Supplemental material

Cryopreservation and Culture Duration drive Pro-Fibrotic Signatures in Human Precision-Cut Lung Slices (PCLS)

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Methods

Donor Tissue Procurement and Preparation of PCLS

Human lungs from one subject were obtained from the National Disease Research Interchange (NDRI). Human biospecimen research was approved by the Brigham and Women's Hospital Institutional Review Board (2010P002594, 2019P003592) and Institutional Biosafety Committee (2020B000172). PCLS were generated and treated by the commercial research organization (CRO), the Institute for In Vitro Sciences (IIVS, Gaithersburg, MD, USA). Upon receipt, each lung was visually inspected for tissue quality. Suitable lobes were inflated with a pre-warmed agarose solution (0.8% in HBSS supplemented with Ca^{2+} and Mg^{2+}) to ensure uniform filling, and then allowed to gel in a cooled environment. Following inflation, the tissue was sectioned into uniform blocks (approximately 1 - 1.5 cm thick) and cored using an 8-mm diameter cylindrical blade. Cores were maintained in a cold slicing buffer until slicing.

Precision-cut lung slices (PCLS) were prepared using a Krumdieck tissue slicer. The slicer was pre-cooled with Slicing Buffer, and cores were mounted individually in the core-loading sleeve. Slices were generated at an average thickness of 600 µm after verifying that a set of five test slices met the designated thickness criteria. From the total cores, 198 slices were prepared; 105 slices were designated for the fresh culture study while the remainder were cryopreserved for repeat studies or downstream applications.

Freshly prepared PCLS were placed in 24-well culture plates, each well receiving 1 mL of PCLS Culture Medium composed of DMEM-F12 (Quality Biologicals), GlutaMAX™, and Antibiotics-Antimycotics. The slices were maintained at an air-liquid interface in a humidified incubator at 37 ± 1 °C. A 2 to 4-day acclimation period before treatment was implemented. During this acclimation period, PCLS were fed the day after acclimation begins and every alternate day thereafter.

PCLS were randomly assigned to five treatment groups with triplicate slices at each time point. Negative Controls were treated with PCLS Culture Medium (vehicle only) evaluated at 0, 6, 24, 48 hours, 7 and 14 days. TGF-β treatment consisted in 1, 5, and 25 ng/mL concentrations with treatment durations at 24 and 48 hours, as well as extended exposures at 7 and 14 days.

The rest of the slices were cryopreserved immediately after harvest in CryoStor® CS10 following a rinse with HBSS. Cryovials were initially frozen at ≤ –60 °C (using controlled-rate

freezing containers) and then stored in liquid nitrogen until further use. For the repeat experiments with frozen-thawed tissue, a subset of PCLS was cryopreserved on the day of slicing. For cryopreservation, three slices were placed in each cryovial containing 1 mL of CryoStor® CS10. After freezing at ≤ –60 °C for at least 4 hours, vials were transferred to a cryobox for long-term storage in liquid nitrogen. For subsequent experiments, cryovials were rapidly thawed in a water bath, and the slices were transferred to 24-well plates containing a pre-warmed acclimation medium. An acclimation period of 2–4 days was observed before commencing treatment, mirroring the protocol used for fresh tissue.

Once in the air-liquid interface culture, 100 µL of the prepared treatment solution were used in apical exposure and applied directly onto the tissue surface, while 900 µL was added to the basal compartment. The treatments were maintained in contact with the slices until the scheduled harvest time. Refeeding, using a conditioning medium (CM) alone or CM plus TGF-β, was performed every 2 days.

At designated time points, the viability of PCLS was assessed using a WST-8 assay. Briefly, after removal of the culture medium, each PCLS was transferred to a 24-well plate containing 500 µL of WST-8 buffer. The buffer was prepared by mixing the assay medium and the CCK-8 kit reagent (at a 10:1 ratio). Following an incubation period of 120 ± 5 minutes under standard culture conditions, a 100 µL aliquot of the supernatant was transferred to a 96-well plate for optical density (OD) measurement at 450 nm. Background readings from the WST-8 buffer without tissue were subtracted, and viability was calculated relative to untreated controls.

Post-assay, each slice was bisected; one half was processed in Trizol for subsequent RNA extraction (and eventual bulk RNA sequencing), and the other half was fixed in 10% neutral buffered formalin for histological processing and H&E staining.

RNA Sequencing Data Analysis

A total of 108 human PCLS RNA-seq samples were generated using an ultra-low input protocol. Quality control was conducted using MultiQC, FastQC, and STAR alignment metrics. Read length was short (2 × 38 bp), yet over 90% of samples had high per-base sequence quality (>Q30) across all positions. Skewer trimming efficiently removed low-quality bases and adapters. Alignment with STAR[1] to the human reference genome (GRCh38) revealed that 92 out of 108 evaluated samples (85.1%) had >85% uniquely mapped reads, supporting high-confidence gene quantification via featureCounts.

Gene-level expression was quantified using featureCounts, which generated raw read counts based on the reference annotation. Counts were normalized using DESeq2 [2] to account for differences in sequencing depth and gene length. Principal component analysis (PCA) was then applied to variance-stabilized counts to reduce data dimensionality and visualize dominant sources of transcriptomic variation. To interpret the underlying biological drivers of variation, we extracted the top 100 genes with the highest absolute loadings on PC1 and performed functional enrichment analysis.

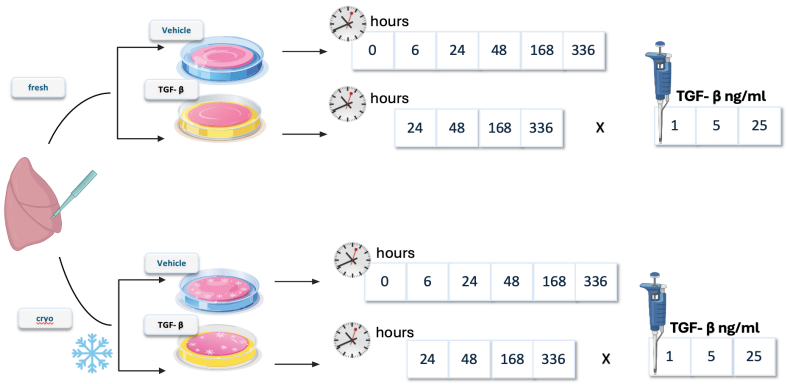
Differential expression analysis was performed using DESeq2, with significance determined based on adjusted p-values (FDR) and fold change thresholds. Further, differentially expressed genes underwent pathway analysis via Gene overrepresentation analysis (ORA) to identify enriched signaling pathways. Gene Set Variation Analysis (GSVA) [3] was also applied to the normalized expression data to compute pathway activity scores (pathway database from msigDB [4] in an unsupervised manner, thereby uncovering subtle pathway-level changes across treatment groups that might not be apparent from individual gene expression levels. In parallel, a heatmap using ComplexHeatmap R package [5], was generated based on the most variable genes across samples. This heatmap, constructed using hierarchical clustering and variance filtering, enabled the visualization of distinct expression patterns and facilitated the identification of candidate biomarkers as well as underlying biological mechanisms.

To further dissect the cellular heterogeneity within the bulk RNA sequencing data, the MUSIC [6] deconvolution algorithm was implemented. By leveraging publicly available reference single-cell RNA sequencing dataset of human pulmonary fibrosis patients [7], MUSIC was able to deconvolve the bulk transcriptome into its constituent cell type signatures, providing valuable insights into the cellular composition and the contributions of different cell types to the pro-inflammatory responses observed.

Data Availability

The bulk RNA-seq transcriptomic dataset will be publicly available at NCBI GEO upon publication.

A.


fresh
Vehicle
TGF- β
hours
0 6 24 48 168 336
hours
24 48 168 336
X
TGF- β ng/ml
1 5 25
cryo
Vehicle
TGF- β
hours
0 6 24 48 168 336
hours
24 48 168 336
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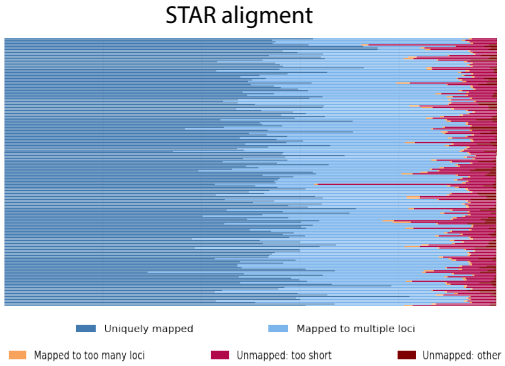
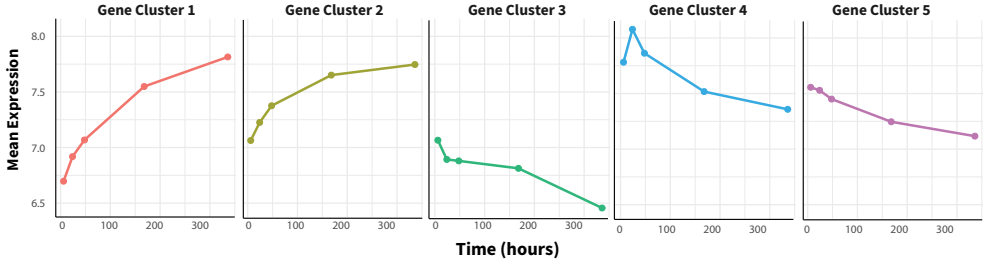
TGF- β ng/ml
1 5 25

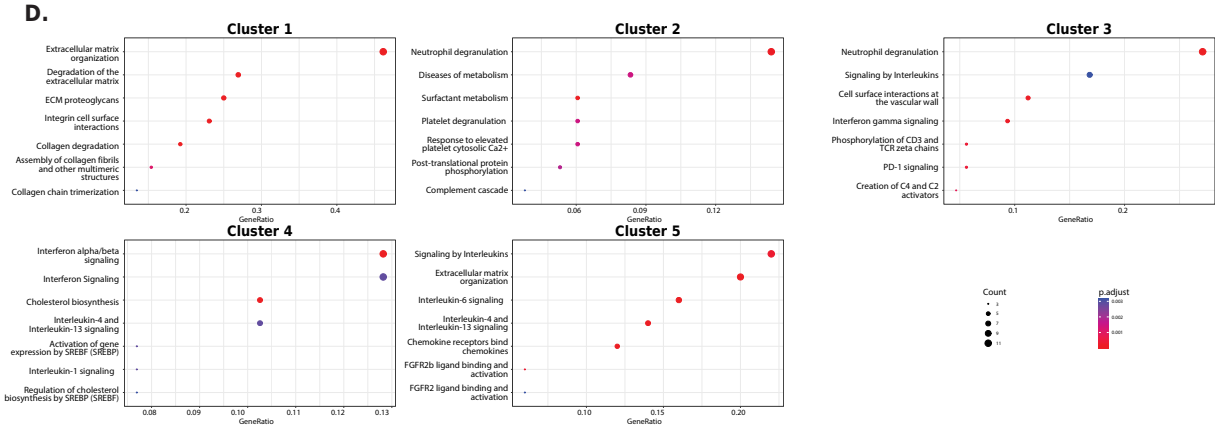
B.


STAR aligment
Uniquely mapped
Mapped to multiple loci
Mapped to too many loci
Unmapped: too short
Unmapped: other

C.


Gene Cluster 1
Gene Cluster 2
Gene Cluster 3
Gene Cluster 4
Gene Cluster 5
Mean Expression
Time (hours)

D.


Cluster 1
Extracellular matrix organization
Degradation of the extracellular matrix
ECM proteoglycans
Integrin cell surface interactions
Collagen degradation
Assembly of collagen fibrils and other multimeric structures
Collagen chain trimerization
GeneRatio
Cluster 2
Neutrophil degranulation
Diseases of metabolism
Surfactant metabolism
Platelet degranulation
Response to elevated platelet cytosolic Ca2+
Post-translational protein phosphorylation
Complement cascade
GeneRatio
Cluster 3
Neutrophil degranulation
Signaling by Interleukins
Cell surface interactions at the vascular wall
Interferon gamma signaling
Phosphorylation of CD3 and TCR zeta chains
PD-1 signaling
Creation of C4 and C2 activators
GeneRatio
Cluster 4
Interferon alpha/beta signaling
Interferon Signaling
Cholesterol biosynthesis
Interleukin-4 and Interleukin-13 signaling
Activation of gene expression by SREBF (SREBP)
Interleukin-1 signaling
Regulation of cholesterol biosynthesis by SREBP (SREBF)
GeneRatio
Cluster 5
Signaling by Interleukins
Extracellular matrix organization
Interleukin-6 signaling
Interleukin-4 and Interleukin-13 signaling
Chemokine receptors bind chemokines
FGFR2b ligand binding and activation
FGFR2 ligand binding and activation
GeneRatio
Count
p.adjust

Supplemental Figure 1. Transcriptomic analysis of fresh and cryopreserved human PCLS over time after treatment with TGF-β1. PCLS were generated from one healthy control donor and either used fresh or used after cryopreservation. PCLS were treated with TGF-β1 or vehicle control and analyzed by bulk RNA-seq at multiple time points.

(A) Study design schematic. PCLS were generated from one healthy control donor and either used fresh or used after cryopreservation. PCLS were treated with TGF-β or vehicle control and analyzed by bulk RNA-seq at multiple time points.

(B) RNAseq QC: percent of reads align to the genome of reference.

(C) Hierarchical clustering revealed 5 Gene Clusters (Figure 1B). Mean expression of gene clusters shown over time across all PCLS samples.

(D) Gene Set Variation Analysis of gene clusters from Figure 1B.

References for supplement

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