

Inhibition of Galectin-1 attenuates lung fibroblast activation and proliferation in lung fibrosis

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ABSTRACT

The purpose of this study was to investigate the parenchymal changes in idiopathic pulmonary fibrosis (IPF) caused by massive fibroblastic infiltration and proliferation in lung tissue. Galectin-1 (Gal-1) has been reported to be involved in angiogenesis and fibrosis via modification of TGF- β receptor signaling pathways. However, it remains unknown whether Galectin-1 plays a critical role in IPF. In the current study, we aimed to identify Gal-1 as a crucial fibrotic protein in IPF process. Murine lung fibroblast was pre-treated using Gal-1 inhibitor OTX-008 or overexpression of Gal-1 and then activated using transforming growth factor- β (TGF- β). Adult male C57BL/6J mice were conducted intratracheal injection of bleomycin (BLM) for lung fibrosis. Mice were conducted OTX-008 administration. Gal-1 expression, fibroblast activation and proliferation, extracellular matrix (ECM), lung fibrosis, lung histology and pulmonary function were investigated respectively. We demonstrated that Gal-1, as a positive pro-fibrotic marker, could promote lung fibroblast activation and proliferation. Inhibition of Gal-1 reduced fibroblast activation and proliferation through negative regulation of TGF- β /Erk1/2 and AKT pathway. In vivo, Gal-1 inhibition ameliorates lung fibroblast accumulation and protects lung histology and function. Gal-1 is verified to be a pro-fibrotic gene in IPF pathogenesis, which promotes fibroblast activation and proliferation via TGF- β /Erk1/2 and AKT pathway. Moreover, inhibition of Gal-1 in lung fibrosis model attenuates lung fibroblast bioactivity and reduces ECM, leading to improved pulmonary histology and function. Hence, knockdown of Gal-1 in IPF may be a promising target therapy.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is an unknown etiologic disease characterized by chronic and progressive lung fibrosis (1). The fiber-derived injury repair leads to massive fibroblast activation and accumulation eventually forming the myofibroblastic foci, which immensely affects lung function (2,3). It is universally cognized that extensive endothelial cell damage in alveolar and permanent stimulation of inflammation elicits abnormal proliferation of lung fibroblasts, contributing to an overwhelming generation and deposition of ECM in pulmonary parenchyma and subsequently resulting in disordered histology and dysfunction of the lung (4,5). Considering the incomplete molecular mechanism in IPF pathogenesis, the present clinical treatment for IPF hardly improved the survival rate except for lung transplantation (4). Hence, novel studies concerning anti-fibrotic therapeutic strategies have been sprung up in researches. Gal-1 is one of the members in the Galectin family and localized in cytomembrane and cytoplasm, which has been verified to exert a regulated role in cell proliferation, migration and growth (6,7). Importantly, Gal-1 was reported to be involved in angiogenesis via binding with N-glycans of vascular endothelial growth factor (VEGF) receptor 2 to further regulate the VEGF-A effect (8). Besides, a significant role of Gal-1 is that it promotes SMAD family member 2 (SMAD 2) induced mesenchymal activity in various cells, such as cancer cells, stellate

cells and especially fibroblast (9-11). Bennett and his colleagues found that Gal-1 expression showed a significant increase in bronchoalveolar lavage of patients with idiopathic pulmonary fibrosis (12), suggesting a potential role of Gal-1 in IPF. Administration of TGF- β induced the differentiation of fibroblasts to myofibroblasts in various organ-derived fibroblasts (13,14). Previous studies showed that Gal-1 increase by TGF- β stimuli could promote fibrosis through enhancing nuclear retention of Smad2 (9). However, the underlying molecular mechanism of Gal-1 in the IPF model is elusive. In the present study, we demonstrate the first evidence of Gal-1 as a potent accelerant in BLM-induced lung tissue, together with the specific mechanism in lung fibroblasts exposed to TGF- β . Both in vivo and in vitro data indicate that Gal-1 inhibition attenuates lung fibrosis via reduction of lung fibroblast to myofibroblast and ECM deposition.

Materials and Methods

Cell treatment

The murine lung fibroblasts (LFs) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and seeded in dulbecco's modified eagle medium (DMEM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, Rockville, MD, USA). Growing to 80%

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confluence in the flask, cells were pretreated with Gal-1 inhibitor (30 mM; OTX-008; MedChemExpress, Hangzhou, China) or overexpression of Gal-1 before stimulation of TGF- β (10 ng/ml, Sigma, St. Louis, MO, USA). Primers 5'-TCTGTACAGCTATTTTATTTTCAAT-3' and 5'-AGACAGTCGATAAAATAAAAAGTTA-3' were used for the Mut-gene of the Gal-1. Then cells were harvested using lysis buffer or TRIzol for western blot or RT-PCR analysis.

Animals and Modeling

Adult male C57BL/6J mice (20-22 g) were randomly assigned to three different experimental groups. Saline injection group (Saline), BLM-induced lung fibrosis group (BLM) and BLM-induced lung fibrosis+OTX-008 injection group (BLM+INH) were established in the study. Briefly, we anesthetized mice using 1% pentobarbital sodium (50mg/kg, I.P.). Then mice were exposed the trachea and BLM (3.5mg/kg, Invitrogen, USA) diluted in PBS was injected slowly into trachea using a micro-syringe (Saline group only injected isopycnic saline). 2 h after BLM injection, OTX-008 (Sigma, 20 mg/kg, I.P., q.d, St. Louis, MO, USA) dissolved by 2% dimethylsulfoxide was conducted for 7 days. All operations to animals were approved by the Animal Ethics Committee of Xi'an Jiaotong University.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from lung tissue using a TRIzol reagent (Beyotime Biotechnology, Shanghai, China) following the manufacturer's protocol. Reverse transcription was conducted using qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA). RNAs level was detected using perfecta SYcBR Green Supermix (Quanta Biosciences, Beverly, MA, USA). Melting curve was employed to show each RNAs level. GAPDH was used for normalization. The data of relative mRNA expression levels were quantified by the $2^{-\Delta\Delta Ct}$ methods. The primers of RNAs are listed at Table 1.

Western blot analysis

Protein was collected and diluted with a loading buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein and then transferring was conducted. α -SMA, phosphor-AKT (p-AKT), AKT, phosphor-Erk1/2 (p-Erk1/2), Erk1/2, collagen I, fibronectin and Gal-1 antibodies were purchased from Abcam (Cambridge, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) antibody was obtained from Proteintech (Rosemont, IL, USA). The expression of each protein was visualized using an enhanced chemiluminescence system (ECL; Tanon, China).

Immunofluorescence (IF) and Immunohistochemistry (IHC)

Cell and extracted lung tissue were fixed with 4% paraformaldehyde (PFA). Tissue was embedded into paraffin and cut into 5 μ m sections. After immuno-block, cell and sections were incubated with primary antibodies including α -SMA, Gal-1, p-Erk1/2, and p-AKT and secondary antibodies. The nucleus was counterstained with diamidine phenyl indoles (DAPI) followed by the capture of fluorescence images using a fluorescence microscope.

Hematoxylin-Eosin Staining and Masson Staining

The section was performed deparaffinage and hydration conducted hematoxylin-eosin staining using a HE staining Kit and Masson staining Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocols. The histologic images were collected using a microscope.

Wound healing assay

Cells were seeded in 12-well plates in advance and treated with the accordingly factors. When cells overgrew fully, we used tips to make a wound and wait 24 h for cell healing. The images were captured using a microscope.

Trans-well assay

Cell were seeded in chambers with trans-well containing FBS-free DMEM, then we inserted the chambers into a 24-well plate filling DMEM with 10% FBS and accordingly factors. After 24 h, cells were fixed using 4% PFA and stained with crystal violet (Keygen, Nanjing, China) for 20 min. Then images were collected at a microscope.

Lung function measurement

Lung function were measured using FlexiVentTM system (Scireq, Canada). Mice underwent mechanical ventilation in the condition of 150 breaths/min, 10 ml/kg tidal volume, and 3 cm H₂O positive end-expiratory pressure. Total lung capacity was examined at deep inflation perturbation (30 cm H₂O). Lung compliance was measured using a snapshot perturbation maneuver and repeated 3–4 times per 30 s. Then lung tissue resistance was detected by broadband low-frequency forced oscillation measurements (1–20.5 Hz) and repeated 3–4 times per 30 s.

Statistical analysis

Data in the study were exhibited as the means \pm standard deviations (SD). Statistical difference between two groups was analyzed using Student's t-test and among more than two groups was assessed via one-way ANOVA. All of the analysis was operated in Statistic Package for Social Science (SPSS) 21.0 software (IBM, Armonk, NY, USA). P < 0.05 is identified as statistical significance.

Results

Gal-1 promotes TGF- β induced lung fibroblast differentiation and ECM deposition

To verify whether the expression of Gal-1 alters in lung fibrosis, we first examined both RNA level and protein level in control and BLM-injected mice. The RNA result exhibited a remarkable increase of Gal-1 expression in the BLM group (Figure 1A); moreover, we consistently witnessed a significant increase of Gal-1 protein after BLM employment in the lung (Figure 1B). Furthermore, we administrated overexpression of Gal-1 (OE) or inhibition of Gal-1 (INH) in vitro cultured LF before TGF- β stimuli to certify the role of Gal-1 expression in fibrogenesis. IF co-staining of Gal-1 and myofibroblast marker α -smooth muscle actin (α -SMA) showed that both Gal-1 and α -SMA expression increased in LF treated with TGF- β and Gal-1 OE elevated Gal-1 and α -SMA expression increase compared with the TGF- β group, whereas Gal-1 INH reduced Gal-1 and α -SMA expressions in TGF- β treated LF (Figure 1C). The result indicated that Gal-1 promoted the differen-

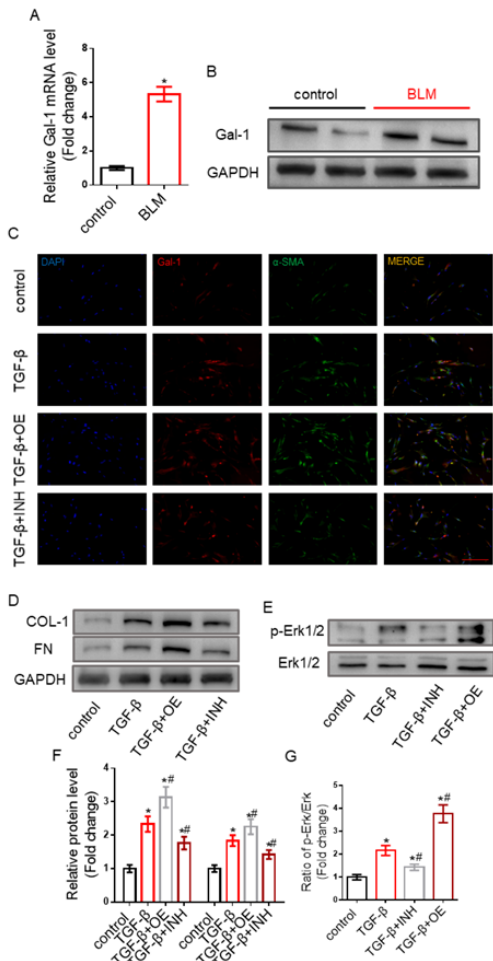


Figure 1. Gal-1 promotes TGF-β induced lung fibroblast differentiation and ECM deposition. (A) Representative RNA level of Gal-1 in lung at 21 days after BLM induction or not. (B) Representative protein level of Gal-1 lung at 21 days after BLM induction or not. (C) Representative IF staining of α-SMA and Gal-1 in lung fibroblast in control, TGF-β, TGF-β+OE (Gal-1 overexpression) and TGF-β+INH (Gal-1 inhibition) group, (magnification: 100×, bar=200 μm). (D-E) Representative protein levels of COL-1, FN, p-Erk1/2, and Erk1/2 in the control, TGF-β, TGF-β+OE and TGF-β+INH group. (F-G) Quantification of COL-1, FN, p-Erk1/2, and Erk1/2. “*” means vs. control group and “#” means vs. TGF-β group with statistical significance.

tiation from fibroblast to myofibroblast. In addition, we measured the ECM protein expressions including collagen I (COL-1) and fibronectin (FN) as well as the related Erk/MAPK signaling pathway. Western blotting exhibited that TGF-β treatment markedly increased the protein expressions of COL-1 and FN (Figure 1D and 1F). Importantly, OE of Gal-1 enhanced the representative ECM markers in LF with TGF-β treatment, yet INH of Gal-1 decreased the levels of COL-1 and FN in TGF-β stimuli. Consistently, the phosphorylation of Erk pathway showed an increased level after TGF-β administration, OE or IHN of Gal-1 expression increased or decreased the phosphorylation of Erk1/2 protein, respectively (Figure 1E and 1G). Taken together, Gal-1 regulates LF differentiation and ECM expressions through Erk/MAPK signaling pathway.

Expression of Gal-1 accelerates proliferation and migration lung fibroblast via regulation of AKT pathway

Further, we measured whether Gal expression affects

the proliferation and migration of LF treated with TGF-β. Wound healing assay showed that TGF-β treatment increased proliferation of LF and OE of Gal-1 promoted TGF-β induced cell proliferation. However, INH of Gal-1 reduced TGF-β induced LF proliferation at 24 h after wounding treatment (Figure 2A). For migration measurement, the Trans-well assay showed that TGF-β increased the amount of traversed cells in under layer, OE of Gal-1 improved TGF-β induced cell migration while INH of Gal-1 reduced the number of migratory LF (Figure 2B). To verify the mechanism of the proliferation and migration in LF, we detected the AKT pathway in LF. AKT pathway is reported to be involved in the proliferation and migration of fibroblast with TGF-β. Protein level exhibited an increased expression of phosphorylated AKT (p-AKT) after TGF-β treatment and higher expression of p-AKT in the TGF-β+OE group. However, the INH of Gal-1 decreased the TGF-β-induced p-AKT activation in LF (Figure 2C and 2D), indicating the important role of Gal-1 expression in the proliferation and migration via the regulation of the AKT pathway.

Silence of Gal-1 attenuates the formation of myofibroblast foci and ECM generation

We next evaluated the effect of Gal-1 inhibition in the lung fibrosis model. Firstly, fibrogenesis markers including COL-1 and FN as well as Gal-1 expression were measured using RT-PCR and western blot, showing that both RNA and protein levels of COL-1 and FN in BLM injected mice were significantly increased, however, INH of Gal-1 decreased the expressions of COL-1 and FN, accordingly reduced Gal-1 protein expression (Figure 3A and 3B). To analyze the region of myofibroblast foci and the changes

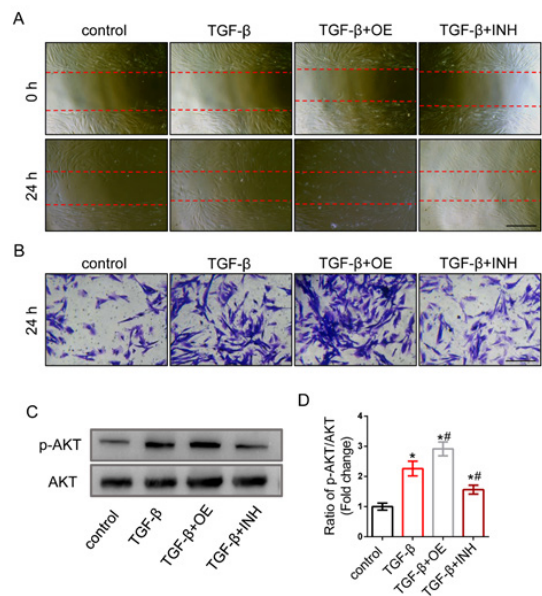


Figure 2. Expression of Gal-1 accelerates proliferation and migration of lung fibroblast via regulation of AKT pathway. (A) Images of wound healing in control, TGF-β, TGF-β+OE and TGF-β+INH group at 0 h and 24 h post operation, (magnification: 200×, bar=100 μm). (B) Images of trans-well assay in control, TGF-β, TGF-β+OE and TGF-β+INH group at 24 h post operation, (magnification: 200×, bar=100 μm). (C) Representative protein levels of AKT and p-AKT in control, TGF-β, TGF-β+OE and TGF-β+INH group. (D) Quantification of p-AKT and AKT. “*” means vs. control group and “#” means vs. TGF-β group with statistical significance.

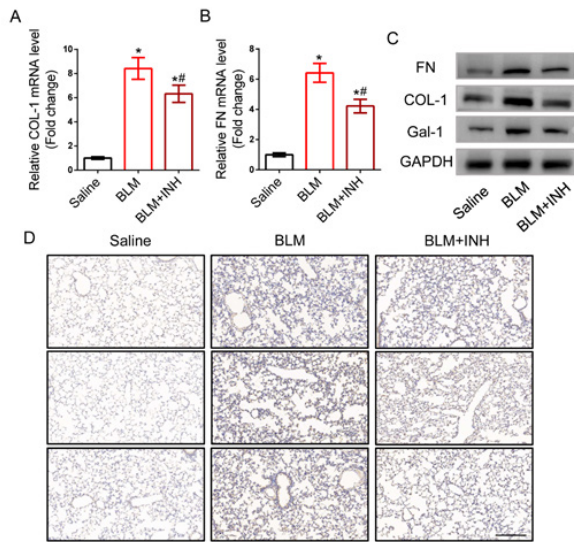


Figure 3. The silence of Gal-1 attenuates the formation of myofibroblast foci and ECM generation. (A-B) Representative RNA levels of COL-1 and FN in lung tissue at 21 days after BLM induction. (C) Representative protein levels of COL-1, FN, and Gal-1 in lung tissue at 21 days after BLM induction. (D) Representative IHC of α -SMA, p-Erk1/2, and p-AKT in lung tissue at 21 days after BLM induction, (magnification: 100 \times , bar=200 μ m). “*” means vs. Saline group and “#” means vs. BLM group with statistical significance.

of TGF- β related pathway, we conducted IHC staining to measure the phosphorylation of Erk and AKT, displaying that massive positive region of myofibroblasts in lung tissue associated with high expression of p-Erk and p-AKT. However, INH of Gal-1 reduced the phosphorylation of Erk and AKT protein and mitigated α -SMA positive area (Figure 3C). The results demonstrate that Gal-1 inhibition in lung tissue attenuates fibrosis pathogenesis and ECM deposition.

Inhibition of Gal-1 protects histology and function in lung fibrosis

Furthermore, we investigated the effect of Gal inhibition treatment on the protection of lung histology and function. Lung histology and lung fibrosis degree were visualized using HE staining and Masson's trichrome staining, respectively. BLM-induced lung fibrosis markedly increased the destruction of alveolar structure and aggravated the fiber accumulation in parenchyma. However, Gal-1 inhibition decreased the disorder of tissue and relieved the degree of fibrosis in the lung (Figure 4A and 4B). Moreover, lung function including total lung capacity, compliance, and lung resistance was measured, showing that BLM injection severely damaged lung function, which decreased total lung capacity and lung compliance in mice while increasing lung resistance. INH of Gal-1 reversely improved decreased total lung capacity and lung compliance and reduced increased lung resistance in BLM-induced lung fibrosis, indicating a protective role of Gal-1 inhibition in damaged lung function (Figure 4C-4E). Taken together, Gal-1 inhibition protects lung tissue and function from fibrotic damage.

Discussion

The current study provided new evidence that Gal-1

as a fibrogenic gene regulates fibroblast differentiation, proliferation, and migration in the pulmonary fibrosis model. We also found that the phosphorylated Erk pathway played a major role in the regulation of Gal-1 fibrinogenesis, and activation of the AKT pathway acted as a key on the proliferation and migration of fibroblasts. Positive and negative regulation of Gal-1 expression showed that Gal-1 acted as a promoter of the fibrosis process in lung fibroblasts in vitro, involved in myofibroblast transformation and ECM secretion, and accelerated cell proliferation and migration. Besides, in the pulmonary fibrosis model, we successfully blocked the increase of Gal-1 in the lung associated with decreased fibrous protein through an inhibitor of Gal-1 OTX008. Our data demonstrated that inhibition of Gal-1 in the lung reduced activation of the Erk and AKT pathways, which is consistent with the results of in vitro experiments. Moreover, we witnessed that the number of myofibroblasts decreased after Gal-1 inhibition, which was consistent with the improved tissue structure and ECM deposition in histology by HE and Masson staining. The characteristic pathological feature of IPF is the formation of fibroblast foci and extensive ECM-enriched fibroblast/myofibroblast area in lung parenchyma. The progressive dilation in lung tissue leads to disorders of alveolar structure and disruption of pulmonary exchange. Therefore, the number of fibroblast foci is closely related to the prognosis of IPF, where activated myofibroblasts drive the progression of fibrosis. Consistent with previous reports (15-17), an increase of Gal-1 was found in pulmonary fibrosis. Against Gal-1 increase showed an important relationship between Gal-1 accumulation and phenotypic changes of fibroblasts. Our study supported Gal-1 served as an important downstream factor in TGF- β -induced activation of myofibroblasts in the lung. TGF- β is well known as a major fibrogenic factor and has been shown to induce fibroblasts to myofibroblasts in pulmonary fibrosis, characterized by overhigh positive α -SMA expression and excessive ECM synthesis including COL-1, COL-3, and

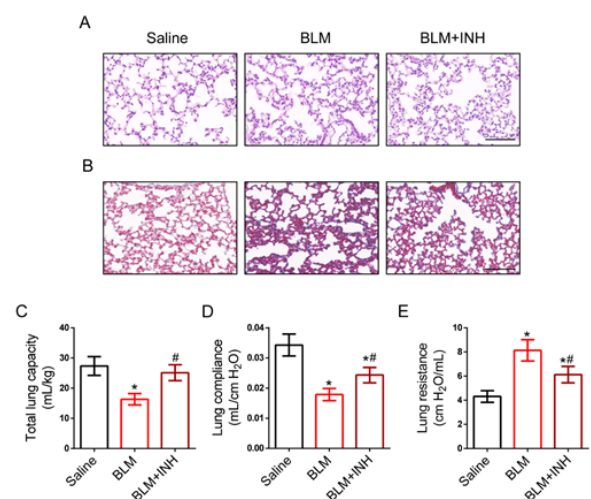


Figure 4. Inhibition of Gal-1 protects histology and function in lung fibrosis. (A) Representative HE staining of lung tissue at 21 days after BLM induction, (magnification: 200 \times , bar=100 μ m). (B) Representative Masson staining of lung tissue at 21 days after BLM induction, (magnification: 200 \times , bar=200 μ m). (C-E) Representative total lung capacity, compliance, and lung resistance at 21 days after BLM induction.

FN (18,19). However, the molecular signals involved in TGF- β mediated fibroblast differentiation are incomplete. In addition to the classical Smad family, non-classical TGF- β signaling such as the MAPKs and AKT pathways have been shown to be involved in fibrogenesis of lung fibroblasts (20-22). The present study found that Gal-1 is one of the missing molecules associated with TGF- β and non-classical pathways. Activation of lung fibroblasts with TGF for 24h resulted in activation of the AKT and Erk pathways as evidenced by elevated phosphorylated AKT and Erk1/2 protein levels. The stimulation was associated with increased Gal-1 expression, suggesting that Gal-1 protein is associated with the transformation of fibroblasts into myofibroblasts. We also confirmed that changes in Gal-1 expression were consistent with ECM markers COL-1 and FN, further suggesting that alteration of Gal-1 expression regulated TGF- β mediated fibroblast differentiation and biological activity. Previous studies have demonstrated that induction of Gal-1 by TGF- β 1 accelerated fibrosis through enhancing nuclear retention of Smad2 (9). Our results further strongly supplemented the mechanism by which Gal-1 regulates non-classical TGF- β pathways. Increasing researches showed that Gal-1 played a fibrotic promoter in several disease models, such as subretinal fibrosis (11), diabetes (15), and chronic hepatitis (23). Our data also confirmed the pro-fibrotic effect of GAL-1 on pulmonary fibrosis. By jointly inhibiting the Erk and AKT pathways, reduced Gal-1 levels attenuated the formation of myofibroblast foci and ECM accumulation in lung parenchyma. Similarly, the study reported by Maeda et al. has demonstrated that regulating Gal-1 levels in hepatic stellate cells inhibited liver fibrosis through the Erk1/2 signaling pathway. Taken together, the present study provides a novel gene Gal-1 that plays a pro-fibrotic role in lung fibroblast after TGF- β induction via activation of Erk and AKT signaling pathways (24). However, Gal-1 inhibition *in vivo* reduces the formation of myofibroblast foci and ECM release in lung tissue via inhibiting Erk and AKT signaling pathways, leading to a protective effect on pulmonary histology and function in the terminal. Considering the deficiency of our study, several unresolved questions remain to be investigated in the future. Firstly, we failed to prove other targets that play a synergistic or antergic effect with Gal-1 in lung fibrosis. Secondly, the downstream molecular mechanism concerning Gal-1 and TGF- β non-classical pathways is still an open question. We further need more researches about Gal-1 and other molecular mechanisms in lung fibrosis.

Conclusion

Herein, we proved that inhibition of Gal-1 reduces fibrosis levels through inhibiting myofibroblast activation and alleviates ECM via blocking Erk1/2 and AKT pathways. Therefore, Gal-1 as a promoter of lung fibrosis and fibroblast differentiation may be a promising therapeutic target in IPF treatment.

Conflict of Interests

The authors declared no conflict of interest.

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