SINGLE NUCLEI RNA-SEQ PROFILING OF FIBROTIC HUMAN PRECISION-CUT LIVER SLICES: A NOVEL METHOD FOR EVALUATING ANTI-FIBROTIC DRUG CANDIDATES

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BACKGROUND

Severe liver fibrosis and cirrhosis increase the risk of liver-related and all-cause mortality, highlighting the need for a better understanding of the mechanisms behind fibrotic processes and novel therapies to target them. Human precision-cut liver slices (PCLivS) are a translational model bridging the gap between cell-based models and *in vivo* models of liver fibrosis, providing a translational assay platform for investigating fibrogenesis in small sections of intact fibrotic human tissue. Changes in bulk gene and protein expression in PCLivS treated with putative anti-fibrotic agents have previously been used to gain insight into drug-related effects on liver fibrosis, however, these methods lack the precision to evaluate the role of individual cell types in fibrotic pathways.

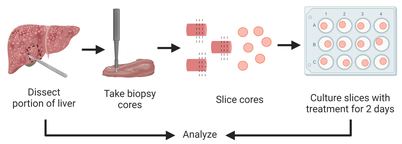
Here, we describe a novel method for evaluating the effects of anti-fibrotic agents on individual cell populations present in fibrotic human liver tissue cultured *ex vivo* using single nuclei RNA-Seq (snRNA-Seq).

METHODS

Precision-cut liver slices

Human rejected donor livers with fibrosis were obtained from a commercial supplier. All livers were transported on ice in UW or HTK solutions with less than 24 hours of cold ischemia time. PCLivS were generated and cultured for 2 days in the presence of vehicle (DMSO) or an ALK5 inhibitor (ALK5i; R-268712; 1 µM) that blocks TGF-β signaling, a well-known driver of fibrotic scar formation. Viability of sentinel slices was determined using an alamarBlue assay. Bulk tissue gene expression changes after culture and treatment were measured using a NanoString nCounter MAX with PlexSet reagents and a custom gene panel.

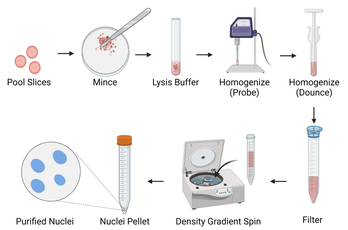
Figure 1. Generation and culture of PCLivS



Single nuclei isolation

A method was developed and optimized to isolate intact nuclei from slices using a combination of detergent-based lysis, mechanical disruption, and filtration. The crude nuclei suspension underwent density gradient centrifugation to remove debris and enrich for nuclei.

Figure 2. Isolation of single nuclei



snRNA-Seq

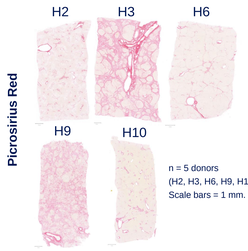
Transcriptomic analysis of PCLivS single cells was performed using 10x Chromium Next GEM 3' technology via snRNA-sequencing. Sequenced libraries were processed by cell ranger, aligned, filtered and normalized. Custom annotation of cell types was performed using gene markers established from recently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

[MacParland et al, 2018; Ramachandran et al, 2019].

RESULTS

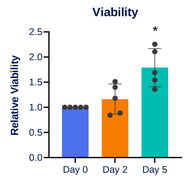
Characterization of PCLivS Platform and Nuclei Isolation

Figure 3. Donor livers had mild to severe fibrosis



n = 5 donors (H2, H3, H6, H9, H10).
Scale bars = 1 mm.

Figure 4. PCLivS are viable up to 5 days in culture

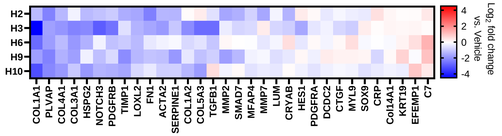


Viability of sentinel PCLivS from fibrotic liver determined by alamarBlue assay (signal/mg tissue) and reported as relative to viability of sentinel slices from the same donor on Day 0.

n = 5 donors (H2, H3, H6, H9, H10). *p<0.05.

- Picrosirius red staining shows a range of fibrosis in explants from patients with a reported history of cirrhosis
- PCLivS remained viable for duration of culture

Figure 5. ALK5 inhibition reduced fibrogenic gene expression by bulk tissue analysis



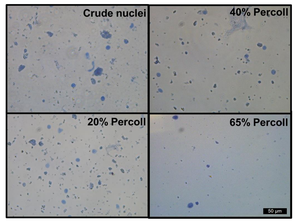
PCLivS were treated with 1 µM of an ALK5 inhibitor for 2 days. Each row is the mean of n = 3 slices from a single donor. n = 5 donors (H2, H3, H6, H9, H10).

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

Figure 6. Isolation of single nuclei from cultured PCLivS

% Percoll	# Nuclei	% Yield
0 (crude)	2,376,000	- - - -
20%	1,320,000	55%
40%	1,280,000	53.9%
65%	800,000	33.7%

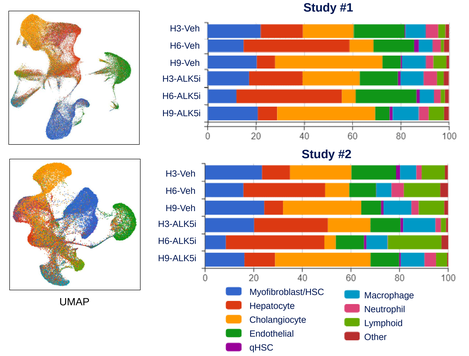
Table shows the total number of nuclei and percent yield at varying Percoll densities. Panels display trypan blue staining of nuclei after centrifugation with Percoll. Images at 40X magnification.



- Crude nuclei isolation results in abundant cellular debris
- Post-lysis density gradient centrifugation (Percoll) can separate low density debris from higher density nuclei
- 65% Percoll achieves best balance of maintaining sufficient nuclear yield while removing significant amounts of debris

Evaluation of Sequencing Quality and Cell Annotation

Figure 7. Purified nuclei resulted in high quality sequencing data and reproducible cell annotation

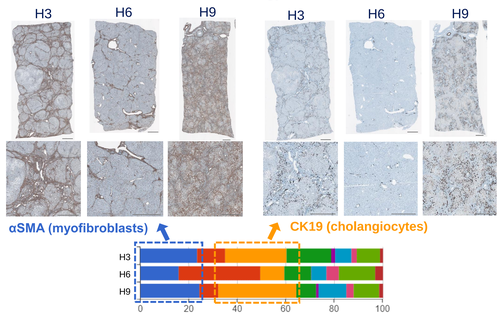


Uniform manifold approximation and projection (UMAP) plots of filtered and normalized unique barcodes from 10x-sequenced single nuclei preparations of PCLivS samples prepared from the same donors in two independent studies. Cell type annotation for major cell type categories is indicated by color legend. Relative proportion of cell types for individual replicate samples across the two studies are shown in the bar graphs. n = 3 donors (H3, H6, H9).

ALK5i, ALK5 inhibitor; Veh, Vehicle (DMSO)

- Major cell populations of myofibroblast/HSC, hepatocytes, and cholangiocytes were identified
- Additional smaller cell populations (aggregated into larger categories for clarity) were also identified
- Relative proportions of major cell categories were consistent for replicate samples across the two independent studies with small variations

Figure 8. Annotated cell type profiles from PCLivS cultures were consistent with donor tissue histology

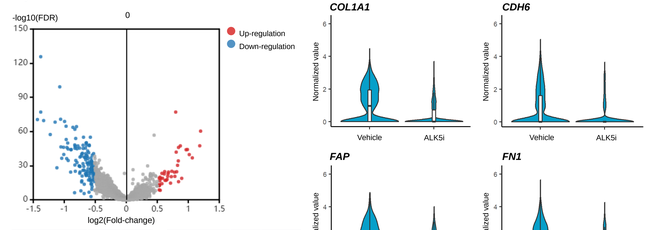


Immunohistochemistry (IHC) for αSMA (myofibroblasts) and CK19 (cholangiocytes) in adjacent tissue from the original explant compared to the cell type profiles determined by snRNA-Seq from cultured, vehicle-treated PCLivS. n = 3 donors (H3, H6, H9). Scale bars = 1 mm.

- Donor-dependent differences in relative abundance of myofibroblasts and cholangiocytes, determined in cultured slices by snRNA-Seq, matched abundance observed by IHC in the original explants

Evaluation of Differentially Expressed Genes in Myofibroblasts

Figure 9. snRNA-Seq analysis identified specific genes down-regulated in hepatic myofibroblasts with ALK5 inhibition



Gene Name (down)	Log2FC	-log10 FDR
GAS7	-1.433	70.5
PLOD2	-1.38	125.7
FAP	-1.38	77.1
CDH6	-1.333	69.6
FN1	-1.226	57.3
RUNX1	-1.148	68.2
LINC00536	-1.119	28.1
COL1A1	-1.073	99.2
AC107021.1	-1.059	70.7
VCAN-AS1	-1.054	46.4
CCDC102B	-1.041	36.4
AC113386.1	-1.012	46.1
GPC6	-1.008	39.8
PALM2AKAP2	-0.995	68.8
CASC15	-0.971	64.6
LDLRAD4	-0.969	52.9
SLC7A11	-0.961	36.2
ITGA1	-0.957	45.9
AP000331.1	-0.945	49.8
ZEB1	-0.939	44.6
UACA	-0.937	34.5
PIEZO2	-0.897	24.3
CACNA1C	-0.892	43.9
PKNOX2	-0.889	44.1
PXDN	-0.861	63.6

Gene Name (up)	Log2FC	-log10 FDR
GNA14	1.199	60.3
FGF7	1.185	47.4
PLCB1	1.065	36.8
SLC7A2	1.016	39.7
TNFRSF11B	0.986	44.1
CCBE1	0.965	44.0
STX17-AS1	0.894	18.8
LPAR1	0.872	47.4
NAMPT	0.843	46.0
ACSL4	0.843	15.9
ITGB8	0.839	34.1
ADGRL2	0.827	30.3
ADAMTSL1	0.813	24.6
CFH	0.805	41.9
PLCG2	0.797	77.1
NR4A3	0.784	20.5
AC083837.1	0.735	25.0
THBS2	0.726	20.3
BX322234.1	0.714	14.6
IL6	0.681	24.3
HGF	0.674	22.9
PDZRN3	0.668	20.6
SDK1	0.658	17.9
KAZN	0.643	17.0

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

Volcano plot showing all significant differentially expressed genes of ALK5i-treated vs vehicle-treated PCLivS in myofibroblast cluster. Top 25 up- and down-regulated gene tables are shown with log2 of fold change (log2FC) and -log10 of false discover rate values (-log10FDR) shown. Violin plots of individual fibrogenic genes are plotted for ALK5i vs vehicle (DMSO) for myofibroblast cluster. Table showing top GO: Biological Process terms enriched for down-regulated genes. n = 5 donors (H2, H3, H6, H9, H10).

ALK5i, ALK5 inhibitor.

- 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 down-regulated genes (log2 fold change > 0.5; FDR < 0.05)
- Among the top down-regulated genes are many known fibrogenic markers, including *COL1A1, CDH6, FAP, and FN1*
- Pathway enrichment analysis of down-regulated genes identified many biological processes associated with extracellular matrix (collagen) structural organization and signaling

CONCLUSIONS

- **A method was developed to isolate single nuclei from fibrotic precision-cut liver slices (PCLivS) post-culture**
- **Single nuclei RNA-Seq analysis of PCLivS successfully identified unique cell populations, including hepatocytes, cholangiocytes, and myofibroblasts**
- **Differential gene expression analysis of myofibroblasts identified individual genes and pathways related to extracellular matrix organization and signaling that were down-regulated with ALK5 (TGF-β receptor kinase) inhibition**
- **snRNA-Seq analysis of fibrotic PCLivS may be useful for better understanding the mechanism of action of anti-fibrotic therapies in hepatic disease**

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