

Investigating collagen deposition in Idiopathic Pulmonary Fibrosis using Precision Cut Lung Slices

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Background:

Idiopathic Pulmonary Fibrosis (IPF) is the most common cause of idiopathic interstitial pneumonia (IIP)¹. IIP form a group of interstitial lung diseases characterised by varying inflammation and/or fibrosis severity. IPF's mechanism is not fully understood however damage to alveolar epithelium and abnormal tissue repair are thought to be key factors². Chemotherapy or immunosuppressive agents like bleomycin or methotrexate, can also cause lung fibrosis and inflammation. In the UK, IPF has a prevalence of 50 per 100,000, rising over the recent decades^{3,4}. IPF often occurs in middle-aged or elderly males and has a poor prognosis because of progressive morbidity and a low median survival of approximately 3 years². Treatment options for IPF are limited. Nintedanib and Pirfenidone are contemporary anti-fibrotic agents that can be effective in slowing down disease progression however patients still suffer respiratory decline, and these agents cannot reverse previously injured lung tissue². Consequently, understanding the pathogenesis of IPF, particularly how dysregulated extracellular matrix deposition may accelerate the disease, may reveal pathways that can be targeted with future anti-fibrotic therapies.

Aims:

This project aimed to investigate collagen deposition in IPF using human precision-cut lung slices (hPCLS) as an ex-vivo model. To do this, we used immunofluorescence (IF) staining of hPCLS treated with transforming growth factor beta 1 (TGF- β 1) and bleomycin (BLEO) to explore whether the early deposition and organisation of collagen types I (COL1) and III (COL 3) are altered by these fibrotic markers.

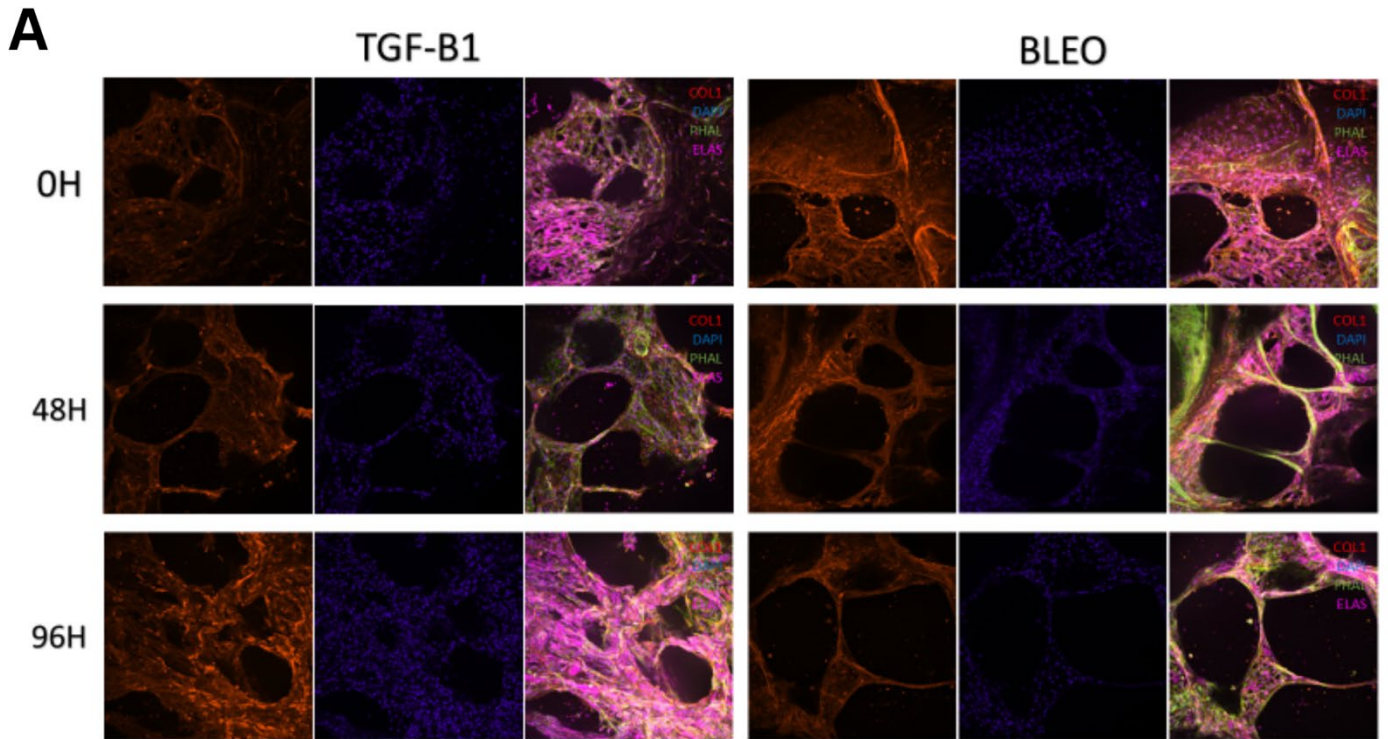
Materials and methods:

Adult lung tissue was obtained from lung resection specimens within 24 hours of surgery and kept in media supplemented by antibiotics to reduce pathogenic risk. Live lung tissue was inflated using agarose to retain structure and subjected to sectioning using a vibratome, followed by treatment with TGF- β 1 (5 ng/ml) and BLEO (50 μ g/ml), or untreated samples for up to 120h. hPCLS were then fixed every 24 hours until the end of the assay and subjected to immunostaining using antibodies to COL1 and COL3. Primary antibodies were incubated overnight at 4^oC in conjunction with an elastin (ELAS) stain (4',6'-diamidino-2-phenylindole), DAPI (to label nuclei) and fluorescently conjugated phalloidin (to label F-actin). Secondary antibodies were incubated the following day. Images of stained hPCLS were captured using a Nikon spinning disk confocal microscope and processed using ImageJ software. Statistical analysis was performed using Prism GraphPad and $p < 0.05$ was taken as statistically significant.

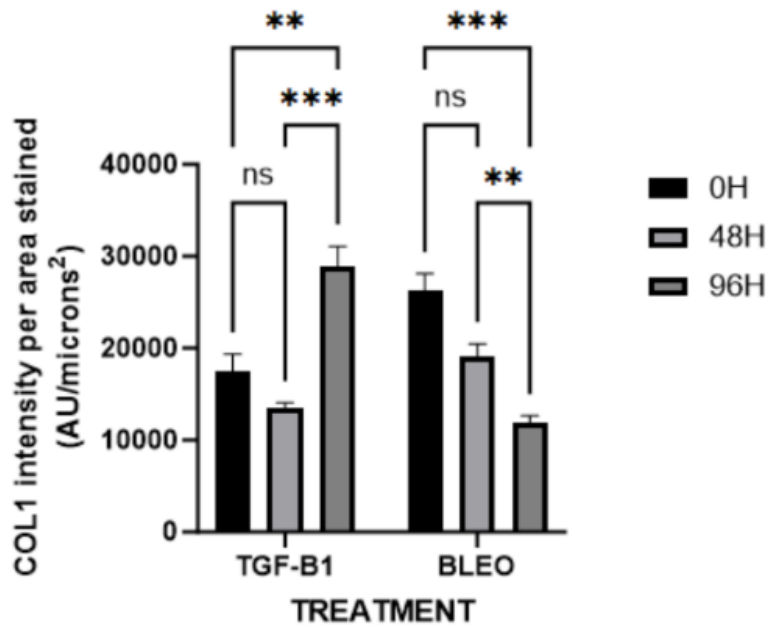
Results:

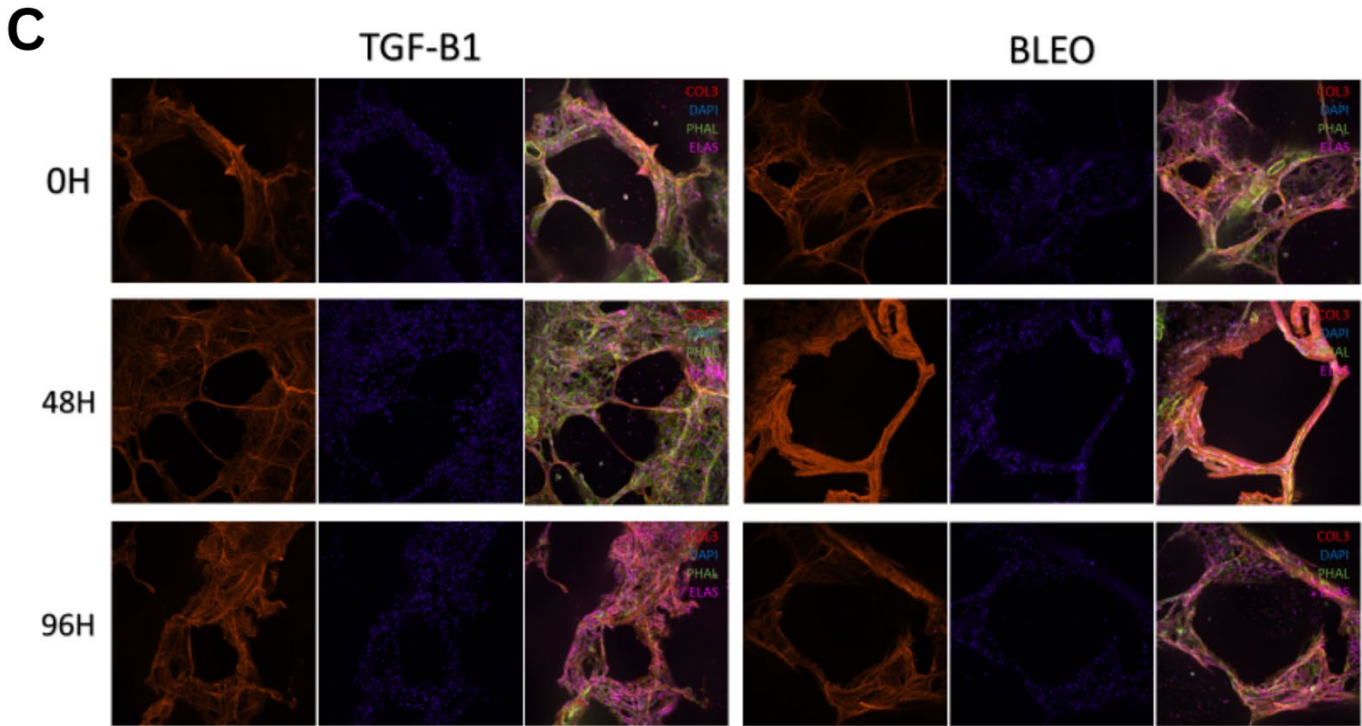
Collagen deposition in fibrosis-induced human lung tissue assays

The deposition of COL1 and COL3 staining in hPCLS (Figures 1A and 1C) was quantified by calculating the area (mm^2) and the fluorescence intensity (AU) for each condition and dividing intensity by area to determine AU/mm^2 . COL1 deposition in TGF- β 1-treated hPCLS was significantly higher at 96H compared to 48H ($p=0.0006$) and 0H ($p=0.0026$) (Figure 1B). This suggests that excess COL1 deposition can be triggered in normal hPCLS 96H after an initial TGF- β 1 insult. Conversely, COL1 deposition in BLEO-treated hPCLS declined over time and was significantly lower at 96H compared to 48H ($p=0.0016$) and 0H (0.0003) (Figure 1B). COL3 deposition in TGF- β 1 and BLEO treated hPCLS showed no significant difference over time. Together, these results indicate that COL1 is the primary collagen deposition upon early TGF- β 1 treatment and that BLEO is not sufficient to induce COL1 deposition in ex vivo hPCLS.



B COLLAGEN 1 DEPOSITION IN HPCLS TREATED WITH TGFβ1 AND BLEOMYCIN AT 0, 48 AND 96 HOURS





D

COLLAGEN 3 DEPOSITION IN HPCLS TREATED WITH TGFβ1 AND BLEOMYCIN AT 0, 48 AND 96 HOURS

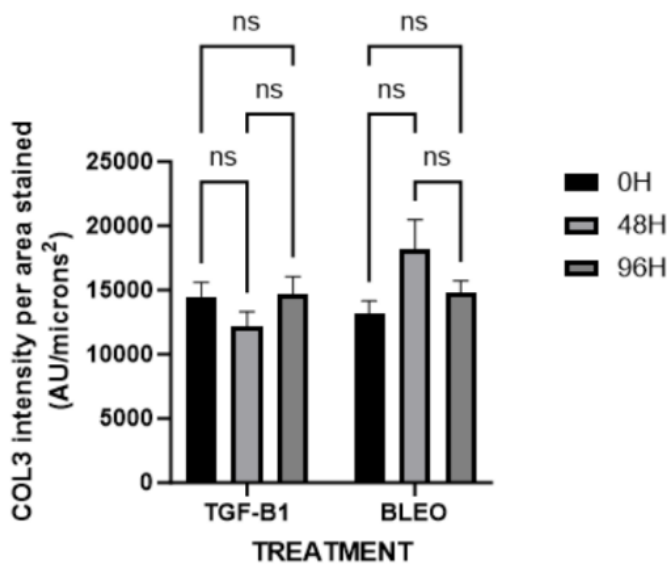


Figure 1A-D: Collagen deposition in TGF-β1 and BLEO treated hPCLS over time.

Representative images and quantification of IF staining in hPCLS (n=1) treated with TGF-β1 and BLEO at 0H, 48H and 96H.

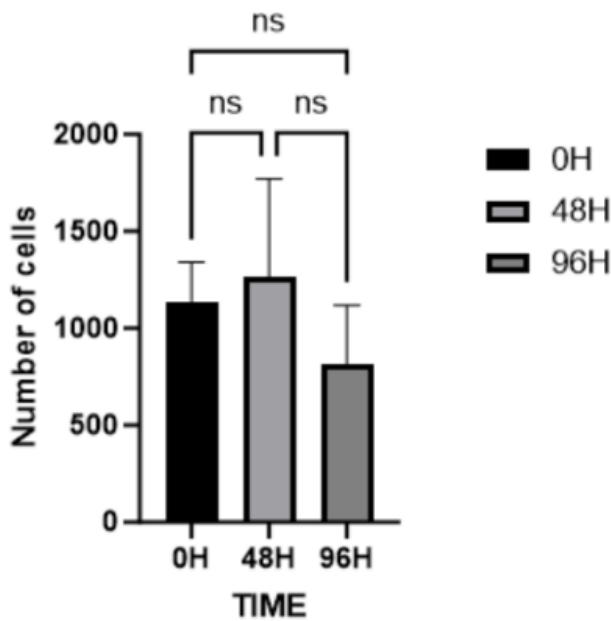
A - COL1 and DAPI IF stained images of hPCLS treated with TGF-β1 (left) and BLEO (right) at 0H (top row), 48H (middle row), and 96H (bottom row). Composite images also display phalloidin (PHAL) and elastin (ELAS) staining.

B - Quantification of IF staining images seen in (A).

C - COL3 and DAPI IF stained images of hPCLS treated with TGF-β1 (left) and BLEO (right) at 0H (top row), 48H (middle row), and 96H (bottom row). Composite images also display PHAL and ELAS staining.

D - Quantification of IF staining images seen in (C).

CELL COUNT IN HPCLS TREATED WITH BLEOMYCIN AT 0, 48 AND 96 HOURS



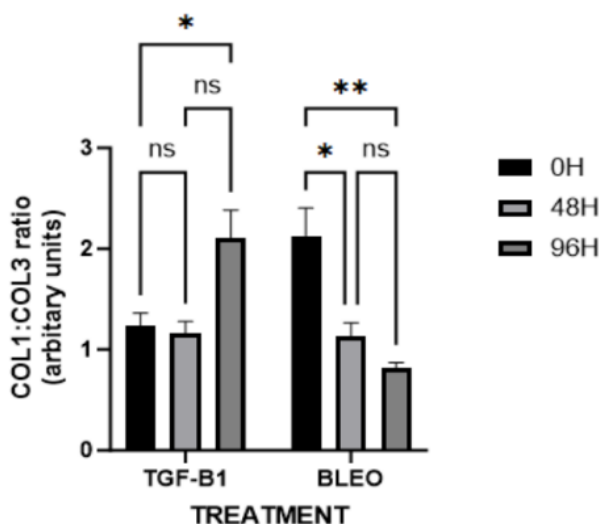
We hypothesised that reduced COL1 deposition seen in BLEO-treated hPCLS may be due to cell death induced by high concentrations of BLEO (50ng/ml). To investigate this, we recorded cell counts in BLEO-treated hPCLS at 0, 48 and 96H (see Fig 2). No significant difference was seen however there was a trend towards reduced cell count at 96H. Cell counts at further timepoints with a larger sample of hPCLS would be needed to investigate the potential effects of high-dose BLEO.

Figure 2: Cell count in BLEO treated hPCLS over time.

Quantification of nuclei in DAPI IF stained images of BLEO COL1 hPCLS (n=1) at 0H, 48H, and 96H.

Orientation of collagen fibrils in fibrosis-induced human lung tissue assays

RATIO OF COLLAGEN 1:COLLAGEN 3 DEPOSITION IN HPCLS TREATED WITH TGFβ1 AND BLEOMYCIN AT 0, 48 AND 96 HOURS



Previous studies report that changes in the ratio of COL1 to COL3 deposition may be associated with changes in the microstructure of collagen. Kottmann et al showed that there is an increased COL1:COL3 ratio in hPCLS sections with usual interstitial pneumonia (UIP), the histopathology underlying IPF, compared to healthy controls⁵. Moreover, they reported association of these changes with differences in the microstructure of collagen they observed in UIP compared to controls⁵. Consequently, we aimed to explore both the COL1:COL3 ratio and orientation of collagen fibres in our samples to determine whether increased collagen deposition induced similar changes.

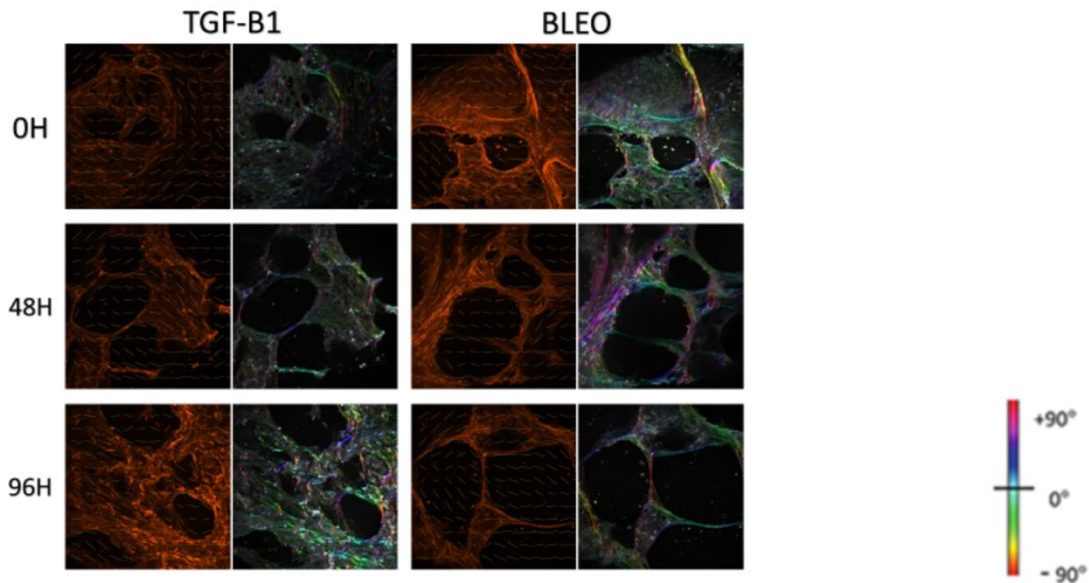
Firstly, we analysed the COL1:COL3 ratios from images shown in Figures 1A-D. There was no significant difference between COL1:COL3 ratios at 0H versus 48H in TGF-β1-treated hPCLS (Figure 3). However, at 96H, the COL1:COL3 ratio was significantly higher than 0H (p=0.0295). In BLEO-treated hPCLS, there was a significantly higher COL1:COL3 ratio than at 48H (p=0.0232) and 96H (p=0.0049). These results further indicate that TGF-β1 induces a pro-fibrotic effect by 96H.

Figure 3: Ratio of COL1:COL3 deposition in TGF-β1 and BLEO treated hPCLS over time.

Quantification and ratio of COL1 and COL3 deposition in hPCLS (n=1) treated with BLEO at 0H, 48H, and 96H.

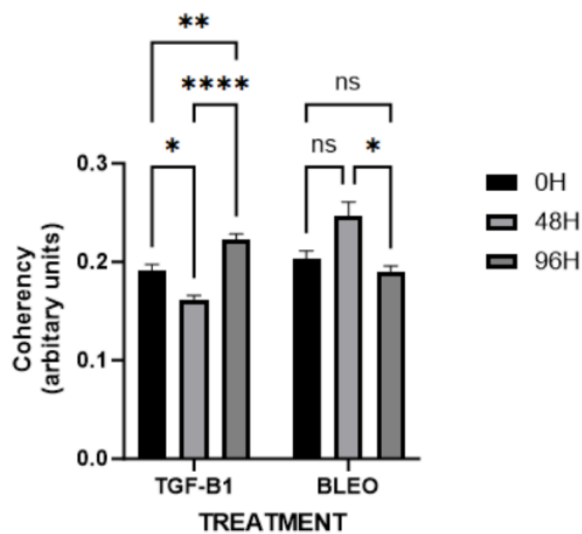
Finally, the orientation of COL1 and COL3 fibres was analysed using OrientationJ (ImageJ plug-in). The coherency collagen fibres were quantified from the images acquired of. Coherency is the degree to which local features, in this case collagen fibres, are orientated⁶. A coherency of 1 would indicate that collagen fibres have a single dominant orientation whereas 0 would indicate a uniform magnitude in all orientations. COL1 fibres shows significantly higher coherency at 96H compared to 48H ($p < 0.00061$) and 0H ($p = 0.0048$) (Figures 4A-B). This suggests that increased COL1 produced in lung fibrosis may be deposited more uniformly and linearly than COL1 in healthy lungs. COL1 deposition in our BLEO-treated hPCLS was significantly lower at 96H compared to 48H ($p = 0.0128$) only. COL3 fibre orientation in TGF- β -1 and BLEO treated hPCLS showed no significant difference between timepoints (Figures 4C-D)

A

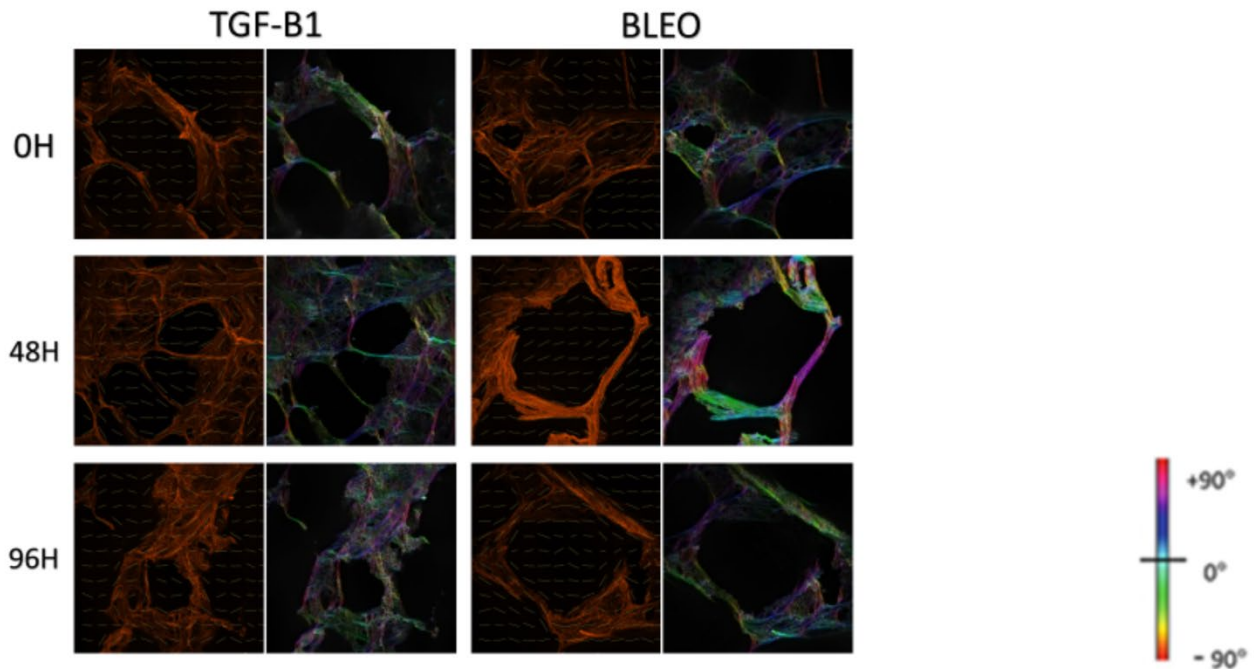


B

COLLAGEN 1 FIBRE ORIENTATION IN hPCLS TREATED WITH TGF- β 1 AND BLEOMYCIN AT 0, 48 AND 96 HOURS



C



D

COLLAGEN 3 FIBRE ORIENTATION IN HPCLS TREATED WITH TGFβ1 AND BLEOMYCIN AT 0, 48 AND 96 HOURS

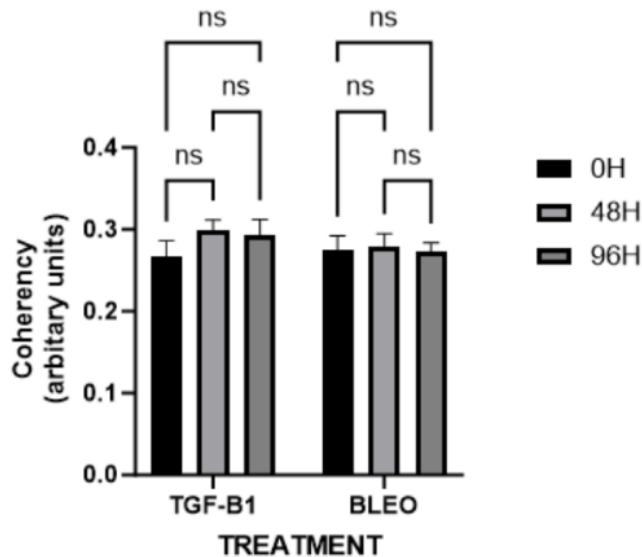


Figure 4A-D: Collagen fibre orientation in fibrosis treated hPCLS treated with TGF-β1 and BLEO over time.

Representative vector and colour survey images and quantification of IF staining in hPCLS (n=1) treated with TGF-β1 and BLEO at 0H, 48H and 96H.

A - COL1 IF stained images of hPCLS treated with TGF-β1 (left) and BLEO (right) at 0H (top row), 48H (middle row), and 96H (bottom row). Colour map for colour survey images on bottom right displays angle of orientation.

B - Quantification of vectors/orientation of IF stained images seen in (A).

C - COL3 IF stained images of hPCLS treated with TGF-β1 (left) and BLEO (right) at 0H (top row), 48H (middle row), and 96H (bottom row). Colour map for colour survey images on bottom right displays angle of orientation.

D - Quantification of vectors/orientation of IF stained images seen in (C)

Conclusion and future directions:

Collagen type 1, and not type 3 collagen 3, is induced in hPCLS models following TGF-β1 treatment. Moreover, collagen type 1 showed greater alignment under these conditions. These findings may indicate that TGF-β1 treatment of hPCLS may represent a valuable tool to study IPF progression. It is important to note that this study's data was generated from technical replicates from one donor and future work should incorporate expanded biological replicates

by using multiple patient samples to increase donor cohort and the generalisability of the results. Additional data be used to aid the development of novel therapies against IPF.

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