Single nuclei RNA-seq profiling of fibrotic human precision-cut tissue slices: a model for evaluating anti-fibrotic therapies in lung and liver

Steve Ho, Mahru An, Vikram Rao, Richard Ahn, Paul Wolters, Scott Turner, Martin Decaris, Johanna Schaub | Pliant Therapeutics, Inc., South San Francisco, CA, USA

BACKGROUND

Severe fibrosis increases the risk of lung- and liver-related mortality, highlighting the need for a better understanding of the mechanisms behind fibrotic processes and novel therapies to target them. Human precision-cut tissue slices (PCTS) represent a translational model that bridges the gap between cell-based and *in vivo* models of fibrotic disease by allowing for *ex vivo* investigation of fibrogenesis in small sections of intact fibrotic human tissue. Changes in bulk gene and protein expression in PCTS treated with putative anti-fibrotic agents have previously been used to gain insight into drug-related effects on lung and liver fibrosis, however these methods lack the precision to evaluate the role of individual cell types in fibrotic pathways. Here we describe a novel method for evaluating the effects of anti-fibrotic agents on individual cell types in fibrotic human tissue slices cultured *ex vivo* using single nuclei RNA-sequencing (snRNA-Seq).

METHODS

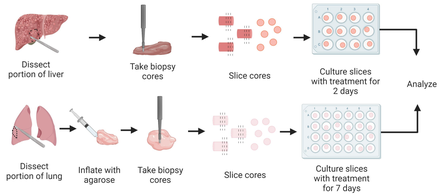
Precision-cut lung and liver slices

Human fibrotic donor tissue was obtained from patients with idiopathic pulmonary fibrosis (IPF) or rejected organ donors (fibrotic livers). All tissues were received within 24 hours cold ischemia time. Precision-cut liver or lung slices (PCLivS and PCLS, respectively) were generated and cultured for up to 7 days in the presence of transforming growth factor-beta (TGF-β) type I receptor inhibitor (ALK5i; R-268712) or vehicle (DMSO). Bulk tissue gene expression changes after culture were measured using a NanoString nCounter MAX with PlexSet reagents and a custom gene panel.

Single nuclei isolation

A method was developed and optimized to isolate intact nuclei from tissue slices using a combination of detergent-based lysis, mechanical disruption, and filtration to achieve a purified nuclei suspension.

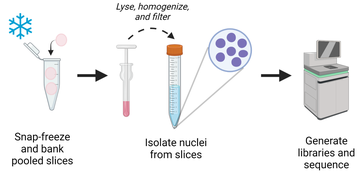
Figure 1. Generation and culture of PCLivS and PCLS



snRNA-Seq

Transcriptomic analysis of PCTS single cells was performed using 10x Chromium Next GEM 3' technology via snRNA-sequencing. Sequenced libraries were processed by cell ranger, aligned, filtered and normalized. Custom annotation of cell types was performed using gene markers established from recently published data sets.

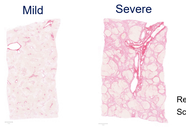
Figure 2. Single nuclei isolation and sequencing workflow



[MacParland et al, 2018; Ramachandran et al, 2019; de Rooij et al 2023; Adams et al, 2020; Tsukui et al, 2020)].

LIVER

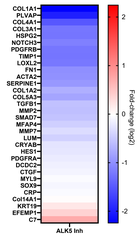
Figure 3. Donor livers had mild to severe fibrosis



Representative images
Scale bars = 1 mm.

- Picrosirius red staining shows a range of fibrosis in explants from cirrhotic livers

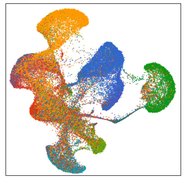
Figure 4. ALK5 inhibition reduced fibrogenic gene expression in PCLivS by bulk tissue analysis



- ALK5 inhibition resulted in reduced expression of myofibroblast markers (*ACTA2, PDGFRB*), fibrillar collagens (*COL1A1, COL3A1*) and markers of TGF-β signaling (*SERPINE1, SMAD7*)

PCLivS were treated with 1 μM of an ALK5 inhibitor for 2 days. Each row is the mean of n = 3 slices from a single donor.
n = 5 donors

Figure 5. Purified nuclei resulted in high quality sequencing data and cell annotation

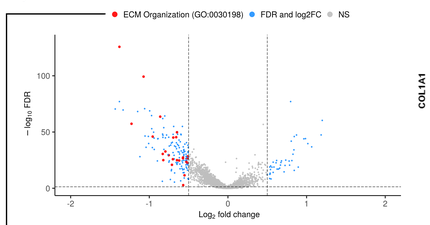


- Major cell populations of myofibroblast/HSC, hepatocytes, and cholangiocytes were identified
- Additional smaller cell populations (aggregated into larger categories for clarity) were also identified

Uniform manifold approximation and projection (UMAP) plots of filtered and normalized unique barcodes from 10x-sequenced single nuclei preparations of PCLivS samples. Cell type annotation for major cell type categories is indicated by color legend. n = 5 donors

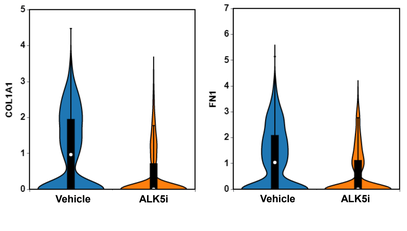
Figure 6. snRNA-Seq analysis identifies specific genes downregulated in hepatic myofibroblasts with ALK5 inhibition

Myofibroblasts: ALK5i vs vehicle



COL18A1,COL15A1,COL27A1,TGFB1,ITGA2,SERPINE1,ITGA1,TNC,FN1, PLOD2,LAMB1,COL1A1,VCAN,COL1A2,COL4A2,COL5A1,COL4A1,ADAM12, COL5A2,PXDN,COL8A1,ITGAV,TGFBI, ADAMTS6

Volcano plot showing all significant differentially expressed genes of ALK5i treated vs vehicle treated PCLivS in myofibroblast cluster with log2 of fold change (log2FC) and -log10 of false discover rate (-log10FDR) shown on axes. Differentially expressed genes defined as: log2FC > 0.5; FDR < 0.05. NS indicates log2FC less than 0.5. Violin plots of individual fibrogenic genes in normalized values are plotted for ALK5i vs vehicle (DMSO) for myofibroblast cluster. Table showing a selection of relevant GO: Biological Process terms enriched for downregulated genes. n = 5 donors.



Myofibroblasts

GO: Biological Process (Down)	Adj. P-value
extracellular structure organization (GO:0043062)	1.42E-16
external encapsulating structure organization (GO:0045229)	1.42E-16
extracellular matrix organization (GO:0030198)	5.11E-16
collagen fibril organization (GO:0030199)	4.60E-10
supramolecular fiber organization (GO:0097435)	7.55E-06
collagen-activated signaling pathway (GO:0038065)	6.01E-05
endodermal cell differentiation (GO:0035987)	5.01E-04
endoderm formation (GO:0001706)	8.02E-04
collagen-activated tyrosine kinase receptor signaling pathway (GO:0038063)	0.00161
negative regulation of smooth muscle cell migration (GO:0014912)	0.01343
negative regulation of transforming growth factor beta receptor signaling pathway (GO:0030512)	0.01343

- Differential gene expression analysis of ALK5 inhibitor-treated versus vehicle-treated PCLivS samples was performed on the myofibroblast/HSC cluster
- Volcano plot shows 41 significantly up- and 142 significantly downregulated genes (log2 fold change > 0.5; FDR < 0.05)
- Among the top downregulated genes are many known fibrogenic markers, including *COL1A1, CDH6, FAP, and FN1*
- Pathway enrichment analysis of downregulated genes identifies many biological processes associated with extracellular matrix (collagen) structural organization and signaling

LUNG

Figure 7. Fibrosis in donor lungs

Representative images
Scale bars = 1 mm.

- Picrosirius red staining shows a range of fibrosis in explants from IPF lung

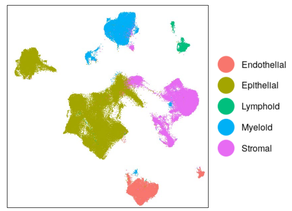
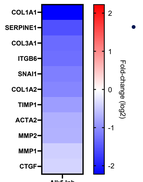


Figure 8. ALK5 inhibition reduced fibrogenic gene expression in PCLS by bulk tissue analysis



- ALK5 inhibition resulted in reduced expression of fibrillar collagens (*COL1A1, COL3A1*) and markers of TGF-β signaling (*SERPINE1*)

Data represent mean of 5–7 independent IPF tissues with ≥ 3 slices analyzed per patient tissue

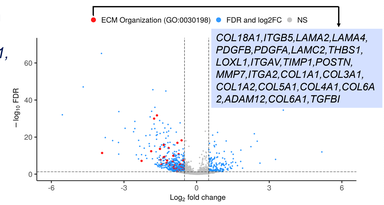
Figure 9. Purified nuclei resulted in high quality sequencing data and cell annotation

- PCLS snRNA-seq revealed the presence of cell populations relevant to fibrotic lung disease including myofibroblasts (stromal), type II alveolar epithelial cells, aberrant basaloid cells (epithelial) and macrophages

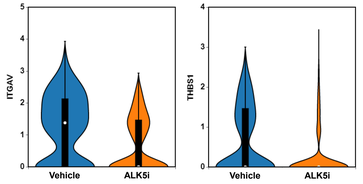
UMAP plots of filtered and normalized unique barcodes from 10x-sequenced single nuclei preparations of PCLS samples. Cell type annotation for major cell type categories is indicated by color legend. n = 4 donors.

Figure 10. snRNA-Seq analysis identifies specific genes downregulated in lung aberrant basaloid cells and fibroblasts with ALK5 inhibition

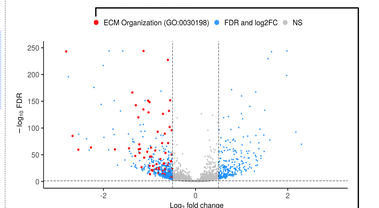
Aberrant Basaloids: ALK5i vs vehicle



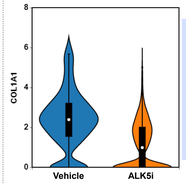
COL18A1,ITGB5,LAMA2,LAMA4, PDGFB,PDGFA,LAMC2,THBS1, LOXL1,ITGAV,TIMP1,POSTN, MMP7,ITGA2,COL1A1,COL3A1, COL1A2,COL5A1,COL4A1,COL6A 2,ADAM12,COL6A1,TGFBI



Fibroblasts: ALK5i vs vehicle



ITGB1,COL18A1,SPARC,ECM2, COL16A1,ITGB5,ELN,COL12A1, SERPINE1,PLOD3,PLOD2,PLOD1, LOXL1,LOXL2,CREB3L1,CTSK,BSG, TIMP2,ITGAV,TIMP1,ADAMTS6, POSTN,COL27A1,MMP1,MMP2,BGN, P3H1,P3H4,HSPG2,GREM1,COL8A1, GAS6,COL15A1,CD151,DPT, ADAMTS12,THBS1,ADAMTS10, FBLN5,ADAMTS14,SERPINH1,LUM, FN1,ITGA11,MFAP2,TGFBI



Aberrant Basaloids

GO: Biological Process (Down)	Adj. P-value
extracellular structure organization (GO:0043062)	1.72E-09
external encapsulating structure organization (GO:0045229)	1.72E-09
extracellular matrix organization (GO:0030198)	2.50E-09
collagen fibril organization (GO:0030199)	1.84E-05
regulation of cell migration (GO:0030334)	0.002495
supramolecular fiber organization (GO:0097435)	0.00369

Fibroblasts

GO: Biological Process (Down)	Adj. P-value
extracellular matrix organization (GO:0030198)	2.38E-45
collagen fibril organization (GO:0030199)	1.61E-32
extracellular structure organization (GO:0043062)	5.97E-26
external encapsulating structure organization (GO:0045229)	5.97E-26
supramolecular fiber organization (GO:0097435)	1.58E-21
regulation of epithelial to mesenchymal transition (GO:0010717)	1.78E-04

- Differential gene expression analysis of PCLS after 7-day culture showed ALK5 inhibition led to downregulation of genes related to fibrogenesis (COL1A1) and latent TGF-β activation (e.g. ITGAV, THBS1) in distinct cell populations
- Pathway enrichment analyses indicate downregulation of several matrix organization processes

Volcano plot showing all significant differentially expressed genes of ALK5i treated vs vehicle treated PCLS in aberrant basaloid and myofibroblast clusters with log2 of fold change (log2FC) and -log10 of false discover rate (-log10FDR) shown on axes. Differentially expressed genes defined as: log2FC > 0.5; FDR < 0.05. NS indicates log2FC less than 0.5. Violin plots of individual fibrogenic genes in normalized values are plotted for ALK5i vs vehicle (DMSO). Table showing a selection of relevant
GO: Biological Process terms enriched for downregulated genes. n = 4 donors.

CONCLUSIONS

- **snRNA-Seq analysis identified unique cell populations in PCLivS (e.g. hepatocytes, cholangiocytes, myofibroblasts) and PCLS (e.g. myofibroblasts, macrophages, alveolar epithelial cells)**
- **Differential gene expression analysis of (myo)fibroblasts in PCLivS and PCLS following treatment with an ALK5 (TGF-β receptor kinase) inhibitor demonstrated downregulation of genes related to fibrogenesis**
- **Transcriptomic analyses of fibrotic PCTS at the single cell level may be useful for interrogating disease biology and developing novel therapeutics**

Contact information: SHo@pliantrx.com

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