Title Page:

Contextualizing Hepatocyte Functionality of Cryopreserved HepaRG® Cell Cultures

Jonathan P. Jackson, Linhao Li, Erica D. Chamberlain, Hongbing Wang, and Stephen S. Ferguson

Life Technologies, Cell System Division, ADME/Tox, Durham, NC: JPJ, ED, and SSF

JPJ currently with Qualyst Transporter Solutions (QTS)
EDC currently with Quest Pharmaceutical Services (QPS)
SSF currently with the Division of the National Toxicology Program, National Institute of Environmental Health Sciences (NIEHS)

Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy,
20 Penn Street, Baltimore, MD 21201: LL and HW
Running Title Page:

Running Title: Hepatocyte Function of Cryo-HepaRG Cultures

Corresponding Author: Stephen S. Ferguson

Address: 111 TW Alexander Dr., RTP, NC 27709

Phone: (919) 541-3799

Email: stephen.ferguson@nih.gov

Number of Text Pages: 45

Number of Tables: 5

Number of Figures: 12 (plus 2 supplemental)

Number of References: 44

Number of Words in Abstract: 227

Number of Words in Introduction: 512

Number of Words in Discussion: 1,457

Nonstandard Abbreviations: Cryopreserved HepaRG® Cells (Cryo-HepaRG), HepaRG® cells (HepaRG), primary human hepatocytes (PHH), sandwich cultured primary human hepatocytes (SC-PHH), Aryl Hydrocarbon Receptor (AhR), Constitutive Androstane Receptor (CAR), Pregnane X Receptor (PXR), Farnesoid X Receptor (FXR), Carboxy Dichlorofluorescein Diacetate (CDFDA), 5-(and-6)-carboxy-2′,7′-dichlorofluorescein (CDF) multidrug resistance-associated protein 2 (MRP2), phenobarbital (PB), flavine monoxygenase (FMO), UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), sodium/taurocholate co-transporting polypeptide (NTCP), organic anion transporting polypeptide 1B1 & 1B3 (OATP1B1/3), ATP-Binding Cassette, Sub-Family B, Member 11 (ABCB11), green fluorescent protein (GFP).
Abstract:

Over the last decade, HepaRG® cells have emerged as a promising alternative to primary human hepatocytes (PHH) featured in over 300 research publications. Most of these reports employed freshly differentiated HepaRG® cells that require time-consuming culture (~28 days) for full differentiation. Recently, a cryopreserved, pre-differentiated format of HepaRG® cells (termed here as Cryo-HepaRG) has emerged as a new model that improves global availability and experimental flexibility; however, it is largely unknown whether this format of HepaRG® cells fully retain their hepatic characteristics. Therefore, we systematically investigated hepatocyte functionality of Cryo-HepaRG cultures in context with the range of interindividual variation observed with PHH in both sandwich culture and suspension formats. These evaluations uncovered a novel adaptation period for the Cryo-HepaRG format and demonstrated the impact of extracellular matrix on Cryo-HepaRG functionality. Pharmacologically-important drug metabolizing alleles were genotyped in HepaRG® cells and identified poor metabolizer alleles for CYP2D6, CYP2C9, and CYP3A5 which are consistent with higher frequency alleles found in individuals of Caucasian decent. We observed liver enzyme inducibility with AhR, CAR, and PXR activators comparable to that of sandwich cultured PHH. Finally, we show for the first time that Cryo-HepaRG supports proper CAR cytosolic sequestration and translocation to hepatocyte nuclei in response to phenobarbital treatment. Taken together, these data reveal important considerations when using this cell model and demonstrate that Cryo-HepaRG is suitable for metabolism and toxicology screening.
Introduction:

The liver is a major organ involved in the detoxification of both endobiotic and xenobiotic chemicals. Primary human hepatocytes (PHH) are a well-accepted in vitro liver model for prediction of drug metabolism and toxicity, due to their proper maintenance of metabolism, transport, and receptor signaling pathways. However, the pronounced interindividual variability and high cost of PHH has led to the emergence of alternative cell models such as the hepatoma-derived HepG2 and the immortalized Fa2N-4 for screening purposes. To date, these immortalized models have been associated with insufficient hepatocyte differentiation and low metabolic functionality (Hariparsad et al., 2008; Donato et al., 2010).

In recent years, freshly differentiated HepaRG® cells have emerged as a promising alternative to PHH for in vitro drug-drug interaction and toxicology studies. To reach phenotypic maturity, HepaRG® cells grow to confluence and differentiate over 4 weeks (from progenitor cells) into co-cultures of hepatocyte-like and cholangiocyte-like cells (Gripon et al., 2002). Since this model was discovered, many studies have shown that freshly differentiated HepaRG® cultures exhibit cellular interactions, drug metabolism/transport, and drug induction responsiveness comparable to that of PHH culture (Grime et al., 2010; McGill et al., 2011; Gerets et al., 2012; Le Vee et al., 2013; Szabo et al., 2013). A cryopreserved format of differentiated HepaRG® cells (Cryo-HepaRG) has recently become available, improving the global availability and experimental flexibility of this model. However, the impact of detachment, cryopreservation, and re-plating on HepaRG® function has not been comprehensively evaluated. It is known that disruption of cellular interactions during liver isolations results in PHH dedifferentiation (Godoy et al., 2013). Therefore, it is important to understand the consequences of detachment/reattachment on Cryo-HepaRG. To date, the effect of culture time on Cryo-HepaRG metabolic competence (post-reattachment to monolayers), liver enzyme induction, and uptake transport has not been characterized or compared to interindividual variation across large numbers of sandwich cultured primary human hepatocytes (SC-PHH) and suspensions of PHH. Finally, no immortalized liver cell line alternative to PHH has been found to properly model the constitutive androstane receptor (CAR) activation pathway where CAR is sequestered in the cytosol of
hepatocytes and translocates to the nucleus upon activation by phenobarbital, a hallmark feature of functional PHH.

In the current study, we evaluated Cryo-HepaRG and found them to resemble freshly differentiated HepaRG after 7-10 days in culture. We observed bile canaliculi formation over time, a hallmark of hepatocyte polarization, analogous to PHH cultures with morphologies (i.e. cords of hepatocyte-like cells) stabilizing after 7-10 days in culture. We monitored the temporal dynamics of metabolic competence in cultured Cryo-HepaRG and observed an adaptation period with an initial loss of metabolic competence that was restored to suspension Cryo-HepaRG levels after 7-10 days in culture. Metabolic activities, liver enzyme induction, and uptake/efflux transport in Cryo-HepaRG were compared with numerous lots of SC-PHH and suspension PHH to provide a broader context to their functionality. Our results reveal the impact of extracellular matrix overlay with Cryo-HepaRG functionality, provide genotyping analysis of pharmacologically-important poor metabolizer alleles, and demonstrate that Cryo-HepaRG can properly sequester CAR in the cytosol and translocate it to the nucleus upon PB treatment.
Materials and Methods:

Materials: Cryopreserved HepaRG® Cells (Cryo-HepaRG), William’s E Medium (WEM), Collagen I Coated 96-well plates, GlutaMAX™ Supplement, HPRG770, HPRG720, and HPRG740 medium supplements, Cryopreserved Hepatocyte Recovery Medium (CHR™), Geltrex™ Matrix, Carboxy Dichlorofluorescein Diacetate (CDFDA), and PHH were obtained from Life Technologies (Carlsbad, CA). Serum-free hepatocyte culture supplement ITS+ was obtained from BD Biosciences (San Jose, CA). Phenacetin, acetaminophen, coumarin, 7-hydroxycoumarin, bupropion, hydroxybupropion, paclitaxel, 6α-hydroxypaclitaxel, diclofenac, 4'-hydroxydiclofenac, mephenytoin, 4'-hydroxymephenytoin, dextromethorphan, dextrophan, testosterone, 6ß-hydroxytestosterone, midazolam, 1-hydroxymidazolam, benzylamine, benzylamine N-oxide, 7-hydroxycoumarin glucuronide and sulfate, omeprazole (OMP), phenobarbital (PB), rifampicin (RIF), dimethyl sulfoxide (DMSO), aflatoxin B1, ketoconazole, benzonaphthoflavone, hyperforin, 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO), taurocholate, and estrone sulfate were obtained from Sigma-Aldrich (St. Louis, MO) or other commercial sources.

Culturing of Cryo-HepaRG: Cryo-HepaRG were thawed and plated (1 x 10⁵ cells/well) on 96-well flat-bottom collagen (I) coated cell culture plates following the Life Technologies standard plating protocol. Briefly, supplemented culture media (WEM supplemented with HPRG770 and GlutaMAX™) was warmed to 37°C (water bath). Media (9 mL/plate) was transferred to a 50 mL conical tube. Cryo-HepaRG were thawed at 37°C (water bath) until a small amount of ice crystals remained. Cryo-HepaRG were aseptically transferred to the 50 mL conical tube and centrifuged for 2 min at 357 x g at room temperature. Media was aspirated and cells were resuspended in 5 mL of HPRG770-supplemented media and cells were counted and assessed for viability with Trypan Blue (0.05%) using a Countess™ (Life Technologies, Carlsbad, CA). Cell densities were adjusted to 1.25 x 10⁶ cells/mL, and 80 µL was delivered to each well (pre-wetted with 45 µL of plating media) of cell culture plates using a multi-channel pipettor. Plated cells were subsequently allowed to settle in cell culture plates for ~10 min in the biosafety cabinet and moved to a humidified incubator at 5% CO₂ and 37°C. Supplemented media were changed, as needed, for each assay type >1 hr after plating, and culture
media were renewed every 2-3 days, unless otherwise stated. All HepaRG and PHH cell culture images were captured using a Zeiss Axiocvert inverted research microscope equipped with phase-contrast optics, a 3CCD camera, and imaging computer with image analysis software. Freshly differentiated HepaRG® cells were grown and differentiated from progenitor cells as previously described (Gripon et al., 2002).

PHH: Cryopreserved PHH were thawed and recovered using CHRM™, then directly assayed in suspension form or plated using WEM (Hepatocyte Plating Supplement Pack, serum-containing, Life Technologies, Carlsbad, CA) at optimal density (e.g., 0.8 x 10⁶ cells/mL) in 24-well culture plates. Cryopreserved and fresh hepatocytes were allowed to attach for 4-6 hr in a humidified incubator at 5% CO₂ and 37°C, prior to overlay with 0.35 mg/mL Geltrex™ in serum-free WEM supplemented with ITS⁺, GlutaMAX™, 1% Pen/Strep, 15 mM HEPES buffer, and 100 nM dexamethasone. Culture media were replaced daily. PHH reference data from lot characterizations across hundreds of donor preparations (cultured and suspension formats) represent approximately equal numbers of male and female donor preparations. For Hu8033, primary hepatocytes were derived from a female donor.

CDFDA Staining: Cryo-HepaRG were thawed and plated (3 x 10⁵ cells/well) onto 24-well culture plates using WEM supplemented with HPRG770 and GlutaMAX™. Media was replaced the next day with WEM supplemented with HPRG720 and GlutaMAX™ supplement. Media was refreshed every two days thereafter. After 10 days in culture, media was aspirated from plates and cell monolayers were washed with warm Hanks’ Balanced Salts Solution (HBBS). HepaRG® cultures were incubated for 15 minutes with cell culture media containing 5 µM CDFDA. After incubations, fluorescence photomicrographs were captured as described above with a fluorescent light source.

Drug Metabolism Assays: Cryo-HepaRG were cultured for 0 (suspension), 1, 2, 3, 4, 10, or 22 days (media were renewed every 2-3 days) in HPRG720 (metabolism media supplement) and GlutaMAX™ prior to conducting in situ incubations for metabolic activities, with the exception of induction assays (as described below). Primary human hepatocytes were cultured 48-72 hr in WEM (ITS⁺, GlutaMAX™, 1% Pen/Strep and 100 nM dexamethasone) prior to in situ metabolism assays with
probe substrates. The following final substrate concentrations were used in cell culture media: 100 µM phenacetin (CYP1A2), 5 µM coumarin (CYP2A6), 500 µM bupropion (CYP2B6), 20 µM paclitaxel (CYP2C8), 25 µM diclofenac (CYP2C9), 250 µM S-mephenytoin (CYP2C19), 15 µM dextromethorphan (CYP2D6), 250 µM chlorzoxazone, 10 µM midazolam (CYP3A4/5), 200 µM testosterone (CYP3A4/5), 250 µM benzydamine (FMO), or 100 µM 7-hydroxycoumarin (SULT, UGT). All in situ metabolism assays were conducted in single probe format as previously described (Jackson et al., 2009). All cell suspension metabolism assays were performed in single probe format as previously described (Smith et al., 2012). All Cryo-HepaRG metabolism assays for all substrates examined were conducted for 1 hr. PHH metabolism assay incubation periods were substrate-dependent (15min-phenacetin, 20min-bupropion, 45min-paclitaxel, 15min-diclofenac, 30min-(S)-mephenytoin, 15min-dextromethorphan, 15min-chlorzoxazone, 14min-testosterone, 10min-midazolam, 30min-ethoxycoumarin, 30min-benzydamine). Metabolism assay samples were collected and stored frozen at –80°C until they were processed for LC-MS/MS analysis. LC-MS/MS analysis of metabolism assay samples were conducted as previously described ((Smith et al., 2012), (Jackson et al., 2009)) and included standard curves with at least 6 calibration standards along with 12 quality control samples (at 3 different concentrations) dispersed in the beginning, middle, and end of analytical runs to assess quantitative continuity throughout the run.

Induction Assays: To evaluate P450 induction properties, Cryo-HepaRG were maintained in WEM supplemented with HPRG740 (no DMSO) and GlutaMAX™ supplement beginning on Day-3 of culture (initially plated in HPRG770-supplemented media). Dosing with 50 µM omeprazole, 1 mM PB, or 10 µM RIF was initiated on day three of culture and renewed once daily over the ~72 hr treatment period. Inducers were dissolved in 100% DMSO and diluted 1/1000 in cell culture media for a final DMSO concentration of 0.1%. The extent of induction was evaluated with in situ metabolism assays for CYP1A2, CYP2B6, & CYP3A4 enzymatic activity compared with vehicle control levels. Probe substrates phenacetin, bupropion, and testosterone were dissolved in 100% DMSO and applied to cell cultures (as described above) for 60 minutes. Assay media were subsequently transferred to round bottom polypropylene plates (96-well) for LC-MS/MS analysis as described above. For gene
expression assays, established (inventoried, validated) TaqMan® assays for CYP1A2, CYP2B6, CYP3A4, and GAPDH were used. TaqMan® universal PCR Master Mix and dNTPs were obtained from Applied Biosystems (Life Technologies, Foster City, CA) and used per the manufacturer’s protocol. Complementary DNA (cDNA) was generated from isolated total RNA from cell cultures using an ABI PRISM® 6100 Nucleic Acid Prep Station and ABI chemistry (ABI, Foster City, CA) following the manufacturer’s protocol for cell culture lysates. Total RNA was quantified using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). A High Capacity cDNA Archive Kit (Life Technologies) was used to synthesize cDNA from 200 ng of total RNA, using random hexamers, following the manufacturer’s protocol.

Cytotoxicity Assays: For cytotoxicity assays, plating media were replaced on Day-2 in culture with WEM supplemented with HPRG720 or HRG770 and GlutaMAX™ supplement. Media were refreshed every two days thereafter. HepaRG cultures were treated with aflatoxin B1 on Day-7 in culture. 24 hours after treatment, ATP content assays were performed. ATP levels were determined using CellTiter-GLO™ Luminescent Cell Viability Assay according to the manufacturer’s instructions (Promega) using a FLUOstar Omega luminometer (BMG Labtechnologies). Dose response curves were modeled with the Hill equation, as previously described (Beam and Motsinger-Reif, 2011).

Uptake Transport Assays: Plated uptake assays were performed on Day-4 of Cryo-HepaRG culture. Cell culture medium was aspirated from culture plates and cell monolayers were washed three times with warm HBBS plus HEPES buffer. Cells were pre-incubated with the third wash in a 37°C humidified incubator with 95% air/5% CO2 for 10 minutes. Buffer was subsequently replaced with dose solution containing varying concentrations of 3H radiolabeled taurocholate, estradiol-17β-glucuronide, or estrone sulfate prepared in HBSS plus HEPES buffer, and incubated for 30 minutes. After incubation, dose solutions were aspirated and HepaRG® or PHH cultures were washed three times with ice cold buffer and placed at -80°C for a minimum of 15 min prior to cell lysis with 0.1% Triton X solution. Samples were analyzed using a Microbeta Trilux (Perkin Elmer) scintillation counter. Primary hepatocyte uptake transport assays were performed analogous to HepaRG®
cultures, with the exception that 10 min incubations were performed, and cultures were maintained for 18-24 hr (post plating), prior to uptake assays.

CYP3A4 Immunohistochemistry: CYP3A4 antibody was purchased from Abcam (Cambridge, MA). Treated cell cultures (24-well) were washed with PBS, fixed with 500 µL of 2% paraformaldehyde solution with 0.1% saponin in PBS for 30 min at room temperature. Fixed cells were washed 3 times with PBS for 1 min on a micro-orbital shaker (~200 rpm). Subsequently, blocking was performed with 300 µL of Image it FX signal enhancer and incubated at room temperature overnight. Blocked cells were washed 3 times with 500 µL/well of warm PBS for 1 min on an orbital shaker (~200 rpm). Cells were further incubated with primary antibody at 1/200 dilution for 1 hr at 37°C and imaged with a Zeiss Axiovert inverted research microscope as described above.

Protein Quantification Assays: Protein quantification assays were performed using Thermo Scientific Pierce BCA Protein Assay Kit following the manufacturer’s instructions and absorbance was quantified using a FLUOstar Omega plate reader (BMG Labtechnologies).

Genotyping Analysis: Genomic DNA was isolated from HepaRG® cells utilizing an ABI PRISM® 6100 Nucleic Acid Prep Station and ABI chemistry following manufacturer’s instructions (Life Technologies). Genotyping of HepaRG® Cells genomic DNA was conducted using TaqMan® Drug Metabolism Genotyping Assays on an ABI 7900 Real-Time PCR instrument following manufacturer’s instructions.

Translocation of CAR in HepaRG® cells: An adenovirus expressing enhanced yellow fluorescent protein tagged hCAR (Ad/EYFP-hCAR) construct was generated and functionally characterized, as previously reported (Li et al., 2009a). Cryo-HepaRG were thawed and seeded on 24-well BioCoat plates in WEM containing HepaRG® Induction Medium Supplements HPRG740 (serum-containing) & HPRG750 (serum-free). Twenty-four hours after plating, HepaRG® cells were infected with Ad/EYFP-hCAR (5 µL virus per well) for 12 hr, followed by treatment with vehicle control (0.1% DMSO) or PB (1 mM) for an additional 8 hr. Treated cells were then subjected to confocal microscopy analysis with a Nikon C1-LU3 instrument based on an inverted Nikon Eclipse TE2000 microscope (Tokyo, Japan).
Localization of hCAR was quantitatively characterized as nuclear, cytosolic, and mixed (nuclear +
cytosolic) expression by counting 100 Ad/EYFP-hCAR-expressing HepaRG® cells from each
treatment group.

Statistical Analyses: Multiple T tests comparisons of mean enzymatic activities were determined in
GraphPad Prism v.6.07, analyzing each row individually without assumption of consistent standard
deviations, correction for multiple comparisons using Holm-Sidak method, and an alpha of 0.05.
Tukey’s pairwise comparisons of mean response data were performed after initial oneway ANOVA
using JMP 11.0.0 (SAS, NC) at an alpha of 0.05.
Results:

Culture Model Evaluation

Hepatocyte morphology is known to be a useful indicator of monolayer integrity/differentiation status with in vitro liver models (Hamilton et al., 2001). Therefore, we assessed Cryo-HepaRG culture morphologies compared with freshly differentiated HepaRG® and SC-PHH (Figure 1A, 1C). In initial cell biology evaluations with Cryo-HepaRG, we observed a rapid (<10 min) attachment to cell culture plates compared with the ~4-6 hr generally required for PHH. After 1-2 hr in culture, Cryo-HepaRG appeared rounded (Figure 1D) and lacked characteristic cobblestone appearance and cord-like networks of hepatocyte-like cells observed with fully/freshly differentiated HepaRG (Figure 1A). Cobblestone-like networks are common to epithelial cells in confluent monolayers that auto-assemble, resembling structures found in vivo within the liver (Godoy et al., 2013). Their absence after only a few hours in culture indicates that Cryo-HepaRG were undergoing a significant transition to their new culture environment. While Cryo-HepaRG began to resemble fully differentiated HepaRG ~24 hr post attachment morphologically (Figure 1E), 7-10 days were required for full recovery of morphology (Figure 1F, 1B). Initial experiments to optimize Cryo-HepaRG cell seeding densities using metabolic competence (Supplemental Figure 1) and cell morphologies resulted in selection of ~100,000 cells/well (96-well format). Seeding densities greater than ~100,000 cells/well resulted in overcrowding of monolayers and forced HepaRG cells to intermittently rise out of plane. Analogous to PHH cultures (LeCluyse et al., 1994) and freshly differentiated HepaRG® (Antherieu et al., 2010), Cryo-HepaRG appeared to form bile canalicular structures after ~3 days in culture. In general, these structures were more rounded between multiple cells compared with longer linear or y-like structures, common to SC-PHH. Bile canalicular formation was further evaluated using CDFDA staining. CDFDA is converted to CDF in hepatocytes, and CDF is a substrate of bile efflux transporter MRP2 in liver (Hoffmaster et al., 2004). MRP2 and other apical efflux transporters are generally thought to be internalized upon disruption of cell-cell interactions during isolation of PHH. Under suitable culture conditions, hepatocytes re-polarize in culture and canalicular transporters (i.e. MRP2) migrate back to the canalicular membrane over time (2-4 days). CDFDA staining of Cryo-HepaRG cultures
demonstrated clear localization of CDF between hepatocytes that perfectly overlaid with canalicular domains in phase contrast images (Figures 1G-I). Approximately 3 days in culture were required to form bile canaliculi in Cryo-HepaRG. Collectively these observations demonstrate that Cryo-HepaRG re-polarize over time in culture analogous to PHH isolated from human livers, and do not immediately re-form fully differentiated monolayers.

**Phase I & Phase II Drug Metabolism Competence Characterization & Temporal Dynamics**

As a marker of hepatocyte differentiation, we evaluated the metabolic competence of Cryo-HepaRG after 10 days in culture with a panel of 12 pharmacologically-important drug metabolizing enzymes (Table 1). Enzymatic activities (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5, flavin monoxygenase (FMO), UDP-glucuronyltransferase (UGT), and sulfotransferase (SULT) were determined in situ and compared to the ranges of interindividual variation observed in SC-PHH under comparable assay conditions. Following 10 days of culture, CYP1A2 activity (phenacetin O-deethylase) was observed at 2.78 +/- 0.728 pmol/min-million cells in Cryo-HepaRG compared with a mean activity of 7.18 +/- 7.78 (near 25th percentile, Figure 2A in red) compiled from a panel of 52 donor preparations of SC-PHH. Cryo-HepaRG CYP1A2 mean activity was statistically different from the SC-PHH mean activity, but the magnitude was less than 2-fold from the median CYP1A2 activity from the 52 different SC-PHH preparations. Notably, both plated Cryo-HepaRG and SC-PHH fall profoundly short of in vivo-like levels at ~6% of median CYP1A2 activity observed across all 212 PHH suspension preparations evaluated (48.0 pmol/min-million cells), which of course, assumes PHH suspensions approximate in vivo levels.

CYP3A4/5 activity in Cryo-HepaRG was evaluated utilizing two probe substrates (testosterone and midazolam) following 10 days in culture under metabolism media (Day-10). Testosterone 6ß-hydroxylase activity was 248 +/- 50.9 pmol/min-million cells in Cryo-HepaRG while midazolam 1-hydroxylase activity was 28.4 +/- 2.39 pmol/min-million cells. Interestingly, the testosterone 6ß-hydroxylase activity in Cryo-HepaRG was ~6.8-fold higher than median and was significantly higher
(P<0.0001) than the mean, median, and top of the range observed with 52 donor preparations of SC-PHH (1.47-178 pmol/min-million cells, mean of 55.2 +/- 49.4). While initially appearing super-physiological, this level of CYP3A4/5 activity was lower than median testosterone 6β-hydroxylase activity observed across 195 suspensions of PHH (407 pmol/min-million cells). Consistent with the elevated CYP3A4/5 basal activity observed across 52 Cryo-HepaRG preparations, CYP2B6 activity levels also exceeded the range observed with SC-PHH (P<0.05) at 17.9 pmol/min-million cells compared with 0.21-13.1 pmol/min-million cells. Although CYP3A4 and CYP2B6 exhibited disproportionally high levels of activity in Cryo-HepaRG, it is important to note that these enzymes are also among the most inducible drug metabolizing enzymes in human liver. Less inducible enzymes such as CYP2A6, CYP2C8, CYP2C9, CYP2C19, and CYP2D6 were not statistically (P<0.05) different from the mean activities of 52 SC-PHH preparations evaluated (Table 1). Generally, all in situ measured activities were around 10% of the median activities observed with PHH suspensions. CYP2D6 activity in Cryo-HepaRG was near the low end of the range compared with SC-PHH and is further discussed in the genotyping analysis described below. CYP2E1 activity was also evaluated in Cryo-HepaRG cultures under these conditions (data not shown); however, the potent inhibitory effects of DMSO on CYP2E1 prohibited accurate evaluation of CYP2E1 metabolic competence. Enzymatic activity data for FMO, UGT, and SULT were also determined for Cryo-HepaRG and reported in Table 1. While no FMO, UGT, or SULT data were available in SC-PHH under these assay conditions, Cryo-HepaRG exhibited ~10% FMO and ~30% SULT activity levels compared to PHH suspensions, while UGT activity levels were comparable between Cryo-HepaRG and PHH suspensions.

As demonstrated in Table 1, SC-PHH lose a substantial proportion of drug metabolism competence over time in culture from their initial suspension format. We hypothesized that a similar phenomenon may occur with Cryo-HepaRG that has not been reported previously and may impact metabolism-related research findings. Therefore, we compared CYP1A2, CYP2B6, and CYP3A4/5 enzymatic activities of Day-4 Cryo-HepaRG (Table 1) with Day-10 Cryo-HepaRG against the activities observed across 52 SC-PHH preparations (Figure 2A-C). Here we observed that Day-4 CYP1A2 activity in
Cryo-HepaRG fell below the 25th percentile of SC-PHH range compared with Day-10 activity that rose ~3-fold to levels near the 25th percentile of SC-PHH (Table 2, Figure 2A). CYP2B6 and CYP3A4 showed similar trends shifting from lower activities at Day-4 (near the 25th percentile and average of the distributions, respectively) to levels above the SC-PHH range by Day-10 with increases of 19.7-fold and 3.8-fold, respectively (Figure 2B & 2C). One possible explanation for the observed increases in P450 activity (normalized to cell numbers plated) over time in culture could be cellular proliferation occurring between Day-4 and Day-10. While proliferation is unlikely in contact-inhibited confluent monolayers (Gripon et al., 2002), we measured total protein content over time as a marker of cell number and observed no statistically significant changes in total protein content across the time points examined (Supplemental Figure 1). Therefore, increased drug metabolism competence over time along with morphological observations appears to reflect and improved hepatocyte differentiation status rather than an increase in the number of HepaRG cells.

To further characterize the temporal dynamics of metabolic competence with Cryo-HepaRG cells, we generated specific activity data with suspensions of Cryo-HepaRG in their initial form for comparison with cultured Cryo-HepaRG at Day-4 and Day-10 (Table 2). In general, Cryo-HepaRG suspensions and Day-10 cultures of Cryo-HepaRG (in HPRG720 metabolism media) produced comparable metabolic activities across 9 metabolizing enzymes, consistent with cell morphology observations. A good correlation (Pearson Correlation with R=0.998) of enzymatic activities was observed between Cryo-HepaRG suspensions and Day-10 cultures of Cryo-HepaRG (Figure 3A). These data coupled with the Day-4 enzymatic activity data suggest that Cryo-HepaRG cells (under metabolism media) initially lose much of their metabolic competence, but recover metabolic competence comparable suspension levels over ~10 days. For the cytochrome P450s evaluated, Day-10 metabolic competence was on average 8.6-fold higher than Day-4, while FMO, UGT, and SULT activities were 2.0-, 3.8-, and 1.8-fold higher on Day-10 relative to Day-4, respectively. We extended this study out to 22 days with activity data shown in Figure 3C that were normalized to respective Day-4 activities in Figure 3D. Metabolic competence appeared to achieve maximal levels at ~10 days in culture across the metabolic pathways assessed and was largely maintained at comparable levels until Day-22.
Notably, Day-16 enzymatic activities were lower than metabolic activities on Day-10 and Day-22 across the panel of enzymes. It is not clear whether this decrease was biologically important as monolayer morphology appeared unchanged after Day-10 and time points on both sides of Day-16 were generally comparable.

Due to the observed lower P450 activities with Day-4 Cryo-HepaRG, we further evaluated the loss of activity from suspensions through the first 96 hours in culture. As hypothesized, Cryo-HepaRG cultures appear to undergo a similar loss of activity (~10-fold for CYP3A4) to that observed with PHH (Smith et al., 2012) as they transition from suspension to cell culture monolayers after removal from the liver (Figure 3B). The observed ‘half-life’ of CYP3A4/5 testosterone 6ß-hydroxylase activity was 34.1 hr +/- 1.02 hr which is consistent with the time frame observed with PHH (~29 hr) (Smith et al., 2012). Minimum activity level was observed after ~72 hr in culture as later time points appeared to produce higher activities. In summary, physiologically-relevant levels of drug metabolism activities were observed in Cryo-HepaRG cultures when compared against SC-PHH. However, Cryo-HepaRG metabolic competence did not appear to be a static, intrinsic property of Cryo-HepaRG post-differentiation/cryopreservation, as Cryo-HepaRG metabolism levels exhibit marked temporal dynamics over the initial 10 days in culture that is important to understand in metabolism-related research.

To more comprehensively contextualize Cryo-HepaRG metabolic competence, we calculated Cryo-HepaRG Day-4, Cryo-HepaRG Day-10, and SC-PHH Day-4 enzymatic activities as percentages of mean suspension PHH activities (Figure 4). From these data, PHH suspension activities far exceeded (P<0.05) those observed with SC-PHH for all enzymes investigated. This was also true for Day-4 Cryo-HepaRG. For Day-10 Cryo-HepaRG, mean activities were also lower and generally statistically significant (P<0.05) compared to mean and median PHH suspension activities. However, due to high interindividual variability observed in CYP3A4/5 (P=0.058 for OHMDZ and P=0.064 with 6ßT), CYP2C19 (P=0.19), and UGT (P=0.15) activities, statistical significance was not reached at the P<0.05 level. These data also show significant (P<0.05) increases for all evaluated enzymatic activities from Day-4 to Day-10 with Cryo-HepaRG cultures in metabolism media. Day-10 Cryo-
HepaRG were also statistically (P<0.05) indistinguishable from SC-PHH across this panel of metabolic activities except for the higher observed activities of CYP2B6 and CYP3A4/5 as described above. Taken together, these data demonstrate that Day-10 Cryo-HepaRG cultures were largely comparable to SC-PHH with elevated levels of CYP2B6 and CYP3A4/5, while Day-4 Cryo-HepaRG were generally lower in metabolic capacity than SC-PHH with the exception of CYP3A4/5 (6ßT).

Effects of Overlay with Extracellular Matrix on Metabolism & Cell Culture Morphologies

Overlay of PHH cultures with extracellular matrices (e.g. Geltrex, Matrigel) in ‘sandwich’ configuration is standard practice with PHH to improve longevity, cuboidal three-dimensionality, and hepatocyte differentiation (LeCluyse et al., 1994). Therefore, we assessed the impact of Geltrex overlay on cultures of Cryo-HepaRG cells in serum-free HPRG750-supplemented induction media (contains no DMSO). Figures 5A & 5B show Cryo-HepaRG cell morphology photomicrographs with and without Geltrex overlay (0.35 mg/mL). Overlay of Cryo-HepaRG cultures led to a marked change in Cryo-HepaRG morphology, producing improved homogenous monolayers within a single focus plane. Overlay appeared to shift the cell populations towards more three-dimensional morphologies analogous to SC- PHH (i.e. smaller nuclear-to-cytosolic ratios), and qualitatively appeared to produce higher proportions of hepatocyte-like cells (fewer cholangiocyte-like cells). These characteristics are generally associated with improved hepatocyte functionalities; therefore, we further evaluated the effects of overlay on metabolic competence. CYP1A2, CYP2B6, and CYP3A4 were examined in situ, and the results are shown in Figure 5C. Approximately 2- to 3-fold increases in basal metabolic activities were observed in the presence of Geltrex overlay consistent with cell morphology observations. To further evaluate the effects of Geltrex overlay on Cryo-HepaRG cultures, immunohistochemistry was performed for CYP3A4, a marker of differentiated hepatocytes (Figures 5D-G). Immunohistochemistry for CYP3A4 was able to effectively stain hepatocyte-like HepaRG cells in standard Cryo-HepaRG cultures (Figure 5D vs. 5E). Upon overlay with Geltrex, the CYP3A4 staining became more prevalent across the culture with increased fluorescence coverage overall and
larger areas of CYP3A4 staining (Figure 5F and 5G). Taken together, these data indicate that overlay of Cryo-HepaRG improved their hepatocyte functionality under the conditions examined.

Genotyping of Important Human ADME Genes HepaRG®

CYP2D6 and CYP2C9 enzymatic activities (Table 1) in Cryo-HepaRG cultures yielded lower levels of metabolic activity (near the lower end of the ranges of interindividual variation) compared with SC-PHH. HepaRG® cells are known to contain only a single copy of chromosome 22 that encodes for CYP2D6 that could contribute to the lower CYP2D6 activity (Gripon et al., 2002). However, these low levels of CYP2D6 and CYP2C9 could arise from the presence of poor metabolizer alleles in cells originating from a female Caucasian donor. Therefore, we assessed common poor metabolizer alleles in a panel of drug metabolizing enzymes (CYP2C9, CYP2C19, CYP2D6, CYP3A5) known to be important for interindividual variation in drug clearance (Ingelman-Sundberg et al., 2007). Genotyping analyses revealed the presence of multiple P450 SNPs (summarized in Table 3). As suspected from enzymatic activity data, HepaRG® cells contain poor metabolizer alleles for CYP2D6 (*2 and *9). These results suggest that HepaRG® cells are deficient in CYP2D6 metabolism. The small observable dextromethorphan conversion to dextrorphan could be the product of other enzymes including CYP3A4/5 as previously reported (Yu and Haining, 2001). Our genotyping analysis also revealed poor metabolizer alleles for CYP2C9 (*2/*2) consistent with the observed low CYP2C9 activity. For CYP3A5, HepaRG® cells contained two CYP3A5*3 alleles. These are known to be null alleles due to expressed RNA instability and are present in the majority of individuals of Caucasian descent. Therefore, it is likely that CYP3A4/5 activity in HepaRG® cells is predominantly attributable to CYP3A4 activity.

Metabolism-Dependent Cytotoxicity in Cryo-HepaRG Cultures
Aflatoxin B1, a well-known metabolism-dependent hepatotoxicant, has been shown to undergo metabolic activation via CYP3A4 and CYP1A2 producing a highly reactive epoxide metabolite (Essigmann et al., 1977). The well-documented mechanism of action of aflatoxin B1 makes it an attractive probe toxicant to evaluate the utility of Cryo-HepaRG to support evaluation of metabolism-dependent toxicity. HepaRG® cells were cultured in HPRG720 metabolism media for 7 days prior to dosing cultures with aflatoxin B1, ketoconazole (a reversible inhibitor of CYP3A metabolism in humans), or the combination of these two chemicals. Cryo-HepaRG cultures were treated for ~24 hr prior to cell health/stress evaluations (Figure 6). Both LDH and ATP data confirm aflatoxin B1-related cytotoxicity at 5-10 µM half maximal effective concentrations (EC₅₀). Ketoconazole (20 µM) was able to attenuate the observed cytotoxicity (Figure 6A, 6B). Comparison of aflatoxin B1 cytotoxicity under HPRG720 vs. HPRG730 (‘Tox’ media) media supplements, known to modulate P450 basal activities, produced a shift in aflatoxin B1 potency consistent a metabolically-activated toxicity (Figure 6C). These data highlight the utility of the Cryo-HepaRG culture model to assess metabolically-activated toxicity, support previous studies showing the utility of P450 inhibitors to study mechanisms of toxicity (Li, 2009b), and replicate previous studies demonstrating freshly differentiated HepaRG are a sensitive model to study aflatoxin B1 cytotoxicity (Aninat et al., 2006).

**Active Uptake Transport Characterization**

An important and often rate-limiting step in drug clearance is hepatic uptake transport (Shitara et al., 2013). Active uptake transport function was evaluated in cultures of Cryo-HepaRG using tritium-labeled taurocholate (an NTCP substrate), estrone sulfate (an OATP1B1/3 substrate) and estradiol-17β-glucuronide (OATP1B1/3). For these evaluations HepaRG® cells were cultured for 10 days prior to initiating experiments. Active uptake with taurocholate was assessed at multiple concentrations at both 4°C and 37°C to estimate the respective passive and active uptake transport capacity of cultured Cryo-HepaRG (Figure 7A) with representative uptake transport data from a single SC-PHH preparation (Hu8083) shown in Figure 7B. For Cryo-HepaRG, active uptake transport at 37°C was...
readily distinguishable from uptake evaluated at 4°C consistent with competence for active uptake via NTCP. The estimated $K_m$ for taurocholate uptake was 21.1 µM +/- 3.08 µM for Cryo-HepaRG compared with 6.68 +/- 0.564 for Hu8083 SC-PHH fit to a Michaelis-Menten model. The extent of taurocholate uptake in Cryo-HepaRG cultures appeared to be somewhat lower than SC-PHH, but it is notable that the percentages of hepatocytes in these models are different. To further contextualize the extent of taurocholate uptake transport in Cryo-HepaRG cultures, we plotted 1 µM taurocholate uptake transport rates vs. the range of transport rates across 76 donor preparations of SC-PHH (37 female, 39 male) under the analogous assay conditions (Figure 7C). Cryo-HepaRG uptake was comparable to SC-PHH falling above the 25th percentile of the distribution (9.3 pmol/min-mg) but lower than the median rate of SC-PHH taurocholate update (11.8 pmol/min-mg). We further evaluated Cryo-HepaRG uptake transport competence with estrone sulfate (ES) and estradiol-17ß-glucuronide (E2-17ßG), known substrates for OATP uptake transporters OATP1B1 and OATP1B3 (Figure 7D, 7E, Table 4). Cryo-HepaRG cultures produced some active uptake with estrone sulfate at both 0.5 and 40 µM concentrations (Figure 7D & 7E). Lower proportions of active uptake were observed with E2-17ßG (~2-fold over 4°C control, data not shown). Reference SC-PHH data were not available for ES uptake. However, median E2-17ßG (1 µM) uptake (37°C) across 76 lots of SC-PHH preparations was 2.92 pmol/min-mg compared with 0.357 +/- 0.0181 pmol/min-mg (0.5µM) under comparable culture conditions with Cryo-HepaRG (Table 4). The presence of serum in cell culture media did appear to elevate uptake transport with both ES and E2-17ßG. Geltrex overlay of Cryo-HepaRG cultures did not appear to have a profound effect on uptake transport, but uptake did appear marginally higher in the presence of overlay. Collectively these data demonstrate that Cryo-HepaRG cultures were competent for active uptake transport, but have a somewhat reduced capacity compared with SC-PHH under the culture conditions examined.

**Functional Xenobiotic Signaling Pathways in Cryo-HepaRG:**
Hepatic cell lines such as HepG2 and Fa2N-4 have been used in screening and mechanistic research to represent hepatic function in vitro. However, numerous reports have demonstrated their severely limited differentiation status for mature hepatocyte phenotypes in these cell lines, including xenobiotic metabolism and receptor signaling pathways (Donato et al., 2008; Hariparsad et al., 2008). Therefore, we evaluated Cryo-HepaRG cultures with various selective activators of AhR, CAR, PXR, and FXR, monitoring induction of downstream target genes (Figure 8A-D). After cells were cultured for 48 hr post-thaw, they were treated with inducers for 72 hr at three concentrations prior in situ metabolism assays. Concentration-related increases in CYP1A2 enzymatic activity were observed with the AhR agonist omeprazole. These responses were compared against ranges of induction responses observed in cultures of PHH, and generally showed comparable (and substantially less variable) induction well within the ranges of ‘normal’ response (Figure 9). Additionally, CYP1A2 mRNA was induced over three independent assay plates with an average of ~350-fold over 0.2% DMSO control (Figure 10A). Induction of CYP1A2 mRNA was larger (fold-over-control) compared with enzymatic activity responses; a phenomenon common to SC-PHH (unpublished observations). It is also notable that for induction assays, metabolism incubations with phenacetin required ~1 hr to produce sufficient levels of CYP1A2 specific metabolite acetaminophen. This is comparable to the incubation time frame (30-60 min) effective with SC-PHH that produces robust activity levels yet avoids substrate depletion. By comparison, ~24 hr are commonly required to observe specific metabolite levels with de-differentiated cell lines such as HepG2 (Granata et al., 2006).

Induction of CYP2B6 is known to be a marker of CAR and PXR activation with both receptors activating response elements within the CYP2B6 upstream regulatory regions (Wang et al., 2003). While there is crosstalk between these pathways, CYP2B6 induction is more responsive to CAR activators compared with PXR activators (Faucette et al., 2006). With SC-PHH, CYP2B6 induction is generally a sensitive marker of hepatocyte culture integrity with lower quality lots failing to support CYP2B6 induction in response to PB (unpublished observations over hundreds of donor preparations of SC-PHH). In Cryo-HepaRG cultures, CYP2B6 was clearly inducible in a concentration-related manner with both direct (CITCO) and indirect (PB) activators of CAR at sufficiently low concentrations.
to observe induction and minimize the contributions of PXR to CYP2B6 induction (Figure 8B). Induction of CYP2B6 in response to indirect activators (i.e. PB and phenytoin) has not been observed with any immortalized hepatic cell lines (i.e., HepG2, Huh-7, Fa2N-4) to date, which has been attributed to insufficient expression and cell signaling functionality to sequester CAR within the cytosol or translocate it to the nucleus (Sueyoshi et al., 2008), (Templeton et al., 2011). CYP2B6 induction in Cryo-HepaRG cultures (3 independent lots) was further contextualized against the range of responses observed in SC- PHH and showed comparable (and substantially less variable) CYP2B6 induction in response to PB (Figure 9). CYP2B6 mRNA induction with PB over three independent assay plates produced ~15-fold induction responses that were larger but consistent with enzymatic activity induction (Figure 10B). Consistent with the CYP1A2 induction evaluations, 1 hour bupropion substrate incubations were effective in Cryo-HepaRG, further supporting their metabolic competence akin to SC-PHH and distinguishing them from de-differentiated cell lines that are generally known to have undetectable enzymatic activity levels.

Two agonists of PXR, RIF and hyperforin, were used to evaluate the competence of Cryo-HepaRG cultures (Figure 8C). From these data, Cryo-HepaRG cultures were clearly responsive to PXR agonists as has been reported previously with freshly differentiated HepaRG® cultures (Andersson et al., 2012). Analogous to the CYP2B6 induction evaluations with Cryo-HepaRG, lower concentrations of inducers (hyperforin and RIF) were used to activate PXR while minimizing the potential for crosstalk with the CAR pathway. Induction of CYP3A4/5 metabolic activity compared favorably with the range of responses observed with SC- PHH (Figure 9). The inter-lot variation with Cryo-HepaRG (3 independent lots) was profoundly lower than the variability observed across 52 SC-PHH preparations. CYP3A4 mRNA was also effectively induced with 10 µM RIF over three independent assay plates. The ~60-fold mRNA induction produced was larger than the fold changes observed at the enzymatic activity level (Figure 10C).

The farnesoid X receptor (FXR) is an important regulator of bile homeostasis, and plays an important role in liver function (Teodoro et al., 2011). We assessed FXR functionality in Cryo-HepaRG cultures with the agonist chenodeoxycholic acid (CDCA) and measured induction of ABCB11 mRNA.
expression (Figure 8D). ABCB11 is a FXR target gene encoding for the bile salt export pump (BSEP) which is heavily involved in bile acid homeostasis and plays an important role in biliary efflux of bile acids that can be quantitatively modeled in vitro with SC-PHH (Plass et al., 2002),(Liu et al., 1999). With CDCA treatment we observed a concentration-related induction of ABCB11 in cultures of Cryo-HepaRG consistent with reports in SC-PHH (Yu et al., 2002). These data suggest that HepaRG® cells support the FXR signaling pathway.

We further explored the effects of cell culture media supplements and Geltrex overlay with Cryo-HepaRG on liver enzyme inducibility (Figure 11). Here we compared CYP1A2, CYP2B6, and CYP3A4/5 enzymatic induction responses with both the HPRG740 (serum-containing) and HPRG750 (serum-free) supplemented media in the presence and absence of Geltrex overlay. Cultures were treated for 72 hr with prototypical AhR, CAR, and PXR activators. In general, comparable fold induction responses were observed with either HPRG740 or HPRG750-supplemented media using prototypical inducers in the presence or absence of Geltrex overlay. However, with overlay, serum-free HPRG750 supplemented media supported more robust induction responses for CYP1A2 and CYP2B6, while the more balanced CYP3A induction by RIF and PB was consistent with induction responses in SC-PHH using serum-free induction media. Analogous to overlay experiments with HPRG720-supplemented metabolism media, the use of Geltrex overlay appeared to elevate basal levels of metabolism. In addition, the use of Geltrex overlay in general gave higher induction responses for CYP1A2, CYP2B6, and CYP3A4/5 with either HPRG740 or HPRG750-supplemented media. These data indicate that overlaying cultures of cryopreserved (and potentially freshly differentiated) HepaRG® cells results in higher baseline metabolic capacities and comparable fold induction responses. The use of serum-containing or serum-free media supplements alone did not result in a substantial change in induction responsiveness with these prototypical inducers in Cryo-HepaRG cultures.

CAR Translocation in HepaRG® Cells:
To date, no immortalized hepatic cell line has been shown to effectively support the CAR signal transduction pathway characterized by appreciable levels of CAR expression, cytoplasmic retention of expressed CAR, and nuclear translocation of CAR in response to activators such as PB. Previous reports with overexpressed CAR-GFP fusion protein (transfected via expression vectors) in HepG2 cells demonstrated that the CAR fusion protein spontaneously accumulates in cell nuclei, resulting in constitutive activity that renders CAR non-responsive to its activators like PB which rely on cytosolic sequestration and nuclear translocation as an activation mechanism (Kobayashi et al., 2003). Proper CAR sequestration and translocation are hallmark features of hepatocyte functionality that has only been shown in vivo or with cultures of PHH to date. To evaluate the function of the CAR cytoplasmic retention mechanism within Cryo-HepaRG, cells were thawed, plated, and infected with adenovirus expressing enhanced yellow fluorescent protein-tagged hCAR (EYFP-hCAR). Cells were cultured in serum-containing and serum-free media conditions treated with vehicle or with 1 mM PB. Fluorescent images were captured ~8 hours after treatment to examine the cellular localization of EYFP-hCAR within HepaRG® cells (Figure 12). Fluorescent images and cell counts demonstrated that EYFP-hCAR was predominantly localized to the cytoplasm of HepaRG® cells (70% cytosolic, 15% nuclear and 15% mixed) without activation. In contrast, over 90% of EYFP-hCAR accumulated in the nuclei of PB-treated HepaRG® cells. For the first time in a hepatic cell line, these data demonstrate the existence of a functioning CAR cytoplasmic retention mechanism, as well as the ability of CAR to translocate to the nucleus in response to activation by PB in Cryo-HepaRG.
Discussion:

Although over 300 publications have been reported with HepaRG® cells, the majority of research has focused on freshly differentiated HepaRG® rather than the newer cryopreserved format (Cryo-HepaRG). Cryo-HepaRG, while significantly more accessible and experimentally flexible, differ from freshly differentiated HepaRG® due to their detachment from freshly differentiated monolayers (disrupting cell-cell/cell-matrix interactions important for hepatocyte function), cryopreservation, thawing, and re-attachment. HepaRG® cell cultures (fresh and cryopreserved) have been claimed to be comparable to SC-PHH, yet comparisons have been limited in functional coverage and broader contextualization to ranges of interindividual variation. In this report we address these limitations and identify an initial adaptation period with Cryo-HepaRG to recover functionality. We provide broader contextualization of Cryo-HepaRG metabolic competence, uptake transport, and liver enzyme inducibility to ranges of interindividual variation observed with PHH, as well as genotyping analysis of pharmacologically-important drug clearance alleles. To our knowledge, this is the first report of the effect that extracellular matrix overlay has on Cryo-HepaRG drug metabolism, transport, and liver enzyme induction. Finally, we demonstrate for the first time that Cryo-HepaRG can sequester CAR in the cytosol of hepatocytes and translocate CAR to hepatocyte nuclei upon PB treatment, analogous to CAR disposition in liver and PHH.

It is well known that hepatocyte cell-cell interactions are key drivers of hepatocyte functionality with PHH that are disrupted during cell isolations from liver (Hamilton et al., 2001). This can result in de-differentiation and reduced drug metabolizing enzyme expression/activity, attributed to the loss of cell-cell interactions and cell-matrix interactions shifting these epithelial cells to more proliferative states. A similar process was observed with the Cryo-HepaRG. Freshly differentiated HepaRG cultures disrupted during detachment/cryopreservation appeared to require a form of reorganization into organized, differentiated ‘epithelia’ upon reattachment. However, unlike PHH, Cryo-HepaRG were able to recover their fresh-like state for multiple weeks in culture. Both freshly differentiated
HepaRG and Cryo-HepaRG appeared to form two distinct cell types with 'cords' of hepatocyte-like cells. These networks did not immediately reform but adaptively matured over 3-4 days, stabilizing by ~7-10 days to fresh-like topologies that persist for at least 22 days. While the mechanisms of how Cryo-HepaRG cultures recover fresh-like topologies are not clear, possible mechanisms might include migration of hepatocyte-like cells in culture or some form of cellular transdifferentiation (Cerec et al., 2007) whereby hepatocyte-like and cholangiocyte-like cells switch identities. After ~10 days of recovery/re-differentiation, we observed enzymatic activities with Cryo-HepaRG were restored to levels comparable with Cryo-HepaRG suspension activities that were substantially higher (~7-fold on average) than Day-4 activities across the enzyme panel. Since drug metabolizing enzymes are established markers of hepatocyte differentiation, lower Day-4 metabolic competence, coupled with cell morphology observations, indicates an initial loss of cellular differentiation with Cryo-HepaRG. A more detailed look at the temporal kinetics of CYP3A4/5 activity loss during the initial 72 hours revealed a 'half-life' of ~34 hours with Cryo-HepaRG cultures (~10-fold decrease) that is analogous to the initial loss of metabolic competence in PHH (~1-3 hr half-life in suspensions, ~20-30 hours in cultures) (Smith et al., 2012). However, PHH cultures do not recover their respective suspension enzyme activity levels under standard conditions (serum-free, no DMSO); instead, activity levels stabilize after the initial loss of metabolic competency to ~10% of their suspension levels as they adapt to their new culture environment, which lasts until they begin to reach the end of their longevity (typically ~1 week). Presumably, this lack of recovery in PHH is the result of the limitations of standard in vitro culture models that lack tissue-like three-dimensionality and dynamic flow (Godoy et al., 2013). In the case of Cryo-HepaRG, the use of high concentrations of DMSO in metabolism/differentiation media likely contributes to the observed differences.

In broader context, the metabolic competence with both SC-PHH and Cryo-HepaRG were lower than suspension PHH levels, which may limit their ability of these models to generate physiologically-relevant metabolites or model metabolic clearance rates. However, these models do effectively model liver enzyme induction. In vivo, liver enzymes are often zonally enriched with higher constitutive levels in Zone 3 and lower, more inducible levels in Zone 2. With Cryo-HepaRG, we observed a similar
phenomenon with varied media supplementation that may reflect ‘zonal’ modeling. It is not clear how this ‘zonality’ with Cryo-HepaRG relates to changes observed during their re-adaptation period.

One possible explanation for the recovery of metabolic competence with Cryo-HepaRG is that cellular proliferation might be occurring. This, however, unlikely since we observed no evidence of increasing cell numbers and HepaRG are thought to be contact inhibited (Gripon et al., 2002). Total protein levels confirmed a lack of evidence for profoundly increased cell numbers over time with Cryo-HepaRG (supplemental Figure 2). Additionally, we observed disproportionately higher levels of certain P450s across the panel of enzymes over time suggesting a simple proliferation mechanism was not solely responsible for the recovery of metabolic competence. This also suggests that metabolic competence with Cryo-HepaRG was not a static, intrinsic property. The disproportionately high levels of CYP2B6, CYP3A4/5, and UGT with Cryo-HepaRG (Day-10) were seemingly super-physiological when compared to activities in SC-PHH. However, these levels were lower than median suspension PHH levels (Figure 4). A probable explanation for these disproportionate levels of inducible enzymes is a combination of hepatocyte re-differentiation and induction by the cell culture media that mimics constitutively induced Zone 3 of a liver lobule. Previous reports have shown that higher levels of DMSO can induce CYP3A4 (Nishimura et al., 2003). Further research to explore the consequences of disproportionate metabolic competence on metabolite profiles is warranted.

Overlay of Cryo-HepaRG cultures with Geltrex, an extracellular matrix commonly used to improve longevity and quality with PHH, yielded a surprising effect on Cryo-HepaRG morphologies. We observed higher apparent hepatocyte-to-cholangiocyte ratios along with corresponding elevations in basal metabolic competence (~2-3-fold). This ‘sandwich’ culture approach is not required with HepaRG cells, but the high concentrations of DMSO that are used to differentiate HepaRG cells (Gripon et al., 2002) can also inhibit some P450 enzymes (e.g. CYP2E1). CYP2E1 is involved in many examples of metabolically-activated toxicity (i.e. acetaminophen), and overlay of Cryo-HepaRG may support more mature differentiation and require lower DMSO levels (Easterbrook et al., 2001; Trafalis et al., 2010). Recent reports have utilized HepaRG® cells in three-dimensional culture configurations and observed similar increases in metabolic capacity/longevity. However, these
models can be complex to generate, highly variable, and difficult to normalize (Malinen et al., 2014; Mueller et al., 2014). Overlaying Cryo-HepaRG may provide a solution for screening and high content imaging that obviates the need for confocal microscopy by creating a more co-planar environment.

Cryo-HepaRG cells are an attractive cell model for in vitro screening due to their functionality and year-over-year availability from a single genetic background, but pose a challenge for xenobiotic metabolism research as they exhibit poor metabolizer alleles for CYP2D6 and CYP3A5 (and, to a lesser extent, CYP2C9). While these polymorphisms may limit the utility of current HepaRG models, they do reflect a substantial proportion of the population due to the prevalence of these alternate alleles. Further efforts to genetically alter these alleles could provide additional tools for research that better reflect population diversity.

In our hands Cryo-HepaRG cells responded to aflatoxin B1 (which produces a CYP3A-mediated genotoxic epoxide metabolite (Kirby et al., 1996)), analogous to previous reports with freshly differentiated HepaRG (Josse et al., 2008). This appeared to be dependent on CYP3A metabolism as an attenuation was observed with CYP3A inhibitor ketoconazole. This functionality coupled with the substantially reduced lot variation of Cryo-HepaRG make this an attractive model for toxicology screening.

One of the hallmarks of a functional in vitro liver model is the ability to respond to prototypical hepatic receptor activators to induce liver enzymes. Cryo-HepaRG cells were clearly able to support prototypical responses to AhR, CAR, PXR, and FXR activators comparable to SC-PHH. One notable difference with Cryo-HepaRG cultures was the reduced magnitude of CYP2B6 induction in response to rifampicin, and further research to explore this mechanism is warranted. Geltrex overlay had little if any effect on the fold induction of liver enzyme levels or the muted CYP2B6 rifampicin response, but did elevate basal levels of metabolic capacity. CAR activators PB (indirect) and CITCO produced induction responses suggestive of a highly functional CAR pathway in Cryo-HepaRG. Further exploration of this mechanism confirmed that Cryo-HepaRG cultures support nuclear sequestering of
CAR in the cytosol and translocation to hepatocyte nuclei upon PB treatment. CAR translocation was not observed in the cholangiocyte-like cells but was observed with and without serum.

In conclusion, this report provides extensive evidence demonstrating the functionality of Cryo-HepaRG and contextualizes various hepatocyte functionalities to ranges of response observed with SC-PHH. The work identifies important temporal dynamics, reveals the beneficial effects of extracellular matrix overlay, provides important genotyping data, and provides the first evidence for a functional CAR translocation pathway with Cryo-HepaRG.
Acknowledgements:

The authors would like to thank Manda Edwards and Ashley Ganoe for their laboratory support with overlay experiments. We also thank Patricia (Miki) Pawlowski and Jasminder Sahi for their contributions to reference primary hepatocyte uptake transport data, and acknowledge Alex Merrick, Sreenivasa Ramaiahgari, Jessica Bonzo, Rafal Witek, and Mark Powers for their efforts in the preparation of this manuscript.
Authorship Contributions:

Participated in research designs: Jackson, Ferguson, Wang, Li, Chamberlain

Conducted experiments: Jackson, Ferguson, Li, Chamberlain

Contributed new reagents or analytical tools:

Performed data analysis: Jackson, Ferguson, Wang, Li

Wrote or contributed to the writing of the manuscript: Ferguson, Jackson, Wang, Li, Chamberlain
References:


KM, and Hengstler JG (2013) Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. Arch Toxicol 87: 1315-1530.


Yu A and Haining RL (2001) Comparative contribution to dextromethorphan metabolism by cytochrome P450 isoforms in vitro: can dextromethorphan be used as a dual probe for both CTP2D6 and CYP3A activities? Drug Metab Dispos 29: 1514-1520.

Footnotes:

a) The laboratory research was funded by Life Technologies (Thermo-Fisher) research and development. Resources for data analysis and manuscript preparations were supported by the Division of the National Toxicology Program, National Institute of Environmental Health Sciences, National Institutes of Health, and by Qualyst Transporter Solutions, Inc.

b) Some of the data presented were part of a poster abstract at the 2011 International Society for the Study of Xenobiotics meeting in Atlanta, GA. Jackson, JP, Edwards, M, Chamberlain, E, and Ferguson, SS. 2011. Cryopreserved HepaRG™ Cells: An Alternative In Vitro Screening Tool for Human Hepatic Drug Metabolism, Induction of Metabolism, & Safety Applications. ISSX, Atlanta GA. Poster.

c) Stephen S. Ferguson, 111 TW Alexander Dr., RTP, NC, 27709. Email: stephen.ferguson@nih.gov

d) ¹Jonathan P. Jackson, ²Linhou Li, ³Erica Chamberlain, ⁴Hongbing Wang, ⁵Stephen S. Ferguson
Figure Legends:

Figure 1: Morphological Evaluation, Seeding Density Optimization, and Bile Canaliculi Staining. Cell morphology photomicrographs with HepaRG® cell cultures at various stages of culture. A) Freshly differentiated HepaRG® after ~4 weeks in culture. B) Cryo-HepaRG after 10 days in culture (post plating). C) Sandwich cultures of cryopreserved PHH (3 days in culture). D) Cryo-HepaRG in culture ~6 hr post plating. E) Cryo-HepaRG ~24 hr in culture. F) Cryo-HepaRG ~7 days in culture. G) Phase contrast brightfield image of Cryo-HepaRG matched to CFDA staining (H), and the merged image (I) highlighting MRP2 efflux function indicative of a polarized monolayer functional for biliary efflux.

Figure 2: HepaRG® Metabolic Competence Contextualized to PHH Sandwich Cultures. Box and whisker plots show ranges of metabolic competence for CYP1A2 (A), CYP2B6 (B), and CYP3A4/5 (C) from PHH sandwich cultures (52 donor preparations) with violet diamonds representing Cryo-HepaRG metabolic activity at Day-4 and red diamonds representing Day-10 Cryo-HepaRG. Error bars show standard deviations of 8 replicate wells from a single lot, box and whiskers show the PHH distribution (52 donor preparations) including: minimum/maximum of the range, mean (crosshair), 25th and 75th percentiles, and median of the distribution. Data were generated using probe substrates phenacetin, bupropion, and testosterone for CYP1A2, CYP2B6, and CYP3A4/5, respectively. LC-MS/MS methods were used to quantify metabolite concentrations from in situ incubations of spent culture media to calculate specific activities. Statistical significance (multiple t tests, P<0.05) between SC-PHH and Cryo-HepaRG indicated with an asterisk (*).

Figure 3: Metabolic Competence (Phase I and Phase II) Comparisons over Time in Culture. Cryo-HepaRG cultures were evaluated for metabolic competence at various times in culture over a range of specific activities with a panel of probe substrates reflective of various metabolic pathways (P450, FMO, UGT, and SULT). Cryo-HepaRG were thawed and plated using HPRG770-supplemented media then switched and maintained in HPRG720-supplemented media (metabolism media) after ~24 hr in culture in sandwich cultures on collagen (Type I) coated plates. A) Pearson correlation
analysis relating observed specific activities from suspension Cryo-HepaRG (pre-plating) compared with Day-10 cultures of Cryo-HepaRG over a range of Phase I and Phase II drug metabolizing enzymes. B) & C) Evaluation of the temporal dynamics of Cryo-HepaRG activity over time in culture. D) Loss of CYP3A4/5 with Cryo-HepaRG in culture during the initial days of culture fit in GraphPad Prism 6.07 using non-linear regression. Data represent mean and standard deviation of 8 independent replicate wells (A, B, & C) and 9 independent replicate wells in (D).

Figure 4: Panel of Liver Enzyme Activities Characterizing Cryo-HepaRG vs. PHH: Metabolic activities with Cryo-HepaRG cultures and SC-PHH were contextualized to mean activities from ranges of interindividual variation observed with suspensions of PHH. Data represent mean and standard deviation percentages of PHH suspension activities (mean) reflective of liver-like levels of enzymatic activities. Statistical significance (multiple t-tests, P<0.05) between mean suspension PHH enzymatic activities vs. other models indicated via asterisk (*).

Figure 5: Cell Morphologies & Metabolic Competence of Cryo-HepaRG with Geltrex Overlay. Cryo-HepaRG were cultured without (A) or with (B) Geltrex overlay (0.35 mg/mL) yielding a more co-planar culture in the presence of Geltrex overlay and more cuboidal cell morphologies consistent with a more three-dimensional configuration. C) After 4 days in culture, metabolic competence for CYP1A2, CYP2B6, and CYP3A4/5 was assessed in Cryo-HepaRG with and without Geltrex overlay in HPRG750-supplemented serum-free culture media. D) Phase contrast image of HepaRG® without Geltrex overlay matched to E) Immunohistochemistry for CYP3A4-immunoreactive protein without Geltrex overlay after 7 days in culture in HPRG720-supplemented ‘metabolism’ media. F) Phase contrast of image of HepaRG® with Geltrex overlay matched to G) Immunohistochemistry for CYP3A4-immunoreactive protein with Geltrex overlay after 7 days in culture in HPRG720-supplemented ‘metabolism’ media. Data represent mean of 3 independent replicate wells and error bars represent the standard deviations of the respective mean responses. Asterisk (*) indicates P<0.05 (Tukey’s Pairwise Comparison) relative to respective No Overlay control.
Figure 6: Metabolism-dependent Cytotoxicity in Cryo-HepaRG. Cryo-HepaRG were cultured for 3 days in HPRG720-supplemented media prior to initiating cytotoxicity experiments. A) & B) Aflatoxin B1 was applied to Cryo-HepaRG cultures in concentration-response for 24 hours and assayed for LDH leakage (A) (CytoTox-One, Promega) and ATP content (B) (CellTiter-GLO, Promega) in the presence/absence of ketoconazole (20 µM). Data represent the mean and standard deviation of 3 independent replicates for each treatment group. C) Concentration-response of aflatoxin B1 in Cryo-HepaRG cultures (7 days in culture) using HPRG720 (metabolism media) and HPRG770-supplemented media after 24 hours of treatment with aflatoxin B1. Data represent mean of 3 independent replicate wells and error bars represent the standard deviations of the respective mean responses.

Figure 7: Active Uptake Transport in Cryo-HepaRG. Cryo-HepaRG were cultured for 10 days in HPRG720-, HPRG740-, and HPRG750-supplemented media and subsequently evaluated for active uptake transport with taurocholate and estrone sulfate. A) Concentration-response curves for taurocholate uptake at 37°C and 4°C in Cryo-HepaRG cultures. B) Representative concentration-response curves for taurocholate uptake at 37°C and 4°C in SC-PHH (Hu8083). C) Box and whisker plot of taurocholate uptake distributions (at 37°C) for 76 donor preparations of sandwich cultured primary human hepatocytes (black) and Day-10 cultures of Cryo-HepaRG (red diamond). D) & E) Estrone sulfate uptake in Cryo-HepaRG in culture for 10 days in metabolism media (HPRG720), serum-containing induction media (HPRG740), and serum-free induction media (HPRG750) media supplements (Cryo-HepaRG were initially plated in HPRG770-plating/maintenance media and changed ~24hr post plating) and assayed for estrone sulfate at 0.5 and 40 µM concentrations. Data represent mean of 3 or 4 independent replicate wells and error bars represent the standard deviations of the respective mean responses.

Figure 8: P450 Enzyme Induction as a Marker of Hepatocyte Functionality with Cryo-HepaRG. Cultures of Cryo-HepaRG were maintained in serum-free induction media (HPRG750) for ~24hr prior to initiating induction experiments with prototypical receptor activators for AhR, CAR, PXR, and FXR. Receptor activators were applied at 3 concentrations to monitor concentration-responsiveness and
were applied at ‘lower’ concentrations less likely to cross-talk with other receptor pathways (e.g., CAR & PXR with PB). A) AhR agonists β-napthoflavone and omeprazole produced induction of CYP1A2 enzymatic activity compared with vehicle control metabolic activity indicating a functional AhR receptor pathway in Cryo-HepaRG cell cultures. B) CAR direct activator CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) and indirect activator PB effectively induced CYP2B6 enzymatic activity at lower concentrations suggesting a functional CAR pathway in Cryo-HepaRG cell cultures. C) PXR activators hyperforin and rifampicin (RIF) were effective in inducing CYP3A4/5 enzymatic activity consistent with a functional PXR receptor pathway in Cryo-HepaRG cell cultures. D) ABCB11 (BSEP protein) gene expression was evaluated with FXR agonist chenodeoxycholic acid (CDCA) in concentration-response in Cryo-HepaRG cell cultures and showed a clear induction response in both HPRG750 (serum-free) and HPRG740-supplemented induction media. E) Summary of relationships between sentinel gene targets and hepatic receptor pathways. *Cytotoxicity was observed at 200 µM concentrations of CDCA in Cryo-HepaRG cultures. Data represent the mean of 3 independent replicates and error bars indicate standard deviations of the mean.

Figure 9: HepaRG® and PHH Induction Response Comparison (Enzymatic Specific Activities). Three separate lots of Cryo-HepaRG cultures (red diamonds) were contextualized to ranges of induction response observed with sandwich cultures PHH (52 lots for CYP2B6 & CYP3A4, 11 lots for CYP1A2) for induction of enzymatic activities level (box and whisker plots). P450 specific activities were evaluated after 72 hr of treatment with 50 µM OMP for CYP1A2 (phenacetin O-deethylation activity), 1 mM PB for CYP2B6 (bupropion hydroxylase activity), or 10 µM RIF for CYP3A4/5 (midazolam 1-hydroxylation and testosterone 6β-hydroxylation activities). Fold over control values were calculated relative to respective vehicle controls. Statistical analysis of lot variation with Cryo-HepaRG was performed using an ANOVA followed by a Tukey pairwise comparison revealed no statistically significantly differences at the P<0.05 level as shown in Table 5.

Figure 10: P450 mRNA Induction of CYP1A2, CYP2B6, and CYP3A4/5 with Cryo-HepaRG Cultures. Cryo-HepaRG cultures were treated with omeprazole (OMP) at 50 µM, PB at 1 mM, and RIF (RIF) at
10 µM for 48 hours and mRNA expression was quantified via qRT-PCR using gene specific TaqMan assays. A) CYP1A2 mRNA induction in three independent assay plates. B) CYP2B6 mRNA induction in 3 independent assay plates. C) CYP3A4 mRNA induction in 3 independent assay plates. Data represent mean responses of 3 independent replicate wells and error bars indicate standard deviations of mean responses.

Figure 11: Impact of Media & Overlay (Geltrex) on Induction with Cultures of Cryo-HepaRG. In these experiments the effects of Geltrex overlay in Cryo-HepaRG cultures on induction responses were evaluated by assaying enzymatic activities in response to specific receptor activators for AhR, CAR, and PXR monitoring CYP1A2 (A), CYP2B6 (B), and CYP3A4/5 (C) enzymatic activities after 72 hours of exposure. Data represent the mean of 3 independent wells with error bars showing the standard deviations of mean responses. Asterisk (*) indicates P<0.05 (Tukey’s Pairwise Comparison) relative to respective DMSO control groups for each culture media/overlay combination.

Figure 12: CAR Translocation in Cryo-HepaRG Cultures in Response to Phenobarbital. Cryo-HepaRG cultures were plated in HPRG770-supplemented media and allowed to equilibrate overnight for ~24 hours prior to changing media to HPRG750 (A+B) or HPRG740 (C+D) induction media. Cryo-HepaRG were transduced with a GFP-tagged CAR using an adenoviral delivery system and treated with indirect CAR activator PB or vehicle control (0.1% DMSO). CAR translocation was evaluated using a fluorescent microscope after 8 hours of treatment with PB revealing a clear translocation of CAR in both serum-free and serum-containing induction media.
Cryo-HepaRG cells were cultured for 10 days post-plating in HepaRG® Metabolism Media Supplemented culture media in 8 replicate wells. Primary human hepatocytes were directly assayed or cultured for 4-5 days in serum-free induction media with ITS+, 100nM dexamethasone, 15 mM HEPES, 1% Penn/Strep. Drug metabolism enzyme specific activities were evaluated using in situ incubations with probe substrates in cryopreserved human hepatocyte culture. LC-MS/MS methods were used to quantify specific metabolite concentrations. Means were compared using unpaired t tests (i.e. Welch’s correction). NA: Data from SC-PHH not available.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Marker Metabolite</th>
<th>Cell Culture Model</th>
<th>Mean Specific Activity +/- SD (pmol/min-million cells)</th>
<th>Mean Statistical Difference HepaRG vs. SC-PHH</th>
<th>Mean Difference (&gt;2-fold) HepaRG vs. SC-PHH Median</th>
<th>PHH Suspension Median Specific Activities (pmol/min-million cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>Acetaminophen</td>
<td>HepaRG</td>
<td>2.78 +/- 0.728</td>
<td>P&lt;0.0001</td>
<td>No</td>
<td>48.0 (212 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=52)</td>
<td>7.18 +/- 7.78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>7-Hydroxycoumarin</td>
<td>HepaRG</td>
<td>0.851 +/- 0.159</td>
<td></td>
<td>No</td>
<td>32.1 (137 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=53)</td>
<td>1.03 +/- 0.974</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>Hydroxybupropion</td>
<td>HepaRG</td>
<td>17.9 +/- 2.21</td>
<td>P&lt;0.0001</td>
<td>Yes, 8.3X</td>
<td>32.1 (137 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=52)</td>
<td>2.94 +/- 2.78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Paclitaxel</td>
<td>6α-Hydroxypaclitaxel</td>
<td>HepaRG</td>
<td>0.223 +/- 0.035</td>
<td></td>
<td>No</td>
<td>5.24 (149 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=34)</td>
<td>0.517 +/- 0.802</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>4'-Hydroxydiclofenac</td>
<td>HepaRG</td>
<td>3.94 +/- 0.380</td>
<td></td>
<td>No</td>
<td>88.2 (187 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=54)</td>
<td>5.37 +/- 9.54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Mephenytin</td>
<td>4'-Hydroxydiclofenac</td>
<td>HepaRG</td>
<td>1.52 +/- 0.408</td>
<td></td>
<td>No</td>
<td>13.8 (208 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=48)</td>
<td>2.26 +/- 4.71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>Dextromethorphan</td>
<td>HepaRG</td>
<td>0.401 +/- 0.0622</td>
<td>No, P=0.057</td>
<td>No</td>
<td>21.1 (24 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=36)</td>
<td>1.18 +/- 1.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Testosterone</td>
<td>6β-Hydroxytestosterone</td>
<td>HepaRG</td>
<td>248 +/- 50.9</td>
<td>P&lt;0.0001</td>
<td>Yes, 6.8X</td>
<td>407 (195 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=52)</td>
<td>55.2 +/- 49.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Midazolam</td>
<td>1-Hydroxymidazolam</td>
<td>HepaRG</td>
<td>28.4 +/- 2.39</td>
<td></td>
<td>NA</td>
<td>63.7 (132 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=0)</td>
<td>Not Available</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMO</td>
<td>Benzydamine</td>
<td>Benzydamine N-oxide</td>
<td>HepaRG</td>
<td>17.3 +/- 1.74</td>
<td></td>
<td>NA</td>
<td>144 (105 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=0)</td>
<td>Not Available</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UGT</td>
<td>7-Hydroxycoumarin</td>
<td>7-Hydroxycoumarin Glucuronide</td>
<td>HepaRG</td>
<td>346 +/- 49.7</td>
<td></td>
<td>NA</td>
<td>494 (196 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=0)</td>
<td>Not Available</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SULT</td>
<td>7-Hydroxycoumarin</td>
<td>7-Hydroxycoumarin Sulfate</td>
<td>HepaRG</td>
<td>9.23 +/- 2.92</td>
<td></td>
<td>NA</td>
<td>37 (195 donor preps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-PHH (N=0)</td>
<td>Not Available</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: HepaRG Metabolic Competence Summary: Suspension HepaRG vs. Cultures (Metabolism Media)

<table>
<thead>
<tr>
<th>Enzyme (Marker Metabolite)</th>
<th>HepaRG Suspension Activity +/- SD</th>
<th>HepaRG Day-4 Cultures Activity +/- SD</th>
<th>HepaRG Day-10 Cultures Activity +/- SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2 (APAP)</td>
<td>1.26 +/- 0.0896</td>
<td>0.736 +/- 0.135</td>
<td>2.78 +/- 0.728</td>
</tr>
<tr>
<td>CYP2A6 (7OHCMN)</td>
<td>2.92 +/- 0.128</td>
<td>0.0905 +/- 0.0872</td>
<td>0.850 +/- 0.160</td>
</tr>
<tr>
<td>CYP2B6 (OHB)</td>
<td>6.95 +/- 0.306</td>
<td>0.908 +/- 0.160</td>
<td>17.9 +/- 2.21</td>
</tr>
<tr>
<td>CYP2C8 (6a-OHTAX)</td>
<td>0.235 +/- 0.0173</td>
<td>0.0812 +/- 0.0539</td>
<td>0.223 +/- 0.035</td>
</tr>
<tr>
<td>CYP2C9 (4OHD)</td>
<td>4.89 +/- 0.382</td>
<td>0.437 +/- 0.0785</td>
<td>3.94 +/- 0.380</td>
</tr>
<tr>
<td>CYP2C19 (4HMPN)</td>
<td>1.27 +/- 0.0404</td>
<td>Not determined</td>
<td>1.52 +/- 0.408</td>
</tr>
<tr>
<td>CYP3A4/5 (OHMDZ)</td>
<td>25.6 +/- 0.586</td>
<td>4.01 +/- 0.588</td>
<td>28.4 +/- 2.39</td>
</tr>
<tr>
<td>CYP3A4/5 (68T)</td>
<td>377 +/- 13.7</td>
<td>65.1 +/- 8.44</td>
<td>248 +/- 50.9</td>
</tr>
<tr>
<td>FMO (BNO)</td>
<td>10.4 +/- 0.499</td>
<td>8.48 +/- 1.77</td>
<td>17.3 +/- 1.74</td>
</tr>
<tr>
<td>UGT (7OHCMN-Glc)</td>
<td>ND</td>
<td>90.9 +/- 8.22</td>
<td>346 +/- 49.6</td>
</tr>
<tr>
<td>SULT (7OHCMN-Sult)</td>
<td>ND</td>
<td>5.08 +/- 1.02</td>
<td>9.23 +/- 2.91</td>
</tr>
</tbody>
</table>

ND: Not Determined
Table 3: Genotyping Summary for ADME Genes in HepaRG®

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism Assessed</th>
<th>Genotype in HepaRG®</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9*2</td>
<td>*2/*2</td>
<td></td>
</tr>
<tr>
<td>CYP2C9*3</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>CYP2C9*6</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>CYP2C19*2</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>CYP2C19*3</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>CYP2C19*6</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>CYP2D6*2</td>
<td>*2/WT</td>
<td></td>
</tr>
<tr>
<td>CYP2D6*3</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>CYP2D6*4</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>CYP2D6*6</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>CYP2D6*9</td>
<td>*9/WT</td>
<td></td>
</tr>
<tr>
<td>CYP3A5*3</td>
<td>*3/*3</td>
<td></td>
</tr>
<tr>
<td>CYP3A5*6</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>CYP3A5*8</td>
<td>WT/WT</td>
<td></td>
</tr>
<tr>
<td>Transporter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OATP1B1*5</td>
<td>*5/WT†</td>
<td></td>
</tr>
<tr>
<td>OATP1B3 (T334G)</td>
<td></td>
<td>WT/WT†</td>
</tr>
</tbody>
</table>

†Data reported by BioPredic International on Certificates of Analysis. WT indicates the wildtype allele was observed in HepaRG cells.
### Table 4: Uptake OATP1B1/1B3 Transport with Cryo-HepaRG & Overlay Effects

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean ES Uptake +/- SD (37°C) (pmol/mg-min)</th>
<th>Mean ES Uptake +/- SD (4°C) (pmol/mg-min)</th>
<th>Mean E2-17ßG Uptake +/- SD (37°C) (pmol/mg-min)</th>
<th>Mean E2-17ßG Uptake +/- SD (4°C) (pmol/mg-min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5µM HPRG720 Overlay</td>
<td>16.9 +/- 0.689</td>
<td>3.38 +/- 0.363</td>
<td>1.33 +/- 0.161</td>
<td>0.348 +/- 0.127</td>
</tr>
<tr>
<td>0.5µM HPRG740 Overlay</td>
<td>11.0 +/- 1.81</td>
<td>2.92 +/- 0.512</td>
<td>0.602 +/- 0.0489</td>
<td>0.154 +/- 0.0418</td>
</tr>
<tr>
<td>40µM HPRG720 Overlay</td>
<td>226 +/- 13.8</td>
<td>54.0 +/- 5.52</td>
<td>33.1 +/- 8.31</td>
<td>11.6 +/- 7.32</td>
</tr>
<tr>
<td>40µM HPRG740 Overlay</td>
<td>111 +/- 23.8</td>
<td>31.7 +/- 3.09</td>
<td>17.8 +/- 1.82</td>
<td>7.07 +/- 1.45</td>
</tr>
<tr>
<td>0.5µM HPRG750 Overlay</td>
<td>14.8 +/- 0.892</td>
<td>1.95 +/- 0.380</td>
<td>0.438 +/- 0.0707</td>
<td>0.181 +/- 0.0390</td>
</tr>
<tr>
<td>40µM HPRG750 Overlay</td>
<td>183 +/- 7.04</td>
<td>48.0 +/- 16.2</td>
<td>16.1 +/- 1.87</td>
<td>8.32 +/- 1.34</td>
</tr>
<tr>
<td>0.5µM HPRG720</td>
<td>222 +/- 21.0</td>
<td>70.8 +/- 7.65</td>
<td>22.2 +/- 7.21</td>
<td>11.0 +/- 5.35</td>
</tr>
<tr>
<td>0.5µM HPRG740</td>
<td>7.46 +/- 0.112</td>
<td>1.98 +/- 0.276</td>
<td>0.356 +/- 0.0219</td>
<td>0.185 +/- 0.0181</td>
</tr>
<tr>
<td>40µM HPRG720</td>
<td>91.1 +/- 4.47</td>
<td>37.7 +/- 8.63</td>
<td>13.7 +/- 0.889</td>
<td>6.02 +/- 1.07</td>
</tr>
<tr>
<td>40µM HPRG740</td>
<td>148 +/- 0.892</td>
<td>1.95 +/- 0.380</td>
<td>0.438 +/- 0.0707</td>
<td>0.181 +/- 0.0390</td>
</tr>
<tr>
<td>0.5µM HPRG750</td>
<td>8.48 +/- 0.317</td>
<td>1.68 +/- 0.126</td>
<td>0.357 +/- 0.0181</td>
<td>0.244 +/- 0.0229</td>
</tr>
<tr>
<td>40µM HPRG750</td>
<td>129 +/- 3.33</td>
<td>38.2 +/- 7.45</td>
<td>17.5 +/- 2.29</td>
<td>6.50 +/- 1.63</td>
</tr>
</tbody>
</table>
Table 5: Inter-Lot Reproducibility

<table>
<thead>
<tr>
<th>Metabolism Specific Activity</th>
<th>Anova P-Value</th>
<th>Tukey (HSD) Comparison</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2 (APAP)</td>
<td>0.212</td>
<td>L1-L2</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L2-L3</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1-L3</td>
<td>No</td>
</tr>
<tr>
<td>CYP2B6 (OHBP)</td>
<td>0.692</td>
<td>L1-L2</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L2-L3</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1-L3</td>
<td>No</td>
</tr>
<tr>
<td>CYP3A4/5 (OHMDZ)</td>
<td>0.124</td>
<td>L1-L2</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L2-L3</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1-L3</td>
<td>No</td>
</tr>
<tr>
<td>CYP3A4/5 (6βT)</td>
<td>0.247</td>
<td>L1-L2</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L2-L3</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1-L3</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 1

A. Freshly Differentiated HepaRG-4 weeks
B. Cryo-HepaRG, 10 days
C. Primary Human Hepatocytes
D. Cryo-HepaRG, post attachment
E. Cryo-HepaRG, 24hrs
F. Cryo-HepaRG, 7 days
G. Phase Contrast
H. CDFDA Staining
I. Merged
Figure 2

A. Phenacetin O-Deethylation

B. Bupropion Hydroxylation

C. Testosterone 6β-Hydroxylation
Figure 3

A

Cryo-HepaRG Suspension (pmol/min/million cells)

Cryo-HepaRG Plated (pmol/min/million cells)

Pearson R = 0.998
P < 0.0001

B

Metabolism Specific Activity (pmol/min/million cells)

Time in Culture (Days)

0 4 6 8 10 12 14 16 18 20 22

C

CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4-66T, CYP3A4-1MDZ, FMO, UGT, SULT

D

IC50 = 29 hrs +/- 5 hrs

CYP3A4/5 Activity (pmol/min/million cells)

Time (Hrs)

0 20 40 60 80

C

Xenobiotic Metabolism Specific Activity (% of Cyp-4)

Time in Culture (Days)

0 4 6 8 10 12 14 16 18 20 22

CYP1A2, CYP2A6, CYP2B6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4-66T, CYP3A4-1MDZ, FMO, UGT, SULT
Figure 4
Figure 5

A

B

C

D

E

F

G
Figure 6

A

Percent LDH Leakage (%)

Concentration (µM)

B

ATP: Percent of Vehicle Control (%)

Concentration (µM)

C

ATP Concentration (Percent of Vehicle Control)

Concentration (µM)
Figure 8

A

Phenacetin O-de-ethylase (Relative Fold Change)

0.17 μM BNF, 2.5 μM BNF, 22.5 μM BNF

B

Buscopan Hydroxylation (Relative Fold Change)

4 μM PB, 37 μM PB, 333 μM PB

C

Testosterone 6β-hydroxylation (Relative Fold Change)

0.01 μM Hyperforin, 0.1 μM Hyperforin, 1.0 μM Hyperforin

D

ABCB11/BSEP mRNA Expression (Relative Fold Change)

0.1% DMSO, 3.125, 6.25, 12.5, 25, 50, 100, 200 μM CDCA

E

AhR ARNT

CYP1A

Xenobiotic Metabolism

CAR RXR

CYP2B

Xenobiotic Metabolism

PXR RXR

CYP3A

Xenobiotic Metabolism

FXR RXR

ABCB11/CYP7A

Bile Acid Metabolism
Figure 10

A

CYP1A2 mRNA

(Relative-Fold Change)

OMP 50 µM

OMP 50 µM

OMP 50 µM

B

CYP2B6 mRNA

(Relative-Fold Change)

PB (1 mM)

PB (1 mM)

PB (1 mM)

C

CYP3A4 mRNA

(Relative-Fold Change)

RIF (10 µM)

RIF (10 µM)

RIF (20 µM)
Figure 12

A. Treat for 8h & Without Serum

0.1% DMSO

PB 1mM

B. Treat for 8h & With Serum

0.1% DMSO

PB 1mM