Cells isolated from donors with nonalcoholic fatty liver disease exhibit disease phenotypes in 3D bioprinted human liver tissue

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ABSTRACT

The identification of targets and biomarkers and development of therapeutics for nonalcoholic fatty liver disease (NAFLD) may be accelerated by the use of well-characterized primary cell and tissue reagents, as well as improved in vitro human cell-based disease models, including three-dimensional (3D) bioprinted liver tissue. The characteristics of donors from which the cells are isolated, and especially their stage on the NAFLD continuum, are likely to influence the resulting performance in two-dimensional (2D) and 3D models. Evaluation of individual cell type characteristics, and their performance when combined in a tissue coculture model, could enable development of in vitro models more representative of specific patient populations and disease phenotypes.

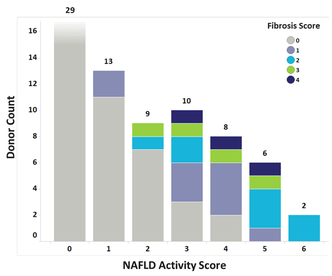
RNA sequencing (RNA-seq) was performed on (5) non-diseased and (5) NAFLD/NASH liver tissues with NAFLD Activity Score (NAS) of 3 or more, revealing clear separation of non-diseased vs. NAFLD/NASH tissues and differential expression of fibrosis related genes. Histological analyses performed on tissue microarrays revealed consistent altered distribution patterns of hepatic stellate cells (HSC), with differential activation of HSC. Hepatocytes and non-parenchymal cells (NPC) (HSC, endothelial cells, and Kupffer cells), were isolated from non-diseased donors and from donors with NAS of 3 or more. The isolated cells were characterized with respect to viability, growth kinetics, cytokine production, and phenotype. 3D bioprinted liver tissue was generated using either NPCs isolated from diseased donors combined with non-diseased hepatocytes, or hepatocytes isolated from diseased donors combined with non-diseased NPCs. 3D bioprinted liver tissue generated using NPCs from a diseased donor exhibited accelerated collagen deposition (by trichrome stain) in comparison to bioprinted liver tissue generated with non-diseased tissue donors. Tissue generated using hepatocytes from a diseased donor exhibited steatosis induction.

Characteristics of the tissue of origin for cells used for in vitro models, including disease status, influence the performance of the cells and the utility of the resulting model. Thus, characterization of cell donors could enable development of in vitro models more representative of specific patient populations and disease phenotypes.

METHODS

Non-transplantable human livers were obtained with consent for research through Organ Procurement Organizations within the United States.

All liver tissues were scored with respect to NAFLD/NASH and fibrosis by a pathologist[1,2]. 77 livers were characterized, covering a spectrum of healthy to diseased:



Whole tissues were dissociated using established methodologies and primary liver cell types were isolated as follows:

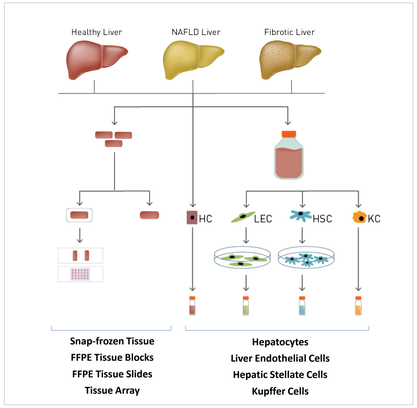
	Hepatocytes	Kupffer Cells	Hepatic Stellate Cells	Liver Endothelial Cells
Isolation Method	Perfusion with enzymatic digestion + percoll gradient	Perfusion with enzymatic digestion + positive selection of CD11b population	Perfusion with enzymatic digestion + Nycodenz gradient	Perfusion with enzymatic digestion + elutriation*

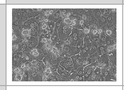
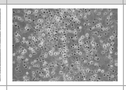
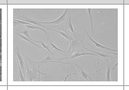
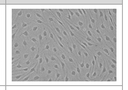
*Some liver endothelial cells were isolated using positive immunoselection with CD146/CD31

1. Kleiner DE, Brunt EM, & Van Natta M. (2005) Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 41(6):1313-1321.

2. Batts KP & Ludwig J. (1995) Chronic hepatitis. An update on terminology and reporting. Am J Surg Pathol. 19(12):1409-1417.

Characterization of liver tissues and primary human cells from non-diseased and NAFLD/NASH donors



		Hepatocytes	Kupffer Cells	Hepatic Stellate Cells	Liver Endothelial Cells
Morphology in Culture					
Characterization Endpoints	**Post-isolation Viability and Yield**	✓	✓	✓	✓
	Post-thaw Viability and Yield	✓	✓	✓	✓
	Growth Kinetics	X	X	✓	✓
	Cell Morphology	✓	✓	✓	✓
	Cell Specific Characterization	Cell attachment efficiency post-plating at 24, 48, 72, 120 hrs	Basal & LPS-induced cytokine secretion (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IFN-γ, TNF-α) Morphologic changes post-LPS treatment	Cell protein expression of αSMA, Desmin, GFAP, CD31**, TE7**	Cell protein expression of CD31, CD146, LYVE1, vWF, CD299, CD45

** Markers generally used as negative controls for population

RESULTS

RNA-seq analysis reveals upregulation of NASH-associated genes in NASH-origin human liver tissues

A pilot RNA-seq study was conducted on NAFLD/NASH-origin whole liver tissue and non-diseased whole liver tissue, to determine whether there was clear differentiation of gene expression patterns in NAFLD/NASH samples. A total of 785 genes were differentially expressed between NAFLD/NASH and non-diseased liver tissues. Key target genes associated with NAFLD/NASH and fibrosis were examined within the data set and demonstrated to be significantly upregulated in NAFLD/NASH-associated tissues (Figure 2).

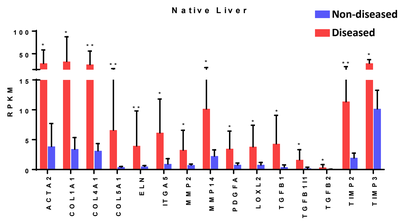


Figure 1: Expression of genes known to be associated with NALFD/NASH from RNA-seq pilot data. Smooth Muscle Actin (ACTA2), Collagens (Col1A1, Col4A1, Col5A1), Elastin (ELN), Integrins (INTGA5), Matrix Metallo-proteinases (MMP2, MMP14), Platelet Derived Growth Factor (PDGFA), Lysyl Oxidase (LOXL2), Transforming Growth Factor Beta (TGFβ1, TGFβ2) and Tissue Inhibitors of Metalloproteinases (TIMP2, TIMP3).

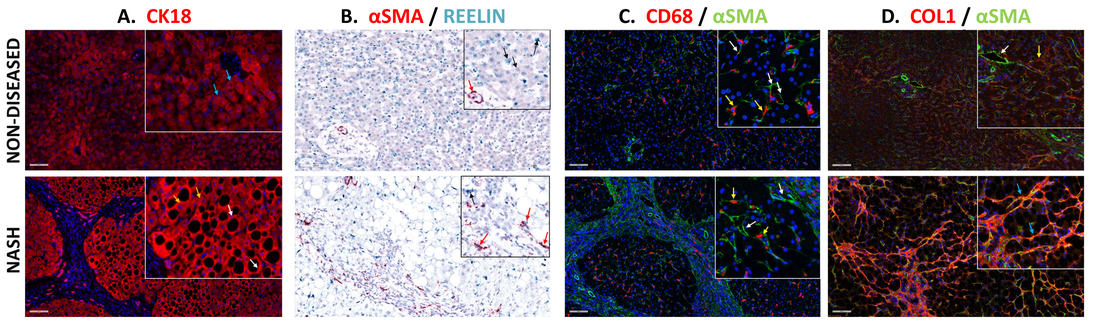


Figure 2: Figure Formalin-fixed, paraffin-embedded human liver tissue arrays were probed with antibodies and in situ hybridization probes. Representative images are shown from non-diseased (NAS 0-1) and NAFLD/NASH (NAS 3+) specimens. **A**: Immunostaining for CK18 (red). Cell-membrane localization (blue arrows), cytoplasmic localization (yellow arrows), and peripheral localization due to displacement by lipid globules (white arrows). **B**: In situ hybridization with probes for αSMA (red) and Reelin (blue) (Advanced Cell Diagnostics, Inc.). **C**: Immunostaining for CD68 (red) and αSMA (green). **D**: Immunostaining for Collagen 1 (red) and for αSMA (green). Compared to non-diseased tissues, NASH-origin liver tissues were characterized by the presence of hepatocytes with disorganized CK18 expression, including increased presence in cytoplasm and disruption of intercellular / cell membrane localization (panel A, white and yellow arrows). In non-diseased tissues, αSMA gene expression was limited to vascular structures, and reelin-expressing HSC were distributed relatively uniformly throughout the lobule along the sinusoids (panel B). In contrast, the NASH-origin tissues exhibited widespread presence of αSMA cells throughout the tissue, with occasional co-expression of αSMA and Reelin (panel B). Reelin+ HSC were infrequently associated with lipid-laden hepatocytes and the αSMA+ cells were frequently associated with the developing bands of fibrosis throughout the lobule, as was the expression of Collagen 1 (panels B-D). CD68+ Kupffer Cells were present with slightly greater frequency in NASH-origin tissues and could be found dispersed throughout the lobules as well as associated with developing fibrosis (panel C).

Bioprinting with Disease-origin Cells

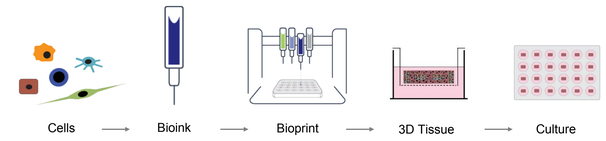


Figure 3: 3D human tissue development using the NovoGen Bioprinter® Platform. Cells reside in heterogeneous and architecturally structured 3D environments in vivo. Using the proprietary NovoGen Bioprinter® Platform, Organovo builds 3D tissues through automated, spatially-controlled cellular deposition to better recapitulate native tissue structure and function.

Bioprinting with Disease-origin Nonparenchymal Cells

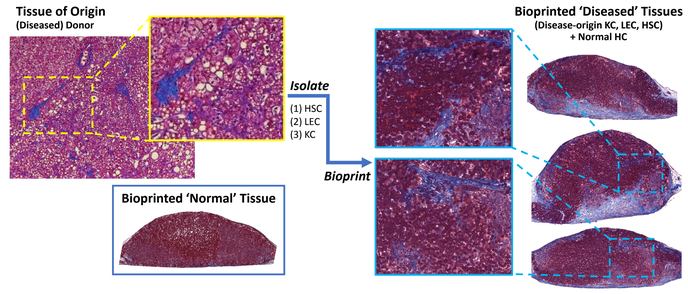


Figure 4: 3D human tissue bioprinted with diseased donor nonparenchymal cells. Hepatocytes (HC) from a non-diseased donor were bioprinted with Liver Endothelial Cells (LEC), Kupffer Cells (KC) and Hepatic Stellate Cells (HSC) from a diseased donor (NAS 3-4). Trichrome staining reveals that tissues bioprinted with diseased donor nonparenchymal cells appear more fibrotic after 2 weeks in culture as compared to tissue bioprinted using normal donor cells (inset).

Bioprinting with Disease-origin Hepatocytes

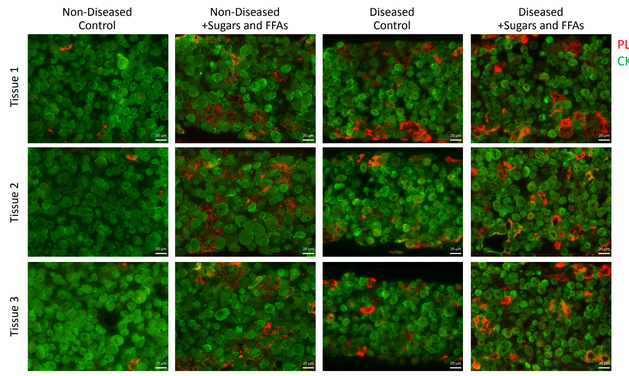


Figure 5: 3D human tissue bioprinted with diseased donor hepatocytes. Hepatocytes from a non-diseased (NAS 0) or diseased donor (NAS 3) were bioprinted with non-diseased liver endothelial cells and hepatic stellate cells and cultured in standard control media or high sugar / free fatty acid media for 2 weeks. Immunofluorescence staining for perilipin 2 (PLIN2), a marker on lipid vesicles, shows an increase in the presence of high sugar / free fatty acid media in tissues with non-diseased hepatocytes. Staining also reveals that tissues bioprinted with diseased donor hepatocytes appear more steatotic in control media as compared to tissue bioprinted using non-diseased donor cells.

CONCLUSIONS

Characteristics of the tissue of origin for cells used to build in vitro models, including disease status, influence the phenotype of the cells and the utility of the resulting model.

- NASH/NAFLD donors exhibit differential expression of key target genes associated with NAFLD/NASH and fibrosis at the mRNA and protein level.
- 3D bioprinted liver tissue generated using NPCs from a diseased donor exhibited accelerated collagen deposition.
- Tissue generated using hepatocytes from a diseased donor exhibited more basal and inducible steatosis induction.