Modeling NAFLD Using 3D Bioprinted Human Liver Tissue

Dwayne Carter[1], Sharon Presnell[1], David Brenner[2], Alice Chen[1] | [1]Organovo, 6275 Nancy Ridge Drive, San Diego, CA 92121, United States
| [2]University of California, San Diego, Department of Medicine, San Diego, CA, United States

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Background

Nonalcoholic fatty liver disease (NAFLD) is a chronic condition that originates as lipid accumulation within hepatocytes (steatosis) and progresses into nonalcoholic steatohepatitis (NASH), characterized by lipid accumulation, inflammation, oxidative stress, and fibrosis. NAFLD is now recognized as the most common cause of chronic liver disease in the western world, with an estimated prevalence of 25% worldwide, and is projected to become the leading indication for liver transplant by 2025. Despite decades of research, the mechanisms of NAFLD progression, therapeutic approaches and non-invasive diagnostics are still resoundingly absent. The study of steatosis and NASH has traditionally utilized rodent models, which are time consuming to generate and do not fully recapitulate the complex phenotypes associated with the human disease. Furthermore, current 2D cell culture models lack relevant liver cell types, do not accurately display diseased phenotypes, and have limited utility due to rapid loss of cell viability and function. To date, there are no current models exploring the role of cell donor heterogeneity and its impact on disease phenotype and the progression of disease. Thus, there is a significant need for a more predictive human multicellular 3D *in vitro* model to study the progression of steatosis into NASH.

Methods

ExVive™ Human Liver Tissue, a human *in vitro* 3D bioprinted liver model comprising primary human hepatocytes, hepatic stellate cells, and endothelial cells exhibits a complex, multicellular architecture similar to that of native liver and retains metabolic competence and liver-specific functions for at least four weeks in culture. To mimic the proposed pathogenesis of NASH via a "Two-Hit Hypothesis", immune competent tissues containing Kupffer cells were exposed to steatogenic cues via a nutrient overload approach of simple sugars and fatty acids, followed by inflammatory stimulation using prototypical inducers.

Technology Overview

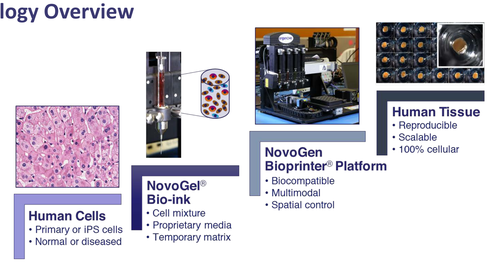

Figure 1: 3D human tissue development using the NovoGen Bioprinter® Platform**.** Cells reside in heterogeneous and architecturally structured 3D environments *in vivo.* Using the proprietary NovoGen Bioprinter® Platform, Organovo builds 3D tissues through automated, spatially-controlled cellular deposition to better recapitulate native tissue structure and function.

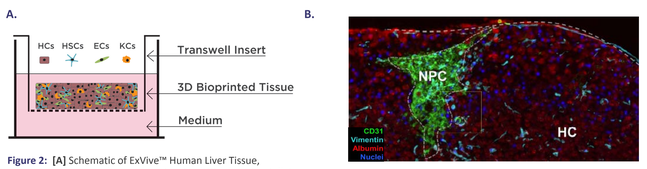

Figure 2: **[A]** Schematic of ExVive™ Human Liver Tissue, comprised of primary human hepatocytes (HCs), hepatic stellate cells (HSCs), and endothelial cells (ECs) bioprinted in a compartmentalized geometry onto the membranes of 24-well transwell culture inserts. **[B]** Representative immunofluorescence image of 3D human liver tissue showing distinct zones of non-parenchymal cells (NPC) in green and parenchymal (HC) cells in red. (Norona, *et al.* 2016) **[C]** ExVive™ Liver tissue exhibits sustained hepatocyte function as indicated by albumin levels versus standard 2D hepatocyte culture, as well as sustained CYP3A4 activity **[D]** (2D = matched hepatocytes, grown on collagen 1 coated plates).

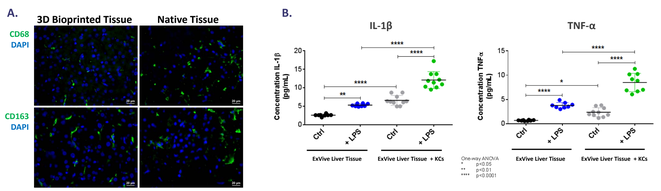

Figure 3: Characterization of bioprinted liver tissue with incorporation of Kupffer cells (KC). **[A]** Kupffer cells in bioprinted liver express prototypical markers such as CD68 and CD168, and a staining pattern similar to native liver. **[B]** ExVive™ Liver Tissue with Kupffer cells exhibited greater cytokine induction after lipopolysaccharide (LPS) treatment. Media samples from tissue treated with LPS (100 µg/mL for 24hr) were analyzed via electrochemiluminesnce.

Steatosis Induction in 3D Bioprinted Liver Tissues

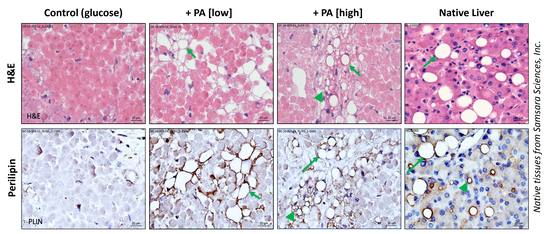

Figure 4: Chronic exposure (21 days) of 3D bioprinted liver tissues to palmitic acid (PA) induces lipid droplet formation. H&E staining of PA-treated tissue shows both macro- (arrows) and micro- (arrowheads) vesicular phenotypes. Perilipin (PLIN5) staining of lipid vesicles further confirms the steatotic phenotype.

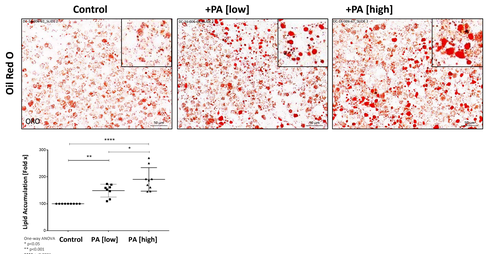

Figure 5: Chronic exposure of 3D bioprinted liver tissues to palmitic acid (PA) induces increased lipid accumulation (steatosis). Oil Red O (ORO) staining of PA-treated tissues shows a dose dependent increase in steatosis.

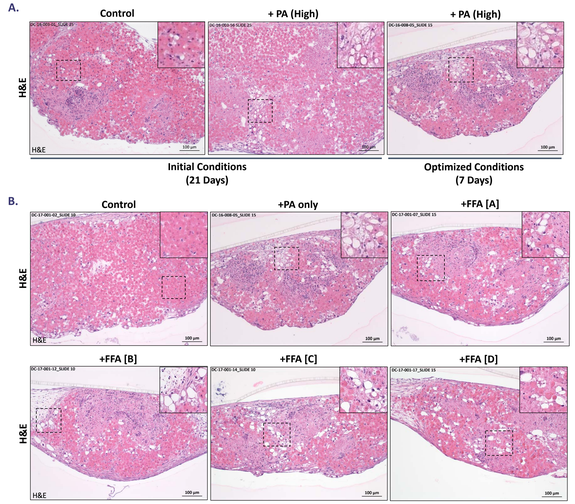

Figure 6: **[A]** Earlier onset of steatosis under optimized conditions. **[B]** Optimized regimen of free fatty acids (FFA) increases degree of steatotic phenotype.

Steatosis Induction in 3D Bioprinted Liver Tissues Continued

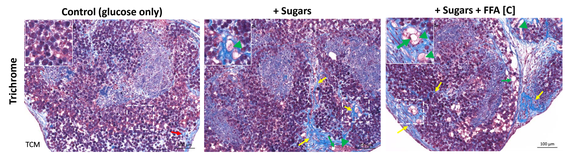

Figure 7: Titration of sugars and fatty acids alone can induce fibrosis (yellow arrows). Addition of sugars and FFAs appear to induce microvesicular (green arrowheads) and macrovesicular steatosis (green arrows).

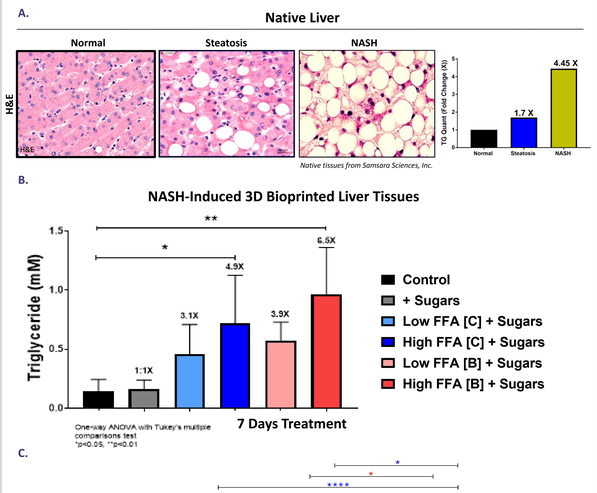

Figure 8: Triglyceride (TG) Quantification in 3D Bioprinted Liver Tissues after FFA Treatment. **[A]** Native human hepatic steatosis and NASH display a 1.7 and 4.5-fold increase in TG quantification when compared to normal livers respectively, n=1 **[B]** 7 days treatment with sugars and low or high doses of FFAs, in immune competent (+ KCs) 3D bioprinted liver tissues results in dose-dependent increases in TG levels and **[C]** significantly increased TG levels over time when compared to untreated glucose only controls. X denotes fold change normalized to controls at timepoint. n = 3 - 4 per group.

NASH Induction in 3D Bioprinted Liver Tissues

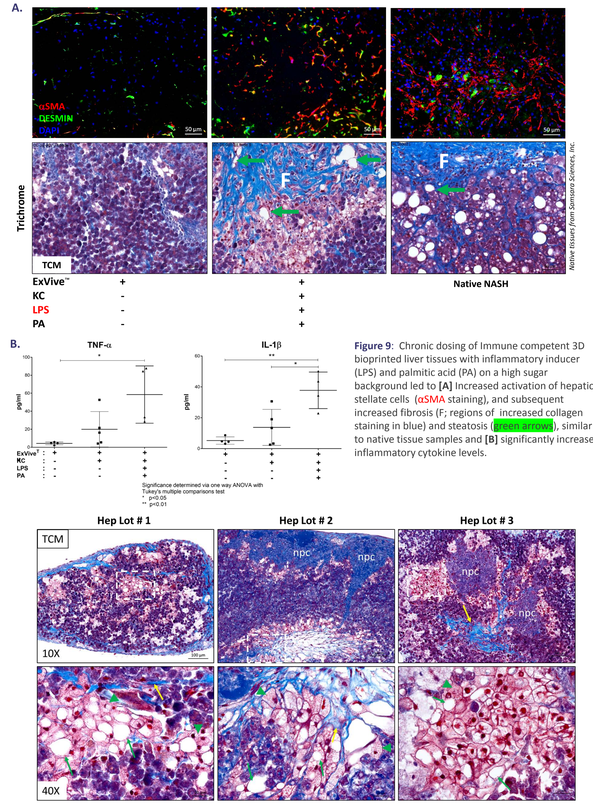

Figure 9: Chronic dosing of Immune competent 3D bioprinted liver tissues with inflammatory inducer (LPS) and palmitic acid (PA) on a high sugar background led to **[A]** increased activation of hepatic stellate cells (αSMA staining), and subsequent increased fibrosis (F; regions of increased collagen staining in blue) and steatosis (green arrows), similar to native tissue samples and **[B]** significantly increased inflammatory cytokine levels.

Figure 10: NASH phenotype is achievable across multiple donors. Chronic dosing with NASH induction conditions in immune competent 3D bioprinted liver tissues comprised of three different hepatocyte donors induces key NASH phenotypes such as steatosis (macrovesicular, green arrows and microvesicular, green arrowheads), and fibrosis (yellow arrows).

Summary and Conclusion

Key features of NASH such as steatosis, increased inflammatory cytokine release, hepatic stellate cell activation, and subsequent fibrogenesis, which are to date, largely lacking in other NAFLD *in vitro* models are attainable in the fully human ExVive™ Liver Tissue via a nutrient overload approach, analogous to a western style diet and inflammatory stimuli. The longevity of the ExVive™ Liver Tissue allows for the testing of several induction strategies such as various dosing and durations of insults (nutrients, inflammatory inducers, xenobiotics), and also has the potential to overlay a range of modulatory approaches to profile prophylactic and treatment oriented drug strategies. Together, these features suggest that the ExVive™ Human Liver Tissue hold promise for the study of complex, chronic conditions such as NASH, which will enable a better understanding of disease processes, discovery of novel therapeutics, potential biomarkers, and the safety assessment of drugs in a disease-relevant background.

Future Directions

- Refinement of induction protocol to achieve optimal steatosis and controlled stimulation of chronic inflammation representative of native disease onset.
- Correlation of *in vitro* model to clinical phenotype.
- Modulation of disease progression *in vitro:* testing reference compounds and client proprietary drug candidates.

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