In vitro hepatocyte function is enhanced in a perfused 3D culture platform

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INTRODUCTION

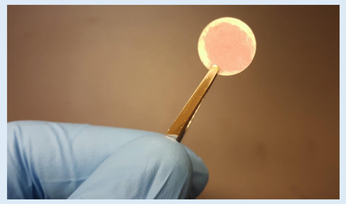
The study of hepatocytes *in vitro* is a critical for understanding liver function, drug metabolism, infection, and disease. Common methods of cell culture utilize immortalized cell lines grown in two dimensions (2D). Cell lines divide readily, but have substantially reduced physiological activity. Cytochrome P450 (CYP) enzyme function is critical for the study of drug metabolism. Due to low levels of CYP enzyme activity, cell lines such as Hep G2 hepatocytes have largely been replaced with primary human hepatocytes (PHH) derived from human donor tissue, which now serve as the gold standard. Despite initially retaining their drug metabolizing ability, de-differentiation of the cells results in a sharp reduction in CYP activity, limiting the duration of drug metabolism studies. While this is sufficient for determination of the metabolic profile for small molecule drugs with short half-lives, slow-clearing drugs cannot be readily assessed for toxicity. Additionally, newer drug development strategies have focused on large-molecules and biologics such as antibodies, which can have half-lives on the order of weeks to months. Alternative models including induced pluripotent stem cell (iPSC)-derived hepatocytes and HepaRG™ cells have been shown to maintain drug metabolizing activity longer than PHH, but are adversely affected by the artificial architecture inherent to 2D cell culture. Satisfactory *in vitro* models of hepatitis C virus (HCV) infection have also been difficult to develop. Virus propagation in PHH is limited by a reduced capacity for cell division and low levels of virus replication. This suggests the morphological, and resulting physiological deficits of 2D cell culture are a hindrance to adequate modeling of hepatic tissue in vitro.

METHODS

Cell Culture

Hep G2 cells (ATCC) were maintained in EMEM supplemented with 10% fetal bovine serum in a humidified incubator at 37 ºC. NoSpin HepaRG™ cells (TRL/Lonza) and Liverpool™ Cryoplateable primary human hepatocytes (BioreclamationIVT, 10-donor, mixed gender) were all cultured in the manufacturer's recommended media. Undifferentiated HepaRG™ cells were grown in Williams E medium with supplement (Biopredic). Differentiation of confluent cultures was induced with 1% DMSO.

3D cultures were generated using Lena Biosciences' patented SeedEZ™ scaffolds:



Cells were seeded in Matrigel® (8 mg/mL) at densities ranging from 100,000 to 500,000 cells per scaffold (depending on plate format and cell type). Cultures were allowed to gel at 37 ºC for 10 minutes and subsequently transferred to Lena Biosciences' Perfused Organ Panel system or to a multiwell plate ("Control"). Cultures in Perfused Organ Panel were either maintained without perfusion ("Static") or subjected to perfusion at a rate of 16 culture volume changes per 24 hour period ("Perfused") for the duration of the study. 2D control cultures consisted of the same number of cells plated in 24-well or 48-well plates.

alamarBlue Assay for Metabolic Function

After 10 days *in vitro* (DIV), alamarBlue reagent was added to Hep G2 cultures at a volume equal to 1/10 of the volume of culture medium. The cultures were returned to the incubator for a period of 2 hours after which a sample of the medium from each condition was transferred to a 96-well plate and read on a Biotek Synergy 4 plate reader for fluorescence (ex/em 545/590 nm). Data were normalized to cell number as determined by total LDH of a set of cell density standards described below.

LDH Assay for Total and Released LDH

Prior to the addition of alamarBlue reagent, a sample of medium was taken to assess LDH release. After the alamarBlue assay, the cultures were lysed with Triton X-100 (1%) for 1 hour at 37 ºC for determination of total LDH. In addition, a freshly plated set of HepG2 3D cultures with densities ranging from 200,000 – 4,000,000 cells/scaffold were lysed and used to generate a standard curve to determine cell number in each culture. A sample of medium from each condition was transferred to a 96-well plate for a colorimetric LDH assay. LDH release data were normalized to cell number.

Albumin ELISA

The supernatants were harvested after the primary human hepatocytes were cultured for 4 days and 7 days. The human albumin concentration in the supernatants were determined using the Human Serum Albumin DuoSet ELISA Kit (R&D Systems) according to the manufacturer's instructions. Samples were analyzed without dilution under each condition.

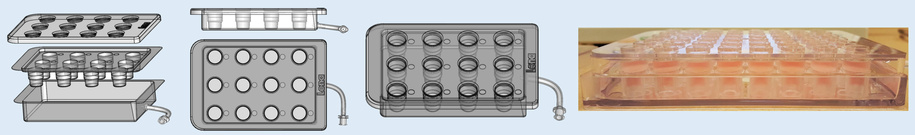
CYP Activity Measurement

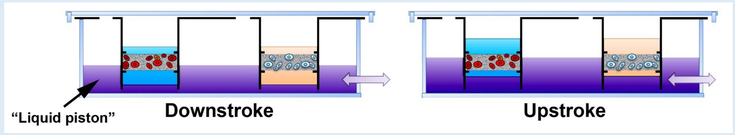
CYP1A1 and CYP2C19 activities were measured fluorescently using EROD and CEC assays, respectively. For the ethyxoyresorufin-O-deethylase (EROD) assay, cells were exposed to 7-ethoxyresorufin (10μM) and salicylamide (inhibitor of phase II metabolism of resorufin, 1.5mM). For the 3-cyano-7-ethoxycoumarin (CEC) assay, cells were exposed to CEC (25μM). CYP1A2, CYP2B6, CYP2C9, and CYP3A4 activities were measured using CYP-Glo assays (Promega) according to the manufacturer's instructions. Omeprazole (20μM) was used to induce CYP1A2. Phenobarbital (1mM) was used to induce CYP2B6. Rifampin (10μM) was used to induce all other CYP enzymes.

Statistical Analysis

Statistical significance was determined by One-way ANOVA and Tukey's post-hoc test or by t-test.

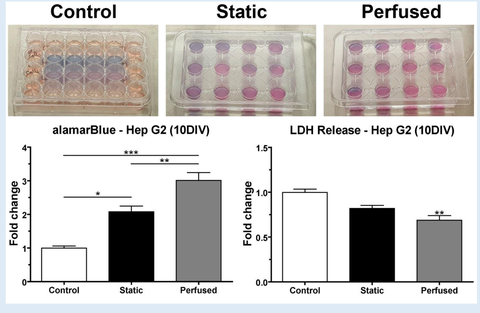
Lena Biosciences' Perfused Organ Panel





Cells seeded in 3D hydrogels supported by a rigid scaffold were maintained in statistically independent wells in their own medium which floats atop a high-density blood-substitute in which medium is immiscible. The blood-substitute readily dissolves atmospheric gases at levels which are orders of magnitude higher than for aqueous medium. Infusion and withdrawal of this blood-substitute acts as a "liquid piston" and introduces perfusion into the system without the need for separate pumps and tubing for each culture condition.

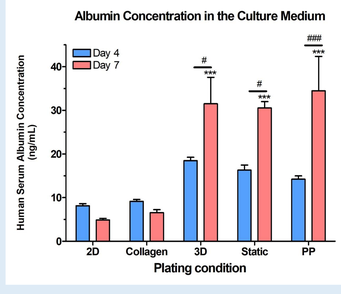
Culturing in Perfused Organ Panel increases metabolic function (respiration) in Hep G2 cells



Culturing Hep G2 cells in the Perfused Organ Panel system consistently resulted in increased metabolic activity as measured by alamarBlue assay. Hep G2 cells maintained in a 12-well system over a 10-day period showed a two-fold improvement in metabolic activity over cultures maintained in a standard multiwell plate when the blood-substitute was introduced ($p<0.05$), and a three-fold improvement when the cultures were perfused ($p<0.001$). Perfused cultures showed 50% more alamarBlue signal than static cultures ($p<0.01$).

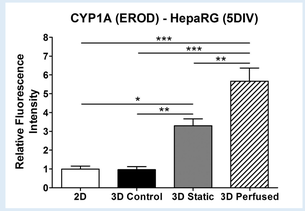
LDH release was also significantly decreased in perfused cultures ($p<0.01$) compared with control cultures, indicating improved cell viability presumably due to increased oxygenation.

Culturing in Perfused Organ Panel increases albumin production in PHH



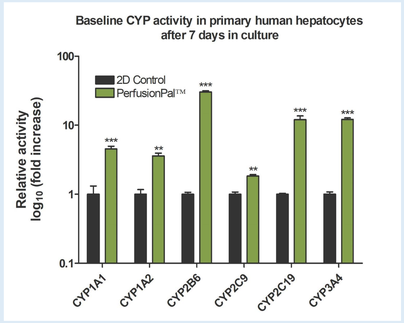
Culturing PHH in Perfused organ panel results in increased albumin production. Compared with 2D controls, PHH maintained in a 48-well system over a trended toward a 50% increase in albumin production after 4 days and showed a three-fold increase after 7 days. This improvement was also noted in the 3D and Static controls, suggesting the simply introducing *in vivo*-like 3D geometry may be sufficient to improve this aspect of PHH culture. Albumin production is an indicator of hepatocyte health and differentiation state. *,# $p<0.05$; ***,### $p<0.001$

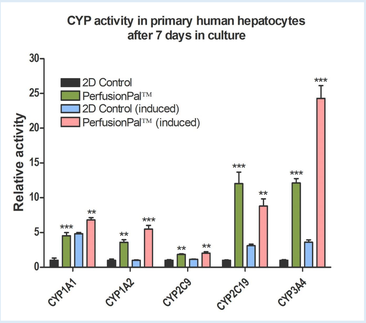
Culturing in Perfused Organ Panel increases baseline CYP1A activity in HepaRG™ cells



Culturing NoSpin HepaRG™ cells in the Perfused Organ Panel system enhances baseline CYP1A activity, and this effect is further increased when perfusion is introduced. HepaRG™ cells maintained in a static system over a 5-day period (n=6 for all 3D conditions and n=3 for 2D) showed a three-fold improvement in CYP1A activity compared with both 2D cultures ($p<0.05$) and 3D control cultures ($p<0.01$). When perfusion was introduced, CYP1A activity increased five-fold compared with 2D cultures ($p<0.001$) and 3D control cultures ($p<0.001$). Further, the measured activity of the perfused cultures was significantly higher than that of the static cultures ($p<0.01$). HepaRG™ cells maintain higher levels of baseline CYP activity, resulting in an assay time of only 2 hours.

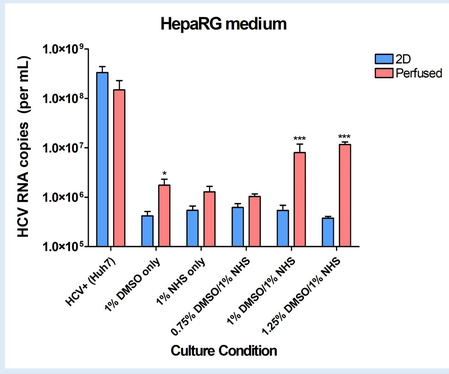
Culturing in Perfused Organ Panel potentiates hepatic CYP activity in PHH for drug toxicity testing

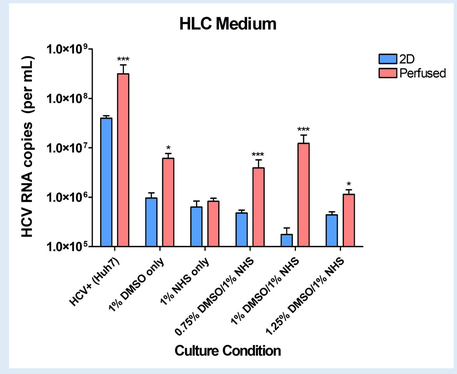




Culturing primary human hepatocytes in the Perfused Organ Panel system consistently enhances baseline and induced CYP450 activity. Primary human hepatocytes (BioIVT Cryoplateable 10-donor Liverpool™) were maintained in a 12-well system over a 7-day period (n=3 for all conditions) in either a multi-well plate ("2D Control") or in Lena Biosciences' Perfused Organ Panel system ("PerfusionPa™I") with a perfusion rate corresponding to 16 culture medium volume exchanges per 24-hour period. Baseline CYP activity (left) was significantly increased for all enzymes tested. Induced CYP activity (right) was similarly enhanced by the perfusion system. The uninduced CYP activities of a number of CYP enzymes in Perfused Organ Panel were higher than the induced activities in 2D. Note the log scale for the left graph. All data are normalized to their respective uninduced 2D control. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Culturing in Perfused Organ Panel increases virus production of HCV in HepaRG™ cells





Culturing in-house differentiated HepaRG™ cells in Perfused Organ Panel significantly increased production of HCV. HepaRG™ cells, differentiated with 1% DMSO for more than two months were grown in 2D or in a 48-well system using various medium conditions. Huh 7.5.1 cells were used as a positive control for infection and virus production. In nearly all of the conditions where DMSO was present, growth in the Perfused Organ Panel significantly elevated the amount of viral replication. Notably, using the HLC medium supplemented with 1% DMSO and 1% NHS showed a nearly 70-fold increase in RNA copies of HCV. *$p<0.05$, ***$p<0.001$

CONCLUSIONS/FUTURE DIRECTIONS

- The functional improvements demonstrate that the Perfused Organ Panel can maintain or drive hepatocytes toward a more differentiated state, allowing them to produce more albumin, metabolize drugs, and model virus infection *in* vitro.
- Taken together, these data show that the Perfused Organ Panel represents an accessible, functional model of perfused 3D tissue, ideal for infectious disease modeling and treatment. In the context of the COVID-19 pandemic, many other tissues have been grown in the Perfused Organ Panel, and it could serve as a platform to study the full-body infection response to SARS-CoV-2. Future studies using the related HCoV-NL63 virus, which also achieves cell entry via ACE2, will demonstrate the utility of the system in this critical area of research.

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