pro Analyzer 3.1. The values from the treatment groups were normalized to the control group.

### Cell proliferation

Cell viability was determined using a CCK-8 assay (Dojindo Molecular Technologies, Inc. Japan). 10  $\mu$ l of CCK-8 assay reagent was added into each well and incubated for 1 h in 5% CO<sub>2</sub> and 37°C. Optical density values were measured using an automatic ELISA reader (BioTek Instruments, USA) at a wavelength of 450 nm.

### SABC assay

Cells were seeded onto the cover glasses until fully attached to the surface and then treated with drugs at the indicated time. The treated cells were fixed with 4% paraformaldehyde (PFA), followed by immunohistochemical staining using an SABC assay, according to manufacturer's instruction.  $\alpha$ -SAM antibody (Abcam, UK) was used at 1:200 dilution in the SABC assay. The cells with brown-yellow

deposition in the cytoplasm were considered to be positively stained. Four fields in each image were analyzed. The expression level of protein was measured as mean optical density (MOD) using Image-pro plus software.

### Semi-quantitative Polymerase Chain Reaction (PCR)

Total RNA was isolated using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA was then reverse-transcribed in a reaction using a reverse-transcription system (Beijing Tiangen, China). The cycle parameters for the PCR reaction were an initial hold at 94 °C for 3 min, followed by 20 cycles at 94°C for 30 s and appropriate annealing temperature (FN at 57°C, Col I at 63°C, and  $\alpha$ -SAM at 60°C) for 30 s, and finally at 72°C for 5 min. Amplification of  $\beta$ -actin was under the same conditions of PCR for its corresponding target gene. The specific primers used for PCR were synthesized at Shenggong Company (Shanghai) and are listed in Table 1. PCR products were analyzed using Gel-Pro Analyzer 3.1. Changes in the expression of target genes were determined using a ratio of target to  $\beta$ -actin following normalization.

### Statistical Analyses

The data were analyzed using SPSS 13.0 software. The values are presented as mean  $\pm$  standard deviation (SD) from three independent experiments. One-way analysis of variance (ANOVA) was used for testing differences among two or more independent groups followed by a LSD (least significant difference) or Tamhane's T2.  $P < 0.05$  was considered statistically significant.

## RESULTS

### IL-1 $\beta$ -induced activation of ERK signaling

Since IL-1 $\beta$  is an important regulator of the ERK signaling pathway, we first investigated the effect of IL-1 $\beta$  on ERK signaling activation in MRC-5 cells using western immunoblotting. The results showed that

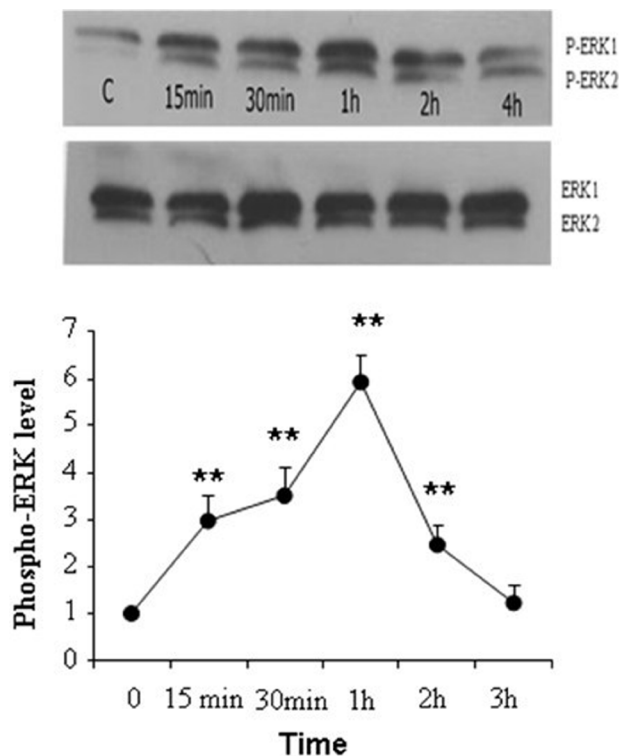


Fig. 1. IL-1 $\beta$  (10 ng/mL) induced a progressive increase of p-ERK level, followed by decrease to the control level 4 h later. Compared with control, \*\* $P < 0.01$ .

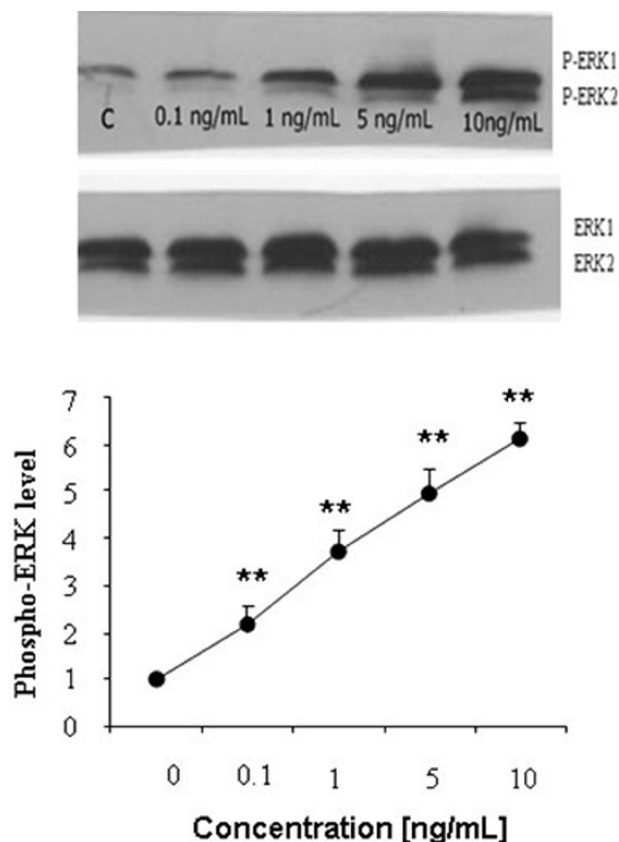


Fig. 2. IL-1 $\beta$  induced a concentration-dependent increase in ERK activation. Compared with the control, \*\* $P < 0.01$ .

IL-1 $\beta$  (10 ng/mL) induced a significant increase in phosphorylation of ERK following 15 min of stimulation, as compared to the control group (Fig. 1). The level of p-ERK reached a peak around 1 h following IL-1 $\beta$  treatment and decreased to the basal level after 4 h treatment. Next, we validated the dose-dependent effects of IL-1 $\beta$  on ERK signaling activation and found that treatment with 10 ng/mL IL-1 $\beta$  for 1 h could induce a 6-fold increase in p-ERK levels, as compared to control (Fig. 2).

#### *The effect of LNT on ERK phosphorylation*

To identify the effect of LNT on IL-1 $\beta$ -induced ERK activation, we observed the p-ERK1/2 level in the LNT group. As shown in Fig. 3, the values of normalized p-ERK1/2 (value in the control group was 1.0) were  $6.367 \pm 0.104$  in the IL-1 $\beta$  group,  $1.220 \pm 0.069$

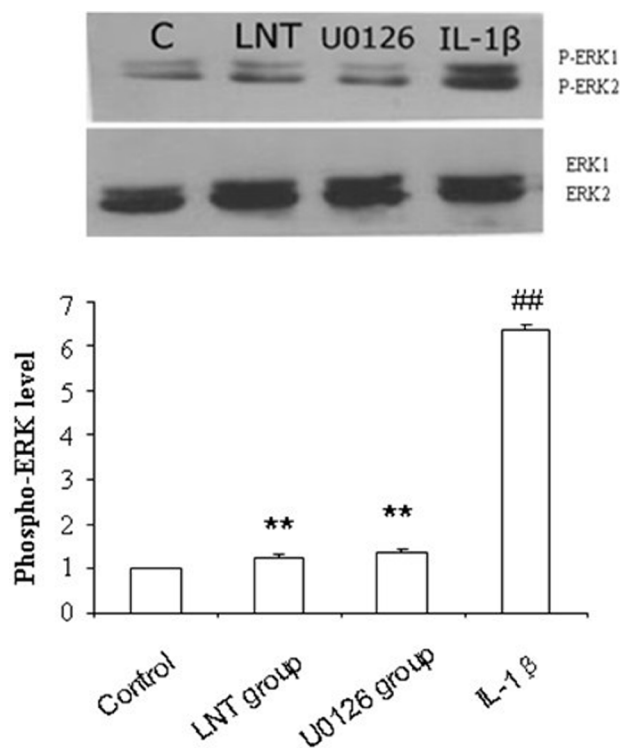


Fig. 3. LNT (4  $\mu$ g/mL) inhibited IL-1 $\beta$ -induced ERK phosphorylation. Compared with the IL-1 $\beta$  group, \*\* $P < 0.01$ ; compared with the control group, \*\* $P < 0.01$ .

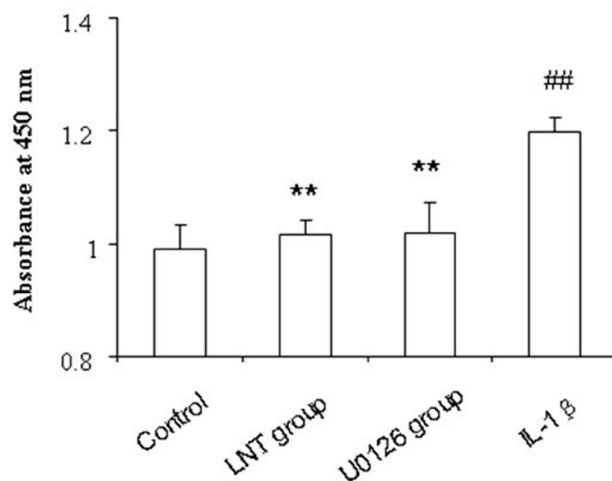
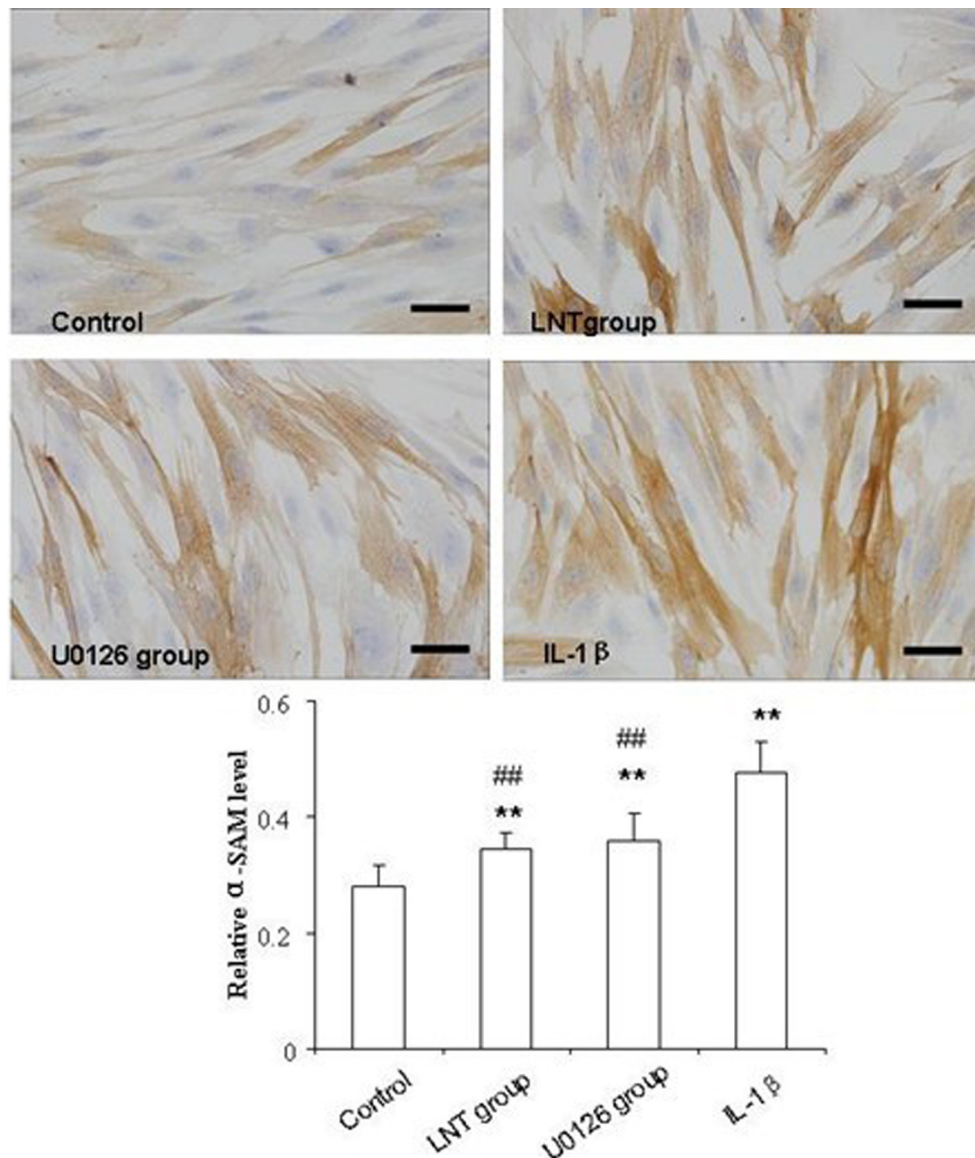


Fig. 4. LNT (4  $\mu$ g/mL) inhibited IL-1 $\beta$ -induced cell proliferation. Compared with the IL-1 $\beta$  group, \*\* $P < 0.01$ ; compared with the control group, \*\* $P < 0.01$ .

in the LNT group (4  $\mu$ g/mL) and  $1.381 \pm 0.054$  in the U0126 group (10  $\mu$ M). These data suggested that LNT could significantly inhibit IL-1 $\beta$ -induced ERK



**Fig. 5.** LNT (4  $\mu\text{g/mL}$ ) inhibited IL-1 $\beta$ -induced  $\alpha$ -SAM expression. Representative images of each group were with a magnification 400 X. Scale bar: 5  $\mu\text{m}$ . Compared with the IL-1 $\beta$  group, \* $P$ <0.05, \*\* $P$ <0.01; compared with the control group, ## $P$ <0.01.

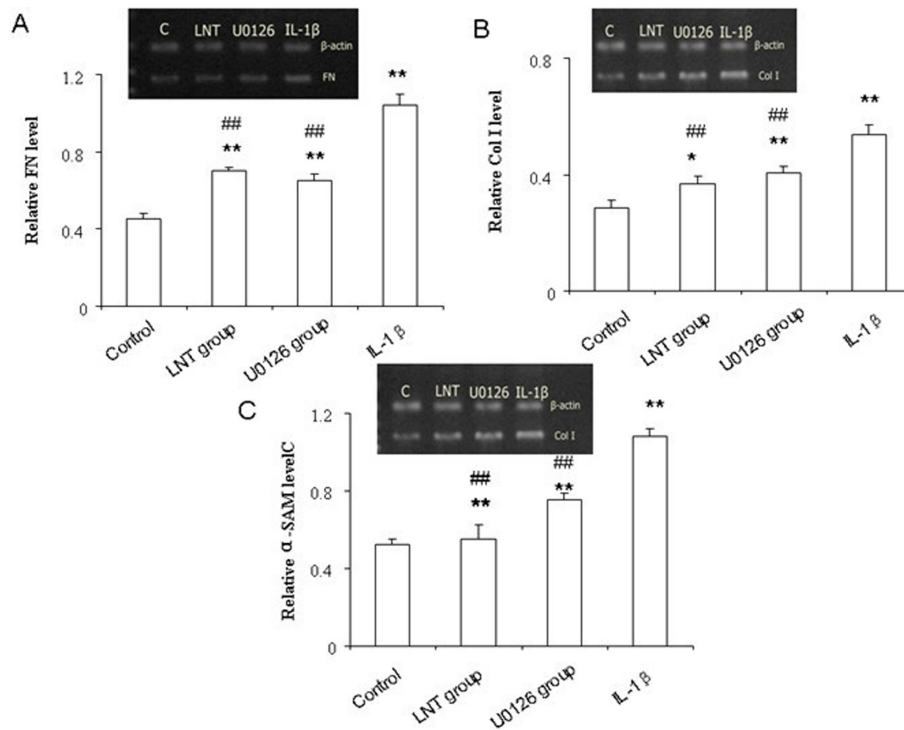
activation ( $P$ <0.01). The inhibitory effect of LNT on IL-1 $\beta$ -induced ERK activation in MRC-5 cells was also confirmed using a comparison of U0126, an MEK inhibitor (Fig. 3).

#### *Effect of LNT on IL-1 $\beta$ -induced cell proliferation*

Next we examined the effect of IL-1 $\beta$  on MRC-5 cell

proliferation. Data using the CCK-8 assay revealed that the treatment with IL-1 $\beta$  for 48 h significantly promoted cell proliferation, as compared to the control group (Fig. 4). Pretreatment with LNT (4  $\mu\text{g/mL}$  LNT) or U0126 (10  $\mu\text{M}$ ) attenuated IL-1 $\beta$ -induced cell proliferation. However, no significant differences were observed between the LNT and U0126 groups ( $P$ >0.05).





**Fig. 6.** LNT (4  $\mu\text{g/mL}$ ) inhibited IL-1 $\beta$ -induced FN expression (A), Col I expression (B) and  $\alpha$ -SAM expression (C). Compared with the IL-1 $\beta$  group, \* $P < 0.05$ , \*\* $P < 0.01$ ; compared with the control group, ## $P < 0.01$ .

**Table 1.** Primer sequences for PCR

Name	Primer sequence	Number of bases
FN forward	TAGCCCTGTCCAGGAGTTCA	346
FN reverse	CTGCAAGCCTTCAATAGTCA	
Col I forward	GTCTTCCTGGCCCCCTCTGGTG	391
Col I reverse	TCGCCCTGTTCGCCTGTCTCA	
$\alpha$ -SAM forward	GCGTGGCTATTCCTTCGTTAC	331
reverse	CATAGTGGTGCCCCCTGATAG	
$\beta$ -actin forward	CCTTCCTGGGCATGGAGTCCT	204
reverse	GGAGCAATGATCTTGATCTT	

#### *Effect of LNT on IL-1 $\beta$ induced fibroblast-to-myofibroblast transdifferentiation*

To identify fibroblast-to-myofibroblast transdifferentiation, the detection of  $\alpha$ -SMA expression was performed using an SABC assay, in which the positive cells present brown-yellow deposition located in the cytoplasm. Compared with the control group, higher

levels of  $\alpha$ -SMA expression were found in the IL-1 $\beta$  group (Fig. 5). The results shown in Fig. 5 demonstrated that IL-1 $\beta$  treatment significantly increased  $\alpha$ -SMA expression. IL-1 $\beta$ -induced fibroblast-to-myofibroblast transdifferentiation in MRC-5 cells could be inhibited by either LNT or U0126 pretreatment, implying a reversal of IL-1 $\beta$ -mediated transdifferentiation by targeting the ERK signaling pathway (Fig. 5).

### *mRNA expression of FN, Col I and $\alpha$ -SAM*

To study the correlation of either FN or Col I and  $\alpha$ -SAM, we assessed the mRNA of these molecules by RT-PCR. As shown in Figs. 6A, 6B and 6C, compared with the control IL-1 $\beta$  induced a significant increase in mRNA levels of FN, Col I and  $\alpha$ -SAM ( $P < 0.01$ ). Furthermore, the IL-1 $\beta$ -induced expression of FN, Col I and  $\alpha$ -SAM was attenuated by pretreatment with either LNT or U0126 (Fig. 6), consistent with the inhibition of IL-1 $\beta$ -induced  $\alpha$ -SAM protein expression using LNT or U0126, as shown in Fig. 5.

### DISCUSSION

The mechanisms underlying the pathological changes associated with asthma remain poorly understood. Structural changes in airway remodeling are thought to be secondary phenomena elicited by inflammation in the asthma airway (Fahy et al., 2000). However, a recent study suggested that airway remodeling was parallel to airway inflammation during asthma or even an elicited continuous airway inflammation reaction (Jin et al., 2011). With asthma, the airway epithelium is more vulnerable to environmental injury and responds to this by impaired healing. However, the relationship to an altered structure and function of the airways is largely unknown.

Holgate et al. (2000) proposed a model of epithelial-mesenchymal communication in the pathogenesis of chronic asthma. In their model, a fiber cell layer, located under the basement membrane of the airway is involved in a functional communication network of epithelium, nerve tissue and extracellular matrix, which is termed the epithelial mesenchymal trophic unit (EMTU). Normally, EMTU is in a stationary state following airway maturation during lung development. When exposed to environmental risk factors, EMTU is activated and the susceptible cell populations produce cytokines and growth factors, such as transforming growth factor- $\beta$  (TGF- $\beta$ ) and epidermal growth factor (EGF). Activation of EMTU is involved in communication between the damaged and stressed epithelium and the underlying fibroblast sheath. Meanwhile, TGF- $\beta$  production

promotes the underlying fibroblasts to differentiate into myofibroblasts that can synthesize more collagen and extracellular matrix components. The active myofibroblasts will release ET1, vascular endothelial growth factor and collagen, finally resulting in airway remodeling and inflammation (Knight et al., 2004; Holgate et al., 2006).

IL-1 $\beta$  is a multifunctional cellular factor, playing central roles in initiating the inflammation. IL-1 $\beta$  can activate lymphocytes and speed cell adhesion and chemotaxis around the inflammatory region, especially associated with the release of inflammatory mediators (Dinarello, 2000). Lin et al. (2008) reported that in asthmatic rats, IL-1 $\beta$  levels in the serum and lavage fluid were increased, contributing to airway remodeling by activating the JNK signaling pathway. Lappalainen et al. (2005) revealed that in respiratory epithelial cells of adult mice, an accumulation of MMP-9 and MMP-12 induced by chronic production of IL-1 $\beta$  could cause lung inflammation, distal airspace enlargement, mucus metaplasia and airway fibrosis. These data implied that IL-1 $\beta$  might be a potent inducer of asthma. Therefore, our present study focused on IL-1 $\beta$ -mediated human embryonic pulmonary fibroblast transdifferentiation. We selected MRC-5 cells as a model to investigate IL-1 $\beta$ -induced fibroblast-to-myofibroblast transdifferentiation in this study. We expect our findings may address the mechanisms underlying asthma pathogenesis, leading to the discovery of novel therapeutic drugs.

Myofibroblasts were characterized using high expression of  $\alpha$ -SMA as one of the markers in distinguishing the differentiation status of myofibroblasts. As one of the components of ECM, Col I is secreted by fibroblasts. Thus, the measurement of Col I is often used for monitoring ECM deposition level. Fibronectin can combine to specific receptors, such as the integrin receptor and growth factor receptor, involved in activating MAPK or other signaling pathways to induce inflammation cellular growth, migration and differentiation, speeding ECM deposition and initiating airway remodeling (Knight et al., 2004). In our study, we observed that treatment with IL-1 $\beta$  at a dose of 10 ng/mL for 48 h, significantly

promoted MRC-5 cell proliferation with increased expression of  $\alpha$ -SAM, FN and Col I. These data indicated that transdifferentiation from pulmonary fibroblasts to myofibroblasts could be induced by IL-1 $\beta$ . Additionally, IL-1 $\beta$ -induced upregulation of FN and Col I expressions might reflect a production of more collagen or ECM deposition that are potential contributors to asthma airway remodeling.

The ERK signaling pathway plays a critical role in chronic asthma airway inflammation, hyperresponsiveness, especially airway remodeling (Duan et al., 2004; Zhang et al., 2004). Our previous study demonstrated that the p-ERK level in the lungs of asthmatic rats was significantly increased and positively correlated with total bronchial wall thickness and smooth muscle thickness (Guan et al., 2007). In this study, we found that IL-1 $\beta$  induced a significant increase in phosphorylation of ERK following 15 min treatment in MRC-5 cells. The level of p-ERK reached a peak around 1 h following IL-1 $\beta$  treatment and decreased to basal levels after 4 h treatment. In addition, IL-1 $\beta$  induced ERK activation in a dose-dependent manner. Treatment with 10 ng/ml IL-1 $\beta$  for 1 h could induce a six-fold increase in p-ERK level, as compared to that of the control. Furthermore, we confirmed that IL-1 $\beta$ -mediated fibroblast-to-myofibroblasts transdifferentiation could be reversed by targeting the ERK signaling pathway, using the ERK inhibitor U0126.

As a T lymphocyte-specific immune adjuvant, lentinan (LNT) plays a critical role in immune regulation (Matsuoka et al., 1997). Our recent study reported that LNT could inhibit IL-1 $\beta$ -induced cell proliferation as well as the expression of  $\alpha$ -SAM, FN and Col I (Jin et al., 2011). In this study, we observed that LNT has a similar effect to U0126 in the inhibition of IL-1 $\beta$ -mediated ERK phosphorylation, cell proliferation, and the expression of FN, Col I and  $\alpha$ -SMA. These data might imply that LNT may have the ability to affect lung fibroblast function, including cell proliferation, phenotypic switching, and ECM deposition, even in asthma airway remodeling, by inhibiting ERK signaling activation.

In conclusion, herein we report that ERK signaling activation is involved in IL-1 $\beta$ -mediated human embryonic pulmonary fibroblast transdifferentiation, phenotypic switching and collagen secretion in MRC-5 cells. These events can be reversed by targeting the ERK signaling pathway using the ERK inhibitor or LNT. This suggests that LNT is a potential agent capable of preventing IL-1 $\beta$ -mediated asthma airway remodeling.

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