Developing a phenotypic *in vitro* model for progression of liver steatosis

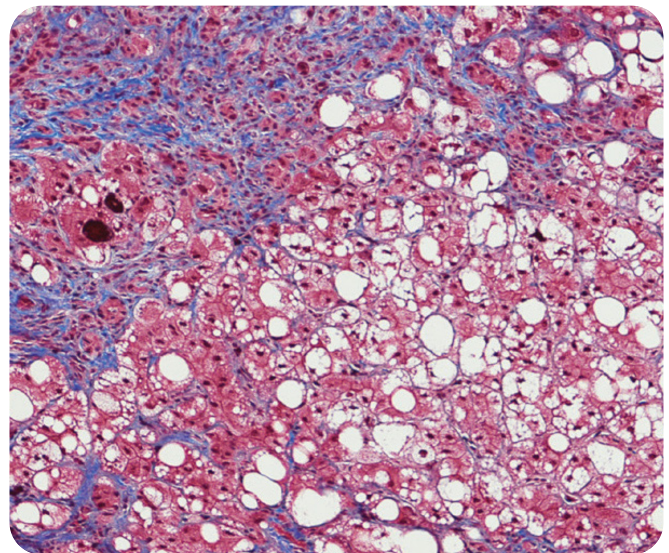
Introduction

Hepatic steatosis, a reversible state of metabolic dysregulation, is the first step in the progression of nonalcoholic fatty liver disease (NAFLD). It is characterized by excessive intracellular lipid accumulation and can progress to nonalcoholic steatohepatitis (NASH) and hepatocellular injury.

The incidence of NAFLD in Western society is approximately 30% in the general population; a figure that increases to 70-95% among individuals with type 2 diabetes and obesity. Although the metabolic syndrome plays a role in most NAFLD patients, a significant patient population is lean with few metabolic conditions. Sedentary lifestyle and a high-fat diet contribute to the accumulation of fat in hepatocytes of NAFLD patients, mainly in the form of triacylglycerols (TAGS). If accumulation exceeds the liver's metabolic capacity, steatotic hepatocytes become more vulnerable to various insults in progressive NAFLD.

Previous research has linked the endoplasmic reticulum (ER) stress response to exacerbated liver steatosis and NAFLD progression. When high levels of saturated fatty acids (FAs) and misfolded proteins alter ER homeostasis, stress signaling pathways, and notably the unfolded protein response (UPR) pathway, are triggered creating a lipotoxic environment in the liver. Disruption of ER homeostasis has been observed in the liver tissues of individuals with NAFLD.

Currently, there are no FDA-approved medicines to treat NAFLD or NASH, and the complex molecular and genetic mechanisms are not completely understood.



There is therefore an unmet clinical need to develop *in vitro* disease models to recapitulate the cellular properties of NAFLD for drug discovery and development.

To address this, Siobhan Malany, from the Conrad Prebys Center for Chemical Genomics in Florida, US, and colleagues developed a model of hepatic steatosis in functional human induced pluripotent stem cell-derived hepatocytes (hiPSC-Hep).[1] Results of the study suggest the model can serve as a translational discovery platform to help understand the molecular pathways involved in NAFLD and facilitate the identification of novel therapeutic molecules based on high-throughput screening strategies.

This article discusses some of the study's main approaches, findings, and potential applications for drug discovery research.

Morphological and functional characterization of hiPSC-Hep

The researchers first set out to determine whether hiPSC-Hep present similar morphological characteristics to primary hepatocytes *in vitro*. hiPSC-Hep were plated on collagen I-coated 384-well plates and visualized using an Operetta CLS™ high-content analysis system (Revvity) at different stages of maturation post thaw. Functional characterization was assessed by quantifying albumin and urea production at the end of the nine-day maturation cycle, analyzed by enzyme-linked immunosorbent assay (ELISA), and read on an EnVision® multimode plate reader (Revvity). The relative expression of common genes was assessed using reverse transcription quantitative PCR (RT-qPCR) analysis and results were compared with expression data from primary hepatocytes seeded for 24 and 48 hours in parallel.

High-content imaging confirmed that the hiPSC-Hep exhibited key hepatic characteristics, such as glycogen storage, bile canaliculi function, and albumin and lipid accumulation, similar to those shown by primary hepatocytes. Gene expression profiling established that the hiPSC-Hep were fully differentiated and resembled the hepatic morphology, phenotype, and functionality of adult primary hepatocytes.

Developing a steatotic phenotype

To confirm the development of a steatotic phenotype in hiPSC-Hep, Malany and colleagues characterized ER stress-induced hepatic metabolic dysfunction in the cells. To achieve this, they developed a multiparametric high-content 384-well platform where hiPSC-Hep were exposed to 25 µM oleic acid (OA) in the presence of varying concentrations of palmitic acid (PA) to mimic nutrient overload, with or without the ER stressor thapsigargin (TG). Lipid accumulation was analyzed using an Operetta high-content analysis system (Revvity) and results are shown in Figure 1.

Imaging analysis revealed that treatment with TG alone did not cause significant lipid droplet accumulation. However, a 2-, 2.7- and 5.6-fold increase in lipid levels was observed when hiPSC-Hep were additionally exposed to 25 µM OA, or a mixture of 25 µM OA with 50 µM or 200 µM PA, respectively, compared with bovine serum albumin (BSA)-treated controls (Fig 1A-C).

To confirm whether TG-induced ER stress altered the capacity of hiPSC-Hep to uptake FAs and/or metabolize them, a fluorescent FA analog labeled with BODIPY was tracked in the cells. Comparison of treatment with a FA mix alone or FA in the presence of TG (TG-FA) revealed that TG-induced ER stress increased exogenous FA analog accumulation, as measured by an increase in cell-associated fluorescence (Fig 1D and E).

To explore whether FA-induced ER stress led to cell death by apoptosis in their model, Malany et al. measured the activity of apoptosis effector caspase 3/7 in treated hiPSC-Hep by live-cell imaging. Results demonstrated that there was no significant change in caspase 3/7 activity or nuclear area in TG-FA-treated versus BSA-treated hiPSC-Hep after 18 hours (Fig 1F and G).

Together, these results show that exposure of hiPSC-Hep to FAs in the presence of an inducer of ER stress triggers a nearly six-fold increase in TAG accumulation, which is comparable to hepatocytes isolated from steatotic human livers versus those obtained from healthy livers. Furthermore, the studies indicate that a reproducible response to lipid accumulation and ER stress can be measured in hiPSC-Hep, without acutely inducing cell death.

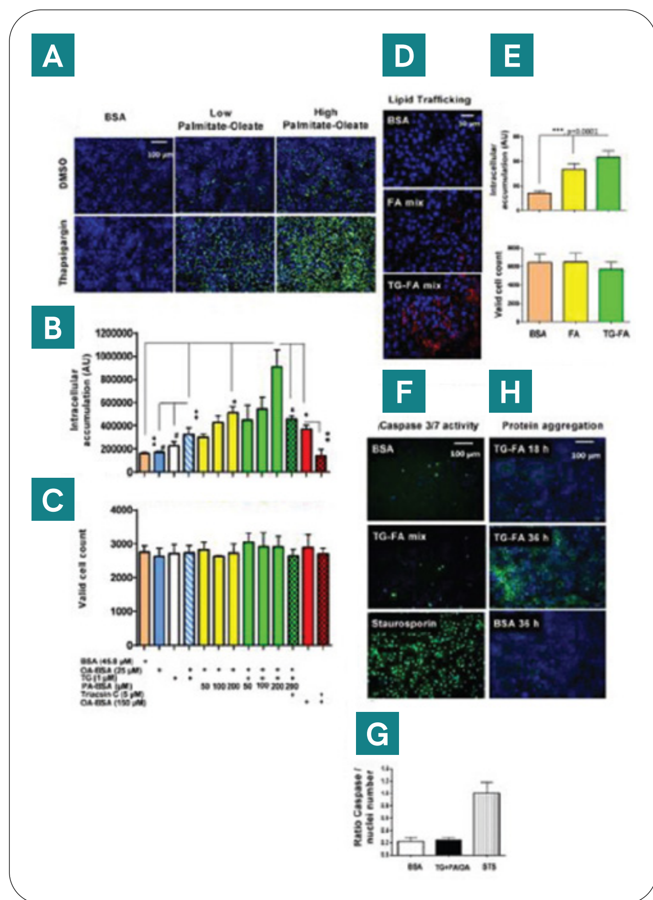


Figure 1: Development of a steatotic phenotype in hiPSC-Hep. (A) BODIPY 493/503 staining of neutral lipid droplet (green) induced by co-treating cells with 50 (low) or 200 (high) µM PA and 25 µM OA with or without thapsigargin (TG) for 18 h compared with BSA-treated cells; nuclei were stained with Hoechst dye (blue). Scale bar: 100 µM. (B,C) Quantification of immunofluorescence in A in the BODIPY channel for lipid accumulation (B) and nuclear channel for number of valid nuclei (C). Data are mean ±s.d. for three experimental determinations, where each determination includes the average of three wells and seven fields per well. *P≤0.01; **P≤0.006; #P≤0.03. (D) 10 µM fluorescent FA C12 analog uptake and accumulation in the BODIPY-FA 558/568 channel (red) in the presence of BSA, FA mix and TG-FA cocktail (TG-FA mix) treatments. Scale bar: 50 µM. (E) Quantification of fluorescent FA analog in BODIPY 558/568 and nuclear channels. (F) Evaluation of caspase 3/7 enzyme activation by confocal live imaging of hiPSC-Hep treated with BSA, TG-FA cocktail or staurosporin control for 18 h in the presence of CellEvent dye (green) and Hoechst (blue). Scale bar: 100 µM. (G) Quantification of caspase 3/7-positive hiPSC-Hep. Data are mean ±s.d for two experimental determinations, where each determination includes the average of three wells and seven fields per well. (H) Evaluation of unfolded protein accumulation by confocal live imaging of hiPSC-Hep co-treated with 10 µM Thioflavin T and TG-FA cocktail and captured at 18 h and 36 h. Images of cells treated with BSA were used as a negative control. Scale bar: 100 µM. (Source: Malany et al.[1])

Transcriptome pathway analysis

To ascertain the genomic events associated with TG-FA treatment and identify which pathways were most affected in hiPSC-Hep, the researchers performed transcriptome and Gene Ontology (GO) enrichment analysis. They observed that the most significantly enriched pathways in the TG-FA phenotype were associated with protein processing in the ER, mineral absorption, metabolic pathways, drug metabolism, and biosynthesis of amino acids. Furthermore, intercellular lipid accumulation was associated with significant upregulation of genes in the ER stress-UPR axis.

Thirty-four modified genes related to three ER stress branches were identified, and RT-qPCR analysis revealed that expression of key ER stress markers was inducible at four hours but reduced at 18 hours post-treatment. This indicates that the cells adapt to restore homeostasis and protect themselves from apoptosis. An additional goal of the model was to induce dysregulation of genes involved in *de novo* lipogenesis and disease progression. RT-qPCR analysis of genes involved in hepatic *de novo* lipogenesis, lipid export and mitochondrial β-oxidation revealed ER stress-associated negative feedback regulation in the model for genes involved in lipid metabolism.

Validation of the model

To validate their model, the researchers used obeticholic acid (OCA), a clinically advanced therapeutic and farnesoid X receptor (FXR) agonist that has previously been shown to benefit NAFLD/NASH patients in clinical trials and improve phenotype and gene signatures in liver-based *in vitro* models. They demonstrate that OCA inhibits the phenotype in the hiPSC-Hep model of steatosis, as shown in Figure 2.

Specifically, when the steatosis stimuli-induced hiPSC-Hep were challenged with OCA, TAG accumulation was dose-dependently reduced. RT-qPCR analysis revealed that the beneficial effect of OCA was specifically mediated by activation of the FXR pathway in hiPSC-Hep treated with TG-FA; OCA treatment upregulates the FXR target genes *SHP* and *FGF19*.

To validate their key results, the researchers used commercially available hiPSC-Hep generated from a different individual than the donor line used for their presented data. They observed similar lipid accumulation and gene expression levels for NAFLD biomarkers in the presence of TG-FA. OCA activity was also similar in the independent hiPSC-Hep donor line.

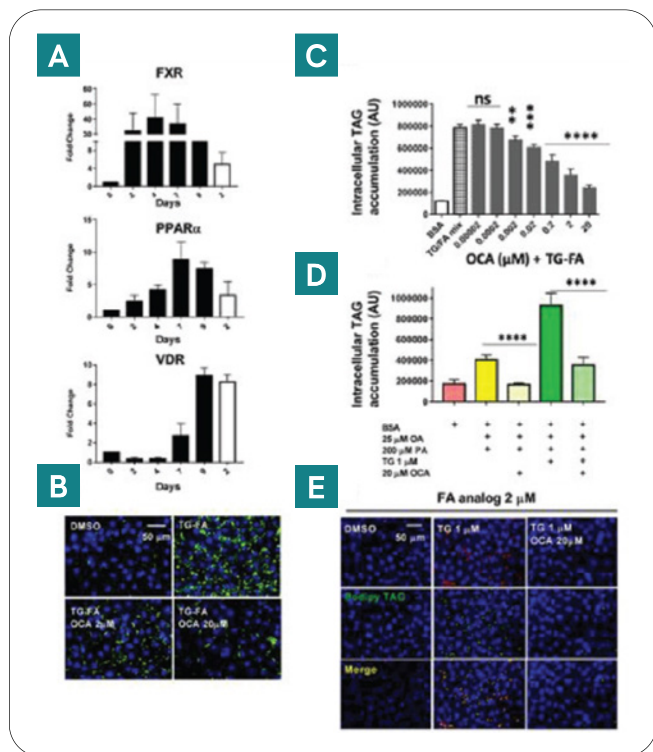


Figure 2: OCA inhibits phenotype in the hiPSC-Hep model of steatosis. (A) RT-qPCR analysis of key nuclear receptors expressed in hiPSC-Hep from day 0 to 9 in culture (black bars) and compared with primary hepatocytes at day 2 in culture (white bars). The fold induction values are relative to day 0. (B) BODIPY labeled neutral lipids (green) show increased prevention of lipid accumulation by 2 µM and 20 µM OCA in TG-FA-treated cells compared with BSA-treated control cells. Nuclei are stained blue. Scale bar: 50 µM. (C) Titration of OCA in 384 w showed dose-dependent reduction of lipid accumulation. Data are mean ±s.d. of three experimental determinations, each performed with triplicate replicates. (D) An independent hiPSC-Hep line (donor 1279) showed similar lipid accumulation induced by FA (yellow bar) and TG-FA (dark green bar) as in Fig. 1A and Fig. S4A and C (donor 1434), and similar inhibition by 20 µM OCA as in C (light yellow and light green bars), compared with BSA-treated cells (pink bar). (E) OCA inhibits the uptake of exogenous fluorescent-labeled BODIPY-C12 FA analog and incorporation into TAG. BODIPY-C12 FA uptake (2 µM, red) by hiPSC-Hep and its incorporation into lipid droplets (green) occurred mainly in TG-treated cells (1 µM). As shown by merged staining (yellow, bottom row), BODIPY-C12 was incorporated into stained BODIPY 493/503-positive lipid droplets within 18 h (middle row), indicating that the FA analog was esterified for lipid droplet incorporation. 20 µM OCA co-treatment with BODIPY-C12 prior to treatment with 1 µM TG prevented FA uptake and TAG synthesis (right column). Scale bar: 50 µM. $^{**}P \le 0.01$; $^{***}P \le 0.002$; $^{****}P \le 0.001$; ns, nonsignificant. (Source: Malany et al.[1])

Conclusion

The present study shows the development and validation of a NAFLD model in hiPSC-Hep, created by ER stress-induced steatosis. The hiPSC-Hep disease phenotype exhibited metabolic changes characteristic of steatosis associated with NAFLD. The researchers conclude that the NAFLD model permits interrogation of molecular pathways involved in disease progression and the discovery of new therapeutics by high-throughput screening technologies.

Reference

1. Parafati M, Kirby R, Khorasanizadeh S, Rastinejad F, Malany S. A nonalcoholic fatty liver disease model in human induced pluripotent stem cell-derived hepatocytes, created by endoplasmic reticulum stress-induced steatosis. *Disease Models & Mechanisms*. 2018;11(9):dmm033530.

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