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Original Article

TRB3 regulates pulmonary interstitial fibrosis through the MAPK signaling pathway

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Abstract: This study aims to investigate the effects of TRB3 on the EMT and MAPK signaling pathways in a bleomycin (BLM)-induced pulmonary fibrosis mouse model. TRB3 adenovirus vector with green fluorescent protein (GFP) and TRB3-siRNA adenovirus vector were constructed for overexpression and down-regulation of TRB3, respectively. The pulmonary fibrosis mouse model was induced by bleomycin, and then treated with adenovirus on the next day. The mice were randomly killed at the 7th (D7), 14th (D14) and 28th (D28) day, respectively. The lung tissues were collected for histopathologic observations, hydroxyproline determination, Immunohistochemistry, western blot and RT-qPCR to detect the expression of TRB3 and EMT-related proteins. Overexpression of TRB3 caused more severe pulmonary fibrosis ($P<0.05$), while downregulation of TRB3 significantly reduced pulmonary fibrosis ($P<0.05$). The expression of MAPK pathway-related and EMT-related genes and proteins was markedly upregulated by TRB3 overexpression ($P<0.05$), but prominently downregulated by TRB3-shRNA ($P<0.05$). In conclusion, exogenous regulation of TRB3 may have effects on bleomycin-induced pulmonary fibrosis in mice.

Keywords: TRB3, MAPK signaling pathway, idiopathic pulmonary fibrosis, EMT

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, irreversible, and progressive lung disease. The prognosis of IPF is very poor, with a median survival time of 2-3 years after diagnosis [1]. Lung transplantation is the only way to cure IPF, but its clinical application is greatly limited by the limited donors and the timing of operation. Therefore, it is very important to explore the molecular mechanisms of the formation and development of pulmonary fibrosis, so as to find specific targets for treatment and to prevent or reverse the progression of fibrosis.

The mechanism of IPF mainly manifests as alveolar epithelial cell injury, a cascade of inflammatory reaction, phenotypic changes of alveolar epithelial cells, and the changes of secretory phenotypes in damaged epithelial cells, which produce proinflammatory factors and profibrotic factors that contribute to the healing of lung injury. Transforming growth factor-β1 (TGF-β1), as the most important profibrotic factor, induces fibroblast migration, proliferation, and differentiation of myofibroblasts, deposition of extracellular matrix (ECM) [2], and epithelial mesenchymal transformation (EMT) [3] to form a characteristic “fibroblast focus”, which further promotes abnormal repair after injury. Many studies have shown that EMT is a key step in fibrosis. It is also proven that EMT is a process controlled by a signaling pathway network [4], through which epithelial phenotype transforms into a phenotype of mesenchymal cells.

Mitogen activated protein kinases (MAPK) are a family of serine/threonine protein kinases, which are widely distributed in mammalian cells. This pathway plays important roles in the pathophysiology of a series of diseases such as inflammation, fibrosis, and tumor. The MAPK signaling pathway plays an important role in the fibrotic process of many major organs, such as myocardial fibrosis, renal fibrosis, and pulmonary fibrosis [5-7]. In IPF, there are three mitogen activated protein kinases (MAPKs) involved in the regulation of lung inflammation and inju-

ry, that is, extracellular regulated protein kinases (ERK), c-Jun N-terminal kinase (JNK), and p38 Mitogen Activated Protein Kinase (p38-MAPK) [7].

Tribbles homologue 3 (TRB3, also known as TRIB3, NIPK), a member of the Tribbles family, is a pseudokinase. Because of the lack of kinase activity, it does not directly regulate gene transcription but plays its role by indirectly assembling coactivating factors and auxiliary repressors for the molecular skeleton in cells by participating in the assembly of multiple protein complexes [8]. It has been shown that TRB3 regulates glycolipid metabolism, apoptosis, stress, and fibrosis through interaction with some proteins such as Smad3 [9], MAPK [10], PI3K and other signaling pathways. As a molecular switch, TRB3 can selectively regulate the activity of MAPK [9]. TRB3 can interact with MAPK kinase and regulate the relative activation of three types of MAPK [11]. It has been reported that the expression of TRB3 is significantly increased in myocardial and renal fibrosis [9, 10, 12], suggesting that TRB3 may play an important role in the regulation of multiple organ fibrosis. A role of TRB3 in the pathogenesis of pulmonary fibrosis has not yet been reported.

The purpose of this study was to explore the changes in the expression of MAPK signaling pathway components and EMT-related molecules in the bleomycin-induced pulmonary fibrosis mouse model regulated by overexpression or down-regulation of TRB3, and to further reveal the role of TRB3 in the pathogenesis of pulmonary fibrosis and its mechanism, so as to find new targets for the treatment of pulmonary fibrosis.

Materials and methods

Animals and establishment of pulmonary fibrosis model

Sixty healthy male C57BL/6 mice (6-8 weeks old, weighing about 20 g) were provided by the Experimental Animal Institutes of Chinese Academy of Medical Sciences, Beijing, China. The animals were randomly divided into 5 groups according to random number table method: control group (Group Mock, n=12), normal mice treated with physiological saline; Fibrosis control group (Group F, n=12), fibrotic mice treated with physiological saline; Fibrosis+GFP group (Group F+GFP, n=12), fibrotic mice treated with adenovirus vector with green fluorescent protein (GFP); Fibrosis+Ad-TRB3 overexpression group (Group F+TRB3, n=12), fibrotic mice treated with adenovirus vector overexpressed TRB3 with GFP; and Fibrosis+Ad-TRB3-shRNA interference group (Group F+Sh-TRB3, n=12), fibrotic mice treated with adenovirus vector with TRB3 Sh-RNA and GFP. Other than Group Mock, the animals in the other groups received endotracheal dripping of bleomycin A5 (3.5 mg/kg, sigma, Germany) to establish a mouse model of pulmonary fibrosis. On the second day after modeling, the corresponding adenovirus (5×10^9 PFU, Tianjin Saier Biotechnology Co., Ltd., China) was given to each mouse by caudal vein injection except the mice in Group Mock and Group F which were given the same dose of physiological saline with the same method. The mice were killed on the 7^{th} (D7), 14^{th} (D14) and 28^{th} (D28) day after treatment. The left lung tissue of each mouse was collected and fixed in 4% Formaldehyde solution (Sigma, Germany) for H&E and Masson examination. Another part of left lung tissue was preserved at -20°C for hydroxyproline determination. The superior lobe of right lung of each mouse was harvested and rinsed with cooled physiological saline. After being cut into pieces, the right lung tissues were quick-frozen in liquid nitrogen and stored at -80°C for RT-PCR. The middle lobe of right lung of each mouse was collected and treated with the same methods for western blot. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Qindao University.

Pathologic and immunohistochemical (IHC) observations

The left lung tissues fixed in 4% paraformaldehyde were made into paraffin blocks and cut into 5 μm thickness serial sections with an interval of 30 μm. After routine dewaxing, the sections were subjected to hematoxylin-eosin (hematoxylin-eosin staining kit, C0105, Beyotime, China) or Masson (Masson Staining Kit, C0105, Beyotime, China) staining following to the instruction provided by manufacturer. Then the histopathologic changes in each sample were observed under an optical microscope

Table 1. RT-PCR primer sequences specific to genes used in this study

Gene	Forward	Reverse	Product size (bp)
Col1a1	5'-GAGACAGGCGAACAAGGTGA3'	5'-CTCAAGGTCACGGTCACGAA3'	399
Col1a3	5'-AGTGGGCATCCAGGTCCTAT3'	5'-GTGCTTACGTGGGACAGTCA3'	480
TRB3	5'-GGAACCTTCAGAGCGACTT3'	5'-TGGCACTCAGGGAGCATC3'	341
β-actin	5'-CCACCATGTACCCAGGCATT3'	5'-CGGACTCATCGTACTCCTGC3'	189

(OLYMPUS CX22, Japan) and photographed at high magnification.

The sections for IHC were incubated with diluted primary antibodies TRB3 (Abcam, USA), E-cadherin (Wanleibio, China), Vimentin (Wanleibio, China), Fibronectin (Santa Cruz, US) and α-SMA (Wanleibio, China) respectively at 4°C overnight and at room temperature for 1 h, followed by incubation with secondary antibodies (HRP-labelled goat anti-rabbit IgG, Tianjin Saier Biotechnology Co., Ltd., China) at room temperature for 20 min. Then the immunoreactivity was developed by DAB kit according to the manufacturer's instruction. Brown granules were regarded as positive in cells or stroma.

Hydroxyproline determination of lung tissue

The hydroxyproline contents in lung tissues were determined using hydroxyproline kit (A030-3, Nanjing Jiancheng Bioengineering Institute, China) strictly according to the instruction provided by the manufacturer.

Total protein extraction and western blot

Total protein in lung tissues was extracted by adding 1 mL RIPA lysate to 250 mg tissue which was then homogenated on ice using a homogenizer. After standing on ice for 30 min, the homogenate was centrifuged at 4°C and 12000 rpm for 10 min. The supernatant was collected for determining the protein concentration using BCA method. After adjusted to the same concentration, each sample was mixed with 5× loading buffer (a ratio of 1:4) and boiled at 100°C for 10 min and then quickly cooled on ice. The same amounts of proteins were separated by SDS-PAGE electrophoresis and transferred onto PVDF membrane (Millipore Company, USA). After blocked with 5% skim milk powder in TBST for 2 h, the membranes were incubated with primary antibodies such as anti-TRB3 (Abcam, USA), anti-E-cadherin (Wanleibio, China), anti-Vimentin (Wanleibio, China), anti-Fibronectin (Santa, USA), anti-p-ERK1/2 (Cell Signaling, USA), anti-ERK1/2 (Cell Signaling, USA), anti-p-p38MAPK (Wanleibio, China), anti-p38MAPK (Cell Signaling, USA) at 4°C overnight with slow shaking. After incubated with the secondary antibody (HRP-labelled goat anti-rabbit IgG, 1:3000, CST, USA) at room temperature for 1 h, the membranes were washed with TBST and stained with enhanced Chemiluminescence (ECL). Then the special bands were analyzed using UVP instrument. GAPDH was used as the internal reference. The grey scale of each sample was generated by the analysis software.

Total RNA extraction and RT-PCR

The total RNA of each sample was extracted using Trizol reagent according to the routine Trizol method. Then the RNA was reverse-transcribed into cDNA. RT-PCR was carried out to detect the expression of mRNA. The specific primer pairs were listed in **Table 1**. The relative content of target genes was calculated by $2^{-\Delta\Delta Ct}$ method.

Detection of Col1a I/III contents in lung tissues

The contents of Col1a I/III in lung tissues of mice were determined using ELISA kit following the procedure provided in the kit.

Statistical analysis

The experimental data were analyzed by SPSS v19 statistical software. The measurement data were expressed as mean ± standard deviation (SD). Independent sample t test was applied to compare the data with the homogeneous variances; otherwise, t test was used. The test level was set as $\alpha=0.05$, and $P<0.05$ was considered a significant difference.

Results

Pathologic changes of lung tissue

As shown in **Figures 1** and **2**, Group Mock showed normal pulmonary alveolar structure. Ho-

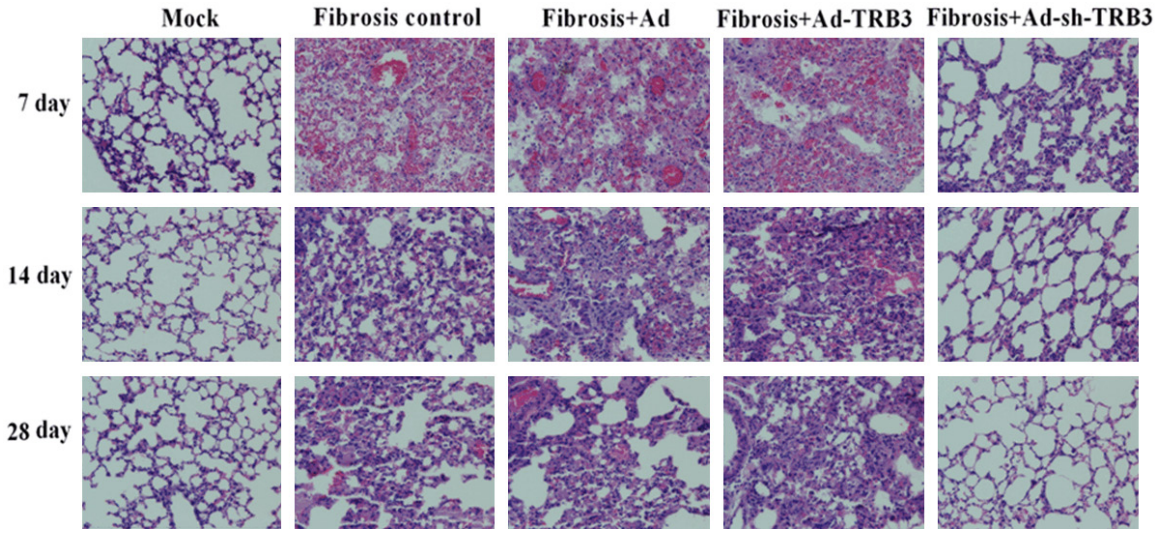

Figure 1. Pathologic changes of lung tissue in each group (H&E staining, 200×).

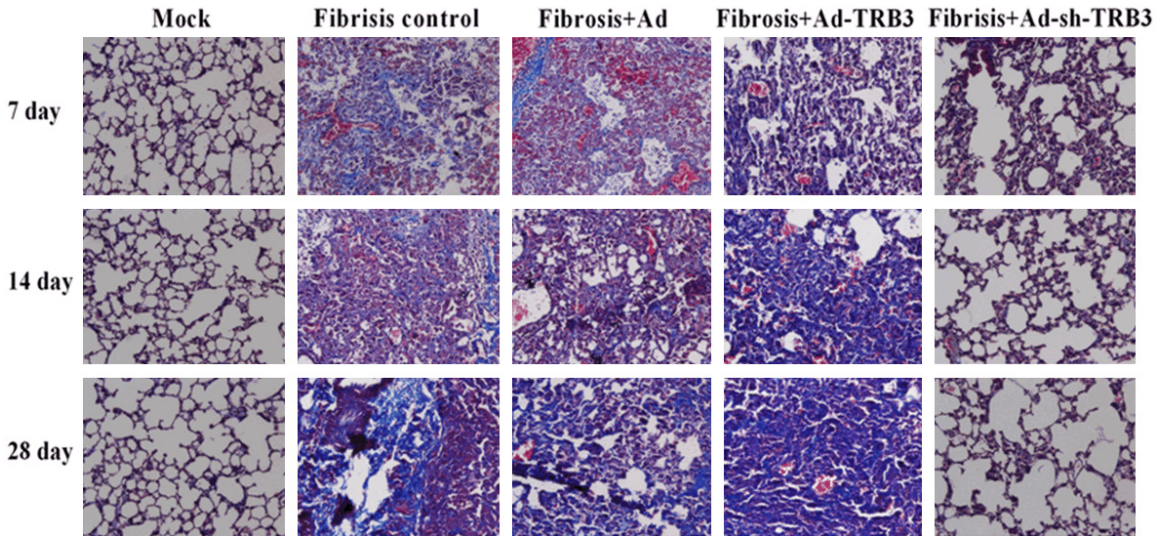

Figure 2. Masson staining of lung tissue in each group (200×).

wever, Group F and Group F+GFP displayed obvious inflammatory cell infiltration but mild fibrosis in alveolar space and alveolar septum on D7, while the alveolar wall was thickened and a large number of fibroblasts were visible in the alveolar septum, alveolar structure was damaged, alveolar spaces were narrowing and fused, and the degree of fibrosis was aggravated on D14 and D28. Compared with Group F and Group F+GFP, alveolar inflammation and fibrosis in Group F+TRB3 were significantly increased at the corresponding time points, while they were prominently reduced in Group F+sh-TRB3 at the corresponding time points.

Determination of hydroxyproline (Hyp)

Hyp was slightly expressed in mouse lung tissues in Group Mock, with no statistical differences among the three time points. Compared with Group Mock, the expression of Hyp on D14 and D28 was elevated significantly in Groups F, F+GFP and F+TRB3, and reached a peak on D28 (**Figure 3**). There was no significant difference in Hyp content between Group F and Group F+GFP at the three time points ($P>0.05$). On D7, the Hyp content in Group F was significantly higher than that in Group Mock ($P<0.05$). Compared with Group F+GFP, the content of

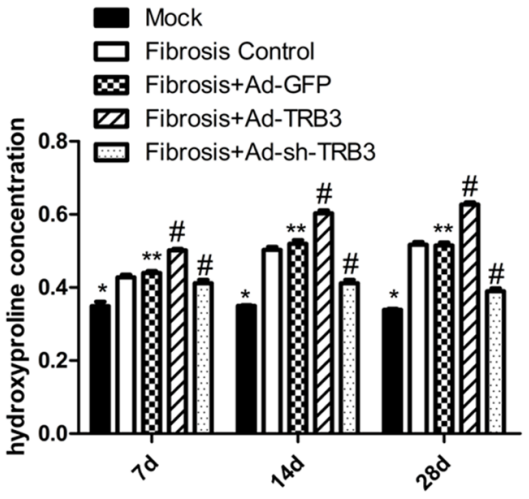

Figure 3. Hyp contents in mouse lung tissues of each group at three time points *P<0.05, *vs.* Group F; **P>0.05, *vs*. Group F; #P<0.05, *vs.* Group F+GFP.

Hyp in Group F+TRB3 was increased greatly (P<0.05), while it was markedly decreased in Group F+sh-TRB3 (P<0.05).

Expression of TRB3, E-cadherin and α-SMA determined by IHC

Expression and location of TRB3 in lung tissues: On D7, the expression of TRB3 in Group Mock was not obvious, but it was weakly positive in Groups F, F+GFP and F+TRB3, mainly located in the blood vessels, peribronchial, and alveolar septum of the lung tissues; and the expression was more remarkable in Group F+TRB3. On D14, a small amount of TRB3 protein was expressed in Group Mock, showing faint yellow granules; while in Groups F, F+GFP and F+TRB3, the positive expression of TRB3 in alveolar septum was higher than that on D7. On D28, the expression of TRB3 in Groups F and F+GFP was higher than that in Group Mock, appearing as diffuse brown granules. The positive expression of TRB3 was the strongest in Group F+TRB3, presenting as dark tan coarse particles in the alveolar septum. There was no positive expression of TRB3 in Group F+sh-TRB3 (**Figure 4A**).

Expression and location of E-cadherin in lung tissues: On D7, E-cadherin was positively expressed in Group Mock, presenting brown granules confined to alveolar epithelium; while in Groups F, F+GFP and F+TRB3, it was also diffusely expressed, appearing as faint yellow granules. The expression of E-cadherin in Group F+sh-TRB3 was similar to that in Group Mock. In Group Mock, E-cadherin expression on D14 was not significant different from that on D28. From D14, the expression of E-cadherin was gradually decreased in Groups F, F+GFP, and F+TRB3, and then was negative on D28, while it was gradually increased in Group F+Sh-TRB3 and reached the highest on D28, displaying coarse and agglomerate distribution (**Figure 4B**).

Expression and location of α-SMA in lung tissues: The expression of α-SMA was weakly positive in Group Mock on D7, presenting as faint yellow granules in alveolar septa, but it was obviously expressed in Groups F and F+GFP, showing diffuse brown granules. It was also positively expressed in Group F+TRB3, which appeared as chrysanthemum-like brown granules. On D14 and D28, the expression of α-SMA showed no difference in Mock group, while it was increased in Group F, F+GFP and F+TRB3. In Group F+TRB3, positive expression of α-SMA displayed as massively distributed tan coarse particles. In Group F+sh-TRB3, the expression of α-SMA at the three time periods was significantly lower than that in Groups F and F+GFP, respectively, but similar to that in Group Mock (**Figure 4C**).

Expression of TRB3, E-cadherin, vimentin, fibronectin, α-SMA, p-ERK1/2, ERK1/2, p38MAPK, p-p38MAPK proteins determined by western blot

Expression of TRB3 protein in lung tissues: A small amount of TRB3 was expressed in Group Mock. Compared with Group Mock, the expression of TRB3 in Group F was significantly increased, with statistical significance (P<0.05). There was no significant difference in TRB3 protein expression between Group F and Group F+GFP (P>0.05, **Figure 5**).

Expression of EMT-related proteins: Compared with Group Mock, the expression of vimentin, fibronectin, and α-SMA was significantly elevated but the content of E-Cadherin was prominently decreased in group F (P<0.05). Compared with Group F+GFP, the expression of vimentin, fibronectin, and α-SMA was obviously upregulated, while the expression of E-Cadherin was notably decreased in Group F+TRB3 (P< 0.05). On the contrary, the expression of vimen-

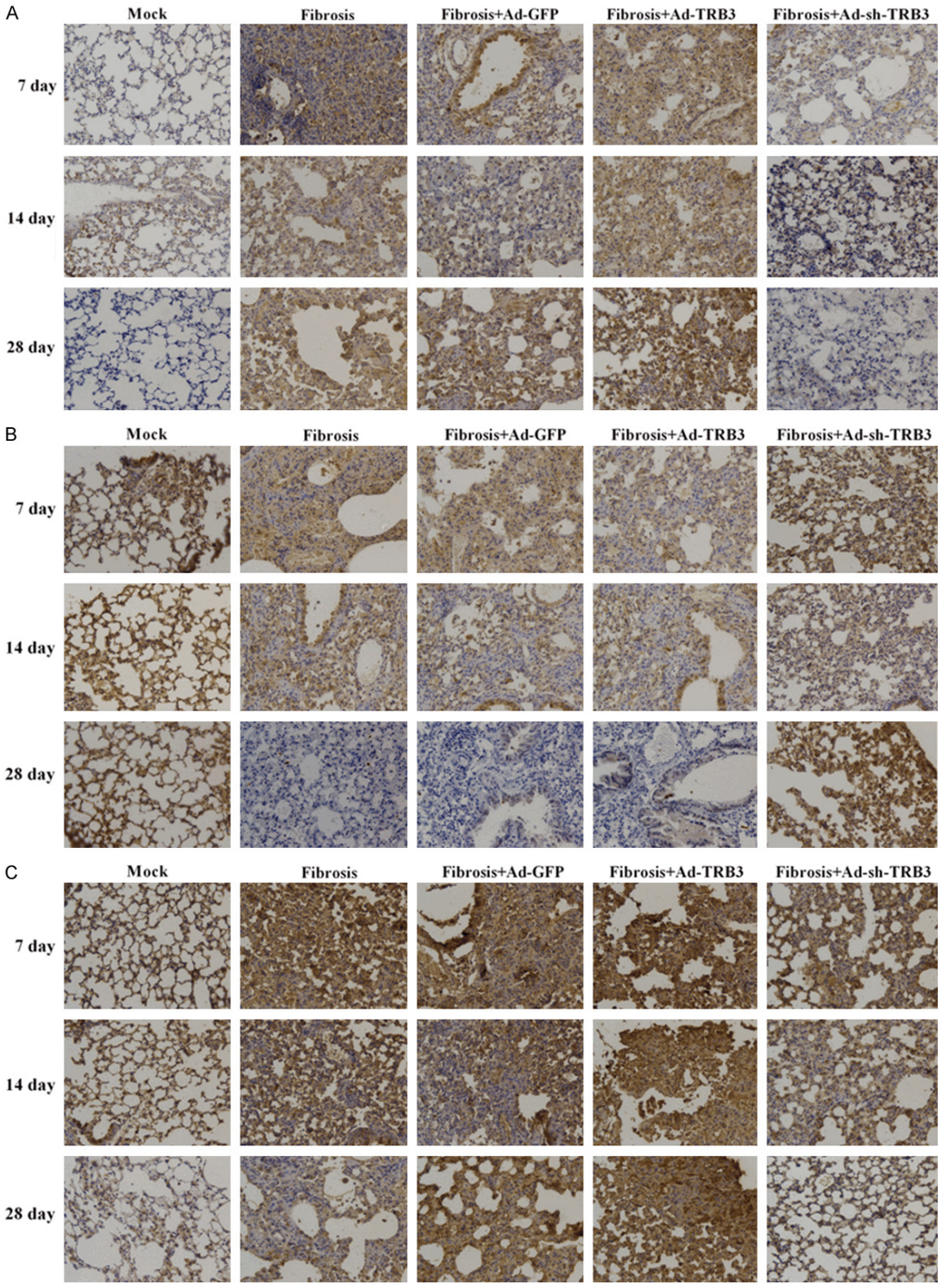

Figure 4. Expression of TRB3, E-cadherin, and α-SMA in mouse lung tissue detected by IHC (200×).

tin, fibronectin, and α-SMA were decreased significantly in group F+sh-TRB3, and the expression of E-Cadherin were increased, compared with that in Group F+GFP (P<0.05, **Figure 5**).

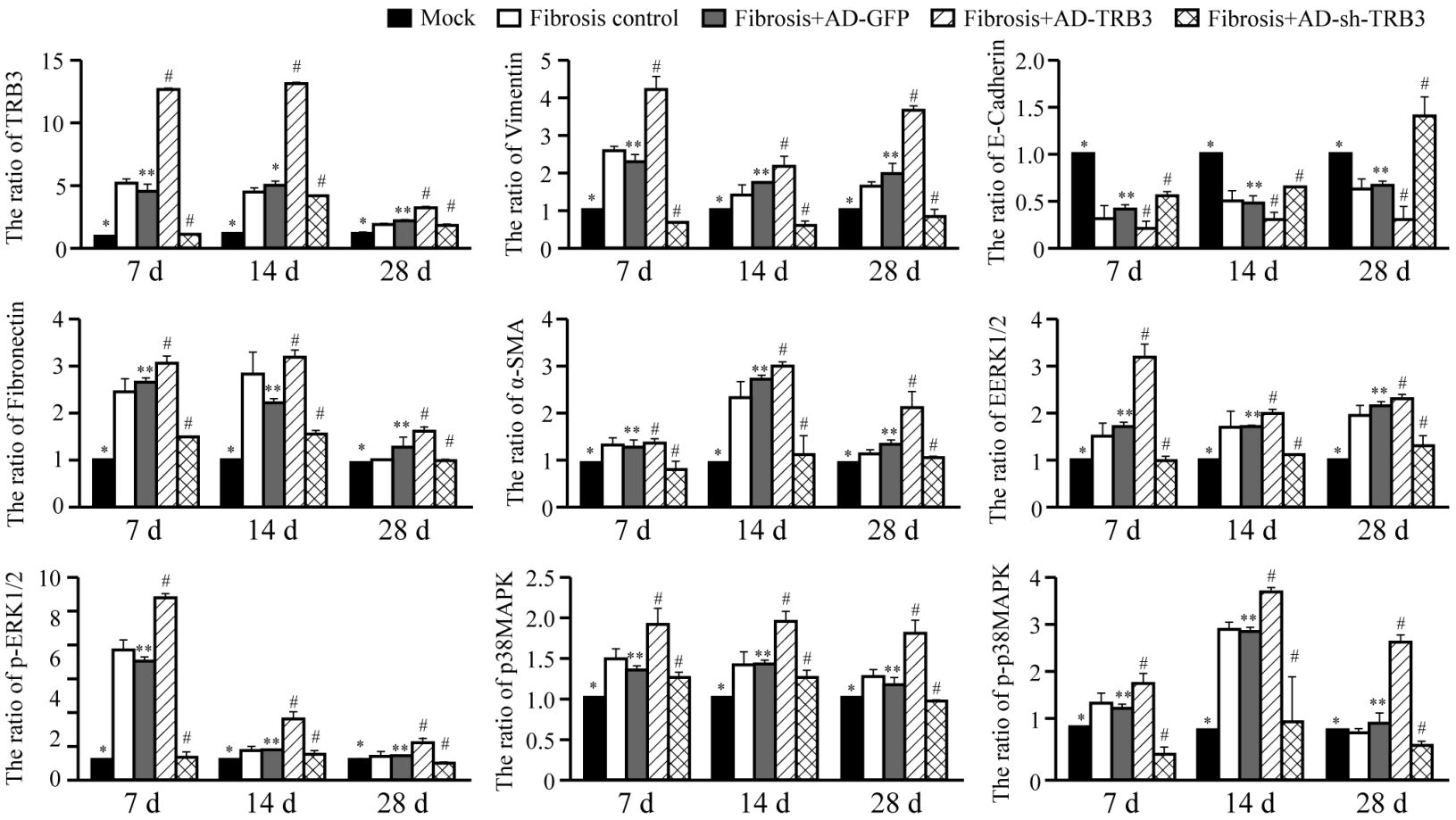

Figure 5. The expression of TRB3, Vimentin, E-Cadherin, Fibronectin, α-SMA, ERK1/2, p-ERK1/2, p38MAPK, and p-p38MAPK in lung tissues at three time points detected by western blot. **P*<0.05, *vs.* Group F; ***P*>0.05, *vs.* Group F; #P<0.05, *vs.* Group F+GFP.

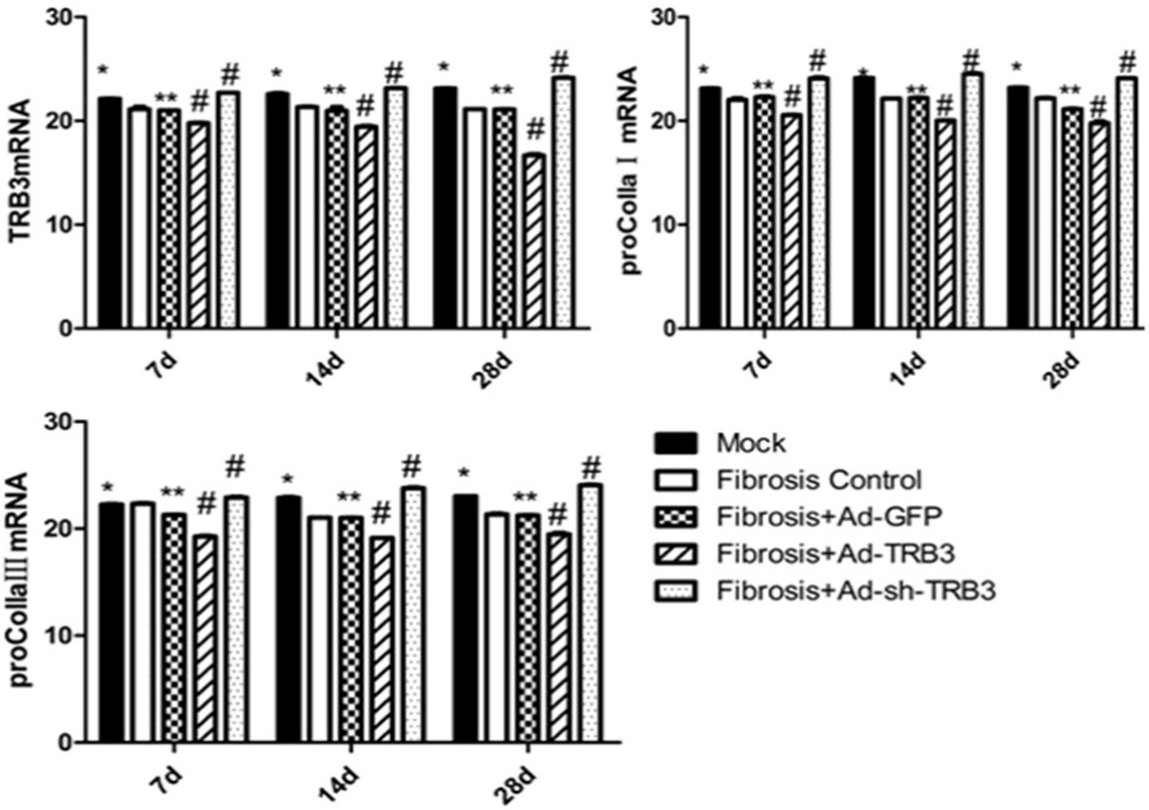

Figure 6. The relative expression of *TRB3* and *proColla I/III* mRNA in lung tissues of each group detected by RT-PCR. **P*<0.05, compared with Group F; ***P*>0.05, compared with Group F; #*P*<0.05, compared with Group F+GFP.

Expression of MAPK-related proteins: The expression levels of ERK1/2, p-ERK1/2, p38MAPK, and p-p38MAPK in Group F were significantly higher than those in group Mock (*P*< 0.05). In addition, the expression of ERK1/2, p-ERK1/2, p38MAPK, and p-p38MAPK was obviously up-regulated in group F+TRB3 as compared with that in Group F+GFP (*P*<0.05). Conversely, the expression of these proteins was significantly down-regulated in Group F+sh-TRB3 as compared with that in Group F+GFP (P<0.05, **Figure 5**). These results suggested that TRB3 could promote fibrosis through the MAPK pathway.

The mRNA expression of TRB3 and proColla I/III

As shown in **Figure 6**, the mRNA expression of *TRB3* and *proColla I and III* was significantly upregulated in Group F as compared with that in Group Mock (*P*<0.05), but it was not significantly different from that in Group F+GFP (*P*>0.05). Compared with Group F+GFP, the expression of *TRB3* and *proColla I/III* mRNA was prominently upregulated in group F+TRB3, but markedly downregulated in Group F+sh-TRB3 (*P*<0.05).

The contents of Colla I and Colla III

The contents of Col1a I and Col1a III in lung tissues of C57BL/6 mice treated with different adenovirus carriers were compared (**Figure 7**). Compared with Group Mock, the contents of Col1a I and Col1a III in Group F were elevated significantly (*P*<0.05). There was no significant difference in the contents of Col1a I and Col1a III between Group F and Group F+GFP (*P*>0.05). However, compared with Group F+GFP, the contents of Col1a I and Col1a III increased notably in Group F+TRB3 but decreased in Group F+sh-TRB3 (*P*<0.05). Furthermore, the contents of Col1a I and Col1a III had an increasing trend following the time points in Groups F, F+GFP and F+TRB3, which reached the highest on D28.

Discussion

The main pathologic features of idiopathic pulmonary fibrosis (IPF) include increased apopto-

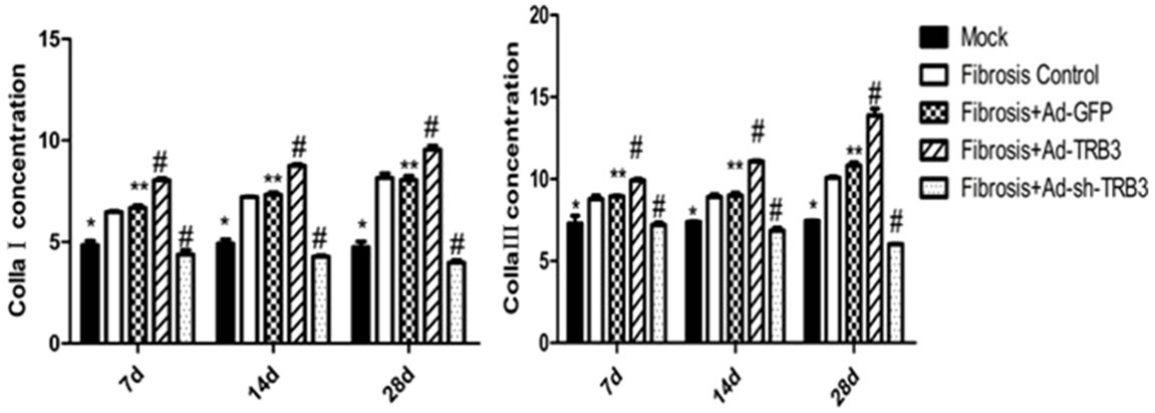

Figure 7. The contents of Col1a I and Col1a III in lung tissues of each group at three time points determined by ELISA. **P*<0.05, *vs.* Group F; ***P*>0.05, *vs*. Group F; #P<0.05, *vs.* Group F+GFP.

sis in type II alveolar epithelial cells, damage of regeneration, differentiation, and migration of type II alveolar epithelial cells, phenotypic accumulation of interstitial cells, and transdifferentiation into myofibroblasts, accompanied with excessive deposition of collagen and related extracellular matrix (ECM) molecules. In the process of EMT, the phenotype of characteristic epithelial cells is lost, such as by the downregulated expression of E-cadherin. The epithelial cells dedifferentiate into myofibroblasts by acquiring interstitial phenotypes such as α-SMA and fibronectin (FN) [13]. ECM is mainly secreted by myofibroblasts that specifically express α-SMA [14]. The main components of ECM are collagen and fibronectin, and the most important components of collagen are Col1a I/III. In this study, a mouse model was established by intratracheal dripping of bleomycin. Then the expression of ECM-related proteins and mRNAs in the lung tissues of the control group, Group F, and Group F+GFP were analyzed by immunohistochemical staining, western blot and RT-PCR. We found that the expression of fibronectin and α-SMA protein and pro-Colla I/III mRNA in Groups F and F+GFP was remarkably higher than that in control group (*P*<0.05), but the expression of E-cadherin was notably less than that in control group. Immunohistochemistry showed that the positive expression of fibronectin and α-SMA in Groups F and F+GFP was significantly stronger than that in control group, while that of E-cadherin was weaker than that in the control group. Thus, it can be confirmed that there is excessive deposition of interstitial ECM during pulmonary interstitial fibrosis. In addition, there was no significant difference in protein and mRNA expression between Group F and Group F+GFP (*P*>0.05), indicating that the empty virus vector and *GFP* gene had no effect on fibrosis.

Studies have confirmed that TRB3 is involved extensively in the development and progression of renal fibrosis and myocardial fibrosis by various signaling pathways [9, 10, 12]. In this study, the relationship between TRB3 and fibrosis was explored. Compared with Group F+GFP at three time points, H&E staining in Group F+TRB3 showed that pulmonary alveoli were obviously damaged, replaced by a large amount of fibroblast proliferation. Masson staining also showed more extensive collagen fibers. The immunohistochemical results revealed that the positive expression of some markers that represent the interstitial phenotypes such as vimentin, α-SMA and fibronectin was significantly higher in Group F+TRB3 than that in Group F+GFP, while the positive expression of markers representing the epithelialphenotype such as E-cadherin was significantly lower than that in Group F+GFP. Group F+TRB3 showed more severe fibrosis, indicating that TRB3 was involved in the development of fibrosis. Then we determined the relationship between TRB3 and fibrosis by western blot and RT-PCR. The results of western blot showed that the expression of fibronectin, vimentin, and α-SMA in Group F+TRB3 was prominently higher than that in Group F+GFP (*P*<0.05), but the expression of E-cadherin was significantly lower (*P*<0.05). Furthermore, the expression of fibronectin, vimentin and α-SMA was significantly decreased in Group F+sh-TRB3 as compared with that in

Group F+GFP (P<0.05), while the expression of E-cadherin was significantly increased (P<0.05). RT-qPCR revealed that the mRNA expression of *colla I/III* in Group F+TRB3 was significantly higher than that in Group F+GFP (P<0.05). On the contrary, the mRNA expression of *colla I/III* in Group F+sh-TRB3 was significantly lower than that in Group F+GFP (P<0.05). Thus, it is suggested that overexpression of TRB3 may further induce pulmonary fibrosis by inducing EMT in type II alveolar cells. Therefore, we believed that the up-regulation of TRB3 can significantly promote the development of fibrosis, but inhibiting the expression of TRB3 can improve bleomycin-induced pulmonary fibrosis.

MAPK signaling pathway plays an important role in the fibrotic processes, such as myocardial fibrosis [15], renal fibrosis [6], pulmonary fibrosis [16], and others. It has been found that the MAPK pathway may play the profibrotic role by mediating TGF-β-induced EMT in the process of fibrosis [17]. TGF-β1 induces EMT through phosphorylation of JNK, p38MAPK, and ERK [18], resulting in the activation of fibroblasts and the proliferation of myofibroblasts [19]. Through activation of ERK1/2 and p38MAPK pathways, TRB3 is involved in the regulation of collagen I and III by AGEs in rat cardiac fibroblasts [20]. Zhang et al. found that TRB3 is involved in diabetic nephropathy fibrosis by regulating TGF-β1 and type IV collagen through ERK1/2-MAPK signaling pathway [12].

In this study, western blot was used to detect the expression of MAPK pathway related proteins such as ERK1/2, p-ERK1/2, p38MAPK and p-p38MAPK in lung tissues. There was no significant difference in the expression of MAPK pathway related proteins between Group F and Group F+GFP (P>0.05), indicating that the empty vector and *GFP* gene had no effect on pulmonary fibrosis. Compared with Group F+GFP, the expression of ERK1/2, p-ERK1/2, p38MAPK, and p-p38MAPK was significantly enhanced in Group F+TRB3 (P<0.05) but notably reduced in Group F+sh-TRB3 (P<0.05). Our results showed that the expression of MAPK signal pathway-related proteins was increased or decreased following the treatment of TRB3 overexpression or downregulation, suggesting that TRB3 promotes pulmonary fibrosis through the MAPK pathway.

The present study only observed the expression of MAPK signaling pathway related proteins ERK1/2 and p38MAPK, but did not include the other cytokines in the MAPK signaling pathway. TRB3 overexpression may promote the production of EMT through the MAPK signaling pathway, and the downregulation of TRB3 may inhibit the production of EMT through the MAPK signaling pathway. This may need more in-depth studies to confirm.

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Disclosure of conflict of interest

None.

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