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ON-CHIP DIFFERENTIATION OF RADIALY VASCULARIZED HEPATIC CORDS MIMICKING THE LIVER LOBULE

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ABSTRACT

Due to a more physiological reproduction of the *in vivo* situation, liver-on-a-chip devices in recent studies have shown higher sensitivity and accuracy for hepatotoxicity testing compared to traditional *in-vitro* liver models [1][2]. In this context, this work presents an original microfluidic device that mimics 3D microstructured and microfluidically vascularized hepatic cords organized radially like the liver lobule. 10 cell-culture chambers seeded in high density are disposed with parallelized flow that emulates blood perfusion and drug clearance. Using this device, on-chip differentiation of Human HepaRG-hepatoblast (HepaRG-HB) to HepaRG-Hepatocytes (HepaRG-HC) has been studied in terms of self-organization capabilities, bile canaliculi formation and albumin expression. Our results indicate that HepaRG-HB are able to proliferate, self-organize and spontaneously differentiate within the cell culture chambers forming directional bile canaliculi, while HepaRG-HC are observed to aggregate and form tissues resembling 2D HepaRG-HC cultures.

KEYWORDS

Liver-on-chip, Microfluidics, Differentiation, HepaRG, Tissue-engineering

INTRODUCTION

Liver-on-a-chip devices in the past two decades have shown promising results as physiological relevant *in vitro* models. Recent studies have shown that liver chips are capable of meeting pharmaceutical industry benchmarks for preclinical Drug - Induced Liver Injury (DILI) models [3]. They have been shown to possess increased sensitivity (87%) over hepatocyte spheroid model (47%) in predictive toxicity of small molecules [1]. Indeed, Liver chips have been shown to have increased expression of genes representing Phase I and Phase II metabolizing enzymes [4]. While primary human hepatocytes remain the main model for preclinical studies, difficulties associated with batch to batch variability, post thaw/long term viability, high cost and low availability can be circumvented by using established cell lines like HepaRG [5].

HepaRG, a hepatic bipotent progenitor cell line, capable of differentiating to hepatocyte-like cells (HepaRG-HC) has shown to possess a gene expression profile of cytochrome P450 close to primary human hepatocytes [6]. Indeed, it has already been demonstrated that HepaRG is an important tool to study liver drug

metabolism and clearance[7]. Different methods of HepaRG differentiation to HepaRG-HC have been reported in the literature [8] with most of them involving a treatment of DMSO, which has been recently found to be pleiotropic [g]. HepaRG cells are observed to spontaneously differentiate once confluent. Multiple works have attempted DMSO-free differentiation by utilizing 3D culture methods which enhance cell-cell interaction [9][10]

The integration of micrometric slits-based barriers in a microfluidic device allows high density packing of cells. This has been studied in previous works [11][12][13]. But the influence of high density packing of HepaRG cells towards spontaneous differentiation and bile canaliculi organization in a 3D microenvironment with flow, has not yet been characterized.

MATERIALS AND METHOD

The microfluidic chip, integrates 10 cell culture chambers (Height - 40 μ m, Width 40 μ m or 60 μ m and Length-1000 μ m) purposely designed to allow the mimicking of hepatic cords (Fig 1). A 2 μ m height slit network connects the cell chamber to the medium flow channels, providing nutrients to the cells by diffusive flow and while protecting them from shear stress (Fig. 2)

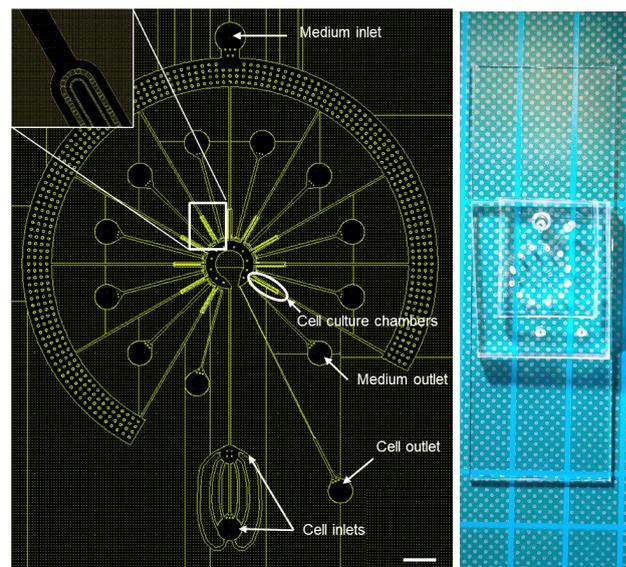


Figure 1: (Left) Schematics view of the microfluidic device mimicking the hepatic lobule, with 10 cell culture chambers (Scale Bar- 1 mm). (Right) Top view of the fabricated PDMS device on a glass slide

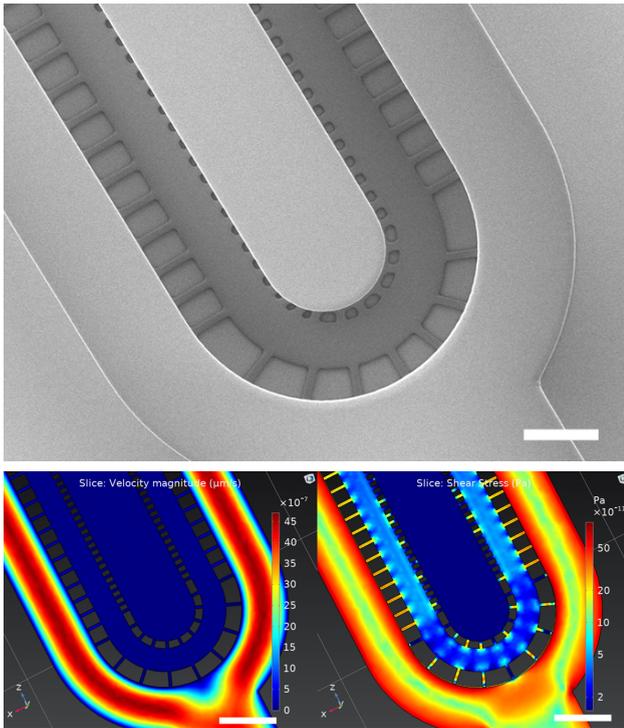


Figure 2: (Top) SEM image of the designed 40µm wide cell culture chamber and network of slits (Scale Bar - 10µm). Simulation of the flow rate (Bottom left) and shear stress (Bottom right) near cell chambers (Scale Bar - 40µm)

The shear stress on the tissue is maintained lower than 0.1 dyne/cm² at a flow rate of 1µl/min due to the geometry and disposition of slits (Fig. 2 Bottom left), which is ideal for hepatocyte culture. High shear stress on hepatocytes in liver chips, negatively affect albumin synthesis and urea production [15].

Chip fabrication:

The multiple photomask of the various levels of the chip was designed using L-Edit (Tanner EDA). On a silicon wafer, SU-8 2002 was spin coated and patterned using the slit-level photomask to a height of 2µm. The alignment marks in the patterned slit layer were covered with tape before spin coating SU-8 2025 to a height of 40µm. The tape was then removed and the channel level photomask was aligned with the wafer and exposed. The mold was hard baked at 180°C for 30 mins. A second mold was fabricated using SU-8 2025 (Height-40µm) containing the design for the second level of PDMS, which interlinks the 10 cell culture chambers outlets for retrieval of medium.

PDMS Sylgard 184 base and curing agent was mixed at a ratio of 10:1 by weight and was used to mold the design from the patterned silicon wafer. Two PDMS chip levels were bonded to each other and onto a glass slide after alignment, using plasma bonding. The Liver chip was sterilized by aspirating with ethanol for 30 mins. Then the fluid inside the device was replaced with PBS and stored until use.

Cell culture on chip

The cell culture chambers were coated with collagen 1 solution (50µg/ml) by aspiration and incubated at 37°C for 1 hour or stored overnight at 4°C. Undifferentiated HepaRG cells obtained from Biopredic International were cultured in a growth medium for 14 days and then in a differentiation medium for 14 days to create HepaRG-HC. HepaRG-HB was obtained by culturing undifferentiated HepaRG cells for 5-7 days on well plates (before confluence).

HepaRG-HB or HepaRG-HC cell suspension (10 million cells/ml) was aspirated into the chambers using differential pressure (below 10 mbar). Once the chambers were filled, the cells were allowed to adhere onto the collagen treated surfaces. The perfusion of the culture was carried out using a micro syringe pump at a flow rate of 1µl/min. HepaRG-HB were cultured in the growth medium and HepaRG-HC was cultured in the differentiation medium. The medium in the syringe was changed every 2-3 days. The chips were fixed at the end of the cell culture duration and an adapted immunostaining protocol was carried out to study the different tissues.

EXPERIMENTAL RESULTS:

1-Self organization of cells:

HepaRG cells within a few hours are observed to attach to the walls of cell culture chambers. Time lapse phase contrast imaging reveals that HepaRG-HB cells proliferate inside the chambers leading to an increased cell density, then they rearrange into a tissue-like structure within a week (Fig. 3 & 4). On the other hand, HepaRG-HC cells behave differently, assembling into a 3D structure in the chamber within 48 hours (Fig 3). They were not observed to proliferate inside the device, but rather shrink into a tissue (Fig- 4 -Right)

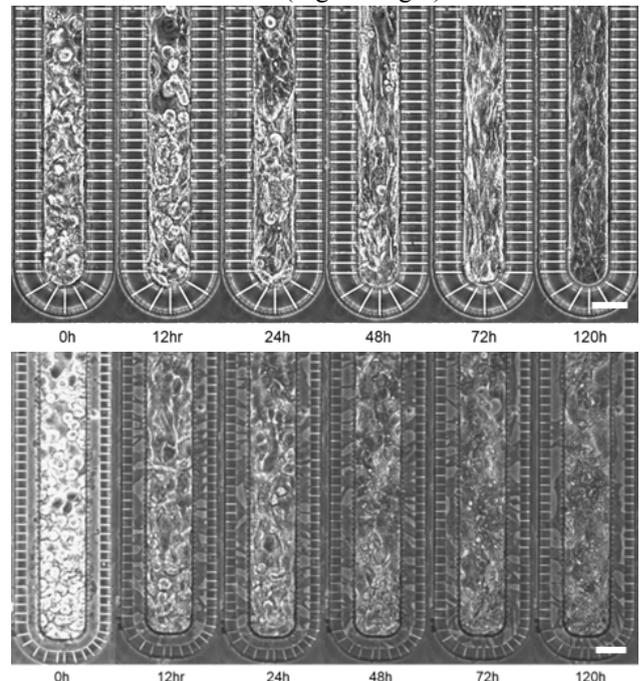


Figure 3: Phase contrast time lapse images over the first 5 days of HepaRG-HB (top) and HepaRG-HC (below) culture on-chip. (Scale bar - 40µm)

2.- Immunostaining Analysis:

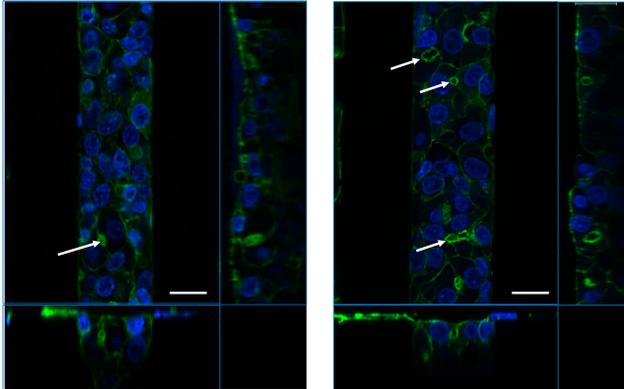


Figure 4: Comparison of the 3D tissue organization when HepaRG-HB (Left) and HepaRG-HC (right) are loaded inside the chip and cultured for 7 and 4 days respectively, by immunostaining nuclei and *f-actin*. (Scale Bar- 20 μ m)

Immunofluorescence staining for *f-actin* in tissues obtained at the end of cell cultures shows points of condensation of the proteins alongside the hepatocyte membrane between cells suggests the possible formation of bile canaliculi (Fig 4 - Arrows).

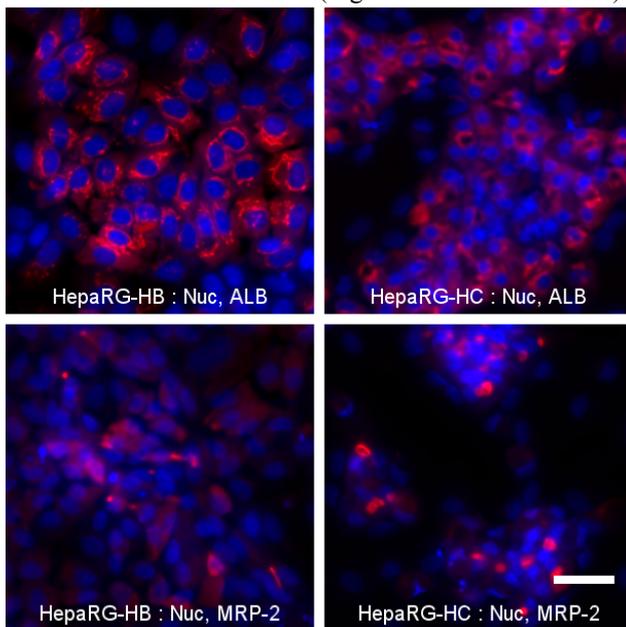


Figure 5: Immunofluorescence staining 2D HepaRG-HB or HepaRG-HC cultured for 7 days in 6 well plates. Nuclei, MRP-2 or Albumin (Scale Bar- 40 μ m)

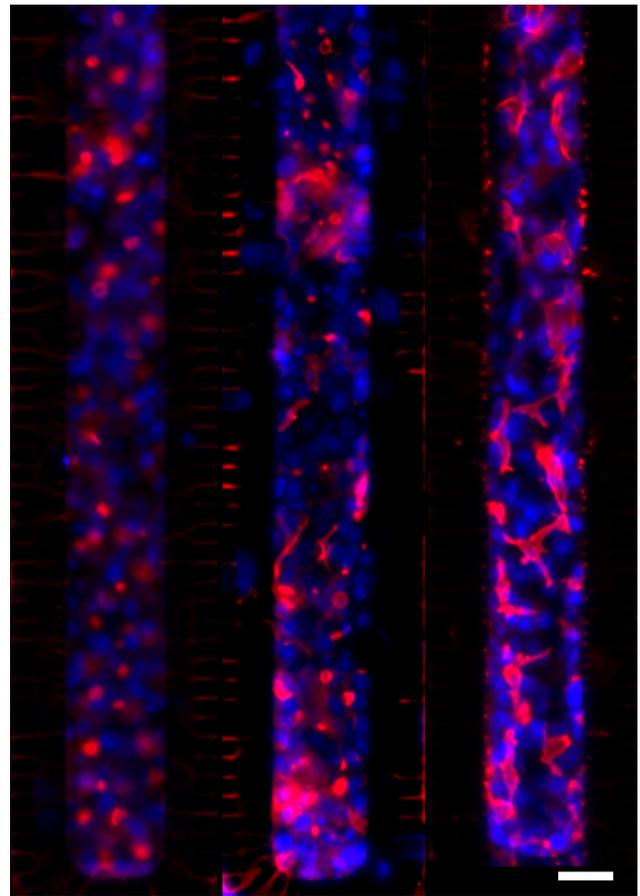


Figure 6: Immunofluorescence staining of HepaRG-HC (Left) and HepaRG-HB (center and right) cultured on chip for 7,7 and 14 days respectively. Nuclei, and MRP-2 (Scale Bar- 20 μ m)

Indeed, staining of the MRP-2, an apical membrane protein involved in bile excretion, confirmed the observations. When cultured for a week in the device, HepaRG-HC forms spherical bile canaliculi between two or three cells (Fig. 6), in line to what was observed in the 2D cultures used as control (Fig. 5). Whereas, HepaRG-HB within the same culture time are observed to form bile canalicular structures with also elongated shapes (Fig. 6). When HepaRG-HB are maintained in culture for a longer period of time, the number of elongated structures increase in number. This reflects a continuous reorganization of cells and/or cellular polarity.

Albumin staining in the case of HepaRG-HB when compared with 2D HepaRG culture (Fig. 5 & 7), indicated that the cells in the tissue are at a hepatoblast state. Indeed, after a week of culture the protein expression still appears to be low; whereas, HepaRG-HC, are clearly characterized by regions of high expression of albumin, similar to 2D HepaRG-HC (Fig. 5 & 7).

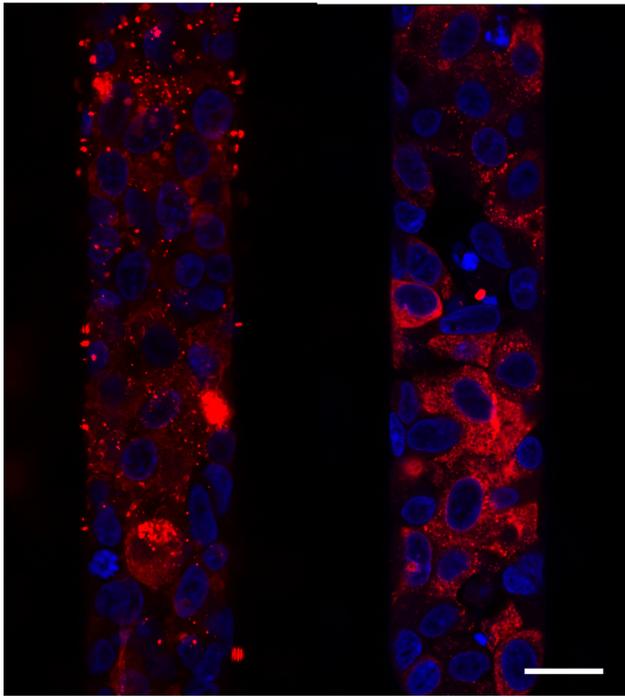


Figure 7: Immunofluorescence staining of Proliferative-HepaRG (Left) and HepaRG-Hepatocytes (right) loaded and cultured for 7 and 4 days on chip respectively. Nuclei, and Albumin. (Scale Bar- 20 μ m)

DISCUSSION

This device architecture showed to be efficient to study differentiation of hepatocytes in a constrained 3D environment. An alternative differentiation strategy for the HepaRG-HC using mechanical compaction in 3D is proposed that doesn't involve DMSO. Use of DMSO is unfavorable since it is a common solvent used in pharmaceutical drug preparations and also known for its repressive capability of albumin production and ammonia detoxification[16]. Transition from growth medium to differentiation medium with DMSO causes significant cell death [17], which is problematic in packed 3D systems like this device, since it interferes with viability studies during toxicological tests. Albumin staining has been used to study the differentiation state of the hepaRG cells and the formation of numerous apical poles is a characteristic of HepaRG-HC, but the tissue needs to be further characterized by the expression of CYP450, hepatocyte nuclear factors and basolateral markers. MRP-2 is an apical transporter protein involved in the excretion of unmodified drugs into bile canaliculi. Concentrated localisation of MRP-2 in the hepatocyte membrane is a marker for acquired hepatocyte polarity. Here, MRP-2 staining was used to study the formation and alignment of apical polarity within the device in different situations and to suggest that self-organization enhances the formation of longer, inter-connected and directional bile canaliculi. But the functionality and integrity of bile canaliculi needs to be further characterized by live-staining excretion assays. Overall, the parallelization of the device equilibrates the various cell culture chambers, which is a significant experimental improvement to the serialized version of hepatic cord

based liver-on-a-chip scaffolds [13]

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