

Rho/Rock cross-talks with transforming growth factor- β /Smad pathway participates in lung fibroblast-myofibroblast differentiation

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Abstract. The differentiation of fibroblasts, which are promoted by transforming growth factor- β (TGF- β)/Smad, is involved in the process of pulmonary fibrosis. The Rho/Rho-associated coiled-coil-forming protein kinase (Rock) pathway may regulate the fibroblast differentiation and myofibroblast expression of α -smooth muscle actin (α -SMA), however, the mechanism is not clear. The aim of the present study was to evaluate the role of Rho/Rock and TGF- β /Smad in TGF- β 1-induced lung fibroblasts differentiation. Human embryonic lung fibroblasts were stimulated by TGF- β 1, Y-27632 (inhibitor of Rho/Rock signaling) and staurosporine (inhibitor of TGF- β /Smad signaling). The α -SMA expression, cell cycle progression, content of the extracellular matrix (ECM) in cell culture supernatants and the expression of RhoA, RhoC, Rock1 and Smad2 were detected. The results demonstrated that α -SMA-positive cells significantly increased following TGF- β 1 stimulation. Rho/Rock and TGF- β /Smad inhibitors suppressed TGF- β 1-induced lung fibroblast differentiation. The inhibitors increased G₀/G₁ and decreased S and G₂/M percentages. The concentrations of the ECM proteins in the supernatant were significantly increased by TGF- β 1 stimulation, whereas they were decreased by inhibitor stimulation. RhoA, RhoC, Rock1, Smad2 and tissue inhibitor of metalloproteinase-1 were upregulated by TGF- β 1 stimulation. The Rho/Rock inhibitor downregulated Smad2 expression and the TGF- β /Smad inhibitor downregulated RhoA, RhoC and Rock1 expression. Therefore, the Rho/Rock pathway and

Smad signaling were involved in the process of lung fibroblasts transformation, induced by TGF- β 1, to myofibroblasts. The two pathways may undergo cross-talk in the lung fibroblasts differentiation *in vitro*.

Introduction

Pulmonary fibrosis is a fatal disease characterized by chronic inflammation and excessive collagen accumulation in the lung, with an unknown pathogenesis (1). Although a number of scientific advances have been made, no definitive and effective drug treatment is available that could improve, or at least inhibit, the progressive course of this disease (2). Therefore, identifying the molecular mechanism and effective molecular targets are important for preventive and therapeutic interventions.

Recent studies have demonstrated that fibroblast-myofibroblast differentiation, induced by transforming growth factor- β (TGF- β)/Smad, was involved in the etiology of pulmonary fibrosis (1,3). Myofibroblasts are the primary collagen-producing cells in fibrosis, which were commonly identified by the expression of α -smooth muscle actin (α -SMA) and intermediate features between the smooth muscle cells and the fibroblasts. In addition to the TGF- β /Smad signaling pathway, the Rho/Rho-associated coiled-coil-forming protein kinase (Rock) system has been shown to play critical roles in organ fibrosis (4,5). The Rho/Rock signaling pathway has been shown to regulate myofibroblasts α -SMA expression and its inhibitors improved the degree of organic fibrosis (6-8).

Whether the Rho/Rock system was involved in the differentiation of lung fibroblasts and interacted with TGF- β /Smad pathway remains unclear. In the present study, human embryonic lung fibroblasts were stimulated by TGF- β 1, Y-27632 and staurosporine. Cell cycle progression, expression of the extracellular matrix (ECM), RhoA, RhoC, Rock1 and Smad2 were evaluated to determine the roles and molecular mechanisms of the TGF- β /Smad and the Rho/Rock signaling pathway in the process of pulmonary fibrosis.

Materials and methods

Cell culture. WI-38 human embryonic lung fibroblasts were provided by the Health Department of Dalian Medical

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University (Dalian, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA, USA), supplemented with penicillin/streptomycin (100 U/ml) and L-glutamine (2 mmol/l) with 10% fetal calf serum (FCS; Gibco-BRL) under conditions of humidified 5% CO₂ at 37°C. When cell confluence reached 70-80%, the medium was replaced with serum-free DMEM for 12 h. The cells were divided into four groups, which were the normal, TGF-β1, inhibitor of Rho/Rock (Y-27632) and inhibitor of TGF-β/Smad (staurosporine) groups. The cells in each group were cultured in DMEM with 10% FCS and stimulated for 48 h with empty vehicle, human recombinant TGF-β1 (5 ng/ml; Sigma-Aldrich, St. Louis, MO, USA), Y-27632 (1 μmol/l; Sigma-Aldrich) and TGF-β1 (5 ng/ml), staurosporine (5 nmol/l; Sigma-Aldrich) and TGF-β1 (5 ng/ml), respectively. All the experiments were repeated 3 times.

Assessment of α-SMA-positive cell percentage by flow cytometry (FCM). The cells were collected and the concentrations were adjusted to 1x10⁶ cells/ml. The cells were permeabilized by adding 500 μl Permeabilization Solution (BD Bioscience, Bedford, MA, USA) to 100 μl of cell suspension for 10 min. Subsequent to washing with phosphate-buffered saline (PBS), the supernatant was discarded and the cell pellets were resuspended in 100 μl PBS with or without 1 μl fluorescein isothiocyanate (FITC)-labeled α-SMA monoclonal antibody (mAb; 1:100). Following incubation in the dark for 30 min at room temperature, the cells were washed with PBS and the supernatant was discarded. The cell pellets were resuspended in 500 μl PBS and analyzed using a flow cytometer (excitation light 488 nm, emission light 530 nm; BD Bioscience). All the data were analyzed with CellQuest software (BD Bioscience).

Staining for α-SMA by immunofluorescence. The cells were cultured on coverslips, washed twice with cold PBS and fixed in cold acetone for 10 min. Subsequent to thoroughly washing with PBS at room temperature 3 times, the cells were blocked with non-immune animal serum for 30 min. The slides were incubated with FITC-labeled α-SMA mAb (1:100) for 90 min at 37°C in a humidified chamber. The slides were visualized with a fluorescence microscope following washing with PBS.

Cell cycle analysis by FCM. The cells were collected and the cell pellets were fixed in 75% ethanol overnight at 4°C, and were washed twice with ice-cold PBS. Each cell pellet (1x10⁶) was resuspended in 2 μl RNase A and incubated at room temperature for 10 min. For each sample, 50 μl propidium iodide-staining solution was added, followed by incubation in the dark for 10 min at 4°C. The cell cycle profiles were determined for 2x10⁵ cells using CellQuest modfit software.

Type I collagen, laminin (LN), fibronectin (FN) and tissue inhibitor of metalloproteinase-1 (TIMP-1) protein expressions by ELISA. The cells were incubated in 6 wells at 1x10⁵ cells/ml in complete culture medium for 24 h, followed by serum-free DMEM for 12 h. After incubation for 48 h, the cell culture supernatant was collected for analysis of type I collagen (Southern Biotech, Birmingham, AL, USA), LN (Sigma-Aldrich), FN (Sigma-Aldrich) and TIMP-1 (Adlitteram

Diagnostic Laboratories, San Diego, CA, USA) using a sensitive ELISA kit.

RhoA, RhoC, Rock1, Smad2 and TIMP-1 mRNA expressions by quantitative polymerase chain reaction (qPCR). The total cellular RNA was isolated with TRIzol and mRNA was examined by qPCR according to the manufacturer's instructions for the Takara RNA PCR kit 3.0 (AMV; Takara Bio, Inc., Shiga, Japan). An equal amount of cDNA from each sample was amplified using primers specific to each gene (Table I). DNA amplification was performed using a thermocycler under the following conditions: For *RhoA*, *RhoC* and *Rock1*, 30 cycles of denaturation at 94°C for 30 sec, annealing at 51°C for 60 sec and extension at 72°C for 60 sec; for *Smad2*, 40 cycles of denaturation at 94°C for 45 sec, annealing at 51°C for 30 sec and extension at 72°C for 30 sec; for *TIMP-1*, 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 60 sec; and for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 35 cycles of denaturation at 94°C for 60 sec, annealing at 60°C for 60 sec and extension at 72°C for 60 sec. The relative expression of the target genes was analyzed by electrophoresis of the PCR products on 1.5% agarose gels by comparing the band density to endogenous *GAPDH*.

RhoA, RhoC, Rock1, Smad2 and TIMP-1 protein expressions by western blotting. The total cell proteins were extracted in lysis buffer containing protease inhibitor. The protein concentrations were measured using a protein assay reagent kit (Pierce Biotechnology, Inc., Rockford, IL, USA). A 7.5% polyacrylamide gel was used to resolve 20 μg of total protein by electrophoresis. Following electrophoresis, the separated proteins were transferred to polyvinylidene fluoride membranes (Pall Corporation, Port Washington, NY, USA). Subsequent to blocking, the membranes were incubated with primary antibodies (mouse monoclonal anti-TIMP-1 and anti-RhoA 1:600; goat polyclonal anti-RhoC 1:800; mouse monoclonal anti-Rock1 1:400; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; and rabbit polyclonal anti-p-Smad2 1:800; New England Biolabs, Ipswich, MA, USA) at 4°C overnight. The blots were washed and incubated with a horseradish peroxidase-conjugated affinity-pure rabbit anti-mouse immunoglobulin G (IgG) antibody (1:1,000), rabbit anti-goat IgG (1:3,000) and goat anti-rabbit IgG (1:3,000; Zhongshan Gold Bridge Biotech, Beijing, China) at 37°C for 1 h. The bands were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology) according to the manufacturer's instructions and blots were quantified by a densitometrical analysis that involved correcting for the background density of each gel. The membranes were re-probed for GAPDH (Proteintech Group, Inc., Chicago, IL, USA) to ensure equal loading.

Statistical analysis. Data analysis was performed using SPSS version 18.0 (SPSS, Chicago, IL, USA) statistical software. Data showed a normal distribution and are expressed as mean ± standard deviation. The results in different experimental groups were analyzed using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Table I. Oligonucleotide primers of the target genes.

mRNA species	mRNA	PCR product (bp)
<i>RhoA</i>		183
Sense	5'-CTGGTGATTGTTGGTGATGG-3'	
Antisense	5'-GCGATCATAATCTTCCTGCC-3'	
<i>RhoC</i>		181
Sense	5'-TCCTCATCGTCTTCAGCAAG-3'	
Antisense	5'-GAGGATGACATCAGTGTCCG-3'	
<i>Rock1</i>		701
Sense	5'-AGTCTGTGGCAATGTGTGAG-3'	
Antisense	5'-CTTCAAGCCGACTAACAGTG-3'	
<i>Smad2</i>		248
Sense	5'-TCTTGATGGTCGTCTCCAGGTA-3'	
Antisense	5'-GAGGCGGAAGTTCTGTTAGGAT-3'	
<i>TIMP-1</i>		652
Sense	5'-TTGAATCCCCACCATGGCCCCCTTTGAGCC-3'	
Antisense	5'-CTGAATTCGCAGGATTCAGGCTATCTG-3'	
<i>GAPDH</i>		448
Sense	5'-ACCACAGTCCATGCCATCAC-3'	
Antisense	5'-TCCACCACCCTGTTGCTGTA-3'	

PCR, polymerase chain reaction; bp, base pair; *Rock*, Rho-associated coiled-coil-forming protein kinase 1; *TIMP-1*, tissue inhibitor of metalloproteinase-1; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

Results

Results of α -SMA expression and staining. In the TGF- β 1 group, the percentage of α -SMA-positive cells increased significantly compared to the normal group ($P<0.05$). In the inhibitor of Rho/Rock and TGF- β /Smad groups, the percentage decreased significantly compared to the TGF- β 1 group ($P<0.05$) (Fig. 1A-D).

Immunofluorescence staining revealed that α -SMA was mainly expressed in cytoplasm. The expression was significantly increased following induction by TGF- β 1, but was suppressed by Y-27632 and staurosporine (Fig. 1).

Results of cell cycle progression. In the TGF- β 1 group, the cell population in the G₀/G₁ phase decreased significantly compared to the normal group ($P<0.05$), but increased significantly in the S phase ($P<0.05$). Following treatment with TGF- β 1 combined with Y-27632 and staurosporine, the cell population in the G₀/G₁ phase increased significantly compared to the TGF- β 1 group ($P<0.05$), but decreased significantly in the S ($P<0.05$) and G₂/M phase ($P<0.05$) (Table II).

Results of ELISA. ELISA showed that the concentrations of type I collagen, LN, FN and TIMP-1 in the cell culture supernatant in the TGF- β 1 group increased significantly compared to the normal group ($P<0.05$). In the inhibitor of Rho/Rock and TGF- β /Smad groups, the concentrations of these factors decreased significantly compared to the TGF- β 1 group ($P<0.05$) (Table III).

Table II. Cell cycle progression in different groups by flow cytometry.

Group	G ₀ /G ₁	S	G ₂ /M
Normal	67.33±4.82	14.97±1.68	17.70±1.98
TGF- β 1	57.53±3.78 ^a	25.16±2.12 ^a	17.31±2.04
Y-27632	72.48±6.31 ^b	18.87±1.38 ^b	8.65±1.07 ^b
Staurosporine	76.74±5.89 ^b	20.64±2.58 ^b	2.62±0.34 ^b

^a $P<0.05$ vs. normal group; ^b $P<0.05$ vs. TGF- β 1 group. Data are presented as %, mean \pm standard deviation. TGF- β 1, transforming growth factor- β 1.

Results of qPCR. The results of qPCR showed that the mRNA expression of *RhoA*, *RhoC*, *Rock1*, *Smad2* and *TIMP-1* in the TGF- β 1 group increased significantly compared to the normal group ($P<0.05$). Compared to the expression in the TGF- β 1 group, *RhoA*, *RhoC*, *Rock1*, *Smad2* and *TIMP-1* decreased significantly in the inhibitor of Rho/Rock group ($P<0.05$) and in the inhibitor of TGF- β /Smad group, except for the *RhoC* expression (Fig. 2).

Results of western blotting. Furthermore, the protein expressions of RhoA, RhoC, Rock1, Smad2 and TIMP-1 were detected using western blotting. The results demonstrated that the protein expression of these factors in the TGF- β 1 group increased significantly compared to the normal group ($P<0.05$). Compared to the TGF- β 1 group, RhoA, RhoC,

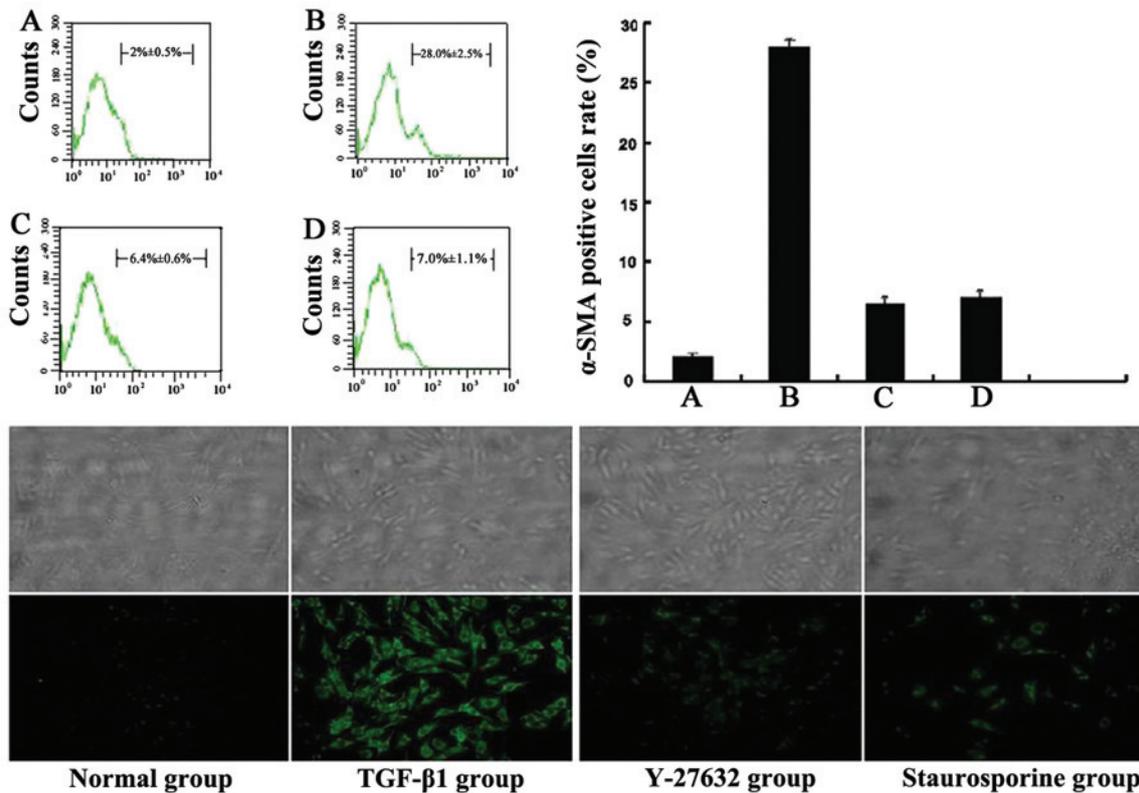


Figure 1. Percentage of α -smooth muscle actin (α -SMA)-positive cells. In the (B) transforming growth factor- β 1 (TGF- β 1) group, the percentage of α -SMA-positive cells increased significantly compared to the (A) normal group (28.0 ± 2.5 vs. $2.0 \pm 0.5\%$, $P < 0.05$). In the (C) inhibitor of Rho/Rho-associated coiled-coil-forming protein kinase and (D) TGF- β /Smad groups, the percentage decreased significantly compared to the TGF- β 1 group (6.4 ± 0.6 vs. 28.0 ± 2.5 and 7.0 ± 1.1 vs. $28.0 \pm 2.5\%$, respectively; $P < 0.05$). Immunofluorescence staining revealed that α -SMA was mainly expressed in cytoplasm. α -SMA was significantly increased following induction by TGF- β 1 (5 ng/ml), but was reduced by Y-27632 and staurosporine.

Rock1, Smad2 and TIMP-1 decreased significantly in the inhibitor of Rho/Rock group ($P < 0.05$) and in the inhibitor of TGF- β /Smad group, except for the RhoC expression (Fig. 3).

Discussion

Pulmonary fibrosis is a chronic, progressive, fibrosing interstitial pneumonia of unknown aetiology. The characteristics of the histopathology include fibroblast foci, interstitial fibrosis and honeycomb changes, which are generated due to fibroblast proliferation and excessive ECM deposition (9). Fibroblast proliferation and matrix deposition may be regulated by a number of mechanisms, such as epithelial-mesenchymal transition, expansion of a local fibroblast population and fibrocyte invasion (10).

In the presence of persisting injurious pathways, TGF- β can initiate the transformation of fibroblast and fibrocyte into myofibroblasts, which are resistant to apoptosis. The resulting deposition of excessive ECM by these myofibroblasts causes the development of pulmonary fibrosis (11). Once fibroblasts become activated, they transform into α -SMA-expressing myofibroblasts that secrete ECM components, factors promoting mesenchymal fibrosis and they induce alveolar epithelial cells apoptosis (12).

The FCM and immunofluorescence results showed that the percentage of α -SMA-positive cells increased significantly following stimulation by TGF- β 1 compared to the normal group. In the inhibitor of TGF- β /Smad group, the percentage

decreased significantly compared to the TGF- β 1 group. These results indicate that TGF- β 1 was involved in regulating fibroblasts activation. The cell cycle progression FCM results showed that the cell population in the G_0/G_1 phase decreased, the S phase increased by TGF- β 1 stimulation, but the G_0/G_1 phase increased and the S phase decreased following inhibition by the TGF- β /Smad pathway. ELISA and western blotting showed that the concentrations of type I collagen, LN, FN and TIMP-1 in the cell culture supernatant of the TGF- β 1 group increased significantly compared to the normal group. In the inhibitor of TGF- β /Smad group, the concentrations of these factors decreased significantly compared to the TGF- β 1 group. The results demonstrated that TGF- β 1 plays important roles in regulating fibroblasts differentiation and proliferation, ECM synthesis and degradation through TGF- β 1/Smad pathway.

In addition to TGF- β 1/Smad, several signaling pathways were involved in the pathogenesis of pulmonary fibrosis. Previous studies have found that the fibrosis organ had abnormal expressions of Rho and Rock, and inhibitors of Rock could improve the models of organ fibrosis (13-15), indicating that the Rho/Rock-mediated pathway may play a role in pulmonary fibrosis. Rho, the small GTPase, and its target protein, Rock, have been identified as major regulators of cell locomotion mediated by reorganization of the actin cytoskeleton. Activated Rock inhibits myosin phosphatase and this in turn induces phosphorylation of the myosin light chain (MLC).

Table III. Expression of type I collagen, laminin (LN), fibronectin (FN) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the cell culture supernatant (mean ± standard deviation).

Group	Type I collagen (ng/ml)	LN (ng/ml)	FN (pg/ml)	TIMP-1 (μg/ml)
Normal	193.47±19.37	9.28±0.64	23.93±7.16	4.75±0.86
TGF-β1	309.29±13.10 ^a	16.68±1.97 ^a	50.60±8.25 ^a	9.32±1.56 ^a
Y-27632	230.88±11.05 ^b	11.72±1.01 ^b	33.23±8.69 ^b	5.23±0.76 ^b
Staurosporine	221.43±15.24 ^b	12.46±1.84 ^b	29.54±8.02 ^b	5.08±0.92 ^b

^aP<0.05 vs. normal group; ^bP<0.05 vs. TGF-β1 group. TGF-β1, transforming growth factor-β1.

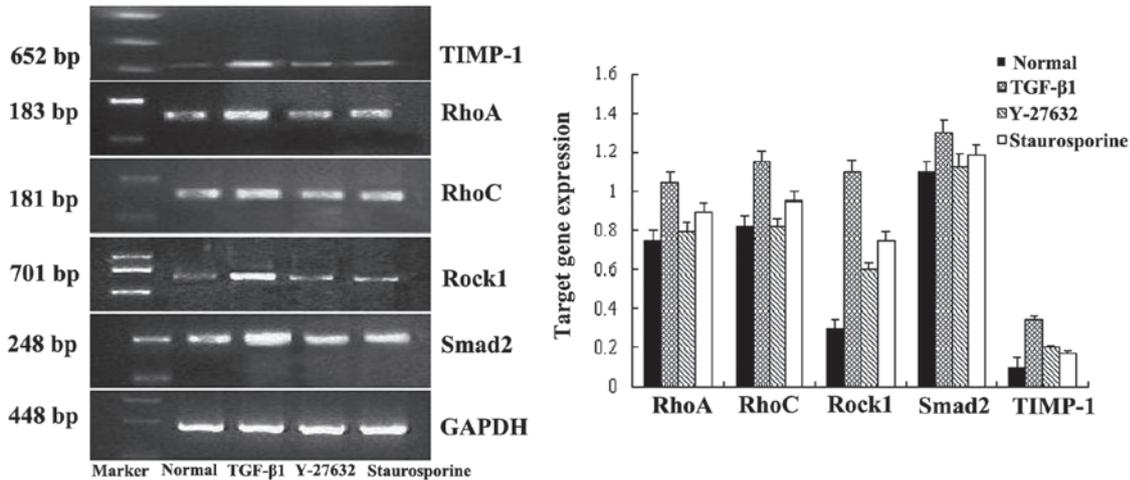


Figure 2. Quantitative polymerase chain reaction showing the expressions of *RhoA*, *RhoC*, Rho-associated coiled-coil-forming protein kinase (*Rock*) 1, *Smad2* and tissue inhibitor of metalloproteinase-1 (*TIMP-1*) in the transforming growth factor-β1 (TGF-β1) group increased significantly compared to the normal group (P<0.05). Compared to the expressions in the TGF-β1 group, *RhoA*, *RhoC*, *Rock1*, *Smad2* and *TIMP-1* decreased significantly in the inhibitor of Rho/Rock (Y-27632) group (P<0.05) and in the inhibitor of TGF-β/Smad (staurosporine) group, except for *RhoC* expression. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

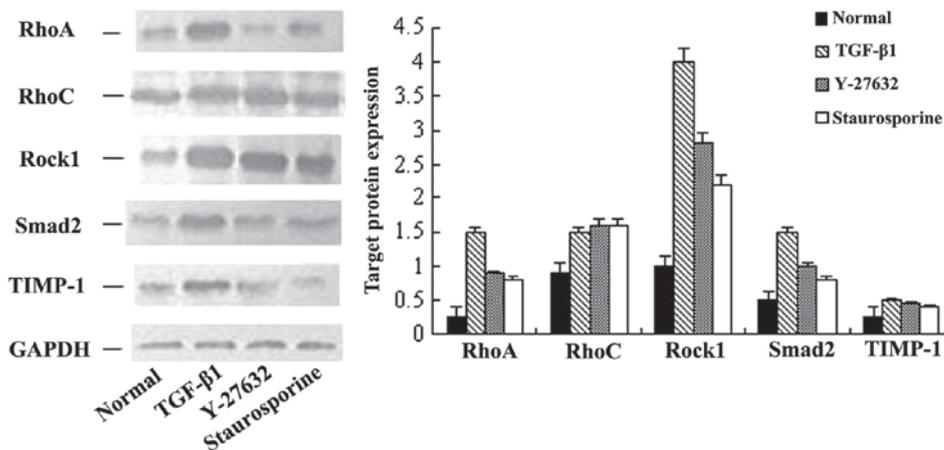


Figure 3. Protein expressions of *RhoA*, *RhoC*, Rho-associated coiled-coil-forming protein kinase (*Rock*) 1, *Smad2*, tissue inhibitor of metalloproteinase-1 (*TIMP-1*) detected by western blotting. The results showed that the protein expression of these factors in the transforming growth factor-β1 (TGF-β1) group increased significantly compared to the normal group (P<0.05). Compared to the TGF-β1 group, *RhoA*, *RhoC*, *Rock1*, *Smad2* and *TIMP-1* decreased significantly in the inhibitor of Rho/Rock (Y-27632) group (P<0.05) and the same in the inhibitor of TGF-β/Smad (staurosporine) group, except for *RhoC* expression. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

In the present study, Y-27632, an inhibitor of the Rho/Rock signaling pathway, was used to observe whether the pathway was involved in the development of fibroblast proliferation and excessive ECM deposition.

Following stimulation by TGF-β1, the lung fibroblasts expression of *RhoA*, *RhoC*, *Rock1* increased, but was decreased when fibroblasts were treated with TGF-β/Smad and the Rho/Rock inhibitor. In the TGF-β1-stimulated fibroblasts,

FCM and immunofluorescence showed the percentage of α -SMA-positive cells decreased following Y-27632 treatment. The cell cycle progression results indicated that Y-27632 prevented the proliferation of fibroblasts induced by TGF- β 1. The concentrations of type I collagen, LN, FN and TIMP-1 in the TGF- β 1-stimulated fibroblasts culture supernatant were reduced due to the inhibition of the Rho/Rock pathway by Y-27632.

The aforementioned results indicated that the Rho/Rock pathway was involved in lung fibroblast proliferation, differentiation and excessive ECM deposition.

According to the study by Shimizu *et al* (13), the Rho/Rock-mediated pathway may contribute to the development of pulmonary fibrosis, and furthermore, Y-27632 inhibited the Rock function at protein levels, resulting in inhibition of muscle and non-muscle MLC 20 phosphorylation. Multiple smooth muscle cell (SMC)-specific differentiation marker genes are regulated by RhoA-induced changes in the actin cytoskeleton. RhoA activity is required for SMC-specific promoter activity as C3 transferase, which ADP ribosylates and irreversibly inactivates RhoA, completely inhibited the activities of the SM22 and α -SMA promoters (16). Y-27632 can inhibit promoter activity, which may be the mechanism for the inhibition of the TGF- β 1-stimulated fibroblast transformation to myofibroblasts.

Excessive ECM deposition was reduced due to Y-27632, which is partly attributed to the decreased amounts of myofibroblasts. In addition, the Rho/Rock pathway was involved in ECM synthesis and decomposition by the mechanisms of the TGF- β /Smad pathway, dependently or independently (17,18).

In the present study, staurosporine reduced the expression of RhoA and Rock1, which were the key factors in Rho/Rock pathway, and Y-27632 reduced the expression of Smad2, which was a critical factor in the TGF- β /Smad pathway. The results indicated that there may have been cross-talk between Rho/Rock and the TGF- β /Smad pathway in the process of lung fibroblasts transforming into myofibroblasts. A previous study has demonstrated that the dominant-negative RhoA or the Rock inhibitor blocked the nuclear translocation of Smad2 and Smad3 due to Smad-phosphorylation inhibition and inhibition of the Smad-dependent Smad binding elements (SBE) promoter activity, whereas constitutively active RhoA significantly enhanced the SBE promoter activity (19). The study by Itoh *et al* (20) indicated that the Rho/Rock pathway plays a role in relaying TGF- β signal transduction to ECM synthesis in retinal pigment epithelial cells in a Smad-dependent and Smad-independent way. Therefore, the results showed that Smad signaling cross-talked with the Rho/Rock pathway during the process of lung fibroblasts differentiation induced by TGF- β 1.

In conclusion, the Rho/Rock pathway and Smad signaling were involved in lung fibroblasts transforming to myofibroblasts induced by TGF- β 1. The two pathways may undergo cross-talk in the lung fibroblasts differentiation *in vitro*. The specific cross points between the two pathways may be the therapeutic targets for treating the disease and the process of pulmonary fibrosis could be more effective.

Acknowledgements

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