

# 1 Disulfiram Exerts anti-pulmonary Fibrosis Effect 2 by Activating PGE2 Synthesis

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24 **Abstract:** Idiopathic pulmonary fibrosis (IPF) is marked with the replacement of normal  
25 alveolar tissue by thicker and harder fibrous material, damaged exchange ability.  
26 Currently, nintedanib and pirfenidone, are the only FDA-approved drugs with limited  
27 efficacy for IPF, which indicated an urgent need to explore new therapies. Disulfiram  
28 (DSF), an acetaldehyde dehydrogenase inhibitor, used as anti-alcohol treatment.  
29 Despite reported with anti-hepatic fibrosis effect of DSF, the underlying mechanism  
30 remains unclear. In our study, DSF exhibited regulative impact on abnormal  
31 proliferation, EMT and ECM production in cell models of IPF including primary DHLF-  
32 IPF cells and TGF- $\beta$ 1-stimulated A549 cells. The absence of COX-2 was restored by  
33 DSF treatment, together with elevated prostaglandin biosynthesis both in vitro and in  
34 vivo models of IPF. Furthermore, the anti-fibrotic effect of DSF was impeded with COX-  
35 2 knockdown or pharmacological inhibition in TGF- $\beta$ 1-stimulated A549 cells, however,  
36 exogenous PGE2 reclaimed with anti-EMT function. In established animal model of  
37 IPF, DSF ameliorated declined lung function and histopathological changes, and  
38 restrained the lung hydroxyproline content. Together, these findings suggest that the  
39 anti-fibrotic effect of DSF was achieved through re-activation of COX-2 mediated PGE2  
40 biosynthesis. The above results suggest that DSF can be applied therapeutically in  
41 fibrotic conditions.

42

43 **Keywords:** Disulfiram; Idiopathic pulmonary fibrosis; Epithelial–mesenchymal  
44 transition; Cyclooxygenase-2; Prostaglandin E2.

**Abbreviations:** DSF, disulfiram. COX-2, Cyclooxygenase-2. PGE2, Prostaglandin E2. IPF, Idiopathic pulmonary fibrosis. EMT, epithelial-mesenchymal transition. ECM, extracellular matrix. TGF- $\beta$ 1, transforming growth factor- $\beta$ . BLM, Bleomycin. DHLF-IPF (IPF) cells, Diseased Human Lung Fibroblasts (Idiopathic Pulmonary Fibrosis) cells. FVC, Forced vital capacity. Cdyn, Dynamic compliance. Re, expiratory resistance. Ri, inspiratory resistance. E-cad, E-cadherin. VIM, Vimentin.  $\alpha$ -SMA, Alpha-Smooth muscle actin. FN, Fibronectin. Col-I, Type I collagen.

## 46 **1. Introduction**

47 Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive fibrosis interstitial  
48 pneumonia, characterized by the excessive accumulation of extracellular matrix and  
49 fibrotic tissue in the lungs [1]. The median survival time is about 2-3 years after  
50 diagnosis [2]. Clinically, Nintedanib and pirfenidone, two anti-fibrosis agents  
51 approved by U.S food and drug administration (FDA), can slow the decline rate of lung  
52 function in patients with IPF, but there are certain some side effects and poor prognosis  
53 [3]. Though the pathogenesis is not well illustrated, Epithelial-mesenchymal transition  
54 (EMT), and ECM deposition are considered major changes in IPF [4]. The morphology  
55 and structure of alveolar epithelial cells changed over the process of self-repair after  
56 injury associated with EMT and ECM deposition [5]. A number of essential cytokines  
57 contribute to EMT in alveolar epithelial cells, and transforming growth factor (TGF- $\beta$ 1)  
58 was identified as the key elements for fibrosis [6]. In vitro study, TGF- $\beta$ 1 induces  
59 morphological change, extracellular matrix deposition, tight junctions destroys  
60 between cells, and gain-of-function with migrate ability in culturing epithelial cells [7].

61 IPF patients are characterized with down regulated COX-2 expression and its  
62 main metabolite prostaglandin E2 (PGE2), which is the terminal product of COX-2  
63 regulation in arachidonic acid metabolic pathway [8]. PGE2 was regarded as an anti-  
64 fibrosis gene and showed the contribution on activation of lung fibroblasts and  
65 excessive deposition of collagen in TGF- $\beta$ 1-induced COX-2 depression [9]. The  
66 differentiation of fibroblasts into myofibroblasts is the fundamental mechanism of the

67 occurrence and development of IPF. The level of PGE2 up-regulation was capable to  
68 reverse differentiation phenotype by inhibiting  $\alpha$ -SMA and collagen deposition [10]. In  
69 addition, PGE2 inhibits the EMT progression by binding to and activating prostaglandin  
70 receptors, indicating that the COX-2/PGE2/EPs axis plays a major role in inhibiting  
71 EMT [11]. Disulfiram, FDA-approved drug for several decades, is a safe, well-tolerated,  
72 inexpensive agent which was supported in alcohol dependence, and it demonstrated  
73 the effects of anti-cancer [12], antiviral [13], as well as metabolic dysfunction  
74 improvement [14]. DSF down-regulates the level of aldehyde dehydrogenase family 1  
75 (ALDH1) in fibroblasts, thereby preventing mucosal fibrosis in human and mouse eye  
76 scar formation [15]. What's more, DSF prevents renal fibrosis [16] and liver fibrosis  
77 [17] via an oxidative mechanism. It's reported that DSF inhibits EMT to reduce cell  
78 metastasis. DSF suppressed the morphological change, EMT-markers expression, cell  
79 migration and invasion in TGF- $\beta$ 1-induced EMT of oral squamous cell carcinoma  
80 (OSCC) cells [18]. DSF further existed excellent anti-tumor activity after complexing  
81 with copper ion, which can dramatically inhibit the EMT, migration and metastasis of  
82 breast cancer cells stimulated with TGF- $\beta$ 1 [19]. However, there was no research on  
83 DSF treating IPF via regulating COX-2/PGE2 signal axis.

84 The aim of our study was to ascertain the effect and mechanism of DSF treatment  
85 on IPF, thereby realizing DSF repositioning in clinic. Together, DSF inhibited the EMT  
86 and ECM in human primary DHLF-IPF cells and TGF- $\beta$ 1 stimulated A549 cells via  
87 activating COX-2/PGE2/EPs axis. In vivo evidence showed that DSF significantly

- 88 repressed EMT and ECM deposition via upregulated PGE2 level in BLM induced IPF
- 89 mice to retard fibrosis progress, suggesting the potential anti-fibrosis effect in IPF.

## 90 **2. Materials and methods**

### 91 *2.1. Cell culture and reagents*

92 Human type II alveolar epithelial cells (A549) were purchased from Fenghui  
93 Biological Technology and cultured with DMEM medium contained with 10% FBS and  
94 1% penicillin/streptomycin. DHLF-IPF cells were contributed by Professor Ren and Dr.  
95 Cao and cultured with F-12K medium contained with 10% FBS and 1%  
96 penicillin/streptomycin. Cells were cultured at 37°C and 5% CO<sub>2</sub>. A549 cells retain type  
97 II alveolar epithelial-like characteristics and can be stimulated by TGF-β1 to transform  
98 into mesenchyma, which is used for the experimental study of IPF[20].

99

### 100 *2.2. Cell viability and cell death*

101 After cultured with TGF-β1 (10 ng/mL) for 24 hours, A549 cells transformed into  
102 mesenchymal-like cell[21], then TGF-β1-induced A549 cells and DHLF-IPF cells were  
103 treated with different concentration gradient DSF for 24 hours at a density of 8×10<sup>5</sup>/mL.  
104 Cell Counting Kit-8 (CCK-8) and propidium iodide/crystal violet (PI/CV) were added to  
105 evaluate cell viability and cell death.

106

### 107 *2.3. Wound-healing assays*

108 TGF-β1-induced A549 cells and DHLF-IPF cells were planted at 10<sup>5</sup> cells per well  
109 in a 6-well plate. Tips were used to scratch the cells in the center of well plate. Images  
110 of the scratch breadth were examined and collected using light microscopy imaging at

111 various time points and analyzed using Image-J software.

112

#### 113 *2.4. Western blot*

114 After lysed with RIPA buffer, the mixture of cells and the mice lung tissues were  
115 then centrifuged to collect the supernatant. The concentration of total protein was  
116 detected by BCA kit, and each lane of the SDS-polyacrylamide gels received equal  
117 protein. According classical western-blot, the results were analyzed by Image-J  
118 software. The antibodies were collected at below.

##### **Antibodies:**

Anti-COL1A1 (COL-I) and anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) were from Santa Cruz Biotechnology (#sc-293182 and #sc-53142), Santa Cruz, CA, USA. Anti-fibronectin (FN) (#610077) was from BD Biosciences, New Jersey, USA). Anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), anti-E-cadherin (E-cad), anti-vimentin (VIM), anti-COX-2 were from Cell Signaling Technology (#19245, #14472, #5741 and #12282, Danvers, MA, USA. Anti-EP1 receptor and anti-EP3 receptor were from Cayman, Michigan, USA (#101740 and #101760). Anti-GAPDH (#ab8245) was from Abcam, Cambridge, UK.

119

#### 120 *2.5. RNA isolation and quantitative real-time PCR*

121 Total RNA was harvested following TRIzol Reagent manufacturer's instructions.  
122 cDNA was obtained by reverse transcription of RNA. Fluorescent labeling was done  
123 using a SuperReal PreMix Plus (SYBR Green) and Real-time quantitative PCR was  
124 performed with the Bio-Rad CFX Maestro System. The expression of mRNA was  
125 normalized to GAPDH expression. Human and mice primer sequences were collected  
126 at below.

##### **Human primer sequences**



GAPDH (F 5' TCCAAAATCAAGTGGGGC 3', R 5' ACTACTAGAACTCCGACA 3'),  
COL1A1 (F 5' GAGGGCCAAGACGAAGACATC 3', R 5' CAGATCACGTCATCGCACAAC 3'),  
ACTA2(F 5' GTGTTGCCCTGAAGAGCAT 3', R 5' GCTGGGACATTGAAAGTCTCA 3')  
CDH1 (F 5' CGAGAGCTACACGTTACGG3', R 5' GGGTGTGCGAGGGAAAAATAGG 3')  
VIM (F 5' AGTCCACTGAGTACCGGAGAC 3', R 5' CATTTCACGCATCTGGCGTTC 3')  
FN1 (F 5' CGGTGGCTGTCAGTCAAAG 3', R 5' AACCTCGGCTTCCTCCATAA 3')  
PTGR1 (F 5' AGCTTGTGCGGTATCATGGTGG 3', R 5' AGCAAGTGTATGACCCTGGTAAT 3')  
PTGER3(F 5' CGCCTCAACCACTCCTACAC 3', R 5' GACACCGATCCGCAATCCTC 3')  
PTGS2 (F 5' CTGGCGCTCAGCCATACAG 3', R 5' CGCACTTATACTGGTCAAATCCC 3')

### **Mice primer sequences**

Gapdh (F 5' CATCACTGCCACCCAGAAGACTG 3', R 5' ATGCCAGTGAGCTTCCCGTTCAG 3')  
Col1a1 (F 5' GCTCCTCTTAGGGGCACT 3', R 5' ATTGGGGACCCTTAGGCCAT 3')  
Acta2 (F 5' GGCACCACTGAACCCTAAGG3', R 5' ACAATACCAGTTGTACGTCCAGA 3')  
Cdh1 (F 5' TCGGAAGACTCCCGATTCAA 3', R 5' CGGACGAGGAACTGGTCTC 3)  
Vim (F 5' CCACACGCACCTACAGTCT 3', R 5' CCGAGGACCGGGTCACATA 3')  
Fn1 (F 5' TCAAGTGTGATCCCCATGAAG 3', R 5' CAGGTCTACGGCAGTTGTCA 3')  
Ptgs2 (F 5' TTCCAATCCATGTCAAACCGT 3', R 5' AGTCCGGGTACAGTCACACTT 3')

127

## 128 *2.6. Lentiviral construction and infection in A549 cells*

129 Three short hairpin (sh)RNA vectors targeting COX-2 (shCOX-2) and a control  
130 vector (shNC) were designed and purchased from GenePharma. Lentiviral particles  
131 were produced by transfecting HEK293T cells with lentiviral plasmids along with  
132 envelope (VSVG) and packing plasmids. For viral infection, A549 cells were plated in  
133 6-well plates, grown to 50-70% confluence, and infected with the presence of 8 µg/ml  
134 polybrene. Following infection for 48 hours, the cells were selected with 5.0 µg/ml  
135 puromycin. Knockdown efficiencies were confirmed via real-time PCR and western blot  
136 analysis.

137

## 138 *2.7. Immunofluorescence microscopy*

139 The cell slides were washed 3 times with PBS and then fixed with 4%  
140 paraformaldehyde for 15 min in culture plates. Cells were permeabilized with 0.5%  
141 Triton X-100 for 15 min at room temperature. Slides were dropped with 10% goat  
142 serum and blocked for 1 h at room temperature. The blocking solution was removed  
143 by absorbent paper, and diluted primary antibody was added to each slide and  
144 incubated in a wet box at 4 °C overnight. The primary antibody was removed by  
145 absorbent paper, followed by fluorescent secondary antibody and incubated for 1 h at  
146 37 °C in a black wet box. Finally, DAPI was added and incubated in the dark for 5 min  
147 to stain nuclei. Slides were sealed with antifade solution containing anti-fluorescence  
148 quencher, and images were observed and collected under a fluorescence microscope.  
149 Pictures were analyzed with Image-J software.

150

## 151 *2.8. PGE<sub>2</sub> measurement*

152 After treated with DSF, the supernatant of cells was collected after centrifuged at  
153 4°C for 5 min at 1000 rpm/min. The remanding cells were stained with purple crystal  
154 to quantitate total protein. The PGE<sub>2</sub> concentration of the supernatants and serum from  
155 mice was determined according to the manufacturer's instructions, and the PGE<sub>2</sub>  
156 concentration in supernatants normalized to the total protein.

157

158 *2.9. BLM-induced IPF in mice*

159 Males C57BL/6J mice weighted 20±2 g (Charles Rive) and housed at 22-24°C  
160 with a 12:12 hr light-dark cycle. Animal experiments were performed according to the  
161 Guidelines on Laboratory Animals of Nankai University and were approved by the  
162 Institute Research Ethics Committee at Nankai University (approval number: 2021-  
163 SYDWLL-000461).

164 The establishment and measured of IPF mice model referred to previous  
165 studies[21]. 50 mg/kg DSF was intraperitoneal injection daily for 14 days beginning 7  
166 days after BLM administration, 0.5% CMC-Na was used as a vehicle.

167

168 *2.10. Histology and immunohistochemistry*

169 Before the lung tissue of mice was embedded in paraffin and sectioned, it was  
170 fixed with 4% paraformaldehyde for 2 days. Tissue paraffin sections were stained with  
171 Hematoxylin-Eosin (H&E) Staining Kit or Masson's Trichrome Stain Kit. Tissue slices  
172 were treated with 3%-hydrogenperoxide solution to remove endogenous enzymes,  
173 infiltrated with 0.5% Triton-100 to permeabilize membrane and blocked by 10% goat  
174 serum. Slides removed the blocking solution, then added the primary antibody  
175 dropwise, and incubated overnight at 4°C. Add the secondary antibody working  
176 solution for 1 hours at room-temperature. Slides were dropped with DAB working  
177 solution and counterstained with hematoxylin. Stained tissue slices were observed  
178 under the microscope. Pictures were analyzed with Image-J software.

179

### 180 *2.11. Hydroxyproline Assay*

181       Accurately weigh the right lung and follow the instructions of the hydroxyproline  
182 test kit purchased from Nanjing Jiancheng. Results were expressed as  $\mu\text{g}$  of  
183 hydroxyproline/mg of lung weight.

184

### 185 *2.12. Human subjects*

186       The lung tissues and serum of IPF used in the study were provided by Professor  
187 Dunqiang Ren (Peking Union Medical College Hospital). The control lung tissues were  
188 derived from the non-tumor infiltrated area of lung cancer patients. Control serum was  
189 obtained from patients without pulmonary fibrosis. The study complied with medical  
190 ethics (Approval number: NKUIRB2021106).

191

### 192 *2.13. Statistical analysis*

193       All data were presented as the means  $\pm$  SEM of at least three independent  
194 experiments ( $n \geq 3$ ). The Student's t test was used to compare two groups and two-way  
195 ANOVA was used for multiple group comparisons. Statistical significance was  
196 considered at  $P < 0.05$ . The graphical representation and statistical analysis were  
197 performed using GraphPad Prism (Version 8.3.0).

198 **3. Results**

199 *3.1 DSF inhibited viability and migration of DHLF-IPF and TGF- $\beta$ 1-induced A549 cells.*

200 Cell culture models and human lung primary cells are beneficial for exploring the  
201 mechanism of EMT, lung fibrosis and the associated treatment strategies. TGF- $\beta$ 1 is  
202 a prototype mediator for fibroblast differentiation into myofibroblasts, induction of  
203 alveolar epithelial cells transformation into mesenchymal cells, as well as the  
204 phenotypic mediator for extracellular matrix [22]. Therefore, we stimulated alveolar  
205 epithelium A549 cells were stimulated with TGF- $\beta$ 1 (10ng/ml) for 24 h to establish an  
206 EMT model *in vitro*.

207 Next, to determine the cultured cell treatment dose of DSF, cell death and cell  
208 viability assay were performed to assess the induction of cell viability in cultures  
209 following treatment with DSF at the indicated dose. After treating with DSF for 24 h in  
210 TGF- $\beta$ 1-induced A549 cells and DHLF-IPF cells, cell viability and cell death were  
211 measured with CCK8 (**Figures 1A and 1D**) and PI/CV (**Figures 1B and 1E**),  
212 respectively. The half-maximal inhibitory concentrations ( $IC_{50}$ ) of DSF in DHLF-IPF  
213 cells and TGF- $\beta$ 1-induced A549 cells were 14.84  $\mu$ M (**Figure 1A**) and 20.99  $\mu$ M  
214 (**Figure 1D**) respectively.

215 Both DHLF-IPF cells and TGF- $\beta$ 1-induced A549 cells showed dose and time-  
216 dependent responses to DSF treatment. In a following antifibrosis study, we used 5  
217  $\mu$ M DSF for DHLF-IPF cells and 15  $\mu$ M for TGF- $\beta$ 1-induced A549 cells to avoid  
218 interference from cytotoxic effects according to  $IC_{50}$ .

219 TGF- $\beta$ 1-induced A549 cells were characterized with EMT phenotype and  
220 generated a migratory phenotype. Thereby, we evaluated the effect of DSF on cell  
221 migration via an *in vitro* wound healing assay, and results revealed that cell migration  
222 rates were significantly reduced in both primary DHLF-IPF cells (**Figures 1C**) and  
223 TGF- $\beta$ 1-induced A549 cells (**Figures 1F**). Together, these results suggested that DSF  
224 inhibited cell viability in a dose-dependent manner, accompanied with cell migration  
225 impeded during EMT progress.

226

227 **Figure 1. DSF inhibited viability and migration of DHLF-IPF and TGF- $\beta$ 1-induced**

228 **A549 cells.** Primary DHLF-IPF cells and TGF- $\beta$ 1-induced A549 cells and were

229 exposed to indicated dose of DSF for 24 h. Cell viability (**A and D**) and cell death (**B**

230 **and E**) were determined by a CCK-8 staining assay and PI exclusion assay,

231 respectively. The half-maximal inhibitory concentration ( $IC_{50}$ ) was calculated by cell

232 viability (**A and D**). The width of the scratch was photographed and quantified at 0, 12,

233 and 24 h post scratching of DHLF-IPF cells (**C**) or at 0, 24, and 48 h post scratching of

234 TGF- $\beta$ 1-induced A549 cells (**F**) (magnification 40 $\times$ ) by a wound-healing assay. The

235 width of gap was measured with Image-J software (Three independent analyses were

236 performed) and calculated with GraphPad Prism. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ .

237

238 *3.2 DSF reversed EMT and ECM in DHLF-IPF and TGF- $\beta$ 1-induced A549 cells.*

239 Since the unexpected wound-healing capacities seen in the context of DSF, the

240 effect of regulatory effects of DSF on EMT and ECM-related biomarkers in DHLF-IPF

241 cells and TGF- $\beta$ 1-induced A549 cells were further investigated.

242 DSF (5  $\mu$ M) depressed the mRNA expression of mesenchymal markers *CDH2*,  
243 *VIM* and *ACTA2*, as well as extracellular matrix *COL1A1* (**Figure 2A**), as well as the  
244 protein levels of VIM,  $\alpha$ -SMA and FN in DHLF-IPF cells (**Figures 2B and**  
245 **Supplementary Figure 1A**). Accordingly, DSF (15  $\mu$ M) were added to TGF- $\beta$ 1-  
246 induced A549 cells in the presence of TGF- $\beta$ 1 for 24 h, the mRNA level of epithelial  
247 marker *CDH1* was increased, *CDH2* and *COL1A1* were reduced significantly  
248 compared with TGF- $\beta$ 1 group (**Figure 2C**). Similarly, the protein expression of  $\alpha$ -SMA,  
249 VIM and FN were depressed whereas the epithelial marker E-cad was not significantly  
250 increased (**Figures 2D Supplementary Figure 1B**). What's more, western blot  
251 analysis was supported by immunofluorescence results showing a significant decrease  
252 in cellular VIM expression occurred after 24 h treatment of DSF in TGF- $\beta$ 1-induced  
253 A549 cells (**Figure 2E**). The reverse change in mesenchymal proteins and epithelial  
254 marker, as well as the reduced ECM deposition suggested that the process of EMT  
255 was disrupted by DSF.

256

257 **Figure 2. DSF reversed EMT and ECM in DHLF-IPF and TGF- $\beta$ 1-induced A549**  
258 **cells. (A)** The mRNA levels in DHLF-IPF cells including *CDH2*, *VIM*, *ACTA2* and  
259 *COL1A1* were detected by qPCR. **(B)** The protein expression of VIM,  $\alpha$ -SMA and FN  
260 were measured with western blot in DHLF-IPF cells. After induced with or without 10  
261 ng/ml TGF- $\beta$ 1 for 24 h, TGF- $\beta$ 1-induced A549 cells were treated with DSF (15  $\mu$ M) for  
262 another 24 h. **(C)** mRNA levels of *CDH1* and *CDH2* and *COL1A1* were detected by

263 qPCR. **(D)** The protein expression of E-cad, VIM,  $\alpha$ -SMA and FN were measured with  
264 western blot. **(E)** Immunofluorescence staining of VIM were performed and nuclear  
265 staining with DAPI in TGF- $\beta$ 1-induced A549 cells (magnification 400 $\times$ , bar=50  $\mu$ m). \* $P$   
266 <0.05, \*\* $P$ <0.01, \*\*\*\* $P$ <0.0001.

267

268 *3.3 DSF inhibited TGF- $\beta$ 1-induced EMT through restoring COX-2 regulated PGE<sub>2</sub>*  
269 *biosynthesis.*

270 During IPF development and progress,  $\alpha$ -SMA is considered as a gold standard  
271 and regarded as a marker of active fibrogenesis [23]. Firstly, the disordered structure  
272 (H&E), significant fibrosis (Masson's staining),  $\alpha$ -SMA and FN positive expression in  
273 the lung tissues was observed compared with those in non-IPF through the histological  
274 alterations in human lung tissues with IPF (**Supplementary Figure 2A**), suggesting  
275 the EMT progress and ECM deposition. We then reanalyzed a public dataset (GEO  
276 accession #: GSE10667), and found that *PTGS2* mRNA was significantly reduced in  
277 IPF lung tissues (**Figure 3A**), which made us curious about the relationship between  
278 the COX-2 and EMT, thus we measured the differences of COX-2 and  $\alpha$ -SMA in  
279 comparable regions of lung tissue from IPF patients using immunohistochemistry  
280 (**Figure 3B**) and confocal microscopy (**Figure 3C**). Immunohistochemistry was used  
281 to examine the spatial location of COX-2 and  $\alpha$ -SMA in lung tissues from IPF patients  
282 (**Figure 3B**). In case #1 IPF lung tissue (left), it showed that low COX-2 expression  
283 located in a  $\alpha$ -SMA-positive tissue area. On the contrary, case #2 showed high positive  
284 of COX-2 and lack of  $\alpha$ -SMA expression (**Figure 3B**), demonstrating the potential



285 negative relationship between the expression of COX-2 and  $\alpha$ -SMA. Furthermore,  
286 limited co-localization between COX-2 and  $\alpha$ -SMA was present in IPF patients via  
287 immunofluorescence microscopy (**Figure 3C**). In addition, COX-2 metabolite PGE<sub>2</sub>  
288 production in serum was detected via Elisa assay, and result revealed that it was  
289 decreased isolated in serum from IPF patients compared with healthy donors (**Figure**  
290 **3D**), suggesting COX-2/PGE<sub>2</sub> axis may play an essential role in IPF development and  
291 progression.

292 COX-2 is the rate-limiting enzyme in the metabolic conversion of arachidonic acid  
293 (AA) into various prostaglandins (PGs) including prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) [24].  
294 Although some studies showed that PGE<sub>2</sub> had pro-inflammatory actions, accumulating  
295 data suggested that the COX-2/PGE<sub>2</sub> plays a vital role in ameliorating fibrosis and  
296 avoiding respiratory damage in IPF [25].

297 To further confirm our conjecture, COX-2 inhibitors Rofecoxib was performed in  
298 our following experiments. The administration of COX-2 inhibitors Rofecoxib did  
299 promote EMT through re-expression of VIM,  $\alpha$ -SMA and FN in IPF cells  
300 (**Supplementary Figure 3B and 3C**), as well as the depressing PGE<sub>2</sub> production in  
301 A549 cells (**Supplementary Figure 3D**), suggesting the loss of COX-2 promoted EMT.  
302 In view of diminished COX-2 expression in fibroblasts with a resultant defect in the  
303 antifibrotic mediator PGE<sub>2</sub> production in IPF, we tested whether DSF treated IPF  
304 through activating COX-2 to induce PGE<sub>2</sub> production. We treated DHLF-IPF cells with  
305 DSF (5  $\mu$ M), and detected relevant indicators through western blot and Elisa. Results

306 exactly suggested that the level of COX-2, PGE<sub>2</sub> receptor-3 (EP3) (**Figure 3E and**  
307 **Supplementary Figure 3A**) and PGE<sub>2</sub> content (**Figure 3F**) in supernatant was  
308 increased in primary DHLF-IPF cells with DSF treatment. Likewise, DSF induced COX-  
309 2 (**Figure 3G and Supplementary Figure 3E**) expression and PGE<sub>2</sub> receptors  
310 (PTGER1 and PTGER3) (**Figure 3H**), which increased prostaglandin E2 (PGE<sub>2</sub>) level  
311 (**Figure 3I**), and subsequently improved EMT through the downregulation of  $\alpha$ -SMA,  
312 VIM and FN (**Figure 3G and Supplementary Figure 3E**) in A549 cells.

313         Given the significant COX-2 expression difference and relevance of IPF, we  
314 further evaluated the direct roles of COX-2 in IPF. Then, COX-2-targeting shRNA  
315 (shCOX-2) or corresponding controls (shNC) were used to establish a stable COX-2-  
316 knockdown cell line in A549 cells (**Supplementary Figure 3F**). Cell morphology and  
317 protein results revealed that DSF had limited interference on TGF- $\beta$ 1 induced shCOX-  
318 2 A549 cells, which showed no significant changes in cell migration morphology  
319 (**Figure 3J**) and the expression of EMT and ECM markers (**Figure 3K and**  
320 **Supplementary Figure 3G**) compared with corresponding shNC cells. To sum up,  
321 these conclusions suggested that DSF may mediate COX-2 expression to play its role  
322 in the treatment of IPF.

323         After determining the role of COX-2, we continue to explore the function of its  
324 downstream product PGE<sub>2</sub> in IPF. Unsurprisingly, TGF- $\beta$ 1 inhibited EMT and COX-  
325 2/PGE<sub>2</sub> signaling pathway, while DSF treatment reversed this phenomenon. Likewise,  
326 exogenous PGE<sub>2</sub> (5  $\mu$ M) treatment for 24 h in the presence of TGF- $\beta$ 1 activated EP1

327 and reversed the expression of VIM,  $\alpha$ -SMA and FN, though did not increased COX-2  
328 expression in TGF- $\beta$ 1 induced A549 cells compared with DSF treatment (**Figure 3L**  
329 **and Supplementary Figure 3H**). Together these data strongly suggested that the  
330 expression of COX-2 made important contribution to the pathogenesis of pulmonary  
331 fibrosis. DSF is associated with upregulation of COX-2, which in turn promotes PGE<sub>2</sub>  
332 synthesis and secretion to improve EMT and ECM.

333

334

335 **Figure 3. DSF inhibited TGF- $\beta$ 1-induced EMT through restoring COX-2 regulated**  
336 **PGE<sub>2</sub> biosynthesis. (A)** Reanalyzed of *PTGS2* mRNA from a public dataset (GEO  
337 accession #: GSE10667) with Wilcox. tests. **(B)** The images showed the expression of  
338 COX-2 and  $\alpha$ -SMA staining in IPF patient tissues.  $\alpha$ -SMA staining (green frames) and  
339 COX-2 expression (red frames) were observed in fibroblastic foci (magnification 200  
340  $\times$ ). **(C)** Immunofluorescence analysis showed the expression of  $\alpha$ -SMA (green) and  
341 COX-2 (red) staining in IPF patient lung tissues. IPF lung tissues area positived for  
342 COX-2 presented  $\alpha$ -SMA negativity (scale bar=75  $\mu$ m or 25  $\mu$ m). **(D)** The prostaglandin  
343 E<sub>2</sub> (PGE<sub>2</sub>) content from no-IPF (n=12) and IPF (n=9) patient serum was assayed by  
344 ELISA kit. **(E)** DHLF-IPF cells were treated with DSF (5  $\mu$ M) for 24 h, then the protein  
345 expression of COX-2 and EP3 were measured with western blot. **(F)** The PGE<sub>2</sub> content  
346 in the supernatant was measured by ELISA kit after DHLF-IPF cells were treated with  
347 DSF (5  $\mu$ M) for 24 h. After induced with or without 10 ng/mL TGF- $\beta$ 1 for 24 h, TGF- $\beta$ 1-  
348 induced A549 cells were treated with DSF (15  $\mu$ M) for another 24 h. **(G)** The protein

349 expression of COX-2,  $\alpha$ -SMA, VIM and FN and were measured with western blot in  
350 A549 cells. **(H)** The mRNA levels of PGE<sub>2</sub> receptors (*PTGER1* and *PTGER3*) were  
351 detected by qPCR and normalized with *GAPDH* in A549 cells. **(I)** TGF- $\beta$ 1-induced  
352 A549 cells were treated with DSF (15  $\mu$ M) for 24 h, then the prostaglandin E2 (PGE<sub>2</sub>)  
353 content in the supernatant was assayed by ELISA kit. **(J)** Cell morphology changes  
354 were observed and photographed with a light microscopy (magnification 100 $\times$ ). **(K)**  
355 Both shCOX-2 and shNC A549 cells were treated with DSF (15  $\mu$ M) for 24h, then the  
356 protein levels of E-cad, VIM,  $\alpha$ -SMA and COX-2 were assessed with western blot. **(L)**  
357 TGF- $\beta$ 1-induced A549 cells were treated with DSF (15  $\mu$ M) or PGE<sub>2</sub> (5  $\mu$ M) for 24 h.  
358 The protein expression was measured with western blot. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <  
359 0.001, \*\*\*\* $P$ <0.0001.

360

361 *3.4 Anti-fibrotic effect of DSF by boosting PGE2 biosynthesis in BLM-induced IPF*  
362 *model.*

363 Finally, experimental models of fibrosis *in vivo* are available for defining the  
364 complexity of matrix metabolism in the intact tissue and validating the findings from  
365 cell culture and *in vitro* systems. IPF mice model was established using 2mg/ml BLM  
366 by atomized drug delivery device for 7days. Mice were sacrificed at the endpoint after  
367 DSF treatment for 14 days. BLM mice treated with or without DSF showed limited  
368 differences, but their body weights were lower than those of blank mice **(Figure 4A)**.  
369 For pulmonary function, DSF (50 mg/kg) treatment attractively relieved respiratory  
370 system dysfunction in the preclinical model via enhancing FVC, Cdyn and depressing

371 Re, Ri compared with BLM treated group (**Figure 4B**).

372 H&E staining was used to observe changes in mice lung tissue pathological  
373 structure. The alveolar structure in the BLM group was blurred or even disappeared,  
374 and the alveolar shape was incomplete combined with obvious fibrotic foci. Meanwhile,  
375 the cell nucleus was deeply stained and the cell proliferation increased wildly. On the  
376 contrary, the most of the intact alveolar structure of BLM-mice treated with DSF (50  
377 mg/kg) was retained, and the fibrotic foci were reduced significantly with no obvious  
378 cell proliferation (**Figure 4C**). Masson's trichrome staining further suggested the  
379 collagen deposition in lung tissues, which showed a significant increase of collagen  
380 around the fibrotic foci in BLM-mice lung tissue compared with the control mice (**Figure**  
381 **5D and 5E**). In addition, collagen deposition in lung sections also quantified from the  
382 hydroxyproline content (**Figure 5F**), both were strikingly decreased with DSF (50  
383 mg/kg) treatment in fibrotic foci of BLM mice compared with those in the control group.

384 At the end point of the experiment, mice lung tissues were removed for further  
385 tissue proteins, mRNA and IHC staining analysis to evaluate the effect of DSF on BLM-  
386 induced IPF. DSF (50 mg/kg) reduced the mRNA levels of mesenchymal markers (*Vim*,  
387 *Acta2*), ECM markers (*Fn1*, *Col1a1*) and increased epithelial marker (*Cdh1*) compared  
388 to the BLM group (**Figure 4H**). Simultaneously, the protein levels in lysates of whole  
389 lung tissues were analyzed, and DSF (50 mg/kg) treatment effectively suppressed Col-  
390 I and FN expression and increased E-cad expression (**Figure 4I and**  
391 **Supplementary Figure 4A**). Similarly, immunohistochemistry results further showed

392 that BLM induced the level of FN, VIM and  $\alpha$ -SMA in mice as compared with control  
393 mice, whereas DSF (50 mg/kg) treatment significantly reduced BLM-induced the  
394 overexpression of FN, VIM and  $\alpha$ -SMA expression (**Figure 5J and 5K**).

395 Further we explored the mechanism of DSF *in vivo*, we confirmed the mRNA level  
396 of *Ptgs2* (**Figure 5H**) and the protein of COX-2 positive expression (**Figure 5J and 5K**)  
397 in whole lung tissue and lung section respectively *in vivo* experimental IPF mice. In  
398 addition, the content of PGE<sub>2</sub> in mice serum was increased in DSF group compared  
399 with BLM-only group (**Figure 5G**). These data indicated that DSF (50 mg/kg) treatment  
400 significantly reduced BLM-induced EMT progression and ECM deposition *in vivo*,  
401 accompanied with pulmonary function reparation and COX-2 reactivation to mediate  
402 PGE<sub>2</sub> biosynthesis, thus ameliorating IFP progression.

403

404 **Figure 4. Anti-fibrotic effect of DSF by boosting PGE<sub>2</sub> biosynthesis in BLM-**  
405 **induced IPF model. (A)** The body weight of each mouse was monitored and recorded  
406 daily. **(B)** Pulmonary function parameters including forced vital capacity (FVC), dynamic  
407 compliance (C<sub>dyn</sub>), expiratory resistance (R<sub>e</sub>) and inspiratory resistance (R<sub>i</sub>) among  
408 different treatments were measured after treated with DSF for 14 days. Lung sections  
409 were stained with H&E **(C)** or Masson's trichrome **(D)** for collagen accumulation  
410 (representative image, magnification 200×, bar=100 μm), and Masson's trichrome  
411 staining was quantified **(E)** by Image-J software compared to blank group. **(F)** The  
412 hydroxyproline content in lung tissues among different groups were analyzed and  
413 quantified. **(G)** The prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) content in the mice serum were detected  
414 by ELISA kit. **(H)** The mRNA levels of *Cdh1*, *Acta2*, *Vim*, *Col1a1*, *Fn1* and *Ptgs2* in  
415 lung tissues were performed by qPCR and normalized with *Gapdh*. **(I)** Western blot  
416 was used to analyze the expression of E-cad, FN and Col- I in lung tissues. **(J)**  
417 Immunohistochemistry staining of FN, VIM and α-SMA and COX-2 in the lung tissues  
418 (magnification 200 ×, scale bar = 100 μm). **(K)** The positive area on lung sections was  
419 quantified by Image-J software, normalized to blank control. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<  
420 0.001, \*\*\*\**P*<0.0001.

421  
422 **Figure 5. Scheme.** We find that COX-2/PGE<sub>2</sub> is negatively expressed in IPF patients.  
423 The decrease of COX-2 promotes abnormal cell proliferation, induces the epithelial-  
424 mesenchymal transition (EMT) of alveolar epithelial cells, activates fibroblast  
425 differentiation, and reduces the production of collagen. Whereas, disulfiram exerts the

426 effect of inhibiting cell proliferation and migration, decreasing EMT of alveolar epithelial  
427 cells, as well as preventing fibroblast activation. On the other hand, DSF also  
428 ameliorates lung function, collagen deposition and pathology injure in BLM induced  
429 IPF mice.

430

#### 431 **4. Discussion**

432 This study revealed the anti-IPF pharmacological activity of the anti-alcohol abuse  
433 drug disulfiram (DSF) [26] that rarely explored. In our research, we utilized human  
434 primary DHLF-IPF cells and TGF- $\beta$ 1 induced EMT cells as the *in vitro* model. Besides,  
435 intratracheal injection of BLM into mice induced IPF mice model to estimate the anti-  
436 fibrotic effect of DSF *in vivo*. Our results proved that DSF inhibited the proliferation and  
437 migration in IPF cell model, improved IPF mice respiratory function and prevented lung  
438 fibrosis. Meanwhile, DSF increased epithelial proteins, reduced mesenchymal proteins  
439 and excessively deposited extracellular matrix proteins *in vitro* and *in vivo*. Notably,  
440 DSF regulated EMT by activating PGE<sub>2</sub> biosynthesis, and the anti-IPF pharmacological  
441 activity of DSF have not reported so far.

442 The formation mechanisms of IPF mainly include the transformation of alveolar  
443 epithelial cells to mesenchyme [27], activation of myofibroblasts [28], deposition of  
444 extracellular fibrous protein [29], secretion of cytokines [30] and so on. TGF- $\beta$ 1 is a  
445 recognized pathogenic factor for pulmonary fibrosis [31]. We have demonstrated for  
446 the first time that DSF exhibited admirable effect on improving EMT and degrading



447 extracellular matrix protein on TGF- $\beta$ 1 induced pulmonary fibrosis cell models and  
448 DHLF-IPF cells.

449 Previous studies confirmed that the expression of COX-2 and PGE<sub>2</sub> was down-  
450 regulated in myofibroblasts and IPF patients [8], while  $\alpha$ -SMA is highly expressed in  
451 lung fibrous foci [8]. Likewise, our analysis of lung pathology in IPF patients also found  
452 that  $\alpha$ -SMA and COX-2 were not co-localized and the expression of PGE<sub>2</sub> was  
453 decreased in the serum. These results proved that COX-2/PGE<sub>2</sub> was a possibility  
454 target for IPF.

455 Numerous studies showed that TGF- $\beta$ 1 induced COX-2 and PGE<sub>2</sub> expression [32].  
456 TGF- $\beta$ 1 induced the expression of COX-2 and increased the synthesis of PGE<sub>2</sub> in  
457 prostate cancer cells [33]. TGF- $\beta$ 1 induced COX-2 expression to train EMT in human  
458 bronchial epithelial cells [34]. TGF- $\beta$ 1 increased COX-2 and PGE<sub>2</sub> receptor EP2  
459 expression in breast cancer cells [35], and supported that PGE<sub>2</sub> was a mediator to  
460 incite angiogenesis and cell migration, and selective EP2 inhibitors reduced the  
461 expression of PGE<sub>2</sub> [35]. Conversely, Peedikayil E Thomas et.al explained that PGE<sub>2</sub>  
462 showed significant effect on inhibiting TGF- $\beta$ 1 induced myofibroblast differentiation,  
463 including modulating cell morphology, cytoskeleton, and cell adhesion-dependent  
464 signals [36]. In addition, transcriptome analysis of TGF- $\beta$ 1 induced myofibroblasts  
465 differentiation process found that PGE<sub>2</sub> reversed the expression of 363 (62%) TGF- $\beta$ 1  
466 up-regulated genes and 345 (50%) TGF- $\beta$ 1 down-regulated genes [37]. Our results  
467 revealed that TGF- $\beta$ 1 reduced COX-2 and PGE<sub>2</sub> expression, and COX-2 silence A549

468 cells are more susceptible to TGF- $\beta$ 1, thus aggravating EMT development. We  
469 observed that exogenous addition of PGE<sub>2</sub> improved EMT and ECM induced by TGF-  
470  $\beta$ 1. These results indicated the important role of COX-2/PGE<sub>2</sub> in IPF.

471 In recent years, the application research of DSF has been ever more extensive  
472 [38]. DSF alone or chelated with divalent metal ions exerted anti-cancer activity [39].  
473 In addition, DSF was realized as a narrow-spectrum antibacterial agent [40, 41]. DSF  
474 dose-dependently inhibited the level of PGE<sub>2</sub> and COX-2 protein expression in the  
475 aqueous humor of uveitis rats whatever oral [42] or topical eye medication [43]. DSF  
476 eye drops administration inhibited the deposition of fibrotic protein in ocular scar  
477 formation in mice. Mechanically, DSF mainly suppressed inflammation factors to  
478 improve fibrous lesions [15]. Studies have shown that DSF inhibits the secretion of  
479 inflammatory factors and type I collagen in rat unilateral urethral obstruction model  
480 [44]. What's more, the main metabolite of DSF, diethyldithiocarbamate (DDC),  
481 suppressed the inflammation and fibrosis-related parameters in non-alcoholic fatty  
482 liver by regulating lipid metabolism and oxidative stress in rodents, including the  
483 inhibition of collagen deposition and expression of  $\alpha$ -SMA protein in liver [45]. PGE<sub>2</sub>  
484 often served as an effective pro-inflammatory mediator and participated in the  
485 inflammatory diseases [46]. The above studies proved that DSF inhibited the  
486 inflammatory factors PGE<sub>2</sub> and COX2 protein. On the contrary, we verified DSF  
487 increased COX-2 and PGE<sub>2</sub> in EMT cells induced by TGF- $\beta$ 1, human primary DHLF-  
488 IPF cells, and IPF mice. To determine the role of COX-2 in the treatment of IPF with

489 DSF, shCOX-2-A549 cells were induced EMT with TGF- $\beta$ 1 and processed by DSF.  
490 We found that DSF failed in improving the EMT and ECM parameters in the shCOX-2  
491 EMT cell model. Instead, it played an anti-fibrotic effect by inducing the expression of  
492 COX-2. PGE<sub>2</sub> is the main production mediated and catalyzed via COX-2 [47]. We  
493 concluded that exogenous addition of PGE<sub>2</sub> significantly improved EMT model of TGF-  
494  $\beta$ 1 induced IPF. Therefore, we believed that DSF prevented EMT and treated IPF by  
495 inducing COX-2/PGE<sub>2</sub> axis expression.

496 The actual strategy to increase PGE<sub>2</sub> in lung tissue during IPF was limited. The  
497 inhibitor of 15-prostaglandin dehydrogenase (15-PGDH), the PGE<sub>2</sub> degrading  
498 enzyme, indirectly increased PGE<sub>2</sub> content, thereby destroying TGF- $\beta$  signaling and  
499 inhibiting myofibroblasts growth and differentiation [48]. In order to reduce the adverse  
500 side effects of elevated PGE<sub>2</sub> on other organs, Ivanova V et al. employed liposomes  
501 to deliver PGE<sub>2</sub> into the lungs by inhalation to treat pulmonary fibrosis [49].  
502 Nonetheless, this study [50] emphasized that IPF was an interspecific lung disease  
503 (ILD), and PGE<sub>2</sub> was significantly elevated in ILD patients. It is pointed out that the  
504 COX-2/PGE<sub>2</sub> axis has dual functions. On the one hand, activation of COX-2/PGE<sub>2</sub> axis  
505 aggravated IPF induced by streptococcus pneumonia, but on the other hand, it also  
506 exists therapeutic effect on non-malignant IPF [50]. Our research based on  
507 experimental IPF induced by TGF- $\beta$ 1 and BLM. DSF mobilized COX-2/PGE<sub>2</sub> axis and  
508 exhibited excellent anti-IPF effect. Furtherly, it is necessary to explore the anti-fibrosis  
509 effect of DSF in different IPF classification, and the role of COX-2/PGE<sub>2</sub> induced by

510 DSF in systemic organs.

511 From a broader perspective, our research illustrated the potential of drug  
512 repositioning, provided new mechanism insights, and determined new IPF treatment  
513 target and clinical trial inspiration. DSF, an old, safe and public domain drug may help  
514 save IPF patients worldwide.

515

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517 performed *in vitro* studies, Xiaolin Pei, Fangxu Zheng, Yin Li, Tianjiao Li designed,  
518 performed and analyzed *in vivo* experiments. Xiaolin Pei, Xiao Han and Ya Feng  
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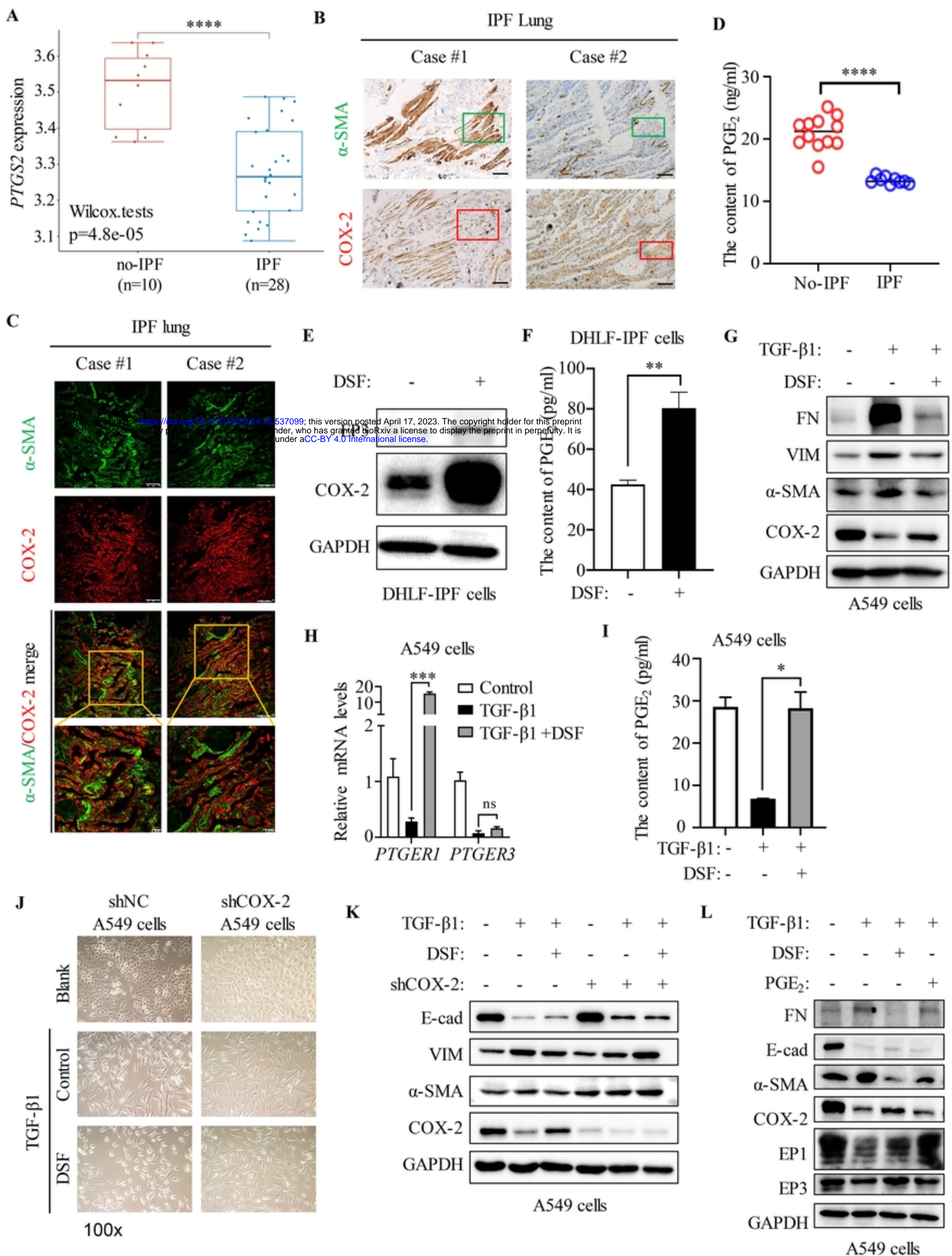
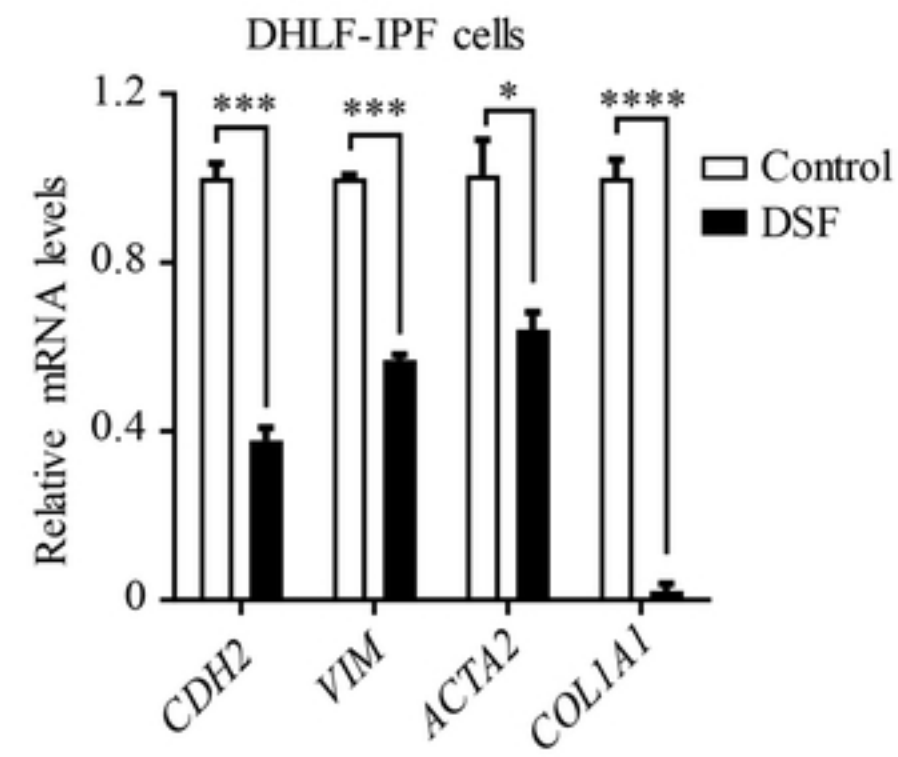
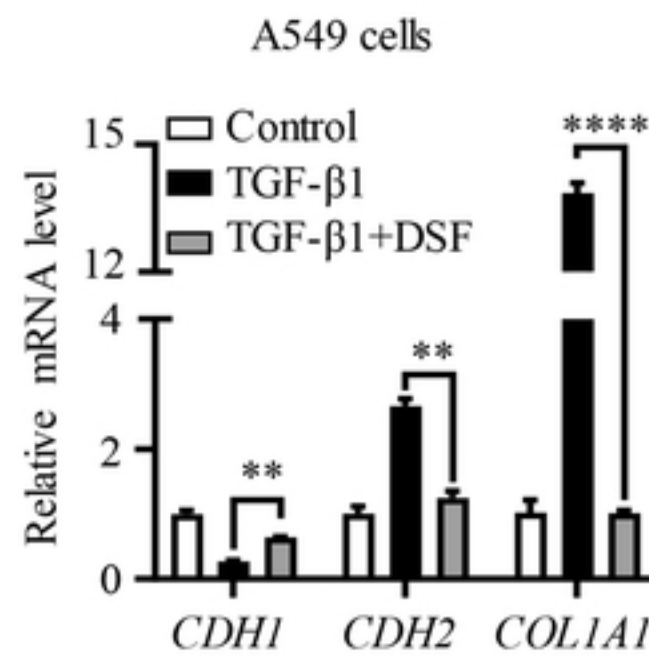
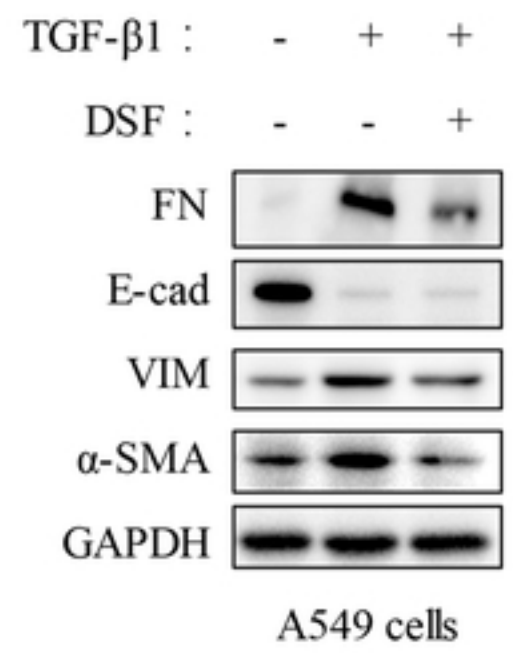
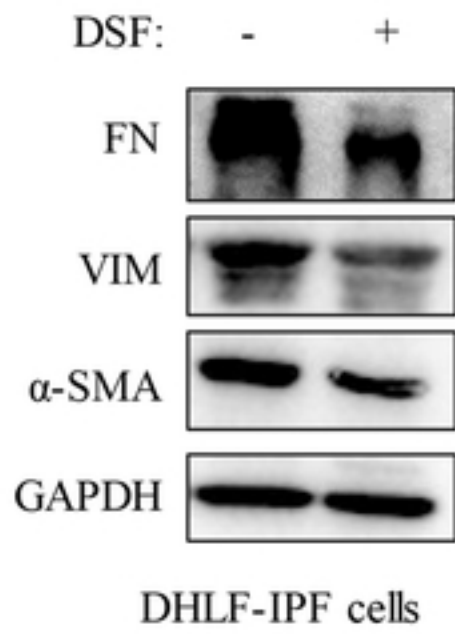
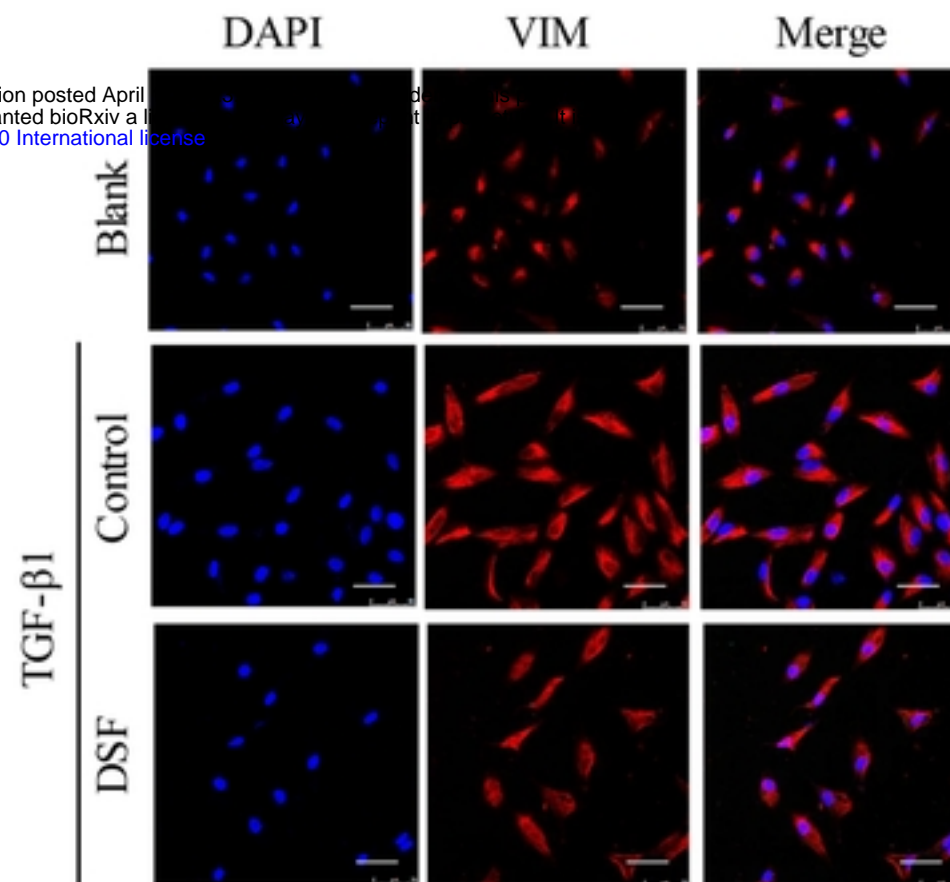


Figure 3

**A****C****D****B**

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**E**



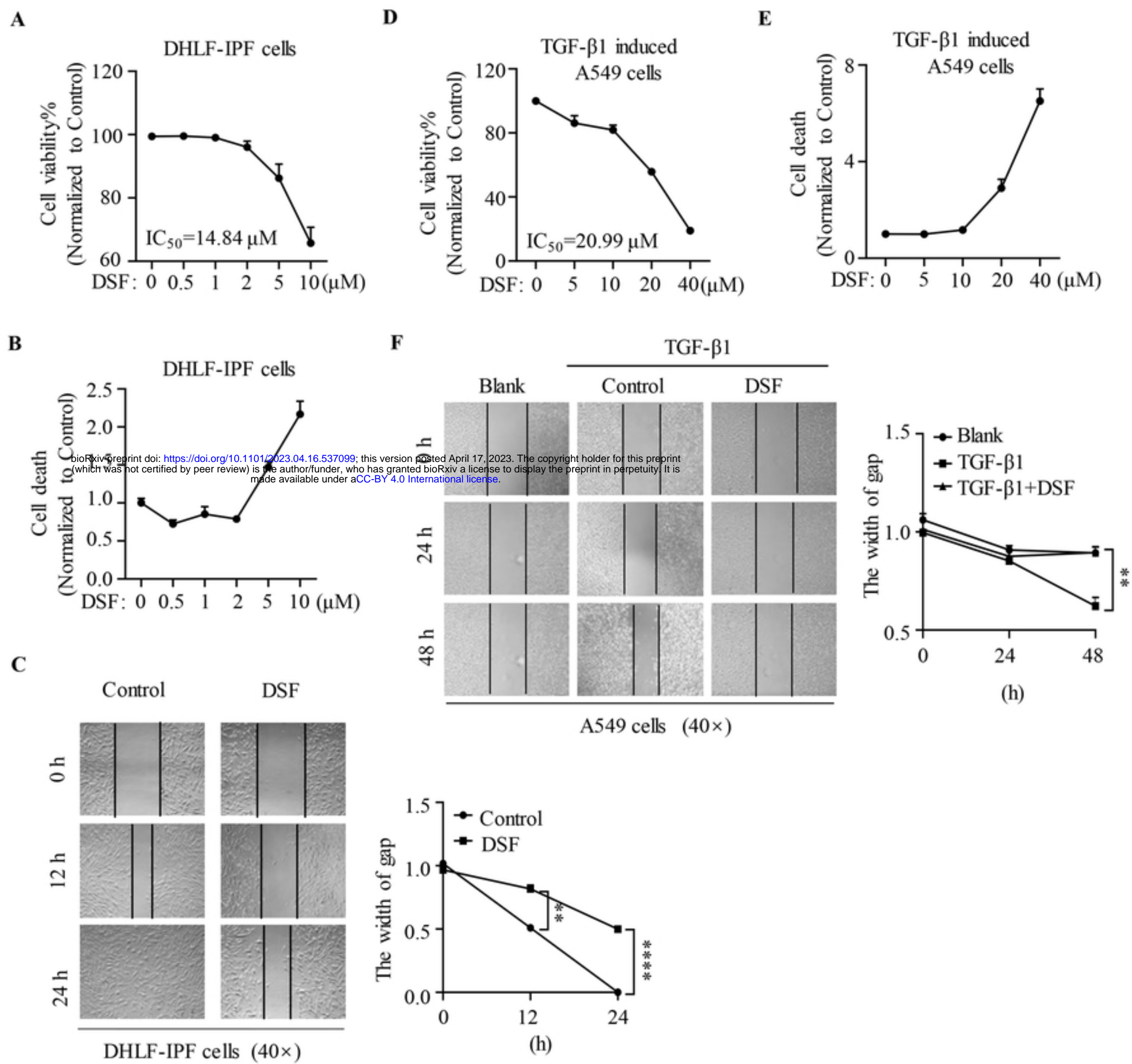
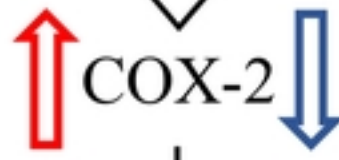
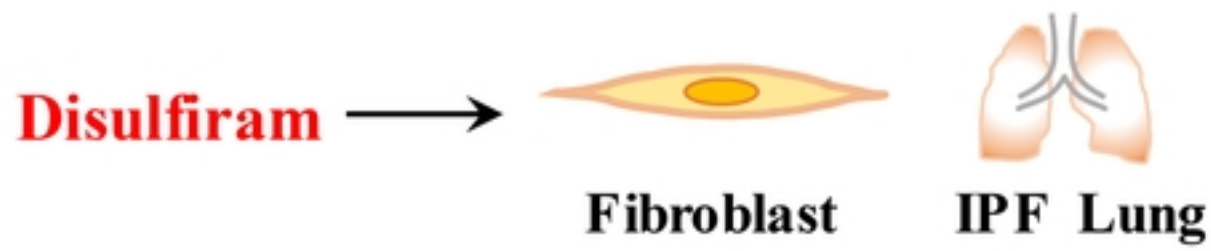
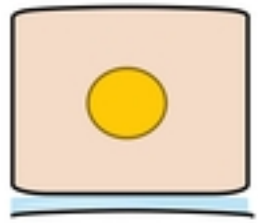


Figure 1

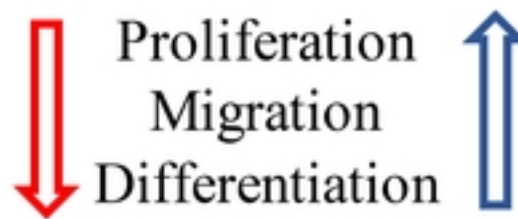
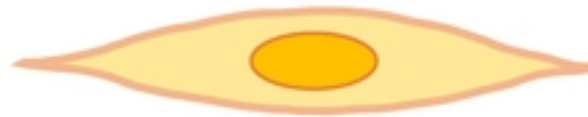


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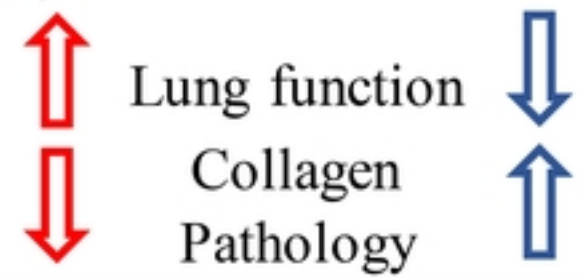
**Alveolar epithelial**



**Fibroblast**



**BLM-mice**



→ Represents alterations in IPF  
→ Illustrates the effect of Disulfiram

Figure 5



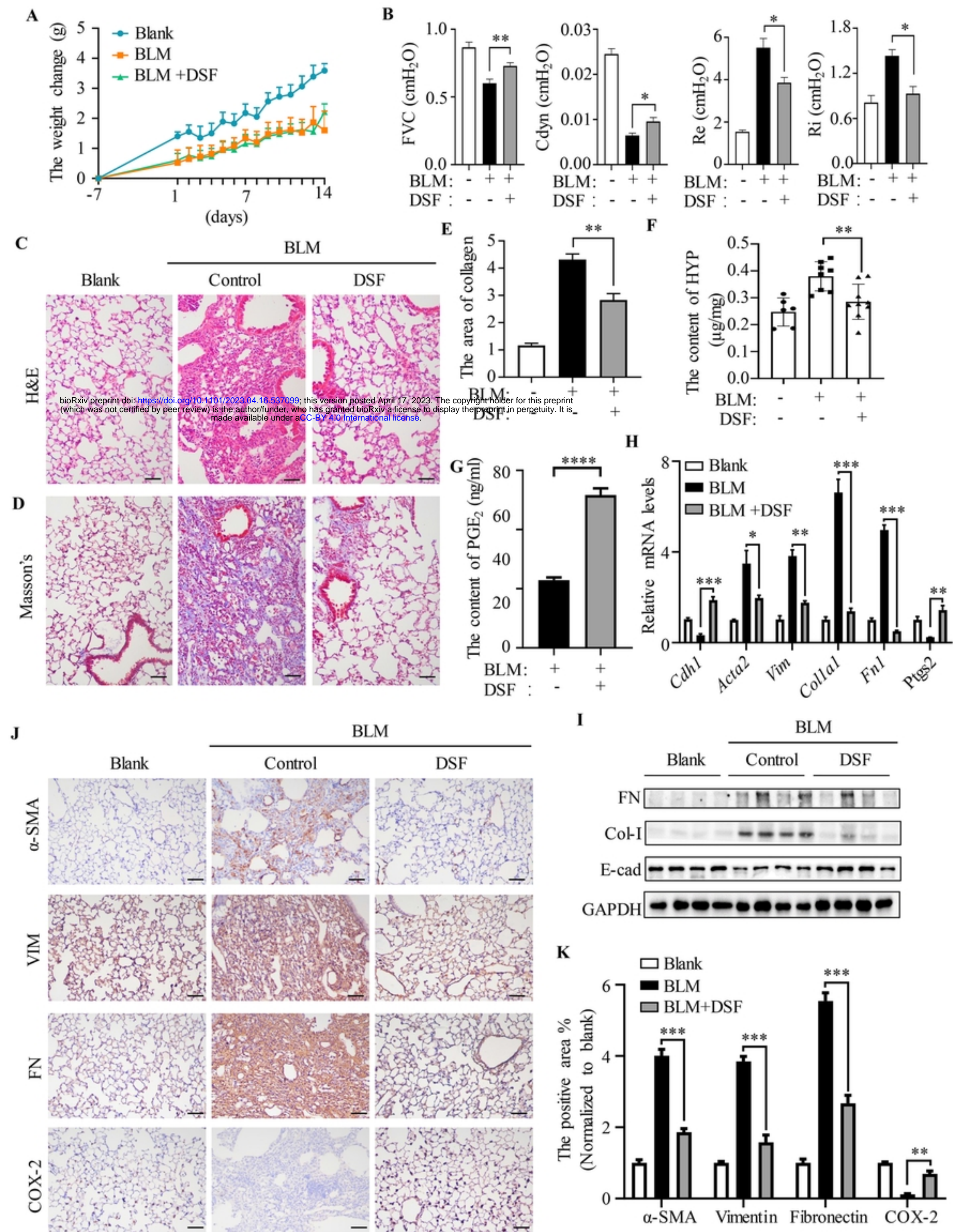


Figure 4