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Characterization of rat or human hepatocytes cultured in microphysiological systems (MPS) to identify hepatotoxicity

Shih-Yu Chang [a], Jenna L. Voellinger [b], Kirk P. Van Ness [b], Brian Chapron [b], Rachel M. Shaffer [a], Thomas Neumann [c], Collin C. White [a], Terrance J. Kavanagh [a], Edward J. Kelly [b], David L. Eaton [a,*]

[a] Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA 98195, USA
[b] Department of Pharmaceutics, University of Washington, Seattle, WA 98195, USA
[c] Nortis, Inc. Seattle, WA, 98102, USA

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ABSTRACT

The liver is the main site for drug and xenobiotics metabolism, including inactivation or bioactivation. In order to improve the predictability of drug safety and efficacy in clinical development, and to facilitate the evaluation of the potential human health effects from exposure to environmental contaminants, there is a critical need to accurately model human organ systems such as the liver *in vitro*. We are developing a microphysiological system (MPS) based on a new commercial microfluidic platform (Nortis, Inc.) that can utilize primary liver cells from multiple species (*e.g.*, rat and human). Compared to conventional monolayer cell culture, which typically survives for 5–7 days or less, primary rat or human hepatocytes in an MPS exhibited higher viability and improved hepatic functions, such as albumin production, expression of hepatocyte marker HNF4α and canaliculi structure, for up to 14 days. Additionally, induction of Cytochrome P450 (CYP) 1A and 3A4 in cryopreserved human hepatocytes was observed in the MPS. The acute cytotoxicity of the potent hepatotoxic and hepatocarcinogen, aflatoxin B_1, was evaluated in human hepatocytes cultured in an MPS, demonstrating the utility of this model for acute hepatotoxicity assessment. These results indicate that MPS-cultured hepatocytes provide a promising approach for evaluating chemical toxicity *in vitro*.

1. Introduction

There has been growing interest over the past decade in developing advanced *in vitro* and/or animal-free models such as *in silico* PK/PD modeling, pathway-based toxicity modeling, and microphysiological systems (MPS) (Esch et al., 2015; Huh et al., 2011) to improve the predictability of pharmacological and toxicological outcomes, and to reduce the use of experimental animals for systems-level analyses. MPS represents an interconnected set of cellular constructs designed to recapitulate the structure and function of human organs, frequently referred to as "organs-on-chips", "*in-vitro* organ constructs", or "organoids" (Wikswo, 2014). MPS platforms utilize a microfluidic device with flow driven by either a perfusion or pneumatic pump through 3D cell constructs imbedded in an extracellular matrix (ECM) gel. Microfluidics that have small volumes (microliters to attoliters) of fluids are integrated in these systems and are used to create microenvironments with dynamic fluid flow and gradients recapitulating human organs (Esch et al., 2015).

Currently, the most commonly used *in vitro* model for *in vitro* preclinical pharmacology and predictive toxicology is monolayer cell culture in plastic flasks or plates using mammalian cells, also known as conventional two-dimensional (2D) culture. Although conventional 2D culture has provided significant contributions to biological research, these basic approaches have numerous limitations, including limited nutrient and metabolite transportation by diffusion, poor mimicry of extracellular concentrations *in vivo*, and difficulties recapitulating heterogeneous tissue microenvironments (Wikswo, 2014). Unlike conventional 2D culture, MPS cultures have the potential to provide an optimal microenvironment for heterogeneous cell growth and differentiation that more closely mimics the physiological responses of tissues *in vivo* (Esch et al., 2015; Huh et al., 2011; Griffith et al., 2014; Huh et al., 2010). Furthermore, in contrast to standard 2D cell culture models, MPS are capable of modeling cell-cell, drug-cell, drug-drug, and organ-drug interactions *in vitro*. By potentially recapitulating *in vivo* physiology and

Abbreviations: MPS, microphysiological system; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; BNF, beta-naphthoflavone; Rif, rifampin; EROD, ethoxyresorufin-O-deethylase; CYPs, cytochrome P450s; HNF4α, hepatocyte nuclear factor 4 alpha; MDZ, midazolam; AFB, aflatoxin B_1; BSEP, bile salt export pump; MRP2, multidrug resistance-associated protein 2.

* Corresponding author at: Department of Environmental and Occupational Health Sciences, 4225 Roosevelt Way NE Suite 100, University of Washington, Seattle, WA 98105, USA.
E-mail address: deaton@uw.edu (D.L. Eaton).

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biochemistry, MPS may be able to more accurately predict chemical toxicity than traditional 2D cultures (Knudsen et al., 2015).

The liver is the main organ that metabolizes drugs and environmental chemicals and the most important organ when it comes to predictive evaluation of compounds for efficacy and toxicity, including both detoxification and bioactivation of drugs and other xenobiotics. In order to improve the predictability of drug safety and efficacy in clinical development, and to have a clear perspective of the potential human health effects from exposure to environmental chemicals, there is a critical need to accurately model liver function using the MPS approach. Here we describe the development and basic validation of rat and human 'liver-on-chip' *in vitro* models using a commercial MPS (Nortis, Inc.). The major goals of this study were to characterize how primary hepatocytes function in the MPS culture system compared to conventional 2D culture, and to validate this liver MPS as a potentially new *in vitro* approach for assessing chemical toxicity and metabolism that allows direct comparison of human and experimental animal responses. As a proof of concept experiment to demonstrate liver MPS bioactivation of a hepatic toxin, we exposed the liver MPS to AFB and evaluated cell viability and toxicity.

2. Materials and methods

2.1. Sources of rat and human primary hepatocytes

Freshly-isolated human hepatocytes or cryopreserved human hepatocytes were purchased from Triangle Research Labs (TRL; donors HUM4037, HUM4038, HUM 4055A, HUM4080 and HUM4096A), or received from The Liver Tissue Cell Distribution System (LTCDS; NIH service; donors 14-018, 15-001,15-002,15-004). All demographic information on hepatocyte donors is listed in Supplementary Table S1. Primary rat hepatocytes were isolated by two-step collagenase digestion following isolation of the liver from male Sprague Dawley® rats (Charles River Laboratories, Seattle, WA) (Shulman and Nahmias, 2013).

2.2. Conventional 2D and MPS cell cultures

Cell culture supplies and materials were from Thermo Fisher Scientific, except for the aflatoxin toxicology experiments, where tissue culture medium from Triangle Research Labs (TRL)/Lonza was used. Plating medium composed of William's E medium supplemented with 5% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin, 100 nM dexamethasone, 1 μg/mL of Gibco® Fungizone®, 100× diluted ITS + (final concentration of insulin/transferrin/selenium was 6.25 μg/mL), and 0.2 mM glutaMAX supplement (Thermo Fisher) was used. The maintenance media were the same formula as plating medium but without FBS.

Primary rat or human hepatocytes were cultured in 2D configuration on 24-well plates coated with type I collagen, or were seeded into Nortis ™ devices for MPS culture (Fig. 1). For the 2D culture, primary rat or human hepatocytes were seeded at a density of 2.1×10^5 cells/cm^2 into type I collagen coated plates. After 4 h of plating cell, the hepatocytes in 2D plates were switched to ice- cold Matrigel® containing (Corning®, Oneonta, NY, Cat#356234, lot#5131014, final conc. 0.23 mg/mL) maintenance medium. For MPS culture *via* chamber seeding (Fig. 1A), 0.2 to 0.3 mL of a cell suspension at a density of 1×10^6 hepatocytes/mL mixed with type I collagen (final conc. is 2–3 mg/mL, Ibidi USA, Inc. Madison, Wisconsin) was injected into each device *via* ablumenal ports. All devices were pre-coated with 0.1 mg/mL of collagen type I in PBS with 0.1% acetic acid at 37 °C for 1 h. For MPS culture *via* channel seeding (Fig. 1B), 2 to 5 μL of a cell suspension at a density of 1×10^7 hepatocytes/mL was injected into each chip with pre-gelled collagen type I (6 mg/mL) *via* the injection port using a sterile 5 μL Hamilton #65 syringe. After 4 h of seeding cells, the hepatocytes were switched to maintenance medium by initiating the lumenal or ablumenal flow at flow rates between 5 and 30 μL/h using an infusion syringe pump (KD Scientific Inc. model# KDS220). Following maintenance of cells at 37 °C in a 5% CO_2 incubator overnight, cells were subsequently maintained in an incubator in a humidified atmosphere at 37°C in 5% CO_2/95% air. The effluent media from the MPS cultures and conditioned media from 2D cultures were collected at various time points and stored at −80 °C for later measures of LDH or ALT, and albumin.

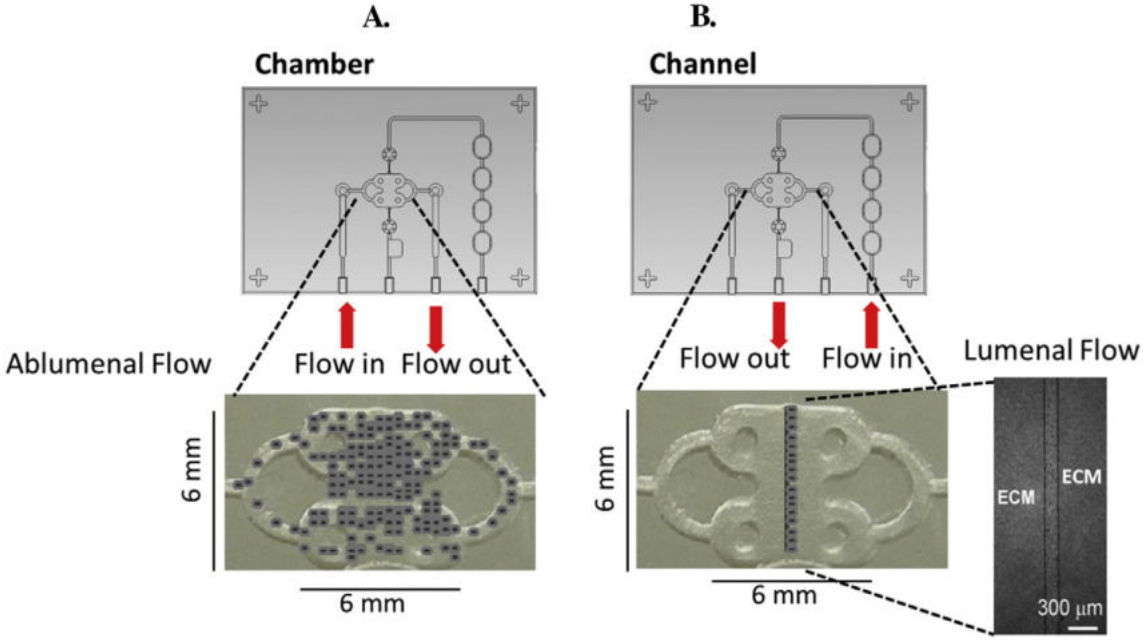


Fig. 1. The methods used in populating hepatocytes inside the MPS. Methods of injecting cells included (A) chamber seeding (ablumenal flow) and (B) channel seeding (lumenal flow) with high concentration (3 mg/mL), or low concentration (1.3 mg/mL) of type I collagen, or Matrigel™ (0.2 mg/ml). Extracellular matrix (ECM) such as type I collagen (6 mg/mL) were used for filling the MPS chips prior to channel seeding. Grey dots represent seeded hepatocytes.

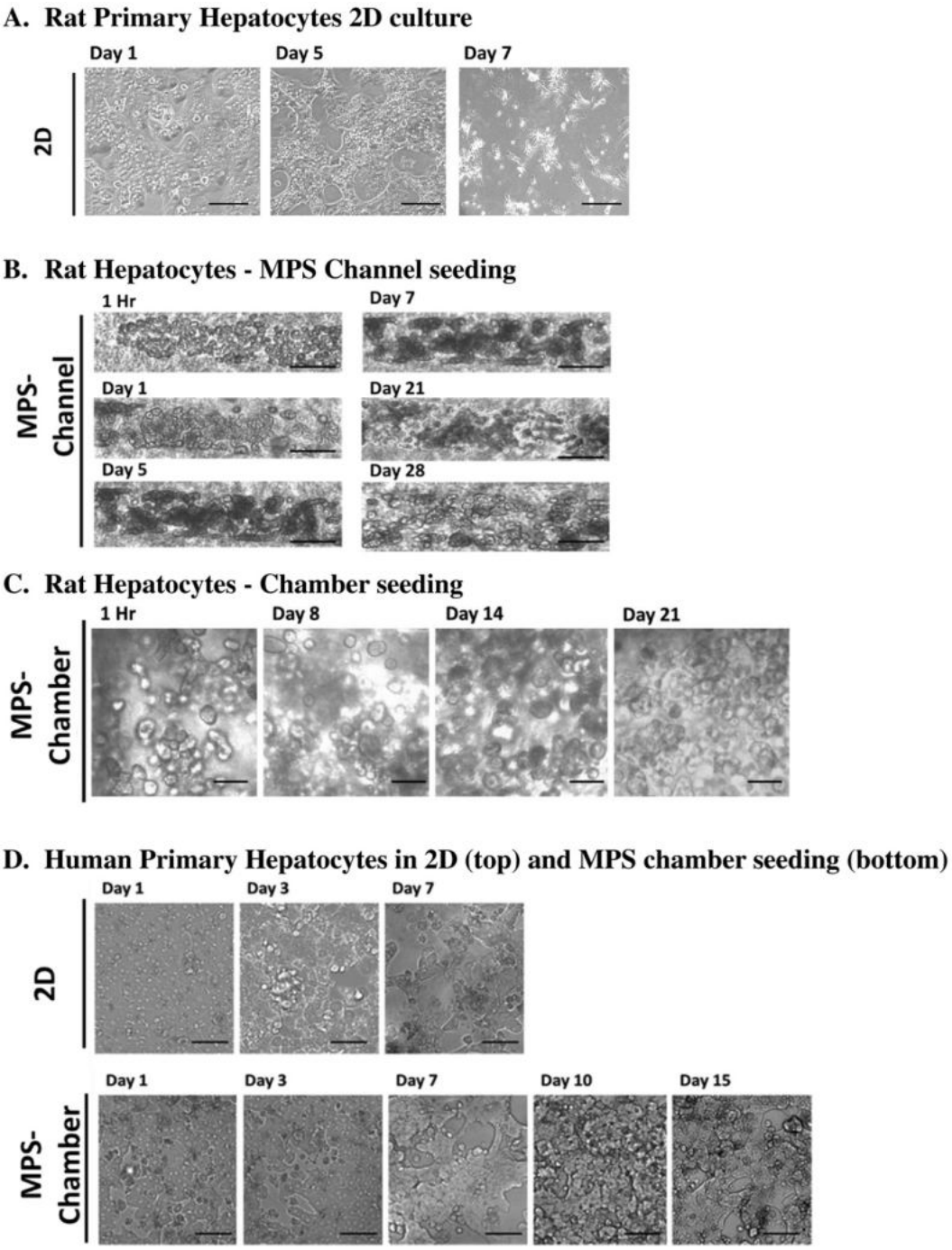


Fig. 2. Morphologies of primary hepatocytes cultured in 2D or MPS over time. Phase contrast pictures showed (A) primary rat hepatocytes cultured in conventional 2D culture (6-well or 24-well plate) and MPS, including (B) seeded in the channel, and (C) seeded in the chamber. (D) Phase contrast pictures showed primary human hepatocytes (donor: HUM4038) in conventional 2D culture (top) and MPS culture (bottom; bar = 100 μm; 200× magnification). Representative pictures were from 3 to 4 independent experiments (cell preparations; human donors included HUM4037, HUM4038, and HUM4055A) with 4–5 technical replicates per experiment.

For the aflatoxin B_1 exposure experiments, the extracellular matrix compartment of the chip was filled with Type I collagen (rat tail tendon) with a 450 μm-wide filament in place. After collagen polymerization, the filament was removed to create the lumen in which the cells would be cultured. Cryopreserved primary hepatocytes (TRL/Lonza; Research Triangle Park, North Carolina) were thawed out and placed in 50 mL of Cryopreserved Human Hepatocyte Thawing medium (TRL/Lonza). After centrifuging the cells at 100*g* for 8–10 min at room temperature, the cell pellet (~1×10^7 cells) was resuspended in 1 mL of Human Hepatocyte Plating Medium containing fetal bovine serum (TRL/Lonza). A sterile 5 μL Hamilton #65 syringe was used to inject the hepatocytes into the injection ports with the aid of an inverted microscope to verify that cell seeding was uniform. Typically, 1 μL of cell suspension (~10,000 cells) was injected into each device followed by placement into a humidified CO_2 incubator at 37 °C for 4 to 6 h to allow the cells to attach to the collagen. After cell attachment, the medium was replaced with serum free medium (TRL/Lonza Hepatocyte

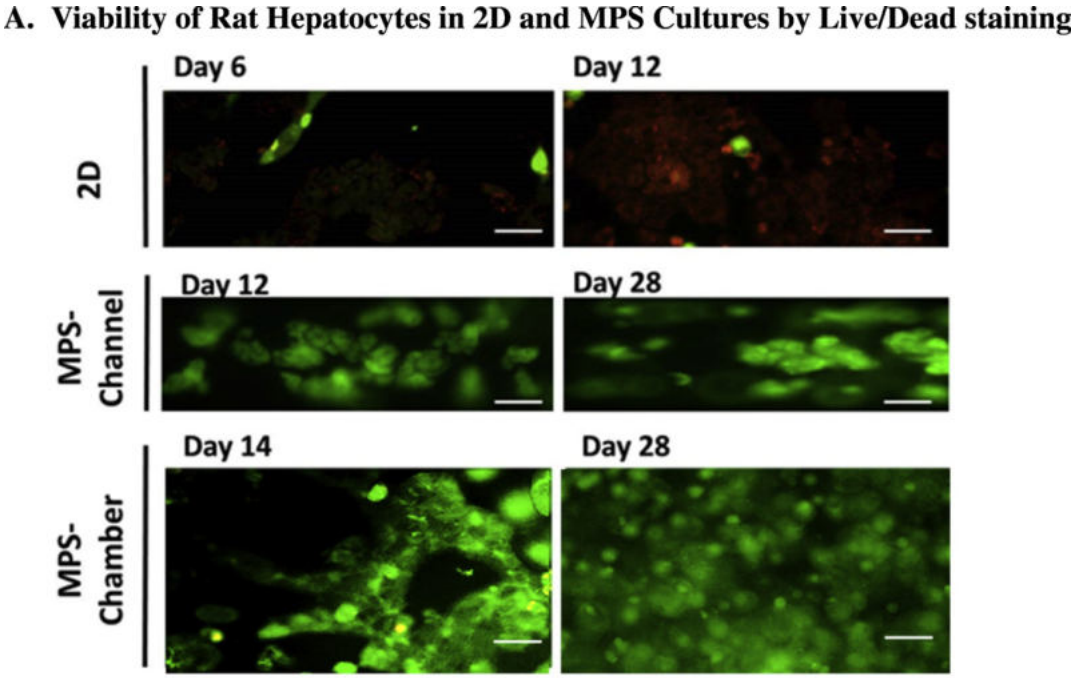


Fig. 3. Viabilities of rat hepatocytes cultured in 2D or MPS over time. (A) Live/Dead® vital staining of rat hepatocytes at different cultured period, up to 28 days (red = ethidium homodimer-1stained dead cells; green = live cells that can metabolize calcein AM to calcein; bar = 50 μm; 200× magnification). (B) Lactate dehydrogenase (LDH) releasing from rat hepatocytes cultured in 2D or MPS over time. LDH releasing measurements were used to represent viability percentage over time (% to seeding number = 100 ± 8% of viability). Results presented as mean ± SD from 3 to 4 independent experiments (cell preparations), including 5–6 MPS chips on different days (over 24 h collection of effluxes) and 6 wells of 2-well chamber slides in total (*t*-test, $p < 0.01$).

Maintenance Medium) and the cells were overlaid with Matrigel® (Corning, Oneonta, NY, Cat#356234, lot#5131014). To achieve this, the devices were connected to a Cole Parmer 74,900 Series 10-Syringe Infusion Pump and Hepatocyte Maintenance Medium with 0.23 mg/mL Matrigel® was perfused through the lumen at 10 μL/min for 20 min followed by a reduction of the flow rate to 0.5 μL/h overnight. The following day, the perfused medium was replaced with Hepatocyte Maintenance Medium without Matrigel® and the flow rate increased to 30 μL/h for 4 h to replace the Matrigel®-containing medium. Then, the flow rate was decreased to 0.5 μL/h and the cells were allowed to recover for 4–5 days before treatment.

2.3. Live/Dead® staining

The Live/Dead® viability/cytotoxicity kit (Life Technologies) was used to distinguish viable cells from dead cells according to the manufacturer's specifications. Briefly, 5 μL of 4 mM calcein AM, 20 μL of 2 mM ethidium homodimer-1 (EthD-1) and 100 μL of 5 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) were diluted in 10 mL of pre-warmed D-PBS. Adequate volumes of diluted reagents were added to 2D culture plates or perfused through the MPS chips at 0.5 μL/min for 20 min and then incubated for 10 min at 37 °C. After the staining procedure, MPS devices or 2D plates were imaged using fluorescent microscopy (Nikon Eclipse Ti-S

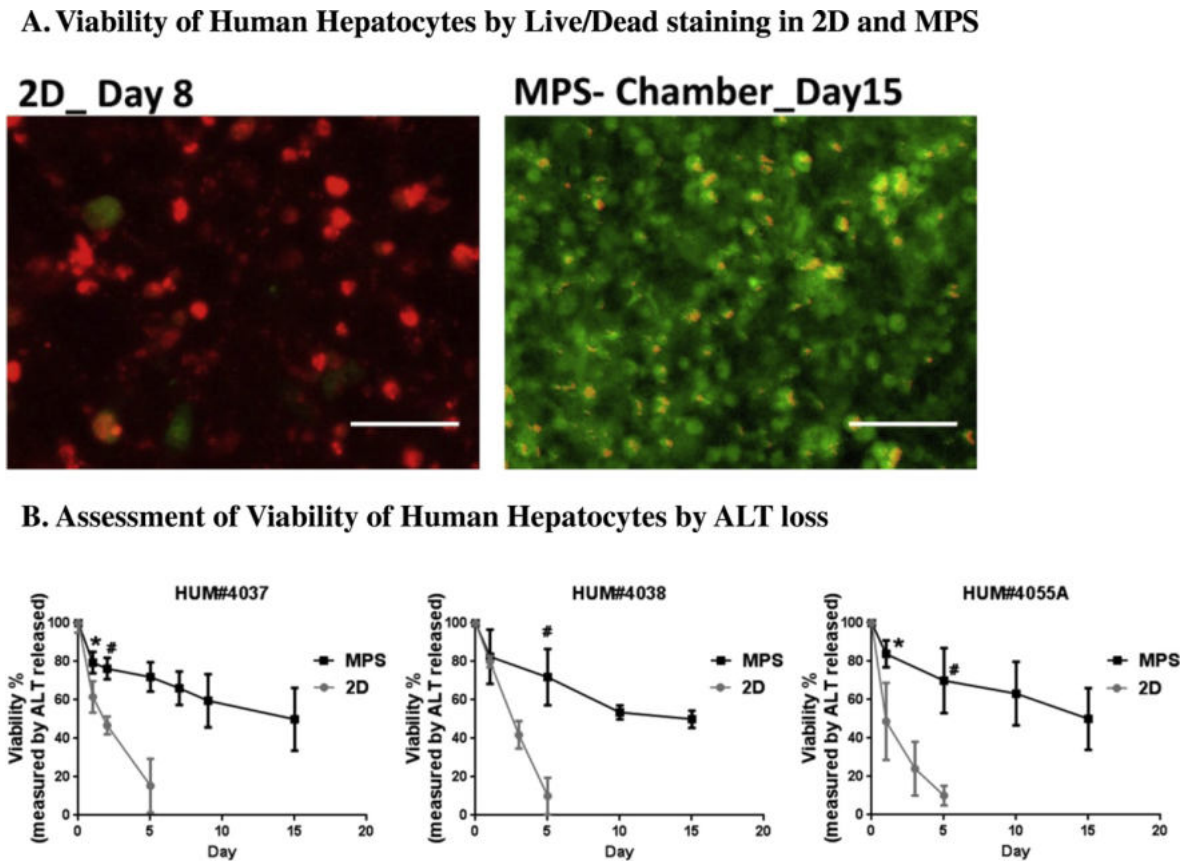


Fig. 4. Viabilities of human hepatocytes cultured in 2D or MPS over time. (A) Live/Dead® vital staining of freshly isolated human hepatocytes from donor 14-018 cultured in 2D on day 8 and in MPS on day 15 (MPS chamber culture with low concentration of type I collagen; bar = 50 μm). **(B)** Accumulative alanine transaminase (ALT) releasing from human hepatocytes cultured in 2D or MPS. Human hepatocytes were from the donors of HUM#4037, HUM#4038 and HUM#4055A, cultured up to 15 days. Results presented as mean ± SD from 3 independent experiments (cell preparations) with total 3 MPS chips and 3 wells of 2-well chamber slides in. Each sample had triplicated data in each assay (*t*-test, *, $p < 0.05$, #, $p < 0.01$).

inverted microscope equipped with a spinning disk confocal apparatus, 3i-Intelligent Imaging Innovations, Denver, CO) to localize red and green fluorescence.

2.4. Immunocytochemistry (ICC) staining

Primary antibodies- HNF4α (mouse monoclonal [K9218], ab41898, Abcam) and BSEP (mouse monoclonal (F-6), sc-74500. Santa Cruz Biotechnology) were used. Cells were fixed with 4% formaldehyde in PBS. After antigen retrieval with warm citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) for 20 min, samples were permeabilized with PBST (PBS plus 0.1% Tween 20) for 1 h, and blocked with 1% bovine serum albumin (BSA) and 10% serum in PBSGT (10 mg/mL glycine, 0.1% Tween 20) for an additional 1 h. The samples were then incubated with primary and secondary antibodies with washing following a standard ICC protocol. Secondary antibodies were goat anti-mouse IgG (H + L) secondary antibody, Alexa Fluor® 488 conjugate (Thermo Fisher Scientific). Diluted ProLong Gold Antifade Mountt with DAPI reagents (catalog# P-36931, ThermoFisher Scientific) were used in the final step of staining. Cells were imaged using fluorescent microscopy using the Nikon Eclipse Ti-S microscope (described above) for detecting the intensity of green and blue fluorescence.

2.5. Lactate dehydrogenase (LDH) release

Cell viability was assessed through lactate dehydrogenase (LDH) release using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) following the manufacturer's suggested protocol. Standard curves (amount of LDH per hepatocyte) and volume of medium were used to normalize the results. In order to normalize the percent cell death (% = experimental LDH release/maximum LDH release) in conditioned medium or effluents at different time points, the maximum LDH release of the MPS model was obtained from lysate of MPS devices that had been freshly-seeded with the same volume of hepatocytes used in the experiments. Results of viability % were calculated from the equation - viability $\%_{(\text{time} = n)} = 100\% - \sum$ cell death$\%_{(\text{time} = n)}$.

2.6. Alanine aminotransferase (ALT) and albumin levels

ALT activity kit (Sigma-Aldrich Co. Catalog # MAK052-1KT) was used following the manufacturer's protocol. ALT activity was determined by a coupled enzyme assay, which results in a colorimetric (570 nm) product, proportional to the pyruvate generated. Standard curves of pyruvate were included for normalizing ALT activity. Human Albumin ELISA Kit (ab108788, Abcam) was used to measure albumin level in effluents of MPS cultures and conditioned medium from 2D cultures. For purposes of comparing 2D and MPS cultures, activity assessment was normalized to the amount of cells seeded into the respective *in vitro* systems (1×10^6 cells in 2D *vs.* 0.02×10^6 in MPS).

2.7. Enzyme activity measurements

Various assays were used to measure CYP1A1/2 and CYP3A4 activities. To induce enzyme activities, cells were pre-treated with beta-naphthoflavone (BNF; final conc. 25 μM, Sigma-Aldrich Co. Catalog # N3633), rifampin (Rif; final conc. 5 μM, Sigma-Aldrich Co. Catalog #R3501), and 0.1% DMSO vehicle control for 72 h prior to performing the enzyme

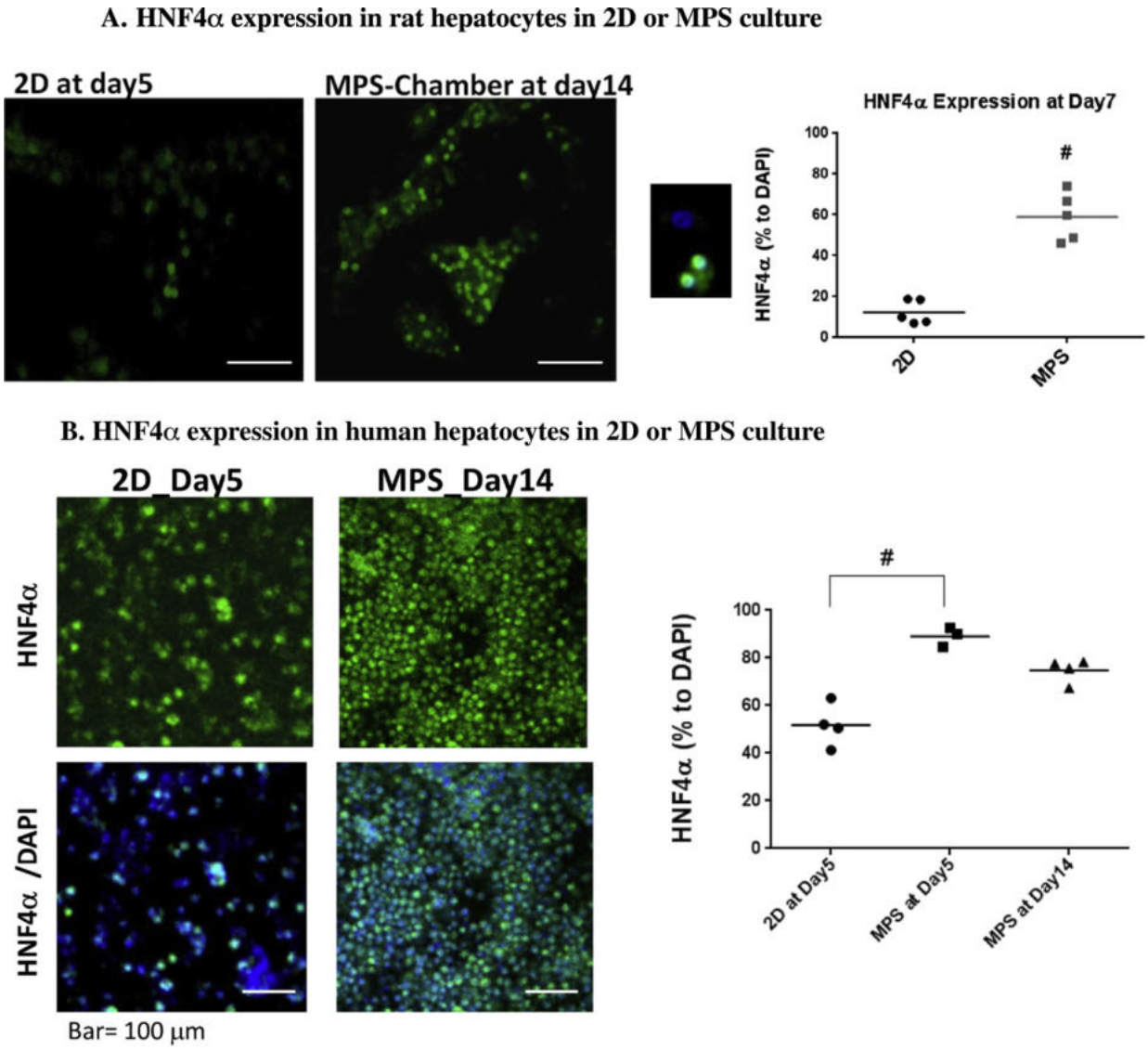


Fig. 5. The protein expression level of HNF4α in primary hepatocytes cultured in 2D or MPS: (A) HNF4α expression in rat hepatocytes cultured in 2D on day 5 and in MPS on day 14 (bar = 100 μm; 200× magnification), and quantitative result of 2D *versus* MPS on day7 (number of HNF4α stained cells compared to number of DAPI-stained nuclei, as %). (B) Human hepatocytes (HUM#4080) cultured in 2D or MPS. Results are presented as mean ± SD from total 5 MPS chips and 5 wells of 2-well chamber slides in 3 independent experiments (cell preparations). Each sample was counted within 10 randomly selected fields under 100× magnification. Significant differences were calculated from 2D *versus* MPS data using *t*-test (#, $p < 0.01$).

activity assays. BNF is an aryl hydrocarbon receptor (AhR) ligand, and commonly used as a CYP1A1/2 inducer (Burke et al., 1994). Rif can activate pregnane X receptor (PXR) and is commonly used as a CYP3A4 inducer (Lehmann et al., 1998).

2.7.1. Ethoxyresorufin-O-deethylase (EROD, CYP1A) kinetic confocal assay

Supplementary Fig. S1 shows a conceptual diagram of the EROD kinetic confocal assay. Cells in an MPS device (Fig. 1A) or in a 2D culture plate (Thermo Scientific™ Nunc™ Lab-Tek™ II Chamber Slide™ System) were incubated with dicumarol (25 μM) (Heinonen et al., 1996), verapamil (100 μM) (Kolwankar et al., 2005), and probenecid (Roth et al., 2012) (water soluble, ThermoFisher Scientific-Invitrogen, Cat# P36400, final conc. 1 mM) in HBSS++ for 30 min prior to the assay. Cells cultured in the MPS were perfused with HBSS++ reaction buffer, which contained 7-ethoxyresorufin (10 μM), dicumarol (25 μM), verapamil (100 μM) and probenecid (1 mM), at a flow rate of 100 μL/min for 30–40 min. Cells in the 2D plate were incubated with 0.5 ml of the same reaction buffer. The average fluorescence intensity in a randomly-selected area (100×) was monitored with an Olympus IMT2 inverted microscope equipped with an argon ion laser, a Nipkow spinning disk confocal unit (Yokogawa CSU10B), a cooled CCD digital camera (Andor USA, Concord, MA), and MetaMorph image analysis software (Molecular Devices, Downingtown, PA) every min starting with the reaction buffer incubation or perfusion (ex = 514 nm, em > 570 nm). Representative images of Δ fluorescence intensity were presented in pseudocolor mode. Final results of fluorescence intensity measurements were analyzed from 10 randomly-selected cells in each area imaged. AUCs were calculated and presented as fold-change to compare vehicle control *vs.* BNF-treated (CYP1A no-induction *vs.* CYP1A induction).

2.7.2. CYP3A4 Glo

CYP3A4 enzyme activity was measured using the P450-GloCYP3A4 kit according to the manufacturer's instructions (Promega, Madison, WI), as modified by Choi et al. (Choi et al., 2014). Briefly, cells were incubated with luciferin-IPA (3 μM) under static conditions (2D), or continuous flow (MPS) at 50 μL/h for 4–6 h at 37 °C. Culture supernatants (2D) and effluent media (MPS) were collected every hr starting 2 h before, during, and after the incubation with luciferin-IPA. Collected samples were incubated with luciferin detection reagent for 20 min at room temperature. Relative luminescence units (RLUs) were measured on a luminescence plate reader (Stratec Biomedical USA, Newbury Park,

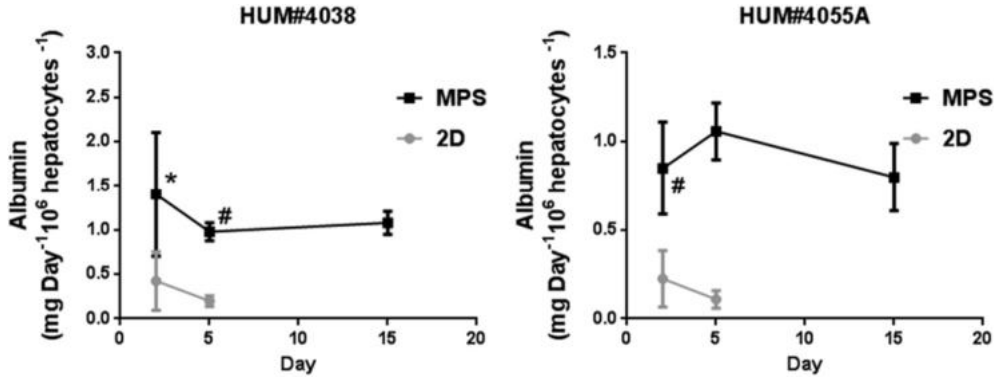


Fig. 6. Albumin production of human hepatocytes cultured in 2D or MPS over time. Hepatocytes from donors HUM#4038 and HUM#4055A were cultured for up to 15 days. Results presented as mean ± SD from total 3 MPS chips, and 3 wells of 2-well chamber slides (2D) in 3 independent experiments (cell preparations). Each sample had triplicated data in each assay (*t*-test, *, $p < 0.05$,#, $p < 0.01$).

CA). The quantification of the luciferin produced was determined by a calibration curve of various concentrations of d-luciferin.

2.7.3. Analysis of midazolam (MDZ) metabolite (1-OH-MDZ) in effluents

Cells cultured in MPS were incubated with media containing 100 μM MDZ for 4 h with a flow rate of 5 μL/min. Effluent was collected and analyzed for MDZ and 1-OH-MDZ by selective ion gas chromatography/negative chemical ionization-mass spectrometry (GC/NCI-MS) according to Paine et al. (Paine et al., 1996).

2.7.4. Visualization of canalicular structures

Chloromethylfluorescein diacetate (CMFDA, CellTracker™ Green CMFDA Dye, ThermoFisher Scientific), a fluorescent substrate for the bile transporter-MRP2, was used to examine canalicular structures in

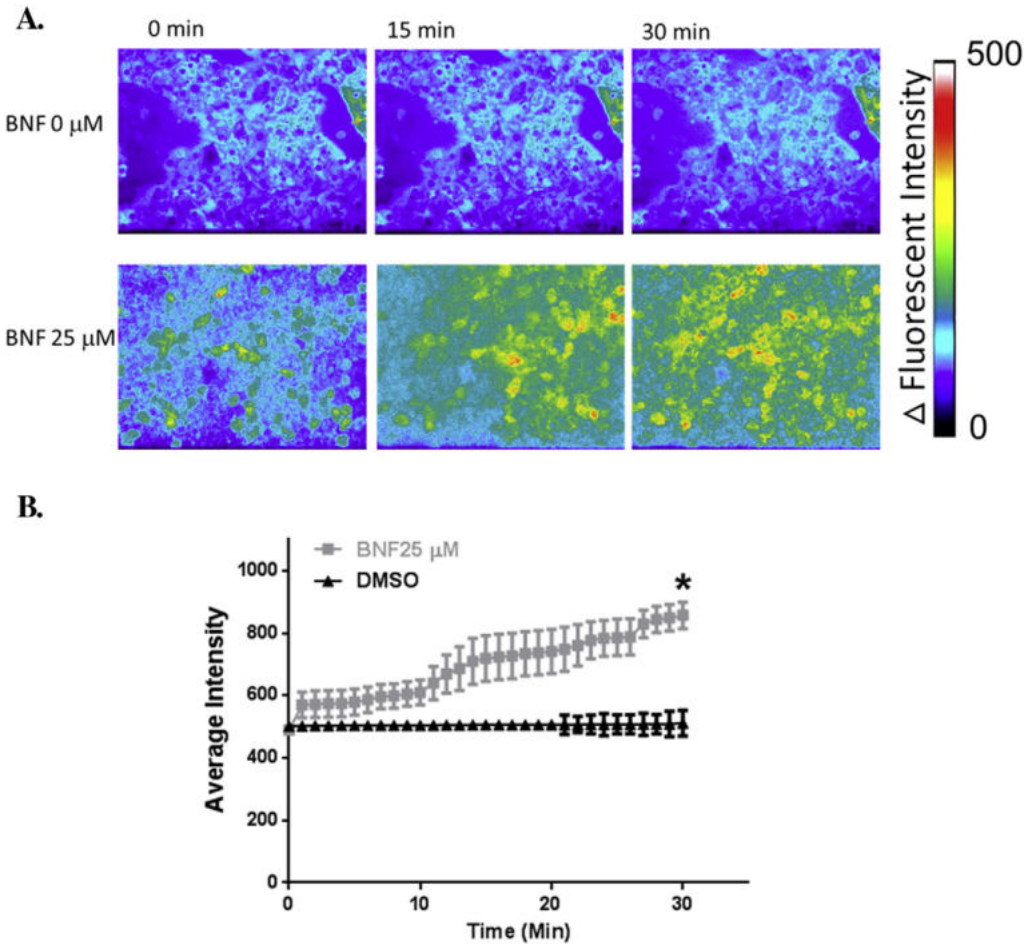


Fig. 7. CYP1A1/2 activity in primary rat hepatocytes cultured in MPS using EROD kinetic confocal assay. Hepatocytes cultured in MPS expressed BNF-inducible CYP1A1/2 after a perfusion of EROD subtract within 40 min. (A) Confocal fluorescence microscopy of EROD activity of rat hepatocytes cultured in MPS with or without prior BNF treatment for 72 h. Fluorescence intensity (represented by the rainbow scale on the right) of CYP1A-activated EROD metabolite (resorufin) was determined at 0, 15 and 30 min after perfusion of substrate. B. Cumulative average fluorescent intensity over time (*t*-test, *, $p < 0.01$). Results are presented as mean ± SD from 3 MPS chips per group in 3 independent experiments (cell preparations). Each sample was analyzed for average intensity of 10 randomly selected cells under 100× magnification.

the liver MPS model (You et al., 2015). The stock solution was diluted to a final working concentration of 25 μM in serum free medium. Pre-warmed CMFDA working solution was perfused through the MPS in a cell culture incubator for 15 min at a flow rate of 5 μL/min, followed by culture medium for 3 min at a flow rate of 5 μL/min. After the procedure, devices were imaged using fluorescence microscopy (as above) to localize CMF green fluorescence.

2.7.5. Aflatoxin treatment

Aflatoxin B_1 (Sigma-Aldrich Co. Catalog #A6636) treatment consisted of a 2-h bolus dose of either 0, 10 or 30 μM AFB in Hepatocyte Maintenance Medium perfused at 30 μL/h followed by replacement with AFB-free medium. This exposure scenario was designed to simulate an acute condition where the liver is exposed to AFB from the consumption of a contaminated food source (Eaton and Gallagher, 1994). The effluent port of each MPS device was fitted with a tube attached to a 1.5 mL Eppendorf tube for the collection of conditioned medium to be analyzed for toxicology-based biomarkers such as LDH. The devices received continued perfusion with AFB-free media for 48 h post-exposure and effluent samples were collected at 24 and 48 h post-exposure. A Live/Dead® viability/cytotoxicity kit (Life Technologies) was used to identify live and dead cells and an LDH assay was used to analyze MPS effluents.

2.8. Statistical analysis

GraphPad Prism 6 was used to plot and analyze results. All results were from three independent experiments of hepatocyte cultures (total $N = 3$). Average ± standard deviation and the two-tailed *t*-test for independent samples were used to compare two groups. *P* values of ≤0.05 were considered statistically significant.

3. Results

3.1. Comparison of cell viability between conventional 2D culture and MPS

Primary rat hepatocytes had high viability and maintained a differentiated morphology (*e.g.*, an epithelial cell-like appearance) for up to 14–28 days in culture in MPS (Fig. 2B and C). By contrast, Live/Dead® staining showed that rat hepatocytes in conventional 2D culture survived for a maximum of 6–8 days (Fig. 3A). Similar results were observed with human hepatocytes. Human hepatocytes maintained differentiated hepatocyte morphology (Fig. 2D) with 80% viability for up to 15 days in culture in MPS compared to a small percent of viable cells at day 8 in 2D culture. Furthermore, based on cell morphology and Live/Dead® staining (Fig. 4A), the few viable cells seen on day 8 in the 2D culture were likely to be non-parenchymal cells.

One advantage of a flow-through system is that one can measure cytotoxicity biomarkers such as LDH and ALT released in effluents collected at different days in MPS cultures and collected conditional media from 2D cultures. These serum markers of hepatocellular injury play a crucial role in evaluating hepatotoxicity in clinical medicine and can also be applied to *in vitro* preclinical studies (Borlak et al., 2014). Cell viability on the day of seeding (day 1, represented as 100 ± 8.0% viability measured using LDH released by lysing whole cells in a parallel MPS device) can be cumulatively subtracted by the measurement of LDH released in effluents on following culture days. The LDH release data (Fig. 3B) show that MPS culture of rat hepatocytes maintained 82.4 ± 7.0% viability up to 28 days, compared to 2D culture which only had 27.9 ± 8.4% viability on day 8. This difference in viability between 2D and MPS was statistically significant ($p < 0.05$) after culturing for 2 days. The level of ALT in effluents or culture media was used as a surrogate for damaged and dead cells when comparing the viability of human hepatocytes for 2D *vs.* MPS culture. Human hepatocytes in conventional 2D culture had a higher level of ALT release (normalized to medium volume and cell seeding number) compared to cells in the MPS culture (Fig. 4B).

3.2. Comparison of hepatic functioning between 2D and MPS cultures using primary hepatocytes

Because the number of cells that can be seeded into the MPS lumen is very small (approximate 2000 cells/device), we utilized the entire MPS extracellular matrix chamber, which can accommodate 20,000–25,000 cells/device, for testing hepatic function.

3.2.1. HNF4α expression

ICC staining of HNF4α, a marker for liver functionality, was performed to compare the conventional 2D culture and MPS. Fig. 5A shows that 59 ± 12% of rat hepatocytes in MPS expressed HNF4α on day 7 compared to only 12.4 ± 6% in 2D culture ($p < 0.01$). Human hepatocytes in MPS culture maintained a higher expression level of HNF4α on day 5, compared to 2D culture (Fig. 5B, $p < 0.01$), and continued to maintain relatively high levels of HNF4α expression on day 14 (75 ± 5%).

3.2.2. Albumin production

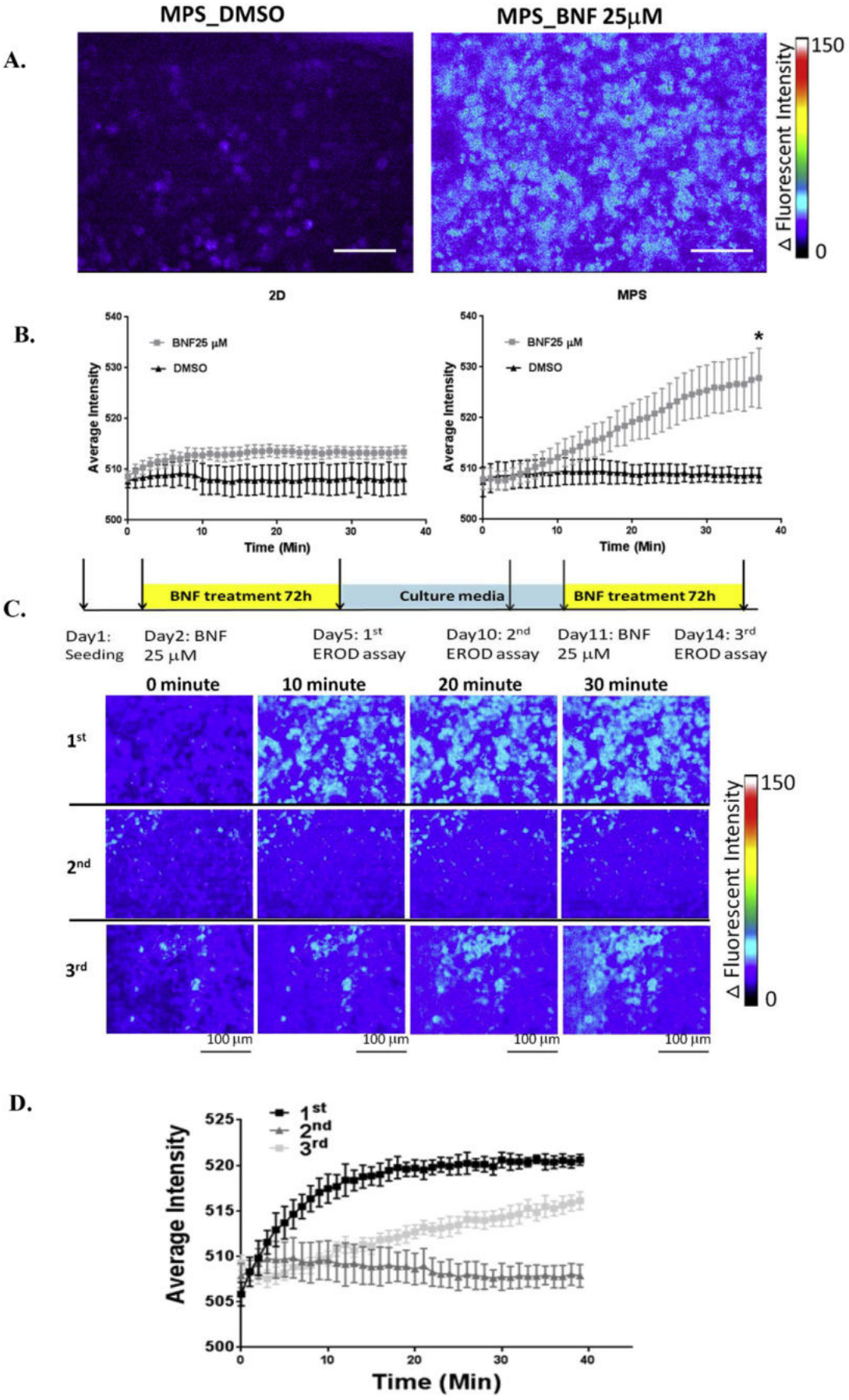
Albumin production is one of the major physiological functions of the liver. ELISA results show that human hepatocytes in MPS culture had higher albumin production (Fig. 6) up to day 15 after normalization to the seeding cell number (10^6 cells: 0.5×10^6 cells for 2D; 0.02×10^6 cells for MPS).

3.2.3. Measurement of CYP1A1/2 activities

CYP1A activities from cells cultured in MPS were measured using the EROD kinetic confocal fluorescence microscopy assay, with real-time confocal monitoring of fluorescence intensity for 30–40 min. The results of this EROD assay, represented as a change in fluorescence intensity over the time period of observation, demonstrated that induced CYP1A enzyme activity was detectable in cells cultured in MPS. Both the rat (Fig. 7) and human (Fig. 8) hepatocytes pre-treated with BNF and cultured in MPS demonstrated BNF-inducible and dose-dependent CYP1A activity on day 7 by 52.7 fold-change in rat cells and 6.3 fold-change in human cells (calculated by comparing AUC). Furthermore, induced CYP1A1/2 activities were measurable in human hepatocytes cultured in MPS on day 5 (1st), day 10 (2nd), and day 14 (3rd) (Fig. 8C). The EROD activity (3rd) after the second induction was lower compared to the first induction (1st EROD).

3.2.4. Measurement of CYP3A4 activities

CYP3A4 is the predominant cytochrome P450 in the liver, and contributes to the metabolism of many drugs, environmental contaminants and some endogenous molecules, such as 25-OH-Vitamin D3 (Werk and Cascorbi, 2014). CYP3A4 and many other biotransformation enzymes are inducible through several different ligand-activated transcription factors, such as the Pregnane X-Receptor (PXR) and the Constitutive Androstane Receptor (CAR). Responsiveness to 'standard' CYP3A4 inducers is an important characteristic of normal liver function, and is a key consideration in pharmaceutical development (Chu et al., 2009). Inducible CYP3A4 activity was measured either by a luminescence reporter assay using the CYP3A4-specific bioluminescent substrate, luciferin-IPA (Choi et al., 2014), or by quantifying midazolam (MDZ) metabolites in effluents before and after induction of CYP3A4 with rifampin (5 μM) for 72 h. Results were calculated as area under the curve (AUC) of metabolite formation for the 6 h following a 4-hr incubation with the parent compound, luceferin-IPA (3 μM). The AUC values were normalized to the cell seeding number (50×10^4 cells in a 24-well plate for 2D; 2×10^4 cells in a MPS chip). Fig. 9A shows that human hepatocytes in MPS had 3-fold higher CYP3A4 activities on day 5 compared to 2D culture. Baseline


A.
MPS_DMSO
MPS_BNF 25μM
150
Δ Fluorescent Intensity
0
B.
2D
MPS
BNF25 μM
DMSO
Average Intensity
Time (Min)
*
C.
BNF treatment 72h
Culture media
BNF treatment 72h
Day1: Seeding
Day2: BNF 25 μM
Day5: 1st EROD assay
Day10: 2nd EROD assay
Day11: BNF 25 μM
Day14: 3rd EROD assay
0 minute
10 minute
20 minute
30 minute
1st
2nd
3rd
100 μm
D.
1st
2nd
3rd
Average Intensity
Time (Min)

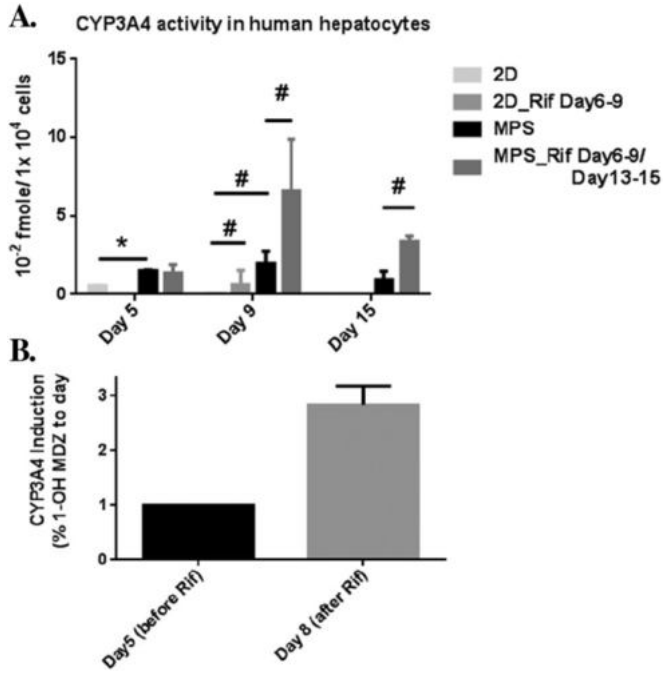


Fig. 9. Measure CYP3A4 activity in primary hepatocytes cultured in 2D or MPS over time. (A) CYP3A4 activity *via* Glo® substrate: the same cells were treated with vehicle or 5 μM rifampin (Rif) for 72 h (days 6–9 and days 13–15 [MPS only]), and were measured enzyme activity at day 5, 9 and 15. Results presented as CYP3A4 metabolite formation normalized to the total culture areas of 2D or MPS (1.9 cm^2 of one well in 24–well plate for 2D; 0.32 cm^2 for MPS) from 3 wells of a 24-well plate or 3 MPS chips per group in 3 independent experiments (cell preparations with donor 15-001, 15-002, and 15-004) (*t*-test, *, $p < 0.05$, #, $p < 0.01$). (B) LC/MS analysis of MPS effluent for midazolam (MDZ) metabolites: Human primary hepatocytes in MPS were pre-treated with rifampin (Rif) 5 μM for 72 h. After induction, 100 μM MDZ in culture medium was perfused though MPS for 4 h. All the effluents before and after Rif treatment were collected and analysis by LC/MS for 1-OH MDZ. The total amount of 1-OH-midazolam formed is presented as the fold change compared to the amount measured prior to Rif treatment. Results presented from 3 chips per group from 1 cell donor (15-004).

CYP3A4 activity was unchanged at day 9, compared to day 5, but at day 15 it had declined to 60% of day 5 baseline activity. Rifampin–treated human hepatocytes in MPS (5 μM during days 6–9, and days 13–15) had approximately 3-fold increased CYP3A4 activity compared to the DMSO-treated control at both time points, although the overall activity of both baseline and induced CYP3A4 at day 15 was reduced by about half when compared to day 9 (Fig. 9A). Qualitative LC-MS/MS data of midazolam hydroxylase activity (Fig. 9B) showed that the CYP3A4 metabolite 1-OH-MDZ increased by about 3-fold after rifampin treatment for 72 h. All results of CYP3A activity support that CYP3A4 activity was maintained through day 15 in cells cultured in MPS.

3.3. Visualization of canalicular structure and cell polarity in primary hepatocytes cultured in MPS

The primary human hepatocytes cultured in MPS maintained canaliculus-like structures (Fig. 10A) up to 15 days in culture. Immunocytochemistry (ICC) for the presence of Bile Salt Exporter Protein (BSEP; Fig. 10B) show that cells cultured in MPS expressed the BSEP and a bile duct-like structure on day 15 in culture, indicating that primary human hepatocytes maintained cell polarity in MPS culture. In order to investigate whether hepatocytes formed canalicular structures, we used chloromethyfluorescein diacetate (CMFDA), a fluorescent substrate for the bile transporter Multidrug Resistance-associated Protein (MRP2), to examine canalicular structures in our liver MPS model (Reif et al., 2015). Fig. 10C shows canalicular structures in human MPS at day 15 in culture by CMFDA staining.

3.4. Aflatoxin toxicity assessment in human liver MPS

While 2-D culture condition provides a simpler means to assess AFB toxicity we have observed large variability in biotransformation enzyme expression levels and activities between donor preparations and relatively low viabilities after a week in culture in 2-D (Fig. 3B) (Gross-Steinmeyer et al., 2005). Previous evaluations of conventional 2-D cultures with primary human hepatocytes to study AFB-mediated cytotoxicity have demonstrated that the use of the Matrigel-overlay maintains higher constitutive and inducible expression of AFB activating biotransformation enzymes such as CYP1A2 and CYP3A4 and we have added this feature to our culture system (Gross-Steinmeyer et al., 2005). Primary hepatocyte culture CYP 1A2 and 3A4 activity from isolated human hepatocytes is dramatically reduced within the first 48 h of culture followed by a steady increase in CYP3A4 then CYP1A2 activity (Gross-Steinmeyer and Eaton, 2012; Hewitt et al., 2007). With overall higher hepatocyte viability with 3-D cultures compared to 2-D over time, we can extend our primary cell culture periods with 3-D cultures to regain metabolic enzyme activity while not sacrificing a large reduction in the viable cell mass.

To demonstrate that the liver MPS model can be used to assess hepatotoxicity for compounds that require metabolic activation, we evaluated the response of human hepatocytes in MPS culture to the known human hepatotoxin, aflatoxin B_1 (AFB). AFB is both a potent hepatotoxin and carcinogen that requires metabolic activation by hepatic CYP1A2 or 3A4 to form the highly reactive aflatoxin 8,9-epoxide (AFBO), which forms protein and DNA adducts (Eaton and Gallagher, 1994). Primary hepatocytes were cultured in a 450 μm diameter tube formed within the Type I collagen matrix that fills the extracellular matrix lumen of the Nortis MPS device to assess aflatoxin bioactivation. This cell culture format has a wider channel than the Nortis MPS devices used in previous experiments (120 μm lumen diameter; Fig. 1 and 2A). The wide channel devices provides for fairly homogeneous and consistent cell seeding densities and a lumen size that minimizes cell-damaging fluid shear forces, and also provides for greater cell numbers for quantitative evaluation. We utilized a Nortis chip with a 450 μm wide by 6 mm long lumen channel and seeded the lumen with primary hepatocytes through the injection ports, then continuously perfused cell culture media through the channel.

Live/Dead staining of the hepatocytes indicated a dose-response in relation to AFB exposure with an increase in red staining (dead cells) and/or decreased overall cell mass due to the sloughing and washing away of dead cells from the lumen (Fig. 11). Analysis of the MPS device effluents for LDH activity at 24 and 48 h post-exposure using the Promega 96® Non-radioactive Cytotoxicity kit showed high variability between devices with a modest increase in LDH at the 30 μM exposure (data not shown). These data demonstrate that this system can support

Fig. 8. CYP1A1/2 activity in primary human hepatocytes cultured in MPS using EROD kinetic confocal assay. Human hepatocytes cultured in MPS expressed BNF-inducible CYP1A1/2 after a perfusion of EROD substrate within 40 min. (A) EROD activity of human hepatocytes cultured in MPS with or without BNF treatment for 72 h. Cells from donor of HUM#4038 cultured in MPS expressed BNF-inducible CYP1A1/2 after a perfusion of EROD subtract for 40 min (bar = 100 μm) (B) Results presented as mean of average intensity from 3 MPS chips per group in 3 independent experiments (cell preparations). Each sample was analyzed for average intensity of 10 randomly selected cells under 100× magnification (*t*-test, *, $p < 0.01$). (C) Repeated BNF-induction of CYP1A1/2 in human hepatocytes cultured in MPS up to 14 days. An experimental flow chart of repeated BNF treatment and dates of performing EROD assays is shown. Hepatocytes were from donor of HUM#4038 cultured in MPS. Repeated BNF-induced CYP1A1/2 activity measured for 0, 10, 20, and 30 min on days 5, 10, and 14 (bar = 100 μm). (D) Results presented as mean of average intensity from 3 MPS chips per group in 3 independent experiments on 1st, 2nd and 3rd EROD assays.

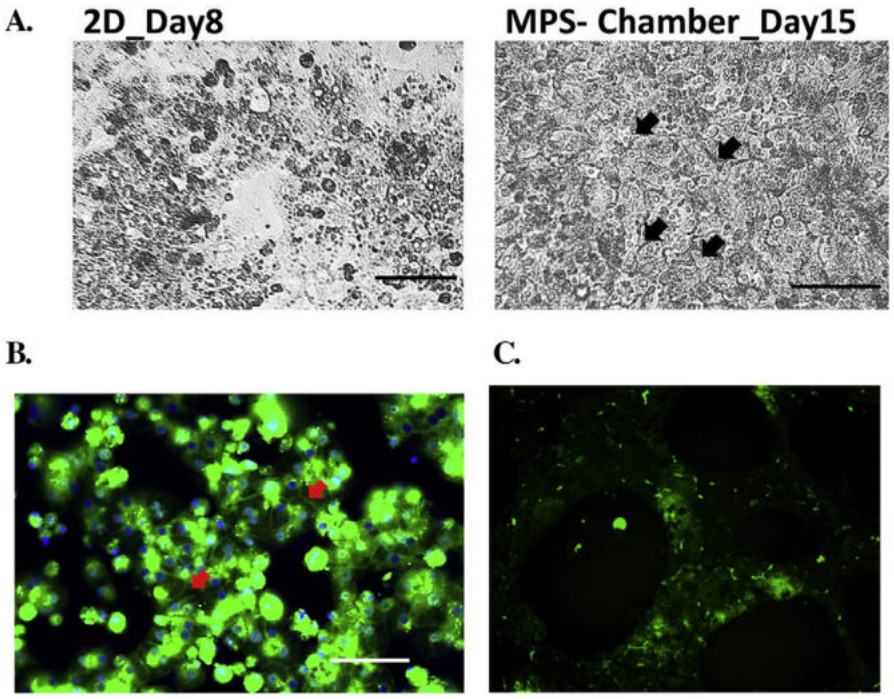


Fig. 10. Canalicular–like structure in human hepatocytes cultured in MPS on day 15. (A) Phase contrast pictures showed human hepatocytes from donor 14-018 in 2D culture on day 8 and in MPS on day 15. (B) ICC of BSEP in hepatocytes from donor 14-018 in cultured in MPS on day 15 (bar = 100 μm; 200× magnification). (C) Canalicular-like structures (arrows) were detected by CMFDA tracer in HUM4097B in culture in MPS on day 15. Representative pictures were from 3 technical replicates.

healthy hepatocyte growth for at least eight days with sufficient CYP activity to drive oxidative metabolism with the formation of toxic reactive metabolites and a promising means to evaluate the hepatotoxicity of toxicants that require bioactivation.

4. Discussion

MPS platforms offer a new opportunity for reducing animal usage and provide an improved *in vitro* culture system for predicting the toxicity and efficacy of drugs and environmental chemicals. Many laboratories, research institutes, and governmental organizations are participating in developing various organ-on-chips with different scaffolds, models, and cell types (Sutherland et al., 2013). However, there are many challenges to the broader use of MPS culture, including costs, scaling the flow rate for integrating multiple organs-on-chips, adsorption problems for hydrophobic compounds, and scarcity of primary human cells. In order to facilitate the usage of MPS culture for toxicity prediction, more validation studies are needed to demonstrate that MPS culture provides benefits over conventional 2D culture. Towards this end, new MPS approaches and platforms should be characterized by determining 1) if the microenvironment in MPS supports longer term viability of cells that continue to exhibit *in vivo*-like function, and 2) if the results using MPS culture better recapitulate the human

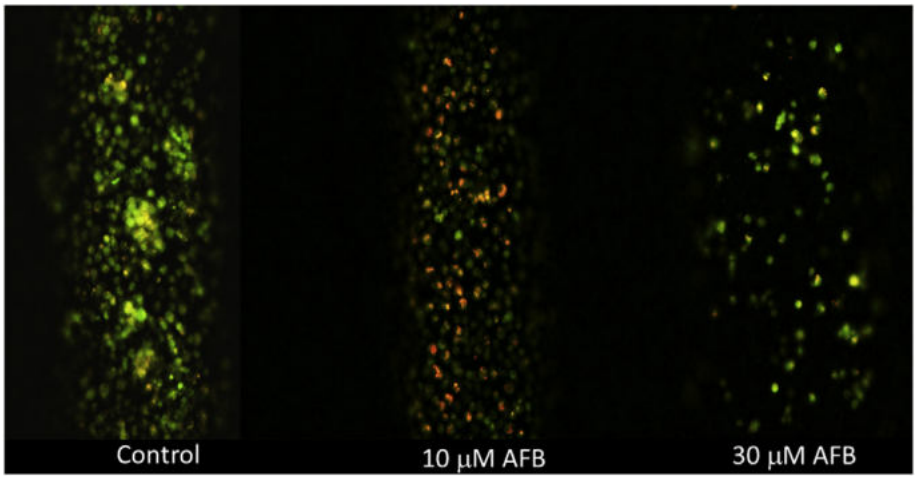


Fig. 11. Aflatoxin toxicity in human MPS. Representative images of primary hepatocytes cultured in the 450 μm wide lumens of Nortis chips exposed to 0, 10 and 30 μM AFB for 2 h followed by 48 h of culture with a 30 μL/h perfusion rate. Chips were stained with a Live/Dead® fluorescent stains with calcein AM staining live cells (green) and ethidium bromodimer-1 marking dead cells (red). A dose-response was observed with an increase in red-stained cells (dead) at 10 μM AFB, and reduced cell mass at the 30 μM, suggesting cell death and sloughing.

response to drugs or environmental chemicals, compared to results using conventional 2D culture.

In this study, the results from our liver MPS model show that MPS culture offers an adequate microenvironment to maintain viability of primary rat and human hepatocytes with crucial hepatic functionalities for at least 14 days. By comparison, 2D culture could only sustain cell viability for 7 days. Additionally, human hepatocytes cultured in the MPS also formed canalicular structures and maintained high levels of HNF4α expression, and albumin synthesis. Levitt et al. determined that the baseline *in vivo* rate of albumin synthesis in human liver is 10.5 g/day (Levitt and Levitt, 2016). Assuming a hepatocyte number of 140×10^{6} cells/g of liver (Sohlenius-Sternbeck, 2006), and further assuming the weight of human liver of 1500 g (range, 838–2584 g (Molina and DiMaio, 2012)), it is possible to estimate the approximate rate of albumin production by human liver *in vivo*, in units of μg of albumin per 1×10^{6} cells per day, that can then be compared with the MPS:

$$\text{Human albumin production rate } in\ vivo = \frac{10.5\ \text{g/day}}{140 \times 10^{6}\frac{\text{cells}}{\text{g}} \times 1500\ \text{g}} = 50\ \mu\text{g per } 1 \times 10^{6}\ \text{cells}^{-1}\ \text{day}^{-1}$$

In this study, the rate of albumin synthesis in MPS and 2D primary culture of isolated human hepatocytes was in the range of 1–2 mg/ml per 10^{6} cells. When adjusted for the flow rate (30 μl/h, or 0.72 ml/day) this converts to an albumin synthesis rate of 700–1400 μg of albumin per 10^{6} cells per day. This value is similar to that reported by Hutson et al. (4–5 mg of albumin per gram of liver per hr., which converts to approximately 650–850 μg albumin per 10^{6} cells per day) in primary cultures of rat hepatocytes (Hutson et al., 1987). Our estimated rate of albumin synthesis in the MPS system is approximately 15–30 times greater than the estimated rate of albumin synthesis by human liver *in vivo* as estimated above. However, the flow through system used for the MPS experiments is non-recirculating, so the input concentration of albumin to the hepatocytes is essentially zero. Regulation of albumin synthesis *in vivo* is complex and depends upon hormones and availability of amino acid pools (Hutson et al., 1987). In our MPS system, it is possible that albumin synthesis is greatly up-regulated, perhaps explaining the apparently higher than 'normal' rate of albumin synthesis, compared to *in vivo*. There are also many assumptions involved in adjusting the *in vivo* rate units that allow for comparison to the MPS, which could substantially underestimate or overestimate the true rate of albumin synthesis per million hepatocytes *in vivo*. It should be emphasized that the main points of this experiment were to 1) demonstrate the ability of these cells to perform an essential physiological function of hepatocytes, and 2) maintain that function at a relatively constant rate over a period of weeks. It is evident from the data in Fig. 6 that albumin synthesis actively occurs in the MPS system, as well as in primary hepatocyte cultures in 2D, but that the rate is maintained much better in MPS for prolonged period of time (2 weeks), relative to 2D sandwich cultures of hepatocytes in Matrigel.

Another important physiological role of the liver is the inducible expression of drug metabolizing enzymes, including CYP1A activity. Here we measured CYP1A activity by EROD and demonstrated that it is highly inducible at mRNA and protein by a range of polycyclic aromatic hydrocarbons and heterocyclic aromatic amines/amides. We used 3-methylcholanthrene (3-MC), BNF and omeprazole (brand names: Prilosec, Rapinex) typical CYP1A inducers that are widely used as the positive controls in primary hepatocytes cultures (Chu et al., 2009; Ma and Lu, 2007). Merredith et al. studied the induction of rat CYP1A *in vivo vs. in vitro* using precision-cut liver slices, and reported a 21-fold increase in CYP1A mRNA by BNF treatment *in vitro* and 2.6-fold increase in EROD activity in dexamethasone (50 mg kg^{-1} day^{-1}) dosed rat *in vivo* (Meredith et al., 2003). Another rat *in vivo* study demonstrated that BNF (80 mg kg^{-1} day^{-1}) caused a 2-fold increase in CYP1A activity in liver (Elsherbiny and Brocks, 2010). In humans, Madan et al. found that *in vitro* treatment of primary hepatocytes culture with BNF (33 μM) increased in EROD activity by 2.3 to 56-fold (average 13-fold, $n = 28$) whereas rifampin caused a 2.3-fold increase in CYP1A2 activity (Madan et al., 2003). Diaz et al. reported a 2- to 10-fold induction of the CYP1A2 protein and CYP1A-dependent activities *in vivo* in liver biopsies from cancer patients before and after 4 days of treatment with omeprazole (Diaz et al., 1990). Two- to 10-fold increases in RNA expression and a minimum 2-fold increase in enzyme activity were considered as acceptable responses to positive controls of CYPs inducers (Chu et al., 2009). The magnitude of induction of CYP1A and CYP3A mRNA following dexamethasone exposure (0.1 μM) was substantially lower than with other CYP3A inducers noted above (Meredith et al., 2003). In this MPS study, we also included 0.1 μM dexamethasone in culture medium, which facilitates relatively higher basal levels of CYPs activity. BNF-treatment increased CYP1A activity by 53-fold in rat cells and 6.3-fold in human cells *in vitro* using MPS culture. Even though CYP3A4 activity in human hepatocytes decreased on day 15 of MPS culture, hepatocytes in MPS retained the rifampin-induced activity of CYP3A4. Furthermore, maintenance of CYP metabolic capacity was demonstrated in this MPS system through our AFB experiments. Since CYP3A4 expression is controlled by multiple different ligand-activated transcription factors (especially PXR and CAR) for which endogenous molecules can serve as activators, it is likely that addition of such ligands to the flow-through medium would provide long-term maintenance of *in vivo* levels of CYP activity.

We have also demonstrated here that human hepatocytes exposed to AFB (a toxicant that requires bioactivation for toxicity to occur) in an MPS culture successfully identified hepatotoxic effects of the highly reactive AFB-8,9-epoxide formed during CYP-dependent AFB metabolism.

However, one pitfall of the current approach is that we used highly purified hepatic parenchymal cells, and thus only one of four major cell types in the liver predominates in this system. A possible next step of our study is to create a liver MPS model that is composed of multiple hepatic cells types, including sinusoidal endothelial cells, Kupffer cells, and stellate cells (Vernetti et al., 2015), with acinus and vascular structures, for capturing immune responses. A liver MPS model with multiple cell types may also prolong viability and functionality for long-term *in vitro* culture (You et al., 2015; Berger et al., 2015; Trask et al., 2014; Bale et al., 2014). We reviewed *in vitro/ex vivo* human hepatic cell systems used in biotransformation and transporter studies from traditional assays to the most recently advanced three-dimension (3D) cultures (Chang et al., 2016).

Why is the MPS culture superior to the conventional 2D culture? Numerous studies (Choi et al., 2014; Kim et al., 2012; Trietsch et al., 2013; Kim and Ingber, 2013) have reported that a microfluidics system can maintain cell viability and functionality for a longer period in culture compared to cell culture without flow. Furthermore, ECM gels, such as collagen or Matrigel®, are often used in MPS culture. Using ECM gels can enhance cellular function and improve tissue organization, thereby better mimicking the natural architecture of tissues, cell structure and polarization (Kleinman et al., 2003; Lutolf and Hubbell, 2005). Several studies have found that ECM can provide an appropriate mechanical microenvironment for cell growth, tissue differentiation and maintenance of tissue functionalities (Jeon et al., 2014; van der Meer et al., 2013; Sung and Shuler, 2009). The combination of ECM gel and the microfluidic system makes MPS a more representative *in vitro* organ-specific, biochemical and mechanical microenvironment for organoid type culture. A representative example is the kidney-on-a-chip microphysiological system that mimics renal physiology by reconstructing the proximal tubule of the kidney (Kelly et al., 2013; Weber et al., 2016).

Even though MPS culture holds substantial promise for the future of pharmacology, toxicology and systems biology, there are still challenges that must be addressed (Wikswo, 2014). For example, using polydimethylsiloxane (PDMS), a major material for fabricating MPS modules, can potentially confound the delivery of drug or toxicants in the MPS culture if the compounds are highly hydrophobic (Berthier et al., 2012) because PDMS has been found to adsorb hydrophobic compounds (log $p > 2.62$; (Wang et al., 2012)). Researchers have been working to solve this adsorption problem by using chemical surface modifications such as glass coatings (Wong and Ho, 2009).

Once individual organ MPS are well-fabricated and tested, the next step is to connect several organs into a multiple-organ MPS and then validate these systems with well-known toxicants. In order to address these challenges, we and several other groups are continuing to develop these systems with support from the Defense Advanced Research Projects Administration (DARPA), the National Center for Advancing Translational Sciences of the National Institutes of Health (NIH-NCATS), FDA, and the Environmental Protection Agency (EPA) (Sutherland et al., 2013; Andersen et al., 2014).

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Transparency document

The Transparency document associated with this article can be found, in the online version.

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