Research Square

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Wnt8b Regulates Myofibroblast Differentiation of Lung-Resident Mesenchymal Stem Cells via the Activation of Wnt/β-catenin Signaling in Pulmonary Fibrogenesis

Chaowen Shi
Nanjing University Medical School
Xiang Chen
Nanjing University Medical School
Wenna Yin
Nanjing University Medical School
Jiwei Hou
Nanjing University Medical School
Zhaorui Sun
Nanjing University Medical School Clinical College: East Region Military Command General Hospital
Xiaodong Han (hanxd@nju.edu.cn)
Nanjing University https://orcid.org/0000-0003-4512-3699

Research

Keywords: Lung resident mesenchymal stem cell, Wnt8b, Wnt/β-catenin signaling, Idiopathic pulmonary fibrosis

Posted Date: May 6th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-466908/v1

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Version of Record: A version of this preprint was published at Differentiation on April 1st, 2022. See the published version at https://doi.org/10.1016/j.diff.2022.03.004.

Abstract

Background

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and fatal lung disease that is characterized by enhanced changes in stem cell differentiation and fibroblast proliferation. Lung resident mesenchymal stem cells (LR-MSCs) are important regulators of pathophysiological processes including tissue repair and inflammation, and evidence suggests that this cell population also plays an essential role in fibrosis. Our previous study demonstrated that Wnt/β-catenin signaling is aberrantly activated in the lungs of bleomycin-treated mice and induces myofibroblast differentiation of LR-MSCs. However, the underlying correlation between LR-MSCs and the Wnt/β-catenin signaling remains poorly understood.

Methods

We used mRNA microarray, immunohistochemistry assay, qRT-PCR, and western blotting to measure the expression of Wnt8b in myofibroblast differentiation of LR-MSCs and BLM-induced mouse fibrotic lungs. Immunofluorescence staining and western blotting were performed to analyze myofibroblast differentiation of LR-MSCs after overexpressing or silence Wnt8b. Moreover, we analyzed the effects of Wnt8b inhibition on BLM-induced pulmonary fibrosis by Sirius Red Staining and immunohistochemical staining.

Results

We found that Wnt8b was highly expressed by LR-MSCs undergoing myofibroblast differentiation. *In vitro*, Wnt8b promoted LR-MSCs differentiate into myofibroblasts via activating Wnt/β-catenin signaling. Moreover, siRNA-mediated inhibition of Wnt8b prevented Transforming growth factor (TGF)-β1-induced myofibroblast differentiation of LR-MSCs *in vitro* and ameliorated pulmonary fibrotic lesions.

Conclusions

Our study identified Wnt proteins and Wnt/β-catenin signaling in pulmonary fibrosis *in vitro* and *in vivo*, and highlighted Wnt8b as a potential therapeutic target in pulmonary fibrosis. Moreover, these finding might provide a new perspective in the development of treatment strategies for IPF.

Background

Idiopathic pulmonary fibrosis (IPF) is a progressive and usually fatal lung disease characterized by stem cell differentiation, fibroblast proliferation and extracellular matrix remodeling, which results in irreversible distortion of the lung's architecture [1, 2]. Although its causes remain to be elucidated fully, our group did a lot of work to understand the myofibroblast differentiation of lung resident mesenchymal stem cells (LR-MSCs) involved in the biological processes of IPF [3–5]. Tissue mesenchymal stem cells (MSCs) are generally believed to exist in most mammalian organs, including the lung, and to play a significant role in the development of fibrotic diseases [6]. MSCs are adult connective tissue progenitor cells with

multilineage differentiation potential that can be induced to differentiate into various cell types [6]. These LR-MSCs possess typical characteristics shared by other tissue-specific MSCs, including self-renewal ability, multipotent differentiation capacity, and autocrine or paracrine properties [7, 8]. Importantly, these cells can be driven by local profibrotic factors to undergo a myofibroblast transition that participates in the development of pulmonary fibrosis [9]. However, the roles of LR-MSCs involved in the progression of IPF are largely unknown.

Our previous study demonstrated that Wnt/β-catenin signaling is aberrantly activated in the lungs of bleomycin-treated mice and induces myofibroblast differentiation of LR-MSCs [10]. This profibrotic pathway is mediated by Wnt proteins, a family of 19 secreted glycoproteins [11]. Aberrant activation of Wnt/β-catenin signaling that is mediated by altered expressions of Wnt proteins has been reported to be implicated in various diseases, including renal, pulmonary, and liver fibrosis [12–14]. Recent studies demonstrated that the expression levels of Wnt ligands were low in normal lungs but markedly elevated in the lungs of IPF patients [15–17]. Therefore, these Wnt proteins with altered expression may play critical role in myofibroblast differentiation of LR-MSCs involved in the development of IPF. However, the exact mechanisms of Wnt proteins in the development and progression of IPF remains unknown.

In the present study, we examined gene expression patterns of myofibroblast differentiated LR-MSCs by microarrays. We identified extremely increased expression of Wnt8b in the differentiation of LR-MSCs and lung fibrosis. In particularly, we demonstrated that Wnt8b could promote LR-MSCs differentiate into myofibroblasts by activating Wnt/β-catenin signaling. Silence of Wnt8b could suppress transforming growth factor (TGF)-β1-mediated myofibroblast differentiation of LR-MSCs. Furthermore, targeted inhibition of Wnt8b could evidently suppress the development of pulmonary fibrosis. Thus, our study identified Wnt proteins and Wnt/β-catenin signaling in pulmonary fibrosis *in vitro* and *in vivo*, and highlighted Wnt8b as a potential therapeutic target in pulmonary fibrosis.

Materials And Methods

Antibodies and reagents

The antibodies against mouse β-actin (ab8277), rabbit polyclonal antibody against mouse β-catenin (ab32572), rabbit polyclonal antibody against mouse alpha smooth muscle actin (α-SMA, ab5694), rabbit monoclonal antibody against mouse Vimentin (ab92547), rabbit polyclonal antibody against mouse Collagen I (ab34710), rabbit monoclonal antibody against mouse ATP-binding cassette protein G2 (ABCG-2) (ab207732) were purchased from Abcam (Cambridge, MA). Rabbit anti-fibronectin antibody (F3648) was obtained from Sigma. Goat polyclonal antibody against mouse Wnt8b (AF3367) was purchased from R&D (Minneapolis, MN). The secondary horseradish peroxidase (HRP)-conjugated antibodies, Alexa Fluor 488– and Alexa Fluor 594–coupled goat anti-rabbit or anti-mouse and donkey anti-goat IgG, fetal bovine serum (FBS), and supplements were obtained from Fisher Scientific. Dulbecco's modified Eagle's medium (DMEM) was obtained from American Type Culture Collection (ATCC,

Manassas, VA). All other chemicals of analytic grade were obtained from Fisher Scientific unless otherwise indicated.

Cell culture and transfection

Isolation of LR-MSCs was performed as previously reported [3, 18]. Freshly isolated LR-MSCs were cultured at a concentration higher than 10^5 cells/ml with DMEM containing 15% FBS, 4% L-glutamine, 1% nonessential amino acids, and 1% penicillin and streptomycin, and maintained in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. The cells were passaged 1:2 using 0.25% trypsin when they reached 70–90% confluence. Transfections were performed with lentiviral protocols provided by GENECHEM (Shanghai, China). The LV-Wnt8b and LV-Wnt8b-RNAi were synthesized by GENECHEM. The transfection efficiency was measured by quantitative real-time PCR (qRT-PCR) and western blot.

Quantitative real-time PCR

For analysis of mRNA, HiScript 1st strand cDNA Synthesis Kit (Vazyme Biotech Co., Nanjing, China) was used for RT-PCR reaction. Gene expression was quantified by SYBR Green Q-PCR Kit (Roche, Germany) using the ABI Prim 7300 Sequence Detection System (Applied Biosystems, Foster city, CA). Specific primers for mRNAs are listed in Table 1. The Ct values were analyzed using the ΔΔCt method and relative changes of mRNA levels were obtained by normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) relative to the control.

Table 1
Primers used for PCR analysis.

Genes	Forward primer	Reverse primer
α-SMA	CCCAGATTATGTTTGAGACCTTC	ATCTCCAGAGTCCAGCACAATAC
Vimentin	CCTGGAGTCACTTCCTCTGGTTG	TCTTGCTGGTACTGCACTGTTGC
Collagen I	CTTCTGGTCCTCGTGGTCTCCCT	AAGCCTCGGTGTCCCTTCATTCC
Wnt8b	CCAGAGTTCCGGGAGGTAG	GAGATGGAGCGGAAGGTGT
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Western blot analysis

Tissue or cell lysates were prepared and separated on SDS-polyacrylamide gels as described previously [4]. The PVDF membrane with transferred proteins was probed with various antibodies, and the signals on the membrane were visualized by an Odyssey Scanning System (LI-COR, Lincoln, NE).

mRNA expression assays and analysis

The mRNA microarray analysis was performed by Affymetrix Mouse Exon ST 1.0 microarray chips (Invitrogen, Shanghai, China). mRNAs with expression values of greater than 2-fold-change compared to

controls were regarded as overexpressed while less than 0.5 (log scale)-fold-change were considered as under-expressed.

Immunofluorescent staining

The immunofluorescence analysis was performed as previously described28. Rabbit anti-Collagen I, mouse anti-α-SMA, Rabbit anti- Vimentin, Rabbit anti-β-catenin, Rabbit anti-ABCG2 and Goat anti-Wnt8b were employed as the primary antibodies. Alexa Fluor 594- or Alexa Fluor 488-conjugated goat anti-mouse/rabbit IgG (Invitrogen) and Alexa Fluor 594-conjugated donkey anti-goat IgG (Invitrogen) were used as the secondary antibody. Nuclei were stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma). The images were captured using a confocal fluorescence microscope (Olympus, Tokyo, Japan).

Induction and treatment of pulmonary fibrosis.

All animal procedures were conducted in accordance with humane animal care standards approved by the Nanjing University Ethics Committee (Nanjing, China) and maintained under specific pathogen-free conditions. The animals were acclimated to the environment for 1 week prior to treatment. The mice were administered with bleomycin (BLM, Nippon Kayaku, Tokyo, Japan) intratracheally at a dose of 5 mg/kg dissolved in a total of 50 μl sterile saline. The control group was similarly treated with 50 μl of sterile saline. The lentiviral vector of LV-Wnt8b and LV-Wnt8b-RNAi were purchased from GENECHEM. Mice were administered with the lentiviral vector intratracheally at a dose of 2×10^8 TU/ml diluted by sterile saline. Seven days later, the LV- Wnt8b and LV- Wnt8b-RNAi groups were administered with BLM intratracheally at a dose of 5 mg/kg dissolved in a total of 50 μl sterile saline. The LV-NC groups were similarly treated with 50 μl sterile saline. The mice were sacrificed for lung collection at day 14 after BLM administration (n = 6 for each time point).

Histopathology

The mouse lungs were inflated with a neutral buffered formalin solution overnight and embedded in paraffin before sectioning into 5 μm-thick slices. The sections were stained with hematoxylin–eosin for structure observation.

Immunohistochemistry and Sirius Red staining

Paraffin-embedded lung tissue was sectioned at 5 μm and then subjected to Sirius Red or immune staining [14]. Briefly, tissue sections were deparaffinized, hydrated, and antigen-retrieved, followed by Sirius Red/Fast Green Collagen Staining Kit (Chondrex, MA), the sections were stained in dye solution and mixed with a dye extraction buffer. The dye solutions were collected and their O.D. values were read by a microplate reader. Or the sections incubated with primary and secondary antibodies for immune staining. Sections stained without primary antibodies served as negative controls. The DAB or AEC Substrate System (DAKO) was used to reveal the immunohistochemical staining.

Statistical analysis

All the experimental data were presented as mean ± S.E. Statistical analysis of data were performed using SigmaStat 3.5 software (Jandel Scientific Software). Comparison between multiple groups was performed by using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test or Student's t test between two groups. A p value of less than 0.05 was considered statistically significant.

Results

LR-MSCs could differentiate into myofibroblasts in vitro

The primary LR-MSCs from mouse lung tissue could differentiate into myofibroblasts in the treatment of TGF- β1, (10 ng/ml) [14, 18]. As shown in the Fig. 1, immunofluorescence assay, qRT-PCR and western blotting were used to measure the expression of collagen I, α-SMA and Vimentin. The major markers of myofibroblast differentiation were increased during the incubation with TGF- β1 for 3 and 7 days.

Wnt8b was upregulated both in myofibroblast differentiation of LR-MSCs and BLM-induced mouse fibrotic lungs

To detect the expressions of the Wnt genes during the myofibroblast differentiation of LR-MSCs, we performed an mRNA microarray with total RNAs isolated from LR-MSCs harvested on day 7 after TGF-β1 (10 ng/ml) treatment. The expressions of 459 mRNAs were changed significantly (2 − ΔΔCT > 2-fold or < − 2-fold, or p-value < 0.05). There are 267 genes up-regulated, among the 267 genes, Wnt8b expression was upregulated (P-value: 0.0274). To confirm the microarray data, immunofluorescence assay, qRT-PCR and western blotting were used to measure the expression of Wnt8b. As shown in Fig. 2 (A-G), the mRNA and protein levels of Wnt8b were significantly increased in both myofibroblast differentiation of LR-MSCs and fibrotic lung tissues. We also confirmed these findings by using immunohistochemistry (IHC) staining in the fibrotic lung tissues (Fig. 2H).

To determine whether the Wnt8b was expressed in LR-MSCs during the development of fibrosis, the fibrotic lung tissue sections were double stained with ABCG-2 and Wnt8b. The results demonstrated that the expression of Wnt8b was upregulated in LR-MSCs (Fig. 2I), indicating Wnt8b involved in the myofibroblast differentiation of LR-MSCs and the development of pulmonary fibrogenesis.

Wnt8b promoted LR-MSCs differentiate into myofibroblasts via activating Wnt/β-catenin signaling

To determine whether Wnt8b plays a role in myofibroblast differentiation of LR-MSCs, we examined the protein levels of β-catenin, Vimentin and Collagen in LR-MSCs which were transfected with LV-Wnt8b or incubated with Wnt3a (10 ng/ml, positive control). Overexpression of Wnt8b or treated with Wnt3a could increase the expressions of β-catenin, α-SMA, Vimentin and Collagen (Fig. 3D, E). Similarly, the

immunofluorescence results showed that the expressions of α-SMA, β-catenin, Vimentin and Collagen were dramatically elevated after transfecting Wnt8b or treated with Wnt3a (Fig. 3F). These data indicated that Wnt8b could promote myofibroblast differentiation of LR-MSCs through activating Wnt/β-catenin signaling.

Silence of Wnt8b could suppress myofibroblast differentiation of LR-MSCs

To further explore the underlying correlation between Wnt8b and LR-MSCs in myofibroblast differentiation, LR-MSCs were transfected with LV-Wnt8b-RNAi followed by incubation in the presence or absence of TGF-β1 (10 ng/ml) for 7 days. LV-Wnt8b-RNAi could successfully restrain the expression of Wnt8b both in mRNA level and protein level (Fig. 4A-E). After LR-MSCs were transfected with LV-Wnt8b-RNAi, TGF-β1-induced expressions of β-catenin, α-SMA, Vimentin and Collagen were significantly suppressed (Fig. 4F, G, H). The data demonstrated that silence of Wnt8b inhibited the myofibroblast differentiation of LR-MSCs induced by TGF-β1, indicating Wnt8b is involved in this differentiation process.

Suppression of Wnt8b could protect mice from BLM-induced pulmonary fibrosis

We further analyzed the effects of Wnt8b inhibition on BLM-induced pulmonary fibrosis. As shown in Fig. 5 (A-C), pulmonary fibrotic lesion and collagen deposition were greatly reduced after LV-Wnt8b-RNAi administration. LV-Wnt8b-RNAi profoundly suppressed the expressions of Wnt8b, β-catenin, Fibronectin and α-SMA as demonstrated by Immunohistochemistry staining (Fig. 5D). In addition, silencing Wnt8b could attenuate the activation of Wnt/β-catenin signaling through impairing the expression of β-catenin. Immunofluorescence staining also revealed that LV-Wnt8b-RNAi retarded myofibroblast activation in BLM-induced pulmonary fibrosis (Fig. 5E and F). These results indicated that inhibition of Wnt8b could repress the activation of Wnt/β-catenin signaling and inhibit pulmonary fibrosis after bleomycin administration.

Discussion

It is generally believed that tissue-specific stem cells exist in most mammalian tissues. The function of these cells is to maintain tissue homeostasis by suppling new tissue-specific cells either during normal tissue cycling or when existing tissue cells are lost as a result of pathological development [19]. These resident MSCs mediate pathogenic processes largely through the secretion of proscarring, proangiogenic, and immunomodulatory factors [7]. Therefore, LR-MSCs are potentially useful in regenerative therapies in damaged lung tissue repair [9]. It is thus worth exploring the trigger that induces the myofibroblast differentiation of LR-MCSs in IPF. In this study, we revealed the role of Wnt8b and demonstrated a mechanism by which LR-MSCs participate in the development of pulmonary fibrosis.

Previously, we and others have evaluated that Wnt/β-catenin signaling to play a critical role in LR-MSCs fate decisions, Wnt ligands could be expressed in these types of cells [3, 20]. Moreover, activating Wnt/β-catenin signaling could induce LR-MSCs to differentiate into myofibroblasts which were the principal components of myofibroblast foci [14]. The Wnt/β-catenin signaling was mainly activated by Wnt proteins. Several Wnt proteins, including Wnt2, Wnt5a, Wnt7b, Wnt8b, Wnt11 and Wnt13, were expressed in both developing and adult lung [21]. In the present work, firstly we performed a mRNA microarray with total RNAs isolated from LR-MSCs treated with TGF-β1 for 7 days to analysis the expression of Wnt genes. Among the up-regulated genes, we found only Wnt8b was upregulated. Our observation in pulmonary fibrosis is consistent with the acknowledged actions of Wnt molecules in other fibrosis. For example, after unilateral ureteral obstruction, all members of the Wnt family except Wnt5b, Wnt8b, and Wnt9b are upregulated in the fibrotic kidneys with distinct dynamics [12].

Although the expression of Wnt ligands have been shown to be up regulated during the development of IPF, the role of Wnt8b in IPF remains unknown. The present studies showed that Wnt8b is a member of class I Wnt family signaling through β-catenin, and Wnts could regulated cell fate and behavior in the Wnt/β-catenin signaling pathway [22]. Wnt8b has been suggested to be a cancer-related gene through the synergistic activation of the β-catenin signaling [23]. To reveal the role that Wnt8b plays in the myofibroblast differentiation of LR-MSCs, we observed localization of Wnt8b expression in LR-MSCs undergoing pulmonary fibrosis in fibrotic lungs (Fig. 2I). In addition, overexpression of Wnt8b could activate canonical Wnt signaling and promote LR-MSCs to differentiate into myofibroblasts (Fig. 3D and F). Recently, the expression of Wnt8b was shown to play key roles in gastric cancer through activation of the β-catenin / transcription factors (TCF) signaling pathway [23]. Also, Wnt8b exerted a significant influence on phosphate induced vascular calcification (VC) by altering the Wnt/β-catenin signaling pathway. Silencing Wnt8b terminates phosphate-induced VC in vascular smooth muscle cells (VSMCs) by inhibiting the Wnt-β-catenin signaling pathway [24]. Here, we confirmed the contribution of Wnt8b inhibition to the maintenance of LR-MSC quiescence and protection of mice from BLM-induced pulmonary fibrosis (Figs. 4 and 5).

However, the means by which Wnt signaling elicits a biological response largely depends on the combination of Wnt ligands with the corresponding receptors that are present. When Wnt ligands bind to the frizzled (Fzd) receptor along with low-density lipoprotein receptor-related protein (LRP), β-catenin accumulated in the cytoplasm and subsequently translocated into the nucleus, which promote the transcription of target genes [11]. In the previous work we found that Wnt7b, Wnt10a and Fzd10 were induced in LR-MSCs following TGF-β1 treatment and fibrotic lung tissues. Fzd10 knockdown reduced Wnt7b and Wnt10a-induced activation of Wnt/β-catenin signaling, which imply that Wnt7b and Wnt10a may be the ligands for Fzd10 [14]. According to a systematic analysis of Fzd receptor expression, wnt8b and frizzled-3a (fzd3a) genetically interact to regulate the patterning of guidance cues in the rostral forebrain of zebrafish [25]. Wnt8b and Fzd8a can functionally interact to transmit posteriorizing signals that determine the fate of the posterior diencephalon and midbrain of zebrafish [22]. The sonic hedgehog (Shh) signaling is reported to regulate the expression of Wnt molecules by recruiting the transcription factor glioblastoma (Gli) to the promoters of Wnt genes [26]. Gli1 could bound to and increased promoter

activity of the Wnt7b and Wnt10a genes, and inhibition of Gli1 suppressed myofibroblast differentiation of LR-MSCs and pulmonary fibrosis [14, 17]. Sine oculis–related homeobox 3 (Six3) directly repressed Wnt8b expression during the neuroretina specification in mouse embryos [27]. SRY-Box Transcription Factor 21 (SOX21) represses Wnt8b expression by interfering with the binding of TCF4/β-catenin complex to the *Wnt8b* enhancer [28]. The other studies suggested that complex and direct interactions between Wnt8b, and fibroblast Growth Factor 17 (Fgf17) could regulate Gli3 expression [29]. Above all these results indicate a complex mechanism by which regulating the expression of Wnt8b in myofibroblast differentiation of LR-MSCs that involved in fibrosis progression and further studies are needed to clarify the detailed mechanisms. Moreover, the comparison between Wnt10a blockade and Wnt8b inhibition in terms of antifibrotic efficacy and side effects remains to be clarified in future studies.

Conclusions

In summary, our results demonstrate that Wnt8b could promote LR-MSCs differentiate into myofibroblasts by activating Wnt/β-catenin signaling. Silence of Wnt8b could suppress TGF-β1-mediated myofibroblast differentiation of LR-MSCs targeted inhibition of Wnt8b could evidently suppress the development of pulmonary fibrosis. These finding provide strong support for further research that might help clarify the pathogenesis of IPF and indicate that Wnt8b may be a promising therapeutic target for IPF treatment.

Abbreviations

ABCG-2: ATP-binding cassette protein G2; ATCC: American Type Culture Collection; α-SMA: alpha smooth muscle actin; BLM: bleomycin; DAPI: 4’,6-Diamidino-2-phenylindole; DMEM: Dulbecco’s modified Eagle’s medium; FBS: fetal bovine serum; Fzd: frizzled; Fzd10: frizzled-10; Fzd8a: frizzled-8a; Fzd3a: frizzled-3a; Fgf17: fibroblast Growth Factor 17; Gli: glioblastoma; Gli1: glioblastoma 1; Gli3: glioblastoma 3; HRP: horseradish peroxidase; IgG: Immunoglobulin G; IPF: Idiopathic pulmonary fibrosis; IHC: immunohistochemistry; LR-MSCs: lung resident mesenchymal stem cells; LRP: lipoprotein receptor-related protein; MSCs: mesenchymal stem cells; PVDF: polyvinylidene fluoride; qRT-PCR: quantitative real-time PCR; SDS: sodium dodecyl sulphate; Shh: sonic hedgehog; Six3: Sine oculi’s–related homeobox 3; SOX21: SRY-Box transcription factor 21; TCF: transcription factors; TGF-β1: transforming growth factor-β1; VC: vascular calcification; VSMCs: vascular smooth muscle cells; WNT: Wingless-type MMTV integration site family; WNT8b: Wingless-type MMTV integration site family, member 8b; WNT3a: Wingless-type MMTV integration site family, member 3a.

Declarations

Acknowledgements

We also thank Dr. Cong Wang for assistance in data analysis of mRNA microarray.

Author contributions

The authors contributed in the following ways: Chaowen Shi, Xiang Chen and Xiaodong Han were involved in the conception and design of the experiments; Chaowen Shi wrote the manuscript; Chaowen Shi carried out most of the experiments, Xiang Chen and Wenna Yin contributed to some experiments; Zhaorui Sun and Jiwei Hou contributed to the manuscript revision. All the authors contributed to the manuscript preparation and gave final approval of the submitted manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (82000069), Natural Science Foundation of Jiangsu Province of China (BK20200891, BK20200314).

Availability of data and materials

All datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics statement

The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006) 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.

Consent for publication

Not applicable.

Competing interests

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author details

[1]Immunology and Reproduction Biology Laboratory & State Key Laboratory of Analytical Chemistry for Life Science, Medical School, Nanjing University, Nanjing, Jiangsu 210093, China. [2]Jiangsu Key Laboratory of Molecular Medicine, Nanjing, 210093, China. [3]School of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu, 212013, China. [4] Department of Emergency Medicine, Jinling Hospital, Medical School of Nanjing University, Nanjing, 210002, China.

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Figures

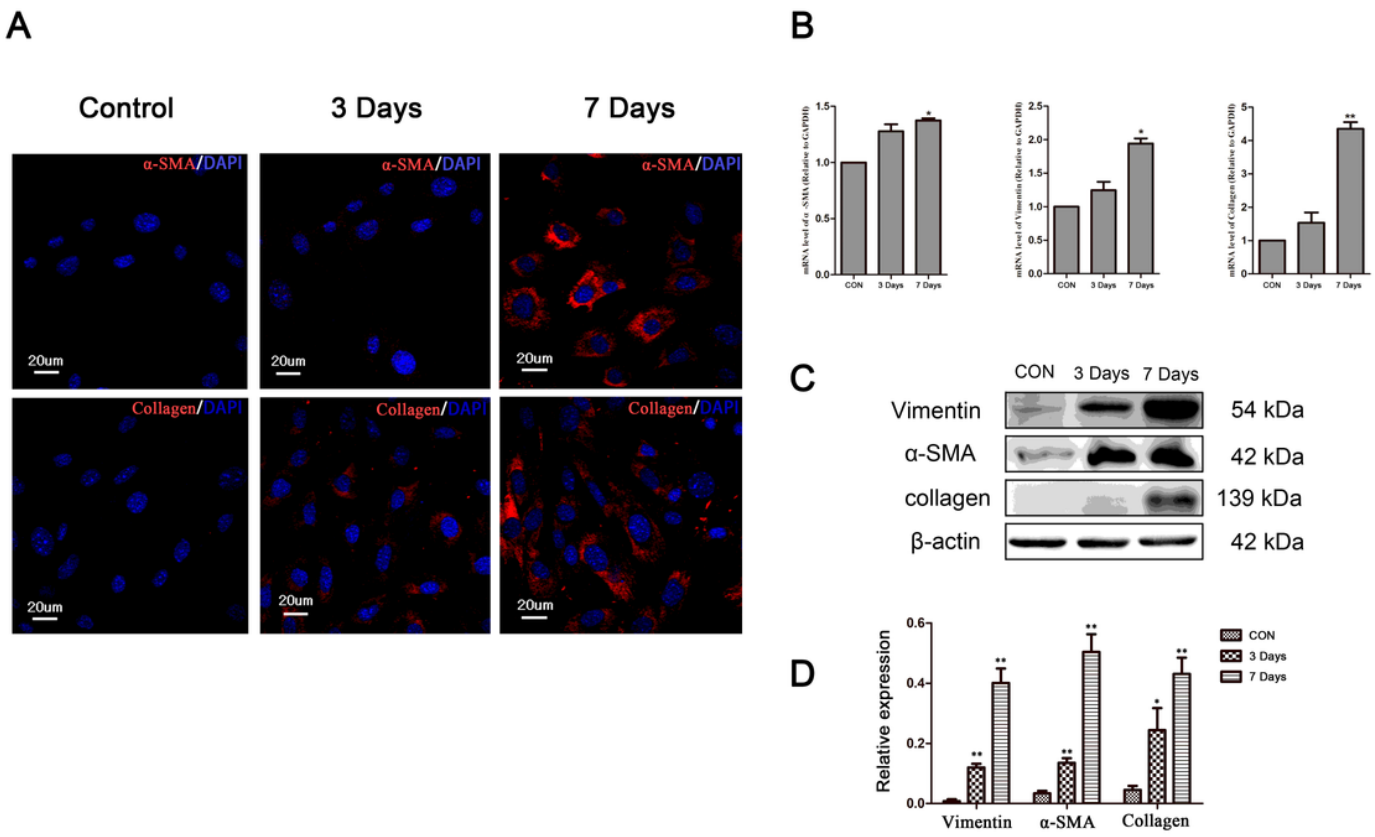

Figure 1

Lung-resident mesenchymal stem cells (LR-MSCs) differentiate into myofibroblasts in the treatment of transforming growth factor-β1(TGF- β1). (A) The expression of α-smooth muscle actin (α-SMA) and Collagen in LR-MSCs treated with TGF-β1 for 3, 7 days were measured by immunofluorescence staining, 400x. Scale bar, 20μm. (B) The mRNA levels of α-SMA, Vimentin, Collagen in LR-MSCs treated with TGF-

β1 for 3, 7 days was determined by Quantitative PCR. (C) The protein levels of α-SMA, Vimentin and Collagen in LR-MSCs treated with TGF-β1 for 3, 7 days were examined by western blotting. (D) quantitation of α-SMA, Vimentin and Collagen abundance. *, p < 0.05; **, p < 0.01; n = 3 experiments.

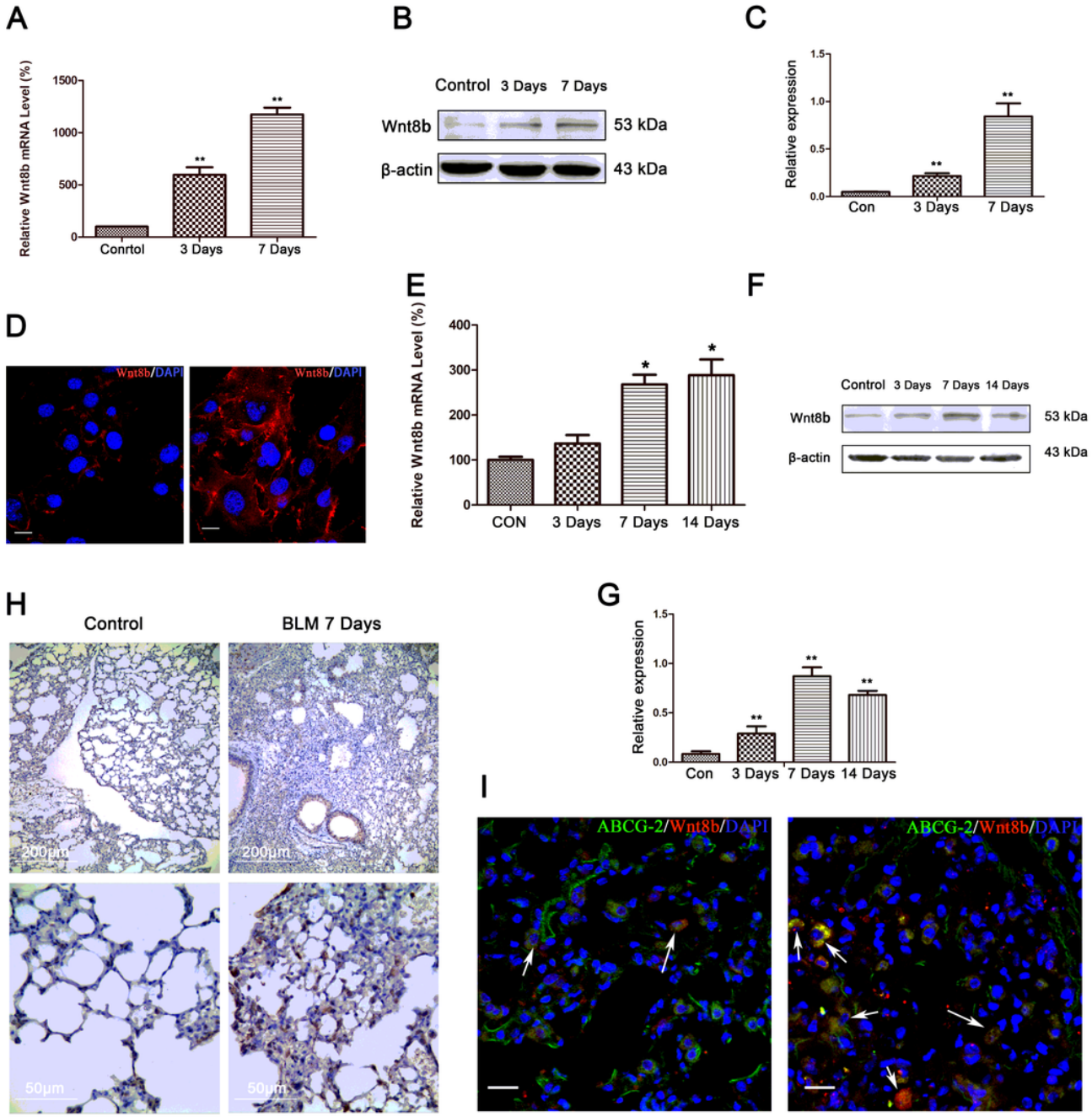

Figure 2

Wnt8b was upregulated both in myofibroblast differentiation of lung-resident mesenchymal stem cells (LR-MSCs) and fibrotic lungs. (A-D) mRNA levels and protein levels of Wnt8b in LR-MSCs treated with TGF-β1 were measured by Quantitative PCR (A), western blotting (B) and immunofluorescence staining,

400x. Scale bar, 20μm (D). Quantitation of Wnt8b abundance (C). *, p < 0.05; **, p < 0.01; n = 3 experiments. E-H Quantitative PCR (E), western blot (F) and immunohistochemistry (H) showed Wnt8b in bleomycin (BLM)-induced fibrotic lung tissue. The quantitation of Wnt8b abundance (G). *, p < 0.05; **, p < 0.01; n = 3 experiments. (I) The levels of of ATP-binding cassette protein G2 (ABCG-2) and Wnt8b in the lung tissues of the BLM-induced pulmonary fibrosis model were examined by immunofluorescence staining, 400x. Scale bar, 20μm.

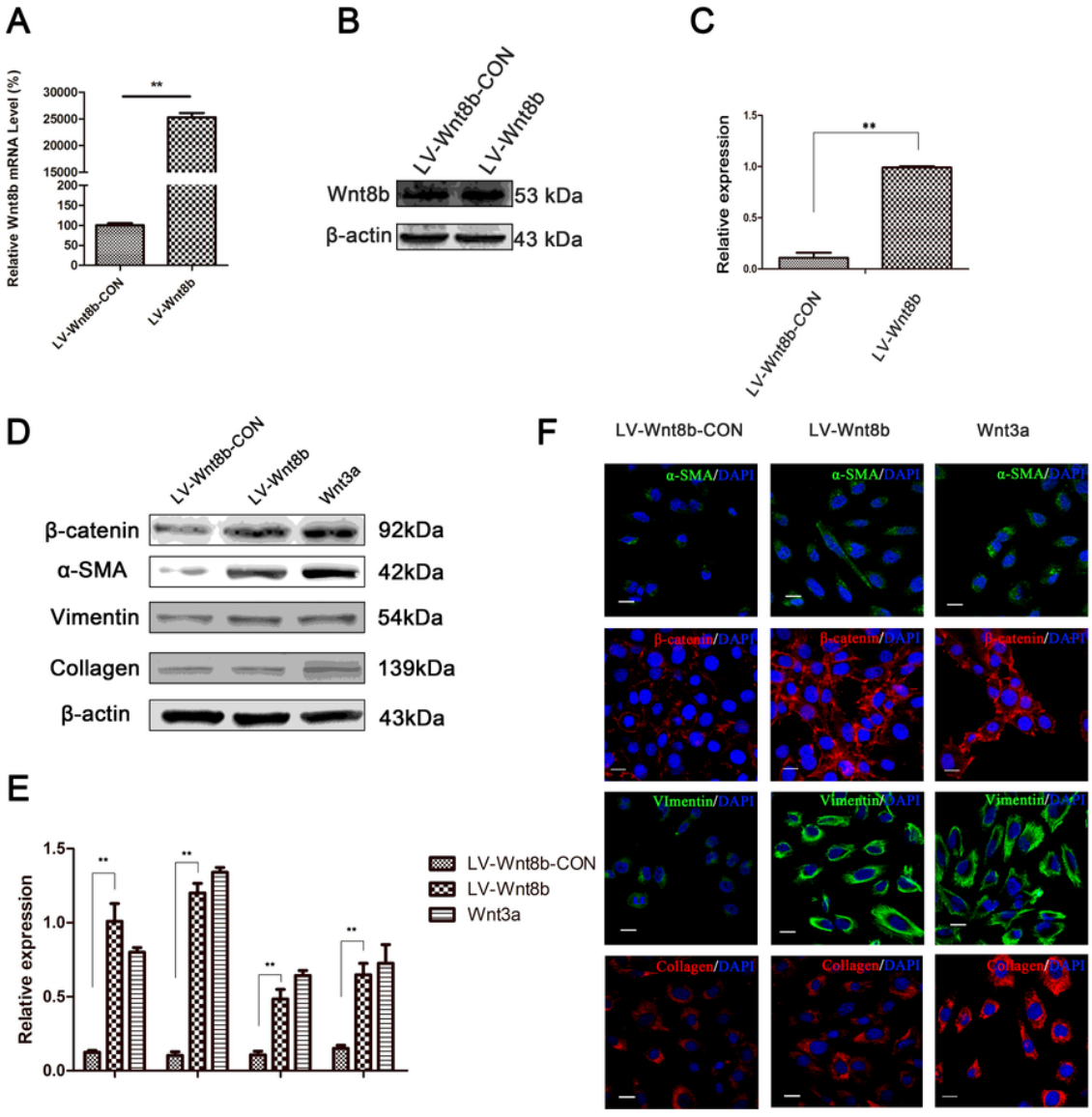

Figure 3

Wnt8b promoted lung-resident mesenchymal stem cells (LR-MSCs) differentiate into myofibroblasts via activating Wnt/β-catenin signaling. (A-C) The mRNA levels (A) and protein levels (B) of Wnt8b in LR-MSCs transfected with LV-Wnt8b. The quantitation of Wnt8b abundance (C). *, $p < 0.05$; **, $p < 0.01$; n = 3 experiments. (D-F) The expression of β-catenin, α-SMA, Vimentin and Collagen in LR-MSCs transfected with LV-Wnt8b or incubated with Wnt3a measured by western blotting (D) and immunofluorescence staining, 400x. Scale bar, 20μm (F). (E) The quantitation of β-catenin, α-SMA, Vimentin and Collagen abundance. *, $p < 0.05$; **, $p < 0.01$; n = 3 experiments.

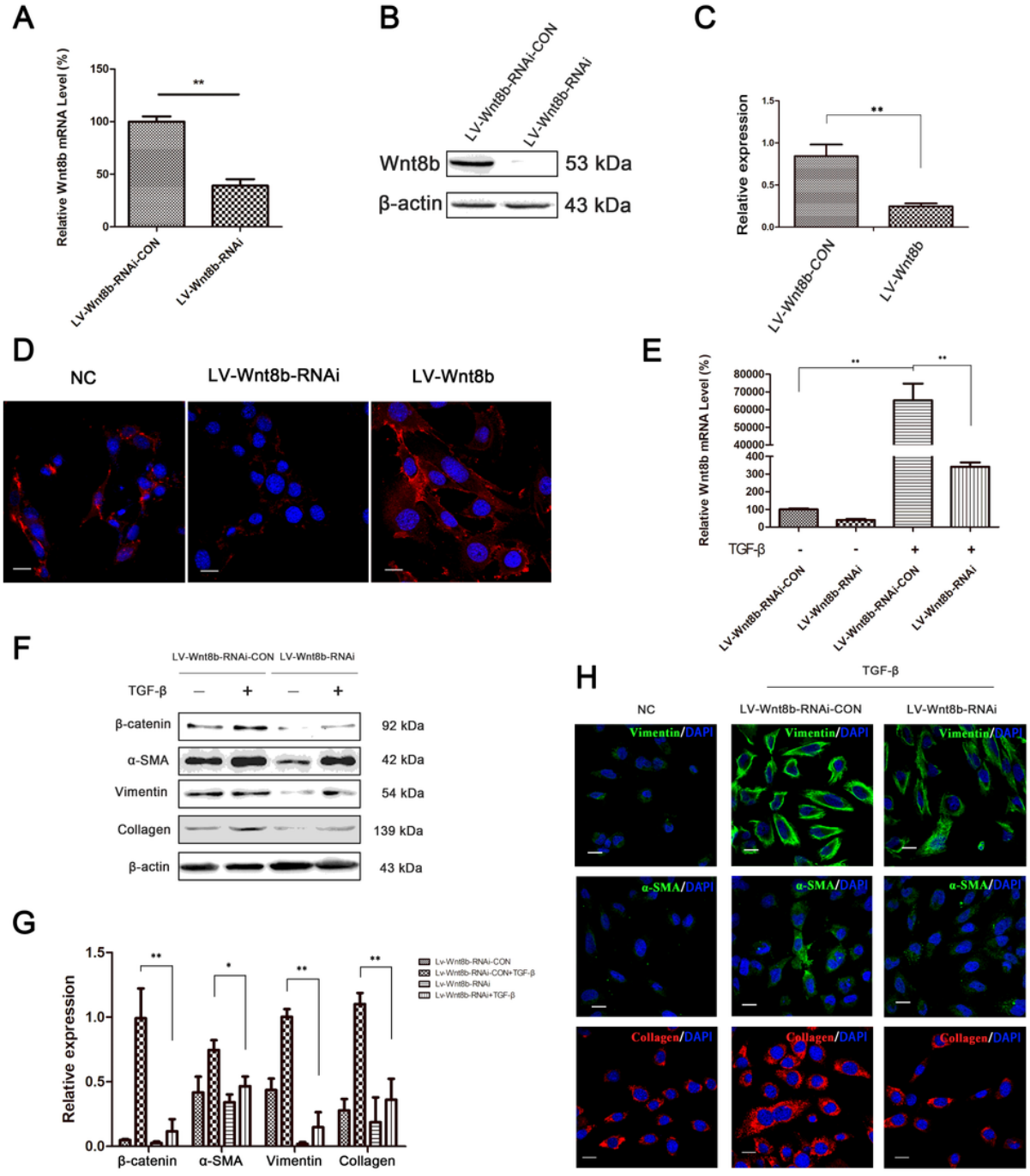

Figure 4

Silence of Wnt8b could suppress myofibroblast differentiation of lung-resident mesenchymal stem cells (LR-MSCs). (A-C) The mRNA levels (A) and protein levels (B) of Wnt8b in LR-MSCs transfected with LV-Wnt8b-RNAi. (C) The quantitation of Wnt8b abundance. *, $p < 0.05$; **, $p < 0.01$; n = 3 experiments. (D) The expression of Wnt8b in LR-MSCs transfected with LV-Wnt8b-RNAi and LV-Wnt8b were detected by immunofluorescence staining, 400x. Scale bar, 20μm. (E-H) LR-MSCs were transfected with LV-Wnt8b-RNAi followed with the treatment of TGF-β1 for 7 days. The mRNA levels of Wnt8b were measured by quantitative PCR (E). The protein levels of β-catenin, α-SMA, Vimentin and Collagen were determined by western blot (F). (G) The quantitation of β-catenin, α-SMA, Vimentin and Collagen abundance. *, $p < 0.05$; **, $p < 0.01$; n = 3 experiments. The expression of Vimentin, α-SMA and Collagen were further examined by immunofluorescence staining, 400x. Scale bar, 20μm (H).

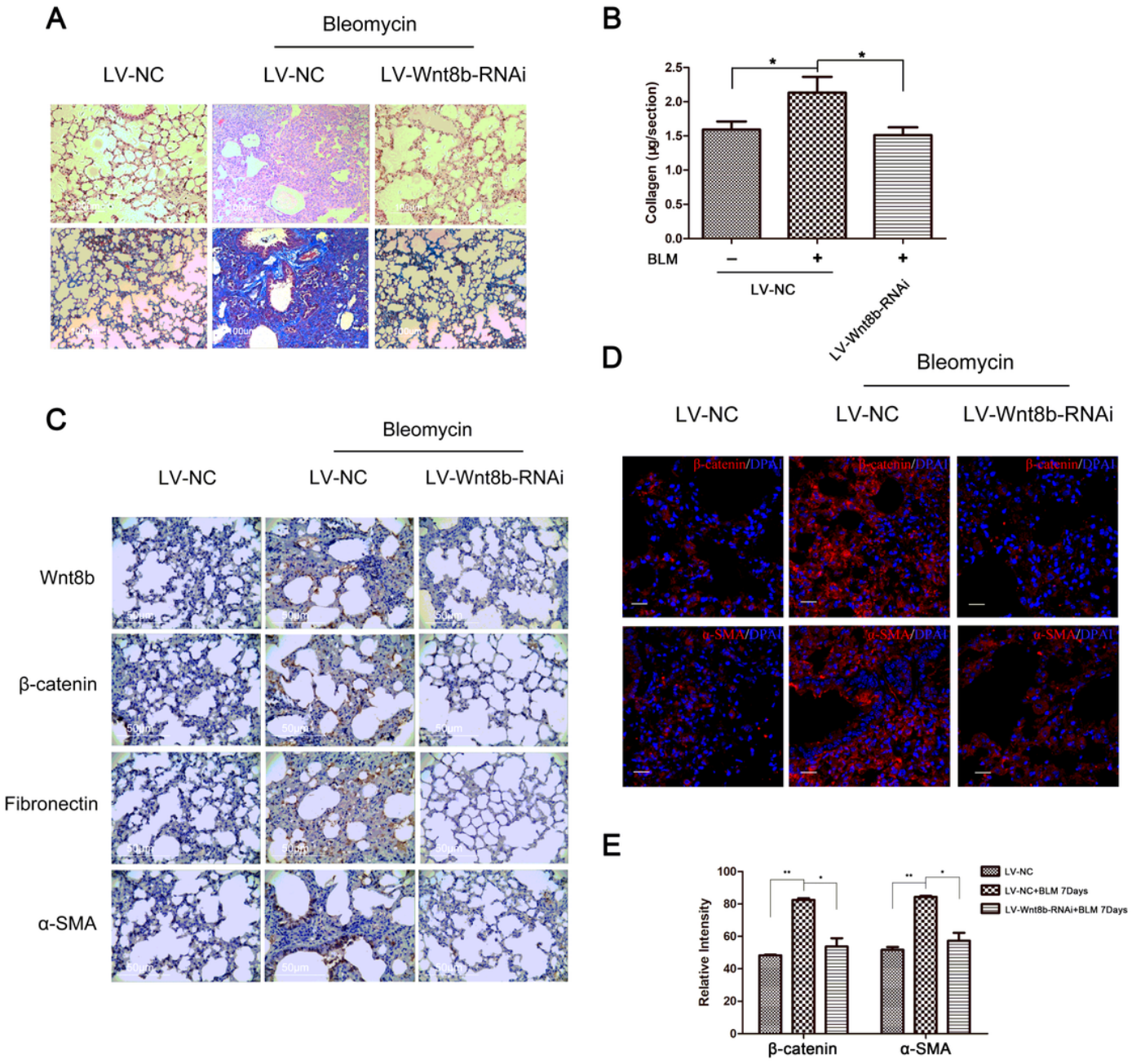

Figure 5

Suppression of Wnt8b could protect mice from BLM-induced pulmonary fibrosis. (A-C) Mice were intratracheally injected with 5 × 108 TU/ml LV-Wnt8b-RNAi or LV-negative control (NC) 7 days after administration of bleomycin (BLM). Mice were killed at day 14 after BLM instillation. Pulmonary fibrosis was determined by hematoxylin–eosin (H&E) staining (A). (B) Quantitative analysis of collagen from lung tissues. The collagen content was measured using Sirius red/fast green collagen staining. n =5 per group. *p<0.05; **p<0.01. (C) The protein levels of Wnt8b, β-catenin, Fibronectin and α-SMA were

measured by immunohistochemistry. (D) The expression of β-catenin and α-SMA were further confirmed by immunofluorescence staining, 400x. Scale bar, 20μm. (E) Immunofluorescence quantification of the expression of β-catenin and α-SMA. *, $p<0.05$; **, $p<0.01$; n=5 mice/group.