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Precision-cut lung slices from bleomycin treated animals as a model for testing potential therapies for idiopathic pulmonary fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a complex lung disease with incompletely understood pathophysiology. Effectiveness of available medicines is limited and the need for new and improved therapies remains. Due to complexity of the disease, it is difficult to develop predictable *in vitro* models. In this study we have described precision-cut lung slices (PCLS) prepared from bleomycin treated mice as an *in vitro* model for testing of novel compounds with antifibrotic activity. We have shown that PCLS during *in vitro* incubation retain characteristics of bleomycin model with increased expression of fibrosis related genes *ACTA2* (α-smooth muscle actin), *COL1A1* (collagen 1), *FN1* (fibronectin 1), *MMP12* (matrix metalloproteinase 12) and *TIMP1* (tissue inhibitor of metalloproteinases). To further evaluate PCLS as an *in vitro* model, we have tested ALK5 inhibitor SB525334 which was previously shown to attenuate fibrosis in *in vivo* bleomycin model and nintedanib which is the FDA approved treatment for IPF. SB525334 and nintedanib inhibited expression of fibrosis related genes in PCLS from bleomycin treated mice. In addition, comparable activity profile of SB525334 was achieved in PCLS and *in vivo* model. Altogether these results suggest that PCLS may be a suitable *in vitro* model for compound testing during drug development process.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive lung disease with median survival of 3–5 years upon diagnosis [1]. It is characterized by excessive tissue scarring and loss of function, eventually leading to organ failure. Lung transplantation is often the only treatment option for end-stage disease patients [2]. The exact mechanism of the disease onset is still not fully understood [3–7], however some risk factors such as smoking, environmental and occupational hazards as well as recurrent viral infections have been described. The process of injury repair is deregulated in IPF leading to overproduction of connective tissue and therefore scarring. There are currently only two approved medicines for the treatment of IPF, nintedanib and pirfenidone, which slow down disease progression [8]. The need for new treatments still remains and a lot of effort is put into this research [9,10].

Several *in vitro* and *in vivo* models are well described and used in IPF research and drug development. However, given the complexity of the disease none of the models encompasses all the pathological processes present in lungs of IPF patients [11]. One of the most used animal models in IPF research is bleomycin model. This model is characterized by acute lung injury with increased inflammatory response and epithelial apoptosis during the first 7 days after bleomycin administration. Fibrosis occurs after approximately 14 days, persists for 3–4 weeks and is followed by resolution phase [12]. Although it is argued over the extent to which bleomycin model reflects human disease and its predictability in drug development, many of the fibrotic changes in lungs of bleomycin-treated mice are also observed in lungs of IPF patients [13].

Precision-cut lung slices (PCLS) are an *in vitro* model growingly used to study lung physiology and pathogenesis [14–18]. Since various cell types of respiratory tract are retained in relevant tissue architecture with preserved extracellular matrix (ECM), PCLS represent phenotypically relevant testing system. For the same reason, PCLS have an advantage over cell culture models that are often used in drug development. Possibility of using PCLS for *in vitro* testing of new therapies for IPF has started to be investigated recently [19–22]. Use of PCLS as a model for IPF could complement limitations of other models, such as

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cell culture, and bring an advantage in development of effective therapies for IPF treatment.

The aim of this study was to characterise PCLS derived from bleomycin treated mice as a potential *in vitro* model for IPF.

2. Material and methods

2.1. Bleomycin-induced pulmonary fibrosis in mice

12 week old C57BL/6 male mice (Charles River, Italy) were used in the studies. Mice were kept in type III polysulfonate cage with ALPHA-dri dust free bedding and cotton nestles and maintained under standard laboratory conditions (temperature 22 ± 2 °C, relative humidity 60 ± 5%, 15 air changes per hour, artificial lighting with circadian cycle of 12 h). Pelleted food and water were provided *ad libitum*. All studies in animals were approved by the ethics committee of Fidelta and were carried out in accordance to 2010/63/EU and National legislation regulating the use of laboratory animals in scientific research and for other purposes (Official Gazette 55/13).

Lung fibrosis was induced by intranasal administration of 30 μg of bleomycin (Enzo Life Sciences). Control group received saline. ALK5 inhibitor SB525334 (BioVision) was administered orally at dose 30 mg/kg 1 h prior to bleomycin challenge and twice daily for the remaining 14 days. 14 days after the bleomycin/saline administration animals were euthanized and lungs were excised. The whole superior right lung was gently removed and placed into Precellys Lysing Kit – Hard Tissue Homogenizing CK28 Tube (Bertin Technologies) and immediately frozen in liquid nitrogen until further RNA isolation. The remaining lungs were stored at room temperature in 10% buffered formalin (Shandon) until further histopathological evaluation.

2.2. Histopathological evaluation of lungs

Lungs were embedded in paraffin and stained according to Crossman's Trichrome for Muscle and Collagen [23].

2.3. RNA isolation from frozen lung tissue

750 μL of Trizol reagent (Thermo Fisher Scientific) was added to the Precellys CK28 Tube containing whole superior right lung lobe and homogenized using Precellys Instrument (Bertin Technologies). Phase separation with chloroform (Kemika) was performed and RNA was further isolated from aqueous phase using NucleoSpin 96 RNA Kit (Macherey-Nagel) following manufacturer's instructions.

2.4. Precision-cut lung slices (PCLS)

Bleomycin/saline treated animals were anesthetized 14 days after bleomycin administration and thorax was gently opened. Plastic feeding tube was inserted into trachea and lungs were filled with 1.5% agarose type I, low EED (Sigma). Lungs were then excised and incubated in Advanced DMEM/F12 tissue medium (Thermo Fisher Scientific) for 30 min on ice to allow agarose polymerisation. Agarose filled lungs were first cut into cylindrical shapes followed by cutting into 250 μm thick slices by use of Krumdieck tissue slicer (Alabama Research & Development). After cutting, PCLS were stored in 500 μL of RNA Later Solution (Ambion) at −20 °C and labelled as day 0 samples. The rest of the obtained PCLS were placed in 700 μL of tissue medium in 24-well plates, treated with SB525334 (BioVision) or nintedanib (Santa Cruz Biotechnology) *in vitro* and incubated for 3 or 5 days at 37 °C, 5% CO_2 and 95% humidity. Compounds were tested at five consecutive five-fold dilutions starting from 5 μM (SB525334) or 10 μM (nintedanib). At the end of incubation supernatants were collected and stored at −20 °C, while PCLS were stored in RNA Later Solution at −20 °C until RNA isolation.

2.5. RNA isolation from PCLS

RNA was isolated from PCLS using protocol adapted from Ogura et al. [24]. PCLS were homogenized using Precellys Lysing Kit – Hard Tissue Homogenizing CK28 Tube (Bertin Technologies) in 750 μL of Trizol reagent (Thermo Fisher Scientific) following manufacturer's instructions. After RNA precipitation with isopropanol and washing with 75% ethanol, RNA pellet was dissolved in NTC buffer (Macherey-Nagel) to reduce the agarose contamination in PCLS samples. The RNA samples were further purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) following manufacturer's instructions.

2.6. PCLS viability

Viability of PCLS was assessed by measuring activity of lactate dehydrogenase (LDH) released from damaged cells into supernatants. LDH Activity Colorimetric Assay Kit (BioVision) was used according to manufacturer's instructions.

2.7. Gene expression analysis

To determine expression of *ACTA2* (α-smooth muscle actin), *COL1A1* (collagen 1), *FN1* (fibronectin 1), *MMP12* (matrix metalloproteinase 12), *TIMP1* (tissue inhibitor of metalloproteinases), *TGFβ1* (transforming growth factor β 1), *TNFα* (tumor necrosis factor α), *IL1β* (interleukin 1β) and *IL6* (interleukin 6), isolated RNA was reverse transcribed into cDNA using Superscript III Reverse Transcriptase (Thermo Fisher Scientific). Analysis of gene expression was performed using specific primers and TaqMan probes (Microsynth) and Brilliant II Qpcr Master Mix (Agilent). Sequences of used primers and probes are shown in Table 1. Gene expression analyses were performed using AriaMX Realtime PCR System (Agilent). Expression of test genes was normalized to *GAPDH* as a housekeeping gene. Relative gene expression

Table 1
Sequences of primers and probes used for real-time PCR analysis of gene expression. F stands for forward primer, R for reverse primer and P for TaqMan probe.

Test gene	Sequences
GAPDH	F: TGTGTCCGTCGTGGATCTGA
	R: CCTGCTTCACCACCTTCTTGA
	P: CCGCCTGGAGAAACCTGCCAAGTATG
ACTA2	F: CTGACGGGCAGGTGATCAC
	R: AATGAAAGATGGCTGGAAGAGAGT
	P: AAACGAACGCTTCCGCTGCCC
COL1A1	F: CCCGCCGATGTCGCTAT
	R: GCTACGCTGTTCTTGCAGTGAT
	P: CTTCCTGCGCCTAATGTCCACCGA
FN1	F: ACAGAGAAGTGACACGCTTCGA
	R: TCCGGTCACCGTGTTGCT
	P: CAGCGCCAGCACCCCTGTGAC
TIMP1	F: GCCCTTCGCATGGACATTTA
	R: CCCCGATCTGCGATGATG
	P: TCTCCACTGTGCAGCCCCTGCC
MMP12	F: GATGTGAGGCAGGAGCTCATG
	R: GGCTTGATTCCTGGGAAGTG
	P: CCCTGCTTACCCCAAGCTGATTTCCA
TGFβ1	F: AAACGGAAGCGCATCGAA
	R: GGGACTGGCGAGCCTTAGTT
	P: CCATCCGTGGCCAGATCCTGTCC
TNFα	F: GGCTGCCCCGACTACGT
	R: GACTTTCTCCTGGTATGAGATAGCAA
	P: CCTCACCCACACCGTCAGCCG
IL1β	F: AATCTATACCTGTCCTGTGTAATGAAAGAC
	R: TGGGTATTGCTTGGGATCCA
	P: CACACCCACCCTGCAGCTGGAGA
IL6	F: ACAAGTCGGAGGCTTAATTACACAT
	R: AATCAGAATTGCCATTGCACAA
	P: TCTTTTCTCATTTCCACGATTTCCCAGAGAA

was calculated using 2^(-ΔCt) equation.

2.8. Statistical analysis

Unpaired *t*-test was used for defining statistical difference in gene expression between PCLS prepared from saline treated and bleomycin treated mice, while one-way ANOVA test followed by Dunnett's multiple comparison post-test were used for defining statistical difference between SB525334 treated or nintedanib treated and non-treated mice and PCLS. All analyses were performed using GraphPad Prism 5.04 software for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The level of significance was set at $p < 0.05$.

3. Results

3.1. Expression of fibrosis-related genes is increased in lungs of bleomycin treated mice and reduced after SB525334 treatment

In order to confirm that fibrosis has developed *in vivo* after bleomycin administration, histological changes of the tissue were estimated. Fibrotic thickening of alveolar walls with damage of pulmonary structure, involving coarse fibrous bands or small fibrous masses were observed in lungs of bleomycin treated animals after 14 days, while saline treated mice had no signs of fibrosis. Increased deposition of collagen was observed along the alveolar walls, within alveolar spaces, around bronchi and vessels in lungs of mice after 14 days of bleomycin administration (Fig. 1). These results confirmed presence of lung fibrosis 14 days after bleomycin administration.

Next, expression of fibrosis-related genes involved in connective tissue remodelling was determined. 14 days after intranasal administration of 30 μg of bleomycin *ACTA2* (α-smooth muscle actin), *COL1A1* (collagen 1), *FN1* (fibronectin 1), *TIMP1* (tissue inhibitor of metalloproteinases) and *MMP12* (matrix metalloproteinase 12) expression was increased in lung tissue (Fig. 2). *MMP12* expression was increased 19-fold in lungs of bleomycin treated mice in comparison to saline treated mice. *FN1* and *TIMP1* expression were increased 11-fold and *COL1A1* expression 7-fold. *ACTA2* expression was increased 1.7 fold, while there was no change in expression of *TGFβ1* between bleomycin and saline treated mice.

SB525334 treatment (30 mg/kg p. o., b. i.d.) reduced expression of most of the analysed fibrosis-related genes (*ACTA2, COL1A1, FN1* and *TIMP1*) (Fig. 2), similarly to previously published data [25,26]. On the other hand *MMP12* expression, was further increased 2.6-fold upon SB525334 treatment (Fig. 2). *TGFβ1* expression was the same in bleomycin and saline treated mice and SB525334 treatment had no impact on its expression (Fig. 2).

3.2. Expression of fibrosis-related genes is increased in PCLS samples prepared from lungs of mice 14 days after bleomycin treatment

Crossman trichrome staining of PCLS prepared from bleomycin treated mice exhibited increased collagen production in fibrotic foci in comparison to PCLS prepared from saline treated mice, confirming that *in vivo* developed fibrosis is present in PCLS upon preparation (data not shown). Expression of *COL1A1* (collagen 1), *FN1* (fibronectin 1), *TIMP1* (tissue inhibitor of metalloproteinases) and *MMP12* (matrix metalloproteinase 12) was increased in PCLS prepared from lungs of mice 14 days after bleomycin treatment in comparison to saline treated mice (Fig. 3). *FN1* had the highest increase in expression of 33-fold over PCLS from saline treated mice. *COL1A1* and MMP12 expression was increased 17- and 10-fold respectively, while *TIMP1* was only slightly increased. *ACTA2* (α-smooth muscle actin) expression in PCLS prepared from bleomycin treated mice did not reach statistically significant increase in comparison to PCLS prepared from saline treated mice, however a trend in increase with 1.7 fold was observed. On the other hand, *TGFβ1* (transforming growth factor β 1) expression did not differ between PCLS prepared from bleomycin and saline treated mice (Fig. 3).

3.3. Expression of fibrosis-related genes remains increased in PCLS samples after 3 days of in vitro incubation

PCLS prepared from mice 14 days after bleomycin treatment were further incubated *in vitro* for 3 and 5 days. Expression of *COL1A1* (collagen 1), *FN1* (fibronectin 1), *TIMP1* (tissue inhibitor of metalloproteinases) and *MMP12* (matrix metalloproteinase 12) remained increased in PCLS prepared from bleomycin treated mice in comparison to saline treated mice after 3 days of *in vitro* incubation (Fig. 4). Additionally, *ACTA2* (α-smooth muscle actin) and *TGFβ1* (transforming growth factor β 1) expression were also increased (Fig. 4). *COL1A1* was the most increased gene with 32-fold increase, followed by 16-fold increase of *MMP12* expression. *TGFβ1* expression was increased 7-fold, while both *FN1* and *TIMP1* expression was increased 5-fold. The lowest increase in expression had *ACTA2* of 2-fold.

Similar results were obtained after 5 days of *in vitro* incubation where expression of *ACTA2, COL1A1, TIMP1, MMP12* and *TGFβ1* was increased in PCLS prepared from bleomycin treated mice (Fig. 4). Among the measured genes only *FN1 expression* did not remain increased after 5 days of incubation (Fig. 4). *COL1A1* expression remained 32-fold increased, while *MMP12* expression was 9-fold increased. *TGFβ1* expression was increased 3-fold, *TIMP1* 6-fold and *ACTA2* 4-fold in PCLS prepared from bleomycin treated mice.

Relative gene expression of all the genes tested was decreased after 5 days of *in vitro* incubation in comparison to 3 days of incubation (Fig. 4).

3.4. Expression of inflammation-related genes in PCLS prepared from lungs of mice 14 days after bleomycin treatment

Inflammation-related genes *TNFα* (tumor necrosis factor α), *IL1β* (interleukin 1β) and *IL6* (interleukin 6) were analysed and there was no difference in *TNFα* and *IL1β* expression, while *IL6* expression was

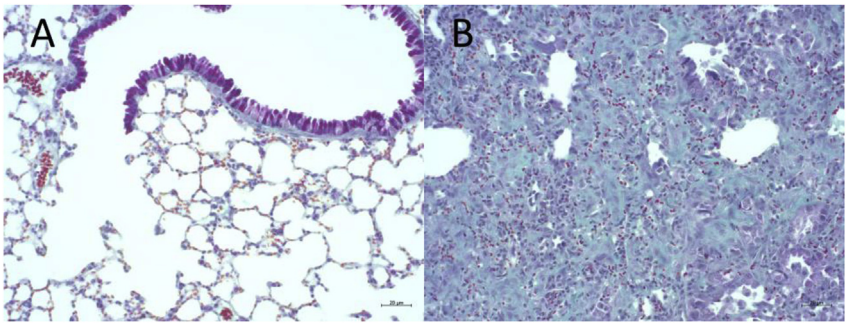

Fig. 1. Crossman trichrome staining of lungs from saline treated (A) and bleomycin treated mice (B) 14 days after administration. Magnification X200.

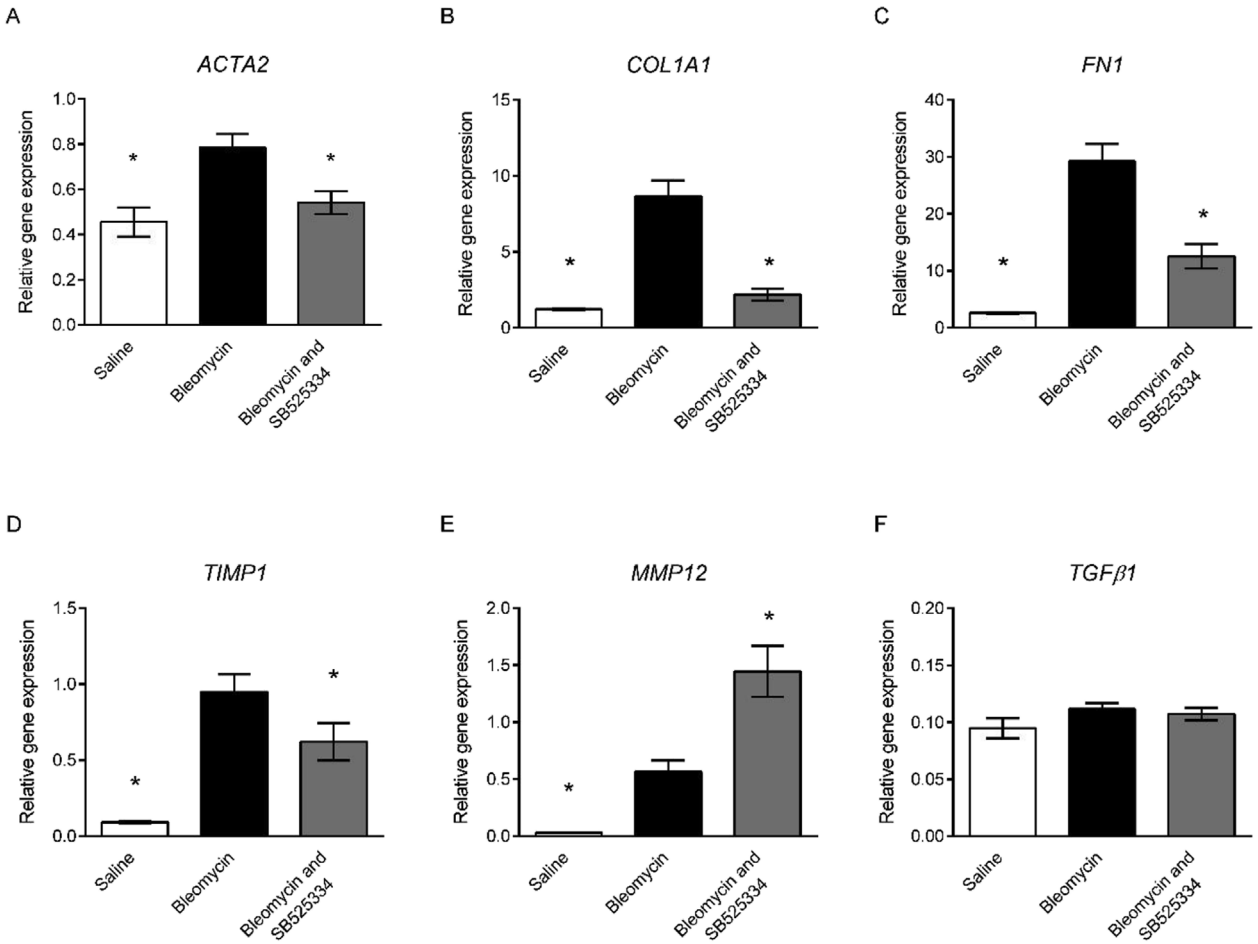

Fig. 2. *ACTA2* (A), *COL1A1* (B), *FN1* (C), *TIMP1* (D), *MMP12* (E) *and TGFβ1* (F) expression in lung tissue 14 days after bleomycin treatment. SB525334 was administered orally at a dose of 30 mg/kg b. i.d. for 14 days. Data are presented as means ± S.E.M. (N = 15). *p < 0.05 vs. bleomycin, one-way ANOVA with Dunnett's post test.

increased in PCLS prepared from bleomycin treated mice in comparison to saline treated mice (Fig. 5).

3.5. SB525334 and nintedanib reduced expression of fibrosis-related genes in PCLS model

To further validate PCLS as an *in vitro* model we incubated PCLS prepared from lungs 14 days after bleomycin administration with ALK5 (activin receptor-like kinase 5) inhibitor SB5253345 or nintedanib. LDH release from the tissue was used as a measure of tissue viability and SB525334 treatment did not change viability of PCLS samples during 3 days of incubation, while 10 μM nintedanib significantly increased LDH release (data not shown). SB525334 concentration dependently inhibited expression of *ACTA2* (α-smooth muscle actin), *COL1A1* (collagen 1), *FN1* (fibronectin 1) and *TIMP1* (tissue inhibitor of metalloproteinases) genes, while inhibition of *MMP12* (matrix metalloproteinase 12) and *TGFβ1* (transforming growth factor β 1) was not observed (Fig. 6). Nintedanib concentration dependently inhibited expression of *COL1A1*, *FN1* and *MMP12*, while no inhibition was observed for *TIMP1* and *TGFβ1* expression (Fig. 7). 10 μM nintedanib inhibited *ACTA2* expression and increased *TGFβ1* expression, however, tissue viability was affected at this concentration, as observed by LDH release.

4. Discussion

Complex and incompletely understood pathophysiology of IPF is difficult to replicate in models for research and drug development [27]. To achieve better predictability during drug development process, further improvement of currently used models as well as development of new models is important. The aim of this study was to develop PCLS prepared from bleomycin treated mice as an *in vitro* model for testing compounds for IPF treatment. PCLS have an advantage over monolayer cell culture because they consist of various cell types with conserved intercellular and cell-connective tissue interactions. So far, PCLS have mostly been used for studying toxicity, immune response and airway contractility [18,28], with a recent study describing PI3K/mTOR inhibitor testing in IPF patients derived PCLS [22].

In this study we aimed to characterise fibrotic changes that occur in PCLS prepared from bleomycin treated mice and to further validate this model by *in vitro* testing of ALK5 inhibitor SB525334, as well as one of the currently used therapies for IPF, nintedanib. Since remodelling and excessive production of connective tissue is one of the key features of IPF, we analysed expression of genes known to be involved in these processes. Connective tissue is composed of fibrous proteins and glycosaminoglycans with collagen being the most abundant component. In IPF patients, activated fibroblasts and myofibroblasts are responsible for excessive production and aberrant deposition of collagen in the lungs, as well as other components such as fibronectin, proteoglycans and tenascin [29]. This leads to formation of scar tissue, followed by decline in lung function of the patients. Aberrant collagen turnover can be used as a biomarker for identification of IPF, as well as for monitoring disease progression [30]. Although *in vivo* bleomycin model has its limitations and does not fully replicate IPF disease in humans, some of the key processes are present in this model. Collagen and fibronectin

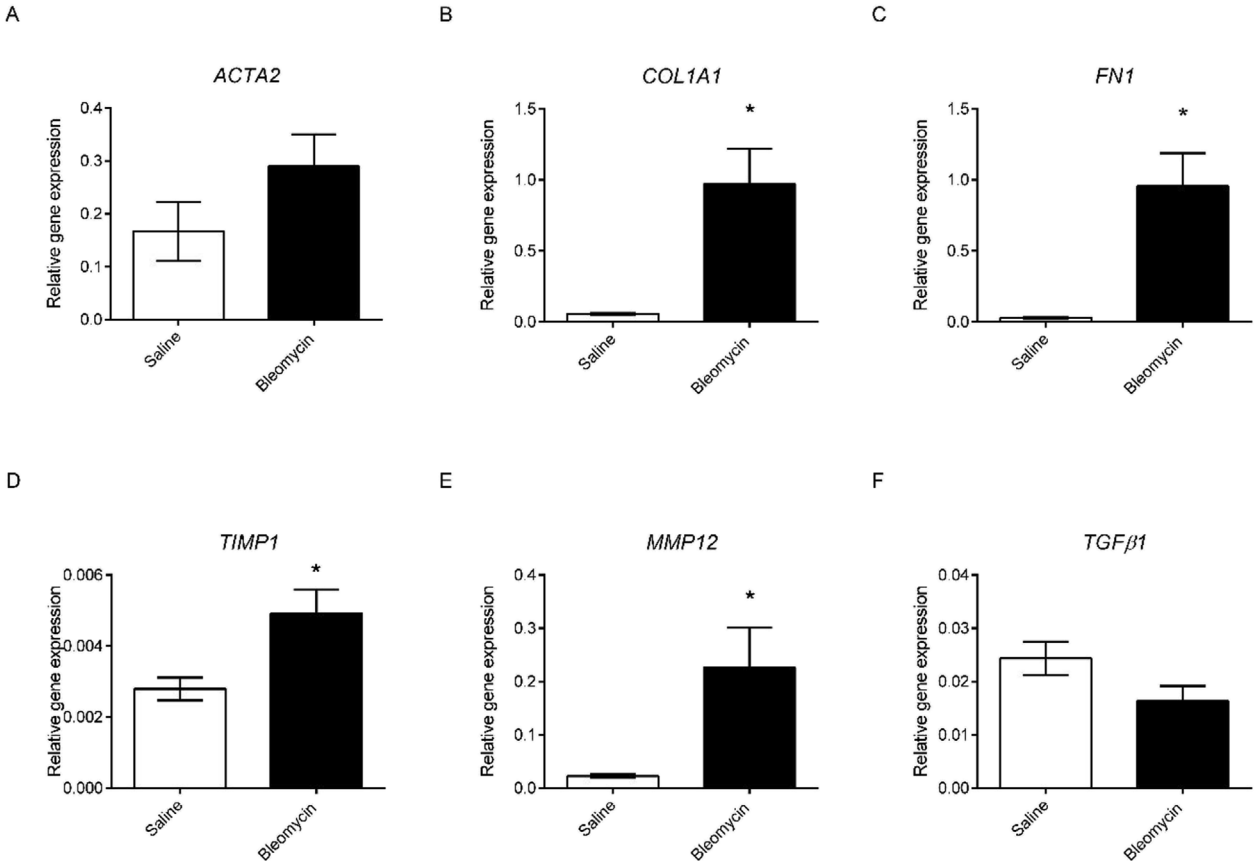

Fig. 3. *ACTA2* (A), *COL1A1* (B), *FN1* (C), *TIMP1* (D), *MMP12* (E) *and TGFβ1* (F) expression in PCLS prepared from lungs of mice 14 days after bleomycin treatment. Data are presented as means ± S.E.M. (N = 6). *p < 0.05 vs. saline, unpaired *t*-test.

are increasingly expressed both in IPF and in bleomycin treated mice [13]. We observed the same increase in PCLS prepared from bleomycin treated mice. *COL1A1* (collagen 1) expression in PCLS was one of the most increased among analysed fibrosis-related genes, which correlates with recently published results of increased collagen 1 turnover in PCLS prepared from *in vivo* bleomycin model [21]. This increased expression remained during 3 and 5 days of *in vitro* incubation, making possible to test compounds on expression of this gene. Similar results were obtained for *FN1* (fibronectin 1) expression. Altogether these results indicate that excessive production of connective tissue is present in PCLS.

In addition to these main components of connective tissue, we also analysed matrix metalloproteinase (MMP) and tissue inhibitor of matrix metalloproteinase (TIMP), important regulators of connective tissue homeostasis. MMP12 is mainly produced by macrophages [31] and is responsible for degradation of collagen IV, the component of basal membrane [32]. Increased expression of MMP12 leads to destruction of basal membrane and subsequent disruption of lung architecture in IPF patients. *MMP12* gene expression was increased in our *in vivo* bleomycin model. This increase was also observed in PCLS prepared from bleomycin treated mice and continued to be increased during *in vitro* incubation of PCLS. The same results were obtained for *TIMP1* expression.

α-smooth muscle actin (α-SMA) is a cytoplasmic protein expressed in myofibroblasts present only in tissue undergoing repair. Myofibroblasts are the key drivers of fibrotic changes with one of the roles being production of connective tissue components. α-SMA expression is one of the hallmarks of IPF disease [29] and its gene expression was analysed in this study, together with TGFβ (transforming growth factor β1), another key pro-fibrotic molecule. *TGFβ1* expression was not increased in PCLS prepared from bleomycin treated mice at day 0 while a trend in increase of *ACTA2* (α-smooth muscle actin) expression was observed. This was in correlation with the results from *in vivo* bleomycin model where no change in *TGFβ1* expression between bleomycin and saline treated animals was observed and only small (1.7-fold) increase in *ACTA2* expression was observed. Since both proteins are important factors in fibrosis initiation it is possible that expression of these genes is increased in the earlier days upon bleomycin administration [33]. In addition, TGF-β1 protein can also be activated from its latent form anchored in extra-cellular matrix, therefore reducing the need for *de novo* synthesis which may explain lack of change on mRNA level. It would be of interest to further determine if the increased expression of fibrosis-related genes that were measured in PCLS model in this study is followed by an increase in protein level as well, as it is known for *in vivo* bleomycin model.

Since we have shown that expression profile of fibrosis-related genes correlates between *in vivo* bleomycin model and PCLS derived from it, we further examined expression of the same genes in PCLS during 3 and 5 days of *in vitro* incubation. All measured fibrosis-related genes (*ACTA2, COL1A1, FN1, TIMP1, MMP12* and *TGFβ1*) were increased in PCLS prepared from bleomycin treated mice after 3 days of *in vitro* incubation and closely resemble to the expression profile observed immediately after preparation of PCLS. The only difference was observed in *TGFβ1* expression which was not increased in PCLS prepared from bleomycin treated mice at the day 0, but was increased in PCLS from mice during both 3 and 5 days of incubation. Fibrosis-related gene expression in PCLS prepared from bleomycin treated mice after 5 days of *in vitro* incubation were lower than in those after 3 days of incubation suggesting decrease of fibrotic changes, possibly because of lack of stimulus in *in vitro* conditions. Altogether, incubation time of 3 days was chosen for testing the compounds since expression profile of measured genes was similar to the one in PCLS prior to *in vitro* incubation while it provides enough time for the compounds to exhibit

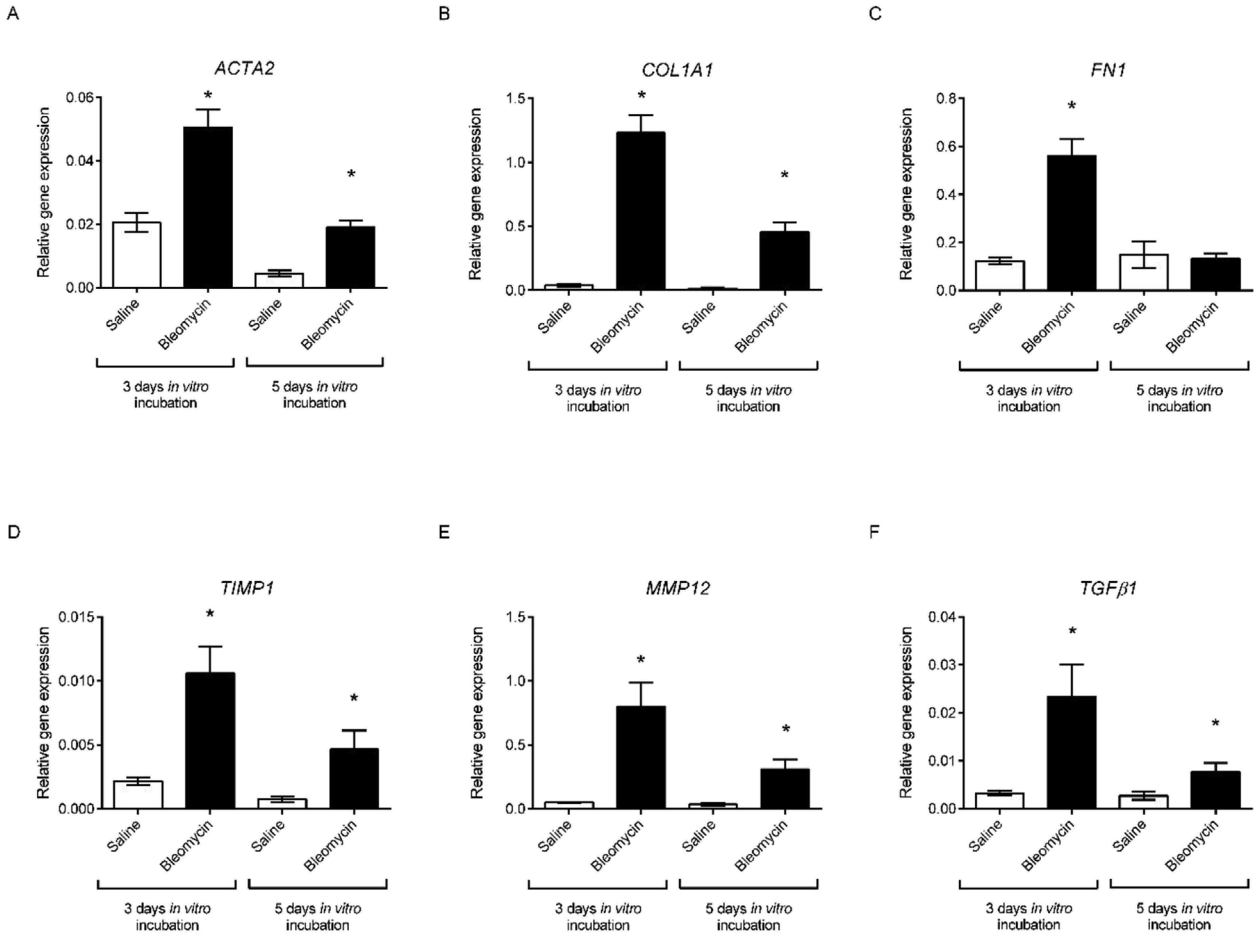

Fig. 4. *ACTA2* (A), *COL1A1* (B), *FN1* (C), *TIMP1* (D), *MMP12* (E) and *TGFβ1* (F) expression in PCLS prepared from lungs of mice 14 days after bleomycin treatment and after 3 and 5 days of *in vitro* incubation. Data are presented as means ± S.E.M. (N = 9 for saline and N = 12 for bleomycin). *p < 0.05 vs. saline at the corresponding time point, unpaired *t*-test.

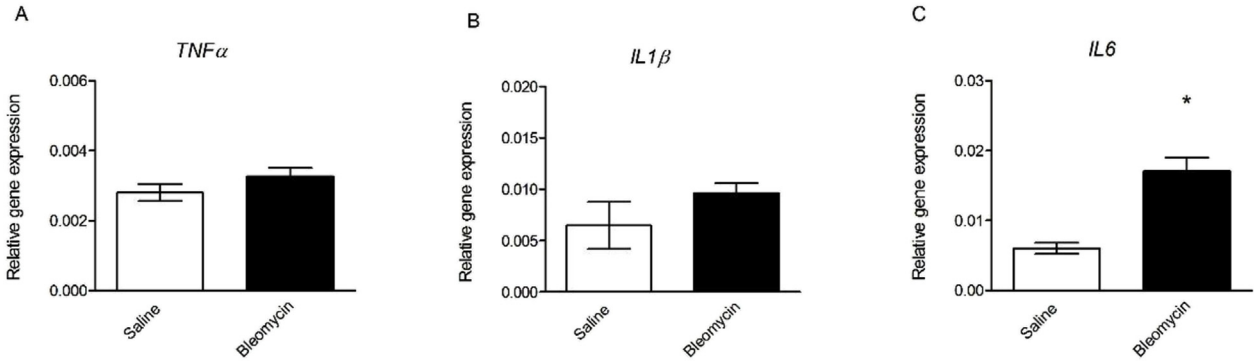

Fig. 5. *TNFα* (A), *IL1β* (B) and *IL6* (C) expression in PCLS prepared from lungs of mice 14 days after bleomycin treatment and after 3 days of *in vitro* incubation. Data are presented as means ± S.E.M. from two separate experiments (N = 11 for saline and N = 22 for bleomycin). *p < 0.05 vs. saline, unpaired *t*-test.

their activity.

Administration of bleomycin to rodents induces severe damage of the lung tissue with consequent inflammation and development of fibrotic foci. The inflammatory phase of bleomycin induced fibrosis usually peaks several days after bleomycin administration, followed by increased fibrotic phase at 14 days after administration [34,35], confirmed by both gene expression and histology (Fig. 1). More extensive histology analysis of *in vivo* bleomycin model and compounds treatment is in press preparation. We analysed gene expression of *TNFα*, *IL1β* and *IL6* as markers of ongoing inflammatory processes in our PCLS model. *IL1β* and *TNFα* were not increased in PCLS from bleomycin treated mice in comparison to PCLS from saline treated mice, indicating that the inflammatory phase in this model is not present or it has declined. This suggests that in this model fibrotic phase is predominant over inflammatory phase, which is important when testing compounds for treatment of IPF.

SB525334 is an ALK5 inhibitor that has been used to attenuate lung fibrosis in *in vivo* bleomycin model where its activity profile is well described [13,25,26,36]. In these studies, SB525334 was shown to inhibit connective tissue remodelling as observed by decreased gene or protein expression of collagen, fibronectin, α-SMA, CTGF (connective tissue growth factor), TIMP and other pro-fibrotic factors. SB525334

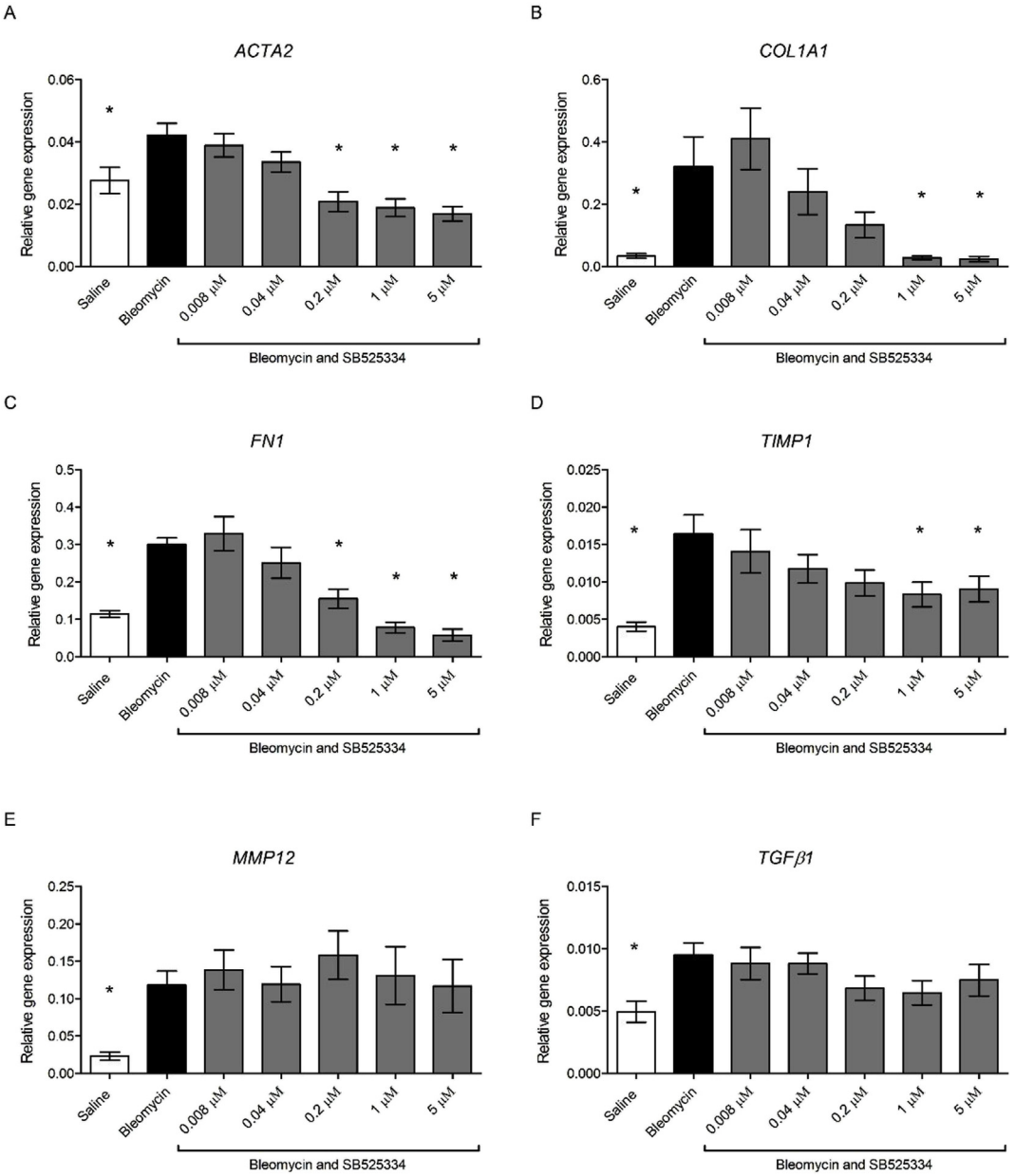

Fig. 6. *ACTA2* (A), *COL1A1* (B), *FN1* (C), *TIMP1* (D), *MMP12* (E) *and TGFβ1* (F) expression in PCLS prepared from lungs of mice 14 days after bleomycin treatment. PCLS were incubated with SB525334 for 3 days. Data are presented as means ± S.E.M. from two separate experiments (N = 14 for saline and N = 11 for bleomycin). *p < 0.05 vs. bleomycin, one-way ANOVA with Dunnett's post test.

was therefore chosen as one of the tool compounds in our PCLS model. SB525334 treatment in PCLS inhibited expression of the same genes (*ACTA2*, *COL1A1*, *FN1* and *TIMP1*) as in *in vivo* bleomycin mouse model. Difference in activity was observed on *MMP12* expression which was further increased upon SB525334 treatment in *in vivo* bleomycin model, however this was not observed in PCLS where SB525334 treatment had no effect on expression of this gene. One possible explanation for the observed difference in SB525334 activity on MMP12 expression are different dosing regimens in *in vivo* and PCLS models, which is an obvious weakness in this study. In *in vivo* model, SB525334 was given in prophylactic regime, while in PCLS model SB525334 was introduced after development of lung fibrosis when inflammatory phase was already finished or in decline. However, ALK5 inhibition in *in vivo* model was shown to have anti-fibrotic effects when dosed in both prophylactic and therapeutic regimens [26,37,38].

For further validation of our PCLS model we tested one of the currently approved therapies for IPF, nintedanib. It is a small molecule inhibitor of several receptor tyrosine kinases (fibroblast growth factor receptor, vascular endothelial growth factor receptor and platelet derived growth factor receptor). Activity of nintedanib was previously described in various *in vitro* and *in vivo* assays, including the *in vivo* bleomycin model in mice [39,40] as well as in clinical studies as reviewed in Ref. [41]. The results of these studies demonstrated that nintedanib inhibited protein or gene expression of TGFβ1, collagen 1, fibronectin 1, CTGF, TIMP1 and others. Similarly, in our *in vitro* PCLS model, nintedanib dose dependently inhibited gene expression of *COL1A1*, *FN1* and *MMP12*. Inhibition of *ACTA2* was not as pronounced, with only 10 μM nintedanib exhibiting an effect. However, the observed effects at this concentration could be due to reduced tissue viability as suggested by increased LDH release. These results confirm that PCLS prepared from bleomycin treated mice can be used for *in vitro* testing of compounds for IPF treatment, since activity of SB525334 and nintedanib was observed in PCLS and expression of important factors that drive fibrosis in IPF patients was reduced. It would also be important to

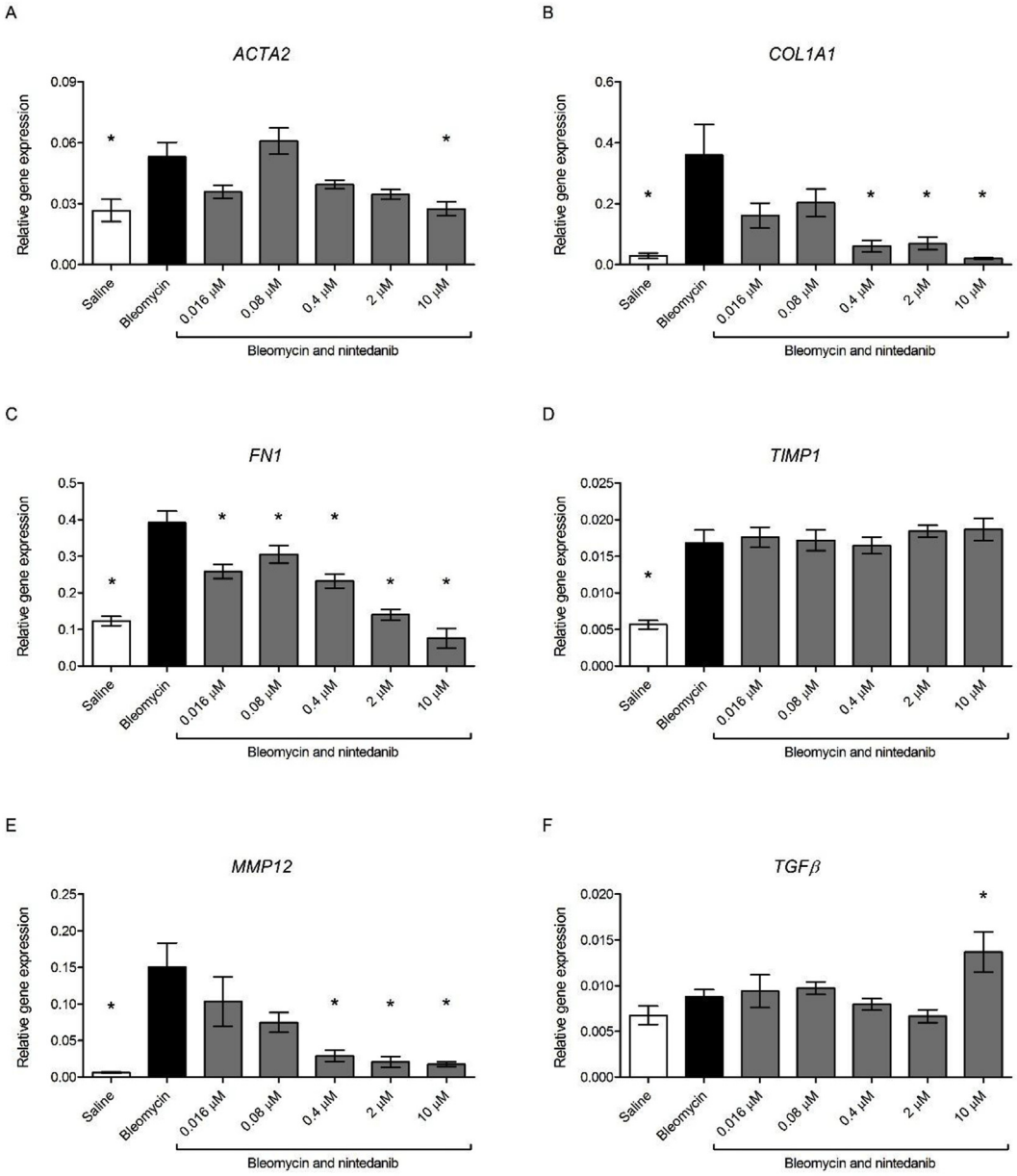

Fig. 7. *ACTA2* (A), *COL1A1* (B), *FN1* (C), *TIMP1* (D), *MMP12* (E) *and TGFβ1* (F) expression in PCLS prepared from lungs of mice 14 days after bleomycin treatment. PCLS were incubated with nintedanib for 3 days. Data are presented as means ± S.E.M. (N = 8 for saline and N = 6 for bleomycin). *p < 0.05 vs. bleomycin, one-way ANOVA with Dunnett's post test.

confirm these changes on protein level as well, either by histology or other methods.

In summary, our results demonstrate that PCLS derived from bleomycin mice and incubated *in vitro* for 3 days have increased expression of fibrosis-related genes *ACTA2, COL1A1, FN1, TIMP1, MMP12* and *TGFβ*, similarly to *in vivo* bleomycin model. These are all important factors that drive fibrosis and are deregulated in IPF patients, with most of them being desirable therapeutic targets. We have shown that PCLS are useful as *in vitro* model for testing the compounds against these factors and can therefore be used to complement other *in vitro* models during drug development.

Declarations of interest

None.

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