DUAL $\alpha_V\beta_6/\alpha_V\beta_1$ INTEGRIN INHIBITOR BEXOTEGRAST (PLN-74809) ATTENUATES PATHOLOGIC FIBROBLASTS IN HUMAN FIBROTIC LUNG EXPLANT TISSUE

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ABSTRACT

Background

Recently published single-cell RNA-Seq analyses of fibrotic human lungs have identified subpopulations of fibroblasts and epithelial cells which appear to drive disease progression, however little is known about the effects of novel anti-fibrotic therapies on these specific cells. Bexotegrast (PLN-74809), a dual inhibitor of TGF-β activating integrins $\alpha_V\beta_6$ and $\alpha_V\beta_1$ currently in development for the treatment of idiopathic pulmonary fibrosis (IPF), has previously been shown to reduce collagen gene expression in fibrotic human precision-cut lung slices (PCLS), however its relative effects on individual cell populations remains unclear. We combined robust nuclear isolation and 10x single nuclei RNA sequencing (snRNA-Seq) to characterize the response of unique cell populations in fibrotic human PCLS, a physiologically relevant *ex vivo* model system preserving the diverse cellular and extracellular composition of the fibrotic human lung, to treatment with bexotegrast.

Methods

PCLS generated from fibrotic human donor lungs were cultured for 7 days in the presence of dual $\alpha_V\beta_6/\alpha_V\beta_1$ integrin inhibitor bexotegrast (200 nM) or vehicle (DMSO). Single nuclei were isolated from n=6 individual slices per treatment from different areas of each donor lung explant and processed for single nuclear barcoding using 10x Chromium Next GEM 3' HT kits. Resulting libraries were sequenced, processed using CellRanger, and analyzed using Seurat. A custom reference based on a published fibrotic lung single-cell RNAseq dataset (1,2,3) was used to annotate identified cell clusters.

Results

snRNA-Seq analysis of fibrotic PCLS identified several key cell populations important to fibrotic disease, including several fibroblast subtypes and aberrant basaloid cells. Fibroblasts from PCLS treated with bexotegrast showed significant reduction in pro-fibrotic genes relative to vehicle-treated PCLS, including *COL1A1* and *FN1*. Subcluster analysis of annotated fibroblasts also identified *CTHRC1*-high expressing cells previously characterized as pathologic collagen-producing fibroblasts (2). Treatment of PCLS with bexotegrast reduced the number of *CTHRC1*-high expressing fibroblasts several fold when compared to vehicle. In addition to its effects on fibroblasts, bexotegrast treatment also reduced the expression of pro-fibrotic genes, including *COL1A1* and *VIM*, in aberrant basaloid cells, $\alpha_v\beta_6$-expressing cells that reside adjacent to fibroblastic foci.

METHODS

Precision-Cut Lung Slice (PCLS) Platform

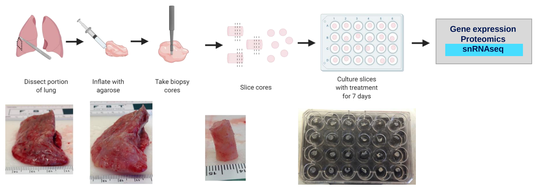


Figure 1. Flowchart of PCLS preparation and culture

- Explanted fibrotic lung tissue from IPF patients were used to evaluate the effects of novel anti-fibrotic agents
- 10x Chromium Next GEM 3' libraries were generated from single nuclei suspensions of n=6 pooled PCLS per donor per treatment group

Experimental Design for Single Nuclei RNA-seq Analysis of Treated PCLS

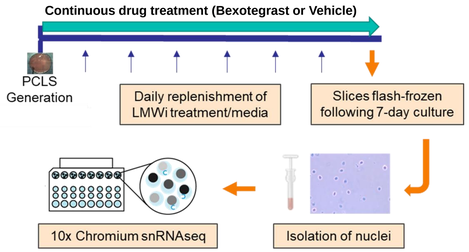


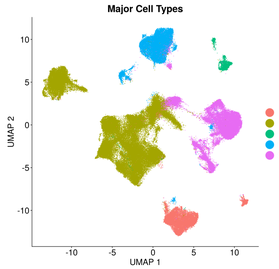
Figure 2. Study design for snRNA-Seq analysis of PCLS tissue. PCLS were generated from explanted lungs from patients with IPF receiving transplants. Replicate slices prepared from each donor tissue were pooled (n=6 slices per treatment)

Summary of snRNAseq Dataset

- 132,657 cells - snRNAseq (10x chromium)
- Bexotegrast and Vehicle (DMSO) treatments were compared after 7-day culture in PCLS from 4 IPF donor lungs
- Longitudinally integrated dataset incorporating frozen explant with cultured PCLS samples guided annotation confidence

RESULTS

Marker Based Annotation Identifies Cell Types Consistent with Previous Datasets

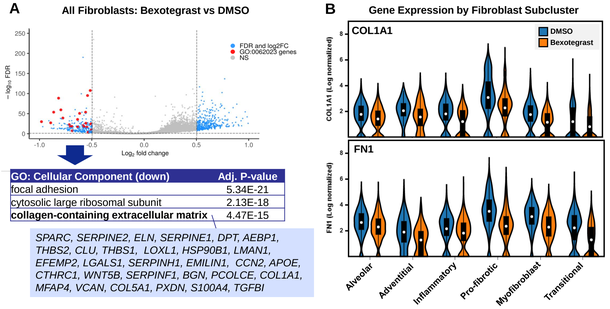


Endothelial	Epithelial
Endothelial	ATI
VE Arterial	ATII
VE Venous	Aberrant basaloid
VE Capillary	Basal cell
VE Peribroncial	Ciliated
	Goblet
Lymphoid	**Stromal**
B cell	Fibroblast-alveolar
T cell	Fibroblast-adventitial
Treg	Fibroblast-inflammatory
NK cell	Fibroblast-pro-fibrotic
Plasma B cell	Fibroblast-transitional
	Myofibroblast
Myeloid	Pericyte
Myeloid 1	SMC
Myeloid 2	
Mast cell	

Figure 3. UMAP representation of >130,000 cells identified in PCLS following 7 days of culture. Primary cell type categories are further broken down to cell subtype categories annotated using a marker-based approach informed by both single cell (1,2) and single nuclear (3) published fibrotic lung reference datasets

- Annotation by marker-based approach informed by published datasets identified major cell types as well as secondary cell subtypes previously characterized in single cell datasets (1,2,3)

Fibrogenic Genes and Pathways are Downregulated by Bexotegrast Treatment in Fibroblasts



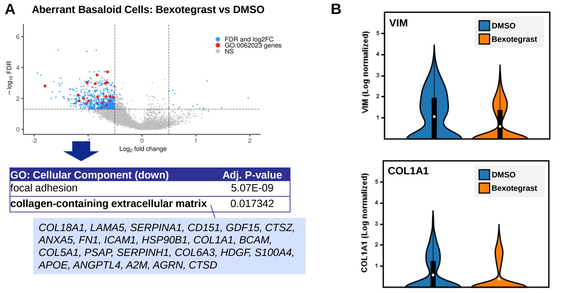
GO: Cellular Component (down)	Adj. P-value
focal adhesion	5.34E-21
cytosolic large ribosomal subunit	2.13E-18
collagen-containing extracellular matrix	4.47E-15

SPARC, SERPINE2, ELN, SERPINE1, DPT, AEBP1, THBS2, CLU, THBS1, LOXL1, HSP90B1, LMAN1, EFEMP2, LGALS1, SERPINH1, EMILIN1, CCN2, APOE, CTHRC1, WNT5B, SERPINF1, BGN, PCOLCE, COL1A1, MFAP4, VCAN, COL5A1, PXDN, S100A4, TGFBI

Figure 4. (A) Volcano plot of differentially expressed genes in bexotegrast vs DMSO treated comparison in all fibroblasts. Thresholds for FDR<0.05 and LOG2FC>|0.5| are shown. Select gene ontology (GO):cellular component terms from pathway enrichment analysis of downregulated genes is shown. Enriched genelist for significantly downregulated collagen-containing extracellular matrix terms is shown and highlighted in the volcano plot. (B) Violin plots for *COL1A1* and *FN1* by treatment and split by fibroblast subcluster.

- Bexotegrast treatment in PCLS downregulated expression of fibrogenic genes
- Pathway enrichment analysis of all fibroblasts further demonstrated an enrichment of GO terms related to fibrogenic mechanisms downregulated with bexotegrast treatment

Fibrogenic Genes and Pathways are Downregulated by Bexotegrast in Aberrant Basaloid Cells



GO: Cellular Component (down)	Adj. P-value
focal adhesion	5.07E-09
collagen-containing extracellular matrix	0.017342

COL18A1, LAMA5, SERPINA1, CD151, GDF15, CTSZ, ANXA5, FN1, ICAM1, HSP90B1, COL1A1, BCAM, COL5A1, PSAP, SERPINH1, COL6A3, HDGF, S100A4, APOE, ANGPTL4, A2M, AGRN, CTSD

Figure 5. (A) Volcano plot of differentially expressed genes in bexotegrast vs DMSO treated comparison in aberrant basaloid cells. Thresholds for FDR<0.05 and LOG2FC>|0.5| are shown. Select GO:cellular component terms from pathway enrichment analysis of downregulated genes is shown. Enriched genelist for significantly downregulated collagen-containing extracellular matrix terms is shown and highlighted in the volcano plot. (B) Violin plots for *COL1A1* and *VIM* by treatment in aberrant basaloid cells.

- Bexotegrast treatment downregulated fibrogenic genes and pathways in aberrant basaloid cells, known to express $\alpha_v\beta_6$
- Pathways enriched for downregulated genes include collagen-containing ECM and epithelial mesenchymal transition

COL1A1-High and *CTHRC1*-High Cells are Identified Within Pro-Fibrotic Fibroblasts

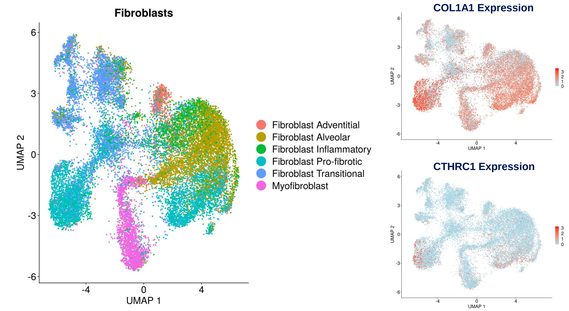
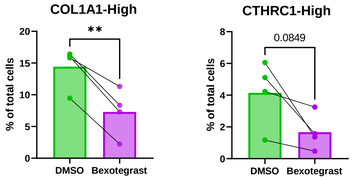


Figure 6. UMAP representation (left) of fibroblast cells subclustered by Louvain clustering and annotated using a marker-based approach informed by published datasets (2,3). UMAPs (right) indicating *COL1A1* and *CTHRC1* expression level.

- Highest *COL1A1*-expressing cells were associated with pro-fibrotic fibroblasts
- *CTHRC1*-expressing cells subpopulations also coincided with pro-fibrotic clusters

Fibrogenic Populations are Attenuated with Bexotegrast Treatment in PCLS



Individual donor data plotted. ** p<0.01 in paired t-test

Figure 7. *COL1A1*-high and *CTHRC1*-high expressing cells were plotted by treatment as percent of total cells per sample. Each dot represents 6 pooled PCLS replicates from each individual donor treated. Individual donor treatment pairs are indicated by solid lines. Statistical comparisons were performed as a paired t-test.

- Bexotegrast treatment in PCLS attenuated *COL1A1*-high fibroblasts
- *CTHRC1*-expressing fibroblasts, a previously described marker of pathologic fibroblasts (2), were also reduced by bexotegrast treatment
- Effects were consistent across all IPF donors treated with bexotegrast

CONCLUSION

snRNA-Seq analysis of fibrotic PCLS treated with dual $\alpha_V\beta_6/\alpha_V\beta_1$ integrin inhibitor bexotegrast revealed clear reductions in pro-fibrotic gene expression within unique cell populations thought to be important to IPF pathology. These data demonstrate the utility of combining single cell transcriptomic techniques with fibrotic human PCLS to evaluate the mechanism of action of novel anti-fibrotic therapies.

References: 1. Adams et al, Sci Adv. (2020); 2. Tsukui et al, Nature Comm. (2020); 3. de Rooij et al, Cardiovasc Res (2023)

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Disclosures: MA, SH, VR, BH, RA, JL, CH, ST, JS, and MD are employees and shareholders of Pliant Therapeutics