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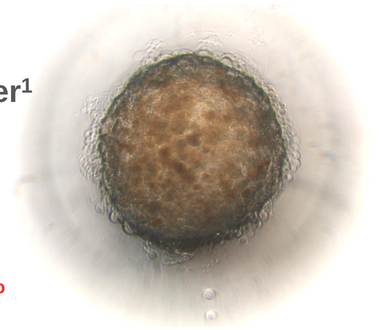
Primary Human Hepatocytes 3D in vitro Culture Model for Studying Hepatic Function



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INTRODUCTION

Primary Human Hepatocyte (PHH) culture provides the closest in vitro model to human liver that can produce a metabolic profile of a given drug very similar to that found in vivo. Hence, PHH culture is the gold standard for studying the in vitro hepatic biology, liver function, and drug induced hepatotoxicity. The conventional way of culturing PHH in 2-dimension (2D) has major pitfalls. The PHH rapidly de-differentiate and lose the hepatic specific functions in a week. Therefore, there is a need for more robust in vitro models that reflect in vivo liver biology more accurately and maintains the liver functions for a longer time. 3-dimensional (3D) hepatic in vitro models have gained a lot of attention for their ability to recapitulate the hepatic function and greater longevity.

Recently we have developed an easy-to-assemble user-friendly in vitro Primary Human Hepatocyte (PHH) 3D-spheroid model. The 3D-hepatic spheroids are viable for at least 4 weeks in culture and remain phenotypically stable, retaining the hepatocyte-specific functions.

MATERIALS AND METHODS

Spheroid culture

Hepatic spheroids were formed using Gibco™ cryopreserved spheroid–qualified human hepatocytes (Catalog No. HMCPSQ) following the user guide [1]. Each well contained PHHs between 750 and 7500 hepatocytes, depending on the experimental conditions. The spheroids formed within 5 days of cell seeding. Starting on day 5, half of the plating medium was changed every 48–72 hours. Following this culture condition the 3D hepatic spheroids can remain viable at least up to 4 weeks.

Metabolic assay

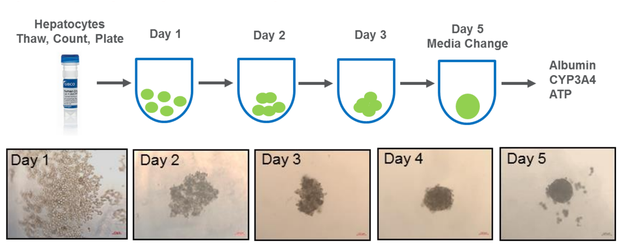
As the liver is the primary site of metabolism for most drugs, primary hepatocytes are the most popular in vitro tool to evaluate hepatic drug metabolism. However, the efficiency of 3D hepatic spheroids for studying drug metabolism is relatively unknown. In order to study the biotransformation of various drugs by Cytochrome P450 (CYP) enzymes in hepatic shperoids, 3000 PHH/well were seeded in Nunclon™ Sphera™ plate and cultured as described above. On Day 9 of the 3D culture 2D hepatocytes were seeded using 80,000 PHH per well as described in the user manual [2]. On day 10, six test articles were evaluated in both 2D and 3D PHH cultures in serum-free Gibco™ William's E Medium. The compounds were selected such that several CYP enzymes that are important for hepatic drug metabolism could be interrogated. Table 1 lists the identity of each compound and the CYP enzymes primarily responsible for their metabolism, the metabolites analyzed, and the drug concentrations tested. Cell culture samples collected from both 2D and 3D cultures were analyzed for metabolite formation using the Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer.

Table 1. Cytochrome P450 enzymes assayed and compounds analyzed in the metabolic assays.

CYP enzyme assayed	Compounds Used	Metabolites Analyzed	Concentration (µM)
CYP2D6	Dextromethorphan	Dextrorphan	150
CYP3A4	Midazolam	1-Hydroxymidazolam	100
CYP1A2	Phenacetin	Acetaminophen	200
CYP2B6	Bupropion	Hydroxybupropion	500
CYP2B6	Testosterone	6β-Hydroxytestesterone	400
CYP2C9	Tolbutamide	4-Hydroxytolbutamide	500

RESULTS

Figure 1. Work Flow of self assembly and characterization of primary hepatocyte into 3D-spheroid



Gibco™ Human Spheroid-Qualified Hepatocytes are seeded in Nunclon™ Sphera™ low attachment U-bottom 96-well Microplates (Catalog No. 174925), where they self assemble into 3D-spheroids by day 5. By day 7 they are ready for further biochemical assays and characterization. The self assembly of the PHH in 3D-spheroid does not require any intermittent handling or maneuvering.

Figure 2. Optimization of Hepatic Spheroids: Variable Cell Numbers.

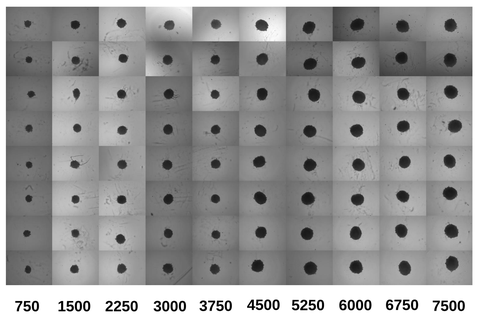
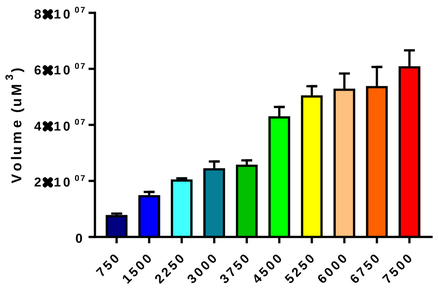
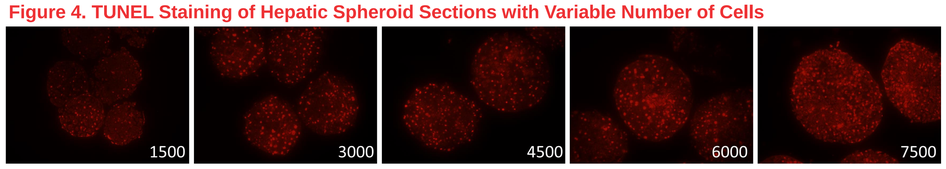


Figure 3. Comparison of Spheroid volumes with variable number of cells.



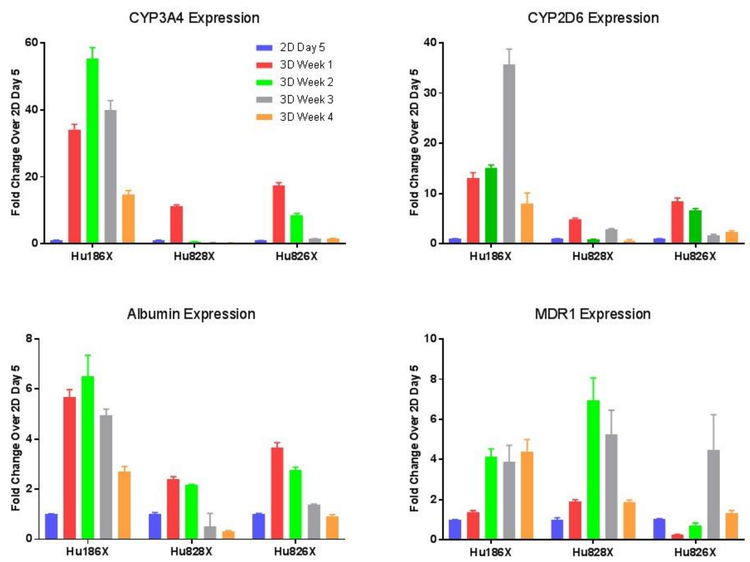
PHH were seeded at different cell densities and the spheroids were imaged used EVOS Cell Imaging System. The diameters of at least 5-spheroids for each size were measured using ImageJ, and the volume of each of the spheroids were calculated.

Figure 4. TUNEL Staining of Hepatic Spheroid Sections with Variable Number of Cells



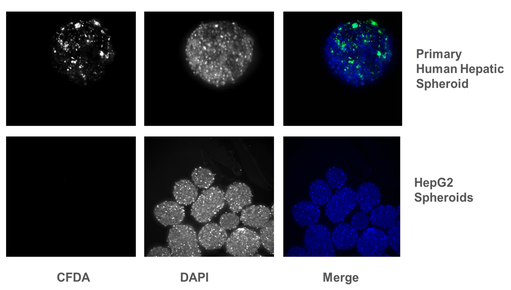
Paraffin fixed sections of hepatic spheroids collected at day 10 with variable number of cells were H&E stained and micrographed at 20X optical zoom. These sections also underwent in situ cell death detection (DNA-strand breaks) using a fluorometric Click-iT™ TUNEL Assay protocol (Invitrogen C10617). The red fluorescence denotes apoptotic cells, which seemed to be homogenously distributed throughout the spheroids of different sizes.

Figure 5. Elevated Gene Expressions 3D Hepatic Cultures compared to conventional 2D culture.



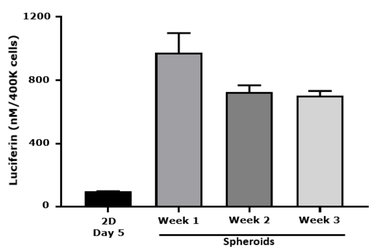
RT-qPCR results for albumin, MDR1, CYP3A4, and CYP2D6 mRNA levels, respectively, of 3 individual lots of Gibco 3D spheroid–qualified human hepatocytes. Gene expression levels of 3D cultures at different timepoints were normalized to day 5 of 2D culture. Each 3D spheroid sample contained a pool of 16 spheroids. Results are ±SEM, n = 3 spheroid pool samples.

Figure 6. Formation of Bile Ducts in Hepatic Spheroids.



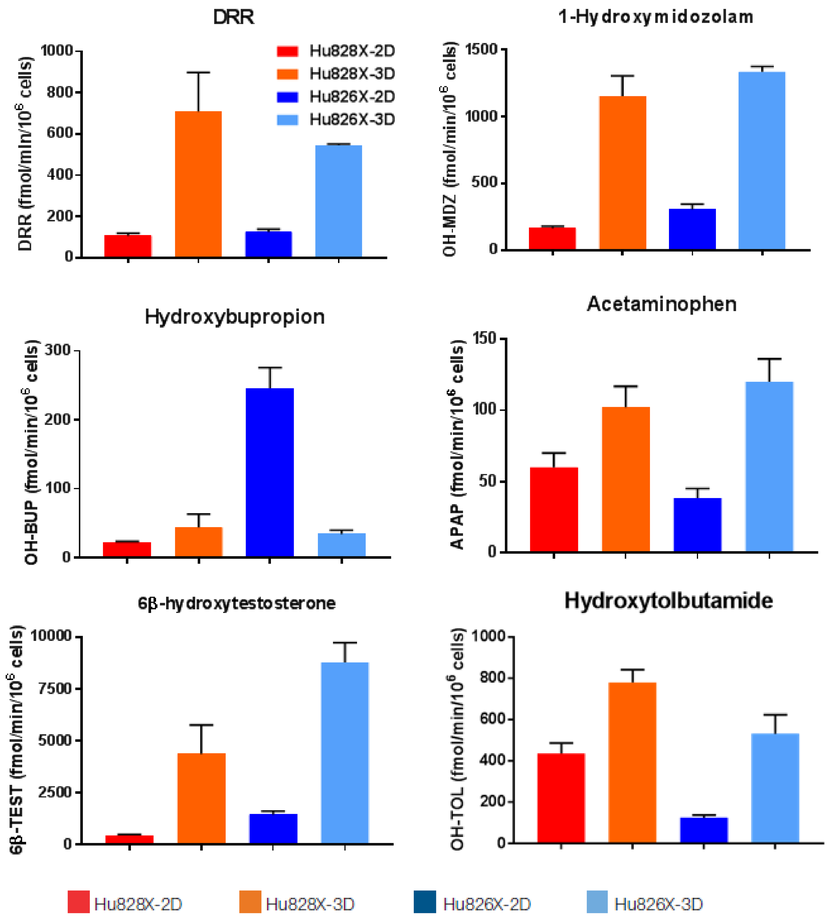
Bile duct formation in the primary human hepatic spheroids was analyzed by treating 7-day old spheroids with bile tracer CFDA.HepG2 spheroids were also treated with CFDA as negative control.

Figure 7. Higher Temporal CYP3A4 activity in 3D hepatic cultures.



CYP3A4 activity was measured using P450 ProGlo assay (Promega). Cyp3A4 activity of the 3D culture was found to be higher than that of 2D culture, indicating higher metabolic function of 3D culture. Results are mean +/- SD, n = 3.

Figure 8. Higher metabolic activities in 3D cultures compared to conventional 2D hepatic culture system.



The metabolites quantified using HRMS were converted to mole amounts based on the standard curves of the respective metabolites. Two different lots of PHH, Hu828X and Hu826X (partially redacted), were used in this assay. Results were normalized to incubation time of individual substrates and number of cells per well in the 2D and 3D cultures. Data is the mean ±SD; n = 3.

CONCLUSIONS

- **Gibco™ Primary Human Hepatocytes (HMCPSQ)** can easily be assembled into a 3D culture in 5 days using either **Gibco™ Hepatic Spheroid Kit (A41390)** or Nunclon™ Sphera™ low attachment U-bottom 96-well Microplates, Gibco™ plating media and plating supplements.
- The Primary Hepatic Spheroids are functionally viable for at 4-weeks, which is a significant progress in primary hepatocyte culture considering the conventional 2D-culture methods.
- The 3D hepatocyte culture requires a significantly lower number of cells than that of the 2D counterpart, which opens new possibilities for high throughput assays using PHH.
- 3D hepatocyte culture are superior with respect to gene expression and metabolic activities compared to conventional 2D hepatic culture system.

FUTURE DIRECTIONS

- Assessment of in vitro CYP Induction in 3D Hepatic Spheroids.
- Coculture of PHH with non-parenchymal cells to establish in vitro liver model.
- 3D culture of PHH isolated from diseased livers (such as NAFLD, NASH and hepatic fibrosis).

REFERENCES

1. Thermo Fisher Scientific. User Guide: Cryopreserved 3D-Spheroid Qualified Human Hepatocytes. Pub. No. MAN0018280.
2. Thermo Fisher Scientific. Thawing and Plating Cryopreserved Hepatocytes. Protocol available at: https://www.thermofisher.com/us/en/home/references/protocols/drug-discovery/adme-tox-protocols/thawing-and-plating-hepatocytes-protocol.html.

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