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Revised version**Anti-fibrotic effects of nintedanib on lung fibroblasts derived from patients with
Progressive Fibrosing Interstitial Lung Diseases (PF-ILDs).**

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26 **Running title:** Anti-fibrotic effects of nintedanib on PPF fibroblasts

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30 **Nonstandard abbreviations:** ILD: interstitial lung disease, PF-ILD: Progressive fibrosing-

31 ILD, IPF: idiopathic pulmonary fibrosis, HP: hypersensitivity pneumonitis, PPFE:

32 pleuroparenchymal fibroelastosis, PBS: phosphate-buffered saline, HLFs: human lung

33 fibroblasts, TGF- β 1: transforming growth factor- β 1, PDGF-BB: platelet-derived growth factor

34 BB, COL1: collagen I, α -SMA: α -smooth muscle actin, SEM: standard error of the mean,

35 ANOVA: one-way analysis of variance.

36

37 **Abstract**

38 The tyrosine kinase inhibitor nintedanib has been recently approved for the treatment of
39 Interstitial Lung Diseases (ILDs) that manifest a progressive fibrosis phenotype other than
40 Idiopathic pulmonary Fibrosis (IPF).

41 Nintedanib reduces the development of lung fibrosis in various animal models resembling
42 features of PF-ILD and *in vitro*, it inhibits the fibrosing phenotype of human lung fibroblasts
43 (HLFs) isolated from patients with IPF. To get insight on the cellular and molecular
44 mechanisms that drive the clinical efficiency of nintedanib in patients with non-IPF PF-ILD,
45 we investigated its effects on the fibrosing functions of HLFs derived from patients with PF-
46 hypersensitivity pneumonitis (PF-HP, n=7), PF-sarcoidosis (n=5) and pleuroparenchymal
47 fibroelastosis (PPFE, n=4).

48 HLFs were treated with nintedanib (10 nM-1 μ M) and then stimulated with PDGF-BB (25-
49 50 ng/ml) or TGF- β 1 (1 ng/ml) for 24 to 72 hours to assess proliferation and migration or
50 differentiation.

51 At nanomolar concentrations, nintedanib reduced the levels of PDGF receptor and ERK1/2
52 phosphorylation, the proliferation and the migration of PF-HP, PF-sarcoidosis and PPFE HLFs
53 stimulated with PDGF-BB. Moreover, nintedanib also attenuates the myofibroblastic
54 differentiation driven by TGF- β 1 but only when it is used at 1 μ M. The drug reduced the
55 phosphorylation of SMAD2/3 and decreased the induction of collagen, fibronectin and α -
56 smooth muscle actin expression induced by TGF- β 1.

57 In conclusion, our results demonstrate that nintedanib counteracts fundamental fibrosing
58 functions of lung fibroblasts derived from patients with PF-HP, PF-sarcoidosis and PPFE, at
59 concentrations previously reported to inhibit control and IPF HLFs. Such effects may contribute
60 to its clinical benefit in patients suffering from these irreversible ILDs.

61

62

63 **Highlights:**

64 Nintedanib blocks PDGF-BB-induced signalling of PF-ILD HLFs.

65 Nintedanib inhibits PDGF-BB-induced proliferation of PF-ILD HLFs

66 Nintedanib prevents PDGF-BB-induced migration of PF-ILD HLFs

67 Nintedanib diminishes TGF- β 1-induced differentiation of PF-ILD HLFs

68 **Keywords (6):** PPF, primary human lung fibroblasts, nintedanib, proliferation, migration,
69 differentiation.

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Journal Pre-proof

72 **1. Introduction**

73 Interstitial lung diseases (ILDs) are defined as a large and heterogeneous group of
74 parenchymal pulmonary disorders of various etiologies characterized by diffuse damages in the
75 lung parenchyma [1,2]. Idiopathic pulmonary fibrosis (IPF), the best described ILD, is a
76 progressive fibrosing ILD (PF-ILD) associated with a high rate of mortality. A proportion of
77 patients with non-IPF ILD can also develop a progressive fibrosing phenotype that leads to
78 decline in lung function, quality of life worsening and early mortality [3]. These ILDs notably
79 include idiopathic non-specific interstitial pneumonia (NSIP), unclassifiable idiopathic
80 interstitial pneumonia, connective-tissue disease ILDs, chronic sarcoidosis, fibrosing
81 hypersensitivity pneumonitis (HP), asbestosis, silicosis or pleuroparenchymal fibroelastosis
82 (PPFE). The estimated prevalence of non-IPF PF-ILD ranges from 6.9 (Europe) to 70.3 (United
83 States) per 100 000 persons and the estimated incidence from 2.1 (Europe) to 32.6 (United
84 States) per 100 000 persons per years [4]. It is estimated that around 30% of all ILDs will evolve
85 toward PF-ILD [5,6].

86 The development of pulmonary fibrosis in non-IPF ILD is probably the consequence of
87 chronic injuries to lung endothelial cells or epithelial alveolar cells associated with immune
88 stimulation or autoimmunity. The repeated damages to alveolar tissues lead to the migration
89 and proliferation of lung fibroblasts and their differentiation into myofibroblasts in fibroblastic
90 foci [2,7]. Pulmonary tissue then acquires a fibrosis phenotype, characterized by self-
91 progression and activation loops that contribute to the accumulation of extracellular matrix
92 (ECM) and end-stage lung remodeling.

93 Nintedanib is a tyrosine kinase inhibitor that has been used for several years in the treatment
94 of IPF. Recently, nintedanib has also been approved in EU, US and many other countries for
95 the treatment of patients that develop other PF-ILD [8,9]. The randomized, double-blind
96 placebo-controlled INBUILD[®] study, which assessed the efficacy and safety of nintedanib,

97 demonstrated that the drug slows the progression of several non-IPF PF-ILDs, established by
98 the annual rate of decline in forced vital capacity (FVC). Nintedanib likely reduces the
99 development of IPF by interfering with key functions of lung fibroblasts. Indeed, *in vitro*,
100 nintedanib inhibits the migration and proliferation of human lung fibroblasts (HLFs) isolated
101 from patients with IPF by competitively blocking the ATP-binding pocket in various tyrosine
102 kinase receptors including the platelet-derived growth factor receptor (PDGFR), the fibroblast
103 growth factor receptor and the vascular endothelial growth factor receptor [10]. However,
104 besides IPF, the effects of nintedanib on the functions of lung fibroblasts isolated from patients
105 suffering from other PF-ILD remain largely unknown.

106 In order to demonstrate the pharmacological activity of nintedanib on non-IPF HLFs, we
107 investigated in the present study the *in vitro* drug effects on the pro-fibrosing functions of HLFs
108 isolated from ILD patients with PF-HP, PF-sarcoidosis and PPFE.

109

110 **2. Materials and Methods**

111 **2.1 Primary cultures of HLFs**

112 PF-HP (median age 58 year; range 46.23-66.91 years) (4 females, 3 males), PF-sarcoidosis
113 (median age 57 year; range 36.57-68.05 years) (2 females, 3 males), PPFE (median age 52.85
114 year; range 29.5-72.53 years) (3 females, 1 male) and IPF (median age 66.94 year; range 56.72-
115 76.06 years) lung samples were isolated from patients undergoing open lung biopsy using the
116 explant method [11]. PF-ILD patients were required to meet at least one of the following criteria
117 for progression of their ILD within 24 months, a relative decline in the FVC of at least 10% of
118 the predicted value, a relative decline FVC of 5% to less than 10% of the predicted value and
119 worsening of respiratory symptoms or an increased extent of fibrosis on high-resolution CT, or
120 worsening of respiratory symptoms and an increased extent of fibrosis [2,3]. IPF (2 females, 7
121 males) were diagnosed according to the ATS/ERS/JRS/ALAT criteria, including
122 histopathological features of usual interstitial pneumonia [12,13]. Control samples (median age
123 64.14 year; range 59.09-71.29 year, males) were from lung cancer patients undergoing
124 lobectomy or pneumonectomy, away from the tumor. The absence of tumor tissue in control
125 samples was verified histologically. This study was approved by the local ethics committee
126 (Ethics Committee CHU Rennes, n° 16.123). Written informed consent was obtained from all
127 subjects. Cell morphology was checked by phase contrast microscopy. All experiments were
128 performed in accordance with relevant guidelines and regulations.

129 **2.2 Cell culture and treatments**

130 HLFs derived from patients with PF-HP (n=7), PF-sarcoidosis (n=5) and PPFE (n=4) were
131 cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, Life Technologies,
132 Courtaboeuf, France) supplemented with 10% fetal calf serum (FCS) (Eurobio scientific, Evry,
133 France), antibiotic-antimycotic solution (Gibco™, Life Technologies) and L-glutamine used at
134 passages 5 and 6. Nintedanib was provided by Boehringer Ingelheim Pharma GmbH (Biberach,

135 Germany). To analyze mRNA and protein expressions, HLFs were seeded for 24 h in 6-well
136 plates, and then starved in FCS-free DMEM for 16 h. To study cell growth, HLFs were serum-
137 starved for 16 h, cultured in DMEM containing 1 % FCS and 0.01-0.3 μ M nintedanib (NTD)
138 for 3 h, and finally stimulated with PDGF-BB (50 ng/ml) (R&D Systems, Bio-Techne, Lille,
139 France) for a further 72 h. To analyze the TGF- β 1-induced myofibroblastic differentiation,
140 serum-starved HLFs were pretreated with nintedanib (0.01-1 μ M) for 3 h and then stimulated
141 with TGF- β 1 (1 ng/ml; Preprotech, Neuilly-sur-Seine, France) for 24 h.

142 **2.3 Proliferation**

143 After nintedanib and PDGF-BB treatments, cells were dissociated with trypsin, centrifuged
144 (1500g, 5 min) and resuspended in DMEM containing 10 % FCS. Proliferation was assessed
145 with the acridine orange/propidium iodide staining (Logos Biosystems, Villeneuve d'Ascq,
146 France) and an automated fluorescence cell counter (Luna™, Logos Biosystems). The dye
147 causes viable nucleated cells to fluoresce green and nonviable nucleated cells to fluoresce red.
148 After cell counting, the relative cell proliferation was calculated by dividing the number of
149 viable cells exposed to nintedanib +/- PDGF-BB by the number of viable untreated cells
150 arbitrarily set at 1.

151 **2.4 Migration assay**

152 HLF migration was assessed in a modified Boyden chamber assay, using a ThinCert®
153 permeable support (6.5 mm, 8 μ m) (Greiner Bio-One, Kremsmünster, Austria) coated with
154 fibronectin (0.1 μ g/ml) (Sigma-Aldrich, St. Quentin Fallavier, France). The medium in the
155 upper compartment (DMEM-0%FCS) contained 0.01-0.3 μ M nintedanib and the medium in the
156 lower compartment contained (DMEM-1%FCS) 25 ng/ml PDGF-BB. Cells were incubated for
157 16 h at 37°C, fixed in 70 % ethanol, and labelled with DAPI. Migration was assessed by
158 counting the number of cells on the lower surface of the filters. The relative cell migration was

159 defined by dividing the number of migrating cells stimulated with PDGF-BB by the number of
160 migrating cells left untreated and arbitrarily set at 1.

161 **2.5 Protein analysis**

162 HLFs were lysed in ice-cold RIPA buffer containing cOmplete™ Protease Inhibitor Cocktail
163 (Roche, Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich). Proteins were
164 separated by electrophoresis on 10 % Tris-glycine SDS polyacrylamide gels and transferred to
165 nitrocellulose membranes (pore size: 0.2 µm, Invitrogen). Free binding sites were blocked by
166 incubation in 5 % milk or bovine serum albumin (BSA) (Sigma-Aldrich, St. Quentin Fallavier,
167 France) for 2 h at room temperature. Membranes were then incubated with the appropriate
168 primary human antibody (Ab) overnight at 4°C. The primary Abs were anti- α -smooth muscle
169 actin (α -SMA) (clone 1A4, Sigma-Aldrich), anti-collagen I (Southern Biotechnology
170 Associates, Birmingham, AL), anti-GAPDH (mab90009-P, Covalab, Bron, France). Anti-
171 phospho p44/42 MAPK (197G2), anti-p44/42 MAPK (9101), anti-phospho SMAD2 (3104),
172 anti-SMAD2 (D43B4), anti-phospho SMAD3 (C25A9), anti-SMAD3 (C67H9), anti-phospho
173 PDGF receptor (PDGFR) β (3161) and anti-PDGFR (28E1) Abs were from Cell Signaling
174 Technology (Massachusetts, USA). Digital images were acquired on a gel imaging system
175 (Chemi doc, BIO-RAD Laboratories, Marnes-La-Coquette, France) equipped with a CCD
176 camera. Western blots were quantified by densitometry using Image Lab™ software (BIO-
177 RAD) and normalized to GAPDH. The relative protein expression in HLFs was determined by
178 arbitrarily setting at 1 the protein expression measured in untreated cells.

179 **2.6 RNA isolation and RT-PCR assays**

180 Total RNAs were extracted using the mRNA extraction kit from Macherey Nagel (Hoerd,
181 France) according to the manufacturer's instructions. RNA concentrations were measured by
182 spectrofluorimetry using a NanoDrop 1000 (Thermo Fisher scientific, Saint-Herblain, France).

183 Then mRNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription
184 kit (Applied Biosystems, Thermo Fisher Scientific). Quantitative PCR (polymerase chain
185 reaction) was performed using the SYBR Green methodology on a CFX384 Real-Time PCR
186 System (Bio-Rad Laboratories) in duplicates. All the primers were provided by Sigma-Aldrich
187 (KiCqStart™ SYBR® Green Primers, St. Quentin Fallavier, France). The specificity of gene
188 amplification was checked at the end of PCR using the comparative cycle threshold method
189 (CFX Manager Software). The mean Cq values were used to normalize the target mRNA
190 concentrations to those of the 18S ribosomal protein by the $2^{-\Delta\Delta Cq}$ method. The mRNA levels
191 were expressed relatively to those measured in unstimulated HLFs, arbitrarily set at 1.

192 **2.7 Statistical analysis**

193 All results are presented as means \pm standard errors of mean (SEM) of the indicated numbers
194 of independent biological experiments performed with cell cultures deriving from different
195 patients. Statistical analyses were performed with Prism 8.0 (GraphPad Software, La Jolla, CA).
196 Significant differences were determined by ANOVA followed by the Dunnett's Multiple
197 comparison *t*-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

198

199 3. Results

200 3.1 Nintedanib blocked PDGF-BB-induced signaling of PF-HP, PF-sarcoidosis and 201 PPFE primary HLFs.

202 PDGF-BB modulates cell functions, notably in lung fibroblasts, by stimulating the PDGFR
203 which, in turn, controls the activation of the ERK1/2 signaling pathway [14]. Our results show
204 that PDGF-BB increased the level of phosphorylation of the PDGFR and ERK1/2 in PF-HP
205 (Fig. 1A-B), PF-sarcoidosis (Fig. 1C-D), PPFE (Fig. 1E-F), IPF and control (Supplementary
206 Fig. 1) HLFs. To explore the effects of nintedanib on the PDGFR signaling pathway in PF-ILD
207 HLFs, cells were first treated for 3 h with 10 nM-300 nM nintedanib and then stimulated for 10
208 min with PDGF-BB. The drug decreased in a concentration-dependent manner the PDGF-BB-
209 induced phosphorylation of PDGFR and ERK in PF-HP (Fig. 1 A-B) and PF-sarcoidosis (Fig.
210 1 C-D) HLFs, as well as in IPF and control HLFs (Supplementary Fig. 1 A-D). Its effects were
211 significant from 10-30 nM and maximal at 300 nM. In PPFE HLFs, nintedanid also reduced
212 PDGFR and ERK phosphorylation but this effect was only significant at 300 nM.

213 3.2 Nintedanib inhibited PDGF-BB-induced proliferation of PF-HP, PF-sarcoidosis 214 and PPFE HLFs.

215 Fibroblast proliferation is an important step of self-sustaining fibrosis that likely promotes
216 the formation of pulmonary fibroblastic foci [15] in the lungs of patients suffering from IPF or
217 non-IPF PF-ILD [2,7]. We thus explored the potential of nintedanib to block cell proliferation
218 induced by PDGF-BB, a potent mitogenic cytokine. HLFs were incubated for 3 h with
219 nintedanib and then stimulated with PDGF-BB for the next 72 h. As expected, PDGF-BB
220 increased the proliferation of PF-ILD HLFs (Fig. 2 A, B, C), IPF and control HLFs
221 (Supplemental Fig. 1 E, G). Nintedanib significantly and dose-dependently reduced the PGDF-
222 BB-induced proliferation of PF-HP, PF-sarcoidosis and PPFE HLFs. At 100 nM, nintedanib
223 totally inhibited the proliferation of all PF-ILD HLFs.

224 **3.3 Nintedanib prevented PDGF-BB-induced migration of PF-HP, PF-sarcoidosis and**
225 **PPFE HLFs.**

226 The migratory cell dynamic is another important function involved in the formation of
227 fibroblastic foci and self-sustaining fibrosis [15,16]. Besides proliferation, PDGF-BB also
228 promotes cell migration through its chemoattractant properties. Figure 3 shows that PDGF-BB
229 significantly increased the migration of the different PF-ILD HLFs. Nintedanib significantly
230 decreased, in a concentration-dependent manner, the migration of PF-HP, PF-sarcoidosis and
231 PPFE stimulated with PDGF-BB. Significant inhibition started at 30 nM and reduction close to
232 baseline was reached at 300 nM. Similarly, the drug strongly reduced the migration of IPF and
233 control HLFs cultured with PDGF-BB (Supplemental Fig. 1 F, H).

234 **3.4 Nintedanib diminished TGF- β 1-induced fibroblast to myofibroblast transition of**
235 **PF-HP, PF-sarcoidosis and PPFE HLFs.**

236 The canonical TGF- β signaling pathway, mediated by the signal transducers and
237 transcriptional modulators SMAD2 and SMAD3, drives the production of myogenic markers
238 linked to myofibroblast differentiation. This signaling pathway controls fibrogenesis by
239 regulating the production of ECM and the development of contractile actin stress fibers. In
240 order to study the effects of nintedanib on the fibroblast to myofibroblast transition, PF-HP, PF-
241 sarcoidosis and PPFE HLFs were treated with the drug for 3 h and then stimulated with TGF-
242 β 1 for the next 24 hours. Figure 4 shows that TGF- β 1 induced the phosphorylation of SMAD2
243 and SMAD3 proteins in all three PF-ILD HLFs (Fig. 4). The cytokine also strongly increased
244 the production of the myofibroblastic markers fibronectin, collagen-1 (COL1) and α -smooth-
245 muscle actin (α -SMA), at both mRNA (Fig. 5) and protein levels (Fig. 6). In these conditions,
246 nintedanib weakly reduced the phosphorylation of SMAD 2/3 in PF-ILD HLFs. Its effects were
247 only significant when nintedanib was used at its highest concentration (1 μ M) (Fig. 4).
248 Similarly, nintedanib poorly limited the expression of the different myofibroblastic markers, at

249 both mRNA and protein levels in non-IPF HLFs (Figures 5 and 6), as well as in IPF and control
250 HLFs (Supplementary Fig. 2). Nintedanib significantly reduced the *FIBRONECTIN*, *COL1A1*
251 and *ACTA2* mRNA levels in PF-HP and PPFE HLFs, mainly at its highest concentration (1
252 μM), and it only prevented the *FIBRONECTIN* gene expression in PF-sarcoidosis HLFs. In
253 addition, 1 μM nintedanib prevented the expression of fibronectin, COL1 and α -SMA in PF-
254 HP HLFs (Fig. 6). At this concentration, the drug also significantly reduced α -SMA protein
255 levels in PF-sarcoidosis and PPFE HLFs. However, nintedanib had no significant effect on
256 COL1 levels in these two cell types. As expected, high drug concentrations significantly
257 prevented protein expression of the three myofibroblastic markers in IPF and control HLFs
258 (Supplemental Fig. 2 C-D and G-H).

259

260 4. Discussion

261 Our study demonstrates for the first time that the tyrosine kinase inhibitor nintedanib, used
262 *in vitro* at pharmacologically relevant concentrations, reduced the proliferation, migration and
263 differentiation of primary HLFs derived from progressive fibrosing (PF)-HP, PF-sarcoidosis
264 and PPFE HLFs.

265 Several reports have previously shown that nintedanib could modulate different signalling
266 pathways and pro-fibrotic functions in HLFs isolated from patients with IPF [17–19] or with
267 systemic sclerosis-associated ILD (SSc-ILD) [20]. The present results show that nintedanib also
268 exerts potent *in vitro* antifibrotic effects on HLFs deriving from patients with three other ILDs
269 that could be associated with a progressive fibrosing phenotype, i.e fibrosing HP, sarcoidosis
270 and PPFE. As described in IPF HLFs, nintedanib strongly inhibited the PDGFR-dependent
271 signaling pathways in the three types of HLFs [19]. Our results suggest that low drug
272 concentrations (30-100 nM) significantly and similarly prevented the proliferation and
273 migration of PF-HP, PF-sarcoidosis and PPFE HLF stimulated with PDGF-BB. Moreover,

274 these concentrations are equivalent to those inhibiting the proliferation and migration of IPF-
275 HLFs [19] and SSc-ILD HLFs [20]. Consequently, nintedanib seems to be equally active to
276 block PDGF effects in IPF and non-IPF HLFs.

277 In addition, we demonstrated that nintedanib partially blocked the switch of fibroblasts to
278 myofibroblasts induced by TGF- β 1 in PF-HP, PF-sarcoidosis and PPFE HLFs. During this step,
279 HLFs gain the expression of contractile and ECM proteins. Specifically, the production of α -
280 SMA allows the contraction of myofibroblasts and contributes to the matrix stiffening [21].
281 Nintedanib significantly reduced the TGF- β 1-induced expression of α -SMA in the three PF-
282 ILD HLFs, but its effects were more potent in PF-HP HLF. In addition, we show that nintedanib
283 specifically reduced COL1 expression in PF-HP HLFs. The production and secretion of
284 collagen is another critical event conferring the stiffness of fibrotic lung tissues. In viable
285 precision-cut lung slices from bleomycin-treated rats and IPF patients, nintedanib modulates
286 type III collagen turnover [22]. In IPF HLFs, nintedanib also inhibited COL1 fibril formation
287 and altered the appearance of collagen fibril bundles [23]. As reported for IPF [17,18] and SSc-
288 ILD HLFs [20], our results clearly show that the concentrations of nintedanib required to block
289 the myofibroblast differentiation are higher than those preventing proliferation and migration
290 of HLFs. Indeed, COL1 and α -SMA expressions were only significantly reduced in HLFs
291 treated with 1 μ M nintedanib. The reasons why different ranges of concentrations of nintedanib
292 are necessary to prevent the proliferation and migration of HLFs on the one hand and the
293 differentiation of HLFs on the other hand remain still unclear since the half maximal inhibitory
294 concentrations of nintedanib for the PDGFR and TGF- β 1 receptor tyrosine kinases are very
295 similar [24,25].

296 It was recently discussed that the fibrotic process controlling the development of non-IPF
297 PF-ILD may be similar to those involved in the progression of IPF [2,7,26,27]. Different recent
298 reports are in agreement with this hypothesis. In fibrotic HP, a single-cell transcriptional

299 analysis had identified a subpopulation of fibroblasts with a high expression of *ACTA2* or
300 *COL1A1* [28]. In fibrotic lung tissues of patients with sarcoidosis, gene and protein expressions
301 in fibroblastic foci were quite similar to those detected in patients with IPF [29]. PPFE, a very
302 rare subtype of interstitial pneumonia [30] is also characterized by the development of an elastic
303 fibre-rich fibrosis in pulmonary parenchyma. The fact that nintedanib similarly inhibited major
304 fibroblastic functions in IPF and other PF-ILD HLFs also supports a common mechanism of
305 fibrogenesis in these heterogeneous fibrotic ILDs. Finally, our results suggest that nintedanib
306 may prevent the decline in lung function in most PF-ILDs, including PPFE, by inhibiting the
307 main functions of HLFs [31].

308 **5. Conclusion**

309 In conclusion, we found that nintedanib, used at pharmacologically relevant concentrations,
310 counteracts *in vitro* key fibrotic functions of lung fibroblasts isolated from patients with PF-
311 HP, PF-sarcoidosis and PPFE. These cellular and molecular effects are likely to contribute to
312 its clinical benefit in patients suffering from these irreversible ILDs.

313

314

315

316 **6. Authorship contributions**

317 *Conceptualization:* AJ, LV, SJ conceived and designed the study.

318 *Performed data analysis:* AJ, CM, TV performed the experiments. AJ analyzed and interpreted
319 the data.

320 *Resources:* FLG, DCC, CLN, BDLT, SR provided lung biopsy. SG and AL provide clinical
321 data. MJ and BC provided HLFs.

322 *Writing - review & editing:* AJ, LV, SJ and LW.

323 All authors read and approved the final manuscript.

324

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Journal Pre-proof

326 **7. References**

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455

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462 9. Competing interests

463 SJ has received fees, funding or reimbursement for national and international conferences,
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465 Astra Zeneca, Bellorophon Therapeutics, Biogen, BMS, Boehringer Ingelheim, Chiesi,
466 Fibrogen, Galecto Biotech, Genzyme, Gilead, GSK, LVL, Mundipharma, Novartis, Olam
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469 that they have no competing interests.

470

471

472 10. Figure Legends**473 Figure 1. Nintedanib blocked the PDGFR signalling pathway in PF-HP, PF-sarcoidosis**
474 and PPFE HLFs.

475 PF-HP (n=7), PF-sarcoidosis (n=5) and PPFE (n=4) HLFs were treated with 0.01-0.3 μ M
476 nintedanib for 3 h and then stimulated with 25 ng/ml PDGF-BB for 10 min. **A,C,E.**
477 Representative Western blots showing the phosphorylation of PDGF receptor and ERK. **B, D,**
478 **F.** Proteins were quantified by densitometry and then normalized to GAPDH. The fold change
479 in the levels of phosphorylated proteins found in stimulated cells was compared to the levels of
480 phosphorylated proteins measured in untreated cells, arbitrarily set at 1. Results are expressed
481 as means \pm SEM of 4-7 independent experiments. Significant differences were determined by
482 ANOVA followed by the Dunnett's multiple comparison *t*-test (* $p<0.05$; ** $p<0.01$; ***
483 $p<0.001$). NTD: nintedanib

484 Figure 2. Nintedanib blocked the proliferation of PF-HP, PF-sarcoidosis and PPFE HLFs
485 stimulated with PDGF-BB.

486 PF-HP (n=7) (**A**), PF- sarcoidosis (n=5) (**B**) and PPFE (n=4) (**C**) HLFs were treated with
487 0.01-0.3 μ M nintedanib for 3 h and then stimulated with 50 ng/ml PDGF-BB for 72 h. Cell
488 proliferation was determined by the fluorescent acridine orange/propidium iodide staining and
489 automated fluorescence cell counting. The relative cell proliferation was calculated by dividing
490 the number of viable cells treated with nintedanib +/- PDGF-BB by the number of viable
491 untreated cells, which was arbitrarily set at 1. All results are expressed as means \pm SEM of 4-7
492 independent experiments. Significant differences were determined by ANOVA followed by the
493 Dunnett's multiple comparison *t*-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). NTD: nintedanib

494

495 Figure 3. Nintedanib decreased the PDGF-BB-induced migration of PF HP, sarcoidosis
496 and PPFE HLFs.

497 PF-HP (n=7) (A), PF-sarcoidosis (n=5) (B) and PPFE (n=4) (C) HLFs were treated with 0.01-
498 0.3 μ M nintedanib for 24 h along with 25 ng/ml PDGF-BB, used as a chemoattractant to induce
499 cell migration. The relative cell migration was calculated by dividing the number of migrating
500 cells treated with nintedanib +/- PDGF-BB by the number of untreated migrating cells,
501 arbitrarily set at 1. Values are expressed as means \pm SEM of 4-7 independent experiments.
502 Significant differences were determined by ANOVA followed by the Dunnett's multiple
503 comparison *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

504 **Figure 4. Nintedanib attenuated the phosphorylation of SMAD2/3 induced by TGF- β 1 in**
505 **PF-HP, PF-sarcoidosis and PPFE HLFs.**

506 PF-HP (n=7), PF-sarcoidosis (n=5) and PPFE (n=4) HLFs were treated with 0.01-1 μ M
507 nintedanib for 3 h and then stimulated with 1 ng/ml TGF- β 1 for 30 min. **A, C, E.** Representative
508 Western blots showing the phosphorylation of SMAD2 and SMAD3. **B, D, F.** Proteins were
509 quantified by densitometry and then normalized to the GAPDH. The fold change in the levels
510 of phosphorylated proteins found in stimulated cells was determined by arbitrarily setting at 1
511 the levels of phosphorylated proteins measured in untreated cells. Results are expressed as
512 means \pm SEM of 4-7 independent experiments. Significant differences were determined by
513 ANOVA followed by Dunnett's multiple comparison *t*-test (* $p < 0.05$, ** $p < 0.01$, * $p < 0.001$).
514 NTD: nintedanib

515 **Figure 5. Nintedanib decreased the mRNA levels of myofibroblastic markers in PF-HP,**
516 **PF-sarcoidosis and PPFE HLFs stimulated with TGF- β 1.**

517 PF-HP (n=7), PF-sarcoidosis (n=5) and PPFE (n=4) HLFs were treated with 0.01-1 μ M
518 nintedanib for 3 h and then stimulated with 1 ng/ml TGF- β 1 for 24 h. **A, B, C.** Relative
519 *FIBRONECTIN*, *COL1A1* and *ACTA2* mRNA levels were measured by quantitative RT-PCR
520 and normalized to endogenous ribosomal *18S* mRNA levels. Data are expressed relatively to
521 the mRNA levels founds in unstimulated HLFs, arbitrarily set at 1. Results are expressed as

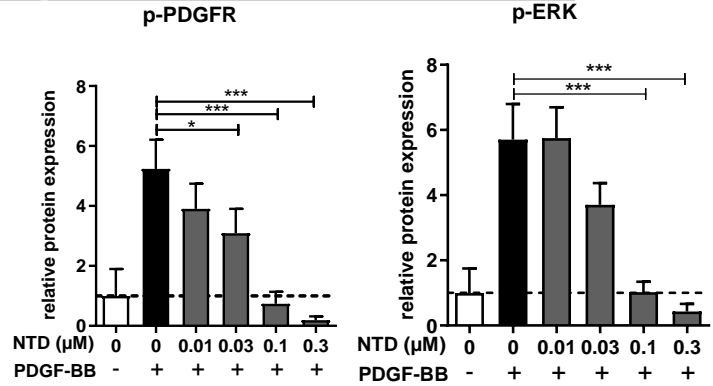
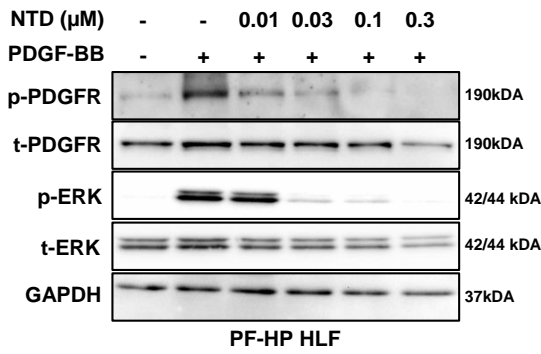
522 means \pm SEM of 4-7 independent experiments. Significant differences were determined by
523 ANOVA followed by Dunnett's Multiple comparison *t*-test (* $p<0.05$, ** $p<0.01$, ***
524 $p<0.001$). NTD: nintedanib

525 **Figure 6. Nintedanib decreased the TGF- β 1-induced differentiation of PF-HP, PF-**
526 **sarcoidosis and PPFE HLFs.**

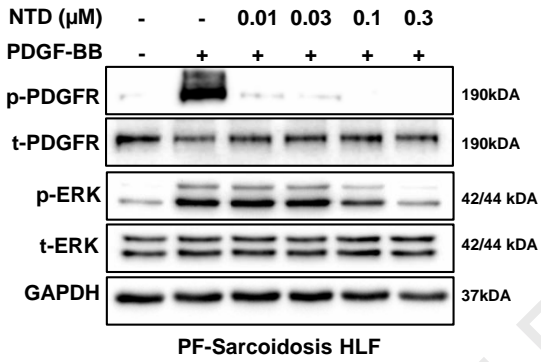
527 PF-HP (n=7), PF-sarcoidosis (n=6) and PPFE (n=4) HLFs were treated with 0.01-1 μ M NTD
528 for 3 h and then stimulated with 1 ng/ml TGF- β 1 for 24 h. **A, C, E.** Representative Western
529 blots of Fibronectin (FN), collagen-1 (COL1) and α -SMA. **B, D, F.** Protein levels were
530 quantified by densitometry and normalized to the GAPDH levels. The fold change in protein
531 expression in stimulated cells was determined by arbitrarily setting at 1 the protein expression
532 measured in untreated cells. Results are expressed as means \pm SEM of 4-7 independent
533 experiments. Significant differences were determined by ANOVA followed by the Dunnett's
534 multiple comparison *t*-test (* $p<0.05$; ** $p<0.01$). NTD: nintedanib

Figure 1

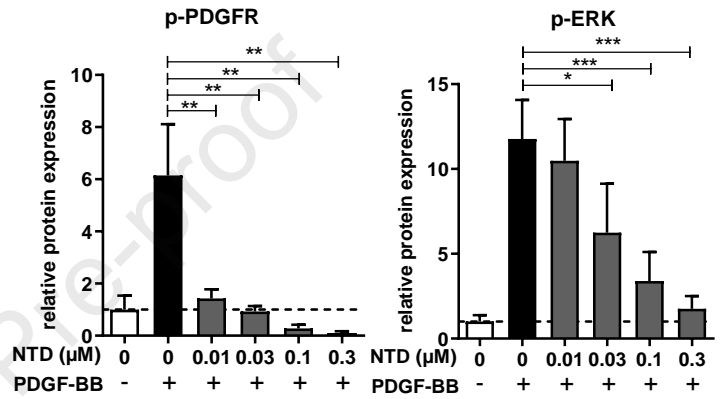
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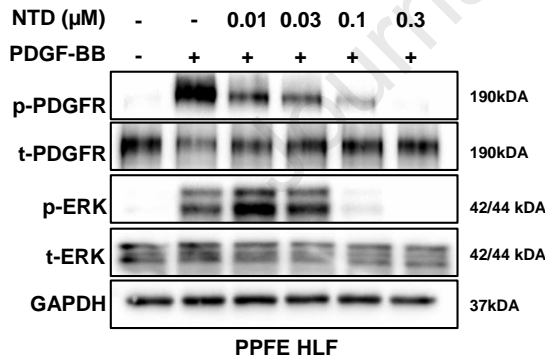
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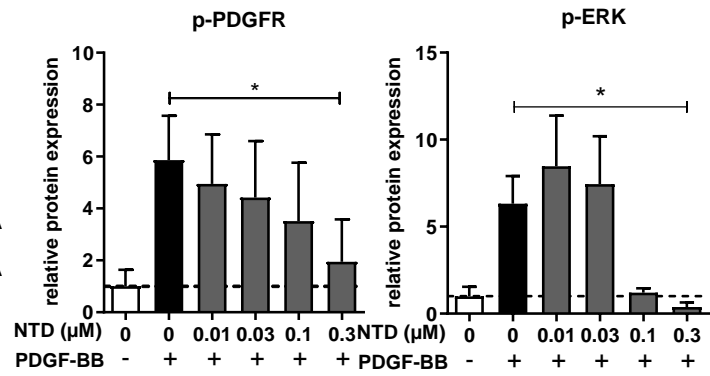
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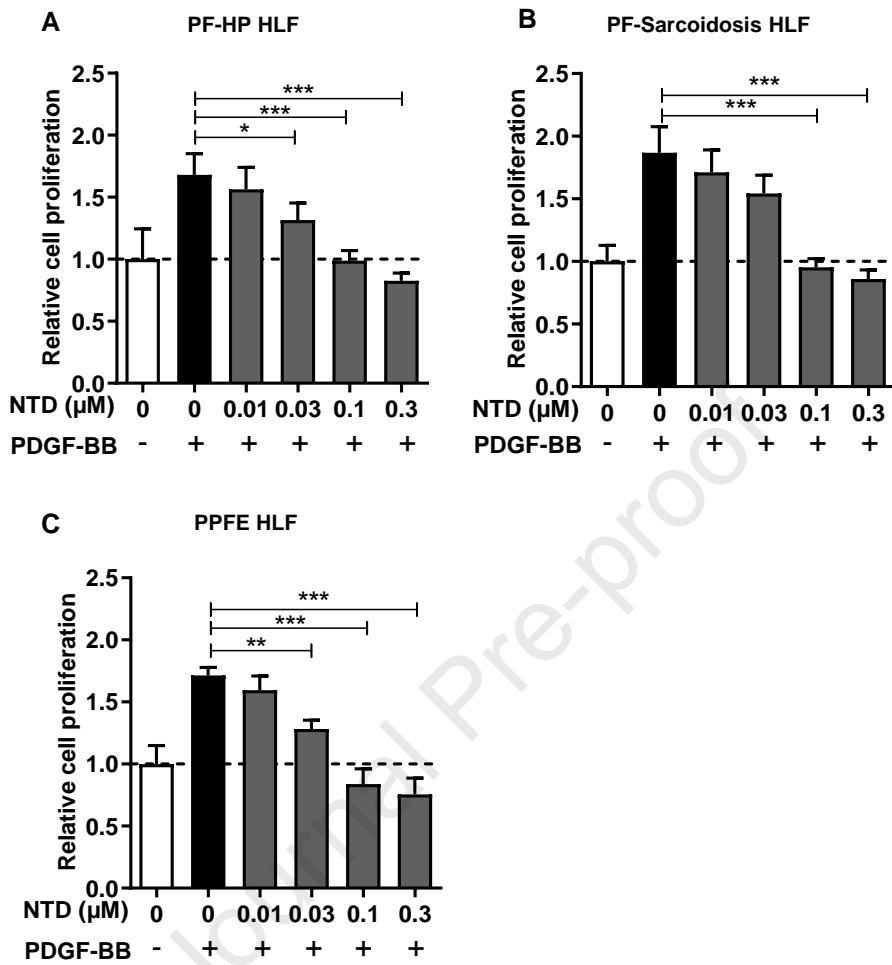


E



F





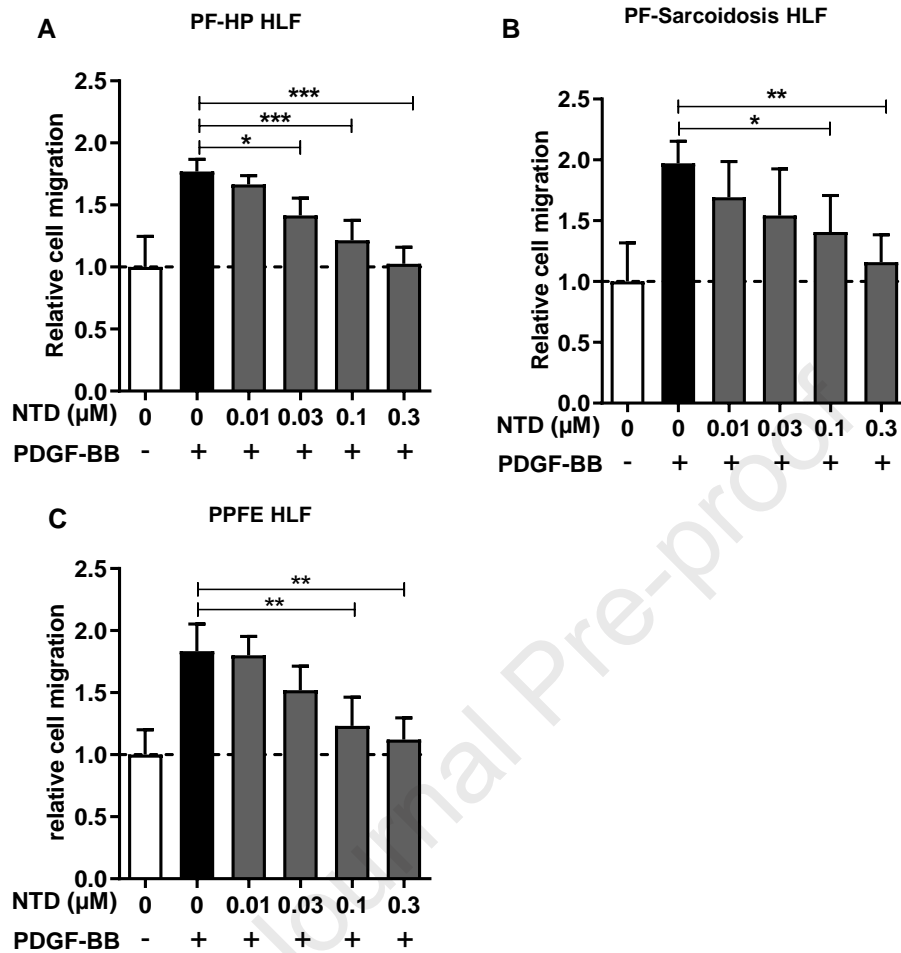


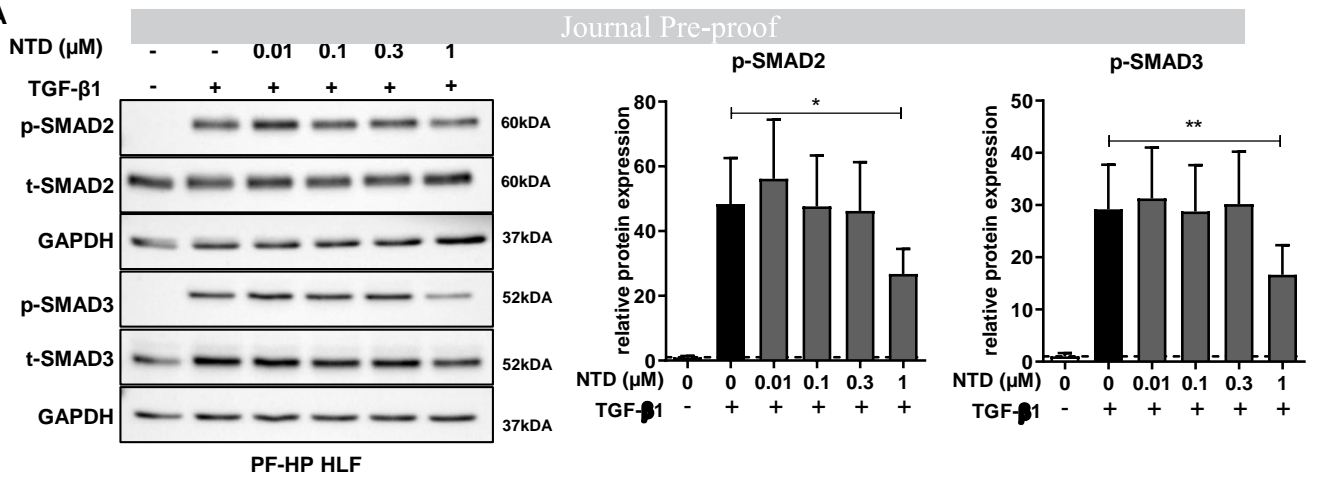
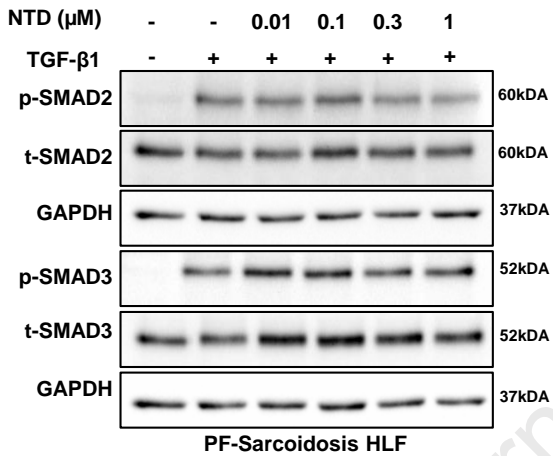
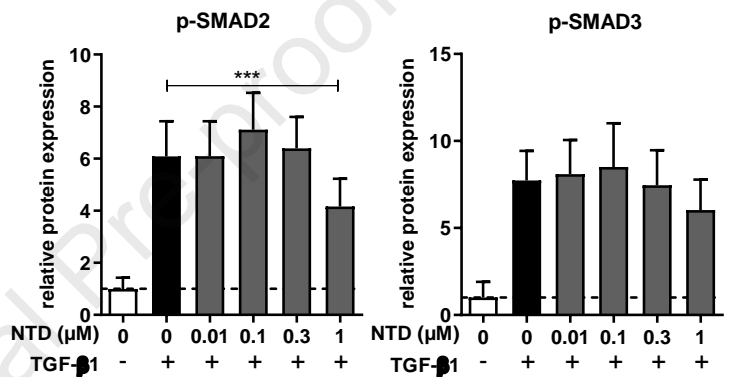
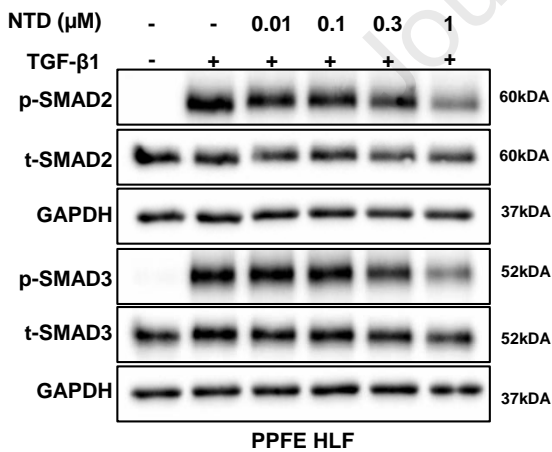
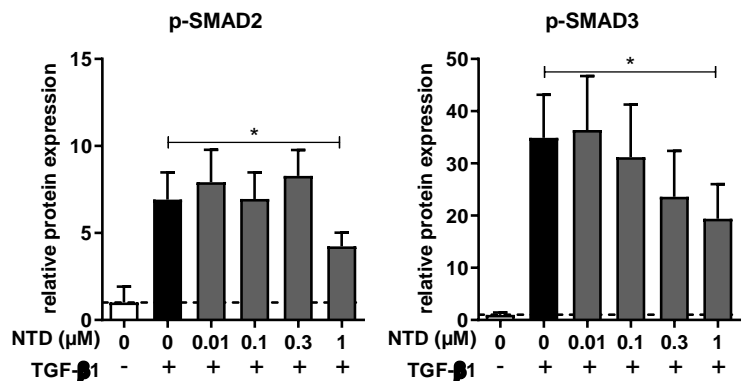
Figure 4**A****C****D****E****F**

Figure 5

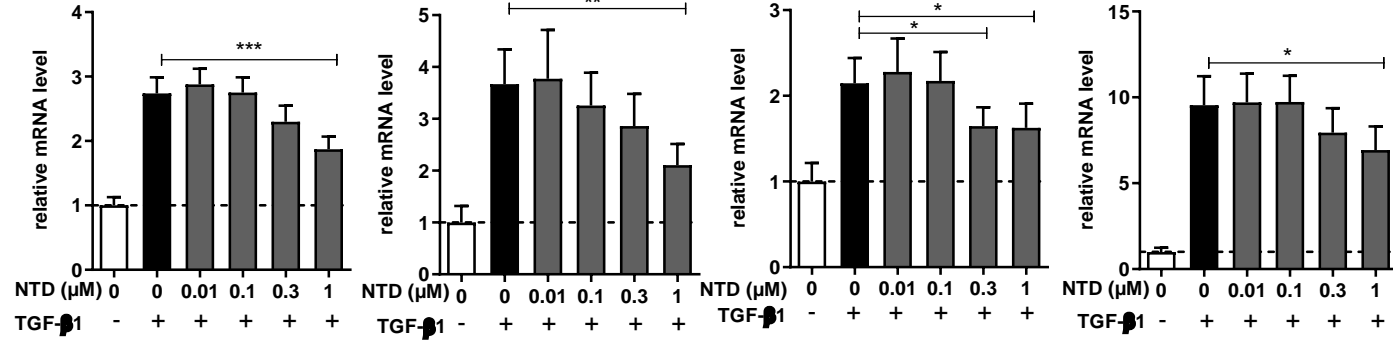
FIBRONECTIN

COL1A1

COL1A2

ACTA2

A



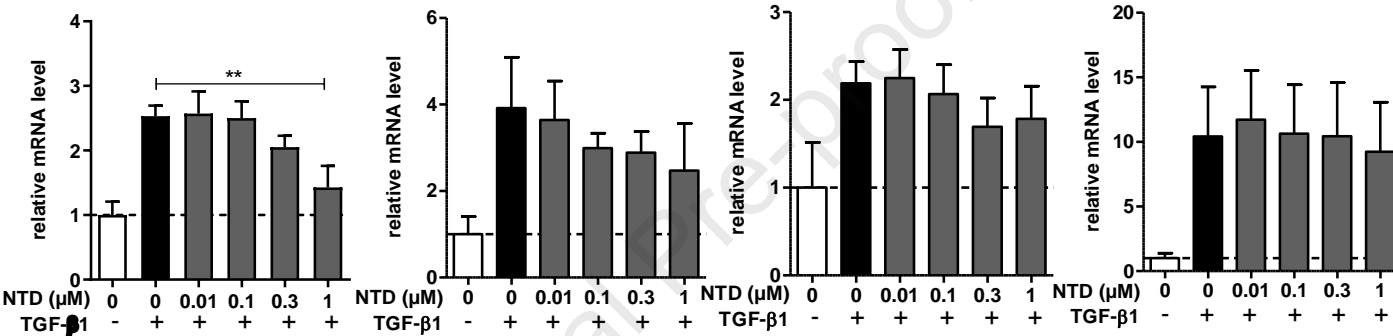
B

FIBRONECTIN

COL1A1

COL1A2

ACTA2



C

FIBRONECTIN

COL1A1

COL1A2

ACTA2

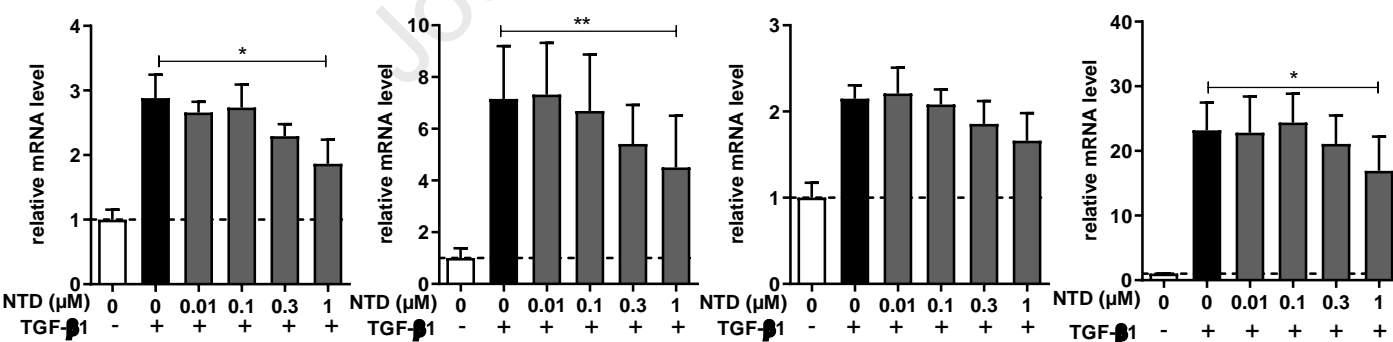
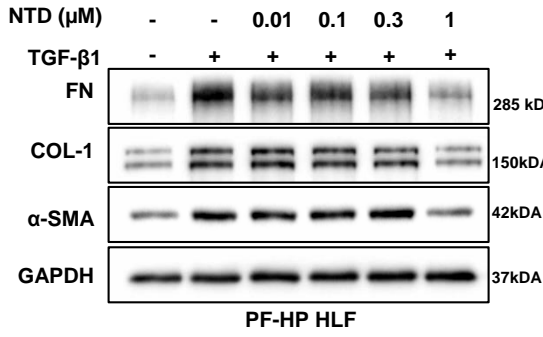
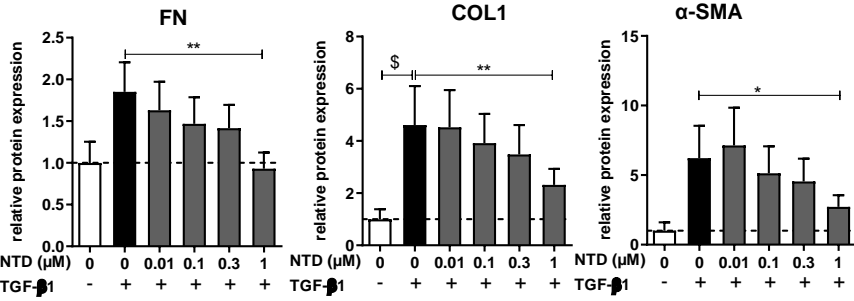


Figure 6

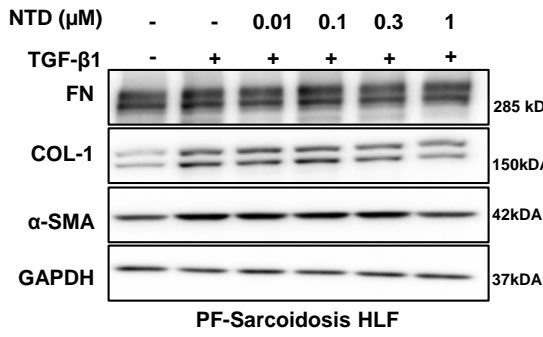
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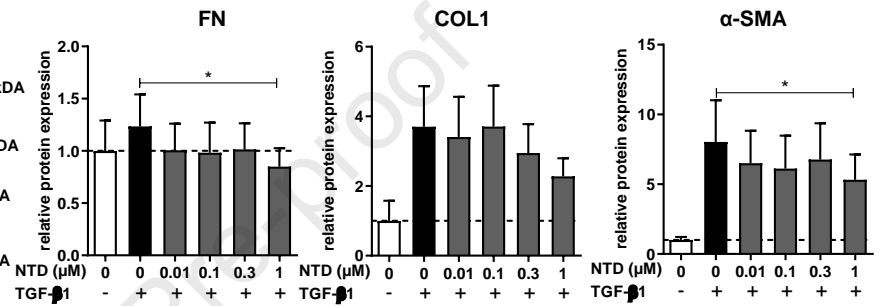
B



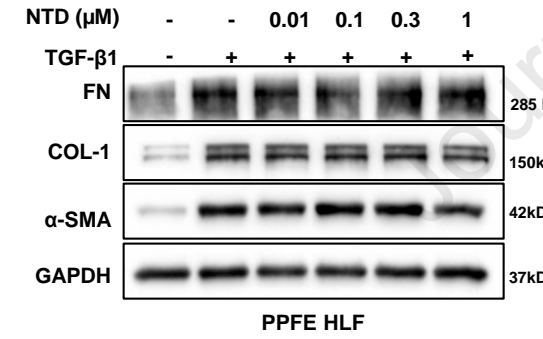
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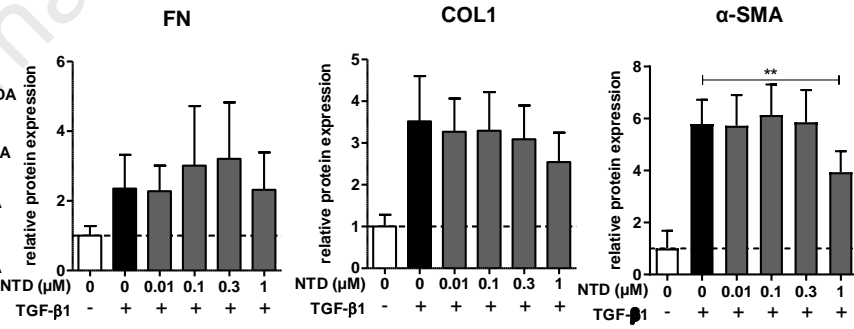
D



E



F



Authors statement

Conceptualization: AJ, LV, SJ conceived and designed the study.

Performed data analysis: AJ, CM, TV performed the experiments. AJ analyzed and interpreted the data.

Resources: FLG, DCC, CLN, BDLT, SR provided lung biopsy. SG and AL provide clinical data. MJ and BC provided HLFs.

Writing - review & editing: AJ, LV, SJ and LW.

All authors read and approved the final manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Journal Pre-proof