

# KL-6 regulated the expression of HGF, collagen and myofibroblast differentiation

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**Abstract. – OBJECTIVES:** KL-6 is a pulmonary epithelial-derived mucin which is secreted mainly by type II alveolar epithelial cells. The level of KL-6 in serum is closely correlated to the clinical activity of various interstitial lung diseases (ILD) and acts as a prognostic factor for ILD patients. Previous studies have showed that KL-6 promoted chemotaxis, proliferation and inhibited the apoptosis of human lung fibroblasts. However, the underlying mechanism remains unknown. In this study, we investigated the function of KL-6 on the expression of hepatocyte growth factor (HGF), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), collagen and  $\alpha$ -smooth muscle actin( $\alpha$ -SMA) in human embryonic lung fibroblasts cell line MRC-5.

**METHODS:** Human embryonic lung fibroblasts were cultured in Eagle's minimum essential medium. The cells plated in 6-well plates was cultured in serum-free medium at 37°C in 5% CO<sub>2</sub> and challenged with recombinant KL-6 at a final concentration of 0, 10, 20, 40 ng/mL.

Five micrograms of total RNA template were transcribed to cDNA by using AMV (Avian Myeloblastosis Virus) reverse transcriptase and random 9 mers as the first-strand primer. Synthesized cDNA was used in PCR experiments. The expression of TGF- $\beta$ 1 and HGF in cell culture supernatants was measured using ELISA kit. Cells incubated with KL-6 for 72h were collected for flow-cytometry analysis. The analysis was done using a Beckman counter device.

**RESULTS:** It was found that KL-6 up-regulated the expression of collagen type I and III in a dose-dependent manner. However, the addition of KL-6 significantly inhibited the production of HGF. As regard to the biological function, KL-6 induced myofibroblast differentiation confirmed by the elevated expression of  $\alpha$ -SMA.

**CONCLUSIONS:** KL-6 is one of the key molecules involved in epithelial-mesenchymal interactions and might contribute to the fibrosis in ILD.

## Key Words:

$\alpha$ -smooth muscle actin, KL-6, Interstitial lung diseases, Transforming growth factor- $\beta$ 1, Hepatocyte growth factor.

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic fibrosis disease with poor prognosis. Although the pathogenesis of IPF is unknown, injury to the alveolar epithelium is one of the earliest histopathologic features<sup>1,2</sup>. KL-6 (Krebs. von den Lungen-6) is a high-molecular-weight glycoprotein classified in humans as MUC1 mucin and is mainly expressed on type II alveolar epithelial cells (AECs), especially in proliferating, regenerating and injured type II AECs. Studies have showed that the expression level of KL-6 in the serum is elevated in a majority of adult patients with different types of interstitial lung diseases (ILD), including IPF<sup>3,4</sup> and act as a prognostic factor for ILD patients<sup>5,6</sup>. Despite the clinical importance of KL-6, the pathophysiological role of KL-6 in pulmonary fibrosis remains poorly understood. In this work, we investigated the function of KL-6 on the expression of hepatocyte growth factor (HGF), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and collagen by fibroblast as well as the effects of KL-6 on the differentiation of fibroblast to myofibroblast.

## Materials and Methods

### Cell Culture

Medical Research Council (MRC)-5 human embryonic lung fibroblasts (obtained from Shanghai Institute of Life Science Cell Resource Center, Shanghai, China) were cultured in Eagle's minimum essential medium (EMEM) (Jinuo Biotechnology Co., Ltd; Hangzhou, China) supplemented with 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin in the presence of 10% fetal calf serum (FCS) (Thermo Scientific HyClone; Logan, UT, USA) at 37°C in 5% CO<sub>2</sub>.

For cytokine treatment, the cells plated in 6-well plates was cultured in serum-free medium at

37°C in 5% CO<sub>2</sub> and challenged with recombinant KL-6 (Sino Biological Inc., Beijing, China) at a final concentration of 0, 10, 20, 40 ng/mL. The cells and conditioned media were collected for further assay after a 48 or 72h incubation.

### **RT-PCR Analysis**

After 48h incubation with recombinant KL-6, cells were lysed and total RNA was extracted using MicroElute™ Total RNA Kit (Omega; Norcross, GA, USA) according to the manufacturer's protocol. RT-PCR experiment was performed using RT-PCR kits (TaKaRa, Kyoto, Japan). Briefly, five micrograms of total RNA template were transcribed to cDNA by using AMV reverse transcriptase and random 9 mers as the first-strand primer. Synthesized cDNA was used in PCR experiments. Primers were designed using the Primer Premier 5.0 Software. The primers used were (name: forward primer, reverse primer): collagen type I (Col1a I): 5'-GCC TAG CAA CAT GCC AAT C -3', 5'-GCA AAG TTC CCA CCG AGA-3', product size: 181bp; collagen type III (Col1a III): 5'-GCT CTG CTT CAT CCC ACT ATT A-3', 5'-TGC GAG TCC TCC TAC TGC TAC-3', product size: 471bp; GAPDH: 5'-AGG TCG GAG TCA ACG GAT TTG-3', 5'-GTG ATG GCA TGG ACT GTG GT-3', product size: 532bp. The PCR products were electrophoresed on 2% agarose gel. The density of each band was measured by densitometry. Relative expression of collagen types I, III mRNA were normalized to the expression of the internal control (GAPDH: glyceraldehyde 3-phosphate dehydrogenase).

### **Measurement of TGF-β<sub>1</sub> and HGF by ELISA**

The expression of TGF-β<sub>1</sub> and HGF in cell culture supernatants was measured using ELISA kit (R and D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

### **Flow-cytometry Analysis of α-Smooth Muscle Actin (α-SMA) Expression**

Cells incubated with KL-6 for 72h were collected for flow-cytometry analysis. After trypsination, cells were washed with flow cytometry staining buffer (eBioscience; San Diego, CA, USA) and fixed with IC fixation buffer (eBioscience; San Diego, CA, USA) in the dark at room temperature for 20 minutes. After two washing steps with permeabilization buffer (eBioscience; San Diego, CA, USA), the cells were resuspended in perme-

abilization buffer and incubated for 60 min in the dark with anti-human α-SMA IgG (Epitomics Inc, Burlingame, CA, USA) or rabbit-IgG isotype control (Epitomics; Burlingame, CA, USA). Then, the cells were washed twice with permeabilization buffer and incubated with fluorescein isothiocyanate (FITC)-labeled second antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted in 100 μL permeabilization buffer for 30 minutes in the dark at room temperature. After one wash with permeabilization buffer and one wash with flow cytometry staining buffer, the cells were resuspended in 500 μl flow cytometry staining buffer. The analysis was done using a Beckman counter device (Beckman Coulter; Fullerton, CA, USA).

### **Statistical Analysis**

The results were expressed as means ± SEM. One-way ANOVA was used to evaluate differences between more than three groups. Bonferroni test or Tamhane test was used to evaluate differences between two groups. All analyses were performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The mean values were considered to be statistically significant when the probability of the event was below 5% ( $p < 0.05$ ).

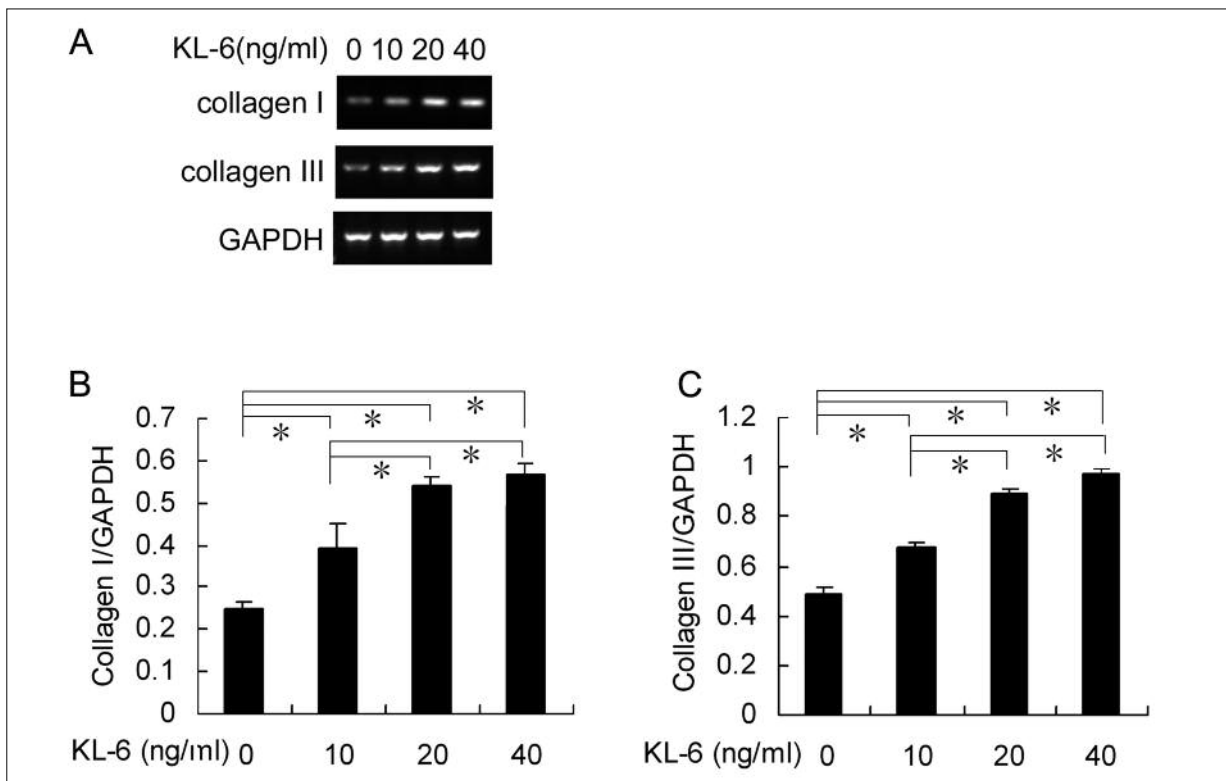
## **Results**

### **KL-6 up-Regulates the Expression of Collagen type I and III**

As shown in Figure 1, KL-6 up-regulated the expression of collagen type I and III in a dose-dependent manner ( $p = 0.000$ ). However, no significant difference for the expression of collagen type I and III was observed when the cells were stimulated with either 20 or 40 ng/ml KL-6 ( $p > 0.05$ ).

### **KL-6 Down-Regulates the Expression of HGF**

The addition of 20 or 40 ng/ml of KL-6 in the culture medium was found to significantly inhibit the production of HGF though the presence of 10 ng/mL KL-6 led to a slight but not significant decrease in the production of HGF by fibroblasts compared to the KL-6-free control group ( $320.25 \pm 13.12$  vs  $361.55 \pm 6.75$ ,  $p = 0.073$ ). However, addition of KL-6 had no effect on the production of TGF-β<sub>1</sub> by human fibroblasts ( $p = 0.076$ ) (Figure 2).



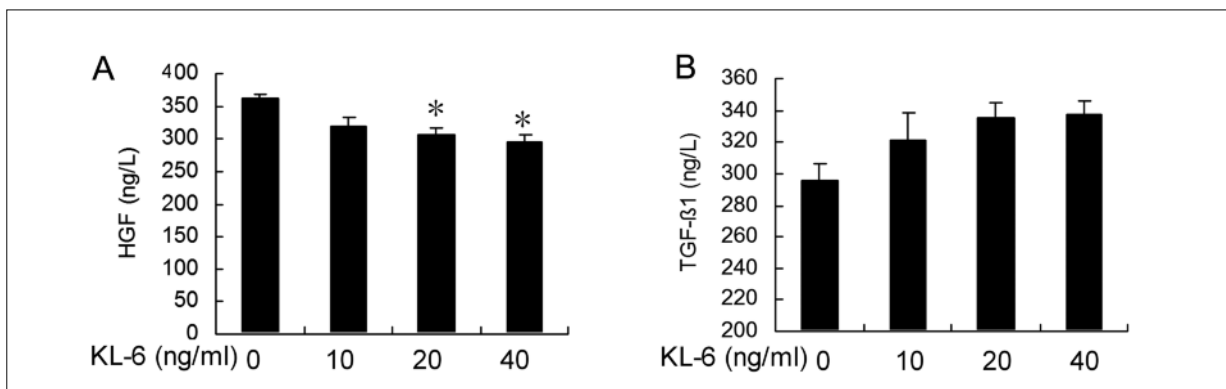
**Figure 1.** RT-PCR analysis of the mRNA expression of collagen I and III in MRC-5 human fibroblasts incubated with KL-6. Total RNA from MRC-5 cells were extracted after 48h of incubation, and then the mRNA level was analyzed by semiquantitative RT-PCR. **A**, The expression of GAPDH, collagen I and collagen III mRNA. Quantification of mRNA was achieved by gel analysis and expressed as a ratio with GAPDH mRNA. **B**, The mean  $\pm$  SEM of six experiments were reported for collagen I (**B**) and collagen III (**C**), respectively. \* $p < 0.05$ .

**Induction of Myofibroblasts Differentiation by KL-6**

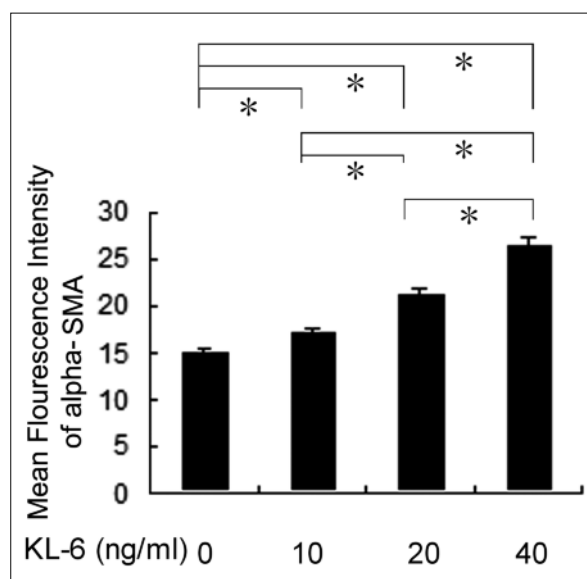
$\alpha$ -SMA is a biomarker for myofibroblasts differentiation. As shown in Figure 3, the mean fluorescence intensity for  $\alpha$ -SMA was increased in the presence of KL-6 in the flow-cytometry analysis.

**Discussion**

IPF is characterized by a sequence of events that start with micro-injuries in alveolar epithelial followed by the formation of fibroblastic foci<sup>6</sup>. Epithelial-mesenchymal interactions play



**Figure 2.** Effects of KL-6 on the production of HGF and TGF- $\beta$ 1 in MRC-5 human fibroblasts under rest and stimulated conditions. MRC-5 cells were cultured with various concentrations of KL-6 for 48h. The supernatants were analyzed by ELISA. Results for six experiments were reported as mean  $\pm$  SEM. \* $p < 0.05$  compared to those in the absence of KL-6.



**Figure 3.** Flow cytometry analysis for the  $\alpha$ -SMA expression in MRC-5 human fibroblasts incubated with KL-6. Results for six experiments were expressed as mean fluorescence intensity (MFI)  $\pm$  SEM and adjusted by subtracting the mean fluorescence of the negative control. \* $p < 0.05$ .

the central role in the lung repair process. Previous studies have showed that the injured type II AECs produced more KL-6 than normal cells and the expression level of KL-6 in serum reflected the disease activity of interstitial pneumonitis such as IPF<sup>7,8</sup>. However, the role of KL-6 in the pathogenesis of pulmonary fibrosis has not been well elucidated. In the present work, we have demonstrated that KL-6 inhibited the production of HGF, promoted the expression of collagen type I and III by fibroblasts and induced myofibroblasts differentiation.

The formation of fibroblastic foci and increased deposition of extracellular matrix (ECM) are the hallmarks of IPF. In the injured tissues, the fibroblasts are activated and differentiate into  $\alpha$ -SMA positive myofibroblasts, which play an important role in synthesis, deposition and remodelling of ECM. In addition, the myofibroblasts induce alveolar epithelial cell injury/apoptosis, perpetuated the damage of the alveolar epithelium and inhibited re-epithelialisation. In this study, it was showed that KL-6 participated in the fibrotic processes through promoting the expression of collagen I and III by fibroblasts and inducing fibroblast-to-myofibroblast differentiation.

HGF is a paracrine factor produced by cells of mesenchymal origin such as fibroblasts and plays

a key role in alveolar homeostasis<sup>9</sup>. HGF has been demonstrated to increase proliferation of alveolar epithelial cells<sup>10</sup>, protect them from apoptosis and inhibit epithelial-myofibroblast transition<sup>11</sup>. It also suppresses the production of  $\alpha$ -SMA induced by TGF- $\beta_1$  in fibroblasts<sup>12</sup> and induces apoptosis of fibroblastic MRC5 cells<sup>13</sup>. The administration of HGF is effective in preventing the fibrosis of the liver, heart, kidney and lung in animal models<sup>14-17</sup>. Marchand-Adam et al<sup>18</sup> reported that basal HGF secretion by IPF fibroblasts was decreased by 50% when compared with control fibroblasts. Taken together with our study that KL-6 inhibited the production of HGF by fibroblasts, it was suggested that the injured AECs secreted large amounts of KL-6 to inhibit the fibroblasts from producing HGF and then promoted lung fibrosis.

## Conclusions

Our results along with previous studies suggest that the injured alveolar epithelial cells released KL-6 to initiate fibroblast-to-myofibroblast differentiation, increased the expression of collagen I and III, and inhibited the production of HGF. These findings indicate that KL-6 is one of the key molecules involved in epithelial-mesenchymal interactions. KL-6 might contribute, at least in part, to the fibrogenesis in ILD. The mechanism by which KL-6 exerts on fibroblasts should be clarified by further studies.

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## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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