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Inhibition of Wnt/ β -catenin signaling suppresses bleomycin-induced pulmonary fibrosis by attenuating the expression of TGF- β 1 and FGF-2

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Abstract

Pulmonary fibrosis is a progressive lung disorder of unknown etiology, which is characterized by alterations in alveolar epithelium function, fibroblast activation, and increased extracellular matrix deposition. Recent studies have demonstrated that PF is associated with uncontrolled production of cytokines after lung injury. In the present study, we found that transforming growth factor- β 1 (TGF- β 1) and fibroblast growth factor 2 (FGF-2) were both upregulated in bleomycin-induced fibrotic lung tissue and primary murine alveolar epithelial Type II (ATII) cells treated with bleomycin. Furthermore, we discovered that TGF- β 1 could induce the differentiation of lung resident mesenchymal stem cells (LR-MSCs) into fibroblasts, which may play an essential role in PF. LR-MSCs incubated with FGF-2 showed modest alterations in the expression of α -SMA and Vimentin. Moreover, in our study, we found that Wnt/ β -catenin signaling was activated both *in vitro* and *in vivo* as a result of bleomycin treatment. Interestingly, we also found that suppression of the Wnt/ β -catenin signaling could significantly attenuate bleomycin-induced PF accompanied with decreased expression of TGF- β 1 and FGF-2 *in vitro* and *in vivo*. These results support that controlling the aberrant expression of TGF- β 1 and FGF-2 *via* inhibition of Wnt/ β -catenin signaling could serve as a potential therapeutic strategy for PF.

Keywords

Alveolar epithelial type II cells (ATII cells); Lung resident mesenchymal stem cells (LR-MSCs); Transforming growth factor (TGF)- β 1; Fibroblast growth factor (FGF)-2; Fibroblastic differentiation

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1. Introduction

Pulmonary fibrosis (PF) is a disease with high mortality for which lung transplantation is considered to be the only definitive therapy (Katzenstein and Myers, 1998). The pathogenesis of PF remains unclear, and its most frequent histopathological patterns include usual interstitial pneumonia, a process which is characterized by heterogeneous areas of dense fibrosis, honeycombing, fibroblast and myofibroblast accumulation and proliferation known as fibroblastic foci, and distortion of the lung architecture (Behr, 2013; King et al., 2011). Recently, emerging concepts suggest that repeated injury to bronchiole-like epithelial cells and hyperplastic type II alveolar epithelial cells induces honeycomb fibrosis (Katzenstein and Myers, 1998; King et al., 2011), and stem cells also play a key role in the development of PF (Chilosi et al., 2010; Mora and Rojas, 2013; Toonkel et al., 2013). Damaged epithelial cells produce various pro-fibrotic factors, including transforming growth factor β (TGF- β) (Pittet et al., 2001; Polosukhin et al., 2012) and fibroblast growth factor (FGF)-2 (Xiao et al., 2012), resulting in epithelial-to-mesenchymal transition (EMT) contributing to PF (Chen et al., 2014; Kim et al., 2006; Willis et al., 2005; Zhou et al., 2012a).

To date, lung MSCs have been shown to be multipotent vascular precursors, capable of fibroblastic, endothelial and pericyte differentiation *in vitro* (Chow et al., 2013). In the development of PF, a variety of cytokines act as functional regulators and exert potent systemic effects (Dinarello, 2004). However, many systemic regulators including cytokines also exhibit local effects including tissue fibrosis, and these include TGF- β (Ask et al., 2008; Penn et al., 2012), interleukin-6 (IL-6) (Le et al., 2014; O'Reilly et al., 2014), insulin-like growth factor (IGF) (Hamaguchi et al., 2008), platelet-derived growth factor (PDGF) (Betsholtz, 2004), and FGF (Maltseva et al., 2001). Cytokines may also exert anti-fibrotic effects, and these include interferon (IFN)- γ (King et al., 2009), hepatocyte growth factor (HGF) (Jinnin et al., 2005), and C-X-C motif chemokine (CXCL10) (Jiang et al., 2010). Thus, it is necessary to investigate the effects of these cytokines on the differentiation of lung MSCs, which could modulate the development of PF.

In our previous study, we found that Wnt signaling plays an important role in PF (Sun et al., 2014). The disordered Wnt/ β -catenin pathway has been reported in PF (Bowley et al., 2007), and activation of the Wnt signaling pathway can induce the activation and proliferation of fibroblasts, promoting the secretion of collagen fibers (Kurayoshi et al., 2007). Whether Wnt signaling participated in pulmonary fibrogenesis *via* activating those pro-fibrotic factors mentioned above remained unclear. In the present study, we intended to explore the relationship between pro-fibrotic cytokines and the activated Wnt signaling in pulmonary fibrogenesis. We investigated the expression of TGF- β 1 and FGF-2 in pulmonary fibrogenesis and explored the effects of these two cytokines in the differentiation of LR-MSCs (Sca-1⁺CD45⁻CD31⁻) into fibroblasts *in vitro*. We measured the levels of TGF- β 1 and FGF-2 in correlation to Wnt/ β -catenin signaling during pulmonary fibrogenesis to clarify the relationship between Wnt signaling and these two pro-fibrotic cytokines.

2. Materials and methods

2.1. Ethics statement

The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals and all experimental protocols were approved under the animal protocol number SYXK (Su) 2009–0017 by the Animal Care and Use Committee of Nanjing University.

2.2. ATII cell line culture

The mouse lung epithelial type II cell line MLE-12 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and maintained in a humidified atmosphere of 5% CO₂/95% air at 37 °C. All of the cells were passaged 1:2 or 1:3 using 0.25% trypsin when they reached 70–90% confluence.

2.3. Isolation of LR-MSCs

Lung single-cell suspensions were prepared from the pooled lungs of at least 5 C57BL/6 mice (4–6 weeks old). In brief, mice were sacrificed by cervical dislocation. The lung parenchyma from the mice was digested by fine mincing with a razor blade, followed by incubation in an enzyme mixture containing 0.2% collagenase I (Sigma, USA), 2.4 U/mL dispase (Sigma, USA) and 0.001% DNase (Sigma, USA) for 1 h at 37 °C with shaking. This suspension was filtered through 100-µm and 40-µm filters, centrifuged, and depleted of red blood cells by lysis. Cells were resuspended in PBS at 1×10^7 cells/mL and stained for stem cell antigen (Sca)-1, CD45 and CD31 followed by sorting using the AutoMACS cell separator system (Miltenyi Biotec, Bergisch Gladbach, Germany).

Freshly isolated LR-MSCs were cultured at a concentration higher than 10^5 cells/mL with DMEM containing 15% fetal bovine serum, 4% L-glutamine, 1% nonessential amino acids, and 1% penicillin and streptomycin, and maintained in a humidified atmosphere of 95% air, 5% CO₂ at 37 °C. The cells were passaged 1:2 using 0.25% trypsin when they reached 70–90% confluence.

2.4. Cell viability assay

Cell viability was measured by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). The principle of CCK-8 is based on the conversion of an orange-colored product from water-soluble tetrazolium salt (WST-8) by dehydrogenases in live cells. ATII cells were cultured in various concentrations of bleomycin (25 µM, 50 µM, 100 µM, 200 µM, 400 µM, and 800 µM) in 96-well plates for 24 h followed by the CCK-8 assay according to the instructions from the manufacturer. Cell proliferation = (the absorbance of experimental group — the absorbance of blank group) / (the absorbance of control group — the absorbance of blank group) × 100%.

2.5. Bleomycin-induced mouse PF model

Male C57BL/6 mice (6–7 weeks old) were purchased from the Medical School of Yangzhou University (Yangzhou, China). All mice were maintained under standard conditions with

free access to water and laboratory rodent food. Following anesthesia with pentobarbital sodium (3 mg/kg), mice received a single, slow intratracheal injection of 5 mg/kg bleomycin (Nippon Kayaku, Tokyo, Japan) dissolved in 50 µl of saline with MicroSprayer (Penn-Century, Wyndmoor, PA). Control mice received 50 µl of saline only. Mice were sacrificed 7 or 14 days after bleomycin instillation. Mouse lungs were obtained for histopathologic analysis and collagen assay (Wang et al., 2014).

For the evaluation of the effect of XAV 939, a small-molecule compound that specifically inhibits Wnt/β-catenin signaling (Wang et al., 2014), 10 days after administration of bleomycin, XAV939 was administered intraperitoneally at 5 mg/kg for 11 consecutive days. Mice were sacrificed 21 days after bleomycin instillation.

2.6. Histology

Lower right lungs were fixed in 4% (w/v) neutral phosphate-buffered paraformaldehyde for 24 h, dehydrated, transparentized and embedded in paraffin. Lung tissues were cut into 5-µm sections which were stained with hematoxylin–eosin (H&E) for structured observation, or with Masson's trichrome stain for detection of collagen deposits, or were subjected to immunohistochemistry analysis.

2.7. Immunohistochemistry analysis

Immunohistochemistry staining for detection of fibroblast activation *in vivo* was performed as previously described (Sun et al., 2014). Briefly, the slides were cleared of paraffin and subjected to antigen retrieval. Next, quenching of endogenous peroxidase activity was achieved by incubation with 3% (v/v) H₂O₂ for 10 min. The primary antibodies were employed as follows: rabbit anti-TGF-β1, rabbit anti-FGF-2 and rabbit anti-β-catenin (Abcam, Cambridge, MA). The secondary antibodies incubated were horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (Boster, Wuhan, China). Immunoreactive protein bands were detected using an Odyssey Scanning System (LI-COR, USA). As a negative control, the primary antibody was replaced with non-immune IgG.

2.8. Flow cytometric analysis

To determine the expression of various surface markers, LR-MSCs following the first passage were incubated with fluorescent antibodies at 37 °C for 40 min in the dark followed by two washes with PBS. Flow cytometry was performed on a FACS Calibur™ flow cytometer and the data were analyzed using Paint-A-Gate software (Becton Dickinson). The antibodies used were: FITC-conjugated anti-Ly-6A/E (Sca-1), PE-conjugated anti-CD45, PE-conjugated anti-CD90, PE-conjugated anti-CD44 and FITC-conjugated anti-CD106 (all from eBioscience, San Diego, CA); APC-conjugated anti-CD31 and PE-conjugated anti-CD29 (Bio-legend, San Diego, CA).

2.9. RNA extraction and quantitative real-time polymerase chain reaction (Q-PCR)

Total RNA was isolated from each sample using Column Animal RNAout according to the manufacturer's instructions. An Easyscript first-strand cDNA synthesis super mix kit (Vazmye, Nanjing, China) was used for reverse transcription polymerase chain reaction (RT-PCR). The sequences of primer pairs used in this assay are listed in Table 1. Q-PCR was

conducted by amplifying 20 µl of diluted cDNA with the SYBR Green Q-PCR kit (Roche, Germany) on a 7300 Real-Time PCR System (ABI, America). Each sample was run in triplicate, and PCR reactions without the addition of the template were used as blank controls. The relative quantification of the expression of the target genes was measured using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. After completion of the PCR amplification, data were analyzed with 7300 Sequence Detection Software (ABI, USA).

2.10. Immunofluorescent staining and Western blot assay

The immunofluorescence analysis was performed as described (Shi et al., 2014). The following primary antibodies were employed: rabbit anti-collagen I and rabbit anti- α -smooth muscle actin (SMA), rabbit anti-E-cadherin (all from Abcam, Cambridge, MA). Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen, USA) was used as the secondary antibody. Nuclei were stained with 1 g/mL DAPI (Sigma). The images were captured using a confocal fluorescence microscope (Olympus, Tokyo, Japan).

For Western blotting analysis, whole cell lysates were separated using SDS/10% PAGE and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Roche, Germany) by standard procedures. After the membranes had been blocked, primary antibodies were added to the membranes followed by incubation at 4 °C for 12 h. After three washes in PBST, the membranes were incubated with the secondary antibody at 37 °C for 1 h. The primary antibodies employed were: rabbit anti-TGF- β 1, rabbit anti- β -catenin, rabbit anti-FGF-2, rabbit anti-collagen I, rabbit anti- α -smooth muscle actin, rabbit anti-vimentin, rabbit anti-cytokeratin 18 (CK18), rabbit anti-cytokeratin 19 (CK19) and mouse anti- β -actin (Abcam, Cambridge, MA). Horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (Boster, Wuhan, China) was used as the secondary antibody. Immunoreactive protein bands were detected using an Odyssey Scanning System (LI-COR, Lincoln, NE).

2.11. Statistical analysis

Experimental results were expressed as mean $\pm$ standard deviation. Differences were analyzed for significance ($P < 0.05$) by one-way ANOVA using SPASS for windows version 11.0 (SPASS, Chicago, IL).

3. Results

3.1. TGF- β 1 and FGF-2 are upregulated in bleomycin-induced PF along with the activation of Wnt/ β -catenin signaling

Following intratracheal spray of bleomycin, alveolar structure was destroyed and collagen deposition started to occur in lung tissues from day 7 to day 14 as assessed by H&E (Fig. 1A) and Masson's trichrome staining (Fig. 1B). Compared with saline controls, the expression of α -SMA and collagen I was markedly increased (Fig. 1C). Epithelial markers such as CK19 and CK18 were significantly downregulated in lungs as a result of bleomycin-induced injury (Fig. 1C). Moreover, the expression of β -catenin was upregulated, suggesting activated Wnt/ β -catenin signaling in the bleomycin-induced PF model (Fig. 2). In addition,

the protein levels of pro-fibrotic cytokines TGF- β 1 and FGF-2 were substantially upregulated (Fig. 2).

3.2. TGF- β 1 and FGF-2 promotes the fibroblastic differentiation of LR-MSCs

The primary LR-MSCs from mouse lung tissues were isolated by MACS as previously reported (Shi et al., 2014). The purity of LR-MSCs was confirmed by the expression of CD44, CD90, CD29 and Sca-1, but not CD45 or CD31 (Fig. 3A).

Next, in order to confirm whether the pro-fibrotic cytokines TGF- β 1 and FGF-2 that were upregulated by injured ATII cells could induce LR-MSCs to differentiate into fibroblasts, LR-MSCs were incubated with FGF-2 or TGF- β 1 for 7 days. Despite the fact that no significant morphological changes were observed in LR-MSCs following incubation with FGF-2 (Fig. 3B), FGF-2 could modestly induce the expression of fibroblast markers α -SMA and collagen in LR-MSCs (Fig. 3C and D). The expression of these markers in LR-MSCs was also quantified by real-time PCR and Western blotting (Fig. 3E and F), which was consistent with the immunofluorescence data, confirming the modest effect of FGF-2 on the differentiation of LR-MSCs to fibroblasts.

In the presence of TGF- β 1, approximately 90% of LR-MSCs showed remarkable morphological changes after incubation with TGF- β 1 (Fig. 3B). Furthermore, TGF- β 1 could substantially induce the expression of α -SMA and collagen in LR-MSCs as demonstrated by immunofluorescence staining (Figs. 4C and 3D). The expression patterns were also confirmed by Real-time PCR and Western blotting (Fig. 3E and F). In conclusion, treatment with TGF- β 1 could strongly induce LR-MSCs to differentiate into fibroblasts. Our results supported that TGF- β 1 may play a critical role in the fibroblastic differentiation of LR-MSCs aggravating PF.

3.3. Blocking Wnt/ β -catenin signaling suppresses TGF- β 1 and FGF-2 in the bleomycin-induced PF model

XAV939 has previously been demonstrated to effectively suppress bleomycin-induced PF *in vivo* by inhibiting Wnt/ β -catenin signaling (Wang et al., 2014). We reproduced this model in the current study and demonstrated again that XAV939 significantly reduced the severity of PF as assessed by H&E (Fig. 4A). And, bleomycin-induced expression of α -SMA, collagen, MMP-7, TGF- β 1, FGF-2 and β -catenin was significantly inhibited by XAV939 (Fig. 4B and C). In contrast, treatment with XAV939 resulted in a marked increase in the expression of E-cadherin in lung tissues (Fig. 4B and C), consistent with the effect of XAV939 on suppressing bleomycin-induced PF.

3.4. Blocking Wnt/ β -catenin signaling suppresses bleomycin-induced TGF- β 1 and FGF-2 expression in the ATII cell line

It has been previously reported that epithelial cells are subjected to injury after intratracheal injection of bleomycin, a trigger of the EMT process (Moeller et al., 2008; Moore and Hogaboam, 2008). To confirm the effect of bleomycin on ATII cell viability, we cultured ATII cells with various concentrations of bleomycin and cell proliferation was measured by

CCK-8. At a concentration higher than 25 μ M, bleomycin could remarkably inhibit the viability of ATII cells (Fig. 5B).

To determine the role of Wnt/ β -catenin signaling on the EMT process of ATII cells, we measured the expression of epithelial and fibroblast markers before and after the addition of DKK-1, which is a stable inhibitor of Wnt/ β -catenin signaling with low *in vitro* cytotoxicity (Shi et al., 2015). DKK-1 could restrain bleomycin-induced EMT process in ATII cells (Fig. 5A). Moreover, bleomycin induced TGF- β 1 and FGF-2 expression, which was suppressed by DKK-1 (Fig. 5C and D). In order to confirm the role of TGF- β 1 and FGF-2 in bleomycin-induced fibrosis, recombinant TGF- β 1 or FGF-2 was administered together with DKK-1. We found that TGF- β 1 and FGF-2 could downregulate the level of E-cadherin and induce the expression of collagen and α -SMA (Fig. 5E), antagonizing the effect of DKK-1. Taken together, these data indicated that administration of exogenous TGF- β 1 and FGF-2 could abrogate the therapeutic effects of Wnt/ β -catenin signaling inhibition in this PF model.

4. Discussion

PF is a fatal lung disease characterized by progressive and irreversible destruction of lung architecture. It is well-accepted that repetitive injury of the alveolar epithelium, in the presence or absence of local inflammation, leads to deregulated wound-repair processes and an accumulation of extracellular matrix (Gross and Hunninghake, 2001). One of the mediators linked to the impaired wound-healing response in PF is the Wnt/ β -catenin signaling pathway (Konigshoff and Eickelberg, 2010; Selman et al., 2008). Here, we report that inhibiting Wnt/ β -catenin signaling could suppress the production of the pro-fibrotic cytokines TGF- β 1 and FGF-2 in bleomycin-treated mice and ATII cells cultured with bleomycin. We provide both *in vitro* and *in vivo* evidence that the two cytokines were suppressed at the mRNA as well as protein levels following inhibition of Wnt/ β -catenin signaling.

The Wnt/ β -catenin signaling pathway has been implicated in the development of experimental and human PF (Chilosi et al., 2003; Konigshoff et al., 2008; Konigshoff et al., 2009). However, the cell-specific mechanism by which Wnt/ β -catenin signaling exerts its effect on cellular function remains elusive. Crosstalk between TGF- β and Wnt/ β -catenin signaling has recently been demonstrated as crucial for the EMT and the development of lung fibrosis (Kim et al., 2006; Li et al., 2011; Zhou et al., 2012b). Verena and colleagues found no EMT-like signature in the transcriptome array of wnt3 α -stimulated ATII cells, which indicates that whereas canonical Wnt signaling alone may not drive EMT processes, other cytokines, such as TGF- β , may exert their pro-fibrotic effects *via* β -catenin activation (Aumiller et al., 2013). In a previous study, we demonstrated that activated Wnt/ β -catenin signaling could induce the expression of its target gene MMP-7 (Wang et al., 2015), which is one of the genes most consistently elevated in fibrotic lungs. MMP-7 has been described as a pro-fibrotic metalloproteinase (Pardo et al., 2008). Several lines of research suggest that MMP-7 may promote a fibrotic response *via* regulatory effects on epithelial repair and release of latent TGF- β (Pardo et al., 2008). However, the relationship between Wnt/ β -

catenin signaling and FGF-2 remain unknown, which needs to be further investigated in future studies.

Furthermore, LR-MSCs are an important regulator of pathophysiological processes including lung repair and regeneration, fibrosis, and inflammation. LR-MSCs have been found to be able to renew and proliferate to form epithelial cells *in vivo* and *in vitro* (Gong et al., 2014), and these cells can be triggered by local factors to differentiate into fibroblasts which contributes to disease progression (Foronjy and Majka, 2012). Therefore, LR-MSCs are potentially useful in regenerative therapies, for example, in damaged lung tissue repair (Tzouvelekis et al., 2011). It is thus worth exploring the trigger that induces the differentiation of LR-MSCs to fibroblasts in PF. Our findings demonstrate that both TGF- β 1 and FGF-2 could induce LR-MSCs to differentiate into fibroblasts, with TGF- β 1 exerting a more pronounced role. Interestingly, Bosse et al. reported synergism between FGF-2 and TGF- β 1 in human bronchial smooth muscle cell proliferation (Bosse et al., 2006). We assume that this synergism also exists in the differentiation of LR-MSCs into fibroblasts, which awaits further experimental validation.

In conclusion, we provide evidence that the expression of the two pro-fibrotic cytokines TGF- β 1 and FGF-2 was correlated to Wnt/ β -catenin activation in alveolar epithelial cells, providing further support for a disturbed autocrine and paracrine signaling loop during PF. Our data support a potential therapeutic strategy for PF by targeting TGF- β 1 and FGF-2 suppression *via* inhibition of Wnt/ β -catenin signaling.

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Fig. 1.

Pulmonary fibrosis was induced in bleomycin-treated mice. Mice ($n = 10$ in each group) received either saline or bleomycin (5 mg/kg body wt in 50 μ l saline) intratracheally. Mice were sacrificed after 7 or 14 days, and pulmonary fibrosis was determined by hematoxylin–eosin (H&E) staining ($\times 200$; A) and collagen was revealed by Masson's trichrome staining ($\times 200$; B). Representative images are shown. C. The expression of CK-18, CK-19, α -SMA and collagen I in lung tissues was measured by Western blotting. Representative gel electrophoresis bands are shown. Quantification of the band intensities is provided.

Fig. 2.

FGF-2 and TGF-β1 were upregulated in bleomycin-treated mice, along with the activation of Wnt/β-catenin signaling. A. The levels of β-catenin, FGF-2, and TGF-β1 in the lung tissues of bleomycin-induced pulmonary fibrosis were examined by immunohistochemistry (×200) (n = 10 per group). Representative images are shown. B and C. The expression of β-catenin, TGF-β1 and FGF-2 was measured by q-PCR and Western blotting. Representative gel electrophoresis bands are shown.

Fig. 3.

FGF-2 and TGF- β 1 induced LR-MSCs to differentiate into fibroblasts. LR-MSCs were cultured in the presence of FGF-2 or TGF- β 1 at 10 nM for 7 days. A. The expression of Sca-1, CD29, CD31, CD44, CD45 and CD90 on LR-MSCs was measured by flow cytometry. Red lines, isotype control staining. Blue lines, specific antibody staining. B. Contrasting images of the fibroblastic differentiation of LR-MSCs after culture with FGF-2 or TGF- β 1 for 7 days are shown. C and D. α -SMA and collagen expression in LR-MSCs cultured with FGF-2 or TGF- β 1 for 0 (Con) and 7 days was analyzed by immunofluorescent staining. α -SMA and collagen I were revealed with secondary Alex594-labeled antibodies; nuclei were revealed with DAPI staining. E and F. The mRNA and protein levels of α -SMA, vimentin and collagen I in LR-MSCs were analyzed by real-time Q-PCR and Western Blot after culture with FGF-2 or TGF- β 1 for 7 days.

Fig. 4.

XAV939 inhibited the development of pulmonary fibrosis and the expression of pro-fibrotic cytokines in bleomycin-treated mice. Mice were treated with XAV939 daily at 5 mg/kg intraperitoneally for various time periods as indicated 10 days after the administration of bleomycin. Mice were sacrificed at day 21 after bleomycin instillation. A. Pulmonary fibrosis was determined by H&E staining ($\times 200$). B. The levels of β -catenin, FGF-2, and TGF- β 1 were examined by immunohistochemistry ($\times 200$) ($n = 10$ per group). Representative images were shown. C. The expression of α -SMA, collagen I, β -catenin, MMP-7, E-cadherin, TGF- β 1 and FGF-2 was measured by Western blotting. Representative gel electrophoresis bands are shown. Quantification of the band intensities is provided.

Fig. 5.

DKK-1 suppressed bleomycin-induced EMT and pro-fibrotic cytokine secretion in ATII cells. **A.** α -SMA and E-cadherin expression in ATII cells cultured with bleomycin and DKK-1 for 0 (Con) and 7 days was analyzed by immunofluorescent staining. α -SMA and E-cadherin were revealed with secondary Alex488- and 594-labeled antibodies; nuclei were revealed by DAPI staining. **B.** ATII cells were cultured in various concentrations of bleomycin (0, 25, 50, 100, 200, 400, 800 μ M) in 96-well plates followed by the CCK-8 assay at 24 h. Six repeats were performed. * $P < 0.05$ vs. control. **C.** FGF2 and TGF- β 1 mRNA levels in ATII cells were examined by real-time PCR analysis. **D.** The expression of the fibrosis and epithelial markers collagen I, α -SMA and E-cadherin, the levels of pro-fibrotic cytokines TGF- β 1 and FGF-2, and the Wnt/ β -catenin signaling relevant proteins MMP-7 and β -catenin in ATII cells were analyzed by Western blot. Representative gel electrophoresis bands are shown. Quantification of the band intensities is provided. **E:** ATII cells were incubated with DKK-1 together with recombinant TGF- β 1 (10 ng/ml) or FGF-2 (10 ng/ml). The levels of E-cadherin, collagen and α -SMA were determined by Western blot

Table 1

RT-PCR primers and products.

Genes	Forward primer	Reverse primer
TGF- β 1	TGCTAATGGTGGACCGCAACAAC	GGGCACTGCTTCCCGAATGTCTG
FGF-2	GACGGCTGCTGGCTTCTAAGTGT	GCCTTCTGTCCAGGTCCCGTTTT
α -SMA	CCCAGATTATGTTTGAGACCTTC	ATCTCCAGAGTCCAGCACATAAC
Vimentin	CCTGGAGTCACTTCCTCTGGTTG	TCTTGCTGGTACTGCACTGTTGC
Collagen I	CTTCTGGTCCTCGTGGTCTCCCT	AAGCCTCGGTGTCCCTTCATTCC
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA