

THE EFFECT OF TGF- β 1 AND Smad7 GENE TRANSFER ON THE PHENOTYPIC CHANGES OF RAT ALVEOLAR EPITHELIAL CELLS

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Abstract: The aim of this study was to investigate whether transforming growth factor- β 1 (TGF- β 1) could induce alveolar epithelial-mesenchymal transition (EMT) *in vitro*, and whether Smad7 gene transfer could block this transition. We also aimed to elucidate the possible mechanisms of these processes. The Smad7 gene was transfected to the rat type II alveolar epithelial cell line (RLE-6TN). Expression of the EMT-associated markers was assayed by Western Blot and Real-time PCR. Morphological alterations were examined via phase-contrast microscope and fluorescence microscope, while ultrastructural changes were examined via electron microscope. TGF- β 1 treatment induced a fibrotic phenotype of RLE-6TN with increased expression of fibronectin (FN), α -smooth muscle actin (α -SMA) and vimentin, and decreased expression of E-cadherin (E-cad) and cytokeratin19 (CK19). After transfecting the RLE-6TN with the Smad7 gene, the expression of the mesenchymal markers was downregulated while that of the epithelial markers was upregulated. TGF- β 1 treatment for 48 h resulted in the separation of RLE-6TN from one another and a change into elongated, myofibroblast-like cells. After the RLE-6TN had been transfected with the Smad7 gene, TGF- β 1 treatment had no effect on the morphology of the RLE-6TN. TGF- β 1 treatment for 48 h resulted in an abundant expression of α -SMA in the RLE-6TN. If the RLE-6TN were transfected with the Smad7 gene, TGF- β 1 treatment for 48 h could only induce a low level of α -SMA expression. Furthermore, TGF- β 1 treatment for 12 h resulted in the degeneration

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Abbreviations used: α -SMA – α -smooth muscle actin; CK19 – cytokeratin19; E-cad – E-cadherin; ECM – extracellular matrix; EGFR – epidermal growth factor receptor; EMT – epithelial-mesenchymal transition; FN – fibronectin; IPF – Idiopathic pulmonary fibrosis; TGF- β 1 – transforming growth factor- β 1

and swelling of the osmiophilic multilamellar bodies, which were the markers of type II alveolar epithelial cells. TGF- β 1 can induce alveolar epithelial-mesenchymal transition *in vitro*, which is dependent on the Smads signaling pathway to a certain extent. Overexpression of the Smad7 gene can partially block this process.

Key words: Epithelial-mesenchymal transition, Gene transfer, Smad7, Transforming growth factor- β 1

INTRODUCTION

In recent years, there has been an observable increase in the incidence of pulmonary fibrosis, partly because of the greater life expectancy, but also somewhat due to the use of bleomycin (BLM), an anticancer drug. Idiopathic pulmonary fibrosis (IPF), the most common pulmonary fibrotic disorder, is a progressive and fatal disease with an unknown etiology. Its pathogenesis uniquely features the presence of fibroblastic foci in the parenchyma of the lungs [1]. These are comprised of aggregates of mesenchymal cells including fibroblasts and cells which exhibit the phenotypic features of myofibroblasts (MFs): α -smooth muscle actin (α -SMA) expression, increased mitogenic capacity, and enhanced extracellular matrix (ECM) production. Increased numbers of fibroblastic foci are associated with the progression of the disease, and with a worse prognosis in IPF/UIP [2]. According to the recently elucidated epithelial/fibroblastic model of IPF pathogenesis, it is considered that the fibroblastic foci underlie areas of unresolved epithelial injury, and are sites where activated fibroblasts and myofibroblasts migrate, proliferate and synthesize ECM proteins [3]. However, the cellular origin of MFs in pulmonary fibroblast foci is still debated. Some experiments suggested that pulmonary MFs were derived from type II alveolar epithelial cells through the epithelial-mesenchymal transition (EMT) process [4].

It is now well recognized that a number of key growth factors are responsible for driving the process of fibrogenesis, including transforming growth factor- β 1 (TGF- β 1), interleukin-1 beta (IL-1 β), and tumor necrosis factor-alpha (TNF- α). In general, it is the level of TGF- β 1 that best correlates with the extent of fibrosis and myofibroblast-like cell induction [5]. Recent evidence from the studies of other fibrotic disorders, including renal [6, 7] and hepatic fibrosis [7], supports a view that TGF- β 1 might play a role in pulmonary fibrogenesis by promoting alveolar epithelial mesenchymal transition. During the process of EMT, membrane-associated adherent junctions and desmosomes are dissociated, whilst at the same time or shortly thereafter, cytoskeletal rearrangement takes place and the amount of mRNA for intermediate filament proteins is increased, facilitating the cell adopting a mesenchymal phenotype [8]. E-cadherin (E-cad) is an epithelial cell transmembrane protein with an extracellular domain which interacts with that of E-cad molecules expressed by adjacent cells. It has

a critical role in establishing firm adhesion, and maintaining cell polarity and epithelial tightness [9]. The cadherin complex suppresses the dissociation of epithelial cells, and thus, the crucial step of EMT is the downregulation of E-cad [10].

Smad7 is an inhibitory Smad that interferes with Smad signaling by preventing the phosphorylation of Smads2/3, which normally occurs following the binding of TGF- β 1/activin to their cell surface receptors [11-13]. The transient introduction of the Smad7 gene using an adenovirus vector was recently shown to be effective in treating tissue inflammatory/fibrotic disorders in the kidneys [14] and liver [15].

To understand the role of EMT in the development of fibroblastic foci in IPF, we examined whether or not TGF- β 1 could induce the EMT process in the rat type II alveolar epithelial cell line (RLE-6TN) *in vitro*, and whether Smad7 gene transfer could block this transition. We also investigated the possible mechanisms of these processes. This was done by transfecting Smad7 onto the *in vitro* cultured RLE-6TN and making a comparison study of the EMT-associated markers at the level of the gene and protein at different treatment time points.

MATERIALS AND METHODS

Cell culture and treatment

The rat type II alveolar epithelial cell line (RLE-6TN), purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), was cultured with DMEM/Ham's F12 medium supplemented with 10% fetal bovine serum (FBS; PAA, the Cell Culture Company), 100 kU/l penicillin and 100 mg/l streptomycin, and maintained in humidified 5% CO₂ at 37°C. To observe the effects of TGF- β 1 on RLE-6TN, serum-starved RLE-6TN were incubated with 3 ng/ml TGF- β 1 (R&D, Minneapolis) for 1, 3, 6, 12 or 24 h. Then the cells were collected to examine the mRNA expression of fibronectin (FN), α -smooth muscle actin (α -SMA), vimentin, TGF- β 1, epidermal growth factor receptor (EGFR), E-cadherin (E-cad) and cytokeratin19 (CK19) using Real-time PCR. Also, for Western Blot analysis, other serum-starved RLE-6TN were incubated with TGF- β 1 for 3, 6, 12, 24, 48 or 72 h. Then the cells were collected to examine the protein expression of FN, α -SMA, vimentin, EGFR, E-cad and CK19. To investigate the effects of Smad7 on RLE-6TN, serum-starved cells transfected with the Smad7 gene were incubated with 3 ng/ml TGF- β 1, and then the cell lysates were harvested for Real-time PCR and Western blot analysis.

Transfection experiments

RLE-6TN cells were transfected at 90%-95% confluence in six-well plates. The transfection mixture was prepared by incubating 10 μ l of liposome (Lipofectamine 2000, Invitrogen) and 250 μ l Opti-MEMI for 20 min at room temperature. pcDNA3.0-Smad7 (provided by Prof. Yun-Jian Huang) and mock pcDNA3.0 were then added to the above mixture and incubated for a further 30 min at room

temperature. Finally, this transfection mixture was added to a six-well plate containing 2 ml of Opti-MEM1 for 4-6 h. The medium was then changed to fresh DMEM/F12 medium containing 20% FBS for a further 48 h. The cells were then passaged into 100-mm dishes in the ratio of 1:10 for a further 48 h. Fresh DMEM/F12 medium with 10% FBS containing G418 (1200 $\mu\text{g/ml}$) was used to select cells, and about 10 days later, positive clones appeared and were then selected.

Morphological observation

RLE-6TN and ST1 cells grown to 60-80% confluence were cultured for 24 h with serum-free medium, and then treated with 3 ng/ml TGF- β 1 for 48 h. The cells were observed with a phase-contrast microscope and photos were taken.

Cell culture and immunofluorescent staining

Cells were seeded into sterile coverslips placed inside 6-well plates. On reaching 60-80% confluence, the medium was changed to serum-free medium, and TGF- β 1 was added to a subset of wells to a final concentration of 3 ng/ml. 48 h later, all the wells were washed twice with cold PBS, and a subset of the wells were fixed with cold methanol at -20°C for 10 min. The coverslips were removed from the wells and placed on glass slides, the cells on the coverslips were incubated with anti α -SMA mAb (1:100) at 37°C for 1 h, and subsequently incubated with goat anti-mouse IgG-cy3 (1:50) at 37°C for 1 h. Hoechst 33258 was used to stain the nuclei for 5 min, and the coverslips were mounted with 50% glycerol. The slides were examined using a ZEISS fluorescence microscope equipped with a digital camera, and photos were taken.

Ultrastructure observation

RLE-6TN cells grown to 60-80% confluence were cultured for 24 h with serum-free medium, and then treated with 3 ng/ml TGF- β 1 for 12, 24, 48 and 72 h. The cells were observed with an electron microscope and photos were taken.

Western blot

Cells were solubilized in a lysis buffer consisting of 62.5 mM Tris, 2% sodium dodecyl sulphate (SDS) and a mixture of protease inhibitors: 5 mg/ml each of leupeptin and aprotinin (Sigma), and 0.4 mM 4-(2-aminoethyl)-benzolsulfonyl fluoride (Pefabloc; Roche). The protein concentration was determined with the Micro BCA™ protein assay kit (Pierce, Rockford, IL). The samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Following blotting onto PVDF membranes (Millipore), non-specific binding sites were blocked by incubating the membrane for 2 h in a buffer consisting of 5% fat-free dried milk in TBS-Tween (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6). Protein bands were detected after incubation with primary antibodies for 1 h at 37°C , and then following overnight storage at 4°C , with secondary antibodies for 1 h by ultrared fluorescence staining (700 nm, 800 nm) using the ODYSSEY Infrared Imaging System (LI-COR Biosciences). The primary

antibody used in the study is shown in Tab. 1. The molecular weights were estimated by comparison with pre-stained SDS-PAGE molecular weight standards (Bio-Rad). A densitometric evaluation of the immunoblotted proteins were performed using ODYSSEY analysis software.

Tab. 1. The primary antibody used for the Western blot analysis.

| Primary antibody | Concentration used | Source |
|------------------|--------------------|-----------------|
| FN | 1:500 | Changdao, CHN |
| α -SMA | 1:1000 | Sigma, USA |
| EGFR | 1:1000 | Santa Cruz, USA |
| Vimentin | 1:1000 | Changdao, CHN |
| CK19 | 1:200 | Santa Cruz, USA |
| E-cad | 1:3000 | BD Company, USA |
| p-Smad2/3 | 1:1000 | CST, USA |
| Smad2/3 | 1:1000 | CST, USA |

Reverse transcription and real-time PCR

Total RNA was extracted from RLE-6TN with TRIzol Reagent (Invitrogen, USA). The RNA samples were quantified by measuring the optic absorbance at 260 nm and 280 nm in a spectrophotometer, with the A_{260}/A_{280} ratio ranging from 1.8 to 2.0. Aliquots of total RNA (2.0 μ g) from each sample were reverse-transcribed into cDNA according to the instructions of the First Strand cDNA Synthesis Kit manufacturer (Invitrogen, USA). Equal amounts of the reverse transcriptional products were subjected to PCR amplification. The amplification was started with 5 min of denaturation at 94°C, followed by 30 cycles (for GAPDH, 25 cycles). Each cycle lasted 45 s at the denaturation temperature of 94°C, 60 s at the relevant annealing temperatures, and 90 s at the extension temperature of 72°C. The final extension was for 10 min at 72°C. After amplification, 100 μ l of each PCR reaction product was electrophoresed through a 1.0% (w/v) agarose gel containing ethidium bromide (0.5 μ g/ml) to check the specificity by PAGE analysis. Then the PCR products were purified using a MinElute PCR Purification Kit (Sangon Biotech, China), and quantified by spectrophotometry at a wavelength of 260 nm. The products served as Real-time PCR standards. The number of copies/ μ l of the standards was calculated according to the following formula: $\text{copies}/\mu\text{l} = 6.7 \times 10^{17} \times C \times 263/660 \times \text{product size (bp)}$, where C means the concentration of DNA. Standards were serially diluted in 1:2, 1:4, or 1:10 log steps. All the calibration curves for the purified PCR products were linear over the whole quantification range, and the intrarun variability was calculated from triplicate samples for all the target genes.

The thermal cycling profile consisted of step 1 at 95°C for 10 min and step 2 at 94°C for 30 s, followed by 30 s at the appropriate annealing temperature. Step 2 was repeated 40 times for all the target genes. The fluorescence product was

detected during annealing/extension periods. As SYBR Green I (Invitrogen, USA) also binds to primer dimers formed non-specifically during all the PCR reactions, a melting curve analysis was used to confirm the specificity of the amplification products: step 1 at 50°C for 15 s; step 2 from 50°C, increasing the temperature by 0.5°C every 15 s up to a final temperature of 95°C. The quantification data was expressed as the ratio of the starting quantity mean (SQm) of the target to the housekeeping gene. All the primers were designed with Primer 5 software and synthesized by Sangon Biotech. The sequences and the amplified lengths are given in Tab. 2.

Tab. 2. The primers used in the Real-time RT-PCR analysis.

| Primers | Sequence (5→3') | Annealing temperature [°C] | size [bp] |
|----------------|--|----------------------------|-----------|
| α -SMA | Sense 5'-AAGAGGAAGACAGCACAGCTC-3' Antisense 5'-GATGGATGGGAAAACAGCC-3' | 60 | 101 |
| FN | Sense 5'-TTACGGTGGCAACTCAA-3' Antisense 5'-GGTCATGCTGCTTATCCC-3' | 53.6 | 419 |
| Vimentin | Sense 5'-TGACCGCTTCGCCAACTA-3' Antisense 5'-CGCAACTCCCTCATCTCCT-3' | 55.9 | 141 |
| TGF- β 1 | Sense 5'-GGCGGTGCTCGCTTTGTA-3' Antisense 5'-GCCACTCAGGCGTATCAG-3' | 57 | 361 |
| EGFR | Sense 5'-TCGGTGCTGTGCGATTTA-3' Antisense 5'-TGGTGGCAGGTGTCTTT-3' | 55.8 | 365 |
| E-cadherin | Sense 5'-TATCCAGGAGGTCTTGAGG-3' Antisense 5'-GCGATGGCAGCATTGTAG-3' | 51.3 | 103 |
| CK19 | Sense 5'-TCGCCTCGCCTCTACTT-3' Antisense 5'-GCTCCTCAGGGCAGTAATT-3' | 55.8 | 415 |
| GAPDH | Sense 5'-ACCACAGTCCATGCCATCAC-3' Antisense 5'-TCCACCACCCTGTTGCTGTA-3' | 55 | 452 |

Statistical analysis

The data is expressed as the means \pm SD. The statistical analysis was performed using One-way ANOVA. A $P < 0.05$ was considered statistically significant.

RESULTS

Smad7 transfection treatment results in the specific and effective upregulation of Smad7 expression

We successfully established the overexpression of Smad7 on two positive RLE-6TN clones (ST1, ST6) and on a mock clone (CRL). The two RLE-6TN clones showed increased mRNA expression of Smad7, 46.5 and 26.6 times that of the control group (Fig. 1A), and increased protein expression of Smad7, 4.4 and 3.6 times that of the control group (Fig. 1B). At the same time, cell indirect immunofluorescence staining demonstrated that the Smad7 transfection was successful (Fig. 1C). In the following experiments, the ST1 with the highest expression of Smad7 protein was selected for further research.

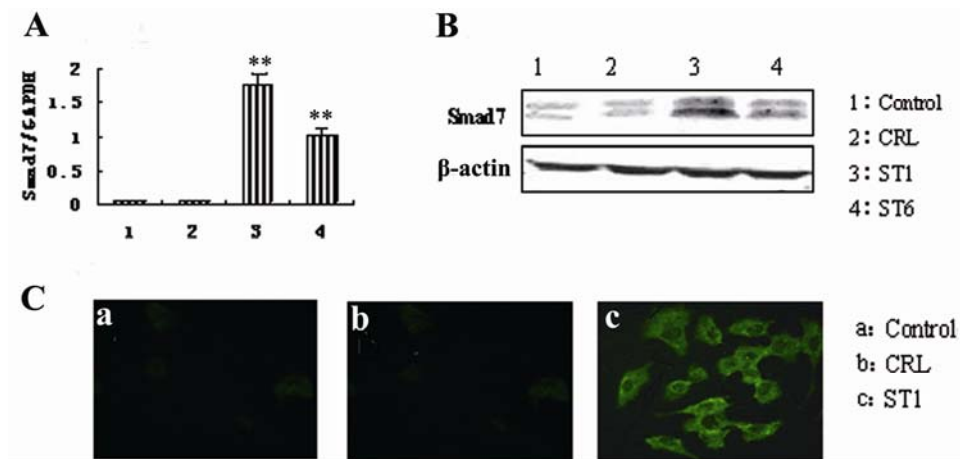


Fig. 1. The effect of Smad7 transfection treatment on the Smad7 expression of RLE-6TN. A – Real-time PCR results showed that the increased mRNA expressions of Smad7 for ST1 and ST6 were respectively 46.5 and 26.6 times that of the control group (** $P < 0.01$). B – Western Blot results showed that the protein expressions of Smad7 were respectively 4.4 and 3.6 times that of the control group. C – Cell indirect immune-fluorescence demonstrated the result of Smad7 transfection: a. Negative control, b. CRL. and c. ST1.

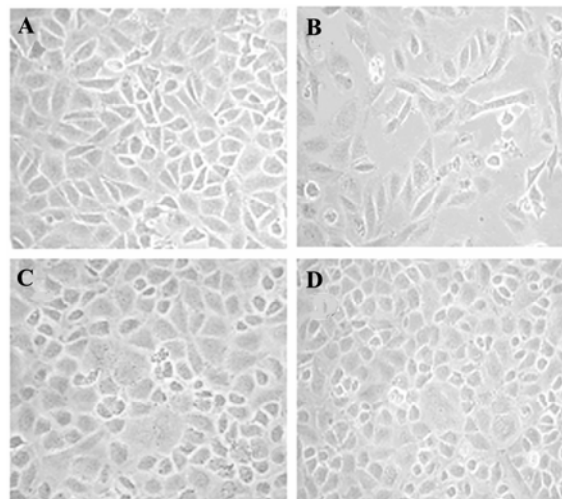


Fig. 2. Morphological changes in RLE-6TN and ST1 in response to TGF- β 1. A – Under control conditions, RLE-6TN showed a pebble-like shape and cell-cell adhesion was clearly observed. B – RLE-6TN showing a decrease in cell-cell contacts and taking on an elongated shape after TGF- β 1 treatment for 48 h. C – Under control conditions, the ST1 showed a pebble-like shape and cell-cell adhesion was clearly observed. D – ST1 showing the same appearance as under control conditions after 48 h TGF- β 1 treatment. Magnification: 200 \times . The figures are representative of the findings of three experiments.

The effects of TGF- β 1 on the cell morphology of RLE-6TN and ST1

Under the phase-contrast microscope, RLE-6TN and ST1 grown in a six-well plate produced a confluent monolayer with a cobblestone appearance. RLE-6TN with TGF- β 1 treatment induced cells separating from each other and taking on an elongated shape (Fig. 2A and B). By contrast, ST1 sustained a confluent monolayer with a cobblestone morphology when TGF- β 1 was added to the medium (Fig. 2C and D).

The effects of TGF- β 1 on the cell phenotype of RLE-6TN and ST1

Under the fluorescence microscope, immunofluorescent staining for α -SMA was not detected in the RLE-6TN under basal conditions (Fig. 3A), but was observable in many cells after TGF- β 1 exposure (Fig. 3B). Immunofluorescent staining for α -SMA was not detected in the ST1 cells under basal conditions (Fig. 3C), but was observable in a few cells after TGF- β 1 exposure (Fig. 3D).

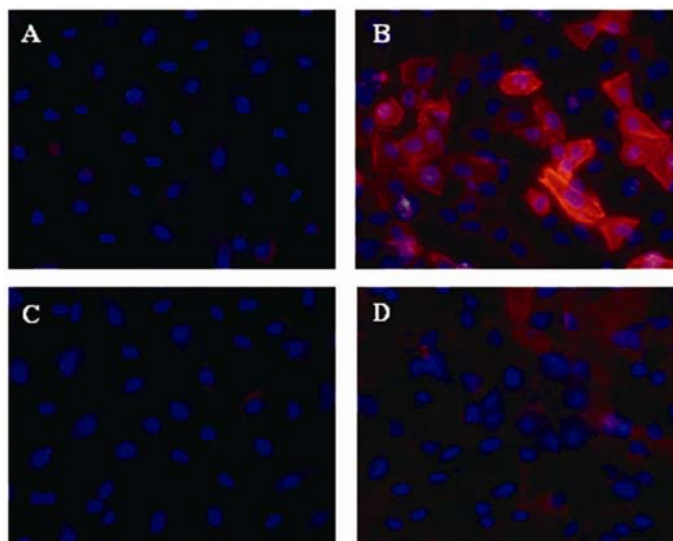


Fig. 3. Phenotypic changes in RLE-6TN and ST1 in response to TGF- β 1. A – Fluorescent staining for α -SMA was not detected in the RLE-6TN under basal conditions. B – TGF- β 1 exposure for 48 h resulted in abundant expression of α -SMA. C – Fluorescent staining for α -SMA was not detected in the ST1 cells under basal conditions. D – TGF- β 1 exposure for 48 h resulted in a low level of α -SMA expression. Magnification: 400 \times .

The effects of TGF- β 1 on the ultrastructure of RLE-6TN

Under the electron microscope, the osmiophilic multilamellar body, the marker of the type II alveolar epithelial cell, was found in the control group of RLE-6TN (Fig. 4A). After TGF- β 1 treatment for 12 h, the bodies became swollen, degenerated, and even disappeared completely with the prolongation of TGF- β 1 treatment (Fig. 4B).

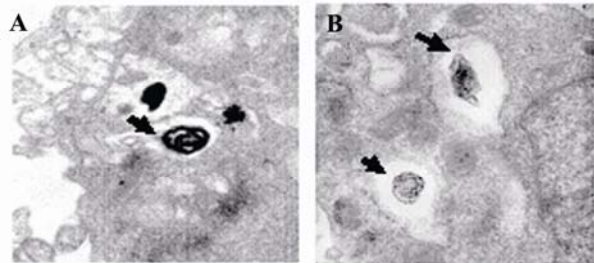


Fig. 4. Ultrastructural changes of RLE-6TN after TGF- β 1 treatment. A – Under control conditions. B – The osmiophilic multilamellar bodies of RLE-6TN became degenerated and swollen (arrows) after TGF- β 1 treatment for 12 h. Magnification: 45000 \times . The figures are representative of the findings of three experiments.

Expression of EMT-related markers of RLE-6TN, ST1 and ST6

Cell lysates from RLE-6TN, CRL (mock), ST1 and ST6 were employed to examine the expression of EMT-associated proteins. The E-cad and CK19 expression of ST1 and ST6 was upregulated when compared with RLE-6TN and CRL ($P < 0.05$), but the FN and vimentin expression simultaneously decreased (Fig. 5).

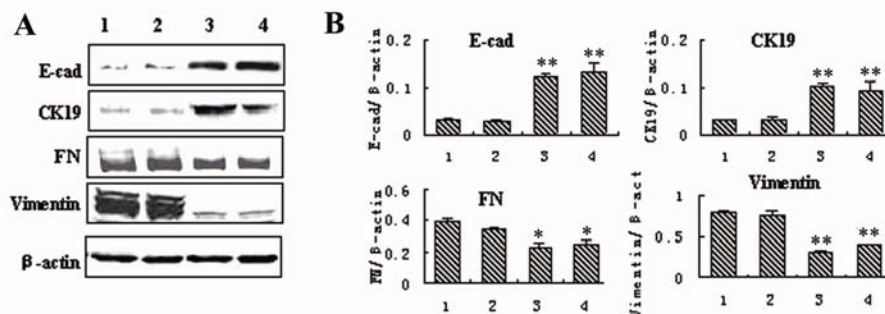


Fig. 5. Western blot analysis of the basal E-cad, CK19, FN and Vimentin protein levels in RLE-6TN (lane 1), CRL (lane 2), ST1 (lane 3) and ST6 (lane 4). The blots shown are from a representative experiment performed in triplicate. Similar results were obtained from three independent experiments. β -actin was used as an internal control.

The effects of TGF- β 1 on the protein expression of the epithelial and mesenchymal markers of RLE-6TN and ST1

Western Blot was used to examine the expression of the EMT-associated markers of RLE-6TN and ST1. Following the addition of TGF- β 1 into the medium of RLE-6TN, the FN and Vimentin expression increased ($P < 0.05$). At the same time, the E-cad and CK19 expressions of RLE-6TN were downregulated (Fig. 6A and C). After Smad7 gene transfer to RLE-6TN, TGF- β 1 treatment led to a slight downregulation of the expression of E-cadherin and

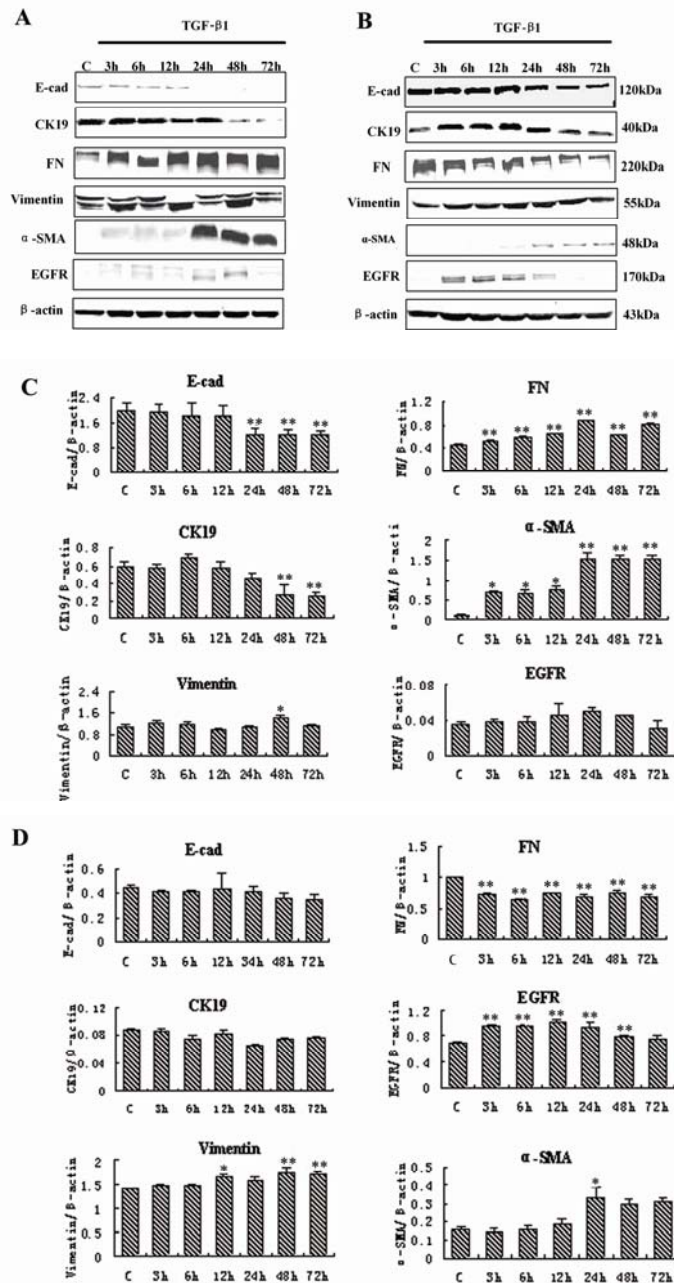


Fig. 6. A Western blot analysis of E-cad, CK19, FN, Vimentin, α-SMA and EGFR protein expression for RLE-6TN (A and C) and ST1 (B and D) with TGF-β1 treatment for 3-72 h. The blots shown are from a representative experiment performed in triplicate. Similar results were obtained from three independent experiments. β-actin was used as the internal control.



Fig. 7. A Western blot analysis of the phosphorylation Smad2 and Smad3 protein expression in RLE-6TN and ST1 with 3 ng/ml of TGF- β 1 for 48 h. The data shown is from a representative experiment performed in triplicate.

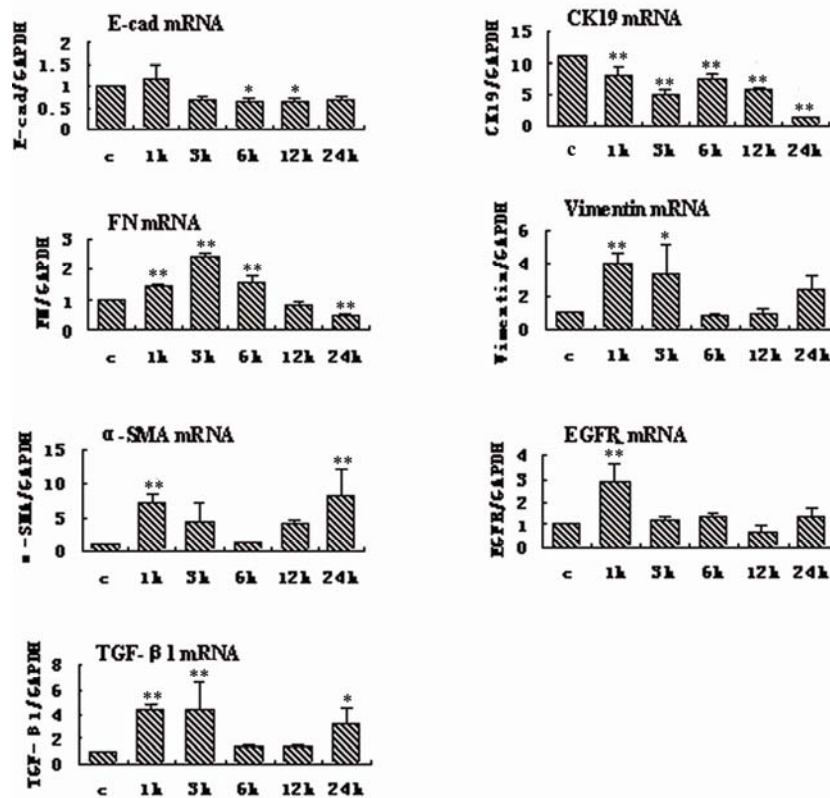


Fig. 8. Quantification of the E-cad, CK19, FN, Vimentin, α -SMA, EGFR and TGF- β 1 mRNA levels by Real-time PCR in TGF- β 1-treated RLE-6TN grown for 1-24 h. The mRNA levels are reported as the E-cad/GAPDH, CK19/GAPDH, FN/GAPDH, Vimentin/GAPDH, EGFR/GAPDH and TGF- β 1/GAPDH ratios and normalized to the control, C. The mean \pm SD of three experiments is given. Compared with the control, C, * P < 0.05, ** P < 0.01.

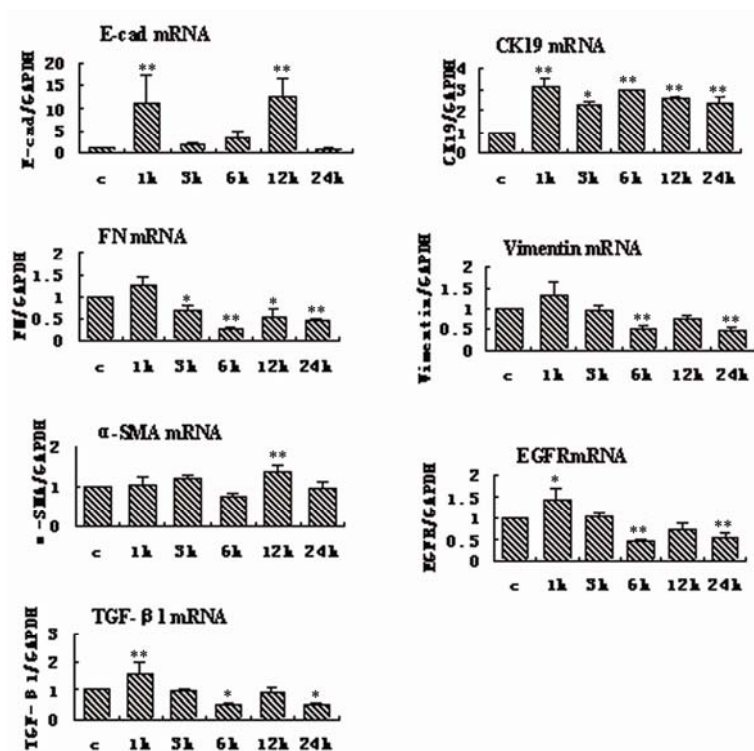


Fig. 9. Quantification of the E-cad, CK19, FN, Vimentin, α -SMA, EGFR, and TGF- β 1 mRNA levels by Real-time PCR in TGF- β 1-treated ST1 grown for 1-24 h. The mRNA levels are reported as the E-cad/GAPDH, CK19/GAPDH, FN/GAPDH, Vimentin/GAPDH, EGFR/GAPDH and TGF- β 1/GAPDH ratios and, normalized to the control, C. The mean \pm SD of three experiments is given. Compared with the control, C, * P < 0.05 ** P < 0.01.

CK19, downregulation of FN expression, and a slight upregulation of the expression of α -SMA and Vimentin (Fig. 6B and D). The expression of β -actin served as a loading control. These results were obtained from three repeated experiments, and one of the representative figures is presented here.

The effects of TGF- β 1 on the phosphorylated Smad2/3 protein expression of RLE-6TN and ST1

TGF- β 1 treatment for 48 h could activate the phosphorylation of the Smad2/3 of RLE-6TN (P < 0.05). However, after Smad7 gene transfer to RLE-6TN, TGF- β 1 could not activate phosphorylation of the Smad2/3 of ST1 (Fig. 7).

The effects of TGF- β 1 on the mRNA expression of the epithelial and mesenchymal markers of RLE-6TN and ST1

Quantification of the epithelial and mesenchymal markers of the mRNA level was performed by Real-time PCR in TGF- β 1-treated RLE-6TN and ST1 grown for 1-24 h. The mRNA expression of the FN, Vimentin and α -SMA of RLE-6TN following the administration of TGF- β 1 was elevated at 3, 6 and 24 h ($P < 0.05$). Meanwhile, the expression of E-cad and CK19 were decreased ($P < 0.05$) (Fig. 8). The mRNA expression of the FN, Vimentin and α -SMA of ST1 following TGF- β 1 treatment was elevated at 1 h, decreased at 3 h, and fluctuated later on but with a general trend of decrease. The expression of E-cad and CK19 was increased (Fig. 9).

DISCUSSION

EMT is a process by which fully differentiated epithelial cells undergo phenotypic transition to fully differentiated mesenchymal cells, often fibroblasts and myofibroblasts [16]. In 1982, Greenburg *et al.* [17] reported that epithelial cells in culture might acquire mesenchymal features, which provided the earliest proof for the process of epithelial-to-mesenchymal transition (EMT). In 1985, Stoke *et al.* [18] observed that conditioned fibroblast medium could induce a ‘scattering’ of polarized epithelial cells into separate, single cells, leading to the isolation of the hepatocyte growth factor (HGF), and demonstrated that EMT might be induced by extracellular stimuli through the activation of kinase-dependent signaling cascades. Since then, numerous studies have focused on tumor metastasis and renal interstitial fibrosis related to the process of EMT. TGF- β 1 was firstly described as an inducer of EMT in normal mammary epithelial cells by signaling through receptor serine/threonine kinase complexes [19]. The mechanism by which TGF- β 1 could induce EMT was TGF- β 1 stimulation of epithelial cells leading to the induction of Smad proteins, which serve both as transcription factors themselves, and as inducers of other transcription factors, including Slug, Snail, Scatter, lymphoid enhancing factors and β -catenin. The transcription factors led to the expression of the “EMT” proteome, including the cellular machinery necessary for junctional disassembly, cytoskeletal rearrangement and cellular motility [20]. This study involved the use of phase-contrast, fluorescence and electron microscopy for observing the phenotypic alterations of RLE-6TN treated with TGF- β 1, with the results indicating that the cells were separated from one another and changed into a short-spindle shape after TGF- β 1 treatment for 48 h. Forty-eight hours after the treatment of TGF- β 1, many of the cells expressed α -SMA. When TGF- β 1 had been added to the medium for 12 h, the osmiophilic multilamellar body, the marker of the type II alveolar epithelial cell, became swollen, degenerated, and gradually disappeared with the extension of TGF- β 1 treatment. The above results showed that TGF- β 1 could induce the phenotypic and ultrastructural alterations of alveolar epithelial cells *in vitro*. At the mRNA level, the

expression of the mesenchymal markers FN, Vimentin and α -SMA upregulated following the treatment of TGF- β 1 for 1 and 3 h, whereas the epithelial markers E-cad and CK19 downregulated. At the protein level, FN, Vimentin and α -SMA were upregulated after TGF- β 1 treatment, while E-cad and CK19 were downregulated. The above results confirmed that TGF- β 1 could indeed induce the EMT process of type II alveolar epithelial cells *in vitro*. In this study, TGF- β 1 could induce the mRNA expression of autocrine TGF- β 1, with the expression phase being identical with that of the EMT-associated markers, demonstrating that TGF- β 1 could stimulate the autocrine loop of TGF- β 1, and then continue to operate the EMT process.

Overexpression of inhibitory Smad7 consistently blocks Smad3-dependent EMT *in vitro*, including retinal pigment epithelium [21] and mammary epithelial cells [22], and in the renal UUO model *in vivo* [23]. Introduction of Smad7 also abolishes upregulation of Snail, α -SMA and extracellular matrix components. Smad7 blocks signaling from both Smad2 and Smad3. In our study, RLE-6TN with Smad7 overexpression (ST1 cells) maintained a confluent monolayer of cobblestone morphology, and the cells were linked closely to one another in response to TGF- β 1 treatment. Forty-eight hours after the treatment with TGF- β 1, there remained a few cells expressing α -SMA under the fluorescence microscope. The basal protein level of the epithelial markers E-cad and CK19 in the ST1 cells was upregulated, while the basal expression of the mesenchymal markers FN and Vimentin was downregulated to different extents. The mRNA expression of the mesenchymal markers mentioned above was elevated at 1 h in response to TGF- β 1 treatment but decreased at 3, 6, 12 and 24 h, especially at 6 and 24 h. The mRNA expression of TGF- β 1 and EGFR was similar to that of those mesenchymal markers, while the mRNA expression of typical epithelial markers were elevated at the corresponding time points. At the protein level, after Smad7 transfection, the expression of FN was downregulated in response to TGF- β 1 treatment. However, the epithelial markers showed no apparent change despite TGF- β 1 treatment, while vimentin and α -SMA were slightly upregulated. These results demonstrated that Smad7 overexpression could maintain the epithelial phenotype, and could partly block TGF- β 1-induced EMT of RLE-6TN. These results also suggested that the expression of EMT markers could be regulated in the levels of post-transcription and protein translation by different factors which remain unclear.

In addition to the primary action of activating the Smad-signaling pathways, TGF- β 1-induced EMT also activated non-Smad signaling pathways, including Rho kinase, Ras, extracellular signal-related kinase (ERK), p38 mitogen-activated protein kinase (MAPK), Notch, Wnt, NF- κ B, and PI3K, which could all affect the extent and reversibility of EMT. Our findings revealed that the expression of phosphorylated Smad2 or Smad3 of RLE-6TN was upregulated markedly in response to TGF- β 1. However, following Smad7 transferring to RLE-6TN, TGF- β 1 had no effect on the expression of phosphorylated Smad2 or

Smad3, which indicated that overexpression of Smad7 could inhibit TGF- β 1-induced cellular Smad proteins, thus blocking the transcription of EMT-associated targeted genes. Our study revealed that TGF- β 1 could induce the increase of mRNA and protein expression of the EGFR of RLE-6TN and ST1, and that the EGFR signaling pathway is probably involved in the process of TGF- β 1-induced EMT.

CONCLUSION

The results of our study suggest that TGF- β 1 induces alveolar epithelial mesenchymal transition *in vitro*, partly through the activation of the Smads signaling pathway. However, the overexpression of Smad7 can block this transition to some extent. Moreover, the process of EMT may involve other non-Smad signaling pathways. The relationships between the different cellular signaling pathways should be further investigated in the future so as to identify the possible mechanism of pulmonary fibrogenesis for effective therapy.

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