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Fibrosis in PCLS: comparing TGF-β and fibrotic cocktail

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Abstract

Introduction Fibrotic cocktail (FC) is a combination of pro-fibrotic and pro-inflammatory mediators that induces early fibrotic changes in organotypic lung models. We hypothesised that transforming growth factor beta 1 (TGF-β1) alone induces a pro-fibrotic effect similar to FC. Our aim was to compare the pro-fibrotic effects of TGF-β1 with FC in human precision-cut lung slices (PCLS).

Methods PCLS from "*healthy*" lung tissue of cancer patients undergoing surgery ($n=7$) were incubated with TGF-β1, FC or control for 72 h. Gene expression markers for myofibroblasts differentiation, extracellular matrix (ECM), as well as TGF-β receptors were assessed (RT-qPCR). ECM proteins expression in lysates and supernatant was assessed by ELISA and immunofluorescence.

Results We found that TGF-β1 significantly increased gene expression of *ACTA2*, *COL1A1*, *CCN2*, and *VIM* compared to control but also compared to FC. FC showed a significant increase of matrix metalloproteinase (MMP) 7 and 1 compared to control, while TGF-β receptor 2 was lower after FC compared to TGF-β1 or control. FC or TGF-β1 showed similar fibronectin protein expression in lysates and supernatants, while type I collagen protein expression in lysates was significantly greater with TGF-β1 compared to control.

Conclusions Our findings show that TGF-β1 induces consistent pro-fibrotic changes in PCLS after 72 h. Compared to TGF-β1, FC treatment resulted in reduced gene expression of TGF-β receptor 2 and increased MMPs expression, potentially mitigating the early pro-fibrotic effects. Selecting specific pro-fibrotic stimuli may be preferable depending on the research question and time point of interest in lung fibrosis studies using PCLS.

Keywords Precision cut lung slices, Fibrotic cocktail, Transforming growth factor beta 1, Ex vivo model, Fibrotic markers, Lung fibrosis, IPF

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Introduction

Fibrosing interstitial lung diseases (ILD) are a subset of ILDs that can present with a progressive phenotype leading to early mortality [1]. Idiopathic pulmonary fibrosis (IPF) is the most severe form of lung fibrosis without inflammation, resulting in the rapid decline of lung function [2]. Most ILDs are characterized by inflammation and fibrosis, making them crucial targets for therapy [3]. Increasingly, it is recognized that treatable traits may be more important in guiding treatment than the specific ILD diagnosis itself [4]. For personalised treatment strategies research models are needed that allow the investigation of specific pathomechanisms, including inflammatory and fibrotic stimuli in combination and separately.

The molecular pathomechanism of lung fibrosis involves several pro-fibrotic factors, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) [5], lysophosphatidic acid (LPA) [6, 7], and connective tissue growth factor (CTGF) [8], that favour an abnormal wound healing response and the development of lung fibrosis [9]. These mediators influence excessive extracellular matrix (ECM) deposition, impaired cell apoptosis or increased senescence, which contribute to sustained scarring and fibrosis progression [10].

Organotypic models including PCLS have provided valuable information about the dynamic interaction between mesenchymal cells and the ECM [11], or about the crosstalk between alveolar fibroblasts and epithelial cells mediated by the transforming growth factor beta 1 (TGF-β1) and non-canonical Wnt pathway [12]. Fibrosis induction in human PCLS has been optimised by various strategies including the addition of matrix metalloproteinase (MMP) inhibitors to TGF-β1 to improve collagen deposition [13], the combination of TGF-β1 with platelet lysate or neutrophil extracellular traps [14].

Alsafadi et al. suggested a specific fibrotic cocktail (FC), that combines TGF-β1 with two pro-fibrotic mediators, PDGF and LPA, and one pro-fibrotic but also inflammatory cytokine, tumour necrosis factor (TNF), to reproduce the physiological changes observed in the early stages of lung fibrosis [15]. The choice of stimuli resulted from current knowledge about fibrosis mediator in lung fibrosis [5, 7, 16]. Notably, the authors state, that the role of TNF in IPF remains incompletely understood [15]. Among all pro-fibrotic factors, TGF-β1 and its signalling pathway represents the major stimulus for the erroneous and permanent repair response in lung fibrogenesis [16]. TGF-β1 is equally a common mediator in fibrosis of other organs [17]. Compared with other fibrotic stimuli, such as PDGF, TGF-β1 promotes a greater ECM production as shown by pro-peptides of type I and type III collagens [18]. In PCLS, TGF-β1 increases ECM deposition and has been used to study anti-fibrotic drugs [19].

FC-induced fibrosis in PCLS is used by various research groups including ours and has led to remarkable progress in lung fibrosis research resulting in numerous publications [20–24]. Comparison of the gene expression in FC stimulated PCLS with IPF stages, FC recapitulates 46% of the observed in vivo changes across all stages. Of these, 11% overlapped with the stage 1–specific signature in IPF, indicating that FC treated PCLS best recapitulates the molecular processes that occur in early IPF [25].

As a limitation of this model, we observe like Alsafadi et al. that some donors respond differently to FC [15]. This is independent of clinical characteristics and possibly due to tissue specific or interindividual susceptibilities [15]. To minimize factors involved in treatment response variations, we hypothesise that TGF-β1 alone may sufficiently induce some of the fibrotic changes in PCLS. This study compared TGF-β1 with FC induced fibrosis in a PCLS model of normal-appearing human lung tissue from cancer patients undergoing surgery.

Methods

PCLS generation

Human control lung tissues ($n = 7$) were collected from normal appearing lung areas of lung cancer resections from patients undergoing surgery at the university hospital, Inselspital, Bern (Ethical approval KEK-BE_2024 – 01841). All patients signed written consent prior sample collection. The patients' characteristics and diagnoses are summarised in Table 1.

Lung tissues (ca. 10 g) were processed to generate PCLS (400 µm thick, ∅ 4 mm) to ensure homogeneity and reproducibility between replicates, following

Table 1 Patient characteristics

Donor	Age (yr.)	Sex	Smoking status	Diagnostic
1	68	F	unknown	NSCLC
2	70	M	unknown	NSCLS
3	54	M	unknown	metastatic melanoma
4	59	M	current smoker	NSCLC
5	46	M	25PY, current smoker	lung metastasis
6	73	M	50PY	NSCLC, COPD
7	64	F	50PY	NSCLC

COPD: chronic obstructive pulmonary disease; F: female; M: male, NSCLC: non-small cell lung cancer; PY: pack-year index

established standard protocols [22]. PCLS were treated with FC (5 ng/ml TGF-β1, 10 ng/ml PDGF-AB, 10 ng/ml TNF, and 5 μM LPA) [15] or 5 ng/ml TGF-β1 only in DMEM supplemented with 0.1% FBS for 72 h. Control PCLS were treated with medium and reagents' diluents. The medium was changed every day. PCLS (4–6 samples per donor) and supernatant were collected for protein and gene expression analysis. Supplemental Table 1 shows the analysis performed in each donor sample.

RNA isolation and gene determination

Total mRNA, cDNA synthesis reverse-transcription reactions and RT-qPCR were performed following our modified previously reported protocol [22]. The gene expression of *ACTA2*, *FN1*, *COL1A1*, *COL3A1*, *CCN2*, *VIM*, *MMP7*, *MMP1*, *TGFBR1*, *TGFBR2*, and *B2M* as housekeeping were quantified by real-time PCR with Fast SYBR™ Green Master Mix (#4385616, Thermo Fisher Scientific). The primer sequences are displayed in Supplemental Table 2 [22, 26].

Protein isolation

PCLS were mechanically disrupted in TissueLyser II (Qiagen) and protein lysate were obtained following previous protocols [22]. Total protein was quantified by Pierce™ BCA protein assay (#23227, Thermo Fisher Scientific) and the samples were stored at -80 °C until use.

ELISA

Human pro-collagen I alpha 1 and human fibronectin were measured in the supernatant and protein lysate from PCLS by ELISA kit (#DY6220-05 and #DY1918-05, respectively, R&D Systems), following the manufacturer's protocol. Dilution determination for the control and treated samples was performed before total sample analysis. The total amount of type I collagen (pg/ml) and fibronectin (ng/ml) in the protein lysate (μg/ml) was assessed by dividing the respective ELISA values by the total protein content measured by BCA protein assay.

Immunofluorescence

Fixed PCLs were immunostained for human fibronectin EDA domain, with DAPI as counterstaining, following previous published protocols [22]. LSM files were processed in Fiji image processing package v1.54f (ImageJ, USA), and the Integrated density (IntDe, in arbitrary units, AU) was subsequently calculated from the positive fluorescent areas of each picture in order to estimate the fluorescence intensity, by following the formula: FN IntDen = Sample IntDen − Negative control IntDen.

Statistical analysis

All results are shown as mean ± standard deviations. Comparisons between groups were assessed by repeated measures one-way ANOVA with Tukey's as post hoc test. Statistical analysis was performed with GraphPad Prism 10 (Insight Partners, USA). Statistical significance was assumed when $p < 0.05$.

Results

TGF-β1 induces gene expression for fibrotic markers in PCLS

The results revealed that TGF-β1 significantly increased the gene expression of the myofibroblast differentiation marker *ACTA2* compared to control ($p < 0.01$) and FC ($p < 0.05$) (Fig. 1A). In addition, mRNA levels of ECM components such as fibronectin extra domain A (*FN-EDA*) ($p < 0.0001$), type I and III collagen (*COL1A1* ($p < 0.0001$), *COL3A1* ($p < 0.05$)), connective tissue growth factor (*CCN2*), and vimentin (*VIM*), were significantly upregulated in PCLS after the addition of TGF-β1 compared to control and FC.

Compared to control ($p < 0.01$) and TGF-β1-treated PCLS ($p < 0.05$), FC-treated PCLS presented increased mRNA levels of *MMP7* and *MMP1* (Fig. 1A), suggesting increased ECM remodelling. FC influenced gene expression of TGF-β receptors, especially TGF-β receptor 2 (*TGFBR2*); FC-stimulated PCLS showed lower expression than control PCLS ($p < 0.05$) and those stimulated with TGF-β1 alone ($p < 0.01$, Fig. 1A), whereas the expression of TGF-β receptor 1 (*TGFBR1*) was higher in TGF-β1-treated PCLS compared to FC-treated PCLS ($p < 0.05$).

TGF-β1 induces protein expression of fibronectin and collagen in tissue lysates and supernatants

PCLS lysates analysed by ELISA revealed that, fibronectin in TGF-β1 treated PCLS increased similar to FC stimulation, while collagen production was significantly greater in PCLS stimulated with TGF-β1 compared to control ($p < 0.05$, Fig. 1B).

We observed that supernatants of FC and TGF-β1 treated PCLS showed similar significant increase of fibronectin and type I collagen compared to control ($p < 0.05$ and $p < 0.01$, respectively; Fig. 1C).

Finally, we stained PCLS for FN-EDA. By semi-quantitative analysis of fluorescence intensity, we found that compared to control, TGF-β1 significant increased fibronectin in PCLS ($p < 0.05$, Fig. 1D).

Discussion

The need for new anti-fibrotic drugs calls for various advanced models to better evaluate pathomechanisms and test drug candidates in preclinical studies, aiming to improve outcome in clinical trials [27]. PCLS carry the biological complexity of the lung, allowing for drug testing while considering effects on different lung cell types [28]. Modelling early fibrotic changes with this

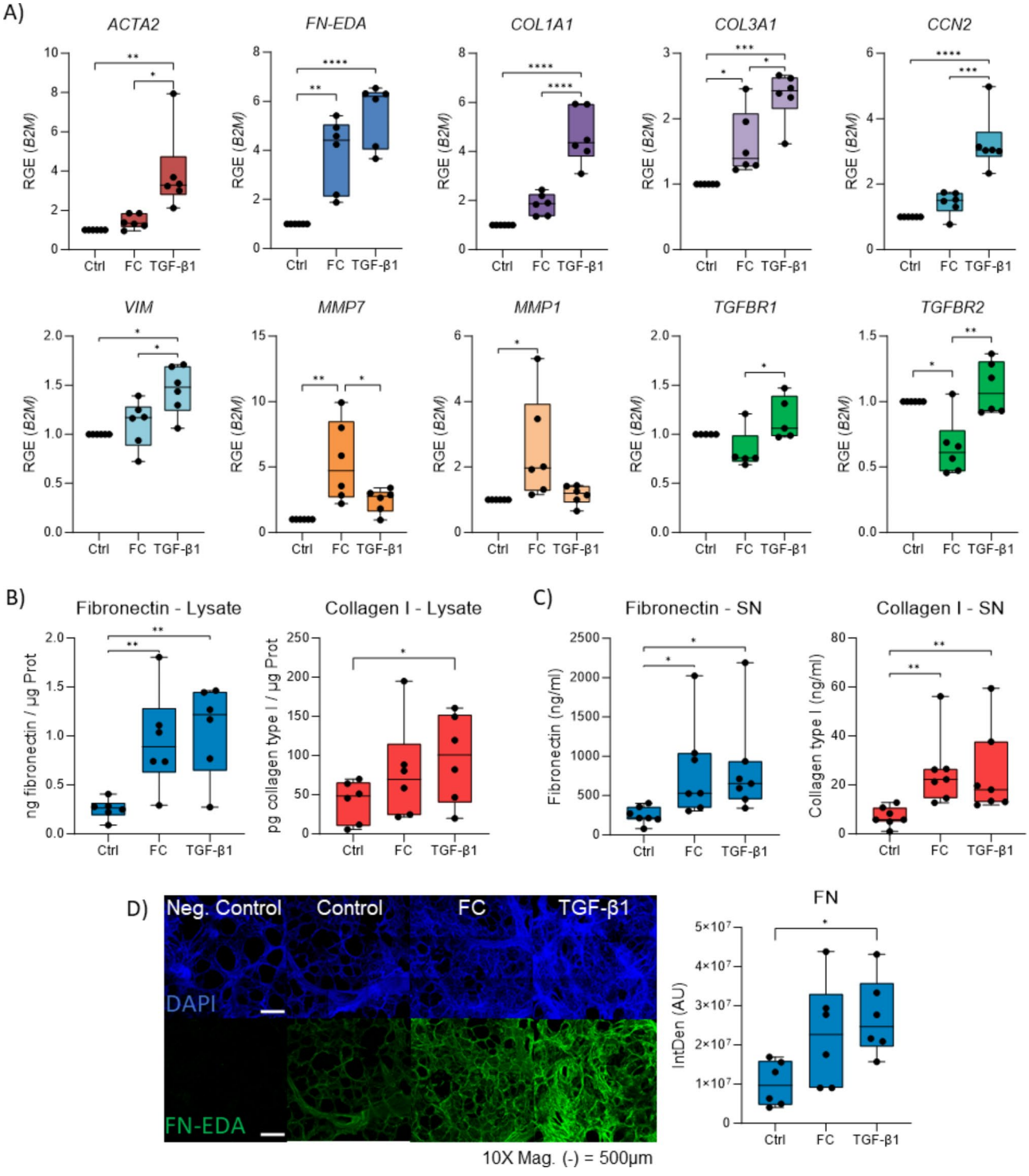

Fig. 1 Fibrotic marker expression in PCLS treated with a fibrotic cocktail (FC) or TGF-β1 alone. (**A**) Gene expression of the myofibroblast marker *ACTA2*, the ECM markers *EDA-FN1*, *COL1A1*, *COL3A1*, *CCN2*, and *VIM*, the ECM remodelling genes *MMP7* and *1*, and the TGF-β receptors *TGFBR1* and *R2* 72 h after treatment with FC or TGF-β1 alone. The gene results are presented as relative gene expression (RGE) to that of the housekeeping gene *B2M* and were normalised to control (Ctrl) condition. (**B**) Concentrations of total fibronectin and type I collagen in PCLS lysate were measured by ELISA. (**C**) Fibronectin and type I collagen release in supernatants (SN) was measured by ELISA. (**D**) Immunofluorescence staining revealed that the protein content of fibronectin extra domain A (FN-EDA) was similar between FC and TGF-β1, as shown by the semi-quantification with IntDen (arbitrary units, AU). Pictures obtained at 10X magnification. (—) = 500 µm. (*) $p < 0.05$; (**) $p > 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$

organotypic model by adding FC to human lung tissue has been an important step for fibrosis research. Our results suggests that the major pro-fibrotic cytokine TGF-β1 can induce significant fibrotic changes, including protein changes in fibronectin and collagen. In selected studies, TGF-β1 might be sufficient to investigate fibrosis for specific questions, while FC remains a more complex model for early lung fibrosis.

While some non-significant results after FC stimulation might be due to interindividual response variability of the tissue in our hands, we speculate that these results could also suggest interference of FC components with TGF-β1 signalling and potentially influence the fibrotic response in the ex vivo model through its receptor regulation [29]. For example, TGFβR2 gene expression was decreased after FC compared to control and TGF-β1, whereas the gene encoding TGFβR1 was significantly higher in TGF-β1-treated PCLS compared to FC. Due to the positive feedback loop between TGF-β1 and its receptors, an increase compared to control would have been expected [30]. Moreover, in previous studies, TNF reduced TGFβR2 protein expression in fibroblasts from systemic sclerosis (SSc) donors at a concentration of 10 ng/ml after 48 h [31]. This might suggest a role for TNF in our finding of reduced TGFβR2 gene expression after FC, which contains TNF.

The controversial role of TNF in lung fibrosis highlights the complex relationship between inflammation and fibrosis in the fibrotic lung. While some reports have shown that TNF promotes myofibroblast differentiation of lung resident mesenchymal stem cells [32], and that TNF polymorphisms are associated with fibrotic hypersensitivity pneumonitis (HP) [33]; others have shown that TNF might have therapeutic uses in the treatment of lung fibrosis, by targeting the pro-fibrotic activity of macrophages [34]. A study showed that the overexpression of TNF in transgenic mice protected the animals from the development of bleomycin-induced lung fibrosis [35]. Moreover, unlike TGF-β1 stimulation, the treatment of alveolar fibroblasts with TNF fails to induce the expression of some markers of fibrotic phenotype (i.e., *COL1A1*$^{+}$; collagen triple helix repeat containing 1, *CTHRC1*$^{+}$; periostin, *POSTN*$^{+}$) [36] and actively promotes collagen degradation by MMPs in isolated human lung fibroblasts [37]. In IPF patients, TNF serum levels were not significantly different compared to healthy controls [38], and immunostaining did not reveal differences in TNF^{+} cells between control lung tissue and normal-appearing lung parenchyma from IPF patients [39]. This underlines the intricate interplay between inflammatory stimuli in a pro-fibrotic environment.

The dynamic interplay TNF and TGF-β1 has been also described in the bleomycin-induced lung fibrosis model, where TNF and MMP showed their major expression during the inflammatory phase, being progressively reduced during the fibrotic phase, where TGF-β1 effects dominate [40]. In bronchial epithelial cells, TNF activates MMPs via transcription factor nuclear factor (NF)-kappa B [41]. Our results revealed increased gene expression of the *MMP7* and *MMP1* in FC-treated PCLS compared to TGF-β1 and controls. Overexpression of MMPs was associated with ECM restructuring and spontaneous emphysema in transgenic mice overexpressing TNF [42]. MMP-mediated ECM disassembly [43] might occur in PCLS treated with FC containing TNF. Interestingly, Khan et al. reported that the addition of MMP inhibitor to TGF-β1-treated PCLS resulted in increased collagen deposition [13].

Despite the observed increase of collagen production in the protein lysate of FC-treated PCLS, no statistically significant difference compared to control was observed. This may be due to half of the donors showing less than a 2-fold change in collagen content after FC treatment, while a consistent response to TGF-β1 was observed in most of the PCLS donors compared to control. Increased levels of MMP-1 after FC and not TGF-β1, might influence collagen at the post-transcriptional level explaining our findings [44] and suggest that some components of the FC may interfere with collagen deposition at this time point.

Overall, our protein results revealed significant changes in fibronectin in FC- and TGF-β1-treated PCLS compared to controls. Protein and gene expression results suggest that, in addition to TGF-β1, other components of the FC might enhance expression of fibronectin, as it has been described for PDGF [5] or for TNF [45]. In our hands, this did not lead to significant increases of fibrosis induction comparing FC to TGF-β1.

In fixed tissue (250 µm-thick slices) analysed with second-harmonic imaging microscopy by Khan et al. did not reveal increased deposition of fibrillar collagen in PCLS after TGF-β1 stimulation after 13 days [13]. In contrast and in parallel to our study, Roach et al. demonstrated that type I and II collagens were upregulated following 7 days of TGF-β1 treatment in an ex vivo lung model by immunostaining and transcriptomic analysis [19, 46].

Our findings confirm the efficacy of FC to induce early fibrotic changes, while our results suggest a comparable response after TGF-β1. We suggest that depending on the experimental set up and analysis, single use of TGF-β1 can be a simplified way to induce fibrotic changes in PCLS.

Our study has limitations. Treatment for 3 days does not give information about later time points. While previous studies using FC have commonly adopted 120 h incubation times for readouts [15, 20, 21, 23], our study focuses on early fibrosis induction. Longer-term studies would be needed to determine how pro-fibrotic

effects evolve over time in both models. In addition, our experimental settings including medium replacement volume and times are modified from those in the original report of FC induced fibrosis in PCLS [15]; this does not allow the direct comparisons of our results with this previous publication. Our study did not evaluate interactions between TGF-β1, PDGF, TNF or LPA to evaluate potential agonistic or antagonistic effects of FC components. While the literature supports an antagonistic relationship between TGF-β1 and TNF [34, 35, 40], future experiments in PCLS exploring the interplay between inflammation and fibrosis will be necessary. Further investigation of the interaction between individual FC components would be interesting, although beyond the scope of this short report. Future characterization of cell-type specific changes by RNA sequencing will be essential to broaden our understanding and pinpoint the key mechanisms and cells involved in the potential interactions between individual FC components.

Conclusions

In summary, our results confirm that TGF-β1 reliably triggers a fibrotic response, reducing sample variability and allowing focused investigation of this molecule. The model's simplicity supports consistent readouts, while complex models may offer insights into broader interactions in fibrosis.

Abbreviations

CTGF	Connective tissue growth factor
CTHRC1	Collagen triple helix repeat containing 1
ECM	Extracellular matrix
EGR1	Early growth response-1
FC	Fibrotic cocktail
FGF	Fibroblast growth factor
FN-EDA	Fibronectin extra domain A
HP	Hypersensitivity pneumonitis
ILD	Interstitial lung diseases
IPF	Idiopathic pulmonary fibrosis
LPA	Lysophosphatidic acid
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-kappa B
PCLS	Precision cut lung slices
PDGF	Platelet derived growth factor AB
POSTN	Periostin
SSc	Systemic sclerosis
TGF-β1	Transforming growth factor beta 1
TGFβR1	Transforming growth factor beta receptor 1
TGFβR2	Transforming growth factor beta receptor 2
TNF	Tumour necrosis factor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-025-03110-2.

Supplementary Material 1

Author contributions

MFC and CM designed the experimental study; TMM, and PD contributed to tissue and clinical data acquisition; CM and MFC contributed to analysis, interpretation of the data and drafting of the manuscript. All the authors critically revised and approved the final manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The study and experimentation in human control lung tissue were approved by the local Swiss Ethical Committee, Bern, Switzerland (approval number: KEK-BE_2024 – 01841). Human samples were collected and handle in accordance with the Declaration of Helsinki, and donors were informed prior to recruitment, and signed a written consent form.

Consent for publication

NA.

Competing interests

The authors declare no competing interests.

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