INTRODUCTION

Pulmonary fibrosis (PF) is a progressive interstitial lung disease with a median survival of 3–5 years. Although the pathogenesis of PF is not fully understood, some researches have suggested that repetitive micro-injuries to the alveolar epithelium initiate the histological changes in PF. These micro-injuries lead to the initial damage and aseptic inflammation of alveolar epithelial-mesenchymal unit and will not be properly repaired. Continuous disruption of the alveolar epithelial cell (AEC) layer promotes destruction of the basal layer.
membrane and the subsequent activation of an intra-alveolar coagulation cascade, including imbalance of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), activation of myofibroblasts, ultimately results in the excessive deposition of extracellular matrix.²

The causes of PF are concerned with age, genomic instability, environmental factors, radiation exposure, smoking, viral infections and so on.²³ In fact, one of the main prognostic factors of PF in epidemiological studies is age.³⁴ An increasing body of evidence has suggested that there is a link between ageing (include cell senescence) and PF pathogenesis.⁴⁻⁶ The established biomarkers of cell senescence, including p16INK4a, p21 and β-galactosidase (β-gal) have been observed in human PF lung tissue.⁷⁻⁸ The pro-ageing stressor is implicated in PF pathogenesis, including DNA damage, oxidative stress, telomere attrition and proteome instability.³⁶ Importantly, elimination of senescent cells has been reported to restore lung structure, compliance and elasticity in aged mice.⁹

Pirfenidone (PFD) is listed as one of only two novel agents conditionally recommended for the treatment of PF, it could attenuate lung fibrosis by suppression of transforming growth factor (TGF-β) and other growth factors. However, treatment-emergent adverse events including upper respiratory infection, bronchitis, gastrointestinal events (nausea and diarrhoea), fatigue, cough and dyspnoea sometimes limit its application.¹⁰ The findings of previous researches on cell senescence and PF suggest that senescence clearance plays a crucial role in PF pathogenesis and improves lung function.⁵ FOXO4-D-Retro-Inverso (FOXO4-DRI), a peptide to perturb FOXO4 interaction with p53, has been found to selectively induce apoptosis of senescent cells.¹¹ More importantly, even the loss of health has occurred, FOXO4-DRI effectively restores tissue homeostasis in aged mice.¹¹ In this study, we examined the effect of FOXO4-DRI on PF using a bleomycin (BLM)-induced PF mouse model.

2 MATERIALS AND METHODS

2.1 Mice

Male C57BL/6J mice were purchased from Beijing Huafukang Bioscience Co. Inc. Mice were used at approximately 6–8 weeks of age. All use of animals in this study were approved by the Animal Care and Ethics Committee of the Institute of Radiation Medicine, Peking Union Medical College and Chinese Academy of Medical Sciences. All experimental procedures in this study were complied with the Guide for the Care and Use of Laboratory Animals and the National Institutes of Health guide for the Care and Use of Laboratory Animals.

2.2 FOXO4-DRI development

FOXO4-DRI was synthesized as previously described.¹¹ It was manufactured at >95% purity and stored at −20°C in 1mg powder aliquots to avoid freeze-thawing artefacts. For in vivo experiment, FOXO4-DRI was dissolved in sterile PBS to generate a 5 mg/ml stock solution. Before intraperitoneal injection, FOXO4-DRI stock solution was diluted in PBS to a concentration of 1 mg/ml.

2.3 BLM-induced PF model and FOXO4-DRI administration

BLM (Solarbio) was diluted in sterile PBS to a concentration of 1 mg/ml. Under 3.5% chloral hydrate anaesthesia, mice were administrated with 5 mg/kg BLM or with 100 μl PBS by intratracheal instillation. Mice were administrated with 5 mg/kg FOXO4-DRI or PBS by intraperitoneal injection on the 14th, 16th and 18th day after BLM administration. For positive control, mice were administrated with 300 mg/kg PFD from the 15th day after BLM administration for 20 times. Twenty-one days after BLM administration, these mice were euthanized.

2.4 Histopathologic evaluation and IHC

Fresh lung tissue was harvested and fixed with 4% formalin, embedded with paraffin, serially sectioned and stained with haematoxylin and eosin. To evaluate collagen deposition, the paraffin-embedded lung tissue was stained using a Masson trichrome kit (Solarbio), according to the manufacturer’s instructions. Collagen deposition was qualified by Image Pro Plus software (Media Cybernetics) on Masson trichrome-stained sections. The data are illustrated as ratio of positive (blue) area relative to the total lung area under 10 microscopic fields as described previously.¹² For the IHC experiments, the primary antibodies of α-SMA and Col1a1 (Bioworld) were incubated overnight at 4°C, after secondary antibody conjunction, a DAB kit was used to detect the positive staining.

2.5 Measurement of hydroxyproline content

Frozen lung tissue was homogenized in water. Hydroxyproline content in the homogenate was measured by a hydroxyproline colorimetric assay kit (Bio Vision), according to the manufacturer’s instructions.

2.6 β-galactosidase (β-gal) staining

Frozen lung sections were stained with a β-gal staining kit (Beyotime Biotechnology) according to the manufacturer’s instructions. Senescent cells were identified as blue-stained under optical microscopy.

2.7 Quantitative real-time PCR

Total RNA was extracted from lung tissue with TRIzol (Life Technologies). cDNA synthesis was carried out with a Revert Aid First
Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer’s protocol. Quantitative real-time PCR was performed using SYBR Green Master (Roche) under an ABI 7500 Sequence Detection System (Thermo). The primer sequences were listed in Table 1.

2.8 Western blot

Lung tissues were homogenized in liquid nitrogen and then lysed in RIPA buffer (Solarbio) plus phenylmethylsulfonyl fluoride (Sigma) on ice. The protein samples were subjected to 10% or 12% SDS-PAGE, transferred to PVDF membranes (Millipore) and incubated with an antibody against β-tubulin (1:1000), p16\(^{INK4a}\) (1:1000), p21 (1:1000), Tenascin (1:500), Thbs2 (1:500), Laminin1 (1:1000), Fibronectin (1:1000), Hmmr (1:500), α-SMA (1:1000), Col1a1(1:1000) and FOXO4(1:500).

2.9 RNA-Seq

Total RNA was extracted from lung tissues, RNA integrity was evaluated and then the libraries were constructed. These libraries were sequenced on the Illumina sequencing platform (HiSeqTM 2500 or Illumina HiSeq X Ten) and 125 bp/150 bp paired-end reads were generated. Raw reads were processed and analysed with the help of OEBiotec Company.

| Gene   | Forward     | Reverse     |
|--------|-------------|-------------|
| TGF-β1 | CAATTCCTGGCCGTCTTACCT | CTGTATTCCGTCTCCTTGG |
| Col1α2 | GCGATTACTCTGGAGTTGAC  | GCGGCTGATGAGTTCTT  |
| Col3α1 | TGGTCTAGGAGGTGAATTGAT | CCAGGACTCAGCAGTATTCC |
| Timp1  | CTTGGTTCCCTGGGCGTCATCT | ACCTGATCGCTCACAAGCAG |
| Mmp2   | AAGATGACCTGCTGTAGCAGAT | AGCCAAATGATGCACCCACCT |
| p21    | CCTGGTGATGTCCGACCTG   | CCATGAGCAGATCAGTATTCC |
| p16    | CGGAGGTGCTTTGCTGACTGT | GTTTCAGGAAAGCGAGG |
| TnF-α  | TGGAAACGGAGCTGAGCTGT | CTCTCAATGACCCGTAGGGC |
| Pai1   | TCCACACAGTCTGAGGACAGC | TGGTACGGGCTTTCCACAAAC |
| Mmp10  | AAAGACCTGAGACCCCCGACACA | GTCGAGCTCATGAGCAGCAT |
| Mmp13  | TTACATGCAGGCTCACATAG | AGCCAAATGATGCACCCACCT |
| IL1a   | AGGGAGTCAGCTATTGACCGG | TGGCAAGACTGATGCTCTTCC |
| IL1b   | TGCAAGCTGCTTGAGTGATG | TGGATGAGCGAGGTAGTTCC |
| Fn1    | CTGACATCCGCTGTCATTTGC | TCAAGCAGCAGCACTACAA |
| Lama1  | ACTATGCGCTACGGGCAAGC | GGCAGGCTTTGGAATATACGA |
| Tnc    | GCTTCCCTATGGAGCTACAAG | TACTGCCGTGAGGTCTCC |
| Hmmr   | ACACCTACCTGAGATTAACCA | GACAGCCTACCATCTCCTT |
| Thbs2  | GCTACTAATGAGGAGCCATAC | TCTCTGACAGCTTCAAC |
| Cyt1   | GTGTTACTCCGCTGAGCT | AGGAAAGCAGCAGAAGTC |
| Gc     | AATTGGCAGAAGGCCCTAA | TTATAGACGACACCTAGG |
| Nrgn   | GACCGATGATTTCTGAGATCC | TCCGCTTCATTTCTTCTT |
| Sele   | GGAATAACAGGAGCCATC | GTCGGTTGCAAGGAATAGG |
| Dpsl5  | GTCAATGCTGTAGTATCTCAG | GAGTAGTGGAAGGTTTG |
| Chil4  | AAGACTTGGCTGTACTGAAA | CGAAAGAATTGTAACCTGAC |
| Gpr176 | TGGCAGCTAGGCTTGGTTA | GCAGAAGAAGCATGTAAGA |
| Krt6a  | GGACAGCATGATGGAGAG | GCAGCATCAGCTCCCTT |
| Mex3a  | CAAGTGAGCCATTGATTTGA | CGCCGGGCTTGTCTATTCA |
| col4a2 | CAGAGGTACCTTTAAGGCCAGGCG | TTTGGCCGAGGTTATCTT |
| Ntrk2  | AAGTTGGCAGACCTTACCT | CGGAAAGAATGAGGTGTT |
| Pappa2 | ACCATGTCATTCCTGCCATAC | GGAACCTTGGAGGTTATCT |
| Tgm5   | AGTGATGAGGCTGTAGGAC | TGAGACCTTGTAGCCTTA |
| Myh2   | TAAAGCGCAATGGCCATTCCTG | GGTCGCGGATAAGGCT |
| β-tubulin | GGATGATGCGTGTGTTGCC | AGTTCATCATCCAATCAGG |

TABLE 1 Primer sequences used in experiments
2.10 | Flow cytometry

The lung cell suspension was prepared as previously described.6 In briefly, mouse lung was harvested, washed with PBS to remove blood, then cut into pieces, which incubated in the DMEM medium containing 0.2 mg/ml Liberase DL (Roche) and 0.03 mg/ml DNase I (Roche) at 37°C for 40 min. 5 × 10⁶ cells were stained with the following antibodies (Biolegend), CD45-BV510, CD31-APC, PDGFα-PE, EPCAM-Percp, PDPN-BV421 and MHCII-APC-Cy7 at 4°C for 30 min. According to previously researches,6,13 CD45−CD31+EPCAM− cells were named as endothelial cells, further marked with PDPN to distinguish vascular endothelial cells(PDPN−) and lymphocytic endothelial cells(PDPN+); CD45−CD31+EPCAM+ cells were named as AECs, further sorted into AEC1(PDPN−) and AEC2(MHCII+); Fibroblasts were marked as CD45+PDGFα+EPCAM− cells. For intracellular staining, the lung cells were fixed and permeabilized using BD Cytofix/Cytoperm buffer first, α-SMA and h-caldesmon antibodies were used to detected myofibroblasts and smooth muscle cells, the mean fluorescence intensity (MFI) of α-SMA and h-caldesmon was detected by a BD Aria III flow cytometer (BD Bioscience).

2.11 | Cell lines, mouse fibroblast isolation and culture

Human lung fibroblast (HLF and MRC5) was purchased from National Collection of Authenticated Cell Cultures (Shanghai, China). For the mouse lung fibroblast (MLF) culture, the lung cells were isolated first as described in the above section, then all the cells were cultured in DMEM plus 20% FBS for 24 h, after suspension cells were removed, the adherent cells could be considered as MLF. Passage 3–6 MLF were used for the remaining experiments. To detect IC50 value, cells were treated with 4 ng/ml TGF-β for 24 h, then co-cultured with a series dilution of FOXO-DRI for 24, 48 and 72 h; CCK-8 assay was executed and the OD value was detected. For apoptosis, 1 × 10⁵ cells were treated as described above, then detected according to the manufacturer’s instructions (BD Bioscience).

2.12 | Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. Significant differences between groups were evaluated by

![Figure 1](image-url)
One-way analysis of variance, followed by a post-hoc student-Newman–Keuls test for multiple comparisons. Differences were considered significant at \( p < 0.05 \).

3 | RESULTS

3.1 | FOXO4-DRI eliminates cell senescence and ameliorates BLM-induced PF

It has been well established that BLM aerosol or intratracheal instillation in mice can lead to PF that recapitulates key features of human PF. In mouse, lung fibrosis appears between 14 and 28 days after a single-dose administration of bleomycin. Therefore, 14 days after the mice were intratracheal instilled with BLM, they were treated with PBS or FOXO4-DRI by intraperitoneal injection for 3 times, and the positive control PFD by oral gavage for 20 times (Figure 1A). Twenty-one days after BLM administration, these mice were euthanized. Senescent cells have been found in the lung with PF, and it has been established that fibrotic lung disease is mediated, in part, by senescent cells. FOXO4-DRI selectively induce senescent cells apoptosis, restores fur density, fitness and renal function in aged mice. Therefore, we first examined whether FOXO4-DRI could clear senescent cells in BLM-induced PF model. As shown in Figure S1A,B, the number of β-gal positive cells was significantly higher than that in control mice at 21\textsuperscript{th} days after BLM administration; FOXO4-DRI treatment substantially reduced the BLM-induced β-gal positive cells, suggesting that BLM causes cell senescence and FOXO4-DRI eliminates senescent cells in the PF model. Senescent cells can develop a senescence-associated secretory phenotype (SASP), which include interleukins, proteases and regulators, chemokines, insoluble factors and so on. SASP can exert profound effects on themselves or on neighbouring cells. As shown in Figure S1C–E, there was significant upregulated expression of PF-related SASP after mice were treated with BLM, and FOXO4-DRI can downregulated these genes expression. p16\(^{INK4a}\) and p21 are the main senescence hallmarks, the results of quantitative real-time PCR and western blot showed that mouse in BLM group exhibited upregulation of p16\(^{INK4a}\) and p21 expression in lung compared with control, FOXO4-DRI significantly downregulated p16\(^{INK4a}\) and p21 expression in the lung of mice with BLM-induced PF (Figure S1E–G). These results suggest FOXO4-DRI clears senescent cells in the PF model and regulates senescence-associated genes.

The strategy of senescent cells clearance had been considered as a nice treatment for PF, so next we explored the effect of FOXO4-DRI on BLM-induced mouse PF model. HE staining revealed that the lungs in control and FOXO4-DRI groups maintained normal histological morphology, which meant normal alveoli and alveolar septum, no abnormal inflammatory cell infiltration and collagen deposition. BLM induced a widening alveolar septum, inflammatory cell infiltration and collagen deposition in the lung, FOXO4-DRI significantly ameliorated BLM-induced morphological changes to positive control level in the lung (Figure 1B). The lung sections were also stained using a Masson trichrome kit to evaluate collagen deposition. As shown in Figure 1B,C, compared with control mice, the collagen deposition (blue-stained areas) in the lung was significantly increased in BLM group, and FOXO4-DRI reduced BLM-induced lung collagen deposition similar to PFD group. Meanwhile, mice in BLM group showed a significantly higher hydroxyproline content in the lung, and FOXO4-DRI decreased the level of hydroxyproline content (Figure 1D).

We next explored the mRNA level of fibrosis relevant genes in lung, a distinct feature of PF is collagen deposition with the upregulation of collagen genes, such as Col1a1, Col3a1 and Col4a2. BLM induced the upregulation of Col1a1, Col3a1 and Col4a2 mRNA expression compared with control, and FOXO4-DRI repressed BLM-induced upregulation of the above genes similar to PFD group (Figure 1E). From collagen deposition, the dysregulation of collagen degradation also contributes to the development of PF. Collagen degradation is mainly performed by matrix metalloproteinases (MMP) family, it has been reported that Mmp2, a major type IV collagen-degradation enzyme, and its inhibitor Timp1 is increased in the lung tissues of BLM-treated mice. In our experiment, BLM induced a remarkable upregulation of Timp1 and Mmp2 mRNA and FOXO4-DRI downregulated their expression similar to PFD group (Figure 1E). These results suggest that FOXO4-DRI represses collagen deposition to ameliorate BLM-induced PF. TGF-β is mainly contribute for the differentiation of fibroblasts into myofibroblasts and mesenchymal transition of epithelial cells. In our experiment, BLM upregulated the expression of TGF-β1 mRNA level, and FOXO4-DRI downregulated TGF-β1 expression in BLM treatment mouse (Figure 1E).

In summary, above data suggests that intratracheal instillation of BLM induces PF in mice, and FOXO4-DRI, similar as positive control PFD, attenuates BLM-induced pathological changes in the lung.

3.2 | FOXO4-DRI improves the impaired ratio in BLM-induced lung cells

In the lung injury-repair, activation of fibroblast-to-myofibroblasts differentiation is one of the most important factor to deposit ECM and support the regeneration of type 2 AECs. The lung is composed of many cell types, including endothelial cells, AECs, fibroblasts and myofibroblasts; endothelial cells can be further sorted as vascular endothelial cells and lymphocytic endothelial cells; AECs can be sorted as AEC 1 and AEC 2. To further explore the effect of FOXO4-DRI on BLM-induced cell injury in lung, we detected the changes of the above cells in lung using a flow cytometer. As shown in Figure 2 and Figure S2, compared with control group, there was significant decrease in the percentage of endothelial cells, AECs, fibroblast, MFI of smooth muscle cells and increase in MFI of myofibroblasts after mice were treated with BLM; compared to BLM group, FOXO4-DRI could elevated the percentage of AEC2 and fibroblast and reduced the MFI of myofibroblasts. Notably, PFD could not increase the cell number of AEC2 in our experiment, also, FOXO4-DRI could not improve the percentage of endothelial cells, which can be rescued by PFD; Above data suggested that FOXO4-DRI may rescue BLM-induced PF through eliminate myofibroblasts and promote recovery of fibroblast and AEC2 number.
3.3 FOXO4-DRI works on the extracellular matrix (ECM) receptor interaction pathway to mitigate BLM-induced PF

FOXO4-DRI ameliorated BLM-induced PF and did not affect the histological morphology of normal lung. To further decipher the protective mechanism of FOXO4-DRI, we performed RNA-Seq and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using lung tissues from control, BLM and BLM+FOXO4-DRI groups. The KEGG pathway enrichment analysis revealed that, compare with control, the significant elevated top genes were mostly involved in ECM-receptor interaction, focal adhesion and PI3K-Akt signalling pathway in BLM group. Compare with those in BLM group, the top genes exhibiting significantly decreased expression were mostly involved in ECM-receptor interaction, cytokine-cytokine receptor interaction, phagosome, chemokine signalling pathway, focal adhesion and PI3K-Akt signalling pathway. (Figure 3A,B). There were 24 overlap
genes involved in all the control, BLM and BLM+FOXO4-DRI group (Figure S3A). We performed quantitative real-time PCR in these overlap genes to verify the results of RNA-Seq analysis. The protein level of ECM-receptor interaction pathway, including Fn1, Tnc, Lama1, Thbs2 and Hmmr was consistently with that in mRNA level, and FOXO4-DRI downregulated these expressions in both

FIGURE 3  FOXO4-DRI downregulates expression of proteins in ECM (extracellular matrix) receptor interaction pathway to mitigate BLM-induced PF. RNA-Seq and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using lung tissues from control, BLM and BLM+FOXO4-DRI groups. (A) The upregulated pathways BLM vs control; and (B) the downregulated pathways BLM+FOXO4-DRI vs BLM; (C) The relative expression of Fn1, Tnc, Lama1, Thbs2 and Hmmr mRNA in the lung tissue; (D) Representative Western blot images of Fn1, Tnc, Lama1, Thbs2 and Hmmr in the lung tissues. (E) Relative grey value of protein expression. All the data were represented as mean ± SEM (n = 5 in panel C, n = 3 in panel E). #p < 0.05 vs control; *p < 0.05 vs BLM

FIGURE 4  FOXO4-DRI alleviates myofibroblast differentiation in BLM-induced PF mouse. IHC experiments were executed using lung tissues as described in Materials and Methods. (A) Representative scanned IHC images of α-SMA and Col1a1 in lung tissues; (B) Representative WB images of α-SMA and Col1a1 in lung tissues; (C) Relative grey value of protein expression. All the data were represented as mean ± SEM (n = 3 in panel C). #p < 0.05 vs control; *p < 0.05 vs BLM
gene and protein level (Figure 3C–E). These results indicated that FOXO4-DRI worked on ECM-receptor interaction pathway in mice to ameliorate BLM-induce PF.

Among these overlap genes, BLM downregulated the expression of Sele, Cyt1, Dpysl5, Gc, Nrgn genes, which are mainly involved in cell adhesion,25 inflammatory response,26 nerve conduction,27 signal transduction,28 material transport and metabolism,29,30 and FOXO4-DRI attenuated the depression of these gene expressions (Figure S3B). BLM also upregulated the expression of Chil4, Pappa2, Gpr176, Krt6a, Mex3a, Ntrk2, Tgm5 and Myh2 genes, which are mainly involved in regulations of signal transduction,31–33 material metabolism34–36 and cell junctions.37 FOXO4-DRI downregulated BLM-induced expressions of Chil4, Pappa2, Gpr176, Krt6a, Mex3a, Ntrk2, Tgm5 to nearly normal levels and elevated the expression of Myh2, a gene associated with tight junction.37 (Figure S3C).

3.4 | FOXO4-DRI alleviates myofibroblast differentiation in BLM-induced PF mouse

Alpha-SMA is used as the myofibroblast marker according to previously reports.38,39 Col1a1 is one of the components of ECM, which upregulated in TGF-β-induced myofibroblast differentiation,40,41 so we detected the expression of both two proteins to further confirm the effect of FOXO4-DRI on BLM-induced myofibroblasts. As shown in Figure 4A, IHC images showed that there was significant increase of α-SMA and Col1a1 positive staining in BLM-induced mice lung section, FOXO4-DRI inhibited the upregulation of α-SMA and Col1a1 proteins. WB results showed a consistently effect of FOXO4-DRI on BLM-induced upregulation of α-SMA and Col1a1 (Figure 4B, C). Above data indicating that FOXO4-DRI could eliminate BLM-induced myofibroblast differentiation.

3.5 | FOXO4-DRI alleviates TGF-β-induced myofibroblast differentiation in vitro

TGF-β is one of the major pro-fibrotic cytokines for myofibroblast differentiation, it was always used to stimulate the fibroblast-to-myofibroblast differentiation in vitro.38,40,42 In this study, we use 4ng/mL TGF-β to stimulate MLF, HLF and MRC5 cell line for 24 h, then different concentration of FOXO4-DRI was co-cultured with cells for 24, 48 and 72 h. As shown in Figure 5, the half maximal

![Figure 5](image-url)
inhibitory concentration (IC50) value of FOXO4-DRI on MLF was 215, 79 and 40 μM, respectively. IC50 value of FOXO4-DRI on TGF-β-stimulated MLF was 145, 54 and 13 μM, respectively, at the time point of 24, 48 and 72 h (Figure 5A); IC50 value of FOXO4-DRI on lung fibroblast compared to the TGF-β-stimulated group. WB results of α-SMA and Col1a1confirmed the rescue of FOXO4-DRI on myofibroblast (Figure 5B, D and F). Above data indicated that FOXO4-DRI is more inclined to kill myofibroblast.

3.6 | FOXO4-DRI induces more apoptotic cells in TGF-β-induced myofibroblasts in vitro

To explore the underlying mechanisms that FOXO4-DRI kills myofibroblast, we detected cell apoptosis in MLF, HLF and MRC5. As shown in Figure 6A–F, after cells were co-cultured with FOXO4-DRI, the apoptotic and dead cells increase 7.3% (MLF), 7.7% (HLF) and 6.4% (MRC5) compared to control groups. TGF-β treatment did not increase cell apoptosis; after cells were co-cultured with FOXO4-DRI, the apoptotic and dead cells increase 8.9% (MLF), 5.5% (HLF) and 8.0% (MRC5) compared to TGF-β groups, suggesting a tendency of FOXO4 induced myofibroblast apoptosis. In senescent cells, there is upregulation of FOXO4 expression, FOXO4-DRI...
perturbs interaction between FOXO4 and p53 and induces cell apoptosis. So the protein level of FOXO4 was detected in our experiment, as shown in Figure 6G,H, FOXO4 protein was upregulated after treatment with TGF-β, FOXO4-DRI reverse the protein expression, which should be attribute to the clearance of FOXO4-riched cells. Above data suggests that FOXO4-DRI is more inclined to kill myofibroblast by inducing cell apoptosis.

4 | DISCUSSION

PF has received attention because of its poor prognosis and its unresponsiveness to traditional therapies. In recent years, the novel strategy of targeting senescent cells to treat PF has been supported by some researches. Hashimoto and his colleagues provide crucial evidence that cell senescence contributes to lung ageing, and elimination of naturally occurring senescent cells promote the recovery of lung structure and function in aged mice. Schafer and his colleagues reported that senescent cell ablation attenuates BLM-induced impairment in lung function. Pan and his colleagues have reported that ABT-263, a senolytic agent, selectively kills senescent cells and reserves radiation-induced PF in mouse. Cumulatively, the strategy of senescent cell elimination exhibits a therapeutic potential on PF.

In this study, we treated the BLM-induced PF mouse model with FOXO4-DRI, and found that besides the elimination of senescent cells and SASP, FOXO4-DRI could attenuate BLM-induced morphological changes and collagen deposition to the similar as the approved medication PFD. Furthermore, FOXO4-DRI increases the percentage of type 2 alveolar epithelial cells (AEC2) and fibroblasts and decreases the myofibroblasts in BLM-induced PF mouse models. Compared to mouse and human lung fibroblast cell lines, FOXO4-DRI is inclined to kill TGF-β-induced myofibroblast in vitro. The inhibited effect of FOXO4-DRI on myofibroblast and ECM proteins lead to a downregulation of ECM-receptor interaction pathway in BLM-induced PF. Above all, FOXO4-DRI ameliorates BLM-induced PF in mouse and may be served as a viable therapeutical option for PF.

Activation of fibroblast-to-myofibroblasts differentiation is one of the most important factor to deposit ECM. In this study, FOXO4-DRI is more inclined to kill the myofibroblasts both in vivo and in vitro. The upregulation expression of FOXO4 protein in TGF-β-stimulated cells may provide the possible reason for the effect of FOXO4-DRI. The formation of ECM also requires the secretion of ECM proteins. ECM is achieved by following a strict layered assembly pattern, which begins with the deposition of fibronectin filaments on the cell surface, a process called fibrillary formation. In this study, FOXO4-DRI downregulated the main ECM protein significantly, which mainly contributed for the reducing of PF degree. Therefore, we summarize the whole process of FOXO4-DRI works on BLM-induced PF as follows, FOXO4-DRI eliminated senescent cells, downregulation of SASP released by senescent cells reduce the inflammatory stimulation to neighbour cells to rescue BLM-induced PF; On the other hand, FOXO4-DRI is more inclined to kill myofibroblasts, downregulated the expression of main ECM proteins, reduced ECM formation and finally inhibited ECM-receptor interaction to mitigate BLM-induced PF.

PFD has been approved in Europe in 2011 for the treatment of IPF and in the USA in 2014. The recommended daily maintenance dose of PFD is 801 mg three times per day (2403 mg/day) with a 14-day titration. Consistently with our results, it has been reported that PFD could ameliorate BLM-Induced PF at administration of 300 mg/kg PFD for 3–4 weeks immediately after BLM treatment. Obviously, PFD needs to be given at very early time point in mouse, and the medication period is long. Compared with PFD, BLM-induced lung fibrosis mouse was treated with 5 mg/kg FOXO4-DRI for 3 times, started from 14th day after BLM treatment; so the advantage over PFD, FOXO4-DRI lies in its short administration time and the low treatment dose. Importantly, the same as other senolytic agents, it can be used to treat PF even when pathological changes have occurred. FOXO4-DRI is a cell-penetrating peptide, which can in theory target any surface-exposed stretch of amino acids to block specific protein-protein interactions, selectively modulate specific signalling events. Thus it have the advantages of target specificity and low toxicity over the reported senolytic agents. In addition, in the treatment of PF, FOXO4-DRI may reduce the risk of cancer progression and metastasis by eliminating senescent cells, which have been demonstrated to contribute to cancer metastasis and relapse. At present, mouse was administrated with FOXO4-DRI by intraperitoneal injection, it will be better to be improved as an oral gavage peptide. Above all, FOXO4-DRI has great potential as a candidate for PF therapy.

ACKNOWLEDGEMENT

This study was supported by the National Natural Science Foundation of China (81903254, 81730086) and CAMS Innovation Fund for Medical Sciences (CIFMS, No. 2021-I2M-1-042).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Xiaodan Han: Data curation (equal); Methodology (equal); Project administration (equal); Resources (equal); Software (equal); Writing – original draft (equal). Tong Yuan: Formal analysis (equal); Methodology (equal); Project administration (equal); Software (equal); Validation (equal); Writing – original draft (equal). Junling Zhang: Conceptualization (equal); Supervision (equal); Writing – review & editing (equal). Yonggang Shi: Methodology (supporting); Supervision (supporting). Deguan Li: Methodology (supporting); Supervision (supporting). Yinpeng Dong: Methodology (supporting); Supervision (supporting). Saijun Fan: Methodology (equal); Project administration (equal); Supervision (equal); Writing – original draft (equal).
REFERENCES

1. Luppi F, Kalluri M, Faverio P, Kreuter M, Ferrara G. Idiopathic pulmonary fibrosis beyond the lung: understanding disease mechanisms to improve diagnosis and management. Respir Res. 2021;22:109.

2. Fernandez IE, Eickelberg O. New cellular and molecular mechanisms of lung injury and fibrosis in idiopathic pulmonary fibrosis. Lancet. 2012;380(9842):680-688.

3. López-Ramírez C, Suarez Valdivia L, Rodríguez Portal JA. Causes of pulmonary fibrosis in the elderly. Med Sci (Basel). 2018;6(3):58.

4. Pardo A, Selman M. Lung fibroblasts, aging, and idiopathic pulmonary fibrosis. Am J Thorac Sci. 2016;13(Suppl 5):S417-S421.

5. Faner R, Rojas M, MacNee W, Agustí A. Abnormal lung aging in chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2012;186(4):306-313.

6. Schafer MJ, White TA, Iijima K, et al. Cellular senescence mediates fibrotic pulmonary disease. Nat Commun. 2017;8:14532.

7. Kuwano K, Kunitake R, Kawasaki M, et al. F22Waf1/Cip1/Sdi1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 1996;154(2 Pt 1):477-483.

8. Lomas NJ, Watts KL, Akram KM, Forsyth NR, Spiteri MA. Idiopathic pulmonary fibrosis. Annu Am Thorac Soc. 2012;9(12):e87732.

9. Hashimoto M, Asai A, Kawagishi H, et al. Elimination of p19ARF-expressing cells reverses tissue fibrosis in the elderly. Ann Am Thorac Soc. 2017;14(8):858-866.

10. Lancaster LH, de Andrade JA, Zibrak JD, et al. Pirfenidone safety and adverse event management in idiopathic pulmonary fibrosis. Eur J Med Sci (Basel). 2020;282(40):29359-29367.

11. Baar MP, Brandt RMC, Putatuv DA, et al. Targeted apoptosis of senescent cells restores tissue homeostasis in response to chemototoxicity and aging. Cell. 2017;169:132-147.e16.

12. Lv XX, Wang X-X, Li KE, et al. Rupatadine protects against pulmonary fibrosis in the elderly. J Thorac Dis. 2017;9(12):8873-8879.

13. Kunitake R, Kawasaki M, et al. F22Waf1/Cip1/Sdi1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 1996;154(2 Pt 1):477-483.

14. Thannickal VJ, Toews GB, White ES, Lynch JP 3rd, Martinez FJ. Mechanisms of pulmonary fibrosis. Ann Rev Med. 2004;55:395-417.

15. Nelson RM, Dolich S, Aruffo A, Cecconi O, Bevilacqua MP. Higher-affinity oligosaccharide ligands for E-selectin. J Clin Invest. 1993;91(3):1157-1166.

16. Bevilacqua MP, Stengelin S, Gimbrone MA Jr, Seed B. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science. 1989;243(4895):1160-1165.

17. Fukada M, Watakabe I, Yuasa-Kawada J, et al. Molecular characterization of CRMP5, a novel member of the collapsin response mediator protein family. J Biol Chem. 2000;275(48):37957-37965.

18. Guo L, Xu JM, Liu L, Liu SM, Zhu R. Hypoxia-induced epithelial-mesenchymal transition is involved in bleomycin-induced lung fibrosis. Biomed Res Int. 2015;2015:232791.

19. Cinetto F, Ceccato J, Caputo I, et al. GSK-3 inhibition modulates metalloproteinases in a model of lung inflammation and fibrosis. Front Mol Biosci. 2021;8:633054.

20. Kim KK, Kugler MC, Wolters PJ, et al. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci USA. 2006;103(35):13180-13185.

21. Scotton CJ, Chambers RC. Molecular targets in pulmonary fibrosis: the myofibroblast in focus. Chest. 2007;132(4):1311-1321.

22. Wolters PJ, Collard HR, Jones KD. Pathogenesis of idiopathic pulmonary fibrosis. Annu Rev Pathol. 2014;9:157-179.

23. Liu RM, Liu G. Cell senescence and fibrotic lung diseases. Int J Mol Sci (Basel). 2012;2015:232791.

24. Nakatani Y, Honda K, Nakatani M, et al. Simultaneous isolation of endothelial and mesenchymal cells in lung tissue culture. Proc Natl Acad Sci USA. 2006;103(35):13180-13185.

25. Overgaard MT, Boldt HB, Laursen LS, Sottrup-Jensen L, Conover CA, Oxvig C. Pregnancy-associated plasma protein A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 gene family member. J Biol Chem. 2001;276(24):21849-21853.

26. Gaudet P, Livstone MS, Lewis SE, Thomas PD. Phyllogenetic-based propagation of functional annotations within the gene ontology consortium. Brief Bioinform. 2011;12(5):449-462.

27. Liang Y, Cucchetti M, Roncagalli R, et al. The lymphoid lineage-specific actin-uncapping protein Rltpr is essential for costimulation via CD28 and the development of regulatory T cells. Nat Immunol. 2013;14(8):858-866.
38. Lagares D, et al. ADAM10-mediated ephrin-B2 shedding promotes myofibroblast activation and organ fibrosis. Nat Med. 2017;23:1405-1415.

39. El Agha E, Kramann R, Schneider RK, et al. Mesenchymal stem cells in fibrotic disease. Cell Stem Cell. 2017;21:166-177.

40. Xie T, Liang J, Liu N, et al. Transcription factor TBX4 regulates myofibroblast accumulation and lung fibrosis. J Clin Invest. 2016;126:3063-3079.

41. Tsubouchi K, Araya J, Minagawa S, et al. Azithromycin attenuates myofibroblast differentiation and lung fibrosis development through proteosomal degradation of NOX4. Autophagy. 2017;13:1420-1434.

42. Xie N, Tan Z, Banerjee S, et al. Glycolytic reprogramming in myofibroblast differentiation and lung fibrosis. Am J Respir Crit Care Med. 2015;192(12):1462-1474.

43. Lehmann M, Korfei M, Mutze K, et al. Senolytic drugs target alveolar epithelial cell function and attenuate experimental lung fibrosis ex vivo. Eur Respir J. 2017;50:1602367.

44. Barnes PJ, Baker J, Donnelly LE. Cellular senescence as a mechanism and target in chronic lung diseases. Am J Respir Crit Care Med. 2019;200:556-564.

45. Sottile J, Hocking DC. Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. Mol Biol Cell. 2002;13(10):3546-3559.

46. Behr J, et al. Efficacy and safety of sildenafil added to pirfenidone in patients with advanced idiopathic pulmonary fibrosis and risk of pulmonary hypertension: a double-blind, randomised, placebo-controlled, phase 2b trial. Lancet Respir Med. 2021;9:85-95.

47. Liu Y, Lu F, Kang L, Wang Z, Wang Y. Pirfenidone attenuates bleomycin-induced pulmonary fibrosis in mice by regulating Nrf2/Bach1 equilibrium. BMC Pulm Med. 2017;17:63.

48. de Keizer PL, Packer LM, Szypowska AA, et al. Activation of forkhead box O transcription factors by oncogenic BRAF promotes p21cip1-dependent senescence. Cancer Res. 2010;70(21):8526-8536.

49. Ohtani N, Takahashi A, Mann DJ, Hara E. Cellular senescence: a double-edged sword in the fight against cancer. Exp Dermatol. 2012;21(Suppl 1):1-4.

50. Demaria M, O’Leary MN, Chang J, et al. Cellular senescence promotes adverse effects of chemotherapy and cancer relapse. Cancer Discov. 2017;7(2):165-176.

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Han X, Yuan T, Zhang J, et al. FOXO4 peptide targets myofibroblast ameliorates bleomycin-induced pulmonary fibrosis in mice through ECM-receptor interaction pathway. J Cell Mol Med. 2022;26:3269–3280. doi:10.1111/jcmm.17333