

## Original Article

# Ouabain ameliorates bleomycin induced pulmonary fibrosis by inhibiting proliferation and promoting apoptosis of lung fibroblasts

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**Abstract:** Idiopathic pulmonary fibrosis (IPF) is a lethal idiopathic interstitial pulmonary disease characterized by progressive deterioration in lung function that commonly affects elderly people. The pathogenesis of the disease is incompletely understood and therefore lacking effective therapy. Ouabain a digitalis has been reported to be able to suppress lung fibroblast activation via downregulating TGF- $\beta$ -smad signal pathway *in vitro*. Here, we investigated the effects of ouabain in pulmonary fibrosis *in vivo*. Pulmonary fibrosis was induced in C57/BL6 mice by an intratracheal instillation of bleomycin (2.0 mg/kg), ouabain (0.6 mg/kg) was given daily via intraperitoneal injection for one week starting at 7 days after intratracheal instillation of bleomycin. Our study showed ouabain significantly reduce  $\alpha$ -SMA, fibronectin and collagen I expression in lung fibrosis animal model. Further, ouabain inhibits cells proliferation and promotes apoptosis of lung fibroblasts *in vitro*. In conclusion, our results indicate ouabain a novel effective drug that inhibits lung fibrosis progression.

**Keywords:** Ouabain, bleomycin, pulmonary fibrosis

## Introduction

Idiopathic interstitial pulmonary fibrosis (IPF) is a chronic, progressive fibrotic interstitial pneumonia occurring in adults and is characterized by the patterns of usual interstitial pneumonia in radiologic and/or histopathologic manifestation. The pathogenesis of IPF, is incompletely understood, and the effective drugs on IPF is lack. So, it is urgent to find new more effective therapeutic strategies for IPF [1, 2]. Accumulation of activated fibroblasts/myofibroblasts and deposition of massive extracellular matrix are crucial processes for fibrotic lung remodeling in IPF. TGF- $\beta$ , one of the major profibrotic cytokines in IPF, plays a pivotal role in activation of fibroblasts [2, 3]. The levels of TGF- $\beta$  were increased in the lungs of IPF patients and in bleomycin-induced pulmonary fibrosis ani-

mal model [2]. TGF- $\beta$  signal pathway regulates epithelial cells injury and fibroblast proliferation and transdifferentiation into myofibroblasts. Thus the suppression of TGF- $\beta$  signal pathway alleviates pulmonary fibrosis [4-6].

Ouabain, a digitalis mainly for heart failure, is an inhibitor of Na, K-ATPase. In addition, Ouabain, has multiply biological functions such as regulating cell proliferation and apoptosis, modulating inflammation, inducing autophagy, etc. [7-9]. Recently, some researches reveal that ouabain inhibits lung fibroblasts activation and epithelial mesenchymal plasticity *in vitro* by blocking TGF- $\beta$ -smad signal pathway including downregulation smad2, TGF $\beta$ RII transcription and smad2 phosphorylation [10, 11]. However, the effects of ouabain on the progress of pulmonary fibrosis remains unknown, so we want

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to know if ouabain could be a new drug for IPF via bleomycin induced pulmonary fibrosis mice model.

In this study, we investigated the effects of ouabain on cell proliferation, apoptosis in lung fibroblasts and evaluated whether ouabain has the anti-profibrotic effects in bleomycin-induced pulmonary fibrosis mouse model.

### Materials and methods

#### Mouse model

C57BL/6N mice (male, 7~8 weeks old, weight 22~24 g) were purchased from Vital River Laboratory Animal Technology Co. Beijing, China. Animals were maintained at a controlled temperature of 24±1°C with a 12:12 h light-dark cycle, and were fed a standard diet. Water was freely available. A dose of 2 mg/Kg bleomycin (Nippon Kayaku Co., Tokyo, Japan) was intratracheally administered. Intraperitoneal dose (0.6 mg/kg) were given from day 7 to day 13. Body weight of the mice were assessed at daily following after ouabain or PBS treatment. Mice were euthanasia on day 14. The lungs were removed for hematoxylin & eosin (HE), Masson trichrome stain, immunohistochemistry stain and gene expression analysis.

#### Cell culture

MRC-5 human fetal lung fibroblasts were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), The cells were maintained in Minimum Essential Medium (MEM)- $\alpha$  (Invitrogen) with 10% FBS (Invitrogen), 100 U/ml penicillin (Hyclone), and 100 mg/ml streptomycin (Hyclone) in a humidified incubator (Thermo Scientific, Waltham, MA, USA) at 37°C in 95% air (21% O<sub>2</sub>) and 5% CO<sub>2</sub>.

#### Western blotting

Western blotting was performed as previously described [12, 13]. The primary antibodies used were anti-mouse  $\alpha$ -SMA (ab124964, Abcam), fibronectin (ab2413, Abcam), and  $\beta$ -actin (ab6267, Abcam). The denatured proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA) using a Mini-Protean electrophoresis module assembly (Bio-Rad) at 80 mV and transferred to nitro-

cellulose membranes (Millipore, Billerica, MA, USA) for 100~120 min using the Mini Trans-Blot electrophoresis transfer cell (Bio-Rad) at 300 mA according to the molecular weight. After incubating with the primary antibodies, the membranes were treated with IRDyeTM800 (green)- or IRDyeTM700 (red)-conjugated affinity purified anti-rabbit or anti-mouse IgG (LI-COR, Lincoln, NE, USA). Positive bands were visualized, and the intensity of the bands was evaluated using a LI-COR Odyssey infrared double-fluorescence imaging system (LI-COR).

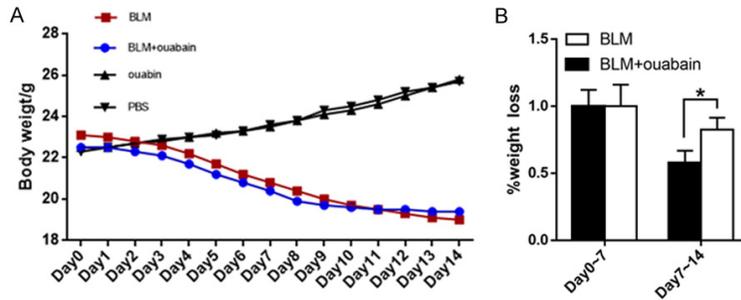
#### Gene expression analysis

Total RNA of lung tissue was homogenized in liquid nitrogen and isolated using the TRIzol reagent (Invitrogen). Reverse transcription was performed on 1  $\mu$ g total RNA with oligo (dT) primers in 25  $\mu$ l reactions using the Omniscript RT kit (Tiangen Biotech, Beijing, China) following the manuals. The primers were purchased from Sangon Biotech, Shanghai, China,  $\beta$ -actin 5'-AGGCCAACCGTGAAAAGATG-3' and 5'-AGAGCATAGCCCTCGTAGATGG-3'; *Fh1* 5'-GTGTAGCACAACTTCCAATTACGAA-3' and 5'-GGAATTTCCGCCTCGAGTCT-3'; *Col1a1* 5'-CCAAGAAGACATCCCTGAAGTCA-3' and 5'-TGCACGTCATCGCACACA-3';  $\alpha$ -SMA 5'-GCTGGTGATGATGCTCCCA-3' and 5'-GCCATTCCAACCATTACTCC-3'. Real-time RT-PCR was performed on an ABI PRISM® 7500 instrument (Applied Biosystems, Foster, CA, USA) as previously described [14].

#### Lung histology

The preparation of mouse lungs for histology was performed as previously described [15]. Briefly, the lung was dehydrated, paraffin-embedded, and cut into 4- $\mu$ m sections. Lung sections were stained with H & E and Masson's trichrome stain for assessment of pathological changes. For immunohistochemistry, the tissue sections were deparaffinized and rehydrated as described above. After a microwave treatment for 20 min in ethylenediaminetetraacetic acid (EDTA) buffer and subsequent cooling, the endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in methanol for 15 min in dark. After blocking in 5% goat serum for 20 min, sections were incubated with antibodies against  $\alpha$ -SMA (ab124964, Abcam), fibronectin (ab2413, Abcam), collagen I overnight at 4°C as described previously [16].

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**Figure 1.** Ouabain treatment rescued BLM-induced weight loss. A. The average body weight of BLM group, BLM+ouabain group and ouabain group from day 0 to day 14. B. The body weight loss has little difference between BLM group and BLM+ouabain group from day 0 to day 7. After ouabain treatment, body weight loss of ouabain group was significantly attenuated. Results are expressed as mean  $\pm$  SEM,  $n = 10$  mice per group, \*,  $P < 0.05$ .

(annexin-V positive, PI negative) from necrosis (annexin-V positive, PI positive) cells.  $2 \times 10^5$  cells under different treatment were resuspended in 100  $\mu$ l binding buffer, and then incubated with 2  $\mu$ l Annexin-V-FITC (20  $\mu$ g/ml) for 15 minutes in dark at room temperature. Each sample was then incubated with 1  $\mu$ l PI (50  $\mu$ g/ml) for 2 minutes, and were immediately analyzed by an Accuri C6 flow cytometer (BD Accuri Cytometers, MI). A minimum of 10,000 events was acquired for each sample.

## Hydroxyproline assay

Total lung collagen was determined by analysis of hydroxyproline on day 14 after bleomycin instillation as previously described [19]. Lung homogenates in 1.5 ml HCl (2 mol/l) were incubated at 110°C overnight. Chloramine T solution (56 mmol/l chloramine T and 10% 1-propanol in citrate/acetate buffer) were added at room temperature for 20 min. The mixture was then incubated with Ehrlich's solution at 65°C for 20 min. After cooling to room temperature, the absorbance was measured at 550 nm by a spectrophotometer and L-hydroxyproline content was determined using a standard curve as previously described [17].

## Cell proliferation assay

For the CCK-8 assay, cells were seeded in 96-well plates ( $2 \times 10^3$  cells/well), and 1% FBS starvation for 24 h. After treated with PBS or ouabain, cells were incubated at 37°C in CO<sub>2</sub> incubator; Cell proliferation indices were measured at 24, 48 and 72 h using a CCK-8 kit (Dojindo Co., Ltd. Japan). The absorbance was measured at 450 nm by a spectrophotometer as previously described [18].

## Cell apoptosis assay

Cell apoptosis assay was performed as previously described [19]. To evaluate the effect of ouabain on MRC-5 cells, annexin-V and propidium iodide (PI) (Apoptosis Detection Kit, annexin-V-FITC, BD, Biosciences Pharmingen, San Diego, CA) were used to distinguish apoptosis

## Statistics analysis

Data are expressed as mean  $\pm$  standard error of the mean (SEM). Two-tailed Student's t-test was performed using two-way analysis of variance. Comparison of the difference among multiple groups were analyzed two-way analysis of variance (ANOVA) with Sidak multiple comparisons test. Kaplan-Meier survival analysis with log-rank test was used to compare survival rates. Statistical analysis was performed using the Prism software (Graphpad Software, CA, USA). For all analyses,  $P$  values  $< 0.05$  were considered as significant.

## Results

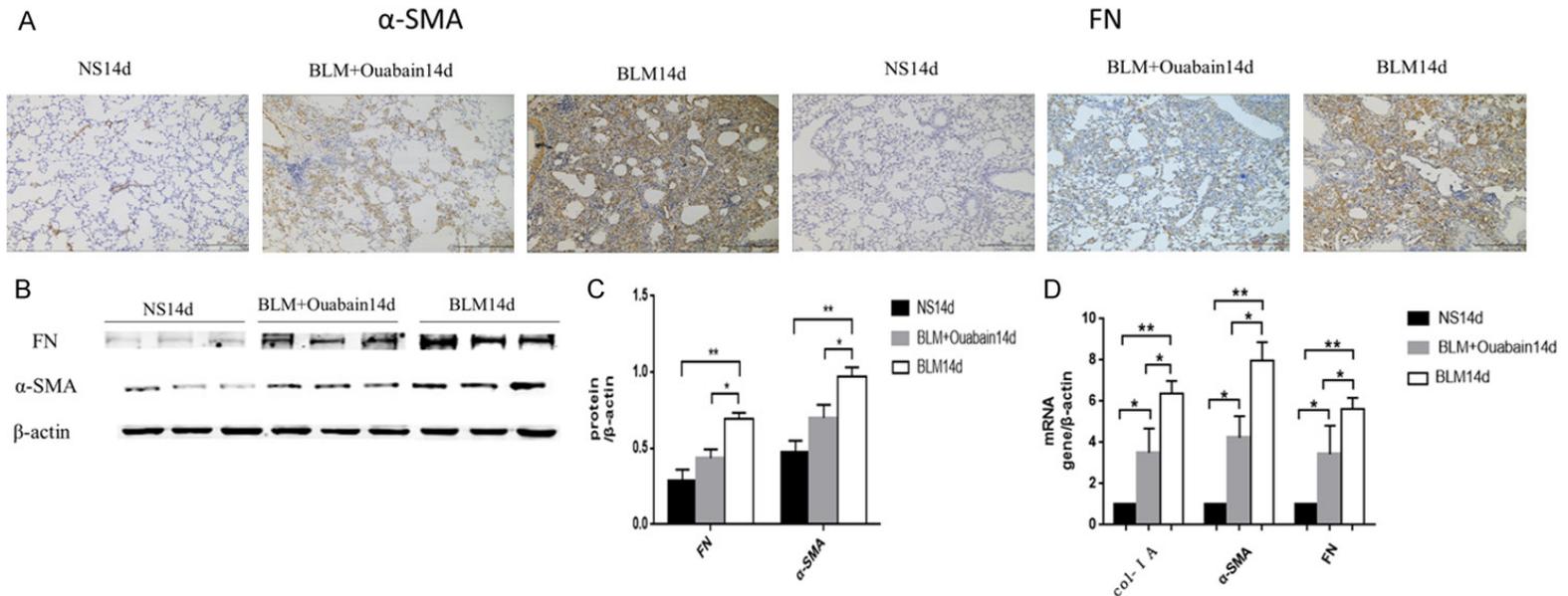
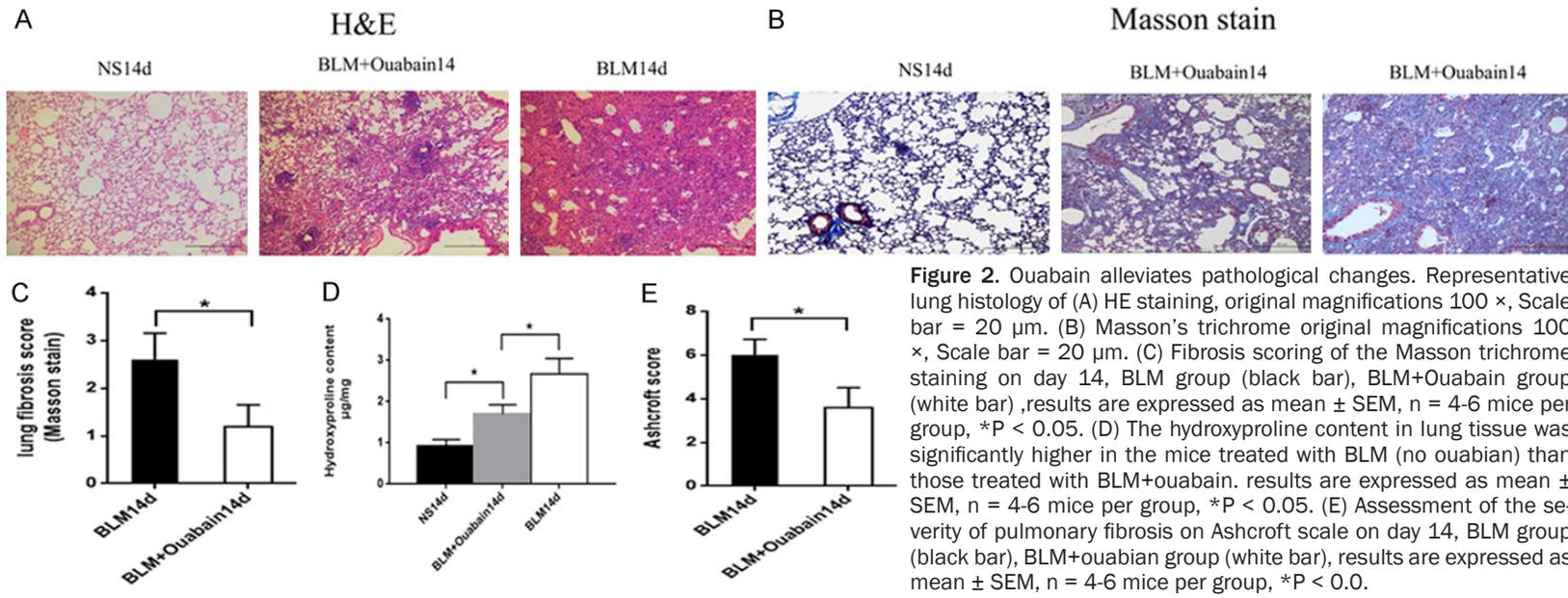
### Ouabain treatment rescued bleomycin-induced weight loss

As shown in **Figure 1A**, the body weight of the mice decreased significantly 5 days after the bleomycin treatment. In addition, compared with the bleomycin group, ouabain (0.5 mg/kg) administration reduced body weight loss from day 7 to day 14. ( $n = 10$ ,  $P < 0.05$ , **Figure 1B**). As expected, ouabain alone did not change mouse body weight.

### Ouabain alleviates pathological changes induced by bleomycin

Bleomycin-induced pulmonary damage and fibrosis in mice was determined by histological analysis. The lungs of bleomycin treated mice have thickening of alveolar septa, collapse of alveolar spaces, loss of alveolar structure, over-

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**Figure 3.** Ouabain suppresses profibrotic proteins expression. A. Representative lung histology immunohistochemistry of  $\alpha$ -SMA and Fibronectin original magnifications 100  $\times$ , Scale bar = 20  $\mu$ m. B. Whole lung tissues western blotting of  $\alpha$ -SMA and Fibronectin. C. Normal saline group (black bar), BLM group (gray bar), BLM+Ouabain group (white bar), results are expressed as mean  $\pm$  SEM, n = 4-6 mice per group, \*P < 0.05 \*\*P < 0.01. D. Relative mRNA expression of collagen 1A,  $\alpha$ -SMA and Fibronectin from lung tissues Normal saline group (black bar), BLM group (gray bar), BLM+Ouabain group (white bar), results are expressed as mean  $\pm$  SEM, n = 4-6 mice per group, \*P < 0.05 \*\*P < 0.01.

proliferation of fibroblasts and infiltration of inflammatory cell infiltration by H & E staining (**Figure 2A**). Masson's staining and hydroxyproline assay showed that bleomycin induced extensive collagen deposition in lung (**Figure 2B**). Ouabain treatment markedly ameliorated these injuries and evidently decreased collagen deposition (**Figure 2C, 2D**). More important, our data showed that ouabain treatment ameliorated bleomycin-induced pulmonary fibrosis as assessed by Ashcroft scoring (P < 0.05 **Figure 2E**).

*Ouabain treatment suppresses  $\alpha$ -SMA, fibronectin, collagen expression in bleomycin-induced lung fibrosis*

The expression of collagen I,  $\alpha$ -SMA and fibronectin in the lung tissue from mice was assayed by immunohistochemistry, Western blotting and qPCR. The results demonstrated that the expression of collagen I,  $\alpha$ -SMA, fibronectin were markedly increased following exposure to bleomycin (**Figure 3**). Treatment with ouabain significantly prevented bleomycin-induced changes the protein level of  $\alpha$ -SMA, and fibronectin (**Figure 3A-C**). Moreover, ouabain treatment significantly decreased the mRNA level of collagen I a,  $\alpha$ -SMA, and fibronectin in the lung tissues (**Figure 3D**).

*Ouabain inhibits the proliferation of lung fibroblasts*

The CCK-8 assay was used to assess the effects of ouabain on the proliferation of MRC-5 cells. 100 nM Ouabain treatment markedly reduced OD values compared with control group at 24 h, 48 h, 72 h (**Figure 4A**). These data indicated that ouabain inhibits lung fibroblasts proliferation.

*Ouabain promotes apoptosis of lung fibroblasts*

Cell apoptosis was detected by Annexin V and 7AAD staining. MRC-5 cells were exposed to

100 nM ouabain for 48 h. As shown in **Figure 4B** and **4C**, ouabain markedly increased early apoptotic and late apoptotic cell populations as well as necrosis cell population compared to control group. These results suggest that ouabain can significantly increase lung fibroblasts apoptosis.

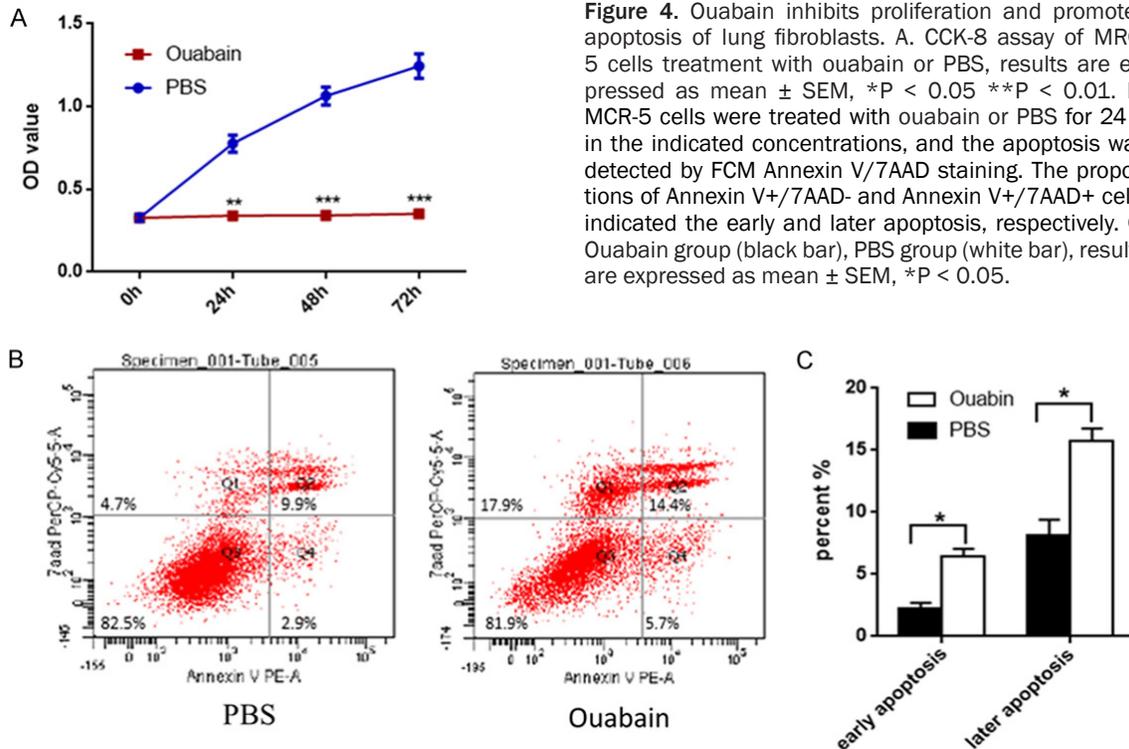
### Discussion

IPF is a fatal interstitial lung disease characterized by alveolar epithelial cells apoptosis, fibroblasts proliferation, myofibroblasts differentiation and excessive ECM deposition. Since the pathogenesis mechanisms remain largely unknown, few drugs are effective for IPF. In this study, we found that ouabain, a digitalis, protects against bleomycin induced mouse pulmonary fibrosis by inhibiting proliferation and promoting apoptosis of lung fibroblasts. Thus, ouabain might be a promising drug for pulmonary fibrosis.

TGF- $\beta$  signaling plays a central role in pulmonary fibrosis [20, 21]. Previous researches indicated that ouabain inhibits lung fibroblast activation and alveolar cell epithelium mesenchymal transition by suppressing smad2 and TGF- $\beta$ RII expression and smad2 phosphorylation [10, 11] in vitro. To examine the anti-fibrotic effect of ouabain in vivo, we used bleomycin-induced pulmonary fibrosis-mouse model. We found that and the expression of collagenI,  $\alpha$ -SMA, fibronectin in the lungs of bleomycin-treated mice. ouabain ameliorates bleomycin induced pulmonary fibrosis.

The activating behaviors of lung fibroblasts from IPF patients including over-proliferation and resistance to apoptosis is involved in the development of IPF [22-25]. One of the therapeutic strategy for pulmonary fibrosis is to inhibit the proliferation and to promote apoptosis of lung fibroblasts. The effect of Ouabain on cell proliferation dependent on drug concentration, However, many studies report ouabain induces cell apoptosis [9, 26-29]. We tested

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**Figure 4.** Ouabain inhibits proliferation and promotes apoptosis of lung fibroblasts. A. CCK-8 assay of MRC-5 cells treatment with ouabain or PBS, results are expressed as mean  $\pm$  SEM, \* $P < 0.05$  \*\* $P < 0.01$ . B. MCR-5 cells were treated with ouabain or PBS for 24 h in the indicated concentrations, and the apoptosis was detected by FCM Annexin V/7AAD staining. The proportions of Annexin V+/7AAD- and Annexin V+/7AAD+ cells indicated the early and later apoptosis, respectively. C. Ouabain group (black bar), PBS group (white bar), results are expressed as mean  $\pm$  SEM, \* $P < 0.05$ .

the effect of ouabain at 100 nM on lung fibroblasts proliferation by CCK-8 assay and apoptosis by flow cytometry. The results show ouabain significantly inhibits growth and promote apoptosis of MRC-5 lung fibroblasts. Several researches indicated the relationship between ouabain and autophagy [9, 30], enhancing autophagy may play an important role in anti-fibrosis.

As we all know, cardiac glycosides is used for heart failure with narrow therapeutic window, since the serious side effects including arrhythmia. According to introduction of ouabain, the LD50 is 1.213 mg/Kg in mouse by intraperitoneal injection. We used dose gradients and found that higher than 0.6 mg/kg dose intraperitoneal injection to mice caused a sudden death easily, moreover, lower than 0.6 mg/kg dose couldn't get satisfied effect. So, we chose 0.6 mg/kg ouabain treatment for mice by intraperitoneal injection. Since the narrow therapeutic window, the dose of ouabain for cure IPF patients needs further explorations [31].

In summary, our data showed that ouabain ameliorates bleomycin-induced pulmonary fibrosis in mouse model by inhibiting proliferation and promoting apoptosis of lung fibro-

blasts. Therefore, ouabain might be a promising novel treatment for pulmonary fibrosis. Further investigation is needed to evaluate the anti-fibrotic mechanisms of ouabain in animal model and its clinical applications.

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### Disclosure of conflict of interest

None.

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