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Young Bok (Abraham) Kang
George Fox University, ykang@georgefox.edu

Temitope R. Sodunke


Jason Lamontagne

Joseph Cirillo

Caroline Rajiv

See next page for additional authors

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Authors

Young Bok (Abraham) Kang, Temitope R. Sodunke, Jason Lamontagne, Joseph Cirillo, Caroline Rajiv, Michael J. Bouchard, and Moses Noh

Liver Sinusoid on a Chip: Long-Term Layered Co-Culture of Primary Rat Hepatocytes and Endothelial Cells in Microfluidic Platforms

Young Bok (Abraham) Kang,¹ Temitope R. Sodunke,¹ Jason Lamontagne,² Joseph Cirillo,¹ Caroline Rajiv,³ Michael J. Bouchard,⁴ Moses Noh¹

¹Mechanical Engineering and Mechanics, Drexel University, Philadelphia, Pennsylvania; telephone: 215- 571- 3471; fax: 215-895-1478; e-mail: mosesnoh@coe.drexel.edu

²Graduate Program in Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, Pennsylvania

³Graduate Program in Biochemistry, Drexel University College of Medicine, Philadelphia, Pennsylvania

⁴Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, Pennsylvania; telephone: 215-762-1898; fax: 215-762-4452; e-mail: michael.bouchard@drexelmed.edu

ABSTRACT: We describe the generation of microfluidic platforms for the co-culture of primary hepatocytes and endothelial cells; these platforms mimic the architecture of a liver sinusoid. This paper describes a progression study of creating such a *liver sinusoid on a chip* system. Primary rat hepatocytes (PRHs) were co-cultured with primary or established endothelial cells in layers in single and dual microchannel configurations with or without continuous perfusion. Cell viability and maintenance of hepatocyte functions were monitored and compared for diverse experimental conditions. When primary rat hepatocytes were co-cultured with immortalized bovine aortic endothelial cells (BAECs) in a dual microchannel with continuous perfusion, hepatocytes maintained their normal morphology and continued to produce urea for at least 30 days. In order to demonstrate the utility of our microfluidic liver sinusoid platform, we also performed an analysis of viral replication for the hepatotropic hepatitis B virus (HBV). HBV replication, as measured by the presence of cell-secreted HBV DNA, was successfully detected. We believe that our liver model closely mimics the *in vivo* liver sinusoid and supports long-term primary liver cell culture. This liver model could be extended to diverse liver biology studies and liver-related disease research such as drug induced liver toxicology, cancer research, and analysis of pathological effects and replication strategies of various hepatotropic infectious agents.

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KEYWORDS: liver sinusoid; liver model; long-term co-culture; liver on a chip; microfluidic platform

Introduction

The liver is the largest internal organ in humans and is involved in drug metabolism and drug detoxification as well as other metabolic activities that regulate blood glucose levels, bile synthesis, and the production of various plasma proteins such as albumin. Because of its overall importance in drug metabolism, the liver is a major focus of pharmaceutical research (Desmet, 2001). Currently, liver biology, liver-related disease studies, and drug discovery and screening research are facilitated predominantly by *in vitro* liver-cell culture models. However, primary liver cells cultured on conventional tissue culture platforms do not maintain their viability for more than a week and do not recapitulate the *in vivo* architecture of the liver (Guillouzo, 1998; Nahmias et al., 2007).

In order to overcome the limitations of conventional liver-cell culture systems and develop a valid *in vitro* liver model that can accurately predict drug-induced liver toxicity, multiple groups have applied microfabrication and microfluidics technologies to liver model development. In one early study, a flat-plate bioreactor was developed to investigate the effect of medium flow and oxygenation on the viability and function of rat hepatocytes co-cultured with fibroblasts (Tilles et al., 2001). A similar bioreactor with an in-line oxygenator was also generated and used to provide physiologic oxygen gradients over a period of 5 days, producing an *in vitro* model of liver zonation (Allen et al., 2005). It is important to note that these two bioreactor systems were based on a mixed co-culture of hepatocytes and fibroblasts, and did not result in successful long-term culture of the hepatocytes. Micropatterned co-cultures without

the introduction of medium flow have also been created to establish a system that facilitated control of cell–cell interactions, which play an important role in maintenance of hepatocyte function (Cho et al., 2010; Khetani and Bhatia, 2008). The results of these studies showed that increasing the heterotypic contact area between hepatocytes and fibroblasts improved the functions of the hepatocytes for at least 4 weeks (Khetani and Bhatia, 2008). More recently, a microfluidic platform was used to study the interplay of the medium flow, production of collagen mRNA, and hepatocyte function. Hepatocytes were cultured under consistent culture medium flow conditions with a collagen overlay to mimic in vivo-like condition for hepatocytes, which are not directly exposed to flow (Hegde et al., 2014). These studies demonstrated that the dynamic interplay of the medium flow and collagen type IV secreted by hepatocytes plays an important role in the maintenance of primary hepatocyte function. During a culture period of 2 weeks, hepatocytes cultured under flow showed better maintenance of hepatocyte function as compared to static cultures without the introduction of medium flow. However, this model did not simulate the complex multi-cellular in vivo microenvironment. Another study in a bioreactor that facilitated the formation of three-dimensional hepatocellular aggregates was also reported; primary rat hepatocytes (PRHs) co-cultured with liver sinusoidal endothelial cells (LSECs) under continuous perfusion in this bioreactor were viable for 1 week (Domansky et al., 2010). The structure of the three-dimensional aggregate in this bioreactor, however, was a randomized cellular mass and not comparable to the organized sinusoidal structure of the liver in vivo.

While microfabrication and microfluidics have great potential to create more in vivo-like liver models, there still remains a need to improve hepatocyte longevity and function in co-culture with liver relevant endothelial cells in microfluidic devices (Khetani and Bhatia, 2008; Ukairo et al., 2013). Previously, we presented a novel liver model using commercially available 6-transwell tissue culture dishes in which primary hepatocytes and endothelial cells were co-cultured on the opposite sides of a microporous membrane for over 30 days (Kang et al., 2013).

In the study described here, we have successfully transferred the layered long-term co-culture configurations of the transwell system to microfluidic platforms with the goal of mimicking the fundamental architecture of the liver sinusoid (Fig. 1a). In vivo, the hepatic sinusoid is a microfluidic system that carries blood from the hepatic artery and portal vein, exposing the liver to oxygen and nutrients; this blood supply can also expose the liver to toxins and infectious agents (Desmet, 2001). The hepatic sinusoid is lined with LSECs and also contains other resident cell types such as Stellate cells and liver-specific macrophages. The hepatocytes are separated from LSECs by a small, extracellular-matrix-protein-enriched, *Space of Disse*. Bile is secreted from hepatocytes and transported to the intestines through bile ducts. Overall, the hepatic sinusoid can be considered to be a fundamental unit where most liver activities occur. This paper presents a progressional study for creating a *liver sinusoid on a chip* system. Each step in this progression was directed towards testing and optimizing cell-culture conditions in microfluidic devices with the goal of attaining increasing levels of similarity to an in vivo liver sinusoid. To achieve this, we first investigated a single-channel configuration in which a layered

co-culture of primary rat hepatocytes and endothelial cells was created with a thin matrigel layer added between the two cell layers (Fig. 1b). Next, a dual-channel configuration was investigated in which two microchannels separated by a porous membrane simulates the sinusoid and a channel to allow removal of secreted factors from hepatocytes (Fig. 1b and c). Finally, the dual-channel configuration was linked to a continuous flow system. In this dual-channel configuration with continuous perfusion, hepatocytes lasted at least 30 days, maintaining their polygonal morphology and detectable hepatocyte functions. In addition to standard function tests, we also demonstrated that our liver model could be used to analyze replication of the hepatitis B virus (HBV), which is dependant on expression of hepatocyte-specific factors. Although there are limitations to the use of in vitro liver models for studying liver biology and drug-induced liver injury, our in vitro liver model still represents important progress in the continuing efforts to generate biologically relevant in vitro liver model systems.

Materials and Methods

Fabrication of Microfluidic Platforms

Soft lithography technique was used to make Polydimethylsiloxane (PDMS) microchannels. Templates for rectangular microchannels (approximately 15 mm long, 1 mm wide, and 80–160 μm high) were fabricated by photolithography. SU-8 2035 (Microchem Corporation, Westborough, MA) was patterned on a 3 in. silicon wafer for this purpose. Stereolithography technique was also used for fabricating microchannel templates for higher dimension (approximately 15 mm long, 1 mm wide, and 200–800 μm high). PhotoSilver 100 (EnvisionTEC GmbH) resin and Envision Tec[®] Perfectory SXGA+ Standard UV Stereolithography system were used for this process. Once the microchannel templates were fabricated using photolithography or stereolithography, PDMS microchannels were made using the replica molding method (Sodunke et al., 2008).

To construct the single-channel device, a PDMS channel was bonded to a 10 cm cell culture dish after air-plasma treatment (Fig. 2a). For the dual-channel device, a microporous membrane obtained from commercially available transwells was cut down to the desired size and placed between two PDMS microchannels. The membrane was made of polyethyleneterephthalate (PET) and is about 10 μm thick with a pore size of approximately 0.4 μm . The two PDMS channels were then bonded together with the microporous membrane in the middle after air-plasma treatment (Fig. 2b). The bonded dual-channel device was then wrapped with Kapton tape (Micronova, Torrance, CA) to seal the device and prevent leakage. Inlets and outlet ports were created by either implanting silicone tubes during PDMS replica molding or attaching them afterward via holes punched into the PDMS channel.

The bottom surface of the microchannel or the PET membrane was coated with collagen type I by introducing a collagen solution (0.198 mg/mL) into the microchannel followed by spontaneous evaporation. The assembled microchannel device was then sterilized under ultraviolet (UV) light (Intensity $\sim 20 \text{ mJ}/\text{cm}^2$) for 30 s.

In order to provide continuous perfusion of culture medium effectively, the dual microchannel was connected to a syringe pump,

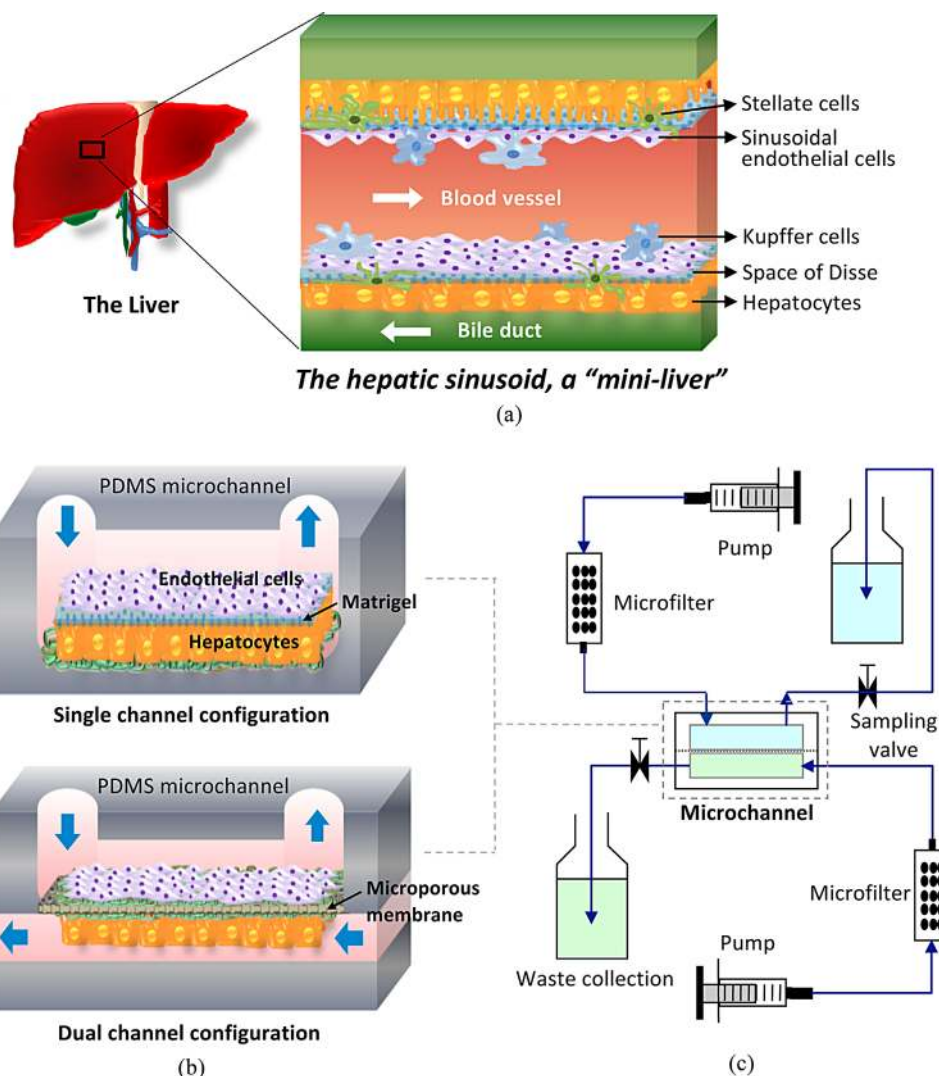


Figure 1. The hepatic sinusoid and the proposed microfluidic cell-culture platforms that mimic the liver sinusoid. (a) The liver sinusoid functional unit is a microfluidic system with several resident cell types (Kang et al., 2013); (b) two microfluidic platforms: single channel and dual channel configurations; and (c) bioreactor circuit for continuous perfusion of media and waste collection.

and a flow rate of 30–40 $\mu\text{L}/\text{h}$ was applied (Fig. 1c). A 0.2 μm sterile filter was placed upstream of the channel inlets to maintain sterile conditions and remove particulates. Flow was applied in counter directions in the dual-channel for effective mass transfer and removal of factors secreted from the cells. The outlet of the microchannel was connected to a waste bottle with a silicone tube (0.0625 in. ID \times 0.125 in. OD, Dow Corning, MIDLAND, MI). Multiple valves were located in the middle of the outlet line for sampling the cell-culture medium (Fig. 1c).

Cell Isolation and Culture

Primary hepatocytes were isolated from the liver of 6–12 weeks old Sprague–Dawley rats as previously described (Clippinger et al., 2009; Seglen, 1993). Approximately $1\text{--}2 \times 10^4$ cells with 70–100% viability were plated in the collagen-coated microchannel. Dulbecco's

Modified Eagle Media (DMEM) (Cellgro, Manassas, VA) supplemented with 1 mM sodium pyruvate (Cellgro), 4 $\mu\text{g}/\text{mL}$ insulin-transferrin-selenium (ITS, Gibco, Grand Island, NY), 5 $\mu\text{g}/\text{mL}$ hydrocortisone (HC, Sigma, St. Louis, MO), 5 ng/mL epidermal growth factor (EGF, BD, Bioscience, San Jose, CA), 1% penicillin-streptomycin, and 10% (vol/vol) fetal bovine serum (FBS, Gemini Bioproducts, West Sacramento, CA) was added to the channel. Cells were maintained at 37°C in 5% CO_2 . For cells cultured in static conditions, the medium was changed daily (Kang et al., 2013).

Primary rat adrenal medullary endothelial cells (RAMECs) were obtained from the Drexel Tissue Engineering and Regenerative Medicine Laboratory (courtesy of Dr. Peter Leikes). Isolation and characterization of RAMECs have been previously described (Manolopoulos et al., 1995; Papadimitriou et al., 1993). RAMECs were cultured on collagen-coated tissue culture dishes in Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum

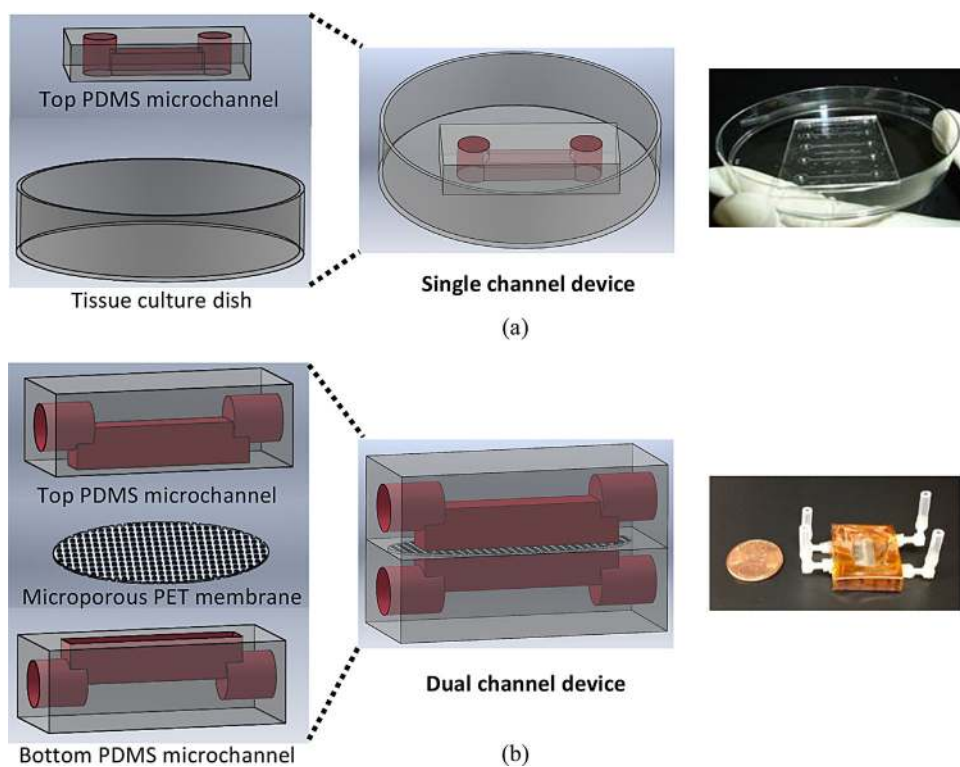


Figure 2. Fabrication of microfluidic cell culture platforms. (a) single channel device was made by attaching a PDMS microchannel (15 mm long, 1 mm wide, and 80–160 μm high) to the tissue culture dish; and (b) dual channel device was made by combining two PDMS microchannels (15 mm long, 1 mm wide, and 200–800 μm high) with a microporous PET membrane in the middle. Digital camera images of the actual devices are shown on the right.

(FBS), nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 5 μg of gentamicin per mL. Cells were maintained at 37°C in 5% CO_2 and cell-culture medium was replaced every 2–3 days. Bovine aortic endothelial cells (BAECs), which are immortalized, microvascular endothelial cells, were also used in co-culture because they are more readily available than primary endothelial cells. BAECs were a kind gift from Dr. Robert Levy (Children’s Hospital of Philadelphia) and the isolation and characterization of BAECs, including their endothelial cell functions, have been previously described (Schwartz, 1978). BAECs were cultured in DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin (Cellgro) at 37°C in 5% CO_2 with medium replacement every 2–3 days as previously described (Kang et al., 2013).

Layered Co-Culture of Primary Rat Hepatocytes and Endothelial Cells

Both single- and dual-channel configurations were investigated in this study (Fig. 1b and c). In the single-channel configuration, primary rat hepatocytes were first plated in the collagen-coated microchannel and then incubated 48 h to allow the cells to adhere and form a complete monolayer. After 48 h, RAMECs suspended in growth medium mixed with 30% (v/v) of matrigel (BD Biosciences) were placed on the PRH monolayer and incubated at 37°C in 5% CO_2 . Culture medium was replaced daily.

For the dual-channel configuration, primary rat hepatocytes were seeded into one channel and cultured for a minimum of 4 h to allow the cells to adhere to the microporous membrane and create a confluent monolayer. After the formation of the PRH monolayer, the device was flipped over and endothelial cells were then seeded into the other channel so that these cells could adhere to the opposite side of the microporous membrane. In order to investigate the effect of continuous perfusion, we tested both static and flow culture conditions. In the case of static condition, medium was replaced every 24 h. For flow culture, the microchannel was connected to a syringe pump, and a flow rate of 30–40 $\mu\text{L}/\text{h}$ of fresh PRH medium was applied to both the top and bottom channels.

RNA Isolation, Reverse Transcription, and PCR Analysis

In order to confirm the differentiation status and identity of primary rat hepatocytes, reverse transcriptase–polymerase chain reaction (RT-PCR) was conducted for cell-type specific markers as previously reported (Kang et al., 2013; Sodunke et al., 2008). To isolate total RNA from cultured cells, the PDMS microchannel was disassembled, and the primary rat hepatocytes were gently scraped and collected from their respective sides of the PET membrane. Total RNA was obtained using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s directions.

Genomic DNA was removed from samples by incubating total RNA with 1 U/ μg RQ1 RNase-Free DNase (Promega, Madison, WI)

at 37°C for 30 min followed by incubation at 65°C for 10 min with stop solution to inactivate the DNase reaction. cDNA was then generated by oligo(dT)-primed reverse transcription, followed by PCR for hepatocyte-specific differentiation markers. Specific primers for hepatocyte nuclear factor 4 (HNF-4), transferrin (TFN), and albumin (ALB) were used as previously described (Kang et al., 2013; Sodunke et al., 2008). The expression of hepatocyte-specific genes was normalized to the level of β -actin expression. The cycling conditions for PCR amplification were: 30 s at 94°C, followed by 30 cycles at 94°C for 30 s, 45 s at the specific annealing temperature for each primer set (Table S1), and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Amplified PCR products were analyzed using agarose gel electrophoresis.

Urea Synthesis Assay

As one test of retention of hepatocyte functions, we measured urea synthesis (Chen et al., 2009; Khetani and Bhatia, 2008). Cell-culture medium samples were collected every 2 days from the PRH-containing side of the dual microchannel and stored at -80°C until assayed. As a control, medium samples were taken from microchannels containing only primary rat hepatocytes without endothelial cells. Urea concentration in the medium was evaluated by a colorimetric endpoint assay utilizing diacetylmonoxime (Urea Nitrogen (BUN), Stanbio Labs, Boerne, TX) as previously described (Kang et al., 2013). Each experiment was performed in duplicate from the same rat liver-cell isolation. Error bars in the plotted graph using quantitative data represent the mean \pm standard error (\pm SEM). The urea concentration was normalized from the measured urea concentration ($\mu\text{g}/\mu\text{L}$) by 1 day of culture as $\mu\text{g}/\mu\text{L}/\text{day}$.

Adenovirus Infection of Primary Rat Hepatocytes

As a final method for analyzing retention of hepatocyte-specific function during the time course of our experiments, we analyzed HBV replication in our system. Recombinant adenoviruses encoding either hrGFP alone (AdGFP) or hrGFP with the HBV genome (AdGFP-HBV) have been described previously (Clippinger et al., 2009; Sodunke et al., 2008). HBV cannot directly infect rat hepatocytes, necessitating the use of a recombinant adenovirus containing a replication-competent copy of the HBV genome. The use of recombinant adenoviruses for similar types of studies in rodent hepatocytes has been previously described (Gearhart and Bouchard, 2010; Sprinzl et al., 2001). For infection, the primary rat hepatocytes were incubated at Day 1 after plating with either AdGFP or AdGFP-HBV for 16 h. After infection, the hepatocytes were washed with $1\times$ phosphate buffered saline (PBS) buffer and fresh primary rat hepatocyte medium was added to the microchannels. Microchannel platforms were connected to a perfusion system and incubated at 37°C in 5% CO_2 . Supernatants from infected cells were collected at the indicated time points and stored at -80°C for analysis by PCR.

Analysis of Secreted HBV DNA

The 1–2 mL supernatants collected from the microchannels were mixed with 30% (w/v) PEG 8000/1.5 M NaCl to reach a final

concentration of 8% (w/v) PEG 8000. Samples were incubated at 4°C for 16 h and then centrifuged at 15,000g for 15 min. The pellet was resuspended in 50 μL PBS and treated with 1.5 μL of DNase I (10 mg/mL) and 0.5 μL of 1 M MgCl_2 for 1 h at 37°C. Samples were then incubated for 1–2 h at 55°C after the following reagents were added: 5 μL of 0.5 M EDTA, 10 μL of 10% SDS, 0.2 μL of 2 M CaCl_2 , and 2.5 μL of proteinase K at 10 mg/mL. After incubation, samples were centrifuged at 15,000g for 15 min. 0.3 μL of 3 M sodium acetate (pH 4.8–5.2) and 75 μL of chilled 100% ethanol were added to samples to precipitate HBV DNA. Samples were kept at -20°C over night, followed by an additional 15 min centrifugation (15,000g). The pellet was washed with 500 μL of 70% ethanol and was air-dried. It was then resuspended in 20 μL of deionized water. The extracted DNA was used for PCR using HBV-specific primers (568 bp) and GFP-specific primers (154 bp) listed in Table S1 (Sodunke et al., 2008). A plasmid containing the HBV genome was used as a positive control for PCR analysis.

Results and Discussion

Layered Co-Culture of Primary Rat Hepatocytes and RAMECs in Single-Channel Configuration Under Static Condition

Prior to layered co-culture studies, primary rat hepatocytes were cultured without endothelial cells in microchannels under static conditions to analyze their viability in the microchannel environment. Primary rat hepatocytes cultured alone made a confluent monolayer in the microchannel by Day 2 after seeding (Fig. 3a). Primary rat hepatocytes maintained a normal phenotype (confluent polygonal shape) for about 4 days before the cells began to detach from the surface, resulting in large empty spaces that were observed by Day 7 (Fig. 3b). This observation is similar to previous observations with primary rat hepatocytes cultured on conventional 6-well culture plates (Kang et al., 2013).

Next, we co-cultured primary rat hepatocytes with RAMECs in the single channel configuration. When primary rat hepatocytes were layered with RAMECs, the primary rat hepatocytes retained their normal hepatocyte morphology at Day 7, unlike in the hepatocyte-only culture (Fig. 3c and d). These results also agree with the findings from others that primary hepatocytes show better viability and function when co-cultured with other cell types, such as fibroblasts (Bhandari et al., 2001; Bhatia et al., 1999; Guillouzo, 1998). Although we did not elucidate a specific mechanism for the increased hepatocyte viability in our system, the results of previous studies suggest that both cell–cell interactions and cell-secreted soluble factors seem to be crucial for maintaining hepatocyte viability and function (Bhandari et al., 2001; Guillouzo, 1998).

To further investigate the extended viability of primary rat hepatocytes co-cultured with RAMECs, we continued to observe the co-cultured cells for 30 days. The layered configuration (primary rat hepatocytes at the bottom and RAMECs at the top) was verified using phase-contrast microscopy. When the RAMECs were in focus, the hepatocytes were not and similarly, when the hepatocytes were in focus, the RAMEC cells were not (Fig. 4a and b). In order to verify the viability of primary rat hepatocytes layered beneath the RAMEC layer, we utilized a recombinant adenovirus that expresses GFP to

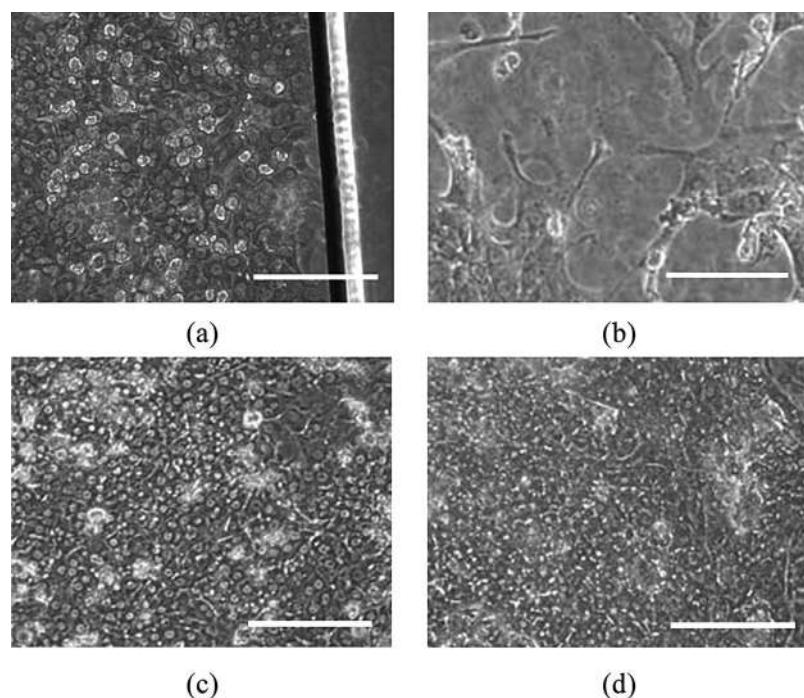


Figure 3. Morphology of PRHs in single channel configuration. (a) PRHs only in the microchannel at Day 2 and (b) Day 7; (c) PRHs only in the microchannel at Day 2 before co-culture with RAMECs; and (d) PRHs co-cultured with RAMECs at Day 7. Scale bar: 200 μm .

infect viable primary rat hepatocytes 30 days after seeding. Importantly, we have confirmed that endothelial cells cannot be infected with adenovirus, so GFP expression is specific to AdGFP-infected primary rat hepatocytes (Figs. 4c and 6b). As shown in Figure 4c, cells expressing GFP were clearly visible 24 h after infection with AdGFP, and 30 days after seeding of the primary rat hepatocytes and RAMECs in the microchannel. Together, these studies show that primary rat hepatocytes retained normal morphology and remained viable for at least 30 days when they were co-cultured with RAMECs in layers in microchannels under static condition even though the cells did not maintain a confluent monolayer at Day 30 over the entire surface of the microchannel. A significant drawback of this co-culture configuration was that over time the RAMECs settled into the empty spaces that formed between hepatocytes, possibly due to non-uniform coating of matrigel between the cell layers. These results suggest that this layered co-culture model does not allow for maintenance of long-term stable layering of hepatocytes and endothelial cells and will eventually resemble a random co-culture monolayer, limiting its use for more physiologically relevant studies.

An additional drawback of this single-channel layered primary rat hepatocytes and RAMECs configuration was that many cells accumulated at the inlet and outlet of the microchannel. We hypothesized that this might be due to rapid depletion of nutrients and growth factors present in the small volume of cell-culture medium within the microchannel. We increased the channel height from 80–160 μm (the height of the original microchannel) to 200–800 μm to increase the volume of cell-culture medium. We observed that the increased channel height of 800 μm allowed a

more homogenous cell layer and resulted in a more stable morphology of primary rat hepatocytes (Fig. S1). Therefore, the microchannel height of 800 μm was then applied to the dual-channel configuration.

Layered Co-Culture of Primary Rat Hepatocytes and BAECs in Dual-Channel Configuration Under Static Condition

Since primary RAMECs can only be passaged for a limited time and thus it is difficult to use them in the subsequent optimization of our microfluidic platform, which would require a large number of endothelial cells, we decided to use immortalized BAECs for the optimization studies. BAECs can be passaged indefinitely and their endothelial characteristics are well characterized (Schwartz, 1978). In order to distinguish endothelial cells from primary rat hepatocytes during co-culture in the microfluidic dual channel, GFP- and red fluorescent protein (RFP)-expressing BAECs cell lines were developed and used (Kang et al., 2013). Primary rat hepatocytes co-cultured with BAECs on the opposite sides of a microporous membrane within a dual-PDMS microchannel made a confluent monolayer. Later on, the hepatocyte cell layer was partially peeled off but remaining primary rat hepatocytes retained their morphology for up to 30 days (Fig. S2). Figure 5a shows primary rat hepatocytes morphology at Day 13 under bright-field microscopy, and Figure 5b shows BAECs expressing RFP at Day 13.

We also investigated urea synthesis as a measure of hepatocyte function. Urea synthesis from oxidation of amino acids or ammonia is a hepatocyte-specific function that occurs in vivo (Vilstrup,

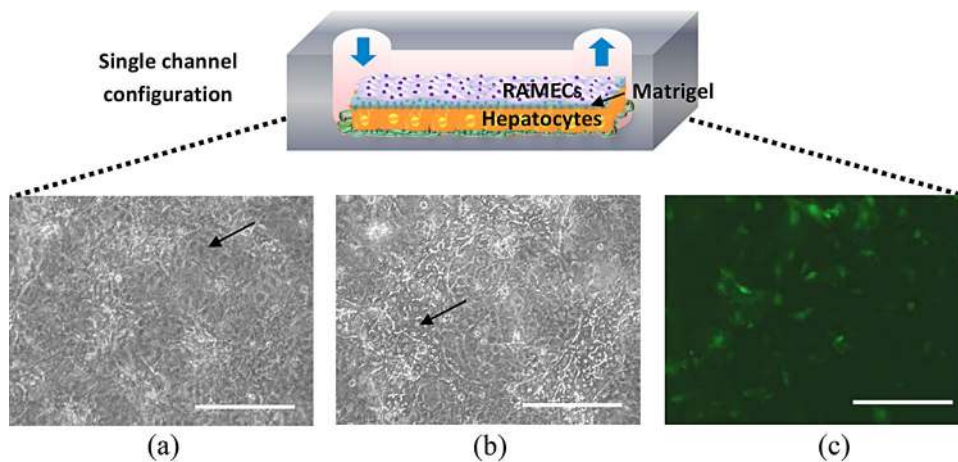


Figure 4. Phase-contrast microscopy of long-term, layered, static co-culture of PRHs and RAMECs in single channel configuration. PRHs co-cultured with RAMECs remained viable for 30 days as verified by GFP expression in cells infected with an adenovirus expressing GFP. (a) RAMECs in focus (arrow) at Day 30; (b) PRHs in focus (arrow) at Day 30; and (c) GFP expression in AdGFP-infected PRHs at Day 30. Scale bar: 200 μm .

1980). We compared primary rat hepatocytes cultured in the absence of BAECs and primary rat hepatocytes co-cultured with BAECs. The urea synthesis level of the primary rat hepatocytes cultured in the absence of endothelial cells rapidly decreased from a

high urea concentration ($>1.0 \mu\text{g}/\mu\text{L}/\text{day}$) in the channel to a negligible level within 1 week and remained at this low level until the end of culture. This correlates with the observation of extensive cell death that occurs in the primary rat hepatocytes-only culture system, as described in Layered Co-Culture of Primary Rat Hepatocytes and RAMECs in Single-Channel Configuration Under Static Condition section. In contrast, the urea synthesis level of the primary rat hepatocytes co-cultured with BAECs under static condition decreased much less during the first 7 days and remained at a stable level ($\sim 0.3\text{--}0.4 \mu\text{g}/\mu\text{L}/\text{day}$) in the channel until the end of culture period (Fig. 5c). The urea amount secreted at the stable urea level was estimated as 15–20 μg per 1 day considering the daily replacement of about 50 μL of medium. The large error in the urea concentration at the early time points may be caused by the large variation in the number of cells that may not have fully attached to the substrate after cell seeding. At later time points, once cell numbers have been stabilized, urea concentration remained at the low levels and showed only small variation.

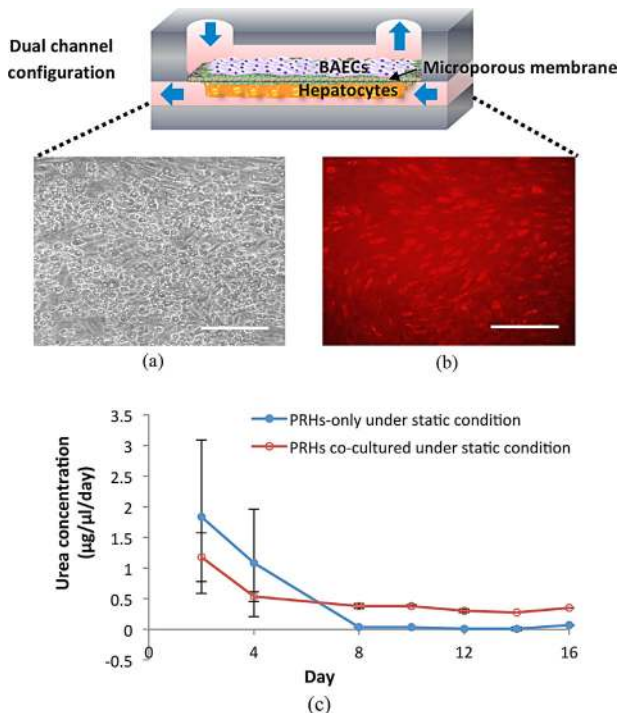


Figure 5. Phase-contrast microscopy and urea synthesis of co-culture of PRHs and BAECs in dual-channel configuration under static conditions. (a) PRHs co-cultured with BAECs at Day 13; and (b) RFP expression of BAECs at Day 13. Scale bar: 200 μm . (c) Urea synthesis in PRHs-only vs. PRHs co-cultured with BAECs under static condition. PRHs used in this experiment were from the same rat. All data was normalized and expressed as $\mu\text{g}/\mu\text{L}/\text{day}$.

An issue with this configuration under static conditions was that the PRH layer sometimes peeled off the microporous membrane. This problem was more noticeable after medium changes, implying that it was related to the sudden shear stress exerted on the cell layer by the flow that was induced during manual medium replacement. We surmised that this limitation could be overcome by applying a continuous perfusion of medium at a low flow rate for the long-term co-culture of primary rat hepatocytes and endothelial cells. Moreover, such a flow condition would better simulate the flow condition of the liver sinusoid and is described below.

Liver Sinusoid on a Chip: Layered Co-Culture of Primary Rat Hepatocytes and BAECs in Dual-Channel Configuration Under Flow Condition

In the next step toward developing the *liver sinusoid on a chip*, primary rat hepatocytes were co-cultured with BAECs in a dual-microchannel under a continuous perfusion, as shown in Figure 6a.

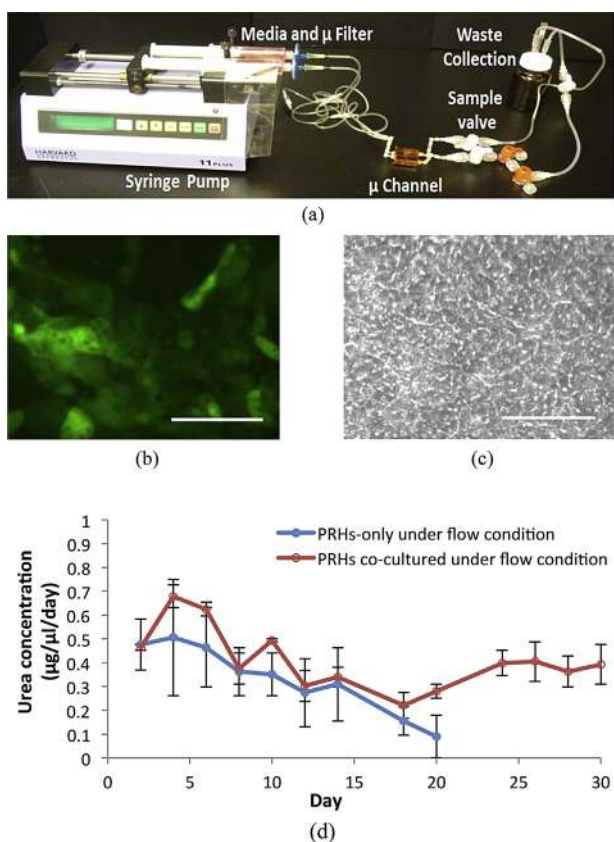


Figure 6. Long-term morphology and urea synthesis of PRHs co-cultured with BAECs in a dual microchannel platform under flow conditions. (a) Continuous perfusion system; (b) PRHs infected with ad-GFP at Day 2 expressed GFP in co-culture of PRHs and BAECs at Day 8; (c) PRHs co-cultured with BAECs at Day 21. Scale bar: 200 μm . (d) Urea synthesis in PRHs-only versus PRHs co-cultured with BAECs under flow condition. PRHs used in this experiment were from the same rat. All data were normalized and expressed as $\mu\text{g}/\mu\text{L}/\text{day}$.

First, we generated a layered co-culture of primary rat hepatocytes and BAECs in a dual-channel configuration as described above. The dual-channel was then connected to a syringe pump for continuous perfusion of cell-growth medium. We tested a range of flow rates (0–100 $\mu\text{L}/\text{h}$) to identify the optimal flow rate. If the flow rate is too high, the cell layer can be peeled and if the flow rate is too low, dead cells cannot be washed out of the channel. A flow rate of 30–40 $\mu\text{L}/\text{h}$ was selected based on our experimental observation and applied to each channel beginning from Day 1. In order to check the viability of primary rat hepatocytes, the cells were infected with AdGFP at Day 1. The AdGFP infected primary rat hepatocytes maintained GFP expression at Day 8 (Fig. 6b). Primary rat hepatocytes co-cultured with BAECs under flow condition maintained a confluent mono layer for over 21 days (Fig. 6c). Peeling of cells from the monolayer or deformation of cell morphology was less evident under flow conditions than with primary rat hepatocytes cultured under static conditions.

We estimated the shear stress applied to the primary rat hepatocytes in both static and flow conditions in the dual-channel configuration. With a steady-state, laminar flow profile, the

maximum shear stress (τ) at the bottom of the channel where the cells are located is expressed as:

$$\tau = \frac{6\mu Q}{h^2 w}$$

where μ is the flow viscosity ($\text{kg}/\text{m} \cdot \text{s}$), Q is the volumetric flow rate (m^3/s), h is the channel height (m), and w is the channel width (m) (Kim et al., 2007b). For the flow rate of 30–40 $\mu\text{L}/\text{h}$ and a channel height of 600–800 μm , the range of the shear stress is determined to be between 7.8×10^{-4} and 1.9×10^{-3} dyne/cm^2 . On the other hand, the shear stress exerted on hepatocytes during medium replacement in static condition can be as high as 0.9–2.8 dyne/cm^2 since the flow rate during media replacement by pipette is estimated to be very high (10.0–16.7 $\mu\text{L}/\text{s}$). Therefore, the shear stress exerted on primary rat hepatocytes under flow condition is about 1,000–2,000 times less than the shear stress exerted on primary rat hepatocytes under static conditions during media replacement. Since the impact of shear stress on cell morphology and viability can be minimized under flow conditions, it is likely to contribute to retaining cell phenotypes for a longer time. It must also be noted that while the physiological range of the shear stress in the liver sinusoid in vivo (<2 dyne/cm^2) (Domansky et al., 2010; Kim et al., 2007b) may be higher than what is seen in our flow system, the actual shear stress that hepatocytes experience in the liver sinusoid is much less than this value due to the presence of the endothelial cell layer that prevents hepatocytes from being directly exposed to the fluid flow.

As seen in Figure 6d, we compared primary rat hepatocytes cultured in the absence of BAECs and primary rat hepatocytes co-cultured with BAECs to investigate urea synthesis of primary hepatocytes cultured in a dual channel under flow condition as a function of time. Urea synthesis in the hepatocyte-only culture under flow condition gradually decreased over 20 days, in contrast to the rapid decline that occurred over 7 days in primary rat hepatocyte-only culture in the static condition (Fig. 5c). By 22 days of culture, few primary rat hepatocytes remained in the dual-channel, flow-exposed, primary rat hepatocyte-only systems, and we stopped our analyses of hepatocyte-specific markers and function. On the other hand, urea synthesis in primary rat hepatocytes co-cultured with BAECs under flow condition showed a slight increase initially and then decreased to a stable level (~ 0.3 $\mu\text{g}/\mu\text{L}/\text{day}$) in the channel that was maintained for at least 30 days (Fig. 6d). The urea amount secreted at the stable urea level was estimated as 216–288 μg per 1 day under flow rate 30–40 $\mu\text{L}/\text{h}$. The decrease in the urea concentration over the time course of the experiment is caused not by hepatocyte differentiation and loss of function, but is likely due to the total level of urea in the sample decreasing because of an increase over time in the number of dead cells, and therefore, a decrease in cells producing urea. We therefore believe that levels of urea detected in our various monoculture and co-culture platforms is mostly related to the number of surviving, differentiated hepatocytes in the various configurations over the time course of our studies. The urea secretion per day from the co-culture system exposed to flow demonstrates that primary rat hepatocytes maintained under flow condition showed higher urea secretion in comparison to static

cultures, as reported in previous studies (Hegde et al., 2014; Park et al., 2005). In addition, prolonged urea secretion supports the conclusion that our culture model maintains hepatocyte function for at least 30 days.

In order to verify that the primary rat hepatocytes co-cultured in the *liver sinusoid on a chip* continued to express specific markers of differentiated hepatocytes throughout the time course of our studies, RT-PCR was performed on total RNA obtained from primary rat hepatocytes co-cultured with BAECs in the dual-channel configuration and exposed to flow. The mRNA expression of hepatocyte-specific differentiation markers such as ALB, TNF, and HNF-4 α was determined by RT-PCR (Costa et al., 2003). Total RNA was isolated from freshly isolated primary rat hepatocytes, as well as from primary rat hepatocytes co-cultured with BAECs exposed to flow for 15 and 21 days. For each study, all hepatocytes that were analyzed were isolated from the same rat. The expression of ALB (105 bp), TNF (121 bp), HNF-4 α (138 bp), and β -actin (133 bp) mRNA was apparent in freshly isolated primary rat hepatocytes and in primary rat hepatocytes that were co-cultured with BAECs for 15 and 21 days in the dual-channel, flow-exposed devices (Fig. 7). The expression level of the three hepatocyte-specific genes was quantified from a gel image and the intensity ratio of each band to the band for β -actin was evaluated. The expression level of the three hepatic-specific genes of primary rat hepatocytes at Day 15 and Day 21 was not changed significantly compared to freshly isolated primary rat hepatocytes. This indicates that primary rat hepatocytes co-cultured with BAECs in the dual-

channel configuration under flow condition retained their differentiation status for at least 21 days.

Functional Analysis of Liver Sinusoid on a Chip Through Support of HBV Replication

To demonstrate the utility of our liver model, we analyzed its use in studying replication of HBV. Because HBV cannot directly infect rat hepatocytes, we used a recombinant adenovirus containing a replication competent copy of the HBV genome (AdGFP-HBV) for these studies. Use of AdGFP-HBV to introduce the HBV genome into non-human cells is an accepted method for studying HBV biology (Gearhart and Bouchard, 2010; Sprinzl et al., 2001). Importantly, HBV is hepatotropic and expression of HBV RNA transcripts is dependent on expression of hepatocyte specific factors. Hence, our ability to detect HBV replication in our microfluidic platform serves as an additional confirmation that the differentiation and functional status of hepatocytes were retained throughout the time course of our studies.

Primary rat hepatocytes were infected with AdGFP or AdGFP-HBV at Day 1 after plating and expressed GFP by day 3 (Fig. 8a and b). Adenovirus infection efficiency resulted in approximately 30–70% of primary rat hepatocyte being infected in our experiments as verified by fluorescent microscopy image of GFP-expressed cells. The GFP expression from the infected cells was maintained for several days and then disappeared gradually (Fig. 8c and d). In order to detect the secretion of replicated HBV

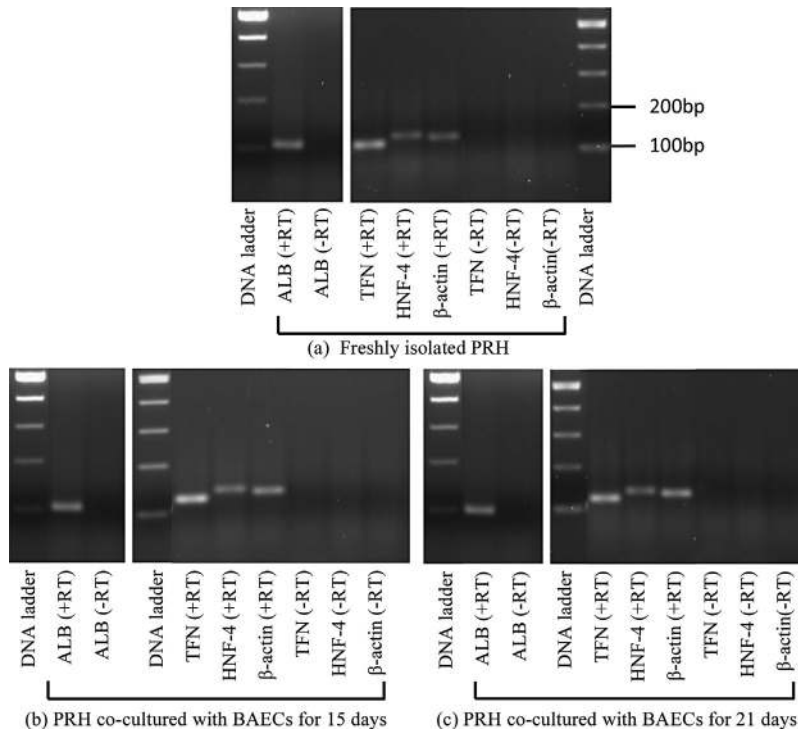


Figure 7. Differentiation of PRHs co-cultured with BAECs in dual channel configuration under flow condition. RT-PCR was performed after total RNA isolation from freshly isolated PRHs (a), PRHs co-cultured for 15 days (b) and 21 days (c) to check mRNA expression of the liver-specific differentiation markers; ALB (105 bp), TNF (121 bp), HNF-4 (138 bp), and β -actin (133 bp). Freshly isolated PRHs and PRHs in co-culture were isolated from the same rat.

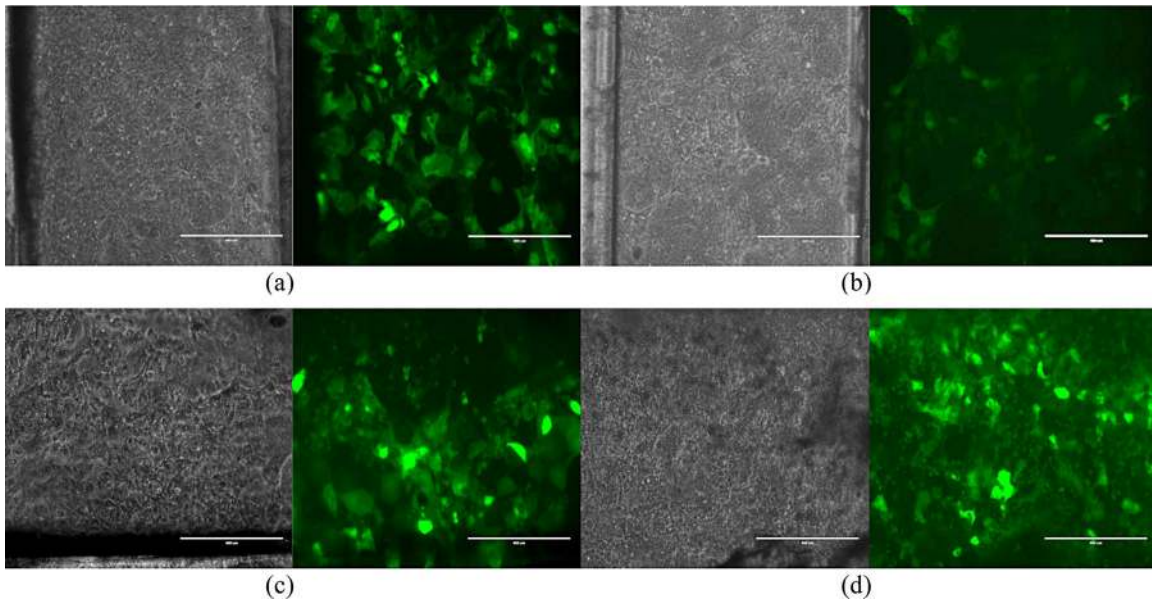


Figure 8. Image of PRHs infected with AdGFP or AdGFP-HBV using the liver sinusoid on a chip. **(a)** PRHs co-cultured with BAECs were infected with AdGFP at Day 1 after seeding. Bright field (left) and GFP image (right) at Day 3. **(b)** PRHs co-cultured with BAECs were infected with AdGFP-HBV at Day 1 after seeding. Bright field (left) and GFP image (right) at Day 3. **(c)** Bright field (left) and GFP image (right) at Day 11 of **(a)**. **(d)** Bright field (left) and GFP image (right) at Day 11 of **(b)**. Scale bar: 400 μm .

into the cell culture media, we used specific primers for regions on the HBV genome. HBV was detected in samples of primary rat hepatocytes infected with AdGFP-HBV (Fig. 9, lanes 1 and 2) and was identical in size to the band from the positive control (lane 4) in Figure 9. These results indicate that primary rat hepatocytes infected with AdGFP-HBV secreted HBV into the culture media. To check contamination of the culture medium samples with recombinant adenovirus used for infection of primary rat hepatocytes, PCR using GFP primers for samples from primary rat hepatocytes infected with AdGFP-HBV was performed. A GFP-specific PCR product from samples of primary rat hepatocytes infected with AdGFP-HBV was not detected (lanes 7 and 8). A positive control for a GFP-specific PCR product was detected when

direct PCR was performed on DNA isolated from AdGFP-HBV (lane 10). Finally, we were unable to detect a HBV-specific PCR product from samples of primary rat hepatocytes infected with AdGFP, confirming that our HBV-specific PCR product was only detected in cells infected with AdGFP-HBV (lanes 3 and 9).

Overall these results verified that primary rat hepatocytes infected with AdGFP-HBV secreted the HBV into culture media in the microchannel platform, indicating that hepatocytes retained their hepatocyte specific phenotype throughout the time course of our studies and could be used for analysis of replication of a hepatotropic virus. These studies also strongly suggest that our *liver sinusoid on a chip* might also be amenable for studies involving HBV infection of primary human hepatocytes that are cultured in our

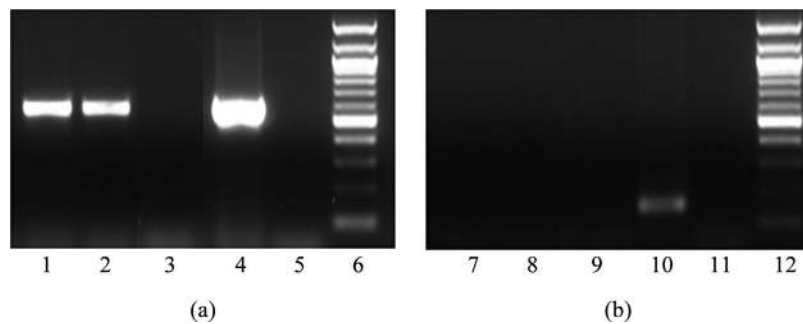


Figure 9. PCR from supernatants of PRHs co-cultured with BAECs on the liver sinusoid on a chip under perfusion system. **(a)** PCR using HBV primers. **(b)** PCR using GFP primers. Lanes 1, 2, 7, and 8: supernatants from PRHs infected with AdGFP-HBV (Day 8), 3 and 9: supernatants from PRHs infected with AdGFP (Day 8), 4 and 10: AdGFP-HBV virus, 5 and 11: water, and 6 and 12: DNA ladder.

microfluidic device and also applied to diverse liver biology studies and liver-related disease research such as toxicology, other hepatotropic infections agents, and drug screening.

Comparison of Different Configurations

Previously, we presented long-term co-culture of primary rat hepatocytes with endothelial cells using commercial transwell devices (Kang et al., 2013). Because of the size of the culture chamber (24 mm diameter), a large amount of cells ($4\text{--}6 \times 10^5$ cells) and culture medium are required in this model. Moreover, the transwell did not support continuous perfusion of medium. In contrast, the microfluidic platforms presented in this paper require much smaller amounts of cells (1×10^4 cells) and culture medium and facilitate continuous perfusion. We investigated both single- and dual-channel configurations, and our results show that the layered co-culture in the dual-channel configuration supports long-term culture of primary rat hepatocytes, and is better than the layered co-culture in the single-channel configuration.

The single-channel configuration has only one microchannel with one inlet and one outlet for supplying culture medium to both primary rat hepatocytes and endothelial cells (Fig. 1). Mass transfer by hepatocytes, such as nutrient and growth factor uptake and oxygen exchange, was limited in this system because hepatocytes were covered with a matrigel layer and an endothelial cell layer. Moreover, factors secreted from hepatocytes may stagnate around the cell layers and negatively affect the cultured hepatocytes (Kim et al., 2007a; Zarrinpar and Loomba, 2012). In contrast, the dual-channel configuration with two inlets and two outlets can support much better mass transfer for hepatocytes, especially when the two different cell types are cultured on the opposite side of the membrane (Fig. 1). Thus, the co-culture system in the dual-channel configuration supports better cell–medium interactions than the co-culture system in the single-channel configuration.

Strictly speaking, the current dual-channel configuration does not perfectly mimic the structure of the liver sinusoid since primary rat hepatocytes and endothelial cells are separated by a relatively thick porous membrane unlike the actual liver sinusoid in which the cells are separated by a very thin, protein-rich matrix, the *Space of Disse*. In addition, our co-culture model uses a non-liver endothelial cell line instead of primary liver sinusoidal endothelial cells, which would be the most physiologically relevant in vitro liver model, and does not include non-parenchymal cells such as Stellate cells and Kupffer cells. These limitations result in an incomplete understanding of the cell–cell interactions in the in vivo system. Including these primary non-parenchymal liver cells in our liver on a chip remains future work, which will move us closer to a model that fully mimics the in vivo liver environment. In terms of recreating *Space of Disse*, we attempted to mimic this structure using a thin matrigel layer but the endothelial cells eventually invaded into the hepatocyte layer, disrupting the layered structure. This result led us to come up with the incorporation of a microporous membrane to physically separate the two cell layers. Although the interaction between hepatocytes and endothelial cells might be limited in this configuration due to the presence of the microporous membrane, the direct exposure of hepatocytes to the continuous perfusion of medium seemed to have a positive effect in the long-term culture. In

this configuration with continuous flow, the hepatocytes are exposed to a consistent level of nutrients, growth factors, and a low level of secreted factors from hepatocytes. The peeling-off of the cell layer due to shear stress is also minimized, and as a result, hepatocyte morphology and function remained for a longer time than in the other systems we tested. In addition, this culture system has less possibility of contamination because it does not require daily medium replacement. Finally, the dual-channel, continuous flow configuration also allows continuous collection of medium or isolation of cells from each channel for the analysis of cell function without contamination from the other cell type.

Among all configurations and conditions tested, the layered co-culture of primary rat hepatocytes and endothelial cells in a dual-channel configuration under flow condition seems to be the best long-term liver model and more closely mimics the structure and microenvironment of the liver sinusoid than the other configurations that we have tested. The cell culture results obtained from the model were highly reproducible. It must also be noted that the current *liver sinusoid on a chip* is clearly distinct from other existing microfluidic in vitro liver models (Cho et al., 2010; Khetani and Bhatia, 2008; Kim et al., 2010) and supports long-term maintenance of hepatocyte functions.

Conclusions

We presented a novel liver model that mimics the architecture and microenvironment of a liver sinusoid, where most liver activities occur. Our *liver sinusoid on a chip* support long-term co-culture of primary hepatocytes and endothelial cells in a layered configuration with or without continuous perfusion of culture medium. This paper describes a progressional study of creating such a *liver sinusoid on a chip* system. Each step in the progressional study was designed to help to optimize cell survival and maintenance of hepatocyte functions while simultaneously achieving a configuration that closely mimics the structure and microenvironment of a liver sinusoid unit. First, we investigated a single-channel configuration in which a layered co-culture of primary rat hepatocytes and endothelial cells was created with a thin matrigel layer added between the two cell layers. Next, a dual-channel configuration was investigated in which two microchannels simulate the blood sinusoid and a lower channel for the removal of the secreted factors from hepatocytes. When primary rat hepatocytes and BAECs were co-cultured on the opposite sides of a microporous membrane in a dual microchannel platform, along with the addition of continuous perfusion, the hepatocytes maintained their normal morphology and hepatocyte-like functions for at least 30 days. In addition, studies of secreted HBV were used to show the effective application of our in vitro *liver sinusoid on a chip* to studies of hepatotropic viruses. We believe that our *liver sinusoid on a chip* closely mimics the in vivo liver sinusoid and supports long-term primary liver cell culture within a microfluidic liver model. This novel liver model could be applied to the study of liver diseases and liver toxicology.

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