12-2013

Layered Long Term Co-Culture of Hepatocytes and Endothelial Cells on a Transwell Membrane: Toward Engineering the Liver Sinusoid

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

Siddhartha Rawat
Drexel University

Joseph Cirillo
Drexel University

Michael Bouchard
Drexel University

Hongseok (Moses) Noh
Drexel University

Published in final edited form as:
Biofabrication. 2013 December ; 5(4): 045008. doi:10.1088/1758-5082/5/4/045008.

Layered Long Term Co-Culture of Hepatocytes and Endothelial Cells on a Transwell Membrane: *Toward Engineering the Liver Sinusoid*

Young Bok (Abraham) Kang[1], **Siddhartha Rawat**[2], **Joseph Cirillo**[1], **Michael Bouchard**[3,*], and **Hongseok (Moses) Noh**[1,*]

[1]Mechanical Engineering and Mechanics, Drexel University, Philadelphia, PA, USA

[2]Graduate Program in Molecular and Cellular Biology and Genetics, Drexel University College of Medicne, Philadelphia, PA, USA

[3]Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA, USA

Abstract

This paper presents a novel liver model that mimics the liver sinusoid where most liver activities occur. A key aspect of our current liver model is a layered co-culture of primary rat hepatocytes (PRHs) and primary rat liver sinusoidal endothelial cells (LSECs) or bovine aortic endothelial cells (BAECs) on a transwell membrane. When a layered co-culture was attempted with a thin matrigel layer placed between hepatocytes and endothelial cells to mimic the Space of Disse, the cells did not form completely separated monolayers. However, when hepatocytes and endothelial cells were cultured on the opposite sides of a transwell membrane, PRHs co-cultured with LSECs or BAECs maintained their viability and normal morphology for 39 and 57 days, respectively. We assessed the presence of hepatocyte-specific differentiation markers to verify that PRHs remained differentiated in the long-term co-culture and analyzed hepatocyte function by monitoring urea synthesis. We also noted that the expression of cytochrome P-450 remained similar in the co-cultured system from Day 13 to Day 48. Thus, our novel liver model system demonstrated that primary hepatocytes can be cultured for extended times and retain their hepatocyte-specific functions when layered with endothelial cells.

Keywords

Liver model; Liver co-culture; Primary Rat Hepatocytes; Liver sinusoidal endothelial cell; Transwell

1. Introduction

The liver is the largest internal organ of the human body and plays an important role in drug metabolism, responses to various toxins, and regulation of numerous metabolite levels in the body [1-3]. Important liver functions include uptake of nutrients and compounds, conversion of drugs or toxins to other biochemical compounds that can inactivate or even make these more toxic, regulation of circulating glucose levels, generation of glycogen, and modulation of circulating lipid levels and storage. A complete understanding of the molecular mechanisms that underlie liver-specific functions, especially functions of the major cell type

*Corresponding authors: michael.bouchard@drexelmed.edu and mosesnoh@coe.drexel.edu.

of the liver, hepatocytes, is very difficult to study *in vivo* due to the complexity of hepatocyte signal transduction pathways, the multiple influences of other liver cells, and the consideration of signals derived from substances release from other organs in the body. In addition, although studies in the livers of non-human primates and non-primates such as rodents have served as models for understanding liver functions, in particular, the response of the liver to various drugs, observations in these model systems are not always applicable to responses in human livers [4]. Drug-induced liver toxicity is a major concern during the development of novel drugs and can result in enormous economic losses when drug toxicity is only realized once a drug is administered to human.

In order to overcome the limitations of *in vivo* animal liver studies, it is important to develop accurate and experimentally tractable *in vitro* liver model systems that facilitate long-term viability of cells and maintenance of liver-specific functions. One of the ways to achieve this would be to mimic the *in vivo* liver sinusoid architecture, the most basic functional unit of the liver (Fig. 1). The liver sinusoid is a capillary lined by Liver Sinusoidal Endothelial Cells (LSECs). Stellate cells that help to maintain the extracellular matrix and Kupffer cells, which are liver macrophages, are also present. There is a small space called the Space of Disse that separates LSECs from hepatocytes. Bile canaliculi are small channels that form between adjacent hepatocytes. Hepatocytes secrete bile that is collected in bile ducts and transported to the intestines or stored in the gall bladder [3,5-7].

Many studies have been conducted to develop an authentic liver model. *In vitro* liver models and bio-artificial livers have been developed for studying liver biology, liver cancer, liver toxicity and drug metabolism as they offer comparatively simpler and more tractable model systems and reduce the cost of conducting such studies in animal model systems. For example, one report showed improved long-term culture of primary rat hepatocytes that were entrapped in Arg-Gly-Asp (RGD)-incorporated hydrogel; the hydrogel was used as a synthetic extracellular matrix of hepatocytes [8]. However, this hepatocyte single culture does not accurately mimic *in vivo* liver sinusoid architecture that consists of hepatocytes (parenchymal cells) and the non-parenchymal cells such as LSECs, Kupffer cells, and hepatic stellate cells. The interaction between the parenchymal and non-parenchymal cells of the liver plays an important role in maintaining hepatocyte function [9-13].

Recently, liver organotypic co-culture systems were developed using synthetic and biodegradable membranes to culture primary human hepatocytes and human umbilical vein endothelial cells [14]. In another study a layered three-dimensional co-culture of primary rat hepatocytes and human LSECs with an intermediate chitosan-hyaluronic acid polyelectrolyte multilayer (PEM) was developed on 6-well tissue culture plates. The chitosan-hyaluronic acid polyelectrolyte multilayer (PEM) was introduced in order to mimic the Space of Disse [13]. A layered tri-culture model of the hepatocyte, hepatic stellate cells and sinusoidal endothelial cells using different size microporous membranes was used to investigate the cell-to-cell communications [15]. However, these reports did not show the long-term co-culture of primary hepatocytes; hepatocytes typically lose hepatocyte-specific functions and de-differentiate shortly after they are isolated from the liver [1,16].

Although some of the previous studies have shown the feasibility of long-term co-culture of hepatocytes with either endothelial cells (ECs) [17] or stellate cells [18], and were able to demonstrate that hepatocytes maintained their functions these systems, none of these long-term hepatocyte co-cultures accurately mimiced the *in vivo* architecture of a liver sinusoid and did not account for bile secretion into bile canaliculi. In this paper, we report a novel *in vitro* liver model system using commercial standard 6-well and 6-transwell tissue culture plates under static condition. Our liver model system mimics the layered organization of the liver sinusoid, and hepatocytes are able to maintain their functions and differentiation status

for at least 57 days. Three different configurations were investigated (Fig. 2) [19]. The first configuration is the simplest design where hepatocytes and ECs are co-cultured in a 6-well plate; a thin layer of matrigel was placed between hepatocytes and ECs to mimic the Space of Disse (Fig. 2a). In the second configuration, the cells were co-cultured similar to the first configuration on a 6-transwell membrane (24 mm diameter, 0.4 μm pore size) (Fig. 2b). This configuration was designed so that the bile can be removed at the bottom of the 6-transwell. It was difficult to achieve completely visualize separated LSECs and PRHs in configurations 1 and 2. Therefore, a third configuration was investigated in which LSECs and PRHs were cultured on the opposite sides of a 6-transwell membrane (24 mm diameter, 0.4 μm pore size) (Fig. 2c). In this configuration, a microporous transwell membrane was used to mimic the Space of Disse. Configuration 3 of our liver model facilitated the layered long-term co-culture of PRHs and endothelial cells on the 6-transwell membrane. We believe that our liver model presented in this paper can serve as an accurate, long-term liver model for fundamental liver biology studies, liver disease research, toxicology studies and drug screening applications.

2. Materials and Methods

2.1. Primary liver cell isolation and culture

Primary rat hepatocytes (PRHs) were isolated from 6-12 weeks old Sprague-Dawley rats as previously described [20]. Animal surgery and hepatocyte isolation complied with all relevant federal and institutional policies. Approximately 4×10^5 cells that were 70–100% viable were seeded on 0.198 mg/ml collagen-coated 6-well or 6-transwell plates. The 6-transwell had a microporous PET membrane of approximately 24 mm diameter, 0.4 μm pore size and 10 μm thickness (Dow Corning). Dulbecco's Modified Eagle Medium (Cat.No. 10-017-CV, Cellgro®) supplemented with 1mM Na Pyruvate (Cellgro®), 4 μg/ml Insulin-Transferrin-Selenium (ITS, Gibco), 5 μg/ml Hydro cortisone (HC, Sigma), 5 ng/ml Epidermal growth factor (EGF, BD Sciences), 1% penicillin-streptomycin, 10% (vol/vol) Fetal Bovine Serum (FBS, Gemini Bioproduct) was used for culturing primary hepatocytes. On the first day after plating hepatocytes, the medium was changed after 2-6 hours. Thereafter the medium was changed every 24 hours.

Primary liver sinusoidal endothelial cells (LSECs) were isolated from the suspension of liver cells derived from the liver perfusion and hepatocyte isolation after removal of the hepatocytes by differential centrifugation, as previously described [21]. Briefly, the supernatant that remained after removal of hepatocytes was centrifuged at 350 g for 10 minutes to pellet the non-parenchymal cells. The pellet was then resuspended in DMEM and added to a 25/50% percoll density gradient (Table 1), followed by centrifugation at 900 g for 23 min. The LSECs accumulated at the interface of the 25% and 50% percoll density gradient and were collected. The cells isolated from the first percoll density gradient were then added to an identical percoll density gradient and the centrifugation was repeated one more time. The purified LSECs at the interface of this 25% and 50% percoll density gradient were resuspended in DMEM followed by centrifugation at 350 g for 10 minutes to remove any remaining percoll solution. The pellet of purified LSECs was finally resuspended in PRH medium and the LSECs were plated. On the first day the medium was changed 2-6 hours after plating; thereafter the medium was changed every 24 hours.

2.2. Bovine aortic endothelial cell culture

The Bovine Aortic Endothelial Cells (BAECs) are an established, immortalized, endothelial cell line that was a kind gift from Dr. Robert Levy (Children's Hospital of Philadelphia). BAECs were cultured in DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin (Cellgro®). Cells were maintained at 37°C in 5% CO_2, and medium

was replaced every 2–3 days. Our ultimate goal was to co-culture primary hepatocytes and liver sinusoidal endothelial cells isolated from the same rat. This required sacrificing two rats with a few days' interval. In order to test our co-culture idea without having to sacrifice many rats we sought to use an established endothelial cell line and chose BAECs because it was readily available to us.

2.3. Medium selection test for co-culture

The medium selection test for co-culture of PRHs and ECs was done so that both the cell types can be cultured in the same medium conditions. PRHs were cultured on 12-well tissue culture plates (22 mm diameter) in a medium that was made by combining PRH medium, LSEC medium and BAEC medium (Table 2) in different ratios; 1:1 mix of PRH and LSEC media, 1:1 mix of PRH and BAEC media, 1:1 mix of LSEC and BAEC media, and 1:1:1 mix of PRH, LSEC and BAEC media. Similarly, the same medium conditions were tested for the culture of BAECs. Based on the observed retention of cell morphology and the longevity of cell survival (data not shown), a modified PRH medium in Table 2 was used for co-culture of PRHs, and LSECs or BAECs.

2.4. Re-plating of primary liver cells

PRHs and LSECs were isolated and purified on the same day; after plating, these cells typically require 24-48 hours to form confluent monolayers. Because we wanted to plate the second cell-type only when the first cell-type has formed a confluent monolayer in configuration 1 and 2, we could not plate both the cell types on the same day. Moreover primary cells can be difficult to re-plate; there is significant cell death during re-plating resulting in low confluence of re-plated cells. To circumvent this issue, we plated one cell type in 6-well tissue culture plate or 6-transwell on day 1, and other cell type was plated on 10 cm tissue culture plate. After the first cell-type formed a confluent monolayer in the 6-well tissue culture plate or 6-transwell, we re-plated the second cell type from the 10 cm plate to the 6-well tissue culture plate or 6-transwell. Since we re-plated the cells from a much larger plate to a smaller plate, we were able to salvage enough cells to form a confluent monolayer. Re-plating of PRHs worked better than re-plating of LSECs (our unpublished observations).

2.5. Layered co-culture of PRHs and endothelial cells

To create a layered co-culture of PRHs and LSECs/BAECs in configurations 1 and 2, PRHs were first plated in collagen-coated 6-well tissue culture plates or 6-transwell tissue culture plates and allowed to adhere. 30% (v/v) growth factor reduced matrigel (BD Biosciences), which was mixed with PRH culture medium, was layered on top of PRHs. 2 hours after matrigel coating, endothelial cells were plated on top of the matrigel to create a co-culture of PRHs and endothelial cells that was separated by matrigel. Cells were incubated at 37°C in 5% CO_2.

For a layered co-culture of PRHs with LSECs or BAECs in configuration 3, both sides of a 6-transwell membrane were first coated with rat-tail collagen. The microporous membrane of a second 6-transwell was then removed by cutting it out. The two 6-transwells were then attached together using poly-parafilm (ThermoFisher) so that both of the 6-transwells shared a common membrane (Fig. 3). We first cultured PRHs on the bottom side of the first 6-transwell membrane for a minimum of 3 hours to allow the cells to attach to the membrane. After attachment of PRHs, the second 6-transwell was removed, and the first 6-transwell was put in a 6-well plate that contained a 1 mm thick supporting ring to increase the gap between the first 6-transwell and 6-well plate. The PRH-plated side of the membrane now faced down, and the increased gap provided by the support ring reduced the possibility that the PRH layer would be damaged by the 6-transwell membrane and 6-well plate.

Endothelial cells were then plated on the topside of the 6-transwell membrane to generate the layered co-culture of PRHs and endothelial cells separated by the transwell membrane. Alternatively, we plated the endothelial cells first and the PRHs second in configuration 3 using the same procedure. To visualize BAECs in the layered co-culture, BAECs were infected with a GFP-expressing lentivirus followed by puromycin selection to generate a BAEC cell line that stably expressed GFP as previously described [30].

2.6. RNA isolation, reverse transcription and PCR analysis

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect hepatocyte-specific mRNA transcripts in cultured PRHs [20]. The long-term co-cultured PRHs were collected from the plate with a plastic scraper. Total RNA was isolated from PRHs using TRIzol reagent (Invitrogen, Life Technologies, USA) according to the manufacturer's directions. Total RNA was then treated with DNase (RQ1 RNase-Free DNase, Promega, USA) and incubated at 65°C for 30 min to remove any DNA contamination. The reverse transcription (RT) of total RNA was done with M-MuLV reverse transcriptase (New England BioLabs Inc.) according to the manufacturer's instructions. The cDNA generated by RT was then amplified by PCR using specific primers [20]. Hepatocyte nuclear factor 4 (HNF-4), transferrin (TFN), and albumin (ALB) were used as hepatocyte specific markers; the primers that were used are listed in Table 3.

PCR was performed in a programmable thermal cycler (Eppendorf) with the following amplification profile: 30 sec at 94°C followed by 30 cycles at 94°C for 30 sec, 61°C for 45 sec and 72°C for 1 min. The samples amplified by PCR were visualized on a 1.0% agarose gel containing 0.001 mg/ml ethidium bromide.

2.7. Urea synthesis assay

Hepatocyte functions were evaluated by monitoring urea synthesis [22-23]. Medium samples from the layered co-cultures were collected every two days and stored at -80°C until assayed. Urea concentration in the media was determined by a colorimetric endpoint assay utilizing diacetylmonoxime (Urea Nitrogen (BUN), Stanbio Labs), according to the manufacturer's instructions. This reaction involves acid catalyzed condensation of urea with diacetylmonoxime in the presence of thiosemicarbazide to give a red-purple color that can be measured by spectrophotometer at 520 nm. The urea concentration was normalized for expression as μg/ml/day.

2.8. Western blot analysis for CYP2E1 expression

Total protein from co-cultured hepatocytes was isolated from the interphase of the phenol-chloroform layer left after the RNA isolation using TRIzol reagent (Invitrogen, Life Technologies, USA) according to the manufacturer's directions. When proteins were not collected after RNA isolation; cells were scraped in Phosphate Buffered Saline (PBS) and were lysed in 0.8% Sodium Dodecyl Sulfate (SDS) buffer (0.8% SDS, 240mM Tris pH 6.8, 10% glycerol). Protein samples were loaded into a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and run at 100 V. The proteins were then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Munich, Germany) at 100 V for 1 hr. The membrane was blocked with 5% nonfat milk in TBST (w/v), and was incubated overnight at 4°C with a rabbit anti-CYP2E1 polyclonal antibody (1:1000 dilution ABCam). The membrane was then washed three times with TBST and incubated with IRDye® 800 CW conjugated, goat anti-rabbit (1:5000, Li-COR) secondary antibody at room temperature for 1 hr. The protein CYP2E1 was detected by Odyssey Infrared Imaging System. For GAPDH detection, membranes were stripped with 0.2 N NaOH, blocked with 5% milk, and then incubated with a GAPDH (14C10) rabbit monoclonal antibody (1:5000, Cell signaling technology®) at room temperature for 2 hrs. The membrane was then washed three times

with TBST and was incubated with IRDye® 800 CW conjugated goat anti-rabbit (1:5000, Li-COR) secondary antibody for 1 hr. GAPDH levels were assessed with the Odyssey Infrared Imaging Systems.

3. Results and Discussion

3.1. PRHs or endothelial cells have low survival when cultured in the absence of other liver cells

PRHs that were plated on 6-well tissue culture plates and 6-transwells tissue culture plates formed confluent monolayers within three days of plating. The adhesion, morphology, viability and differentiation of hepatocytes on 6-transwell plates with a microporous membrane were similar to those of hepatocytes on 6-well tissue culture plate (Fig. 4a, 4b). This result showed that the polyester membrane substrate with 0.4 μm pores in 6-transwell was suitable for hepatocyte adhesion and survival. However, PRHs plated on 6-transwell did not maintain their viability for more than 11 days and; similar results were observed with PRHs that were plated on 6-well tissue culture plates (Fig. 4c and 4d). These data are consistent with previous published reports that show that hepatocytes do not survive more than 7 days when cultured in the absence of other cells [1,4,16,28-29]. LSECs were also plated in 6-well tissue culture plates (or tissue culture plates with 54 mm diameter) and 6-transwell plates; LSECs formed a monolayer on both plates but did not survive beyond day 7 (Fig. 4e, 4f, 4g, and 4h).

BAEC, an immortalized endothelial cell line, formed a confluent monolayer on 6-well tissue culture plates and also in 6-transwell plates; these cells remained viable up to 55 days (data not shown). These results demonstrate that while PRHs and LSECs can be cultured in 6-transwell plates, they do not survive long in the absence of other cells in contrast to an established cell line such as BAEC.

3.2. PRHs and endothelial cells co-cultured in configuration 1

In the first configuration, a layered co-culture of PRHs with BAECs separated by matrigel was created on a 6-well tissue culture plate as described in Materials and Methods. Figure 5a shows a phase-contrast micrograph (10× magnification) at Day 2 of PRHs and BAECs co-cultured on a 6-well tissue culture plate. In this representative picture of the co-culture, PRHs were plated at the bottom of the plate, were layered with matrigel, and then BAECs were plated on top of the matrigel layer. In order to investigate whether the order of cell layering can affect the viability and morphology of PRHs, PRHs were also plated on top of the EC layer. Figure 5b shows the layered co-culture of PRHs and BAECs cells in configuration 1 at day 3, except that the BAECs were plated at the bottom of the plate and PRHs on top of the matrigel. The change in order of plating PRHs and ECs did not alter the viability or morphology of PRHs. PRHs in configuration 1 retained their normal morphology and were viable for 3 days; however, in this configuration it was difficult to observe whether a homogenous layer of matrigel was formed between the PRHs and BAECs and PRHs did not always maintain a confluent monolayer.

3.3. PRHs and endothelial cells co-cultured in configuration 2

In configuration 2, a 6-transwell plate was used to form a layered co-culture of PRHs with BAECs so that bile could be removed from PRHs. PRHs were first plated on the microporous membrane of a 6-transwell plate and then a layer of matrigel was coated on the hepatocyte layer, followed by plating BAECs on the matrigel. The cells retained normal morphology and viability for 3 days. Figure 5c shows a phase-contrast micrograph (10× magnification) of co-culture of PRHs and BAECs on a 6-transwell in configuration 2 at Day 1. The cells in configuration 2 were also plated in the reverse order such that BAECs were

plated at the bottom of 6-transwell and PRHs formed the layer on top of the matrigel. As shown in Figure 5d, changing the order of plating the cells did not affect the morphology or viability of the cells. Similar to configuration 1, it was difficult to tell whether the monolayer formed by matrigel was of uniform thickness and whether the two cell types were completely separated into two monolayers. It was observed at some locations that the endothelial cells plated on top of the matrigel invaded through the matrigel into the PRHs layer.

3.4. PRHs and endothelial cells co-culture in configuration 3

To overcome the limitations of configuration 1 and 2, we designed and generated configuration 3. In this configuration, the hepatocytes and endothelial cells were cultured on opposite sides of the microporous membrane of a 6-transwell, as described in Materials and Methods. Long-term co-culture of PRHs and BAECs was successfully achieved using this configuration. To monitor the viability of BAECs, we generated a BAEC cell line that constitutively expressed GFP, and plated these in configuration 3; expression of GFP is indicative of cell survival. PRHs in configuration 3 retained their normal morphology and were viable for 57 days. Figure 6a is a representative picture of PRHs at Day 15 in co-culture, and Figure 6b is a representative picture at day 38 of the co-culture in configuration 3. For Figures 6a and 6b, BAECs were plated on top of the microporous membrane, and PRHs were plated at the bottom of the membrane. The morphology of PRHs at day 15 and day 38 was similar, and the cells formed a confluent monolayer in the long-term co-culture (Fig. 6a and 6b). We also observed the formation of certain structures between hepatocytes that resemble bile canaliculi (Fig. 6a, 6b). The formation of bile canaliculi may be important for PRHs to maintain morphology and function for a long period and may also be related to hepatocytes polarization. Figure 6c shows the morphology of BAEC cells at day 5 in a 6-transwell single culture. As evident from Figure 6a and 6c, the morphology of BAECs and PRHs is distinct. In order to monitor BAEC cells in co-culture, we utilized GFP-expressing BAECs. Figure 6d shows BAEC cells (circled) in the co-culture system. While green fluorescence can be observed (Fig. 6f), no fluorescence was observed when these cells were visualized using the red filter (Fig. 6e). These results demonstrate that both cell types are viable in the long-term co-culture configuration 3 system.

We next co-cultured PRHs and LSECs in the configuration 3. PRHs were plated at the bottom of the microporous membrane of a 6-transwell, and LSECs were plated on the top side of the microporous membrane. PRHs retained their normal morphology and remained viable for 39 days; Figure 6g is a representative picture of PRHs at Day 21 of the co-culture with LSECs. Overall, these results demonstrate that the configuration 3 is better than the configurations 1 and 2 for a long-term layered co-culture of hepatocytes and endothelial cells.

3.5. Structural and design comparison of configurations 1,2 and 3

In order to mimic the layered organization of the liver sinusoid, three configurations were designed and tested (Fig. 2). From the experimental results reported here, it seems that configuration 3 is a better system than configurations 1 and 2 for the layered long-term co-culture of PRHs and endothelial cells. Although the matrigel layer in configurations 1 and 2 mimics the Space of Disse in the liver more closely than the microporous membrane used in configuration 3, the matrigel layer use in configurations 1 and 2 had critical limitations. One critical limitation that precluded use of these configurations and matrigel was the persistent difficulty in completely separating the cell monolayers; this may be due to non-uniform coating of matrigel that is spread on the cell layer. Cells placed on the matrigel layer were able to invade into the bottom cell layer in configurations 1 and 2. On the other hand, a stiff microporous membrane efficiently separated the hepatocyte and endothelial cell layers in

configuration 3. Although not entirely reminiscent of the Space of Disse, the complete separation of the cell monolayers enabled to create a stable layered co-culture model and also facilitated isolation of either hepatocytes or endothelial cells from this system for evaluation of cell-specific functions. This configuration also allowed ease in analyzing urea synthesis from medium supernatant collected from the channel exposed to hepatocytes.

A potential advantage to configurations 1 and 2 that would be important if matrigel could be used to efficiently separate the two cell types is that the layering of endothelial cells directly on top of the hepatocyte could minimize shear stress on the hepatocytes that results from medium flow during a medium replacement; in configuration 3, hepatocytes are directly exposed to the medium flow. However, mass transport considerations suggest that hepatocytes placed on the bottom of the plate in configurations 1 and 2 are limited in their exposure to the medium because they are covered by both endothelial cells and matrigel. Growth factors and bile secreted from hepatocytes in configurations 1 and 2 may also stagnate around cells, which may be harmful to cell viability. In contrast, the exposure of hepatocytes to medium in configuration 3 may allow for more efficient exposure to oxygen, nutrients, and growth factors. Materials secreted from hepatocytes in configuration 3 can also be released into the surrounding medium and diluted.

Direct cell-to-cell contact between PRHs and ECs cannot take place in configuration 3 because the two cell types are plated on either side of a membrane that is 10 μm thick in section 3.7. Our data suggests that exchange of secreted factors between PRHs and ECs can take place through the microporous membrane, which might be important for long-term survival of hepatocytes.

3.6. PRHs remain differentiated in long-term co-culture in configuration 3

In vivo, hepatocytes are differentiated cells and one of the problems of culturing these cells is that the hepatocytes de-differentiate shortly after plating. To determine whether PRHs that were co-cultured with LSECs in configuration 3 remained differentiated, we assessed the mRNA expression of the established hepatocyte specific differentiation markers albumin (ALB), transferrin (TNF), and hepatocyte nuclear factor 4 (HNF-4) [25]. The expression of ALB (105bp), TFN (121bp), and HNF-4 (770bp) mRNA in PRHs that were co-cultured with BAECs for 28 days indicated that they remain differentiated (Fig.7a). Expression of hepatocyte specific differentiation markers in freshly isolated, unplated PRHs was used as the control. The expression levels of differentiation markers in PRHs that were co-cultured for 28 days was lower as compared to the expression levels in freshly isolated PRHs. This decrease in the expression levels of differentiation markers as compared to the control was most likely due to the loss of cells during cell seeding and long-term co-culture; however, the continued expression of the hepatocyte differentiation markers indicate that the hepatocytes remained differentiated during the long-term co-culture.

To confirm similar long-term expression of hepatocyte differentiation markers when PRHs were plated with LSECs, PRHs were plated on the bottom side of the microporous membrane of a 6-transwell and LSECs were plated on the topside as in configuration 3 followed by analysis of differentiation markers at the indicated times. PRHs were harvested at day 37 of the co-culture, and RT-PCR was performed (Fig. 7b). We were able to detect the mRNA of ALB (lane 2), TFN (lane 4) and HNF-4 (lane 7), suggesting that the PRHs remain differentiated in long-term co-culture with LSECs in configuration 3. Lane 3, 5, 8 represents the control for ALB, TFN, and HNF-4 respectively; PCR was performed without prior reverse transcription to ensure the absence of DNA contamination in the RNA samples.

Similarly, we plated PRHs on the top side of the microporous membrane of a 6-transwell and LSECs on the bottom in configuration 3. PRHs were harvested at day 45, and RT-PCR was performed (Fig. 7b). We detected expression of ALB (lane 10), TFN (lane 12) and HNF-4 (lane 15) mRNA; lanes 11, 13, 16 represents the controls for ALB, TFN, and HNF-4 RT-PCR respectively, where the PCR was done without prior reverse transcription. Overall, these results demonstrate that PRHs remain differentiated in long-term co-culture with LSECs in configuration 3 irrespective of whether PRHs were plated on the tops or bottom of the microporous membrane of the 6-transwell.

3.7. Functional analysis of PRHs in configuration 3

To determine whether PRHs retain normal functions in long-term co-culture with endothelial cells in configuration 3, urea synthesis by hepatocytes was analyzed. We also compared urea synthesis in co-cultures and single cultures. Media of PRHs in co-culture or single culture were collected on different days after seeding, and urea synthesis was analyzed. Urea synthesis in the single culture was high in the beginning but decreased to very low levels by two weeks (Fig. 8). This was consistent with the fact PRHs in single culture were undergoing cell-death (Fig. 4) and as a result of which urea synthesis decreased with time. PRHs that were co-cultured with ECs showed lesser urea synthesis in the beginning as compared to single PRHs culture. In our experiments, day 1 of co-culture corresponds to day 2 of single culture because co-culture was established one day after the single culture when BAECs were plated. Thus, urea synthesis by hepatocytes at day 1 of co-culture was lower as compared to day 1 of the single culture. However, urea concentration reached steady levels after 1 week and remained steady for at least 28 days (Fig. 8).

In order to investigate whether small molecules secreted by PRHs or LSECs pass through membranes with 0.4 μm pore size into the other side during co-culture, samples from both sides of transwell membrane were obtained and urea synthesis was measured. The urea concentration in the media sample from either side of the co-culture was similar (data not shown), suggesting that small molecules such as urea are able to pass through the membrane. These results suggest that exchange of secreted factors can take place through the porous membrane, even though PRHs and LSECs are not in direct contact, and this exchange of secreted factors help PRHs to survive better in long-term co-culture.

3.8. Expression of CYP2E1 in PRHs maintained in configuration 3

The Cytochrome P450, CYP2E1, is an important hepatocyte enzyme that can convert ethanol to acetaldehyde, which is relevant to hepatocyte metabolism of circulating xenobiotic [26,27]. To determine whether PRHs retain CYP2E1 expression when co-cultured with endothelial cells in configuration 3, we analyzed the expression of CYP2E1 in PRHs co-cultured with ECs using western blot analysis. CYP2E1 expression in freshly isolated PRH and in PRHs that were co-cultured with ECs for long-term (up to 48 days) was relatively similar (Fig. 9a,b). PRHs in co-culture and in single culture were from the same rat and thus could be directly compared. Fig. 9a is a representative Western blot and Fig. 9b shows the average of three independent experiments. All the values were normalized to GAPDH, which was used as a loading control. These results also confirm that the co-cultured PRHs from day 1 to day 48 remain viable without obvious changes in hepatocyte function. The lower levels of GAPDH at later time points coincided with an observed slight increase in hepatocyte death at later times of cell collection.

4. Conclusions

We presented a novel liver model that mimics the liver sinusoid and facilitate a long-term culture of PRHs. A key aspect of our current liver model is a layered co-culture of PRHs and

LSECs or BAECs on a transwell membrane. Three different configurations were investigated. In configurations 1 and 2, layered co-culture of PRHs and endothelial cells was attempted by introducing a thin matrigel layer between them. However, these configurations had critical limitations. In particular, it was difficult to achieve complete separation of PRHs and ECs. Other concerns include ineffective removal of secretion (e.g. bile) from hepatocytes and limited interaction of cells placed at the bottom with culture media. On the other hand, the use of a stiff microporous membrane instead of matrigel in configuration 3 and cell culture on the opposite sides of the microporous membranes turned out to be a reliable model. PRHs co-cultured with BAECs or LSECs in this configuration retained their morphology and viability for 57 or 39 days with a confluent monolayer on the 6-transwell, respectively. We also assessed the presence of hepatocyte-specific differentiation markers to verify that PRHs remained differentiated in the long-term co-culture and analyzed hepatocyte function by monitoring urea synthesis. In addition, the expression of cytochrome P-450 remained similar in the co-cultured system from Day 1 to Day 48. In contrast, PRHs cultured in the absence of endothelial cells lost their morphology and viability 4-5 days after plating. Furthermore, endothelial cells also retained morphology and viability for a longer period of time compared to endothelial cells cultured in the absence of hepatocytes. This demonstrates that endothelial cells and hepatocytes have critical roles in influencing the viability and function of each other, and thus supports the importance of including both cell types in long-term liver model systems. Configuration 3 of our liver model supports the layered long-term co-culture of PRHs and endothelial cells, which is difficult to achieve in single cultures. This layered long-term co-culture system is a novel liver model that mimics the layered organization of the liver sinusoid and can be used as a model for studying fundamental liver biology studies, liver disease research, toxicology studies and drug screening applications. This novel model system can now be expanded to incorporate primary human hepatocytes and develop a novel human liver model system.

Acknowledgments

These studies were funded by NSF, Grant #: 0747752 and 0923173 to HN and NIH 1R21AA017458-01A2 to MB and HN. We thank to Lina Maciunas for establishing the BAEC cell line expressing GFP.

References

1. Nahmias Y, Berthiaume F, Yarmush ML. Integration of technologies for hepatic tissue engineering. Adv Biochem Eng Biotechnol. 2007; 103:309–329. [PubMed: 17195468]
2. Lee PJ, Hung PJ, Lee LP. An artificial liver sinusoid with a microfluidic endothelia l-like barrier for primary hepatocyte culture. Biotechnol Bioeng. 2007; 97(5):1340–1346.
3. Desmet, V. The liver, biology, and pathobiology. Arias, I., editor. Lippincott Williams and Wilkins; Philadelphia: 2001. p. 3-15.
4. Guillouzo A. Liver cell models in *in vitro* toxicology. Environ Health Perspect. 1998; 106:511–532. [PubMed: 9599700]
5. Sodunke, T.; Bouchard, M.; Noh, H. Engineering the hepatic sinusoid– a novel mini liver platform; BMES Annu Meeting; Los Angeles, USA. 2008.
6. Cirillo, J.; Hu, CC.; Rawat, S.; Bouchard, M.; Noh, H. Fabrication of dual channels for co-culture of liver hepatocytes and endothelial cells and numerical simulation of oxygen levels; International Biofabrication Conference; Philadelphia, USA. 2010.
7. Braet, F.; Luo, D.; Spector, I.; Vermijlen, D.; Wisse, E. The Liver, Biology, and Pathobiology. Arias, I., editor. Lippincott Williams and Wilkins; Philadelphia: 2001. p. 438-453.
8. Park KH. Improved long-term culture of hepatocytes in a hydrogel containing Arg-Gly-Asp (RGD) Biotechnol. Lett. 2002; 24:1131–1135.

9. Ross MA, Sander CM, Kleeb TB, Watkins SC, Stolz DB. Spatiotemporal expression of angiogenesis growth factor receptors during the revascularization of regenerating rat liver. Hepatology. 2001; 34:1135. [PubMed: 11732003]
10. Nahmias Y, Casali M, Barbe L, Berthiaume F, Yarmush ML. Liver endothelial cells promote LDL-R expression and the uptake of HCV-like particles in primary rat and human hepatocytes. Hepatology. 2006; 43:257. [PubMed: 16440337]
11. Nahmias Y, Schwartz RE, Hu WS, Verfaillie CM, Odde DJ. Endothelium-mediated hepatocyte recruitment in the establishment of liver-like tissue *in vitro*. Tissue Eng. 2006; 12:1627.
12. Abu-Absi SF, Hansen LK, Hu WS. Three-dimensional co-culture of hepatocytes and stellate cells. Cytotechnology. 2004; 45:125–140. [PubMed: 19003250]
13. Kim Y, Larkin AL, Davis RM, Rajagopalan P. The design of *in vitro* liver sinusoid mimics using chitosan–hyaluronic acid polyelectrolyte multilayers. Tissue Eng A. 2010; 16:2731–2741.
14. Salerno S, Campana C, Morelli S, Drioli E, Bartolo LD. Human hepatocytes and endothelial cells in organotypic membrane systems. Biomaterials. 2011; 32:8848–8859. [PubMed: 21871658]
15. Kasuya J, Sudo R, Mitaka T, Lkeda M. Hepatic stellate cell-mediated three-dimensional hepatocyte and endothelial cell triculture model. Tissue Eng A. 2011; 17:361–370.
16. Powers MJ, et al. A microfabricated array bioreactor for perfused 3D liver culture. Biotechnol Bioeng. 2002; 78:257–269.
17. Kim K, Ohashi K, Utoh R, Kano K, Okano T. Preserved liver-specific functions of hepatocytes in 3D co-culture with endothelial cell sheets. Biomaterials. 2012; 33:1406–1413.
18. Riccalton-banks L, Liew C, Bhandari R, Fry J, Shakesheff K. Long-term culture of functional liver tissue: three-dimensional coculture of primary hepatocytes and stellate cells. Tissue Eng. 2003; 9:401–410. [PubMed: 12857408]
19. Kang, YB.; Cirillo, J.; Rawat, S.; Bouchard, MJ.; Noh, H. Layered hepatocytes and endothelial cells on a transwell membrane: toward engineering the liver sinusoid. ASME 2012 conference; Houston USA. 2012. IMECE2012-89413
20. Sodunke TR, Bouchard MJ, Noh H. Microfluidic platform for hepatitis B viral replication studies. Biomed Microdevices. 2008; 10(3):393–402. [PubMed: 18165913]
21. Hwa AJ, Fry RC, Sivaraman A, So PT, Samson LD, Stolz DB, Grifitth LG. Rat liver sinusoidal endothelial cells survive exogenous VEGF in 3D perfused co-cultures with hepatocytes. FASEB J. 2007; 21(10):2564–2579. [PubMed: 17426068]
22. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. Nat Biotechnol. 2007; 26(1):120–126.
23. Chen AA, Khetani SR, Lee S, Bhatia SN, Van Vliet KJ. Modulation of hepatocyte phenotype *in vitro* via chemomechanical tuning of polyelectrolyte multilayers. Biomaterials. 2009; 30:1113–1120. [PubMed: 19046762]
24. Liu TV, Chen AA, Cho LM, Jadin KD, Sah RL, DeLong S, West JL, Bhatia SN. Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. FASEB J. 2007; 21:790–801. [PubMed: 17197384]
25. Costa RH, Kalinichenko VV, Holterman AX, Wang X. Transcription factors in liver development, differentiation, and regeneration. Hepatology. 2003; 38:1331–1347. [PubMed: 14647040]
26. Kölble K. Regional mapping of short tandem repeats on human chromosome 10: cytochrome P450 gene CYP2E, D10S196, D10S220, and D10S225. Genomics. 1993; 18(3):702–4. [PubMed: 8307581]
27. Hayashi S, Watanabe J, Kawajiri K. Genetic polymorphisms in the 5′-flanking region change transcriptional regulation of the human cytochrome P450IIE1 gene. J Biochem. 1991; 110(4):559–65. [PubMed: 1778977]
28. Hewitt NJ. Primary hepatocytes; current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction transporter, clearance, and hepatotoxicity studies. Drug Metab Rev. 2007; 39:159–234. [PubMed: 17364884]
29. Runge D, Runge DM, Jäger D, Lubecki KA, Stolz DB, Karathanasis S, Kietzmann T, Strom SC, Jungermann K, Fleig WE, Michalopoulos GK. Serum-free, long-term cultures of human

hepatocytes: maintenance of cell morphology, transcription factors, and liver-specific functions. Biochem Bioph Res Co. 2000; 269:46–53.

30. Doi K, Hargitai J, Kong J, Tsang SH, Wheatley M, Chang S, Goff S, Gouras P. Lentiviral transduction of green fluorescent protein in retinal epithelium: evidence of rejection. Vision Res. 2002; 42:551–558.
31. Huang J, Karakucuk V, Levitsky LL, Rhoads DB. Expression of HNF4α variants in pancreatic islets and Ins-1 β cells. Diabetes Metab Res Rev. 2008; 24:533–543.

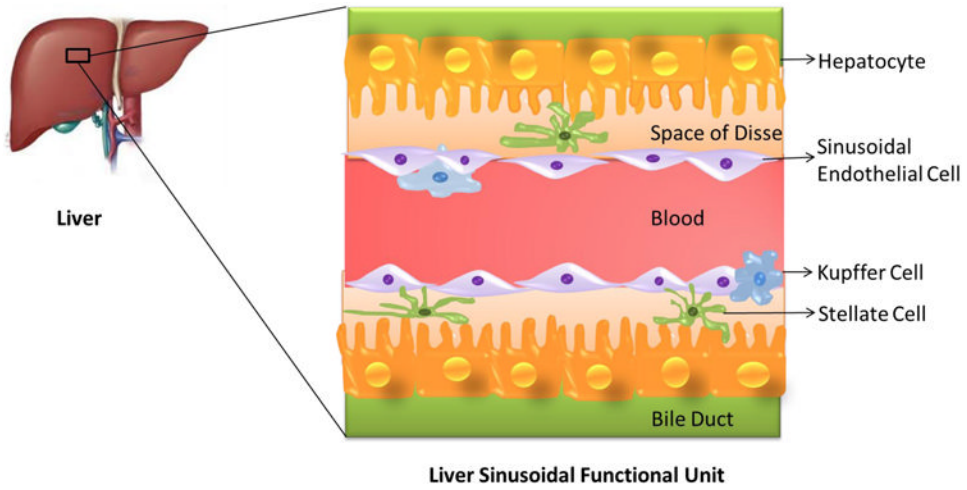

Figure 1.
The liver sinusoid functional unit.

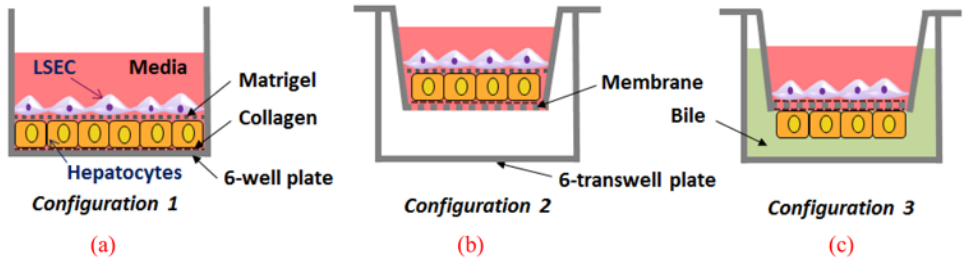

Figure 2.
Schematic diagram of cell culture platforms to mimic the liver sinusoid.

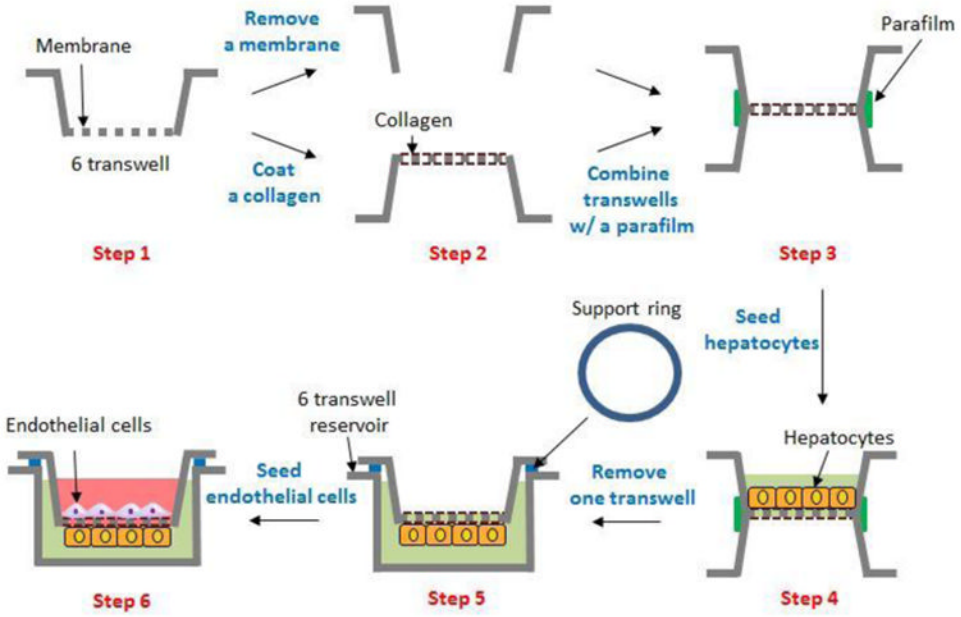

Figure 3.
Conceptual diagram of co-culture structure of hepatocytes and endothelial cells in configuration 3.

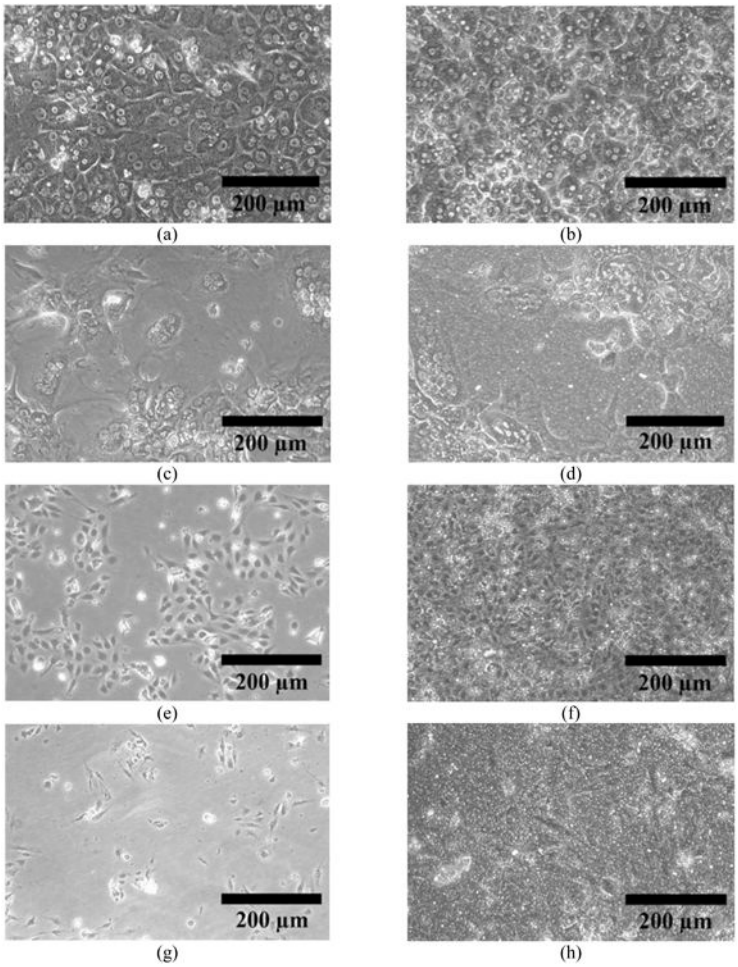

Figure 4.

Phase-contrast micrographs of PRHs and LSECs single culture (a) hepatocytes on 6-well at Day 2. (b) hepatocytes on 6-transwell at Day 1. (c) hepatocytes on 6-well at Day 7. (d) hepatocytes on 6-transwell at Day 11. (e) LSECs on tissue culture plate (54mm diameter) at Day 2. (f) LSECs on 6-transwell at Day 1. (g) LSECs on tissue culture plate (54mm diameter) at Day 7. (h) LSECs on 6-transwell at Day 7. PRHs and LSECs isolated from a different rat at a different day were used in a culture.

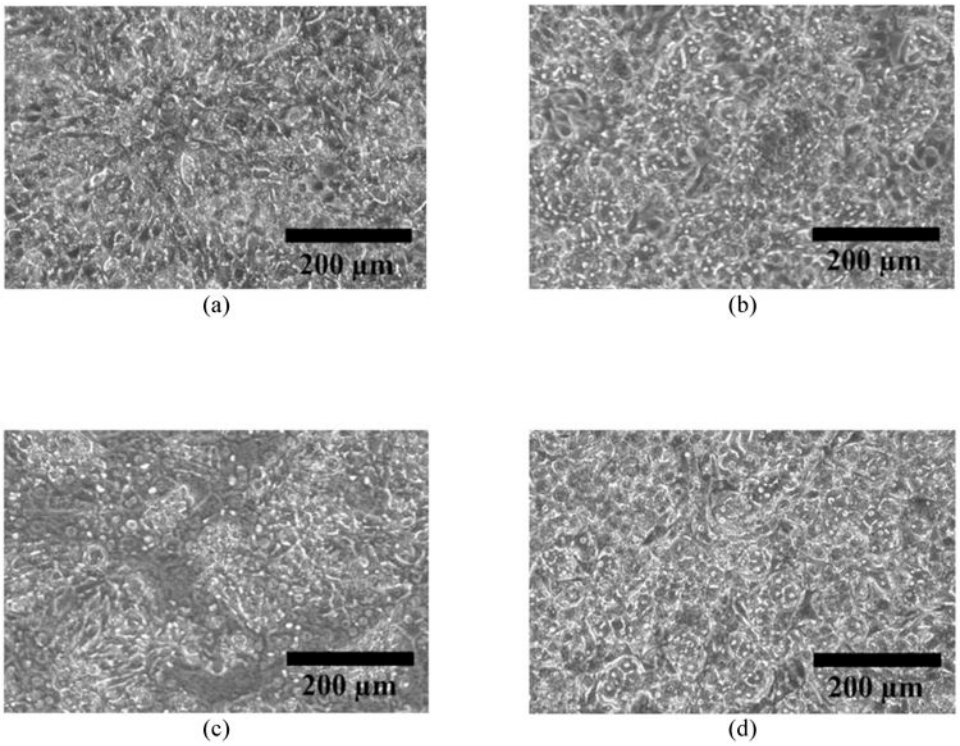

Figure 5.

Phase-contrast micrographs of co-culture of hepatocytes and BAECs on 6-well in configurations 1 and 2. (a) BAECs were layered on matrigel, which was placed on PRHs layer in 6-well (image at Day 2 of co-culture). (b) BAECs were laid on 6-well plate first, followed by matrigel coating and hepatocyte culture (image at Day 3 of co-culture). (c) BAECs were layered on matrigel, which was placed on PRHs layer in 6-transwell (image at Day 1 of co-culture). (d) PRHs were layered on matrigel, which was placed on BAECs layer in 6-transwell (image at Day 2 of co-culture).

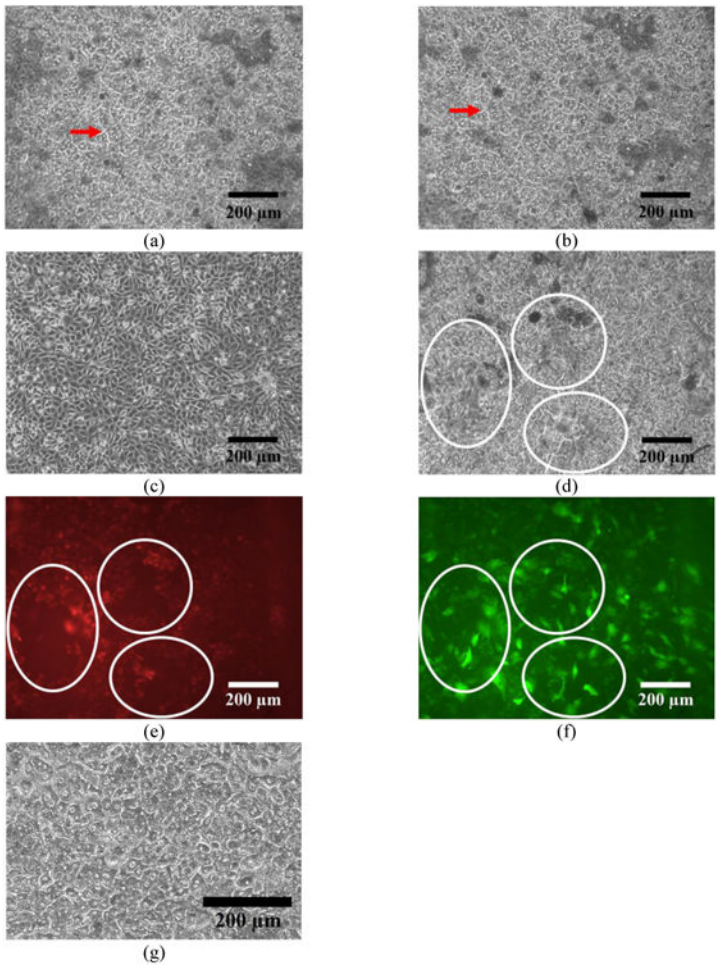

Figure 6.

Phase-contrast micrographs of co-culture of PRHs and BAECs/LSECs on 6-transwell in configuration 3. Comparison of PRHs morphology (a) at D15 and (b) at D38 of co-culture of PRHs on the bottom of membrane and BAECs on the top of membrane. Arrow indicates potential bile canaliculi. (c) BAECs single culture on the 6-transwell at Day 5. (d) long term co-culture of PRHs on the bottom of the membrane and BAECs with ad-GFP on the top of membrane at Day 40. (e) micrograph of red fluorescent field of (d). (f) GFP micrograph of (d). (g) LSECs were layered on top of the membrane and PRHs were placed on the bottom of membrane in transwell after 21 days of co-culture.

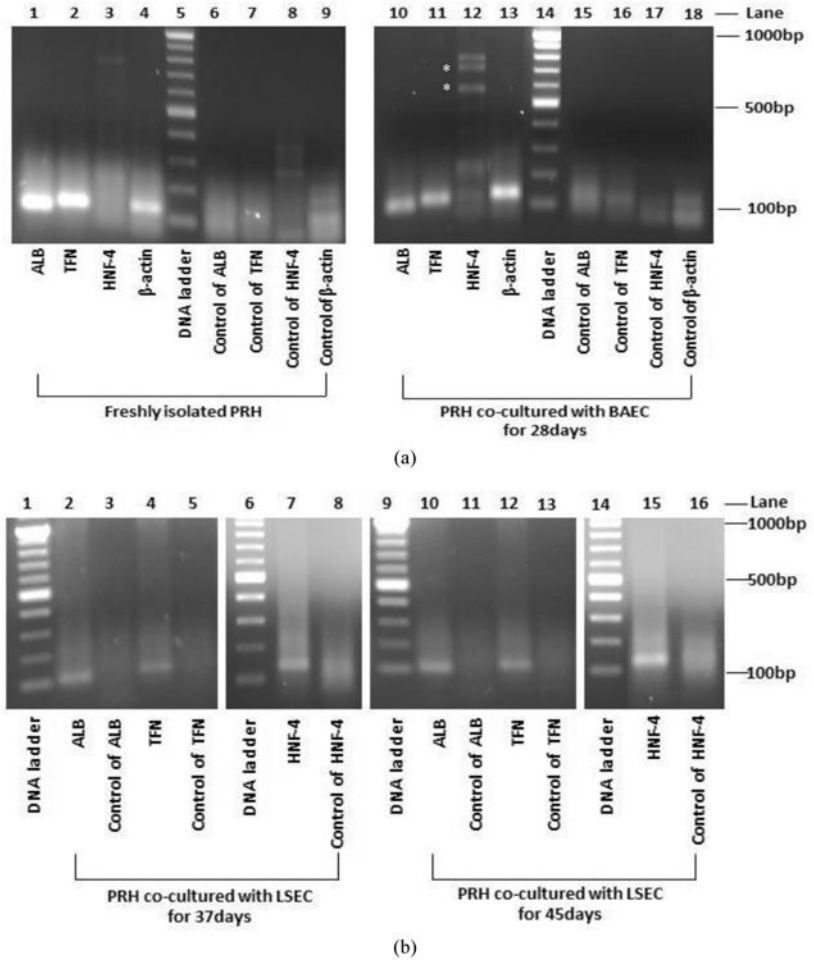

Figure 7.

Confirmation of PRH differentiation on a transwell culture in configuration 3. (a) RT-PCR was performed after total RNA isolation from freshly isolated PRH and PRH co-cultured for 28 days (PRHs on the bottom of membrane and BAECs on the top of membrane) to check mRNA expression of the liver specific differentiation markers; ALB (105bp), TFN (121bp), HNF-4 (770bp) and β-actin (130bp). Fresh PRHs and PRHs in co-culture were isolated from a same rat at a same day. Asterisk * indicates likely detection of alternatively spliced HNF-4 mRNA [31]. (b) RT-PCR was performed on PRHs co-cultured for 37 days (PRHs on the bottom of membrane and LSECs on the top of membrane) and 45 days (PRHs on the top of membrane and LSECs on the bottom of membrane) to check mRNA expression of the liver specific differentiation markers; (ALB (105bp), TFN (121bp), and HNF-4 (138bp). 37 days and 45 daysPRHs co-cultures were from different rats.

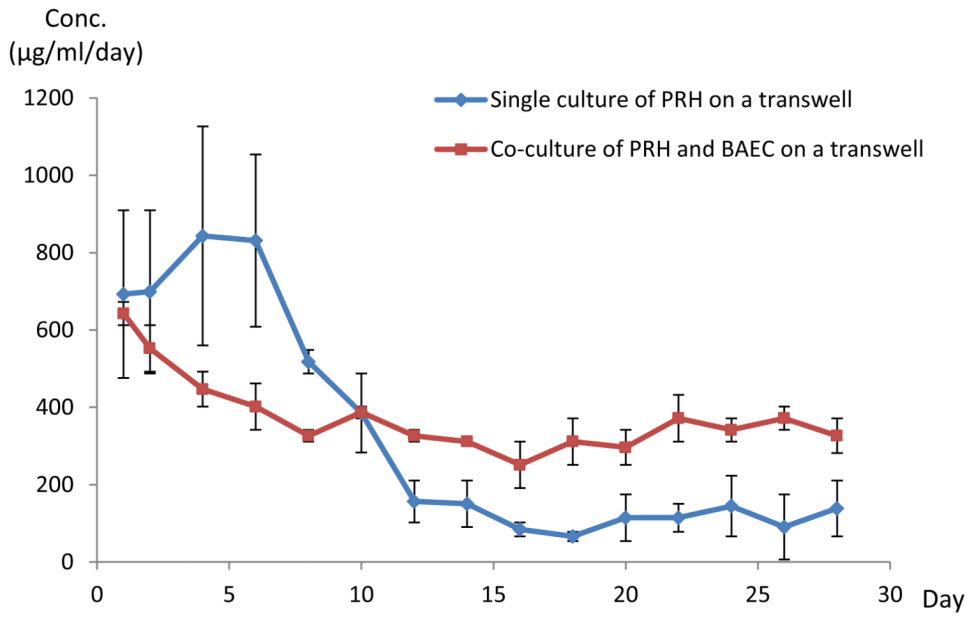

Figure 8.

Urea synthesis analysis of hepatocytes. Media from Single culture of PRHs (blue line), and from long-term co-culture of PRHs with BAECs in configuration 3 (red line), were collected and urea concentration was determined. PRHs used in single culture and co-culture were from same rat; all data was normalized and expressed as μg/ml/day.

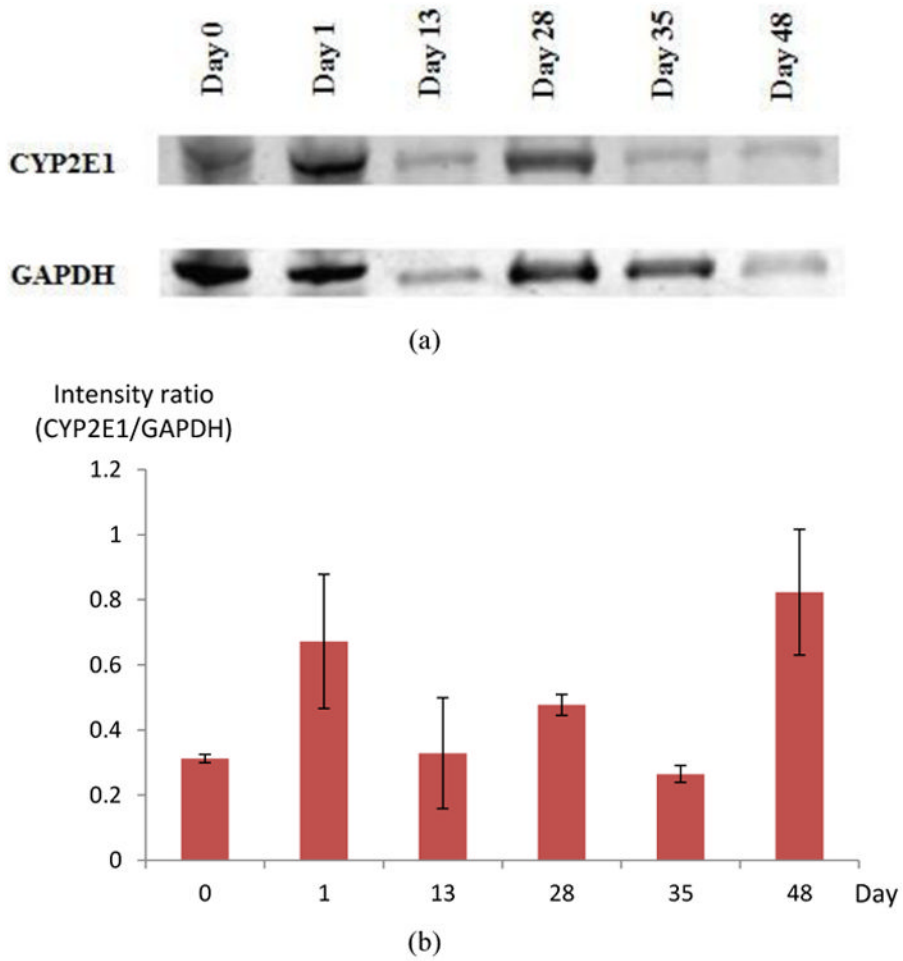

Figure 9.

CYP2E1 protein expression in primary rat hepatocytes co-cultured with BAECs in configuration 3. (a) Representative image of a Western blot analysis of CYP2E1 and GAPDH expression in PRHs co-cultured with BAECs. (b) Bar graph showing the average CYP2E1 expression, in long-term co-cultured PRHs. The CYP2E1 expression was normalized to the GAPDH expression levels and the data represents the average of three independent experiments. Error bars represent +/-SEM

Table 1

Preparation of percoll density gradient.

Material	25%	50%
10× Phosphate Buffered Saline (Cellgro®)	0.375 ml	0.375 ml
Percoll	3.125 ml	6.5 ml
DMEM	6.5 ml	3.375 ml
Bovine Calf Serum(BCS) (Gemini Bioproduct)	2.5 ml	2.5 ml

Table 2
Component of different medium for PRHs and endothelial cells

PRH Medium	LSEC Medium	BAEC Medium	Modified PRH Medium
2 mM Glutamine (Cellgro®)	2 mM Glutamine		
1 mM Na Pyruvate (Cellgro®)	1 mM Na Pyruvate	1% penicillin-streptomycin	1 mM Na- Pyruvate
4 μg/ml Insulin-Transferrin-Selenium (ITS, Gibco)	4 μg/ml ITS		4 μg/ml ITS
10 μg/ml Gentamycin (Gibco)	10 μg/ml Gentamycin	500ml DMEM	5 μg/ml HC
5 μg/ml Hydro cortisone(HC, Sigma)	5 μg/ml HC		5 ng/ml EGF
5 ng/ml Epidermal growth factor (EGF, BD Sciences)	5 ng/ml EGF		1% penicillin- streptomycin
500 ml Williams E (Gibco)	500 ml Williams E 10% FBS	10% FBS	500 ml DMEM 10% FBS

Table 3

Hepatocyte gene-specific primers sequences for PCR.

Primer	Sequence (5′ → 3′)	Size (bp)	Cycle
ALB	AAAGCACTGGTCGGAGCTGTCCG TCGCTGGCTCATACGAGCTACTGC	105	30
HNF-4[a]	CGGGCCACTGGCAAACAC GTAATCCTCCAGGCTCACC	770	30
HNF-4[b]	AGTGCTGCCTTGGACCCAGCCT GGCACACAGGGCACTGACACCC	138	30
TFN	TTACGGGTGCCCCCAAGGATGGAC ATTTCACTGGCGCGCTGTCGATGG	121	30
β-actin	GTCCACACCCGCCACCAGTT GGCCCACGATGGAGGGGAAG	133	30

[a] previous designed primer of HNF-4 [20].

[b] newly designed primer of HNF-4.