Cytochrome P450 enzyme activity is enhanced in hepatocytes grown using a perfused 3D cell culture drug screening system

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INTRODUCTION

A critical requirement for assessing drug toxicity in vitro is cytochrome P450 (CYP) function that is physiologically representative. Due to low levels of CYP enzyme activity, cell lines such as HepG2 hepatocytes have largely been supplanted with primary cells derived from human donor tissue. While these cells retain CYP activity ex vivo, drug screening is limited to short-term assessments due to de-differentiation. This limits their efficacy in testing slow-clearing, large-molecule compounds such as antibody-based therapies and low-turnover drugs. Cell lines in monolayer cultures have been effective in assessing the safety of small molecule drugs, but the disparity between cell lines and *in vivo* tissue biology proves too substantial for current potential therapies. Three-dimensional (3D) cultures have been shown to be more physiologically relevant models of *in vivo* tissue. However, even they fail to fully mimic living tissue and suffer from the lack of active oxygen and nutrient transport. Lena Biosciences has developed a groundbreaking *in vitro* system that facilitates long-term survival of 3D cultures. Cells seeded in 3D hydrogels supported by a rigid scaffold are maintained in statistically independent wells in their own medium which floats atop a high-density blood-substitute in which medium is immiscible. Infusion and withdrawal of this blood-substitute introduces perfusion into a medium-throughput multiwell system without the need for separate pumps for each culture condition. The system serves to more accurately replicate *in vivo* conditions and has broad applications in the testing of anticancer therapies, particularly those that are slow to clear.

METHODS

Cell Culture

HepG2 cells (ATCC) were maintained in EMEM supplemented with 10% fetal bovine serum in a humidified incubator at 37 °C. NoSpin HepaRG™ cells (TRL/Lonza), iCell® Hepatocytes 2.0 (Cellular Dynamics International), and Liverpool™ Cryoplateable primary human hepatocytes (BioreclamationIVT, 10-donor, mixed gender) were all cultured in the manufacturer's recommended media. Rat neural cell cultures were generated by combining cells from two cortical tissue dissociations (BrainBits®, postnatal day 0 and embryonic day 18) and were cultured in Neurobasal medium supplemented with B-27 Supplement and GlutaMAX.

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

Cells were seeded in Matrigel® (8 mg/mL) at densities ranging from 300,000-500,000 cells per scaffold (depending on cell type). Cultures were allowed to gel at 37 ºC for 15 minutes and subsequently transferred to Lena Biosciences' PerfusionPal system or to a multiwell plate ("Control"). Cultures in PerfusionPal 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 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.

Cell Viability Assessment

After 28 DIV, cultures were removed from the PerfusionPal system and were exposed to Calcein AM (in DPBS) for a period of 30 minutes at room temperature followed by multiple rinses with DPBS. Imaging was carried out using Neurolucida software controlling a Nikon Eclipse 80i upright microscope equipped with an Optronics MicroFIRE camera.

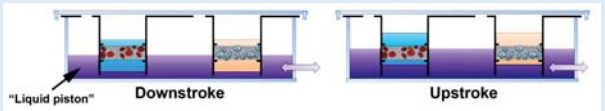
CYP Activity Measurement

CYP1A activity was measured using the ethyoxyresorufin-O-deethylase (EROD) assay. Cells were exposed to 7-ethoxyresorufin (10μM) and salicylamide (inhibitor of phase II metabolism of resorufin, 1.5mM) for times ranging from 2 hours (HepaRG™) to 24 hours (Hep G2) at 37 ºC. After the incubation, 200 μL of medium from each sample was transferred to a 96-well plate and read on a Biotek Synergy 4 plate reader for fluorescence (ex/em 560/584 nm). CYP3A4 activity was measured using a CYP3A4 (Luciferin-PFBE) P450-Glo™ assay (Promega). Cells were exposed to Luciferin-PFBE (40 μM) for 4 hours at 37 ºC. After the incubation, 200 μL of medium from each sample was transferred to a 96-well plate and read on a Biotek Synergy 4 plate reader for luminescence. No inducers were used in these studies.

Statistical Analysis

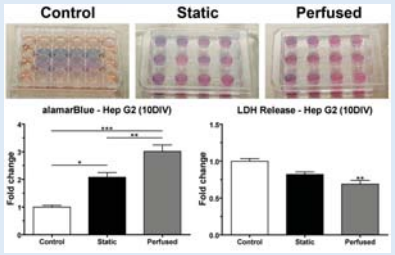
Statistical significance was determined by One-way ANOVA and Tukey's post-hoc test.

Lena Biosciences' PerfusionPal System



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.

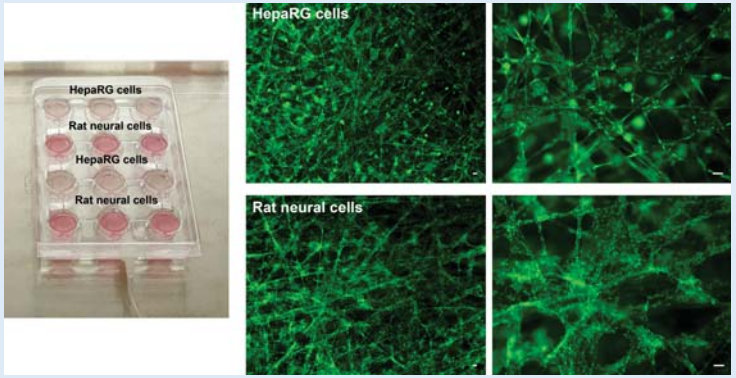
Cultures grown in PerfusionPal have increased metabolic function (respiration)



Culture of HepG2 cells in the PerfusionPal system consistently resulted in increased metabolic activity as measured by alamarBlue assay. HepG2 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 ($p<0.01$).

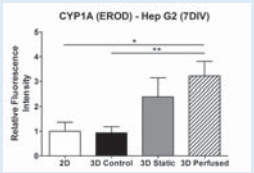
LDH release was also significantly decreased in perfused cultures ($p<0.01$) compared with control cultures, indicating improved cell viability presumably due to increased oxygenation.

PerfusionPal supports simultaneous, long-term culture of cell lines and primary cells



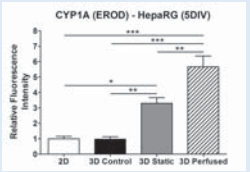
Multiple cell types can be maintained simultaneously in a single PerfusionPal system for a 4 weeks. Primary rat neural cultures consisting of prenatally-derived neurons and postnatally-derived glial cells were cultured alongside HepaRG™ in a PerfusionPal system (6-wells each) for 28 days. Calcein AM images from this time show dense clusters of healthy cells at the nodes where fibers of the SeedEZ scaffold meet. The open structure of the scaffold permits perfused medium to flow between these clusters to deliver oxygen and nutrients more efficiently than can be achieved with diffusion alone. Scale bars: 50μm

Perfusion increases baseline CYP1A activity in Hep G2 cells



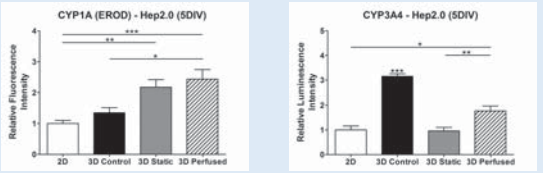
Perfusion of Hep G2 cells enhances baseline CYP1A activity as measured by EROD assay. HepG2 cells maintained in a perfused system over a 7-day period (n=3 for all conditions) showed a three-fold improvement in CYP1A activity compared with both 2D ($p<0.05$) and 3D ($p<0.01$) cultures maintained in a standard multiwell plate. The static condition, though higher, was not significantly different from the 2D or 3D control samples. It should be noted that Hep G2 cells have very low baseline CYP activity and that the cultures were incubated with 7-ethoxyresorufin and salicylamide for 24 hours.

Culturing in PerfusionPal increases baseline CYP1A activity in HepaRG™ cells



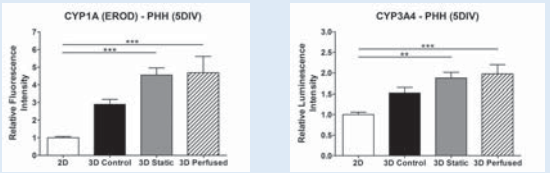
Culturing NoSpin HepaRG™ cells in the PerfusionPal system enhances baseline CYP1A activity, and this effect is further increased when perfusion is introduced. HepaRG™ cells maintained in a static system over a 5-day period (n=6 for all 3D conditions and n=3 for 2D) showed a three-fold improvement in 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 PerfusionPal increases baseline CYP1A activity in Cellular Dynamics iCell® Hepatocytes 2.0



Culturing CDI iCell® Hepatocytes 2.0 in the PerfusionPal system enhances baseline CYP1A activity, but perfusion is required to enhance CYP3A4 activity. Hepatocytes 2.0 maintained in a static ($p<0.01$) or perfused ($p<0.001$) system over a 5-day period (n=6 for all conditions) showed a greater than two-fold improvement in CYP1A activity compared with 2D cultures. Only the perfused condition significantly improved CYP1A activity compared with 3D control cultures ($p<0.05$). Perfusion increased baseline CYP3A4 activity significantly compared with 2D ($p<0.05$) and static ($p<0.01$) conditions. However, the 3D control cultures had significantly higher activity than all other conditions ($p<0.001$) suggesting that culturing in SeedEZ had the biggest impact on CYP3A4 activity.

Culturing in PerfusionPal increases baseline CYP1A and CYP3A4 activity in BioreclamationIVT Liverpool™ cells



Culturing primary human hepatocytes (PHH) in the PerfusionPal system enhances baseline activity of both CYP1A and CYP3A4. Cryoplateable Liverpool™ cells maintained in a static or perfused system over a 5-day period (n=6 for all conditions) showed a greater than four-fold improvement in CYP1A activity ($p<0.001$) and a nearly two-fold improvement in CYP3A4 activity ($p<0.01$ for static and $p<0.001$ for perfused) compared with 2D cultures. The 3D control cultures exhibited higher activity for both CYP1A and CYP3A4 compared with 2D cultures, but the difference was not statistically significant.

CONCLUSIONS/FUTURE DIRECTIONS

- We have demonstrated enhanced activity of CYP enzymes in hepatocytes of four different origins. Depending on cell type, this enhancement can be attributed to the presence of perfusion, simply culturing in the PerfusionPal system, or solely the use of the SeedEZ scaffold for 3D culture.
- We have shown that our system can maintain different cultures simultaneously for long-term drug safety studies which are currently underway.
- The studies will be repeated to generate independent samples and the panel of CYP assays will be expanded.

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