Using 3D human liver microtissues to model NASH progression in vitro for drug discovery and safety testing

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Introduction
Non-alcoholic fatty liver disease (NAFLD) is the most prevalent type of liver disease and currently affects ~30% of the population. With progression to non-alcoholic steatohepatitis (NASH), this disease can eventually lead to liver cirrhosis and failure. To date, there are no approved drugs for NASH treatment and drug development has been impeded by the lack of predictive in vitro models reflecting the complex pathology of NASH. Here, we present a human in vitro NASH model based on 3D microtissue technology. By generating 3D human liver disease microtissues, we were able to recapitulate the primary human hepatocytes, hepatic stellate cells, Kupffer cells (KCs) and liver endothelial cells (LECs), this model includes all the liver cell types that play a crucial role in disease initiation and progression. Upon treatment with free fatty acids and lipopolysaccharides (LPS) in diabetic medium, these microtissues showed key physiological aspects of NASH. Lipotoxic NASH stimuli increased lipid accumulation in hepatocytes as microtissue secretory pro-inflammatory markers, such as TNF-α, IL-6, IL-8, MCP-1, MIP-1α, and IP-10. Furthermore, lipotoxic stress stimuli increased expression of pro-fibrotic markers, such as collagen type I and III, and release of pro-collagen type I. This human 3D NASH model recapitulates key biological aspects of full spectrum of NAFLD diseases, including steatosis, inflammation, and fibrosis. Compatible with high-throughput screening approaches, this model is a powerful tool for assessing efficacy of NASH drugs.

Materials and Methods
We developed a protocol for 10 days treatment with free fatty acids (FFA) and LPS in medium containing high levels of sugars to recapitulate NASH pathogenesis in vitro, and analyzed characteristic markers of NASH (lipid loading, activation of proinflammatory markers, and initiation of fibrosis (Figure 1). To demonstrate lipid-loading within the tissues, we fixed the microtissues with 4% PAF and stained them with Nile Red. Confocal microscopy was performed to visualize lipid-stained microtissues. To measure the triglyceride levels within the microtissues, we used the Glycerol-Triglyceride-Glo™ kit (Promega, not yet commercially available). Release of pro-inflammatory cytokines/chemokines was measured with the Human Magnetic Luminex Assay (R&D systems). Anti-fibrotic TGF-β signaling inhibitor ALK5i and Elafibranor were applied to microtissues with NASH stimuli to investigate their effect on NAFLD and NASH disease progression.

Recapitulating NASH Pathogenesis
Increased lipid loading and triglyceride levels in 3D human liver disease microtissues reflects human NASH disease progression

![Figure 2. NASH stimuil increase lipid loading and the triglyceride levels within microtissues. Nile Red staining in green depicted increased levels of lipids within the NASH-treated human liver disease microtissues as compared to control-treated tissues at day 10. Nuclei are visualized with DAPI (A). Increased levels of triglycerides were observed within the NASH-treated tissues as compared to control-treated tissues. Mean ± SD, n=4 MIts, ***p≤0.001, compared NASH treated samples to control. (B) Histology (H&E) staining demonstrated an increased accumulation of lipids within NASH-treated tissues as compared to the control (C).](image)

Effects of NASH Stimuli
Increased secretion of inflammatory markers elevated in NASH patient serum

![Figure 3. NASH stimuli increase the secretion of pro-inflammatory markers in the NASH treated samples as compared to the control treated samples. Luminex analysis of secreted cytokines and chemokines at day 5 of treatment with NASH stimuli. Mean ± SD, n=4 MIts, ***p≤0.001, compared NASH vs control, one exp. out of n=3.](image)

Increased pro-collagen type I secretion, which is inhibition by ALK5i

Drug Efficacy Testing
Decrease in inflammatory markers secretion in NASH after Elafibranor treatment, similar to clinical findings

![Figure 4. NASH stimuli increase the secretion of pro-collagen type I in NASH-treated samples as compared to control-treated samples. TGF-β1 pathway inhibitor ALK5 significantly decreases secretion of pro-collagen type I as compared to NASH-treated samples. Pro-collagen type I was measured using Pro-Collagen type I kit (Colabo). Mean ± SD, n=4 MIts strongly ≤0.001 vs NASH control, *p≤0.01, *** p≤0.001, NASH vs NASH+ALK5i, 1 exp. of n=3.](image)

Increased deposition of fibril collagen types III, similar to clinical findings

![Figure 5. NASH stimuli increase the deposition of fibril collagen types I and III as detected with Sirius Red staining in NASH-treated samples as compared to control treated samples. TGF-β1 pathway inhibitor ALK5 decreases deposition of fibril collagen as compared to NASH-treated samples.](image)

Summary and Conclusions
- Using our 3D InSight™ microtissue technology, we established a human liver disease model incorporating all liver cell types relevant for NAFLD and NASH pathogenesis.
- Upon treatment with NASH stimuli, 3D human liver disease co-culture model recapitulates key aspects of NASH in patients (e.g., lipid loading, secretion of proinflammatory cytokines, and initiation of fibrosis).
- ALKi inhibitor decreases secretion pro-fibrotic marker pro-collagen type I as compared to NASH-treated samples.
- Elafibranor decreases secretion of pro-inflammatory cytokines/chemokines, similar to clinical data.
- We suggest our NASH model as a promising tool to understand NASH pathogenesis and to test efficacy of novel drug candidates.