Inhibition of Wnt/β-catenin signaling promotes epithelial differentiation of mesenchymal stem cells and repairs bleomycin-induced lung injury

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Wang C, Zhu H, Sun Z, Xiang Z, Ge Y, Ni C, Luo Z, Qian W, Han X. Inhibition of Wnt/β-catenin signaling promotes epithelial differentiation of mesenchymal stem cells and repairs bleomycin-induced lung injury. Am J Physiol Cell Physiol 307: C234–C244, 2014. First published June 4, 2014; doi:10.1152/ajpcell.00366.2013.—Idiopathic pulmonary fibrosis is a progressive lung disorder of unknown etiology. Previous studies have shown that aberrant activation of the Wnt/β-catenin signaling cascade occurs in lungs of patients with idiopathic pulmonary fibrosis. Given the important roles of the Wnt/β-catenin signaling pathway in the development of pulmonary fibrosis, we targeted this pathway for the intervention of pulmonary fibrosis, and our results demonstrated that XAV939, a small molecule that specifically inhibits Wnt/β-catenin signaling, could inhibit the activation of Wnt/β-catenin signaling and attenuated bleomycin-induced lung fibrosis in mice, and thus improved the survival of mice with lung injury. Interestingly, previous investigations have confirmed that endogenous and exogenous mesenchymal stem cells could be recruited to the injured lung, although the exact effects of these cells are debatable. To determine the effect of Wnt/β-catenin signaling in the epithelial differentiation of bone marrow-derived mesenchymal stem cells (BM-MSCs), we established a coculture system that contains BM-MSCs and alveolar type II epithelial cells. The in vitro experiments demonstrated that XAV939 could promote the differentiation of BM-MSCs into an epithelium-like phenotype in the coculture system. We also found that XAV939 could inhibit the proliferation and myofibroblast differentiation of NIH/3T3 fibroblasts. This work supports that inhibition of the Wnt/β-catenin signaling pathway may be exploited for the treatment of idiopathic pulmonary fibrosis for which effective treatment strategies are still lacking.

Idiopathic pulmonary fibrosis; Wnt/β-catenin signaling pathway; XAV939; coculture; bone marrow-derived mesenchymal stem cells; alveolar type II epithelial cells

IDIOPATHIC PULMONARY FIBROSIS (IPF) is a chronic lung disease, whose etiology remains a mystery (15). It is clinically characterized by a progressive loss of lung function leading to dyspnea and restricted respiration (3). The mean survival time is 2 to 3 years after diagnosis (9, 16). The pathogenesis of IPF is not fully understood and no effective drug treatment is available at present. Thus, it is important to unravel the mechanisms underlying the pathogenesis of pulmonary fibrosis to provide effective treatment targets.

Accumulating evidence suggests that the main pathological characteristics of IPF include aberrant proliferation of fibroblasts leading to formation of scar tissues, which may subsequently result in destruction of the lung architecture (16). Activation of the Wnt/β-catenin signaling pathway can increase the activation and proliferation of fibroblasts, promoting the secretion of collagen fiber (19). Being the classical Wnt signal pathway, the Wnt/β-catenin signaling pathway plays an important role in the proliferation, polarity determination, and growth direction of cells in embryonic development (20, 21). Furthermore, the aberrant activation of the Wnt/β-catenin signaling cascade has been observed in the lungs of patients with IPF (17). Taken together, abnormal activation of the Wnt/β-catenin signaling pathway is closely associated with the occurrence and development of pulmonary fibrosis. Hitherto no effective treatment for IPF has been reported. We therefore tested the effect of a Wnt/β-catenin-specific inhibitor in promoting the repair of the injured lung by suppressing the aberrant proliferation of fibroblasts and the development of pulmonary fibrosis. In addition, we also tried to unravel the possible mechanisms in this process.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are characterized by a number of features including easy isolation and culture, as well as multipotent differentiation potential along the osteogenic, chondrogenic, adipogenic, and many other lineages (24). Endogenous and exogenous mesenchymal stem cells can be recruited to the injured lung followed by differentiation into fibroblasts to facilitate the development of pulmonary fibrosis, or by differentiation into alveolar epithelial cells to repair the lung injury (30, 40). BM-MSCs have attracted attention for their antifibrotic effects. However, the role of BM-MSCs in pulmonary fibrosis is still inconclusive (11, 18, 27). BM-MSCs have the potential for multidirectional differentiation, including the capacity of differentiation into the epithelial cell lineage (24, 37), but the mechanism that accounts for the differentiation of BM-MSCs into alveolar epithelial cells remains unclear. Alveolar type II epithelial cells (ATII cells), which form the most important bioactive component of pulmonary epithelium, synthesize and secrete pulmonary surfactant (31). BM-MSCs may be induced by ATII cells to an epithelium-like phenotype. Rojas and colleagues (30) believed that the transplanted BM-MSCs are capable of pro-
motating the repair of lung fibrosis by differentiating into alveolar epithelial cells. However, Hashimoto's research suggested that the transplanted BM-MSCs would differentiate into fibroblasts (11). Previous studies have demonstrated that the Wnt/β-catenin signaling pathway is also involved in the growth regulation of many types of stem cells to maintain these stem cells in a state of self-renewal (26). BM-MSCs express a certain level of the endogenous Wnt protein and regulate their own Wnt/β-catenin signaling to maintain proliferation and function through an autocrine or paracrine loop (8). Taking the effects of Wnt/β-catenin signaling in IPF into consideration, we speculated that a small-molecule compound that specifically inhibits the Wnt/β-catenin signaling may promote the differentiation of BM-MSCs into alveolar epithelial cells, and this process may play a role in the repair of bleomycin-induced pulmonary fibrosis.

XAV939 is a small-molecule compound that specifically targets Wnt/β-catenin signaling. XAV939 can maintain the integrity of the death complex by stabilizing the Axin2 structure via inhibiting the activity of the end anchorage polymerase PARP, eventually leading to the degradation of β-catenin and the suppression of Wnt/β-catenin signaling (13, 28). Unlike the constitutive expression of Axin1, the expression of Axin2 is induced by activated Wnt signaling, and Axin2 is involved in the negative feedback regulation (14). However, Axin2 has the same function as Axin1 (5). Therefore, XAV939 can restrain the abnormal activation of the Wnt/β-catenin signaling pathway without affecting the normal function of the cells. Compared with other Wnt signaling pathway inhibitors, one of the advantages of XAV939 lies in its strong specificity, as it does not inhibit other signaling pathways such as NF-κB and transforming growth factor-β (TGF-β) (13).

In the present study, by setting up a bleomycin-induced mouse pulmonary fibrosis model, we investigated the effects of XAV939 on the activation of Wnt/β-catenin signaling pathways and pulmonary fibrosis development in mice. Furthermore, we established a coculture system containing BM-MSCs and ATII cells to determine the effect of XAV939 on the epithelial differentiation of BM-MSCs. XAV939-mediated myofibroblastic differentiation of NIH/3T3 fibroblasts was also investigated.1

**MATERIALS AND METHODS**

**Ethics statement.** 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 protocol was approved by the Experimentation Ethics Review Committee of Nanjing University (ethics approval no. A9089). All surgery was performed under pentobarbital sodium anesthesia, and all efforts were made to minimize suffering.

**Mesenchymal stem cell culture and flow cytometric analysis.** C57BL/6 mice (6–7 wk old) were bought from the Animal Feeding Center of Yangzhou University (Yangzhou, China). Isolation and culture of mesenchymal stem cells and flow cytometric analysis were performed according to a previously described protocol (37). The cells were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Thermo Scientific, San Jose, CA) with 10% FBS (GIBCO, Paisley, UK; Invitrogen, Carlsbad, CA), 1% l-glutamine, and a 1% solution of penicillin and streptomycin. Cells were seeded at a density of 1 × 10^6 cells/ml onto uncoated flasks and cultured in a humidified incubator at 37°C in 5% CO2. The primary cells were split once they were confluent, and the immediate subculture was defined as passage 1. Passages 2, 3, 4, etc. were thus obtained accordingly following furtherpassaging.

For flow cytometric analysis, 1 × 10^6 passage 5 BM-MSCs were incubated with fluorescence-conjugated primary antibodies at 37°C for 1 h in the dark followed by three washing steps with PBS. Cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) and data were analyzed with BD CellQuest Pro software (Becton-Dickinson). The following antibodies were employed: phycoerythrin (PE)-conjugated CD29 from Bio-Legend (San Diego, CA), PE-conjugated CD44 from Antibexa America (Huntington Sta., NY), PE-conjugated CD34 from eBioscience (Santa Cruz, CA), FITC-conjugated SCA-1, FITC-conjugated CD106, FITC-conjugated CD11B, and FITC-conjugated CD45 from Millipore (Billerica, MA).

**Cell apoptosis was measured by Annexin V-FITC and propidium iodide (PI) staining through flow cytometry.** The cells were transplanted to six-well culture plates at a density of 1 × 10^6 cells/ml and were cultured for 12 h followed by treatment with 10^{-7} to 10^{-3} M XAV939 for 24 h. Cells were collected after centrifugation at 300 g for 5 min. Next, cells were washed twice with cold PBS and were resuspended in 500 μl binding buffer at a concentration of 1 × 10^6 cells/ml. Each cell sample was then stained with 5 μl Annexin V-FITC and 5 μl PI according to the manufacturer’s instructions. The cells were then incubated in the dark at 25°C for 15 min. Samples were acquired on a FACScalibur flow cytometer and analyzed with BD CellQuest Pro software (Becton-Dickinson).

**ATII cell line culture.** The ATII cells from Yili Bio-technology (Shanghai, China) were cultured similarly as described for mesenchymal stem cells except that 5% FBS was used.

**Indirect coculture experiments.** Indirect coculture was established by using cell culture inserts (0.4 μm pore, 4.5 cm2, Corning, NY) as described (37). Both BM-MSCs and ATII cells were plated at 10^5/ml at each plating time. We recorded the changes of cell morphology over a period of 14 days. Next, inserts were removed and BM-MSCs were harvested for RT-PCR, Western blotting, and immunofluorescence staining.

**NIH/3T3 fibroblast culture.** NIH/3T3 fibroblasts (Chinese Academy of Sciences) were cultured in high DMEM (Hyclone) with 10% FBS (GIBCO), 1% l-glutamine, and a 1% solution of penicillin and streptomycin.

**CCK-8 assay.** Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan), which measures cell viability, is based on the conversion of an orange-colored product from water-soluble tetrazolium salt (WST-8) by dehydrogenases in live cells. When NIH/3T3 fibroblasts or ATII cells reached 80% confluence, they were routinely passaged using 0.25% trypsin and were diluted 1:3 at each passage. NIH/3T3 fibroblasts or ATII cells were cultured in various concentrations of XAV939 (10^{-8} to 10^{-4} M) in 96-well plates followed by the CCK-8 assay at 12 h, 24 h, 36 h, or 48 h according to the instruction from the manufacturer.

**FDA and PI staining for morphologic evaluation.** The integrity of the cell membrane was detected using fluorescein diacetate (FDA) (10)PI staining. Cells were placed on a 24-well culture plate at 1 × 10^5 cells/ml in 0.5 ml culture medium and cultured for 12 h followed by treatment with 10^{-2} to 10^{-3} M XAV939 for 24 h. Next, the cells were stained with 5 μg/ml PI and 4 μg/ml FDA, followed by observation under a fluorescent microscope (Nikon ECLIPSE TE2000-S, Tokyo, Japan).

The bleomycin-induced mouse pulmonary fibrosis model. Male C57BL/6 mice (6–7 wk old) were purchased from the Medical School of Yangzhou University. All mice were maintained under standard conditions with free access to water and laboratory rodent food. To set up the bleomycin-induced mouse pulmonary fibrosis model, mice were anesthetized with pentobarbital sodium (3 mg/kg). Next, mice

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1 This article is the topic of an Editorial Focus by Rennolds S Ostrom (27a).
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 instead. Mice were killed on the indicated days. In some experiments, a more severe pulmonary fibrosis model was induced with 10 mg/kg bleomycin.

For the evaluation of the effect of XAV939 on bleomycin-induced pulmonary fibrosis, mice received both bleomycin and XAV939. Specifically, 10 days after administration of bleomycin, XAV939 was administered intraperitoneally at 5 mg/kg for 11 consecutive days. Mice were killed 21 days after bleomycin instillation.

Mouse lungs were obtained for histopathology, collagen assay, RT-PCR, and Western blotting. Type I collagen is the most abundant fibrillar collagen and it is preferentially synthesized in pulmonary fibrosis (22, 33). Therefore an examination of the expression of collagen I can reflect the degree of fibrosis. The effect of XAV939 on the mortality rate in mice that received bleomycin (10 mg/kg) was also studied. Specifically, XAV939 was administered intraperitoneally at 5 mg/kg every day for 7 days beginning on day 10 after bleomycin dosing (10 mg/kg). Mouse survival rate was recorded.

Histology. Lower left lungs were fixed in 4% (wt/vol) neutral phosphate-buffered paraformaldehyde for 24 h, dehydrated, transparentized, and embedded in paraffin. Lung tissues were cut into 5-μm sections that were stained with hematoxylin-eosin (H&E) for structure observation, or with Masson’s trichrome stain for detection of collagen deposits. Determination of hydroxyproline content was carried out using a kit from Nanjing JianCheng Bioengineering Institute (Nanjing, China) according to the instruction by the manufacturer.

RNA extraction and reverse transcriptase polymerase chain reaction. cDNA was generated from RNA extracts derived from cultured BM-MSCs and lung tissues using a reverse transcription kit (Transgen, Beijing, China). GAPDH was used as an internal control. PCR was performed using the primers as shown in Table 1.

Immunofluorescent staining. For detecting the effect of XAV939 on the differentiation of BM-MSCs or NIH/3T3, immunofluorescence microscopy (Olympus, Tokyo, Japan). Western blotting. Proteins were purified from either BM-MSCs or lungs. Western blot analysis of cellular lysates was performed as previously described (35). The following primary antibodies were employed: Rabbit anti-collagen I, rabbit anti-occludin, and rabbit anti-SP-C (all antibodies were purchased from Abcam, Cambridge, MA). Alexa Fluor 488- or 594-conjugated goat anti-rabbit (Invitrogen) was used as the secondary antibody. The images were captured using a confocal fluorescence microscope (Olympus, Tokyo, Japan).

RESULTS

Development of pulmonary fibrosis in bleomycin-treated mice. The development of pulmonary fibrosis in mice treated with 5 mg/kg bleomycin was assessed by H&E (Fig. 1A), Masson’s trichrome staining (Fig. 1B), and the hydroxyproline content assay for collagen (Fig. 1C). We found that the alveolar structure was destroyed with hyperplasia of fibroblasts and collagen deposition from day 7 to day 21. A number of genes were significantly overexpressed after bleomycin administration. These include well-documented Wnt/β-catenin target genes as well as genes associated with epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM) remodeling and fibrosis [i.e., S100A4, cyclin D1, fibronectin, lamin A, vimentin, α-SMA, and collagen I] (Fig. 1, D and E). The upregulation of β-catenin was also confirmed by Western blotting (Fig. 1F).

Effects of XAV939 on pulmonary fibrosis induced by bleomycin. We explored the inhibitory effects of XAV939 on bleomycin-induced pulmonary fibrosis following intratracheal spray of bleomycin (5 mg/kg). XAV939 significantly reduced the severity of pulmonary fibrosis in the lungs compared with saline controls as assessed by H&E (Fig. 2A), Masson’s trichrome staining (Fig. 2B), and hydroxyproline content assay for collagen (Fig. 2C). To confirm the inhibitory mechanisms of Wnt/β-catenin signaling in bleomycin-induced pulmonary fibrosis, the expression of a number of well-documented Wnt/β-catenin target genes was measured. The pulmonary expression of these genes, as well as genes associated with EMT and ECM remodeling and fibrosis, was significantly increased after bleomycin administration. However, the increased expression of these genes was

| Table 1. Primers used for PCR analysis |
|-----------------------------------------|-----------------------------------------|
| **Forward Primer**                      | **Reverse Primer**                      |
| Collagen I                              | CCTGCTTTCTCTGAGAGATCA                   |
| CTGF                                   | GTTTCTGCTGCTCTGAGATCA                   |
| Fibronectin                            | GGATGACTCCATGATCTGT                   |
| Lamin A                                | GGATGACTCCATGATCTGT                   |
| α-SMA                                  | CAGAACCGCCACCCACGAGAA                   |
| Vimentin                               | ACGACACACACTACGAGAT                   |
| Cyclin D1                              | CACACCCACACACGAGAGAA                   |
| S100A4                                 | ATCGACCCACACGAGAGAA                   |
| TGF-β1                                 | ATCGACCCACACGAGAGAA                   |
| CK18                                  | TACAGACCCACACGAGAGAA                   |
| CK19                                  | CCAGACCCACACGAGAGAA                   |
| Occludin                               | ATCGACCCACACGAGAGAA                   |
| SP-C                                  | ATCGACCCACACGAGAGAA                   |
| GAPDH                                  | ATCGACCCACACGAGAGAA                   |

CTGF, connective tissue growth factor; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1.
suppressed in bleomycin-treated mice that received XAV939 (Fig. 2, D and E). Compared with saline controls, expression of vimentin, α-SMA, and β-catenin after bleomycin administration was markedly increased, which was significantly inhibited by XAV939. Epithelial markers such as CK18, CK19, and occludin were significantly downregulated in lungs following bleomycin-induced injury. However, treatment with XAV939 resulted in marked increases of the expression of these proteins in lung tissues, compared with the levels seen in animals that received bleomycin alone (Fig. 2, F and G).

**Effect of XAV939 on survival rate of mice injured by a high dose of bleomycin.** We chose a high dose of bleomycin (10 mg/kg) to investigate the effect of XAV939 on the survival rate after bleomycin-induced injury in mice. Only 20% of mice survived on day 17 after bleomycin treatment. However, mice demonstrated a significantly higher survival rate (65%) following intraperitoneal injection of XAV939 at a daily dose of 5 mg/kg (Fig. 2H).

**Effect of XAV939 on proliferation and myofibroblast differentiation of NIH/3T3 fibroblasts.** Idiopathic pulmonary fibrosis is characterized by aberrant proliferation and recruitment of
Fig. 2. XAV939 inhibited the development of pulmonary fibrosis in bleomycin (BLM)-treated mice. XAV939 was administered daily at 5 mg/kg intraperitoneally for various time periods as indicated 10 days after the administration of bleomycin. A: mice were killed on day 21 after bleomycin instillation. Bleomycin-induced pulmonary fibrotic lesions were determined by H&E staining (×100). B and C: the effect of treatment of XAV939 for 11 days on the level of collagen was determined by Masson’s trichrome stain (×200) (B) and hydroxyproline measurement (*P < 0.05 vs. control, #P < 0.05 vs. bleomycin) (C). D and E: the expression of S100A4, cyclin D1, fibronectin, connective tissue growth factor, TGF-β1, collagen I, lamin A, vimentin, and α-SMA in the injured lungs was analyzed by RT-PCR. Representative gel electrophoresis bands are shown (D), and the expression levels of the mRNAs were quantified by densitometry and normalized to the expression of GAPDH (E). Densitometry data are shown as means ± SD. *P < 0.05 vs. control, #P < 0.05 vs. bleomycin (n = 10 per group). F and G: the levels of vimentin, α-SMA, CK18, CK19, occludin, and β-catenin were examined by Western blotting. Representative gel electrophoresis bands are shown (F), and the expression levels of the proteins were quantified by densitometry and normalized to the expression of β-actin (G). Densitometry data are shown as means ± SD. *P < 0.05 vs. control (n = 10 per group). H: survival of bleomycin-treated mice (10 mg/kg) following treatment with XAV939 (5 mg/kg) for 7 consecutive days was recorded. *P = 0.025 by Fisher’s exact test, bleomycin/saline vs. bleomycin/XAV939 (n = 20 for each group).
fibroblasts and finally by formation of scar tissues leading to
the subsequent destruction of the lung architecture. To explore
the effect of XAV939 on fibroblast proliferation, NIH/3T3
fibroblasts were cultured with various concentrations of
XAV939 and cell proliferation was measured. Our results
demonstrated that XAV939, at a concentration higher than $1 \times
10^{-6}$ M, could remarkably inhibit the proliferation of NIH/3T3
fibroblasts (Fig. 3A). To determine the effect of XAV939 on
myofibroblast differentiation of NIH/3T3 fibroblasts and sup-
pression of the Wnt signaling pathway in the cells, levels of
$\alpha$-SMA, collagen I, and $\beta$-catenin were measured. The results
indicated that the expression of $\alpha$-SMA, collagen I, and
$\beta$-catenin in NIH/3T3 fibroblasts was restrained by XAV939
(Fig. 3, B and C), suggesting compromised myofibroblast
differentiation. We also demonstrated that XAV939, at a con-
centration of $1 \times 10^{-3}$ M, could significantly increase death of
NIH/3T3 fibroblasts (7.28 $\pm$ 0.08% vs. control 5.82 $\pm$ 0.05%,
P < 0.05). No significant differences were observed between
control and exposure at concentrations ranging from $10^{-7}$ to
$10^{-4}$ M (Fig. 3, D and E).

**Impact of XAV939 on apoptosis and differentiation of ATII
cells.** At a concentration higher than $1 \times 10^{-5}$ M, XAV939
could remarkably inhibit the proliferation of ATII cells (Fig. 4A).
XAV939 even at $10^{-6}$ M had no effect on the levels of CK18,
occludin, and SP-C (Fig. 4B), although the expression of
$\beta$-catenin was suppressed significantly (Fig. 4C). After expo-
sure to XAV939 at $10^{-4}$ M for 24 h, no significantly different
levels of cell death were observed. However, significantly
elevated cell death could be observed when concentrations of
XAV939 were raised to $10^{-3}$ M (1.93% $\pm$ 0.03% vs. control,
1.28 $\pm$ 0.03%, P < 0.05; Fig. 4, D and E).

**Identification of BM-MSCs.** Following culture for 5 days, the
cells isolated from mouse bone marrow demonstrated the
morphology of a long spindle, spiral, and radial arrangement

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**Fig. 3.** XAV939 suppressed the proliferation and myofibroblastic differentiation of NIH/3T3 fibroblasts without obvious apoptosis induction. NIH/3T3 fibroblasts were cultured in the presence of XAV939 at $10^{-6}$ M for 24 h unless otherwise indicated. A: cell proliferation was measured using a Cell Counting Kit-8 (CCK-8) assay. Six repeats were performed. *P < 0.05 vs. control. B: expression of $\alpha$-SMA, collagen I, and $\beta$-catenin was measured by immunofluorescent analysis. C: expression of $\alpha$-SMA, collagen I, and $\beta$-catenin was measured by Western blotting. D: cell viability was examined by staining with fluorescein diacetate (FDA) and propidium iodide (PI). E: cell apoptosis was evaluated by flow cytometry. The number in each quadrant represents the percentage of cells in that compartment.
These cells expressed CD29, CD44, CD106, and SCA-1, but not CD11b, CD45, CD34 (Fig. 5B). Taken together, these results suggest that the cells possessed the main features of mesenchymal stem cells.

Impact of XAV939 on differentiation of BM-MSCs cocultured with ATII cells. When BM-MSCs and ATII cells were cocultured for 14 days, BM-MSCs transformed to a paving stone shape resembling epithelial cells and the transformation was further promoted by XAV939 (Fig. 6A). To confirm the differentiation, we measured the expression of some epithelial cell markers of BM-MSCs on day 14 following coculture by RT-PCR and Western blotting. Expression levels of occludin and SP-C were further confirmed by immunofluorescence assay. Increased expression of CK18, CK19, occludin, and SP-C on BM-MSCs following coculture with ATII cells was observed, and the expression was further increased by the addition of XAV939. In contrast, BM-MSCs expressed no epithelial cell markers without coculture (Fig. 6, B–F).

Effect of XAV939 on suppression of β-catenin expression in BM-MSCs. The β-catenin content in BM-MSCs declined following coculture with ATII cells. The addition of XAV939 could further reduce the β-catenin levels in BM-MSCs either in the presence or absence of XAV939. These results suggest that under the condition of coculture with epithelial cells, the Wnt/β-catenin signaling pathway of BM-MSCs was restrained, and XAV939 could further promote such inhibition (Fig. 6, E and F).

DISCUSSION

Pulmonary fibrosis is a systemic disorder that involves many pathogenic factors. Currently, it is particularly desirable to develop new therapeutic approaches to inhibit fibrosis. The lung injury model induced by bleomycin is appropriate for investigating pulmonary fibrosis and the development of effective treatments. Although the model has certain limitations, such as the incomplete simulation of IPF, some of the activated signaling pathways in IPF patients can also be identified in the model (7). The Wnt/β-catenin signaling pathway is associated with fibroblast activation, tissue repair, and collagen synthesis (4, 32). It is reported that the Wnt signaling pathway is activated and the levels of Wnt isoforms are increased in the lungs of IPF and patients with other forms of pulmonary fibrosis (6). Along this line, recent studies have identified
ICG-001, a small molecule that selectively inhibits T-cell factor (TCF)/β-catenin transcription in a Creb-binding protein (CBP)-dependent fashion by repressing the β-catenin-driven gene transcription in a TOPflash luciferase reporter assay and attenuates bleomycin-induced lung fibrosis in mice. Administration of ICG-001 can not only inhibit the occurrence and development of pulmonary fibrosis, but also reverse established fibrosis (12). In this study, we investigated the effect of XAV939 on bleomycin-induced lung fibrosis and tried to reveal detailed pathogenic mechanisms. XAV939 has also been previously used to block the canonical Wnt pathway induced by miRNA 154, which participates in lung fibrosis and seems to upregulate the canonical Wnt/β-catenin signaling (23).

In the present study, we demonstrated that a number of well-documented Wnt/β-catenin target genes were significantly increased after bleomycin administration in the lung, so were genes associated with EMT and ECM remodeling, and fibrosis. These results were consistent with the findings in IPF. Mesenchymal stem cells did not abrogate the histopathologic

Fig. 5. The phenotypes of the bone marrow-derived mesenchymal stem cells (BM-MSCs) were confirmed by microscopy and flow cytometry. Mouse bone marrow cells were collected and cultured for various time points as indicated. The morphology of cells was examined by microscopy and flow cytometry. A: primary cells were cultured for 20 h, 5 days, or passaged for 1, 3, 5, and 7 generations. B: representative flow cytometric analysis of the cell surface markers of BM-MSCs at passage 5 is shown.
changes of HCl-induced acute lung injury and pulmonary fibrosis. We have previously shown that inhibition of Wnt/β-catenin signaling by DKK-1, a natural inhibitor of the Wnt/β-catenin signaling pathway, can promote the differentiation of exogenous BM-MSCs into epithelial cells in vivo and thus regulate lung injury and repair, although only a fraction of the exogenous cells are recruited (34). In addition, recruitment of endogenous mesenchymal stem cells to the injured lung is also observed (40). Therefore, we speculated that inhibition of the Wnt/β-catenin signaling may directly promote the repair of
lungs. As the targeting property and efficiency of DKK-1 are relatively poor due to its relatively large size, perhaps a small-molecule compound that promotes the differentiation of BM-MSCs into alveolar epithelial cells by specifically inhibiting the Wnt/β-catenin signaling may have a more efficient therapeutic potential for the repair of pulmonary fibrosis.

Moeller divided the pathological processes involved in bleomycin-induced rodent pulmonary fibrosis into two stages, namely the early inflammatory reaction stage (7 days) and the subsequent fibrosis stage (apparent on day 14, peak on day 21) (25). To avoid the influence of XAV939 on the role of inflammation, we injected XAV939 intraperitoneally starting from day 10 for 11 consecutive days after the administration of bleomycin by the intratracheal route. Our results showed that XAV939 could inhibit the activation of the Wnt/β-catenin signaling in the lung tissue. Furthermore, XAV939 could inhibit fibroblast differentiation into myofibroblasts, which have a substantially increased potential for the synthesis of ECM, resulting in aggravated fibrosis. Wei and colleagues (38) found that Wnt-3a-induced β-catenin activation could stimulate the proliferation and migration of fibroblasts, collagen gel contraction and myofibroblast differentiation, and enhance profibrogenic gene expression. In the present study, we demonstrated that XAV939 could inhibit myofibroblast differentiation and ECM accumulation in vitro and it can also suppress the proliferation of NIH/3T3 fibroblasts. However, no significantly different levels of apoptosis were observed upon administration of XAV939 within a relevant concentration range. Taken together, these results strongly suggest a direct effect of XAV939 on fibroblasts and suggest that cytotoxicity may not be the major effect mediating the inhibition of myofibroblast differentiation.

Recent studies have shown that bone marrow-derived circulating progenitor cells could accumulate in the lung and contribute to pulmonary fibrosis after lung injury (2). In experimental pulmonary fibrosis in mice, bone marrow progenitor recruitment accounts for ~20% of the fibroblast pool (36). BM-MSCs can be recruited to the damaged tissues and differentiate into specific types of cells involved in tissue repair, but the differentiation direction is uncertain. In this study, we screened the BM-MSCs that were isolated from bone marrow and the results showed that cell surface marker expression was as expected (29). In our in vitro experiment, BM-MSCs differentiated into an epithelium-like phenotype following coculture with alveolar epithelial cells, evidenced by the expression of specific molecular markers of alveolar epithelial cells. XAV939 could significantly suppress Wnt/β-catenin signaling and promote the epithelial differentiation but had little impact on ATII cells. Previous reports suggested that when the Wnt/β-catenin signaling is activated, BM-MSCs can differentiate into fibroblasts or myofibroblasts, and this process can be inhibited by DKK-1 (34). These findings prompted us to propose that inhibition of Wnt/β-catenin signaling could promote the alveolar epithelial cell-mediated differentiation of autologous BM-MSCs into epithelial cells in the damaged lung, and hence the improvement in lung function.

In conclusion, our data demonstrate that XAV939 could significantly improve the pulmonary fibrosis induced by bleomycin through inhibition of the Wnt/β-catenin signaling pathway, and increase mouse survival after bleomycin-induced injury. XAV939 is capable of promoting the differentiation of BM-MSCs to an epithelium-like phenotype in an in vitro coculture system. These results confirm that the Wnt/β-catenin signaling pathway plays an important role in the pathological processes involving pulmonary fibrosis, and that the suppression of Wnt/β-catenin signaling by specific inhibitors could restrain the development of pulmonary fibrosis. We believe that our work has certain clinical significance for developing therapeutic strategies for pulmonary fibrosis.

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**AUTHOR CONTRIBUTIONS**

C.W., H.Z., and X.H. conception and design of research; C.W., H.Z., Z.S., Y.G., C.N., and Z.L. performed experiments; C.W., H.Z., and W.Q. analyzed data; C.W. and W.Q. interpreted results of experiments; C.W. prepared figures; C.W. drafted manuscript; Z.X. edited and revised manuscript; X.H. approved final version of manuscript.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Inhibition of Wnt signaling repairs lung injury.


