

Effect of the alteration of Tribbles homologue 3 expression on epithelial-mesenchymal transition of transforming growth factor β 1-induced mouse alveolar epithelial cells through the Wnt/ β -catenin signaling pathway

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Abstract. The aims of the present study were to elucidate the regulatory effect of exogenous Tribbles homologue 3 (TRB3) expression on the Wnt/ β -catenin signaling pathway and epithelial-mesenchymal transition (EMT) in transforming growth factor- β 1 (TGF- β 1)-induced mouse alveolar epithelial cells (MLE-12) and investigate the underlying regulatory mechanisms. TRB3 expression was upregulated and down-regulated using gene overexpression and RNA interference techniques, respectively. TGF- β 1-stimulated MLE-12 cells were examined for EMT and activation condition of the Wnt/ β -catenin signaling pathway using Cell Counting Kit-8, flow cytometry, western blotting, reverse transcription-quantitative PCR, ELISA and immunofluorescence techniques. During TGF- β 1-induced EMT, TRB3 expression was found to be significantly upregulated ($P<0.05$). In the TRB3 over-expression group, upregulated expression of β -catenin and EMT-related genes and proteins was observed ($P<0.05$), and an increase in fibrosis-related factors in the cell culture supernatant was detected ($P<0.05$); however, the results were the opposite in the TRB3 downregulated group ($P<0.05$). TRB3 may be involved in the regulation of EMT in TGF- β 1-induced MLE-12 cells through the Wnt/ β -catenin signaling pathway.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, fibrosing and interstitial lung disease. The median survival of patients with IPF is 2 to 3 years, with clinical symptoms

of progressively aggravated cough and dyspnea (1). The pathogenesis of IPF is still unknown, and the state of existing therapeutic treatments is poor, barring lung transplantation. Further studies are therefore required to ascertain the mechanism of the disease, in order to explore new therapeutic targets.

Epithelial-mesenchymal transition (EMT) refers to the process through which fully differentiated epithelial cells transform into mesenchymal cells with specific phenotypes under particular pathological and physiological conditions, which is the key step in fibrosis progression. EMT plays an important role in malignant tumor formation and infiltration, tissue remodeling, and fibrosis in multiple organs (2-4). It may play a key role in IPF (5,6). Its controversial role makes this study meaningful as yet.

The initial stage of IPF is the formation of lesions of alveolar epithelial cells, which release multiple cytokines such as transforming growth factor- β 1 (TGF- β 1). This promotes cell apoptosis, migration and extracellular matrix (ECM) formation, which has been widely used to induce EMT *in vitro*. The Wnt/ β -catenin signaling pathway is a classic Wnt pathway, which is highly conserved and plays meaningful roles in embryo and organ development, as well as the maintenance of homeostasis. However, abnormalities in the Wnt/ β -catenin pathway can lead to various lung diseases, including lung cancer and IPF (7,8). One study has demonstrated the close relationship between Wnt/ β -catenin signaling and fibrosis-related factors and EMT during lung fibrosis (9). Tribbles homologue 3 (TRB3) is a member of the putative protein kinase family with a kinase-like domain. TRB3 can react with transcription factors, the type II bone morphogenetic protein (BMP) receptor on the plasma membrane, and members of signaling pathways such as the Wnt/ β -catenin pathway to inhibit mitosis and modulate cell proliferation, apoptosis, migration and morphological alterations. Studies have demonstrated that TRB3 overexpression is involved in renal fibrosis by increasing apoptosis rates in renal tubular epithelial (RTE) cells and by inhibiting cell proliferation (10-12). Nonetheless, the role of TRB3 in lung fibrosis remains to be fully elucidated. In the present study, a new target for IPF treatment was explored by uncovering the role of TRB3 in IPF pathogenesis.

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Materials and methods

Cell culture and grouping. MLE-12 cells [mouse type II alveolar epithelial cells, purchased from American Type Culture Collection (ATCC)] were cultured in DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 2% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in 5% atmospheric CO₂ at 37°C. Cells with 80 to 90% confluency were passaged using 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.) and split 1:4 to 1:6 and transferred to fresh culture flasks.

Cells were divided into eight groups: i) control group; ii) Ad-GFP transduction group (GFP group); iii) Ad-TRB3 transduction group (TRB3 group); iv) Ad-TRB3-shRNA transduction group (shR-TRB3 group); v) group with TGF- β 1 stimulation only (TGF- β 1 group); vi) TGF- β 1 and Ad-GFP transduction group (T+GFP group); vii) TGF- β 1 and Ad-TRB3 transduction group (T+TRB3 group); viii) TGF- β 1 and Ad-TRB3-shRNA transduction group (T+shR-TRB3 group).

Primers were designed based on the TRB3 gene sequence (MGI 3562334) (NCBI Gene-Bank) and the sequences of the primers are listed in Table I. PCR gene (SYBR Green) amplification was performed to construct the Ad-TRB3 recombinant plasmid and Ad-TRB3-shRNA recombinant plasmid. The plasmid carrier was pacAd5 CMV-IRES-GFP, 7.5 kb; MCS: *PmeI*, *ClaI*, *EcoRV*, *EcoRI*, *BamHI*. The recombinant plasmids were then amplified, cultured, purified and packaged with adenovirus.

Six independent parallel experiments were performed for each group. Preliminary results indicated that the optimal concentration of TGF- β 1 stimulation was 10 ng/ml, and the optimal multiplicity of infection (MOI) for adenovirus vector Ad-GFP, Ad-TRB3, Ad-TRB3-shRNA was 100, 800 and 200, respectively, with an optimal transduction time of 48 h.

Cell Counting Kit-8 (CCK-8). MLE-12 cells were cultured in 96-well plates at a concentration of 10³-10⁴/well, and pre-incubated in a cell culture incubator with 5% atmospheric CO₂ at 37°C for 24 h, and then incubated for 12 h in serum-free medium. After the cells attached to the plate completely, three virus vectors and TGF- β 1 recombinant protein were loaded into the wells according to the aforementioned groupings, and CO₂ incubation was resumed. CCK-8 (Dojindo, China) solution was pipetted into each well (10 μ l/well) after 48 h of cultivation, and then incubation was continued. After 2 h, absorbance was measured at 450 nm using a plate reader (Bio-Tek, USA). The experiments were conducted in triplicate.

Flow cytometry. MLE-12 cells were plated into 6-well plates at a concentration of (1-5) × 10⁵ cells/well and cultured in a CO₂ incubator overnight. After the cells attached to the plate completely, the corresponding virus vectors and TGF- β 1 recombinant protein were loaded into the wells according to the aforementioned grouping. After 48 h, the cells were harvested, and the cell suspension was prepared. In a 1.5-ml centrifuge tube, 100 μ l of the cell suspension from each well was mixed with 10 μ l Annexin V-R-PE (Southern Biotech, USA) by clicking the tube gently. The tube was bathed in ice in a dark chamber for 10 min. Next, 380 μ l 1X binding buffer was pipetted into the tube and mixed by clicking the tube gently,

before loading the samples for flow cytometry to evaluate the early-stage apoptotic rates for each group.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol[®] (Invitrogen; Thermo Fisher Scientific, Inc.) 48 h after the cell vectors or recombinant protein treatments. Total RNA (3 μ l) was pipetted into a 33.5 μ l reaction master mix for reverse transcription of cDNA. TRB3, β -catenin, α -SMA (fibrosis-related protein), collagen I/III and β -actin genes were amplified using fluorogenic RT-qPCR (Takara), along with the housekeeping gene. Relative mRNA expression levels of target genes compared to the housekeeping gene were calculated using the $\Delta\Delta$ Ct calculation method. Primers for all target genes are listed in Table I.

Western blotting. Total cell protein content was extracted using cell lysis buffer (Gibco; Thermo Fisher Scientific, Inc.), and sample protein concentrations were determined for each group using a BCA protein assay kit (Wuhan Boster Biological, Co., Ltd.), 48 h after vector or recombinant protein treatment. SDS-PAGE was performed for each group on 10% gels, with 30-50 μ g protein sample per lane, and proteins were transferred from gel to the membrane via the wet method. After 2 h blocking using 5% skim milk powder at 4°C, the membranes were incubated with a dilution of 1:1,000 rabbit anti-mouse TRB3 polyclonal antibody (PcAb; cat. no. ab73547) (anti-mouse TRB3 antibody prepared from rabbit plasma), a dilution of 1:1,000 rabbit anti-mouse β -catenin (PcAb; cat. no. ab16051), a dilution of 1:500 rabbit anti-mouse E-cadherin (PcAb; cat. no. ab202413), a dilution of 1:500 rabbit anti-mouse vimentin (PcAb; cat. no. ab45939), and a dilution of 1:1,000 rabbit anti-mouse fibronectin (PcAb; cat. no. ab2413), respectively, on a 4°C shaker overnight. Membranes were then incubated statically with 1:2,000 goat anti-rabbit IgG (PcAb; cat. no. ab21058) labeled with HRP at room temperature for 1 h. All antibodies mentioned above were obtained from Abcam, USA. Lastly, membranes were photographed using a visible/ultraviolet gel scanning analysis system (UVP, LLC) and LabWorks[™] 4.5 Analysis software (UVP, LLC), and the calibrated band brightness of each group was calculated by dividing the real brightness by the brightness of β -tubulin or GAPDH (internal reference).

Immunofluorescence (IF) assay. Cell coverslips were prepared 48 h after vector or recombinant protein treatment and were fixed using paraformaldehyde for 15 min and washed using PBS. Next, 200 μ l of the corresponding primary antibody (1:500; cat. no. sc-390242; Santa Cruz Biotechnology, Inc.) diluted with 1% donkey serum (Sigma-Aldrich; Merck KGaA) was dripped onto each coverslip, which was then incubated at 4°C overnight. After incubation, the coverslips were washed with PBS and incubated at 4°C for 1.5 to 2 h, after pipetting 200 μ l secondary antibody (1:500; cat. no. sab4600003; Sigma-Aldrich; Merck KGaA) diluted with 1% donkey serum. Then, the coverslips were washed with PBS, stained with DAPI combined with FITC (Sigma-Aldrich; Merck KGaA) and incubated at 4°C for 5 min. The treated cells were moved from the multi-well plate onto microscopic slides and spun. Next, the coverslip was placed onto an object slide, 5 μ l fluorescence protection solution was pipetted onto each slide, and the whole system was sealed with cover glass. Finally, the sample was examined

Table I. Primer sequences for reverse transcription-PCR.

Name	Primer sequence	Product (bp)
TRB3	Forward 5'-GGAACCTTCAGAGCGACTT-3' Reverse 5'-TGGCACTCAGGGAGCATC-3'	341
β -catenin	Forward 5'-AATCCGAGGACTCAATACC-3' Reverse 5'-AGAGTAAAGTATTCACCCA-3'	636
α -SMA	Forward 5'-GTACCCAGGCATTGCTGACA-3' Reverse 5'-GAGGCGCTGATCCACAAAAC-3'	271
Collagen I	Forward 5'-GAGACAGGCGAACAAGGTGA-3' Reverse 5'-CTCAAGGTCACGGTCACGAA-3'	399
Collagen III	Forward 5'-AGTGGGCATCCAGGTCCTAT-3' Reverse 5'-GTGCTTACGTGGGACAGTCA-3'	480
β -actin	Forward 5'-CCACCATGTACCCAGGCATT-3' Reverse 5'-CGGACTCATCGTACTCCTGC-3'	189
TRIB3- <i>Eco</i> RI-S	5'-CCGGAATTCGCCACCATGCGAGCTACACCTCTGGC-3'	
TRIB3- <i>Bam</i> HI-AS	5'-CGCGGATCCGCCGTACAGCCCCACCTCCC-3'	

TRB3, Tribbles homologue 3; α -SMA, α smooth muscle actin; bp, base pairs.

under confocal laser scanning microscopy (magnification, $\times 100$; FV1000; Olympus Corporation) and imaged.

Statistical analysis. SPSS 19.0 (IBM, Corp.) was utilized for database establishment using data from this study. All data are consistent with a normal distribution and are presented as the mean \pm SD. Differences of means between two groups were compared using t-test, while those among multiple groups were compared using ANOVA, with the Least Significant Difference test LSD-t and SNK as post hoc tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Fluorescence detection of viral infection of MLE-12 cells. Twenty-four hours after viral infection, GFP expression could be detected and the fluorescence signal was gradually increased with the prolongation of observation time in the same multiplicity of infection (MOI) value group, which exhibited the best infection efficiency at 48 h, approximately $>90\%$. At the same time, the fluorescence signal increased with the increase in MOI value (Fig. 1). The optimal MOI values of GFP (Fig. 1A), TRB3 (Fig. 1B) and shR-TRB3 (Fig. 1C) groups were 100, 800 and 200, respectively, and they expressed exogenous genes stably and efficiently in target cells. The intensity of the fluorescence signal of TRB3-transfected cells was significantly decreased, while that of TRB3-transfected cells was increased after silencing.

Effect of the alteration of TRB3 expression on proliferation activity and early-stage apoptosis. Proliferation activity assay indicated that the OD450 values of MLE-12 cells in the TRB3 overexpression groups (TRB3 group, T+TRB3 group) were significantly lower than those in the TRB3 downregulated groups (shR-TRB3 group, T+shR-TRB3 group) ($P < 0.05$). After TGF- $\beta 1$ administration, the results demonstrated an enhancing effect of TRB3 overexpression on the role of TGF- $\beta 1$ in inhibiting MLE-12 proliferation activity; while downregulation

of TRB3 expression led to the opposite effect ($P < 0.05$). Early-stage apoptotic rates monitored by flow cytometric assay revealed that with the adenovirus vector only, TRB3 overexpression significantly promoted early-stage apoptosis in MLE-12 cells; while downregulation of TRB3 expression exerted the opposite effect ($P < 0.05$). After TGF- $\beta 1$ administration, the results demonstrated a positive effect of TRB3 overexpression on MLE-12 early-stage apoptosis induced by TGF- $\beta 1$; while downregulation of TRB3 expression inhibited early-stage apoptosis ($P < 0.05$; Table II).

TRB3, β -catenin, α -SMA and collagen I/III mRNA

TRB3 mRNA expression during EMT induced by TGF- $\beta 1$. TRB3 mRNA expression was significantly increased in the TGF- $\beta 1$ -stimulated group relative to the control group ($P < 0.05$; Fig. 2B).

Impact of TRB3 on EMT-related protein and β -catenin expression. The mRNA expression of β -catenin, α -SMA and collagen I/III was significantly increased in the TRB3 overexpression group ($P < 0.05$); whereas expression levels of all three were reduced in the TRB3 downregulated (shR-TRB3) group (Table III).

TRB3 is able to affect EMT induced by TGF- $\beta 1$ and the Wnt/ β -catenin signaling pathway. The mRNA expression of β -catenin, α -SMA and collagen I/III was significantly increased in the TRB3 overexpression combined with the TGF- $\beta 1$ stimulated (T+TRB3) group ($P < 0.05$). The results were the opposite in the TRB3 downregulated combined with TGF- $\beta 1$ stimulated (T+shR-TRB3) group (Table III).

Protein expression levels of TRB3, β -catenin, E-cadherin, vimentin, fibronectin and α -SMA

TRB3 expression during EMT induced by TGF- $\beta 1$. TRB3 expression was significantly increased in the TGF- $\beta 1$ -stimulated group compared to the control group ($P < 0.05$, Fig. 2A).

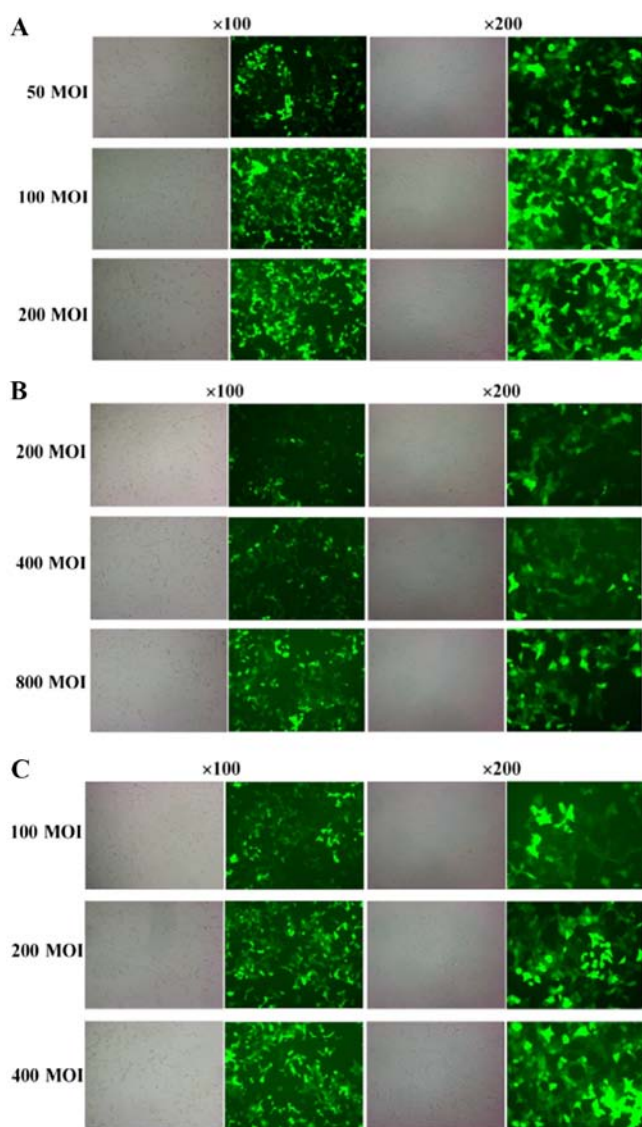


Figure 1. Fluorescence detection of the viral infection of MLE-12 cells. (A) GFP group, (B) TRB3 group and (C) shR-TRB3 group. MOI, multiplicity of infection; TRB3, Tribbles homologue 3.

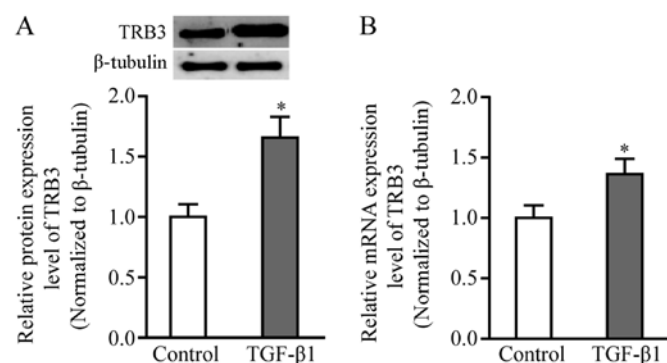


Figure 2. Effect of TGF- β 1 on TRB3 protein and mRNA expression. Impact of TGF- β 1 on the (A) protein expression level and (B) mRNA expression level of TRB3. Data are presented as the mean \pm SD. n=6. *P<0.05 vs. Control. TRB3, Tribbles homologue 3; TGF- β 1, transforming growth factor β 1.

Impact of TRB3 on EMT and β -catenin expression. The expression levels of both β -catenin and EMT mesenchymal

Table II. Effect of TRB3 expression on MLE-12 cell proliferation and apoptosis induced by TGF- β 1 (mean \pm SD, n=3).

Treatment group	OD value	Apoptotic rate (%)
Control	0.4387 \pm 0.0025 ^a	1.56 \pm 0.71 ^a
GFP	0.4227 \pm 0.0060 ^c	1.55 \pm 0.70 ^c
TRB3	0.2487 \pm 0.0330 ^b	10.16 \pm 0.5 ^b
shR-TRB3	0.5480 \pm 0.0291 ^b	1.15 \pm 0.07 ^b
TGF- β 1	0.3537 \pm 0.0189 ^d	3.87 \pm 0.13 ^d
T+GFP	0.3600 \pm 0.0240	3.84 \pm 0.23
T+TRB3	0.2340 \pm 0.0101 ^e	10.21 \pm 0.10 ^e
T+shR-TRB3	0.4213 \pm 0.0114 ^e	6.52 \pm 0.10 ^e

^aP>0.05, compared with the GFP group; ^bP<0.01, compared with the GFP group; ^cP<0.01, comparison between the TGF- β 1 and cell group; ^dP>0.05, compared with the T+GFP group; ^eP<0.01, compared with the T+GFP group. Groups: control group; Ad-GFP transduction group (GFP group); Ad-TRB3 transduction group (TRB3 group); Ad-TRB3-shRNA transduction group (shR-TRB3 group); group with TGF- β 1 stimulation only (TGF- β 1 group); TGF- β 1 and Ad-GFP transduction group (T+GFP group); TGF- β 1 and Ad-TRB3 transduction group (T+TRB3 group); and TGF- β 1 and Ad-TRB3-shRNA transduction group (T+shR-TRB3 group). TRB3, Tribbles homologue 3; TGF- β 1, transforming growth factor β ; OD, optical density.

protein markers were significantly increased in the TRB3 overexpression (TRB3) group, while expression of the epithelial marker, E-cadherin, was decreased dramatically (P<0.05). The results were the opposite in the TRB3 downregulated (shR-TRB3) group (Table IV).

TRB3 affects EMT induced by TGF- β 1 and the Wnt/ β -catenin signaling pathway. The expression levels of both β -catenin and EMT mesenchymal protein markers were significantly increased in the TGF- β 1 stimulation group compared to the control group, while E-cadherin expression was decreased dramatically (P<0.05). Compared to the control group, the expression levels of both β -catenin and EMT mesenchymal protein markers were found to be significantly increased in the TRB3 overexpression combined with TGF- β 1 stimulated group (T+TRB3); while E-cadherin expression was decreased dramatically (P<0.05). The results were the opposite in the TRB3 downregulated combined with TGF- β 1 stimulated (T+shR-TRB3) group (Table IV).

Visualizing expression of TRB3, β -catenin, E-cadherin, fibronectin and α -SMA. Since DAPI revealed the MLE-12 cells, and FITC revealed the different antibodies, Fig. 3A displays the expression of TRB3 in the different groups. The figure shows that the overexpression of TRB3, whether in the control group (left panels) or the TGF- β 1 administration group (right panels), coincides with a significant increase in the expression of β -catenin (Fig. 3B), fibronectin (Fig. 3C), α -SMA (Fig. 3D) and E-cadherin (Fig. 3E) in both the adenovirus vector transduction group (TRB3) and the adenovirus transduction combined with TGF- β 1 stimulated group (T+TRB3) P<0.05. The TRB3 downregulated group had opposing results.

Table III. TRB3, β -catenin, α -SMA and collagen I/III mRNA in MLE-12 cells.

Treatment group	β -catenin	α -SMA	Collagen I	Collagen III
Control	20.34 \pm 0.379 ^a	22.80 \pm 0.178 ^a	21.11 \pm 0.449 ^a	20.56 \pm 0.371 ^a
GFP	20.27 \pm 0.405	22.68 \pm 0.280	21.10 \pm 0.794	20.57 \pm 0.694
TRB3	23.36 \pm 0.319 ^b	24.80 \pm 0.983 ^b	23.71 \pm 0.193 ^b	23.08 \pm 0.489 ^b
shR-TRB3	18.53 \pm 0.285 ^b	21.16 \pm 0.118 ^b	19.61 \pm 0.193 ^b	18.50 \pm 0.145 ^b
TGF- β 1	24.41 \pm 0.128 ^c	25.81 \pm 0.783 ^c	24.67 \pm 0.101 ^c	24.10 \pm 0.554 ^c
T+GFP	24.40 \pm 0.143	25.84 \pm 0.716	24.63 \pm 0.525	24.10 \pm 0.299
T+TRB3	27.43 \pm 0.155 ^d	29.34 \pm 0.124 ^d	26.83 \pm 0.724 ^d	26.12 \pm 0.199 ^d
T+shR-TRB3	22.57 \pm 0.162 ^d	23.17 \pm 0.423 ^d	22.25 \pm 0.657 ^d	22.85 \pm 0.637 ^d

mRNA expression was assessed by RT-qPCR (average Ct value) and data are shown as mean \pm SD (n=6). ^aP>0.05, compared with the GFP group; ^bP<0.01, compared with the GFP group; ^cP>0.05, compared with the T+GFP group; ^dP<0.01, compared with the T+GFP group. Groups: control group; Ad-GFP transduction group (GFP group); Ad-TRB3 transduction group (TRB3 group); Ad-TRB3-shRNA transduction group (shR-TRB3 group); group with TGF- β 1 stimulation only (TGF- β 1 group); TGF- β 1 and Ad-GFP transduction group (T+GFP group); TGF- β 1 and Ad-TRB3 transduction group (T+TRB3 group); and TGF- β 1 and Ad-TRB3-shRNA transduction group (T+shR-TRB3 group). TRB3, Tribbles homologue 3; TGF- β 1, transforming growth factor β 1; α -SMA, α smooth muscle actin.

Table IV. Effect of TRB3 on β -catenin and EMT mesenchymal marker protein expression in TGF- β 1-stimulated MLE-12 cells.

Group	β -catenin	E-cadherin	Vimentin	Fibronectin	α -SMA
Control	350.46 \pm 1.324 ^a	127.23 \pm 1.134 ^a	108.22 \pm 0.542 ^a	113.54 \pm 0.832 ^a	208.35 \pm 0.956 ^a
GFP	351.05 \pm 0.751	127.64 \pm 1.272	108.33 \pm 0.767	115.09 \pm 0.772	208.20 \pm 1.063
TRB3	463.86 \pm 3.663 ^b	51.22 \pm 0.605 ^b	164.66 \pm 0.557 ^b	186.71 \pm 0.930 ^b	359.57 \pm 0.645 ^b
shR-TRB3	166.79 \pm 0.960 ^b	284.61 \pm 0.532 ^b	80.59 \pm 0.536 ^b	40.36 \pm 0.940 ^b	112.61 \pm 0.419 ^b
TGF- β 1	426.82 \pm 2.450 ^c	72.56 \pm 0.550 ^c	145.48 \pm 0.586 ^c	179.46 \pm 2.030 ^c	303.08 \pm 0.697 ^c
T+GFP	397.12 \pm 5.629	72.22 \pm 0.844	145.63 \pm 0.416	182.46 \pm 1.017	303.32 \pm 0.382
T+TRB3	567.28 \pm 1.609 ^d	35.52 \pm 1.147 ^d	216.39 \pm 0.435 ^d	321.55 \pm 1.374 ^d	427.65 \pm 0.432 ^d
T+shR-TRB3	186.02 \pm 1.276 ^d	123.51 \pm 0.532 ^d	90.33 \pm 0.444 ^d	58.50 \pm 0.886 ^d	154.01 \pm 0.719 ^d

Data are shown as the average OD value (mean \pm SD, n=6). ^aP>0.05, compared with the GFP group; ^bP<0.01, compared with the GFP group; ^cP>0.05, compared with the T+GFP group; ^dP<0.01, compared with the T+GFP group. Groups: control group; Ad-GFP transduction group (GFP group); Ad-TRB3 transduction group (TRB3 group); Ad-TRB3-shRNA transduction group (shR-TRB3 group); group with TGF- β 1 stimulation only (TGF- β 1 group); TGF- β 1 and Ad-GFP transduction group (T+GFP group); TGF- β 1 and Ad-TRB3 transduction group (T+TRB3 group); and TGF- β 1 and Ad-TRB3-shRNA transduction group (T+shR-TRB3 group). TRB3, Tribbles homologue 3; EMT, epithelial-mesenchymal transition; TGF- β 1, transforming growth factor β 1; α -SMA, α smooth muscle actin; OD, optical density.

Discussion

The pathogenesis of idiopathic pulmonary fibrosis (IPF) remains ambiguous. Its pathological features are a loss of alveolar epithelial cell membrane integrity and abnormal cell wound healing, resulting in a large amount of extracellular matrix (ECM) deposition. This disrupts normal physiological tissue structure and eventually leads to irreversible pulmonary structural remodeling (1). The transdifferentiation of fibroblasts to myofibroblast after activation, which produces an excessive amount of ECM, is one of the key steps in the progression of fibrotic disease. Transforming growth factor β 1 (TGF- β 1) is the key factor regulating fibroblast activation during the occurrence of fibrotic disease, and it is regulated by intricate intracellular signaling networks (13,14). Research has demonstrated that cells that have undergone EMT exhibit increased proliferation, while NSCLC cell lines with high

EMT gene signature scores (mesenchymal cell lines) were more sensitive to PLK1 (polo-like kinase 1) inhibition than epithelial lines (15).

The Wnt/ β -catenin signaling pathway plays an important role in the pathogenesis of various human diseases. Studies have demonstrated the underlying relationship between TGF- β 1 and the Wnt/ β -catenin signaling pathway. β -catenin and TGF- β pathway signals were found to co-regulate EMT formation, and exert a regulatory effect through transcriptional activation of cAMP response element binding protein (CREB) (16). Akhmetshina *et al* (17) indicated that blocking the Wnt/ β -catenin signaling pathway could serve as a new therapeutic method against fibrosis mediated by TGF- β . TRB3 has been proven to inhibit mitosis of renal tubular epithelial cells, induce cell apoptosis, and suppress cell proliferation activity. TRB3 overexpression could stimulate the classic TGF- β 1 signaling pathway and induce phenotypic transition of

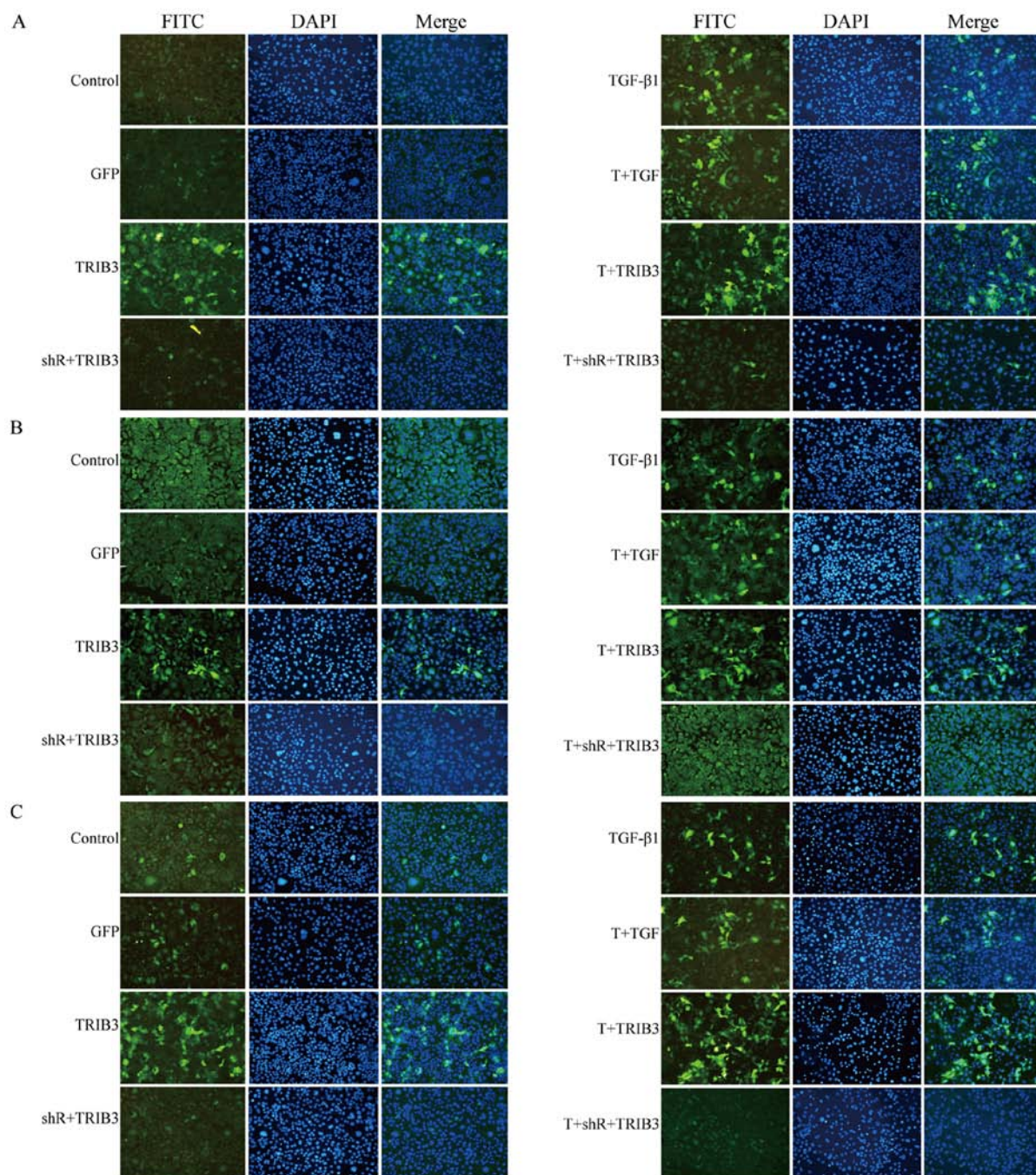


Figure 3. Immunofluorescence (IF) assay showing (A) TRB3, (B) β -catenin, (C) fibronectin protein expression in each group. Magnification, x100.

static fibroblasts; however, TGF- β also induces TRB3 expression. While TRB3 gene knockout led to significantly reduced TGF- β 1-induced fibrosis and collagen synthesis, previous studies demonstrated that in the fibroblasts of systemic sclerosis patients, TRB3 expression was increased in a TGF- β /Smad-dependent manner (18,19). Furthermore, Zhang *et al* (20) revealed that high TRB3 expression was observed in diabetic nephropathy mouse renal tissue, which showed a positive correlation between TGF- β 1 expression and kidney interstitial fibrosis level. A recent study demonstrated the role of TRB3 in regulating fibroblast activation and the onset and development of tissue or organ fibrosis, by stimulating the classic TGF- β signaling pathway (21). Based on the evidence above, we propose the following hypothesis. During

fibrosis, TGF- β 1 is involved in a positive feedback loop, where it can induce upregulation of TRB3 expression and activate the Wnt/ β -catenin signaling pathway. However, TRB3 can in turn affect TGF- β 1 and activate the classic TGF- β /Smad signaling pathway, leading to stimulation of collagen synthesis, and finally the abnormal activation of the TGF- β signaling pathway and the onset of fibrosis.

Our study found low TRB3 gene and protein expression in normal MLE-12 cells, whereas during TGF- β 1-induced EMT, TRB3 gene and protein expression was significantly upregulated. TGF- β 1 enhances all EMT hallmarks. TGF- β 1 administration along with the TRB3 vector promoted EMT to a greater extent; however, TGF- β 1 with shTRB3 altered all values to the levels of the control group. This suggests that inhibition

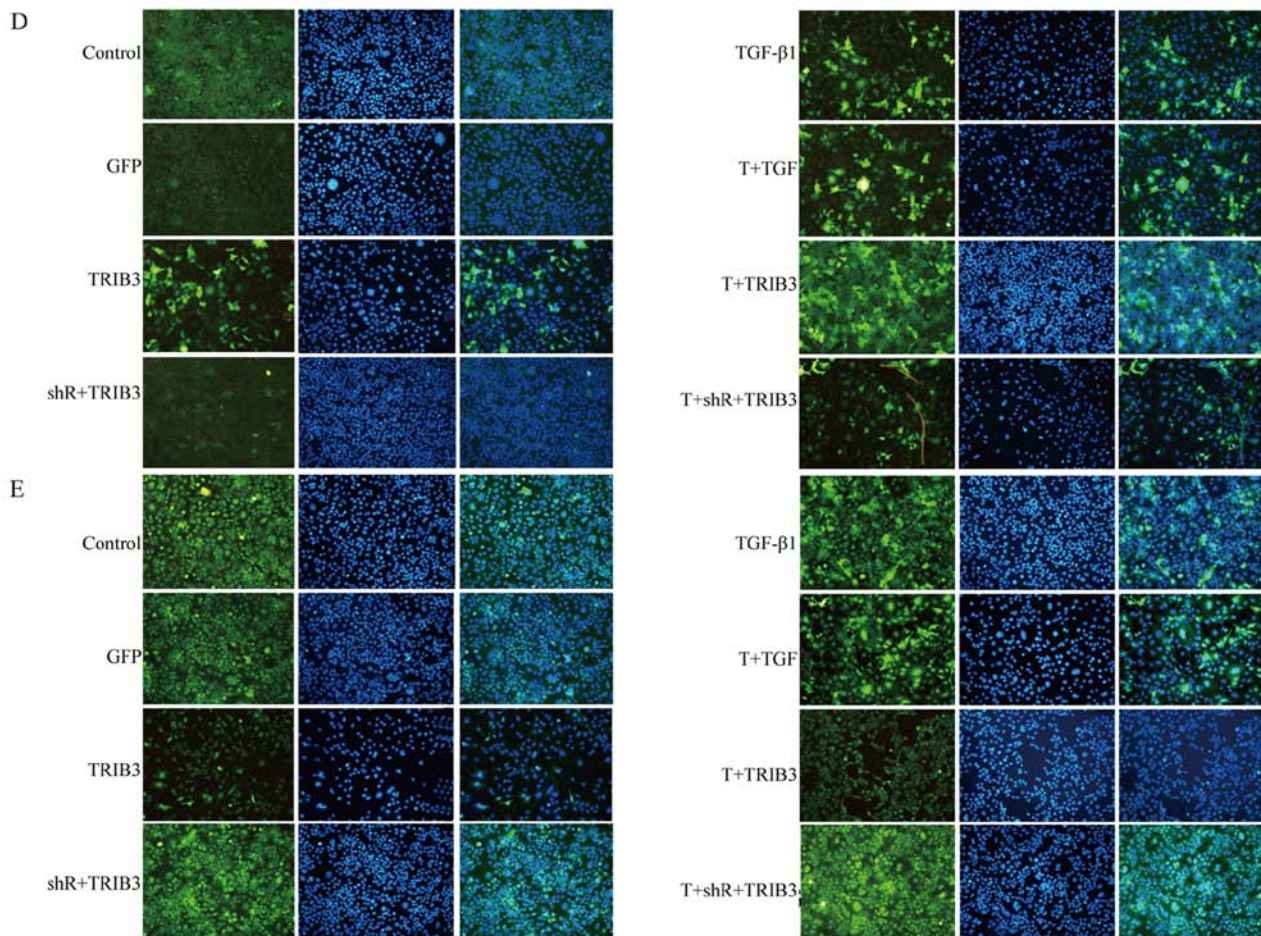


Figure 3. Continued. Immunofluorescence (IF) assay showing (D) α -SMA and (E) E-cadherin protein expression in each group. Magnification, $\times 100$. TRIB3, Tribbles homologue 3; TGF- β 1, transforming growth factor β 1; α -SMA, α smooth muscle actin.

of TRIB3 may interdict the entire pathway of TGF- β 1. In addition, when the results of the Ad-GFP group were compared with those of the control group, no significant expression changes in EMT-related proteins and genes were observed in the Ad-GFP group. This suggests that the adenovirus vector and GFP gene did not affect EMT, while significant upregulation or downregulation of EMT-related genes and proteins was found in the TRIB3 group and the shR-TRIB3 group, respectively. This indicates that EMT is impacted by overexpression or downregulation of TRIB3. One study reported that TGF- β 1 is a key cytokine in the promotion of EMT through the TGF- β 1/Smad signaling pathway, by interacting with Smad signaling protein, and subsequently further promoting related gene and protein expression (17). In our research, when the results of the TGF- β 1 group were compared with those of the control group, the expression levels of EMT-related genes and proteins were significantly increased, and fibrosis-related cytokines in the supernatant also increased, confirming the promotive effect of TGF- β 1 on EMT. This is consistent with the results of the aforementioned study (17). No significant alteration in the expression of EMT-related genes and proteins or fibrosis-related proteins and cytokines was discovered when the results of the T+Ad-GFP group were compared with those of the TGF- β 1 group. These results indicate that the adenovirus vector and GFP gene expression did not affect EMT. However, significant upregulation and downregulation of EMT-related

genes and proteins and fibrosis-related proteins and cytokines were found in the T+TRIB3 group and the T+shR-TRIB3 group, respectively, when compared with the TGF- β 1 stimulation only group. These results indicate that EMT induced by TGF- β 1 could be promoted by TRIB3 expression; while downregulated TRIB3 impaired TGF- β 1-induced EMT, with a probable mechanism of TRIB3 influencing EMT through the TGF- β /Smad signaling pathway.

Various signaling pathways have been demonstrated to be involved in pulmonary fibrosis, including the Smad-dependent signaling pathway and the Wnt/ β -catenin signaling pathway (22). These pathways are interconnected with one other and form an intricate network, modulating the onset of fibrosis, and the Wnt/ β -catenin pathway is an important part of this network. Our research demonstrated that expression of β -catenin, a key protein in the Wnt/ β -catenin signaling pathway, increased significantly during EMT induced by TGF- β 1. Furthermore, TRIB3 overexpression or downregulation affected expression of Wnt/ β -catenin signaling pathway-related proteins. Therefore, we conclude that TRIB3 may influence TGF- β 1-induced EMT through the Wnt/ β -catenin signaling pathway, and fibrosis can be modulated through the Wnt/ β -catenin signaling pathway by altering TRIB3 expression. Only one cell line was used in this study, and this was a limitation. siRNA or shRNA targeting β -catenin or chemicals that exhibit action on GSK3 β should be used in future research. We will also verify our experimental

results using animal models as well. Further study is required regarding the components within the regulatory network of fibrosis development, as well as the differences between *in vitro* and *in vivo* studies.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WY, FW and LM made substantial contributions to the conception and design of the present study. WY and FW collected, analyzed and interpreted the data. FW and LM drafted the manuscript. WY critically revised the manuscript. WY has given final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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