# 3D Spheroidal Human Liver Models for Drug Toxicity Testing

BY

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THESIS

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| | |
|---|---|
| COL | Collagen |
| CYP450 | Cytochrome P450 |
| DILI | Drug-Induced Liver Injury |
| DMSO | Dimethyl sulfoxide |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FDA | Food and Drug Administration |
| FIAU | Fialuridine |
| GA | Growth-arrested |
| HC | 7-hydroxycoumarin |
| HSC | Hepatic Stellate Cells |
| HUM | Human |
| IL | Interleukin |
| LOX | Lipooxygenase |
| MOS | Margin of Safety |
| MPCC | Micropatterned coculture |
| NGA | Non-growth-arrested |
| NIH | National Institute of Health |
| NPC | Non-Parenchymal Cells |
| PEGDA | Poly (ethylene glycol) diacrylate |
| PHH | Primary Human Hepatocytes |

## LIST OF ABBREVIATIONS (continued)

| | |
|---|---|
| RNA | Ribonucleic acid |
| SMA | Smooth Muscle Actin |
| TGF | Total Growth Factor |
| TMB | Tetramethylbenzidine |
| ULA | Ultra-low attachment |
| UV | Ultraviolet |

**SUMMARY**

Drug-induced liver injury (DILI) is the primary cause of preclinical and clinical drug attrition, and drug withdrawals from the market. To mitigate this risk, several model systems are being developed for the prediction of DILI to prevent toxic pharmaceutical drugs from reaching clinical trials. In this study, we optimized a widely used three-dimensional liver model system, namely spheroids. Studies have shown the ability to maintain long term functionality of primary human hepatocytes (PHHs) using monoculture spheroids; however, not all PHH donors can form consistent spheroids, limiting the donor variation available for toxicity screens.

Therefore, this thesis primarily aims to a) characterize a coculture spheroids platform using two relevant cell types that have been previously shown to induce PHH functionality, hepatic stellate cells (HSCs) and murine embryonic fibroblasts (3T3 J2) utilizing a commercially available plate for spheroid formation, and b) fabricating microwells with a photocrosslinkable polymer material to create an efficient high drug screening platform. Towards the first aim, we optimized coculture conditions to include 3T3 J2s and HSCs with PHHs aiming to improve hepatocyte functionality and rate of spheroid formation. Our results suggest a coculture effect (higher functionality) in the spheroids for the majority of functions. Furthermore, we tested all the culture conditions for drug sensitivity using a panel of pharmaceutical drugs with various DILI categories. Contrary to the coculture effect for the functionality, monoculture spheroids showed a higher drug sensitivity in our studies.

Towards the second aim, we fabricated poly (ethylene glycol) diacrylate (PEGDA) microwells enabling multiple spheroid formation per well in a 96 well plate and full media changes, reducing handling time. PEGDA is considered a cytocompatible, non-toxic, and easy to use material. Our results suggest that the platform can successfully be used for future experimentation and optimization.

In conclusion, the thesis provides an understanding of coculture and monoculture spheroids with respect to hepatic functionality and drug sensitivity, opening opportunities to be used in several future applications.

# Chapter 1. 3D spheroidal human liver models for drug toxicity testing

## 1.1 INTRODUCTION

### 1.1.1 Background and Significance

Drug-induced liver injury (DILI) is the primary cause of preclinical and clinical drug attrition, and drug withdrawals from the market [1]. Human in vitro model systems are required to reduce DILI risk in humans and mimic human liver diseases[2] as there is a low concordance of human toxicity with animal models, specifically related to liver toxicity. The cited study suggests a need to obtain test compounds' metabolic transformation from ex vivo or in vitro model systems for DILI evaluation[3]. For example, fialuridine, a candidate hepatitis B pharmaceutical, led to the deaths of 5 out of 15 patients during clinical trials. An additional two patients required life-saving liver transplants due to human toxicity variation compared to animal models [4]. It was later discovered that fialuridine leads to abnormal mitochondrial activity and damage, causing accumulation of micro and macrovesicular fat in humans that was not present in laboratory animals during in vivo studies[5]. Also, as the FDA requires in vivo testing on one rodent and one non-rodent species during the preclinical drug development process, an optimized in vitro platform can serve in selecting the species closest to humans in terms of drug metabolism and predicting drug toxicity[6].

Primary human hepatocytes (PHHs) are considered the "gold standard" for drug metabolism studies[7] compared to cell lines and liver slices as they are amenable to high throughput studies and maintain the highest level of human relevance in terms of metabolism. However, PHHs de-

differentiate in a monolayer format (i.e. tissue culture plastic or glass coated with adsorbed collagen) and lose their hepatic functionality within a week[8]. Different culture techniques have been shown to maintain PHH functionality long-term, allowing for more accurate metabolism and toxicity predictions. One of the most widely used model system for functional maintenance of PHHs and predicting drug sensitivity is spheroids. Studies have shown that spheroids containing only human PHHs maintain hepatocyte functions and stability for five weeks[9]; however, the success rate of the formation of spheroids with only PHHs is below 80% among different donors, with only 65% of the donors maintaining viability long-term above 50% [10]. Thus, studies have incorporated human nonparenchymal cell (NPC) fractions with PHHs in the spheroid format to increase the functionality, stability, and longevity of PHH functions to carry out drug toxicity studies[10-12]; However, NPC fractions' role in drug sensitivity is unclear as there has been no significant improvement in the sensitivity of the model system for predicting hepatotoxicity compared to other studies[6, 11].

3T3 cell lines (mouse embryonic origin) have been widely used in previous coculture spheroid studies. For example, one study seeded primary rat hepatocytes with NIH 3T3 cells on galactosylated poly (vinylidene difluoride). The albumin secretion in the coculture condition was 5.1 times higher than the monoculture spheroids[13]. Another study used similar cell types, NIH 3T3 and rat primary hepatocytes in a polyethylene glycol microwells to demonstrate improved albumin functionality in the coculture condition[14]. Also, it has been shown that direct cell-to-cell contact is necessary for the enhancement of hepatocyte functions, and TGF-β1 seems to be a soluble factor that enhances hepatocyte functionality[15]. Apart from NIH 3T3, 3T3 J2

fibroblasts (a different clone of the 3T3 cell type) have been shown to provide key relevant factors such as extracellular matrix, decorin, and T cadherin that support hepatocytes in vitro[16]. Also, 3T3 J2 has been labeled as high inducers of hepatic functions compared to NIH 3T3[17]. While 3T3 J2 fibroblasts have been utilized in monolayer cultures, sandwich cultures, and micropatterned cocultures to induce hepatic functionality, their utilization in a spheroid format for drug toxicity evaluation is minimal. A study was recently published with the inclusion of 3T3 J2 with primary human hepatocytes in a microtissues format, polymerized collagen microtissue encapsulating primary human hepatocytes[18]. Also, to our knowledge, there has not been a complete comparison of monoculture and coculture spheroids containing PHHs and 3T3 J2s in terms of drug sensitivity. Therefore, we aim to introduce 3T3 J2 with primary human hepatocytes in a spheroid format to increase the model system's functionality and sensitivity and compare them with monoculture spheroids.

Hepatic stellate cells (HSCs), positioned in the liver's perisinusoidal space, make up 5-8% of the human liver, and store vitamin A[19]. Under different types of liver injury (e.g. fatty liver, hepatitis viral infection), HSCs become activated and play a role in liver fibrosis by secreting cytokines and extracellular matrix. HSCs specifically have been used as a coculture cell type in a spheroidal format, rather than the whole NPC fraction. For example, rat hepatocytes with a rat hepatic stellate cell line show overall spheroid growth in serum-free media with hepatocytes[20]; freshly isolated rat hepatocytes with activated stellate cells have shown an increase in functional activity such as cytochrome P450 enzymes[21, 22]; rat hepatocytes with primary rat HSCs were shown to interact via paracrine signaling[23]. HSCs have also been used with the rat hepatocytes to

predict the IC50 (concentration at which 50% of albumin/urea activity is reduced of two toxic drugs producing more consistent values across different dosing duration compared to monoculture spheroids[24]. However, the use of human HSCs with PHHs is minimal. Also, there has been minimal evaluation that directly compares the coculture of 3T3 J2s and HSCs with respect to supporting PHHs in a culture platform and predicting drug sensitivity. Therefore, we aim to introduce HSCs as an alternative cell type to support PHHs in a spheroidal format and perform drug toxicity studies on the model, providing a comprehensive comparison between 3T3 J2 and HSC in a spheroidal format with PHHs. Thus, after creating an optimized spheroid platform with three different conditions, 3T3 J2 coculture, HSC coculture, and monoculture, we dosed them with a set of known hepatotoxins and non-hepatotoxins at specific relevant concentrations to report the formats' sensitivity and specificity as well as the capability to produce more physiological values of drug toxicity.

Most of the recent spheroid studies have been performed in ultra-low attachment (ULA) plates, which are commercially available. While these plates have advantages, like consistent spheroid formation and requiring fewer cells, they also have disadvantages, including loss of spheroids during media change, tedious use and having only one spheroid per well, leading to low detectable concentrations of relevant hepatic markers. Thus, we provided an alternate 3D model system alongside the commercial ULA plate. We fabricated Poly (ethylene glycol) diacrylate (PEGDA) microwells in a 96 well glass bottom plate, each well containing about 80 microwells with 350μm diameter. PEGDA is considered a cytocompatible, non-toxic and easy to use

polymer[25]. We presented this model as a more efficient drug screening model system to be used for drug testing in the future.

### 1.1.2 Purpose of the study

The study's overall aim was to better understand different human liver model systems of 3D cultures, optimizing different conditions for improving the functionality and spheroid formation with a variety of donors and different coculture cell types to ensure reproducibility and donor variation, and their interaction with pharmaceutical drugs. We could completely optimize our spheroids model system with two cell types, including an all human liver spheroid model system with few limitations related to drug sensitivity.

## 1.2 MATERIALS AND METHODS

### 1.2.1 Fabrication of Poly (ethylene glycol) diacrylate (PEGDA) micro wells

PEGDA microwells were fabricated using the concept described in Fan et. al [26]. We utilized UV mediated photo crosslinking method for PEGDA microwell fabrication. However few surface modifications (pending publication) were made by Yong Duk Han, PhD.

### 1.2.2 Hepatocyte cultures

Plateable cryopreserved PHHs were thawed at 37°C for 120 seconds, followed by dilution in human hepatocyte maintenance media[27]. Cells were centrifuged at 500xg for 10 minutes, the supernatant was removed, and the PHHs were then resuspended in human hepatocyte maintenance media[27]. The cells were counted using a hemocytometer, ensuring viability greater than 90%.

***Spheroids:*** 1500 PHHs were seeded per well for monoculture spheroids and 2000 total viable cells for coculture spheroids at varying densities of PHHs and supporting cell types[9]. Cells were seeded in Corning Ultra-Low attachment (ULA) plates followed by centrifugation at 100*g for 2 minutes. 50% media changes for monoculture and coculture conditions were started on day 2, enabling direct model comparisons. Spheroids were maintained in serum-containing media until day 6, after which partial media changes with serum-free media were started for both drug dosing as well as functional assessment.

***Coculture spheroids:*** 3T3 J2s and HSCs were passaged on tissue culture plastic prior to seeding in coculture conditions with PHHs. For the latter part of the optimization, 3T2 J2s and HSCs were growth-arrested by incubating them with 1 µg/ml mitomycin-C (Sigma Aldrich) for 4 hours before

seeding. Different seeding times were considered with 3T3 J2s for the initial part of the optimization. Day 0 seeding signifies same day seeding of PHH and non-growth-arrested (NGA) 3T3 J2 at equal ratios (1:1), and Day 1 seeding correlates to one day after seeding of non-growth-arrested 3T3 J2 with respect to PHHs at equal proportions (1:1). For further optimization, 3T3 J2 were growth-arrested (GA) according to the protocol given above. Different ratios were seeded with respect to PHH (1:1. 2:1 and 3:1 – PHH: GA 3T3 J2). Similarly, HSCs were growth-arrested and seeded with different ratios (1:1 and 9:1 – PHH: GA HSC)

***PEGDA Micro wells:*** For PHH only spheroids, 20,000 hepatocytes were seeded per well in a 96 well plate while coculture conditions consisted of 20,000 PHH along with 20,000 3T3 J2s/HSCs. Media changes started on day 2, with 100% of the media changed every 48 hours. Cells were switched to serum-free media from day 6, and seeding times and ratios were kept consistent with the optimized condition of ultra-low attachment plate spheroids.

### 1.2.3 Functional assessment

Albumin secretion was assessed by sandwich enzyme-linked immunosorbent assay (ELISA; Bethyl Laboratories, Montgomery, TX, USA) with horseradish peroxidase detection and 3,3,5,5-tetramethylbenzidine (TMB; Rockland Immunochemicals, Boyertown, PA, USA). Urea synthesis was assessed using a colorimetric endpoint reaction involving diacetyl monoxime in the presence of acid and heat. Absorbance for both the assays was measured using a Synergy H1 multimode plate reader (BioTek).

Cytochrome P450 (CYP450) enzyme activity was measured by incubating specific substrates with the culture conditions. Luciferin IPA and Luciferin H (Promega Life Sciences, Madison, WI, USA) were incubated with the cells for 3 hours for CYP3A4 and CYP2C9, respectively, followed by a

media collection containing the metabolite, luciferin, which was quantified by measuring luminescence on the Synergy H1 plate reader following manufacture's protocols. Similarly, 5 µM 7-ethoxyresorufin (Sigma Aldrich, St Louis, MO, USA ) and 50 µM coumarin (Sigma Aldrich) were incubated with the cells for 3 hours for CYP1A2 and CYP2A6, respectively. After 3 hours, the media was collected, containing the metabolites resorufin and 7-hydroxycoumarin, which were quantified by measuring fluorescence (excitation/emission: 550/585 nm for resorufin, and 355/460 nm for 7-HC) on the Synergy H1 multimode plate reader.

**1.2.4 Drug dosing**

***Spheroids***: The spheroids were maintained for 10 days before dosing with different drugs. Fialuridine, diclofenac, buspirone was bought from Sigma Aldrich, and troglitazone, phentolamine, and rosiglitazone were purchased from Cayman chemicals. The cultures were treated with two concentrations of the drug, 100 Cmax and 25 Cmax (Cmax is the maximum drug concentration found in human plasma), until day 16 (3 consecutive doses on Day 10, 12 and 14), followed by endpoint analysis for albumin and urea secretions, both shown to be sensitive markers of drug-induced hepatotoxicity previously[28]. Values of Cmax (Troglitazone: 6.387uM, Fialuridine: 1uM, Diclofenac: 8.023 uM, Rosiglitazone: 1.120uM, Buspirone: 0.005uM, Phentolamine: 0.09uM) were taken from published literatures[11, 29] were taken from previous published literature. All the drugs were dissolved in 100% DMSO (Corning Life Sciences) and stored at a stock concentration of 100,000 Cmax. Treated cultures were compared to a control with 0.1% DMSO. The margin of safety (MOS), signifying IC50/Cmax, was set as 100 for our studies. The IC50 (concentration at which 50% of the activity is reduced) value was calculated for the drugs, which reduced the activity of the cells (albumin, and/or urea) by more than 50%

compared to the DMSO control for 100xCmax of the drug. IC50 calculation was done using linear interpolation of drug responses from two different concentrations given above.

### 1.2.5 Data analysis

Spheroids were formed using human PHHs (HUMVNL: Female, HUM4011: 26-year-old male Caucasian, HUM4167: 12-year-old female Caucasian, and HUM4016: 46-year-old male Caucasian, purchased from Triangle Research Labs (Now Lonza Bioscience Solutions). Data with one of the donors is shown in the results.

***Spheroids***: Each spheroid was analyzed separately, with three spheroids analyzed for albumin and urea quantification. For CYPs, at least two spheroids were analyzed independently for each condition. Ten spheroids were dosed with each concentration for the drug dosing experiments and analyzed separately for the endpoint assessments.

Microsoft Excel was used to analyze the data, and GraphPad Prism was used to plot the graphs. Values on the graphs signify the mean value, and error bars represent standard deviation. Statistical significance was determined using the two-way ANOVA test with a 95% confidence interval.

***PEGDA micro wells:*** 3 wells were analyzed for each Albumin, Urea, and CYPs.

## 1.3 RESULTS

### 1.3.1 Comparison of coculture spheroids with different seeding times of non-growth arrested (NGA) 3T3 J2 and monoculture spheroids in ULA plates

Different seeding times of NGA 3T3 J2 were evaluated for coculture spheroids, followed by comparison to monoculture spheroids (**Figure 1**). As shown in **Figure 2A**, during the earlier days of the culture (day 2-16), day 0 seeding of 3T3 J2s better enhanced PHHs albumin secretion than day 1 seeding of 3T3 J2s having a 1.56-fold higher function on day 12. Also, both the coculture conditions exhibited higher albumin secretion compared to monoculture spheroids. Albumin secretion for the monoculture spheroids gradually increased during the latter part of the culture period, having 1.73-fold higher activity compared to day 0 seeded coculture on day 26, which can be attributed to the time taken to form monoculture tight spheroids with improved homotypic interactions. As shown in **Figure 2B**, both the coculture conditions showed similar levels for urea synthesis but were higher than the monoculture spheroids, which remained low for the entire culture period. Contrary to both albumin and urea, in which a coculture effect (higher functionality) was seen, monoculture spheroids performed at par with the coculture spheroids for CYP activity on day 16 and further having 2.61- and 2.68-fold higher activity compared to Day 0 seeded coculture on day 24 for CYP3A4 (**Figure 2C**) and CYP2A6 (**Figure 2D**), respectively. Considering all the above factors, coculture spheroids with same day seeding of 3T3 J2 with PHHs was taken forward along with monoculture spheroids for further optimization.

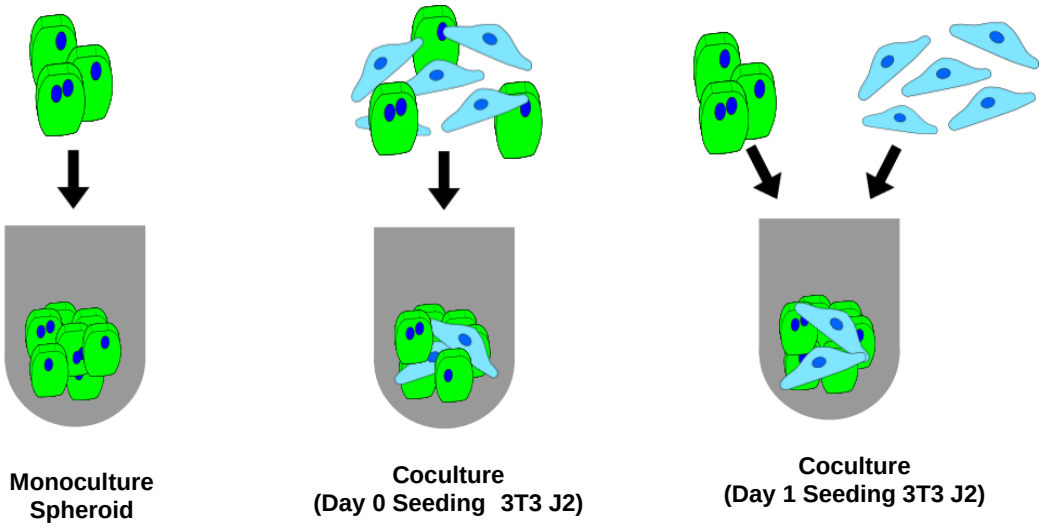


Figure 1. Schematic representing three culture conditions, Day 0 seeding 3T3 J2 coculture, Day 1 seeding 3T3 J2 coculture, monoculture in ULA plates

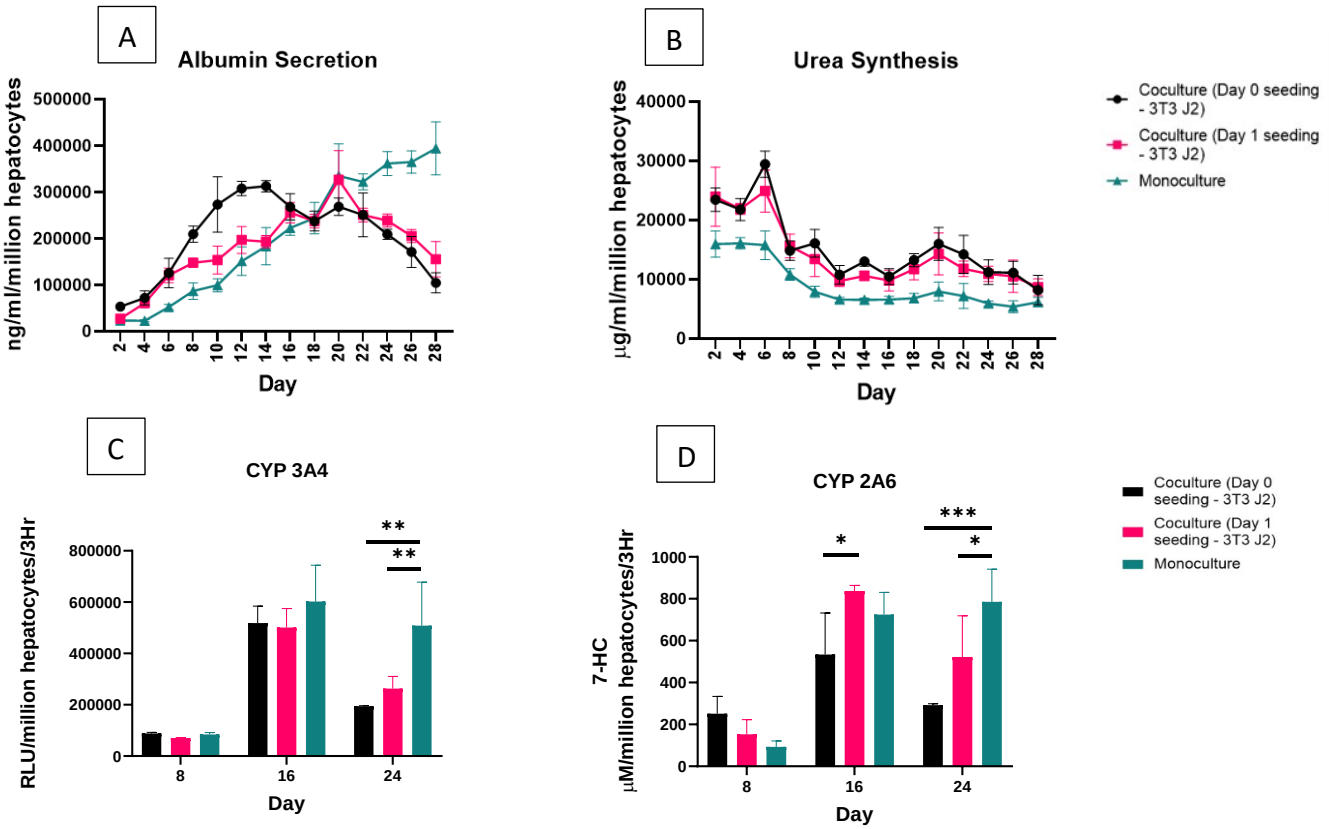


Figure 2: Functions with different seeding times of NGA 3T3 J2 coculture spheroids and comparison with monoculture. (A) Albumin secretion (B) Urea synthesis (C) CYP3A4 (D) CYP2A6. All functions normalized to a million hepatocytes. Two-way ANOVA analysis was performed using the Tukey test. *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001. Findings confirmed with three donors.

### 1.3.2 Comparison of non-growth arrested (NGA) 3T3 J2 and different ratios of growth-arrested (GA) 3T3 J2 coculture spheroids in ULA plates

Non-growth arrested (NGA) 3T3 J2s in coculture with PHHs were compared with different seeding ratios of growth-arrested (GA) 3T3 J2 to assess functionality differences. The morphology of all the three culture conditions remained almost constant throughout (**Figure 3**). We observed no significant differences between NGA and GA 3T3 J2 for inducing albumin function in PHHs until day 20 at a 1:1 ratio (**Figure 4A**). For urea synthesis **(Figure 4B)**, NGA 3T3 J2 coculture spheroids had higher activity than GA 3T3 J2 coculture spheroids for earlier days (days 2-10); however, both the conditions were at similar levels for the remainder of the culture period, the typical time for drug dosing experiments. NGA 3T3 J2 with PHHs had higher levels of CYP3A4 and CYP2A6 than GA 3T3 J2 for one donor (**Figure 4C and 4D**), while another PHH donor had no significant differences (data not shown). While comparing three different ratios of GA 3T3 J2 with PHH, 1:1 PHH: GA 3T3 J2 condition had 1.28-fold and 1.72-fold higher albumin activity on day 18 (**Figure 4A**) than 2:1 and 3:1, respectively. 1:1 PHH: GA 3T3 J2 showed 1.37-fold higher urea functionality on day 18 (**Figure 4B**) than both 2:1 and 3:1. There was no significant difference between the three ratios for CYP2A6 (**Figure 4D**) activity. However, 1:1 PHH: GA 3T3 J2 had a 1.65-fold CYP3A4 (**Figure 4C**) activity compared to 3:1 on day 16. The results suggest that equal proportions of 3T3 J2 and PHHs produce higher functions and be taken for further experimentation. Also, to maintain consistent seeding ratios of all the cell types seeded, the growth-arrested condition was picked over non-growth arrested for the coculture spheroid model.

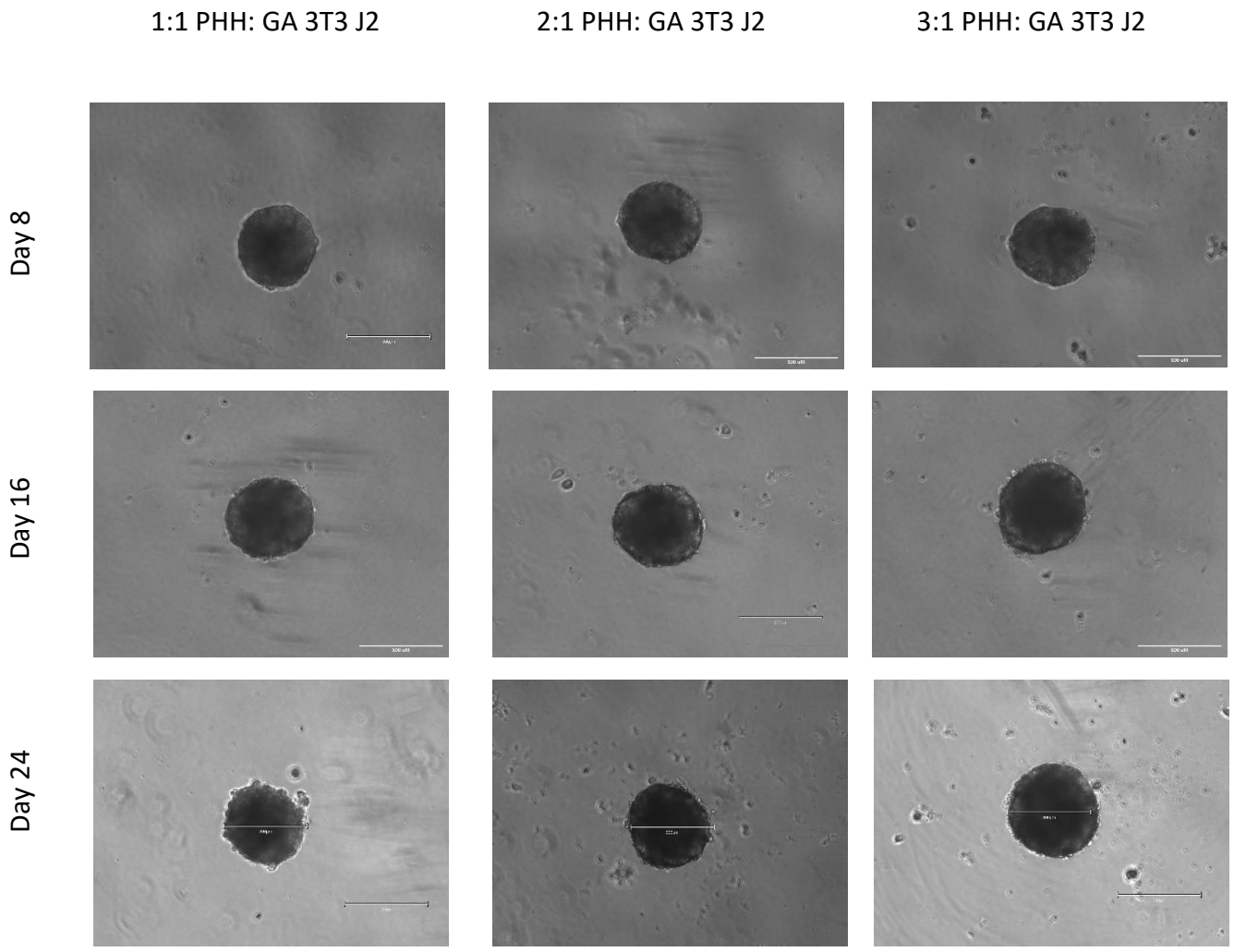


Figure 3: Different seeding ratios of PHH and GA 3T3 J2 in ULA plates. All scale bars are 300 µm.

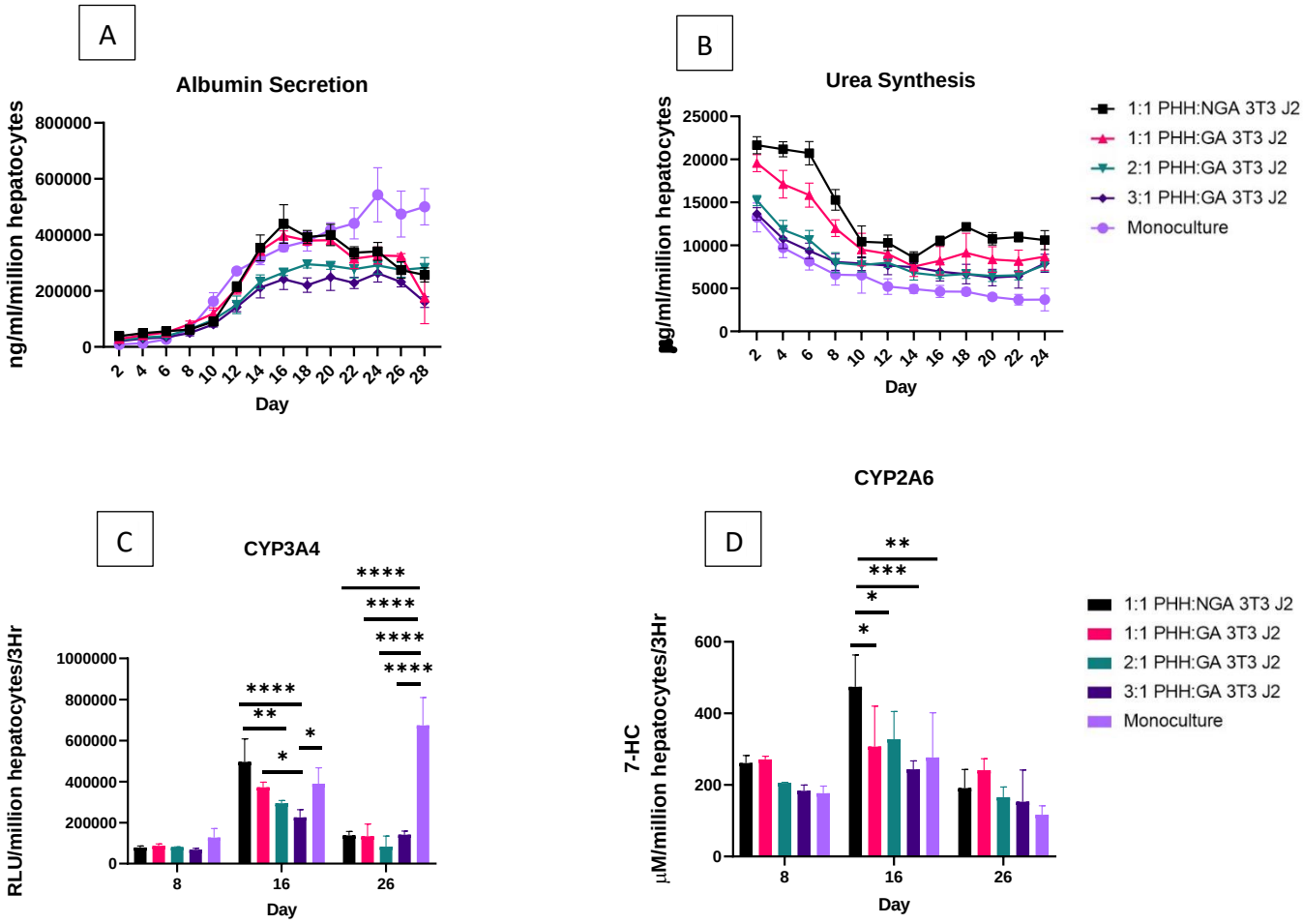


Figure 4: Comparison of NGA 3T3 J2 and GA 3T3 J2 coculture spheroids. (A) Albumin secretion (B) Urea synthesis (C) CYP3A4 (D) CYP2A6. All functions normalized to a million hepatocytes. Two-way ANOVA analysis was performed using the Tukey test. *p<0.0332, **p<0.0021, ***p<0.0002. Findings confirmed with two donors.

### 1.3.3 <u>Comparison between growth-arrested (GA) 3T3 J2 and HSC coculture spheroids in ULA plates</u>

Intending to introduce an all human liver model containing human HSCs and PHHs, we compared GA HSCs with the previously optimized GA 3T3 J2 coculture condition for stabilizing PHH functionality. 1:1 PHH: GA 3T3 J2 maintained more rounded morphology compared to HSC

spheroids (**Figure 5**). For urea synthesis (**Figure 6B**), 3T3 J2 outperformed HSC culture conditions for the entire culture period. Similar to urea expression, 3T3 J2 coculture condition had higher functionality than HSC coculture spheroids for albumin secretion in the earlier days of the culture (until day 18), having 1.52- and 2.51-fold higher activity on day 12 for CYP3A4 (**Figure 6C**) and albumin (**Figure 6A**), respectively. However, HSC coculture spheroids functionality ramped up after that time point, outperforming 3T3 J2 with 3.3- and 1.36-fold higher activity for CYP3A4 and albumin, respectively, on day 26. For CYP2A6 (**Figure 6D**), the 3T3 J2 condition had 5.11-fold higher functionality than HSC spheroids for day 16, with no significant difference between the two coculture conditions on day 26. While selecting the optimized ratio between PHH and GA HSC, 1:1 PHH: GA HSC was chosen over 9:1 PHH: GA HSC as the former condition outperformed the latter for all the functional assessments having 1.22-, 3.44-, and 1.76-fold higher functionality on day 26 for albumin, urea, and CYP3A4 respectively. Thus, for all the further drug dosing experiments, 1:1 PHH: GA HSC was taken forward along with 1:1 PHH: GA J2 and monoculture spheroids

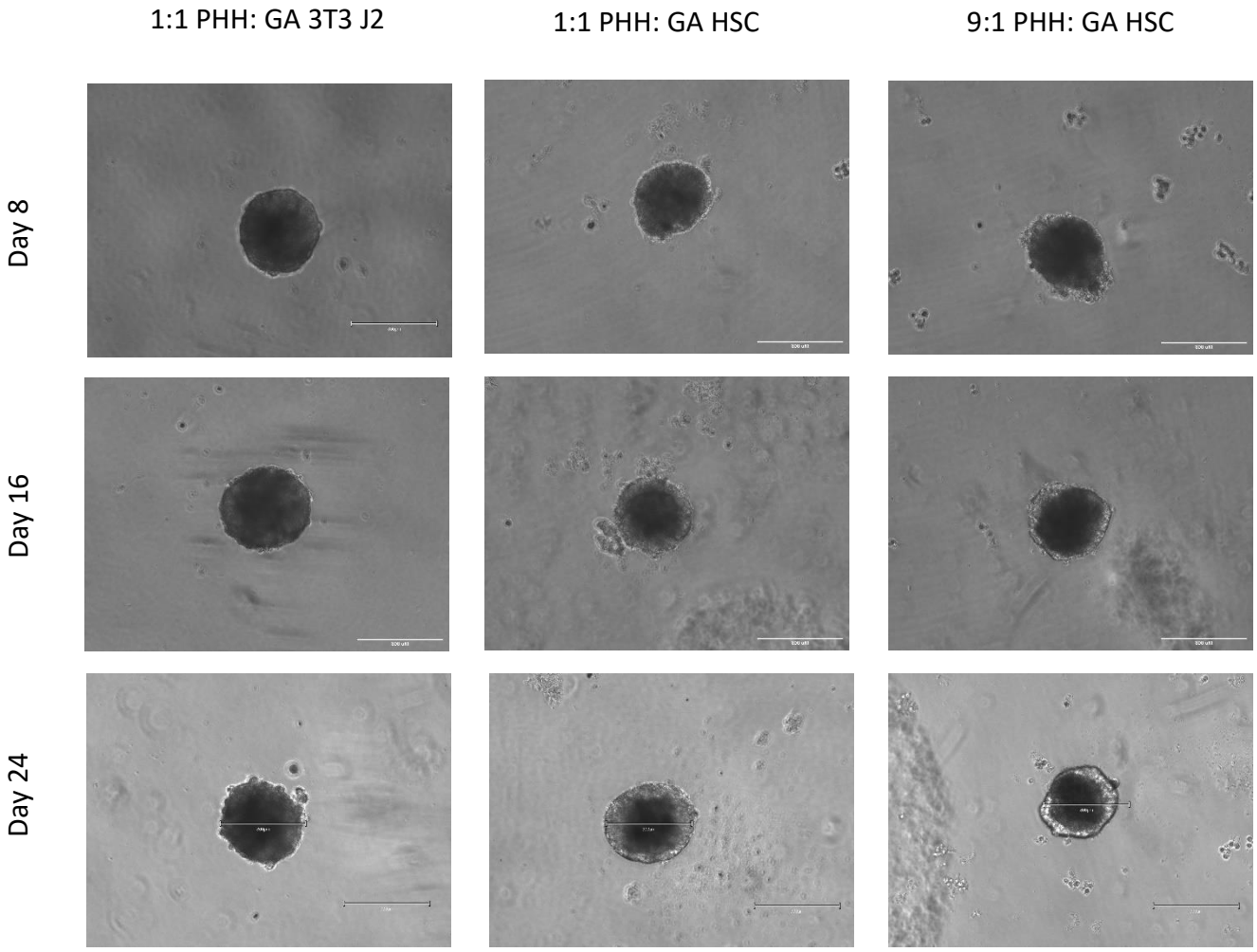


Figure 5 Differences between GA 3T3 J2 and GA HSC coculture spheroids in ULA plates. All scale bars are 300 µm

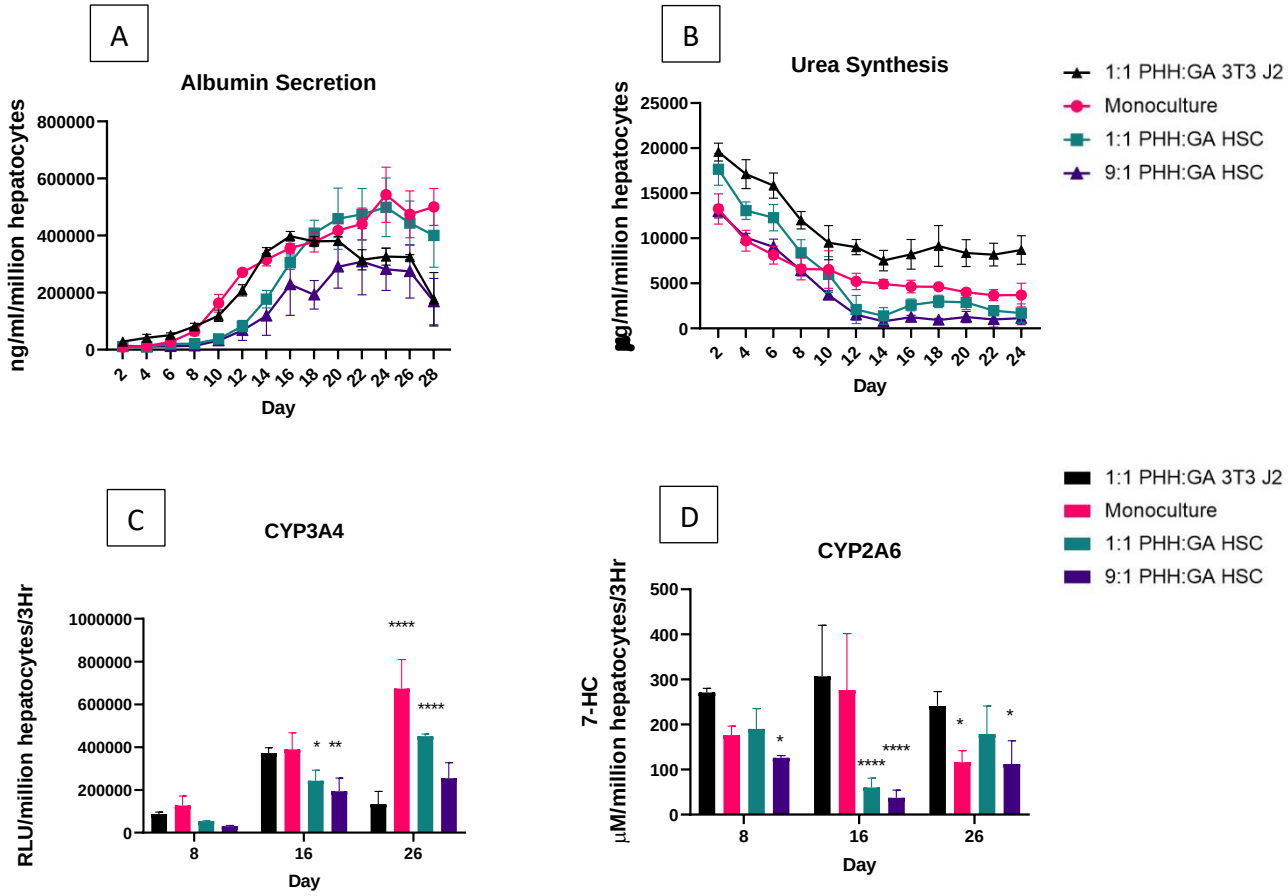


Figure 6 Comparison of GA 3T3 J2 and GA HSC coculture spheroids (A) Albumin secretion (B) Urea synthesis (C) CYP3A4 (D) CYP2A6 All functions normalized to a million cells. Two-way ANOVA analysis was performed using the Dunnett's multiple comparison test. *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001, displayed with respect to 1:1 PHH: GA 3T3 J2. Findings confirmed with two donors.

### 1.3.4 Importance of serum-free media for HSCs to support PHH in a ULA plate spheroids

Optimized serum-free media conditions were compared with cultures maintained in serum-containing media to assess serum-free media's role in HSCs enhancing certain PHH functionality. Also, the monoculture morphology was compared with the coculture and HUM4167 formed very compact spheroids early on without needing any coculture cell type. (**Figure 7**). The serum-

containing HSCs spheroids had 2.02- and 2.31-fold lower albumin activity (**Figure 8A**) on day 12 and day 24, respectively, than the HSC spheroids in serum-free media. Similarly, serum-containing HSCs spheroids had 6.91- and 5.01-fold lower CYP3A4 (**Figure 8B**) activity on day 16 and day 26, respectively.

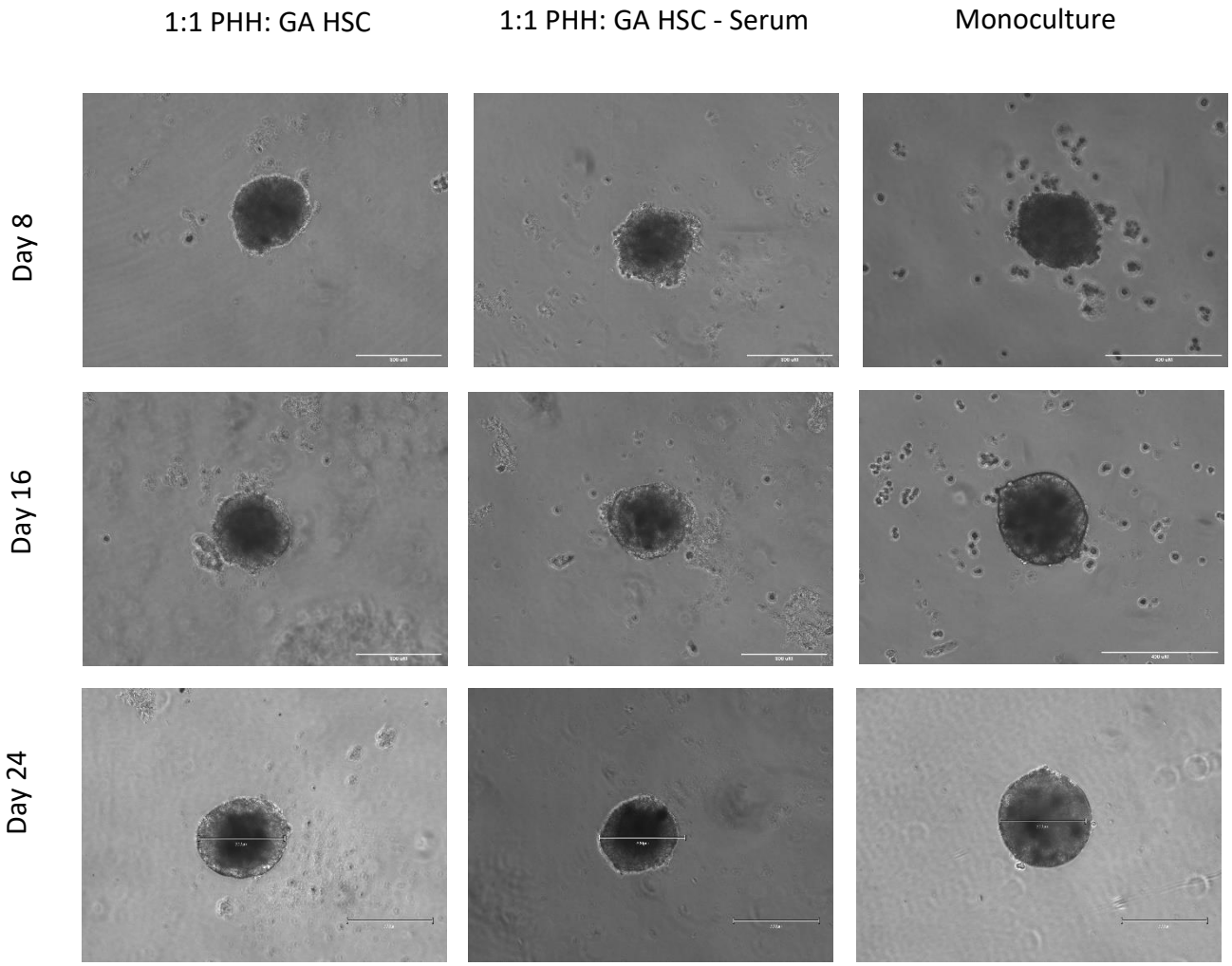


Figure 7: Differences between serum-containing and serum-free GA HSC coculture spheroids in ULA plates. All scale bars are 300µm.

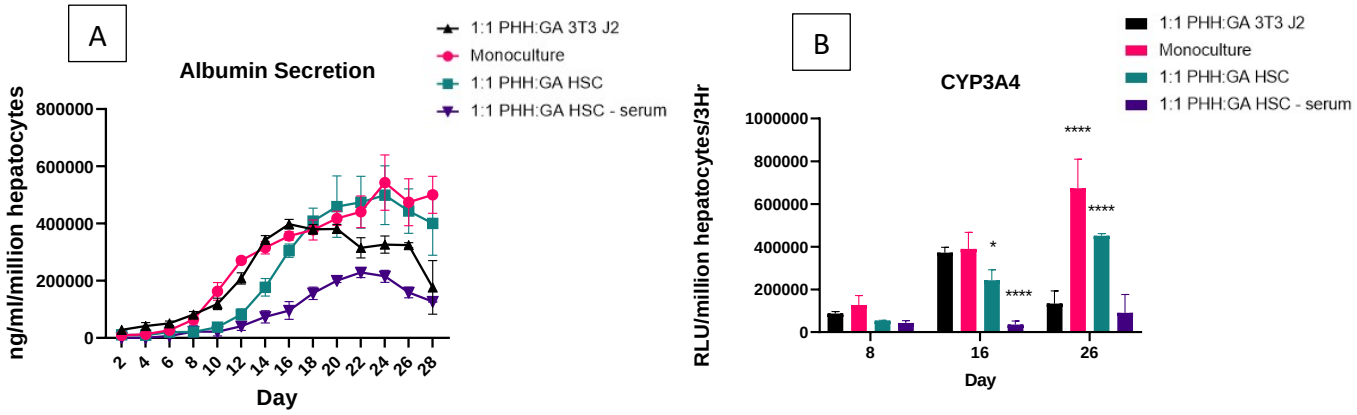


Figure 8 Comparison of spheroids in serum-containing vs. serum-free media. (A)Albumin secretion (B) CYP3A4 All functions normalized to a million cells. Two-way ANOVA analysis was performed using the Dunnett's multiple comparison test. *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001, displayed related to 1:1 PHH: GA 3T3 J2. Findings confirmed with one donor.

### *1.3.5* Drug toxicity in spheroids for GA 3T2 and GA HSC coculture condition, and monoculture in ULA plates

Drug toxicity experiments were performed with two PHH donors (HUM4016 and HUM4167) with six pharmaceutical drugs with different categories listed from the FDA database (https://www.fda.gov/science-research/liver-toxicity-knowledge-base-ltkb/drug-induced-liver-injury-rank-dilirank-dataset). The results from one of the donors are shown in **Table 1.** For diclofenac and troglitazone, all culture conditions showed toxicity; Fialuridine had very limited toxicity in the spheroid format, showing toxicity for only the albumin end point assessment with 3T3 J2 coculture and both endpoint assessments of monoculture condition. Rosiglitazone toxicity was detected only by monoculture culture condition and urea assessment of HSCs' coculture condition. None of our conditions in a spheroid format showed a toxicity for non-hepatotoxins: buspirone and phentolamine.

Table I. IC50 VALUES PRESENTED AS MULTIPLES OF Cmax OF DRUGS FOR DIFFERENT CONDITIONS OF SPHEROID FORMAT. IC50 WAS CALCULATED USING LINEAR INTERPOLATION AS THE VALUE WHICH REDUCES THE ACTIVITY OF CULTURE BY 50%. ALL VALUES ARE LESS THAN Cmax. TABLE CELLS WITH NO VALUESSIGNIFY NO TOXICITY FROM THE DRUG. FINDINGS CONFIRMED WITH ONE DONOR.

| | | 1:1 PHH: GA 3T3 J2 | | 1:1 PHH: GA HSC | | Monoculture | |
|---|---|---|---|---|---|---|---|
| **Drug category** | **Drugs** | Albumin | Urea | Albumin | Urea | Albumin | Urea |
| MOST DILI | Troglitazone | 60.31 | 69.17 | 73.54 | 85.37 | 45.5 | 63.23 |
| MOST DILI | Fialuridine | 97.67 | - | - | - | 23.97 | 43.46 |
| MOST DILI | Diclofenac | 26.58 | 44.26 | 75.98 | 73.32 | 18.63 | 42.48 |
| LESS DILI | Rosiglitazone | - | - | - | 23.39 | 72.37 | 73.63 |
| AMBIGUOUS DILI | Buspirone | - | - | - | - | - | - |
| NO DILI | Phentolamine | - | - | - | - | - | - |

### 1.3.6 <u>Functional maintenance of PHHs in PEGDA microwells</u>

Introducing another format for spheroids, we were able to maintain PHH functions until day 24 (experiment ongoing). Similar to ULA plate spheroids, the 3T3 J2 coculture condition outperformed the HSC coculture condition, having 4.86-fold higher albumin activity (**Figure 11A**) on day 12. Monoculture conditions had lower albumin secretion throughout the culture period. Similarly, for urea synthesis (**Figure 11B**), 3T3 J2 coculture condition outperformed HSCs coculture spheroids and monoculture by 6.86- and 5.42- fold higher activity on day 12. Contrary to albumin activity, the monoculture condition did much better than both the coculture condition having 2.42- and 2.88-fold higher CYP3A4 (**Figure 11C**) activity than 3T3 J2 and HSC

coculture condition on day 24, respectively. Consistent with the ULA plate spheroids, CYP2A6 remained low for HSCs spheroids throughout.

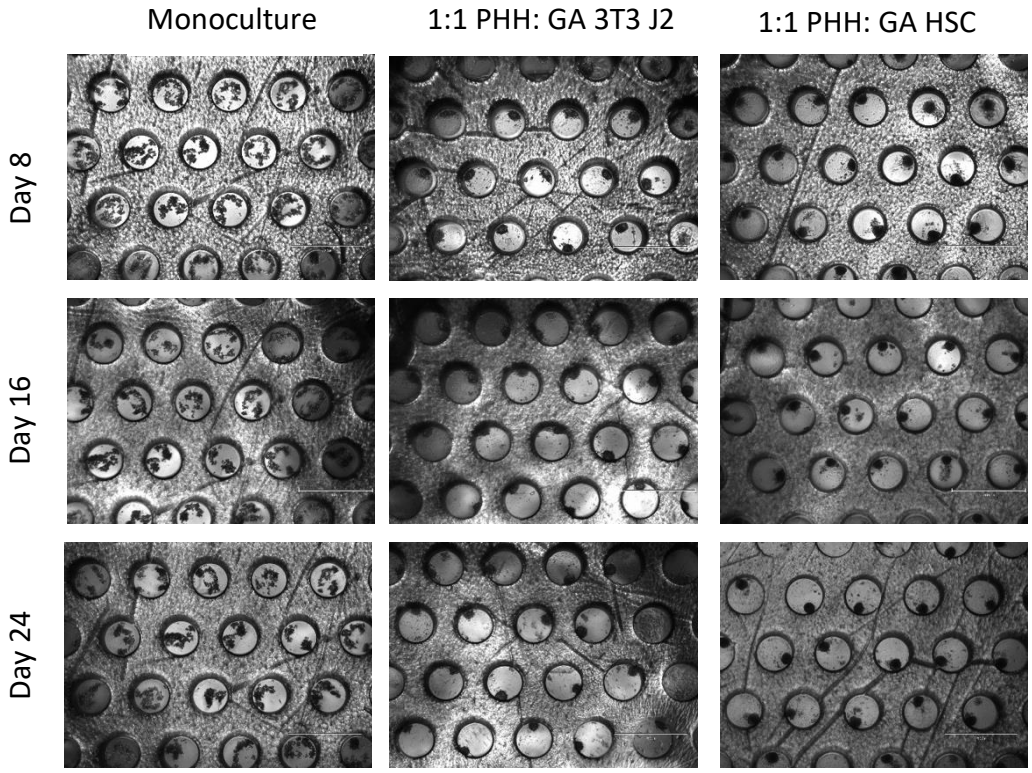


Figure 9 Different culture conditions in PEGDA microwells. All scale bars are 750 µm.

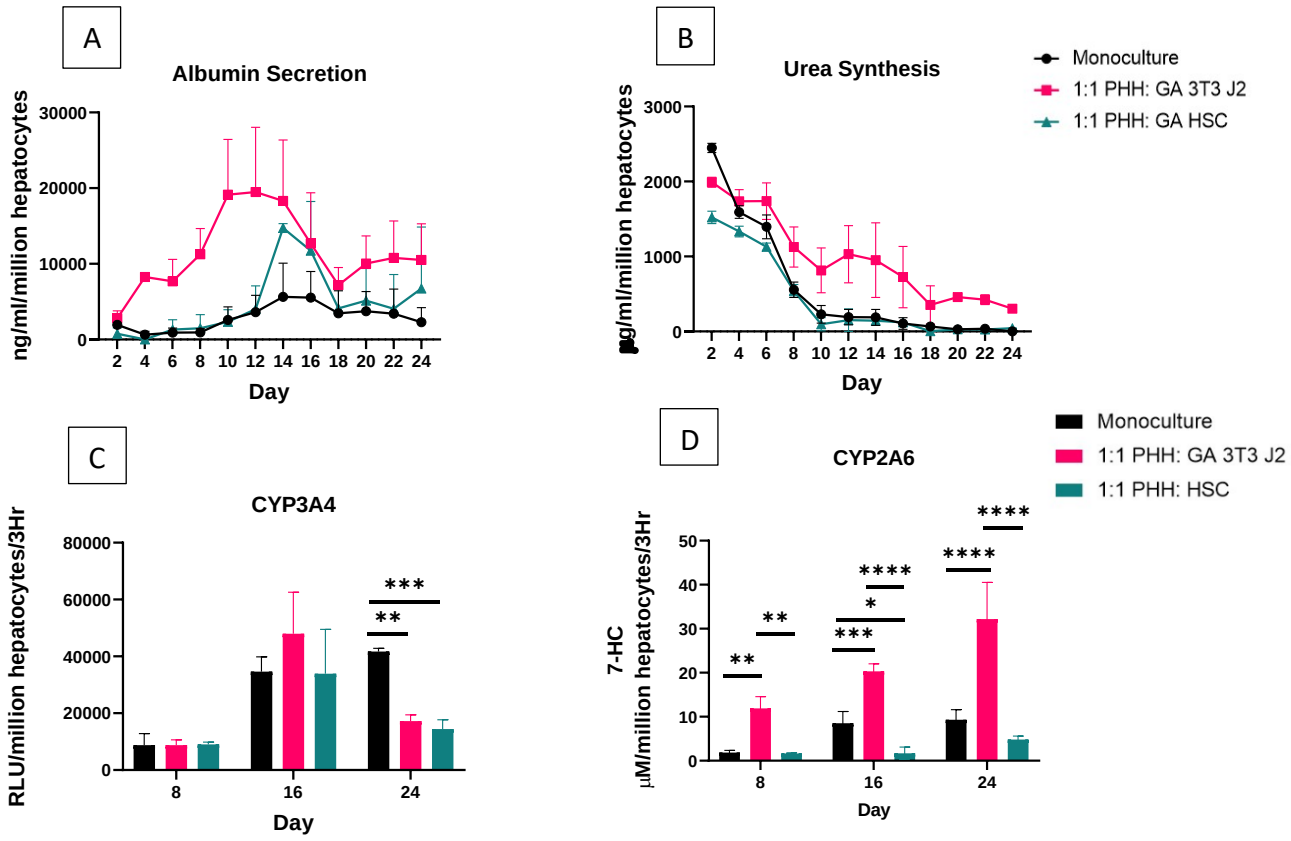


Figure 10: Comparison of monoculture, GA 3T3 J2 and GA HSC coculture spheroids in PEGDA microwells. (A) Albumin (B) Urea (C) CYP3A4 (D) CYP 2A6 All functions normalized to a million cells. Two-way ANOVA analysis was performed using Tukey test. **p<0.0021, ***p<0.0002. Findings confirmed with one donor.

## 1.4 DISCUSSION

The study optimized a spheroid model system with multiple culture conditions to be used in in-vitro drug toxicity screening. Despite the extensive use of monoculture spheroids in recent studies, there is a big disadvantage of donor dependability in monoculture spheroid formation[10]. Thus, our primary focus was to optimize a coculture spheroid condition and compare it with monoculture. 3T3 J2 mouse embryonic fibroblasts and primary human HSCs were used to optimize a coculture spheroid model.

3T3 J2 murine fibroblasts have been shown to maintain relevant hepatic functions of PHHs for up to 4 weeks[8] and used for several applications like predicting drug clearance and drug-drug interactions[27], determining sensitivity and specificity in predicting 45 drugs hepatotoxicity[28] in formats such as micropatterned cocultures (MPCCs), therefore, we introduced 3T3 J2 in a spheroid format with PHHs to predict drug sensitivity as its use for such application is very limited. At the initial stage of the study, we wanted to carefully assess the extent of homotypic and heterotypic interactions and the timeline related to it for maximum functionality. Thus, seeding times and seeding ratios were considered for the culture conditions. It was found that the same day seeding of the fibroblasts and PHHs (day 0) enhanced albumin secretion of the PHHs for at least the first two weeks relative to the condition in which the fibroblasts were seeded onto the PHHs the next day (day 1) (**Figure 2**). We also observed a coculture effect (higher functionality) in the cultures, at least for albumin and urea activity (**Figure 2**). The above finding was confirmed by three different donors in an experiment simultaneously. However, no coculture effect was

seen in one of the PHHs donor for albumin, however, higher functionality of the coculture spheroids was maintained for Urea (**Figure 4**). This can be attributed to the compact monoculture spheroids at an early stage of the cultures (**Figure 7**) which resulted in comparable functions to the coculture spheroid. Thus, coculture spheroids showing higher functionality is dependent on the formation rate of monoculture spheroids. Nevertheless, same day seeding was adopted for all the further experimentation for coculture spheroid conditions, and both the culture conditions, coculture, and the monoculture, were taken forward for further investigation. We tested different ratios of growth-arrested 3T3 J2 with the non-growth arrested 3T3 J2 solely focusing on the higher functionality. Similar activity was observed for non-growth arrested and growth-arrested 3T3 J2 for the entire period of albumin and mid culture period (day 10 to day 14) for urea activity (**Figure 4**). Equal proportions of the growth-arrested fibroblasts and the PHHs yielded the maximum functionality similar to the study by Lu et al. [30]. Thus, to maintain consistent seeding ratios and maintain higher functionality, equal proportions of PHHs and growth-arrested 3T3 J2 were used for further experimentation.

3T3 J2 murine fibroblastic cells having non-human origin can raise concerns of interacting with the PHHs and the dosed pharmaceutical drugs in a non-physiological manner; however, to our knowledge, there has not been any studies published related to it. Therefore, we wanted to create an all human spheroid model system. Studies involving human PHHs and HSCs in a spheroid format are very limited. Moreover, there has not been any direct comparison between 3T3 J2 and HSCs on drug sensitivity level or the use of HSCs coculture for drug toxicity studies with an extensive panel of drugs. Thus, we included both the cell types to form a coculture

spheroid format. We wanted to provide a complete comparison of mouse fibroblasts and human hepatic stellate cells in terms of the capability to support PHH functions. Our study showed that 3T3 J2 maintained better PHH functions compared to HSCs in the cultures' earlier days (**Figure 6**). However, HSCs were able to induce much higher functions, such as albumin, in PHHs for the later part of the culture. One of the possibilities can be the reduction of the activated state of HSC in serum-free conditions, which enhanced the PHH functionality in a coculture condition, but further experimentation is required to prove the above hypothesis. The phenomenon of HSCs supporting the primary cells in a spheroidal serum-free or very limited serum environment can be supported by some studies which showed that fasting media, containing 1% fetal bovine serum and 0.5 g/L glucose compared to normal conditions of 10% fetal bovine serum and 4.5 g/L glucose, efficiently decreased alpha SMA and vimentin in LX-2, an immortalized human hepatic stellate cell line, after treatment with lipopolysaccharide[31], demonstrating the reversion to a phenotype that can be linked with a less activated state after culturing the cells with hepatocytes in a serum-free environment [20]. HSCs that showed myofibroblastic markers, such as Acta2 and Col3a1, in 2D cultures have been shown to be decreased in a spheroid format, containing freshly isolated primary mouse hepatocytes and HSCs, while maintaining the functions of both PHHs and HSCs[32] as well as low mRNA levels of ACTA2, COL1A1, and LOX in coculture spheroids containing HepaRG and HSCs[33]. One thing that is consistent in all the studies was the maintenance of spheroids in a serum-free environment. We also conducted a pilot study in which we compared serum-containing and serum-free media to assess the role of serum in the capability of HSCs to support PHHs. Our results demonstrated extremely low functions of spheroids in serum-containing media (**Figure 8**), which supports the rationale of multiple studies

using serum-free media. However, the reason for HSCs supporting PHHs higher than 3T3 J2 in the later part of the culture is yet to be determined. Observing the potential for the HSCs to support the PHHs in a spheroid format, we considered taking both 3T3 J2 and HSCs for the spheroids' coculture condition forward drug toxicity assessments.

Having all the three culture conditions optimized, we compared all the three conditions' drug sensitivity through evaluating six pharmaceutical drugs under four different categories of DILI. We hypothesized that higher functionality of coculture for albumin and urea in the starting days, typical drug dosing time, would yield higher sensitivity to the drugs. Contrary to which, we discovered that the monoculture spheroids are more sensitive to the drugs (lower values of IC50) than both the coculture spheroid conditions (**Table 1**). Testing a larger drug panel (>30 drugs) in the future will be necessary to calculating sensitivity and specificity metrics for the different spheroid conditions. Nevertheless, the monoculture spheroids showed a toxicity for all the known hepatotoxins for both the endpoint assessments: albumin and urea. There could be a possible hindrance to drugs reaching the PHHs in a coculture format due to the presence of a fibroblast capsule around the PHH spheroids; however, further imaging and drug diffusion studies are needed to prove such a hypothesis. For instance, a future study could include confocal microscopy with immunohistochemical staining studies on the coculture spheroids to assess fibroblasts' position in the self-assembled spheroids that could impact drug exposure. Also, for drug toxicity experiments, one of the donors did not show detectable functions of urea in HSC coculture and monoculture conditions with significantly less functionality of albumin. Thus, there

is a need to carefully select donors for drug dosing experiments, as is typical for studies involving spheroids due to variable rates of spheroid formation by different PHH donors.

Finally, we were able to introduce another format, PEGDA microwells, for the formation of spheroids. The format allowed the formation of multiple spheroids in each well of a 96 well plate resulting in increased enzyme activity. It can also serve for future drug metabolism assays taking advantage of the increased number of cells in each well compared to ultra-low attachment plate spheroids. Also compared to ULA plates, the handling time of the cultures was significantly reduced in PEGDA microwells. The hepatocytes were functional for the entire culture period, with the 3T3 J2 coculture condition having the highest albumin activity (**Figure 10A**) with monoculture spheroids showing higher CYP3A4 activity (**Figure 10C**) for later part of the culture. However, HSCs spheroids did not perform in a similar fashion to ULA plate spheroids. They could reach the level of 3T3 J2 coculture spheroids, but the functionality remained lower throughout the period. One of the possibilities of an increased number of HSCs not able to support the PHHs can we derived from the study which showed an increase in the level of interleukin 6 (IL-6) with an increase in the number of HSCs in a micropatterned coculture format with PHHs and 3T3 J2, which led to the downregulation of hepatic functions such as CYP3A4[34]. However, further investigation of the model system is required to confirm the above hypothesis. Nevertheless, we were able to successfully fabricate microwells in a 96 well plate format with consistent architecture throughout and stability of the polymer with no detachment from the glass substrate. Further optimization of the format requires seeding density of the cells and

fabrication, making it a more robust process. Future work would also involve drug toxicity studies on the format, presenting it as a more efficient drug screening system.

In conclusion, we gained a preliminary understanding of both coculture and monoculture spheroids, their differences in functionality, interaction with the drugs, which gives a strong foundation for further optimization and fabricating a format with the highest drug sensitivity.

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