Optimization of HepaRG Workflow for Use in 3D Spheroid Models

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ABSTRACT

Growing evidence suggest that cells cultured as 3-dimensional (ie. 3D) spheroids exhibit more in vivo-like cellular properties than conventional 2D monolayer cultures which underscores their potential as more physiologically relevant in vitro culture systems. The human hepatic cell line HepaRG, is a well-established model for studying drug metabolism and toxicity, and there have been numerous reports indicating its utility as a 3D model. However, these studies primarily recapitulate the standard monolayer (ie. 2D) workflow, which is based on the dynamic temporal metabolic profile of cytochrome P450 (CYP) enzymes, to culture cells in 3D spheroids. Since gene expression profiles and the behavior of cells frequently differs in 2D and 3D cultures, we hypothesized that the commonly used HepaRG spheroid culture protocol has not been optimized. To address this, we compared the temporal profile of basal CYP3A4 activity in 2D and 3D HepaRG cultures to determine if cells may function differently between these culture formats. HepaRG monolayers were grown on Collagen I coated plates; 3D spheroids were formed in Nunclon Sphera 96-well U-bottom plates. Basal CYP3A4 activity in 2D and 3D culture was measured every 24 hours for 10 days and normalized to cell viability. Consistent with published data, our HepaRG cultures formed spheroids in 2-3 days of culture and the size of the spheroid was directly proportional to the number of cells seeded. Preliminary data confirmed that 2D HepaRG cultures had high CYP3A4 activity in the first 24 hours of culture with a subsequent reduction that slowly recovered to peak activity levels at day 6 of culture. Interestingly, these spheroid cultures have significantly higher basal CYP3A4 activity (~200 fold) everyday of culture compared to 2D cultures. During the aggregation phase of the spheroid culture (ie. Day 1-2) CYP3A4 activity was elevated and reached peak levels at Day 3, when spheroids are fully formed. From Day 3-10, HepaRG spheroids showed relatively constant levels of CYP3A4 activity. In conclusion, these results suggest that the temporal profile of commonly studied metabolic enzymes is different between HepaRG monolayer and spheroid cultures. Moreover, the difference HepaRG spheroids peak activity levels suggest that the spheroid culture method offer more flexibility for experimental design with potentially shorter culture times.

INTRODUCTION

The hepatic stem cell line HepaRG displays the major characteristics of primary hepatocytes but with two main advantages; lack of donor variability and the ability to proliferate. The HepaRG cell line is composed of approximately equal amounts of hepatic-like cells and cholangiocyte-like cells. Based on these features HepaRG cells have become one of the workhorse models for studying drug metabolism and toxicity. However, recent literature suggests HepaRG spheroids can display near in vivo levels of metabolic activity[1]. These studies recapitulate the monolayer workflow to generate spheroid models[2]. Since cell behavior is often different in 3D formats, there is a need to determine if the HepaRG cell culture workflow can be optimized for 3D spheroid applications. Here we describe ongoing efforts to optimize a HepaRG 3D culture workflow based temporal metabolic profiles of CYP enzymes. Further we present important spatial organization of cell types that may have importation considerations for data interpretation.

MATERIALS AND METHODS

Media and Reagents:

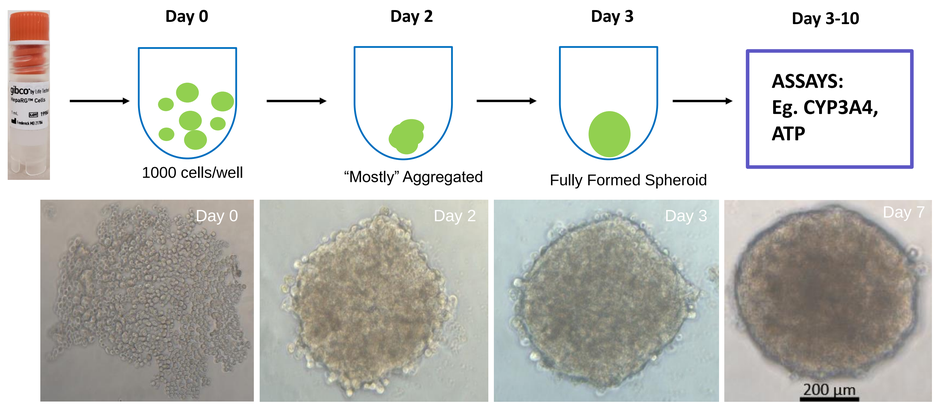
- HepaRG Plating and General Purpose Medium – Williams Media E (WEM) (Gibco™ A1217601), HepaRG Plating and General Purpose Supplement (Gibco™ HPRG770)
- HepaRG Maintenance and Metabolism Medium - WEM (Gibco™ A1217601), Maintenance and Metabolism Supplement (Gibco™ HPRG720)
- HepaRG Differentiated Cells - (Gibco™ HPRGC10)
- Nunclon™ Sphera™ super low attachment U-bottom 96-well microplates (Cat# 174925)
- Gibco™ Collagen 1 Coated 24-well plates (Cat# A1142802)
- WellWash™ Versa Microplate Washer (Cat# 5165050)
- Countess™ II Automated Cell Counter (Cat# AMQAX1000)

HepaRG Spheroid Protocol

1. Cryopreserved HepaRG (Gibco™) vial was thawed quickly in a 37ºC water bath. Upon thawing the cells were promptly transferred into a 15 mL centrifuge tube containing 9 mL Plating and General Purpose Medium.
2. The tube was centrifuged at 500 x *g* for 3 minutes. After centrifugation the supernatant was discarded.
3. The cell pellet was gently re-suspended in 5 mL of HepaRG plating media. Cell counting was performed with Trypan blue using a Countess™ II Automated Cell Counter.
4. Appropriate volume of cell suspension was prepared to contain 1,000 cells/200 µL media. Using a multichannel pipette 200uL of the cell suspension was added into individual wells of Nunclon™ Sphera™ super low attachment U-bottom 96-well microplates.
5. The Sphera™ microplates were centrifuged at 200 x *g* for 2 minutes to allow cells to group at the bottom of the wells.
6. The Sphera™ Microplates were transferred to an incubator (37ºC, 5% CO_2, humidified) and allowed to sit undisturbed in Plating Media for 1 day before changing to Maintenance and Metabolism medium. Media was changed via 3 consecutive 75% media changes using the Wellwash™ versa.
7. Spheroids were maintained in the maintenance and metabolism medium with 3 consecutive 75% medium change every 24 hours.
8. Basal CYP3A4 activity was measured every 24 hours and normalized to viable cell number.

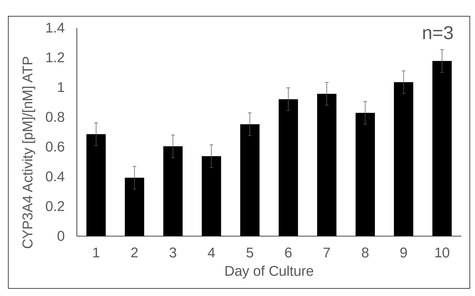
RESULTS

Figure 1. Work Flow of assembly and characterization of HepaRG cells into 3D spheroids.



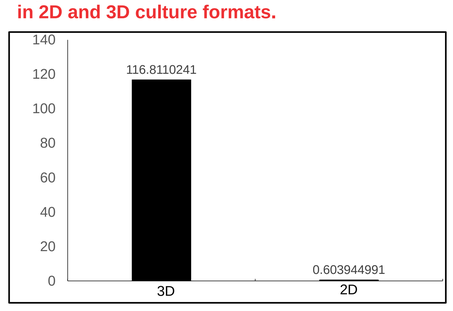
HepaRG cells seeded in Nunclon™ Sphera™ super low attachment U-bottom 96-well microplates self-assembled into spheroids by day 2., and fully formed by day 3 of culture.

Figure 2. Basal CYP3A4 activity temporal profile in monolayer cultures.



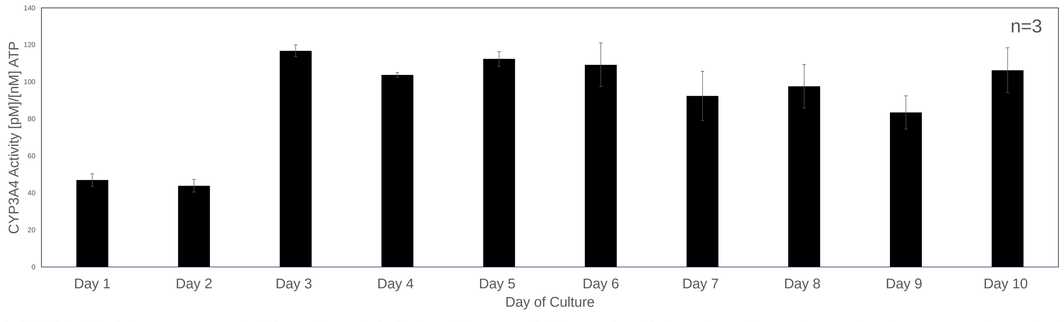
Basal CYP3A4 activity recorded in HepaRG Spheroids every 24 hours for 10 days highlights a dynamic temporal metabolic profile. Enzyme activity was normalized to viable cell number. n = three separate experiments consisting of two technical replicates of 8 pooled spheroids each.

Figure 3. Comparison of HepaRG basal CYP3A4 activity in 2D and 3D culture formats.



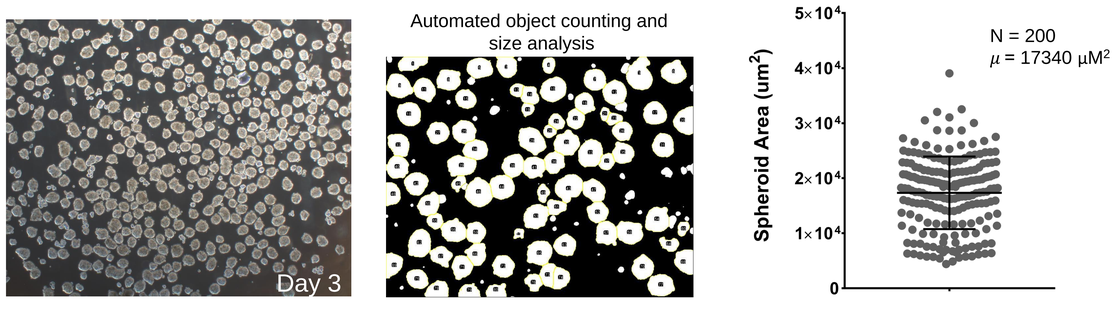
Basal CYP3A4 activity is ~200 times higher in spheroids than monolayer cultures

Figure 4. HepaRG 3D Temporal Metabolic Profile



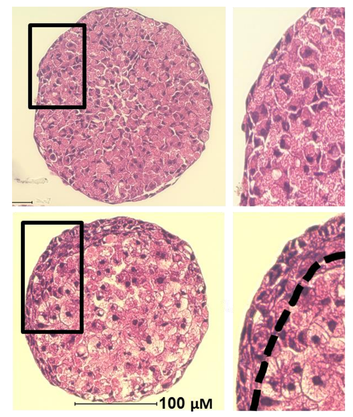
Basal CYP3A4 Activity was recorded from HepaRG Spheroids every 24 hours for 10 days in order to determine the temporal metabolic profile. . . n = three separate experiments consisting of two technical replicates of 8 pooled spheroids each.

Figure 5. HepaRG spheroids can easily be scaledon rocking platforms to generate consistently sized spheroids



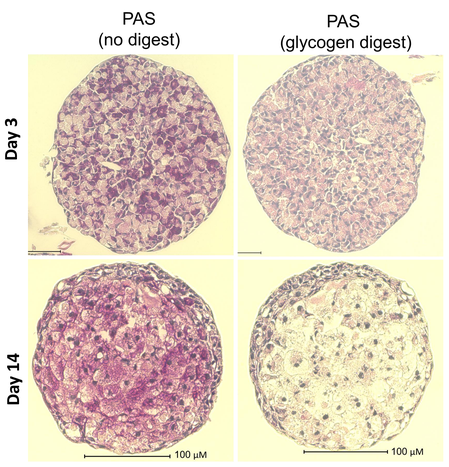
3 Million HepaRG cells were seeded into each well of a Nunclon™ Sphera™ 6-well flat bottom plate and placed on an orbital shaker (85rpm). By day 3 thousands of spheroids have formed with and average size of 17340 µM². This methodology allows researchers to scale up easily for higher throughput screening.

Figure 6. He & E Staining of a Day 14 HepaRG spheroid .



HepaRG spheroids show spatial organization and segregation of cell types that becomes more pronounced over time. The two zoomed in images on the right show one cell type forms the outer shell of the spheroid.

Figure 7. Periodic Acid- Schiff (PAS) staining of HepaRG Spheroid indicates glycogen storage functionality



HepaRG spheroids show increased glycogen storage over time.

CONCLUSIONS

- Gibco™ HepaRG cells can easily be assembled into viable 3D spheroids days using Nunclon™ Sphera™ super low attachment U-bottom 96-well microplates, or using Nunclon™ Sphera™ super low attachment flat bottom 6-well plates on an orbital shaker.
- HepaRG spheroids have basal CYP3A4 activity that is orders of magnitude higher than 2D HepaRG monolayers.
- The 3D HepaRG spheroids show peak basal activity by day 3, as opposed to days 6-10 for 2D monolayers.
- Hepatocyte-like cells in HepaRG spheroids store glycogen suggesting they are functional.
- Based on cell morphology and glycogen storage staining pattern, the spheroids exhibit spatial organization of outer cholangiocyte-like cells and inner hepatocyte-like cells.

FUTURE DIRECTIONS

- Investigate other CYP enzymes in 2D vs 3D HepaRG cultures.
- Benchmark induced and non-induced HepaRG CYP activity against primary hepatocyte cultures.
- Further investigate the organization and ratio of cholangiocyte-like cells to hepatocyte-like cells in 3D HepaRG spheroids.

REFERENCES

1. Ramaiahgari, Sreenivasa C., et al. Three-Dimensional (3D) HepaRG Spheroid Model With Physiologically Relevant Xenobiotic Metabolism Competence and Hepatocyte Functionality for Liver Toxicity Screening." *Toxicological Sciences*, 2017.
2. Hendriks, D., Puigvert, L. F., Messner, S., Moritz, W., & Sundberg, M. I. (2016). 3D hepatic spheroid models for the detection and study of compounds with cholestatic liability. *Toxicology Letters, 258*.