Original Article

Role of Insulin-like Growth Factor II Receptor in Transdifferentiation of Free Silica-induced Primary Rat Lung Fibroblasts*

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Abstract

Objective To study the role of insulin-like growth factor II receptor in free silica-induced transdifferentiation of primary rat lung fibroblasts

Methods Rat lung fibroblasts and rat alveolar macrophages were cultured. A transdifferentiation model of primary rat lung fibroblasts was induced by free silica. Levels of α-SMA protein, IGF-IIR protein and mRNA were measured by immunocytochemistry, Western blot and RT-PCR, respectively. Lung fibroblasts were treated with Wortmannin.

Results The expression levels of α-SMA and IGF-IIR increased with the increasing free silica concentration and decreased after Wortmannin was used.

Conclusion The IGF-IIR plays an important role in free silica-induced transdifferentiation of primary rat lung fibroblasts.

Key words: Transdifferentiation; Lung fibroblasts; Insulin-like growth factor II receptor; Silicosis

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INTRODUCTION

Pulmonary fibrosis is a refractory lung disease characterized by progressive and irreversible destruction of lung architecture that leads to organ malfunction, disruption of gas exchange, and death from respiratory failure. Silicosis is induced by long-term inhalation of air-born dusts containing crystalline silica, in which lung myofibroblasts secrete a large amount of extracellular matrix (ECM) components, such as collagens.

Silica-stimulated macrophages can secrete a lot of cytokines, such as TGF-β_1, TNF-α, and PDGF[1-2]. These cytokines, especially TGF-β_1, induce normal lung fibroblasts to express α-smooth muscle actin (α-SMA), a typical cell marker of lung myofibroblasts[3-4] and secrete extracellular matrix (ECM) components, such as collagens mainly deposited in lung[5-9]. Upon this occasion, lung fibroblasts are transformed to lung myofibroblasts, which is a type of transdifferentiation. Transdifferentiation takes place when a non-stem cell is transformed into a different type of cells or when differentiated stem cells create a differentiation path for the cells outside them. Myofibroblasts secrete ECM and are rapidly proliferated, during which TGF-β_1 plays a key role[10-11].

Insulin-like growth factor type 2/mannose-6-phosphate receptor (IGF-IIR/M6P), a multifunctional

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transmembrane glycoprotein known to regulate diverse biological functions, is a patent therapy target for some diseases, such as lysosomal diseases and cancer, can mediate uptake and processing of M6P-containing cytokines and peptide hormones, such as transforming growth factor-beta, and plays an important role in activation of latent transforming growth factor β (LTGF-β) to active TGF-β, a critical step in the healing process[12-13].

There is evidence that the mannose-6-phosphate/Insulin-like growth factor-II receptor (M6P/IGF-IIR) is up-regulated in isolated culture-activated hepatic satellite cells[14]. IGF-IIR is aberrantly expressed in myocardial infarction scars and cardiomyoblasts[15], which shows that IGF-IIR is a key factor for organ fibrosis. However, no report is available on its role of IGF-IIR in silicosis. In this study, the expression of IGF-IIR in free silica-induced transdifferentiation of lung myofibroblasts was detected and IGF-IIR as a potential therapy target was discussed.

MATERIALS AND METHODS

Chemicals and Reagents

Crystalline silica (99% of particle size less than 5 μm) was obtained from the National Institute of Occupational Health and Poison Control of China, sterilized at 180 °C for 1 h and suspended in DMEM media at the concentration of 100 μg/mL. Wortmannin (98%) was from Acros.

Culture of Rat Lung Fibroblasts

Lung fibroblasts (LF) used *in vitro* experiments were isolated from SPF male SD rats (obtained from the Laboratory Animal Research Center of Zhengzhou University) by two steps of trypsin digestion as previously described. Briefly, lung tissue was separated from the main airways of adult SD rats under sterile conditions. The explants were minced, washed 3 times with D-Hank's solution, and disaggregated with crude bovine pancreatic trypsin. The resulting cell population was resuspended in DMEM (Olarbio Science and Technology, Beijing, China) containing 10% heat-inactivated fetal calf serum (Sijiqing, Hangzhou, China), 100 U/mL penicillin and 100 μg/mL streptomycin. Approximate 1.0×10^6 cells were planted in each of serum dilution bottles with a total media volume of 10 mL. The cells were incubated in 5% CO_2 at 37 °C with the media changed 3 times weekly. The cells were trypsinized and replated after about 5 days of culture.

Culture of Rat Alveolar Macrophages

Rat alveolar macrophages (AM) were collected from the SD rats [Henan Experimental Animal Center by bronchoalveolar lavage (BAL)]. After the rats were euthanized with a pentobarbital overdose, they underwent thoracotomy with their thoracic cavity exposed, the pulmonary vasculature perfused with cold sterile D-Hank's solution via the pulmonary artery, the trachea surgically exposed and cannulated with a blunt 16-gauge needle, the lungs gently inflated with about 10 mL cold isotonic D-Hank's solution. The saline was gently withdrawn from the lungs and the BAL fluid was placed in a 50 mL centrifuge tube. The BAL fluid was centrifuged (800 r/min) to pellet AM which were resuspended in DMEM+10% FCS. The number of AM was counted with a hemocytometer and incubated in 5% CO_2 at 37 °C for 2 h. The medium was then changed with the uncoated cells washed.

Treatment of AM

Crystalline SiO_2 particles were heated at 180 °C for 1 h and diluted in DMEM medium with the concentration of 100 μg/mL into which. 1 μg/cm^2, 5 μg/cm^2, and 10 μg/cm^2 of SiO_2 were added. The AM were incubated with SiO_2 for 24 h. The culture media were collected and centrifuged (1 500 r/min) to remove silica.

Lung fibroblasts were trypsinized and their number was counted with a hemacytometer. The medium was changed by reducing the FCS content in the culture medium and placed into a quiescent state for 24 h. The cells were cultured in AM supernatant.

The FB was cultured in the supernatant of AM at the concentration of 10 μg/cm^2, into which the wortmannin was added at the concentrations of 1 μmol/L, 5 μmol/L, and 50 μmol/L.

Total RNA Extraction and RT-PCR

Total RNA was extracted with the tissue/cell RNA isolation system (Biomed, Beijing, China) according to its manufacturer's instructions. RNA precipitate was washed twice with 75% ethanol by gentle vortexing, centrifuged at 12 000 g, dried under vacuum for 5-10 min, dissolved in 50 μL diethylpyrocarbonate-treated water, and incubated at 55-60 °C for 10-15 min. cDNA was prepared with cDNA first strand synthesis kit (TIANGEN, Beijing,

China) according to its manufacturer's instructions. Thirty-five cycles of were performed. For each cycle, the sample was amplified at 95 °C for 5 min, denatured at 54 °C for 45 s, annealed at 54 °C for 1 min, extended at 72 °C for 45 s and finally extended at 72 °C for 10 min. The RNA samples were demonstrated to have intact 18S and 28S RNA bands on ethidium bromide-strained formaldehyde- agarose gels. The sequences are CCGAGATCTCACCGACTACC and TCCAGAGCGA CATAGCACAG for α-SAM forward and reverse primer, ATGTTCCCTCTCTCTGGCTGT and TCACATTGGTAGCAAATGGTC for IGF-IIR forward and reverse primer, and GGTGCTGAGTATGTCGTGGAGT and CAGTCTTCTGAGTGGCAGTGAT for rat GAPDH forward and reverse primer (SBS Genetech, Beijing, China).

Immunocytochemistry

Lung fibroblasts were cultured on glass culture slides. After growth arrest, the cells were stimulated with test substances for 24 h, washed in D-Hank's buffer, fixed in 20% acetone, and stored at 4 °C. The slides were detected with a monoclonal mouse anti-rat smooth muscle actin antibody (BOSTER, Wuhan, China) and a poloclonal goat antibody against IGF-IIR (Santa Cruz Company, USA) for 2 h at the concentration of 1:500 in a humid chamber containing 1% BSA at room temperature. Nonspecific staining was controlled by omitting primary antibodies and including rat and mouse non-immune serum. The secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse IgG (Zhongshan, Beijing, China) and horseradish peroxidase-conjugated rabbit anti-goat IgG (Zhongshan, Beijing, China) were incubated for 1 h at the concentration of 1:1 000 in 1% BSA at room temperature. The slides were washed with D-Hank's buffer and stained with DAB (Zhongshan, Beijing, China). Negative controls were incubated with secondary antibodies alone. The score was evaluated as previously described[21].

Protein Isolation and Western Blotting

The dissected fibroblast samples were homogenized in lysis buffer containing 2% SDS (Shanghai Solarbio Bioscience & Technology Company, China), 10% glycerol (Shanghai Solarbio Bioscience & Technology Company, China), 2% 2-mercaptoethanol (Solarbio, China), and 0.002% bromphenol blue (Shanghai Solarbio Bioscience & Technology Company, China) in 75 mmol/L Tris-HCl (Shanghai Solarbio Bioscience & Technology Company, China), and heated at 95 °C for 10 min before separation on 10% Tris/Glycine/SDS acrylamide gels (Bio-Rad Company, USA). The proteins were subsequently transblotted to polyvinylidene difluoride (Shanghai Solarbio Bioscience & Technology Company, China) membranes, blocked in 3% BSA for 1 h at room temperature and incubated with poloclonal goat antibody against IGF-IIR (Santa Cruz Company, USA) at 37 °C for 2 h, washed 3 times with TBS/0.05% Tween-20 and incubated with a horseradish peroxidase-conjugated rabbit anti-goat antibody (Zhongshan, Beijing, China) at 37 °C for 1 h. Protein signal was visualized with the Super Signal West Pico Chemiluminescent substrate (PIERCE Company, USA) and detected with the imaging system (Syngene Company, USA). β-actin protein was visualized and detected in the same way.

Statistical Analysis

All data are expressed as mean±SD. The differences of IGF-IIR in mRNA and protein levels of fibroblasts were analyzed by one-way ANOVA and a post hoc Bonferroni's test with SPSS 12.0 software. $P<0.05$ is considered statistically significant.

RESULTS

The cytoplasm was turned into tan-yellow after staining (Figure 1). The α-SMA was lowly expressed in FB without AM supernatant and highly expressed in FB treated with 1 μg/cm^2, 5 μg/cm^2, and 10 μg/cm^2 silica (Table 1).

The cytoplasm became tan-yellow after staining (Figure 2). The IGF-IIR was lowly expressed in FB without AM supernatant and highly expressed in FB treated with 1 μg/cm^2, 5 μg/cm^2, and 10 μg/cm^2 silica. The expressions of IGF-IIR mRNA and protein after treatment with silica are shown in Figures 3 and 4.

Table 1. Immunocytochemistry Showing SMA Protein Level in Lung Fibroblasts (mean±SD)

SiO_2 (μg/cm^2)	*N*	α-SMA
Normal lung fibroblasts	3	0.567±0.075
0	3	0.910±0.060*
1	3	1.140±0.036*
5	3	1.253±0.093*
10	3	1.517±0.029*
F		96.797
P		<0.001

Note. *$P<0.05$ *vs* normal lung fibroblasts.

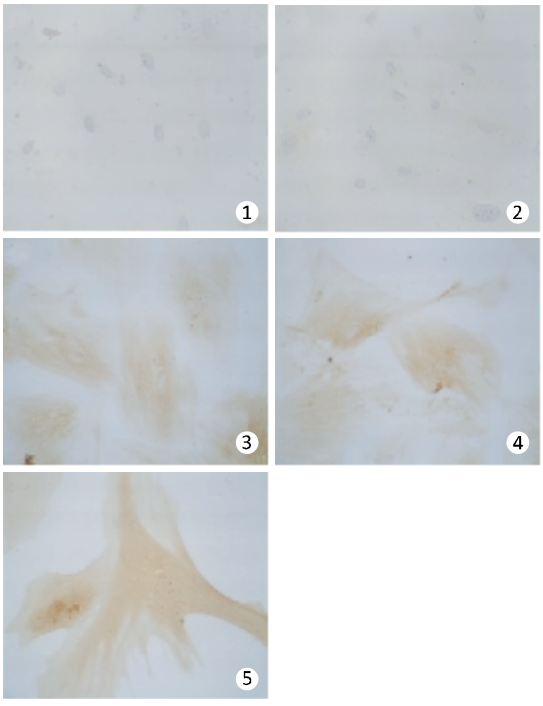

Figure 1. Immunocytochemistry showing expression of SMA of protein in lung fibroblasts (SP, 400×). 1: Normal lung fibroblasts; 2: 0 μg/cm^2 SiO_2; 3: 1 μg/cm^2 SiO_2; 4: 5 μg/cm^2 SiO_2; 5: 10 μg/cm^2 SiO_2

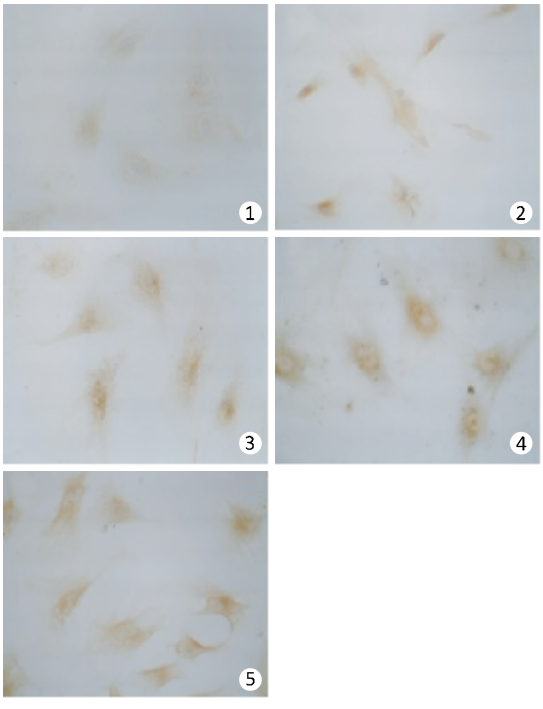

Figure 2. Immunocytochemistry showing expression of IGF-IIR protein in lung fibroblasts (SP, 400×). 1: Normal lung fibroblasts; 2: 0 μg/cm^2 SiO_2; 3: 1 μg/cm^2 SiO_2; 4: 5 μg/cm^2 SiO_2; 5: 10 μg/cm^2 SiO_2

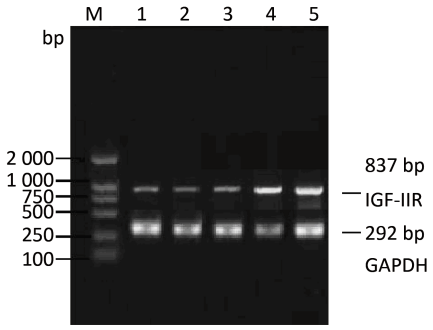

Figure 3. Expression of IGF-IIR mRNA in lung fibroblasts. M: marker; 1: Normal lung fibroblasts; 2: 0 μg/cm^2 SiO_2; 3: 1 μg/cm^2 SiO_2; 4: 5 μg/cm^2 SiO_2; 5: 10 μg/cm^2 SiO_2.

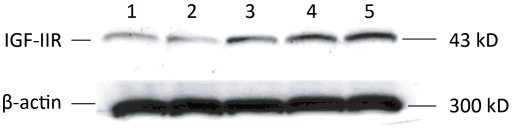

Figure 4. Expression of IGF-IIR protein in lung fibroblasts. 1: Normal lung fibroblasts; 2: 0 μg/cm^2 SiO_2; 3: 1 μg/cm^2 SiO_2; 4: 5 μg/cm^2 SiO_2; 5: 10 μg/cm^2 SiO_2.

The expression levels of IGF-IIR mRNA and protein were higher in 1, 5, and 10 μg/cm^2 SiO_2 than in normal lung fibroblasts (P<0.05, Table 2).

The α-SMA expression level was significantly lower in fibroblasts treated with wortmannin than in those not treated with it (P<0.05, Figure5, Table 3).

The expression level of IGF-IIR mRNA and protein was significantly lower in fibroblasts treated with wortmannin than in those not treated with it (P<0.05, Figures 7 and 8, Table 4).

DISCUSSION

Pulmonary fibrosis is a highly heterogeneous and lethal pathological process[16]. At present, pulmonary fibrosis cannot be reversed. Its mortality is rather high. A number of drugs can delay or inhibit pulmonary fibrosis, such as corticosteroids, immunosuppressive agents, cytokines and growth factor antagonists or inhibitors, antioxidants, anti-inflammatory agents and protease inhibitors[17].

It is known that overexpression of myofibroblasts is critical in pulmonary fibrosis. However, no studies are available on the transdifferentiation of myofibroblasts. α-SMA and collagens are the cell markers of myofibroblasts. Since α-SMA is expressed earlier in rat lung fibroblasts than in collagens, α-SMA was used as the cell marker to identify the transdifferentiation of rat

Table 2. Immunocytochemistry Showing IGF-IIR mRNA and Protein Levels in Lung Fibroblasts (mean±SD)

SiO_2 (μg/cm^2)	*N*	RNA	Protein	
			Immunocytochemistry	Western Blot
Normal lung fibroblasts	3	0.153±0.035	0.153±0.035	0.567±0.075
0	3	0.130±0.040	0.467±0.095*	0.487±0.021
1	3	0.533±0.095*	0.533±0.095*	0.733±0.083*
5	3	0.963±0.074*	0.963±0.074*	1.253±0.093*
10	3	1.597±0.086*	1.597±0.086*	1.517±0.029*
F		231.673	145.359	135.904
P		<0.001	<0.001	<0.001

Note. *P<0.05 *vs* normal lung fibroblasts.

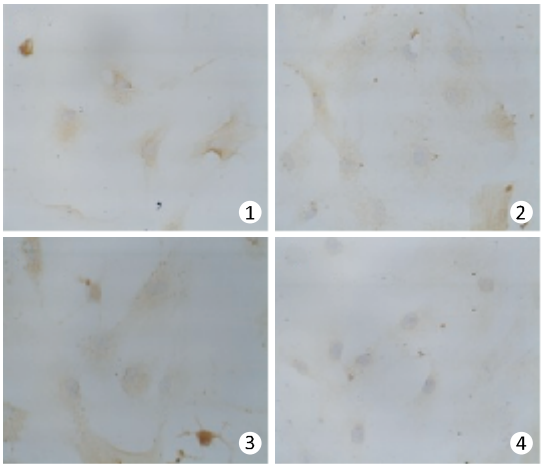

Figure 5. Immunocytochemistry showing expression of SMA protein in lung fibroblasts treated with wortmannin (SP, 400×). 1: 0 μmol/L; 2: 1 μmol/L; 3: 5 μmol/L; 4: 50 μmol/L.

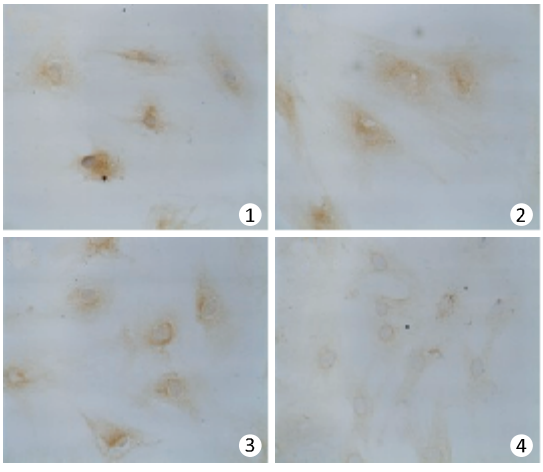

Figure 6. Immunocytochemistry showing expression of IGF-IIR protein in lung fibroblasts treated with wortmannin (SP, 400×). 1: 0 μmol/L; 2: 1 μmol/L; 3: 5 μmol/L; 4: 50 μmol/L.

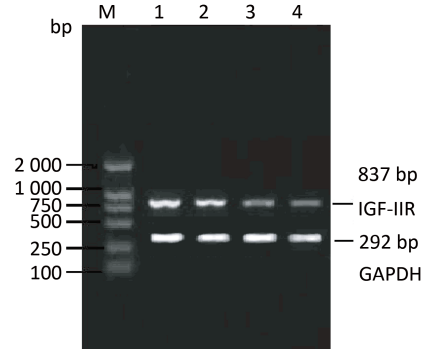

Figure 7. Expression of IGF-IIR mRNA in lung fibroblasts treated with wortmannin. M. DNA marker; 1: 0 μmol/L; 2: 1 μmol/L; 3: 5 μmol/L; 4: 50 μmol/L.

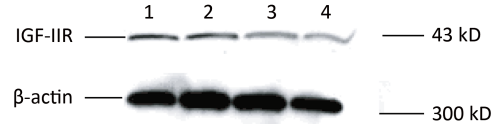

Figure 8. Expression of IGF-IIR protein in lung fibroblasts treated with wortmannin. 1: 0 μmol/L; 2: 1 μmol/L; 3: 5 μmol/L; 4: 50 μmol/L.

Table 3. Immunocytochemistry Showing fSMA and IGF-IIR Protein Level in Lung Fibroblasts Treated with Wortmannin (mean±SD)

Wortmannin (μmol/L)	*N*	α-SMA
0	3	0.601±0.024
1	3	0.482±0.006*
5	3	0.482±0.007*
50	3	0.370±0.003*
F		159.360
P		<0.001

Note. *Contrast to Group 0, P<0.05.

Table 4. IGF-IIR mRNA and Protein Levels in Lung Fibroblasts Treated with Wortmannin (mean±SD)

Wortmannin (μmol/L)	*N*	RNA	Protein	
			Immunocytochemistry	Western Blot
0	3	2.998±0.225	0.419±0.033	2.158±0.133
1	3	1.881±0.331	0.327±0.038*	1.824±0.214
5	3	1.360±0.247*	0.326±0.041*	1.275±0.122*
50	3	0.832±0.062*	0.215±0.012*	1.055±0.112*
F		45.683	19.182	33.807
P		<0.001	<0.001	<0.001

Note. *$P<0.05$ *vs* group 0.

lung fibroblasts in this study, which showed that the expression level of α-SMA increased with the increasing silica concentration, indicating that lung fibroblasts are transdifferentiated to myofibroblasts. The activity of myofibroblasts was increased, which is consistent with that reported in previous studies[18-19]. Moreover, the expression of IGF-IIR was similar to that of α-SMA. The expression levels of IGF-IIR mRNA and protein in myofibroblasts were also similar. Then, the lung fibroblasts were treated with wortmannin, an inhibitor of IGF-IIR before exposed to silica. Wortmannin inhibited the signals of IGF-IIR and PI3K, which can stimulate TGF-β_1[12]. The expression levels of IGF-IIR mRNA and protein and α-SMA were low. As TGF-β_1 plays a critical role in the transformation of lung fibroblasts into myofibroblasts[20-21], IGF-IIR is essential in the activation of latent transforming growth factor β (LTGF-β) to active TGF-β[12-13], indicating that IGF-IIR is related with transdifferentiation of lung fibroblast to myofibroblasts.

The IGF family can be synthesized and secreted by many tissues in humans and is a class of polypeptide with insulin-like anabolic effect and growth-promoting effect. It can also mediate GH to promote growth and metabolism of a variety of tissues and cells. IGF-IIR is a single chain transmembrane glycoprotein, consisted of a N-terminal signal sequence, a cytoplasm outside domain, a transmembrane domain and a cytoplasmic domain with C-terminal. IGF-IIR is also known as an IGF-IIR/ M6P receptor because it has M6P recognition binding sites and can combine with lysosome containing M6P. It was reported that pro-TGF-β cannot become activated TGF-β unless it is bonded to IGF-II/M6P[22]. Thereby, TGF-β activation can be inhibited if IGF-IIR is competitively occupied, which may further delay the progression of fibrosis. According to our results, a high level of IGF-IIR may promote TGF-β activation, thus increasing the TGF-β_1 level and inducing he transdiff-erentiation of lung fibroblasts. When the IGF-IIR is inhibited, the transdifferentiation is delayed. Although we did not measure the TGF-β_1 level in this study, previous reports support our results.

In conclusion, high IGF-IIR expression level in lung fibroblasts is closely related to their transdifferentiation. Effective inhibition of IGF-IIR can delay the transdifferentiation of lung fibroblasts and the progression of silicosis.

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