Optimized Protein Patterning Methods for Human Liver Cultures

BY

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THESIS

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This thesis is dedicated to my grandparents, Sergio, Franca and Clara, to my parents, Rita and Fabio, and my dog Raya, that supported me and believed in me during the accomplishment of this master degree.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
BSC	Biosafety Cabinet
CD	Cluster of Differentiation
CL	Collagen
CYP450	Cytochrome P450
ddH2O	double-deionized water
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
ELISA	Enzyme-Linked Immunosorbent Assay
FN	Fibronectin
HCC	Hepatocellular Carcinoma
H-MPCC	Micropatterned co-culture of PHH and HUVEC
H-MPTC	Micropatterned tri-culture of PHH, HUVEC and 3T3-J2
HUVEC	Human Umbilical Vein Endothelial Cell
L-MPCC	Micropatterned co-culture of PHH and LSEC
L-MPTC	Micropatterned tri-culture of PHH, LSEC and 3T3-J2
LSEC	Liver Sinusoidal Endothelial Cell
L-SIGN	Liver/Lymph node-specific intercellular adhesion molecule-3-grabbing integrin
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor 1
MPCC	Micropatterned co-culture
mRNA	Messenger RiboNucleic Acid
NPC	Non-Parenchymal Cell

LIST OF ABBREVIATIONS (continued)

PA	Polyacrylamide
PBS	Phosphate Buffered Saline
PDMS	Polydimethylsiloxane
PECAM-1	Platelet endothelial cell adhesion molecule
PEN	Penicillin
PFA	Paraformaldehyde
PHH	Primary Human Hepatocytes
RLU	Relative Luminescence Unit
SE-1	Sinusoidal Endothelial – 1 marker
STREP	Streptomycin
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand Factor
µCP	Microcontact Printing

SUMMARY

Organizing cells on extracellular matrix (ECM) proteins is useful for optimizing cell-cell interactions and therefore cell functions *in vitro*. In the case of the liver, micropatterned co-cultures (MPCCs) containing primary human hepatocytes (PHHs) and 3T3-J2 murine embryonic fibroblasts adhered to hard surfaces (e.g. glass and plastic) have been shown to exhibit high levels of hepatic functions for several weeks *in vitro*; the use of PHHs in this model mitigates the differences observed in drug metabolism enzymatic pathways between animal (e.g. rodents) and human livers. Furthermore, MPCCs have been previously augmented with liver sinusoidal endothelial cells (LSECs) or human umbilical vein endothelial cell (HUVECs) non-liver controls to model the interactions between PHHs and endothelial cells as *in vivo*; however, this study was limited to gene expression analysis of the endothelial phenotype, which may not correlate with functional markers.

This thesis seeks to build upon the MPCC platform to a) develop and optimize an alternative technique than currently used for patterning proteins onto softer surfaces that more accurately mimic the stiffness of the liver, and b) determine protein marker expression of the endothelial cells in monocultures and co-cultures with PHHs and/or fibroblasts to complement the published gene expression data set. Towards the first aim, we optimized parameters for a soft lithographic microcontact protein printing method using polydimethylsiloxane (PDMS) stamps and subsequently showed its utility for the creation of highly functional MPCCs on both collagen and fibronectin stamped domains on glass. Additional studies demonstrated the utility of this method for patterning proteins and cells on softer surfaces in the kPa range of stiffness as opposed to the GPa range of stiffness for glass and plastic. Towards the second aim of this thesis, we tested three protein markers (CD31, SE-1, and Factor VIII) for their ability to distinguish

LSECs and HUVECs in pure cultures, co-cultures with either PHHs or fibroblasts, and tri-cultures containing endothelial cells, PHHs, and fibroblasts. Our results showed that while CD31 (immunostaining) and Factor VIII secretion (as assessed via enzyme linked immunosorbent assay) were detected in both endothelial cell types, SE-1 could distinguish LSECs from HUVECs, but only in pure monocultures reliably since this marker was downregulated in co-/tri-cultures containing LSECs, cross-reacted with mouse fibroblast protein(s), and showed upregulation in HUVECs in co-culture with PHHs. Overall, our studies set the stage for the creation and phenotypic characterization of multicellular human liver models on surfaces that mimic liver-like stiffness, which will have robust utility for drug screening and ultimately, regenerative medicine.

CHAPTER 1. PROTEIN PATTERNING VIA AN ELASTOMERIC STAMP

1.1 INTRODUCTION

1.1.1 Importance of ECM proteins pattern and substrate stiffness for *in vitro* cell cultures

Organizing cells on extracellular matrix (ECM) proteins is useful for controlling cell-cell interactions and optimizing cellular functions *in vitro*. For example, it has been shown that cell shape and dimension can affect cell phenotype and differentiation, and that there is a reciprocity between cell morphology and integrin focal adhesion points [1]. Cell pattern enables multi-cellular cultures that better mimic human tissues and organs relative to monoculture studies [2]. Moreover, in tissue engineering and regenerative medicine *in vitro* research there is an increasing need to develop substrate materials for cell culture that are able to efficiently mimic the physiologic nature of the soft tissue for which the studies are being done. In fact, cells are sensitive to the molecular cues within their local microenvironment, such as substrate stiffness [3]. They are known to behave differently on substrates of different stiffnesses in terms of adhesion, migration and differentiation during normal homeostasis and disease states [4]. For example, it has been shown that fibrotic and cirrhotic livers are stiffer than healthier ones [5, 6]; liver stiffness values around 0.2 kPa are considered normal, values between 4 and 12.5 kPa represent tissue fibrotic attitude and values above 20 kPa are directly correlated to cirrhosis [7]. In recent studies it has been shown that soft substrates can support hepatocyte function and phenotype for a longer culture period compared to stiff substrates and promoted cells migration and directionality [8, 9].

Thus, the mechanical environment is recognized as an important factor in this field because in soft tissue studies, the stiffness of the material used for cell culture should be compatible with the stiffness of the native tissue in order to more accurately mimic the behavior of healthy and diseased organs [10].

1.1.2 Techniques involved in ECM protein patterning

Several techniques have been developed to micropattern ECM proteins on cell culture surfaces (e.g. plastic, glass, and gels). One of these techniques is called microcontact printing (μCP) and it is embedded in the field of soft-lithography [11] techniques. This technique has been shown to be an efficient and inexpensive tool for patterning various substrates [12, 13, 14]. There are three principal steps involved in μCP: stamp fabrication from an elastomeric material (e.g. polydimethylsiloxane (PDMS)), inking with a protein solution and transfer of the solution to another substrate via contact with the stamp. The stamp is generally fabricated using an SU-8 polymer master mold manufactured via standard photolithographic procedure [15]. Polydimethylsiloxane is a very elastic and soft organosilicon, inexpensive, it has low autofluorescence, it is hydrophobic, inert, biocompatible and highly permeable to gases and fluids [16]. Once fabricated, the PDMS stamp is inked with a liquid solution (e.g. ECM protein) and the 'ink' is transferred from the stamp to a surface. Precise contact between the elastomeric stamp and the receiving surface is the key in order to achieve a successful transfer of the solution from the stamp to the surface [4] (**Figure 1**).

Another technique uses a PDMS mold to protect regions of a protein-coated surface from oxygen plasma such that once the stamp is removed, the protected protein is maintained,

obtaining micropatterned protein domains for cell attachment [17]. However, this technique is limited in the types of proteins it can pattern and is not compatible with softer surfaces that can be used to mimic the soft tissue-like microenvironment of organs such as the liver [5]. Moreover, it is not known if the plasma causes alteration in polystyrene's properties while it is removing/ablating the protein solution.

A different technique involves the use of a PDMS mold that contains microchannels through which a protein solution can pass while the stamp is positioned on the surface. The channels are filled by capillarity or pressure. This technique is called microfluidic patterning and the solution is added where the stamp does not contact the surface. After the crosslinking or adsorption of the protein to the substrate surface, the stamp is removed from the surface leaving behind the protein pattern. Capillarity provides higher resolution and simplicity relative to pressure, but proteins deplete as the channel is filling, resulting in non-uniform protein patterns [18].

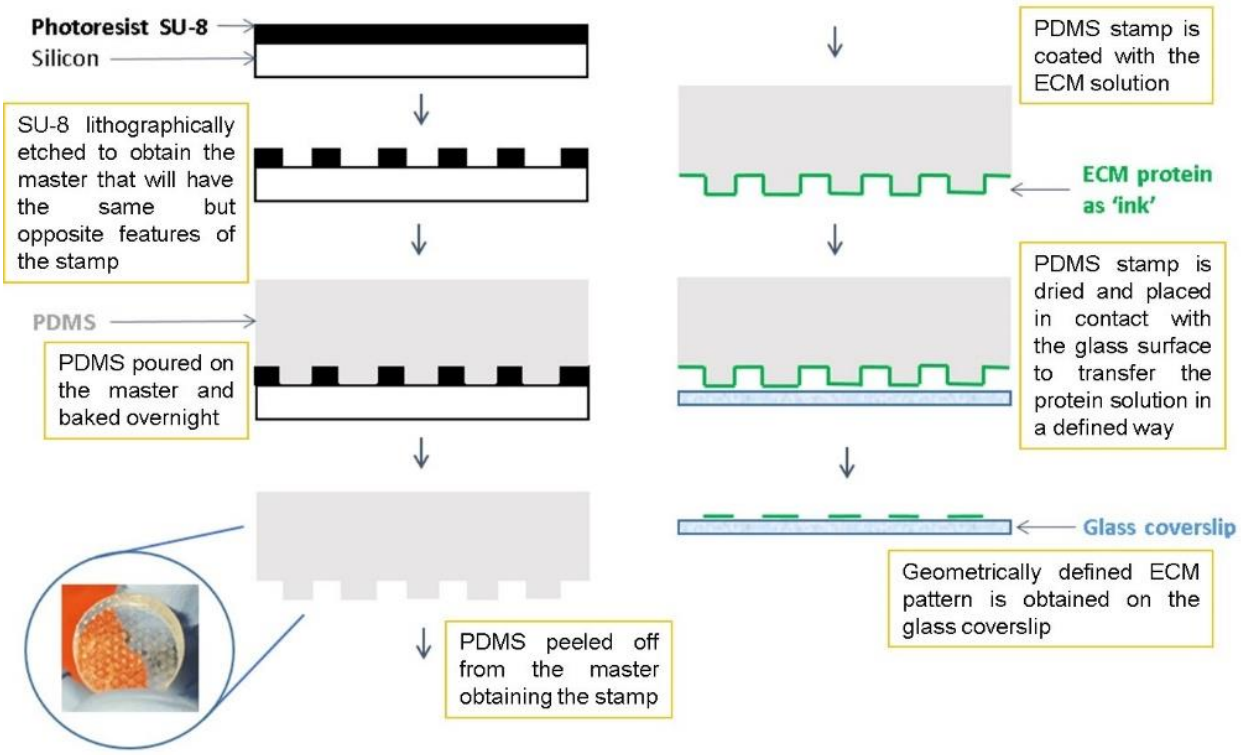

Figure 1. Illustrative representation of the steps involved in microcontact printing.

1.1.3 Purpose of the study

In this study we sought to optimize the steps and parameters usually considered in the microcontact printing technique in order to define patterning protocols for different ECM proteins and for both hard and soft surfaces. With the use of these optimized protocols, we were able to create a human liver model on stamped ECM proteins on glass coverslips that can be used to maintain and support the morphology, viability, and functions of primary human hepatocytes (PHHs) and fibroblasts, at levels comparable to our previous study on

micropatterned co-cultures (MPCCs) of the same two cell types [19]. In addition, we were able to micropattern PHHs on polyacrylamide (PA) gels that could better mimic the stiffness of the liver as compared to hard glass.

1.2 MATERIALS AND METHODS

1.2.1 Microcontact printing of collagen and fibronectin on glass coverslips

To perform the experiments, a 24-well polystyrene tissue culture plate format (Corning) was pre-coated with 200 µL/well of Pluronic® F-127 (SIGMA) diluted in double-deionized water (ddH2O) in order to have a 5% solution. The coating was left overnight at 4°C. The next day the pluronic solution was removed and the wells were rinsed three times with ddH2O. The plates were sterilized under an UV light for at least 1 hour. The polydimethylsiloxane (PDMS) stamps were fabricated starting from a SU-8 patterned master. PDMS solution was obtained by mixing 30 g of PDMS (SYLGARD® 184 SILICONE ELASTOMER KIT) and the curing agent in a ratio 10 to 1. Once the PDMS was properly mixed, it was poured on the master already treated with 100 µL of hexadimethyldisilizane and placed in the vacuum chamber. Hexadimethyldisilizane evaporation is needed in order to help the detachment of the PDMS mold from the master. Vacuum was turned off and on every 15 - 20 minutes until no more bubbles were present. When ready, the master with the PDMS on top was placed in the oven in a flat position in order to obtain a homogenous PDMS mold. It was then cured in the oven (not turned on) for 4 hours and then baked at 60°C overnight. With a scalpel and tweezers, the PDMS mold was removed carefully from the wafer and a mold with protruding pillars was obtained. With the pillars facing up, the mold was punched with a circular cutter in order to create ~ 15 stamps of 15 mm diameter that fit in each well of a 24 well format plate. Each PDMS stamp contained ~ 80 pillars of 500 µm in diameter and 1200 µm center to center spacing.

Final concentration of 50 µg/mL protein solutions were obtained by diluting collagen I from rat tail (Corning) and human fibronectin (Corning Life Science, Manassas, VA) in ddH2O and phosphate buffered saline (1X PBS), respectively.

Glass coverslips (Fisherbrand®) of 12 mm diameter were first exposed to oxygen plasma for 2 minutes. This process is needed both to clean the glass coverslips and also to enhance the hydrophilicity of their surface in order to facilitate the transfer of the ECM protein from the PDMS stamp onto the glass. The PDMS stamps were inked with 200 µL of fibronectin solution on the pillars and a plastic tip was used to help the solution to spread on the top of the stamp in a uniform way. This step is needed because PDMS is a hydrophobic compound that is reluctant in accepting a water based solution. The stamps were 'inked' for 5 minutes, then the excess fibronectin solution was removed by tapping the corner of the stamp (not the pillars) on a tissue paper and they were dried by a stream of compressed air. For collagen, cotton swabs were used to coat the stamp instead of pipetting the solution, followed by compressed air drying. The time of the drying could vary between 20 and 40 seconds. After these steps, the PDMS stamps were placed in conformal contact with the glass coverslips for ~ 2 minutes and a 50 g weight was placed on the stamps to aid in protein transfer across the entire well. After ~ 2 minutes of conformal contact, the coverslips were removed from the stamps with the help of tweezers and carefully placed in the 24 well plate that had been previously coated with the pluronic solution, which aids in repelling cell attachment to the bare plastic underneath the patterned coverslips.

Following execution of the above-mentioned process, the coverslips were washed once with 1X PBS and once with ddH2O to remove excess protein. The sterilization of the plate was done by putting 70% ethanol to cover the coverslips in the wells for at least 1 hour in the bio-safety cabinet (BSC). After this time the coverslips were rinsed three times with ddH2O to

remove the ethanol from underneath the glass surface. At this point the plate was ready to accept the cells, otherwise it was sealed and placed at 4ºC until needed for cell culture experiments.

1.2.2 Microcontact printing of collagen and fibronectin on polyacrylamide gels

Plates containing wells pre-coated with polyacrylamide or PA gels of ~ 25kPa sulfo-SANPAH treated (Softwell, Matrigen) were subjected to the microcontact collagen printing method described in section 1.2.1, except that a concentration of 100 µg/mL of rat tail collagen I was used, along with determination of stamping with or without the 50 g weight placed on the stamp.

1.2.3 Domains morphology assessment and quantification

We wanted to analyze the islands shape before cell seeding and to do so we proceeded by fluorescently tagging the protein after stamping by 1-hour incubation at room temperature (in the dark) with Alexa Fluor 488 carboxylic acid, succinimidyl ester (Invitrogen) diluted in 1X PBS at 20 µg/mL concentration [20]. This is an epifluorescent dye that binds to the ammine groups of the proteins. Then with a fluorescence microscope (green channel, 490 excitation maximum – 525 emission maximum) the patterned islands on the coverslip surface were visualized.

Images of the whole coverslips were acquired using the microscope OLYMPUS IX83P2ZF (Life Science) and islands were quantified and analyzed by the software ImageJ.

1.2.4 Cell selection and seeding

HepG2 (differentiated hepatocellular carcinoma cell line) were purchased from American Tissue Culture Collection (Manassas, VA) and were used to morphologically analyze the attachment of cells to the patterned domains. Furthermore, a live/dead assay (viability/cytotoxicity kit, Invitrogen) was used to detect the cell viability on the islands. HepG2 were thawed in 37ºC water bath for 120 seconds and suspended in hepatocyte seeding media with (formulation described previously [21]). Then the suspended cells were centrifuged at 500 xg for 5 min, the culture medium was dismissed and the hepatocytes were suspended again in the proper hepatocyte medium that was rid of serum. During cell counting, viability was assessed by trypan blue exclusion method and cells were seeded on the patterned coverslips at a density of 1 million cells/mL. Plates were shaken every twenty minutes for four hours to make sure that the ECM islands in the wells could have a uniform coat of seeded hepatocytes. After the circular domains were > 85% filled with hepatocytes, the cells that had not adhered were discarded by rinsing the wells with culture medium (99% 1X DMEM + 1% PEN/STREP) to prevent nonspecific attachment of cells to bare glass surface due to adsorption of serum proteins from the medium. We wanted then to check cell viability on the stamped islands. The live/dead assay was performed before cells fixation (30 - 45 min at room temperature, in the dark) and we observed cell viability (green channel) and death (red channel) under the EVOS FL microscope (Thermo Fisher Scientific).

We proceeded with another cell type, specifically with primary human hepatocytes (PHHs) and we obtained a good pattern of these cells on both collagen and fibronectin domains. In particular, cells from the donor HUM4055A (54 years old white female who died of stroke)

were used and they were seeded at a density of 0.666e6 cells/mL. Viability was again assessed by trypan blue exclusion and culture plates were shaken with the same procedure used for HepG2. The next day, after PHHs were spread to fill in the protein islands, 3T3-J2s cells (murine embryonic fibroblasts), which were cultured and passaged as previously described [20], were seeded at a density of 0.3e6 cells/mL (~ 90k cells per well in a 24-well plate) in order to obtain micropatterned co-cultures (MPCCs) on ECM stamped glass coverslips.

1.2.5 Cell morphology and function assessment

The morphology of the cells was assessed and controlled using the EVOS FL microscope (Thermo Fisher Scientific) with 4x, 10x and 20x phase contrast objectives. Culture supernatants were collected every two days for two weeks and assessed for albumin and urea concentrations using the protocols as previously described [20]. Briefly, human albumin concentration was assessed using a competitive enzyme-linked immunosorbent assay (ELISA) with horseradish peroxidase detection and the substrate 3,3',5,5'-tetramethylbenzidine (TMB, Rockland Immunochemicals, Boyertown, PA), whereas urea secretion was analyzed by a change-color-reaction that involve diacetyl monoxime, acid, and heat (kit from Stanbio Labs, Boerne, TX). Cytochrome P450 (CYP450) enzyme activities were measured via the incubation of the cultures with the substrates for 60 minutes at 37ºC and then luminescence or fluorescence of metabolites were identified using previously described protocols [20]. CYP2A6 enzyme activity was quantified by the modification of coumarin to fluorescent 7-hydroxycoumarin (Sigma-Aldrich, St. Louis, MO), and CYP3A4 activity was measured by cleavage of luciferin-IPA into luminescent luciferin (Promega, Madison, WI) using previously described protocols [20].

Specifically, to measure absorbance, fluorescence and luminescence the BioTek Synergy H1 (BioTek instruments, Winooski, VT) was used.

1.2.6 Data analysis

Each experiment was carried out in triplicate wells for each condition. Data processing and visualization were performed using the ImageJ, Microsoft Excel and GraphPad Prism (La Jolla, CA) softwares. Error bars on graphs represent standard deviation for each condition. Statistical significance was calculated using two-way analysis of variance (ANOVA) with a Dunnett's post-hoc test.

1.3 RESULTS

1.3.1 Morphology of collagen and fibronectin islands on glass coverslips

Rat tail collagen I and human fibronectin could be patterned onto glass coverslips and the island geometry resembled the pillars on the PDMS stamp used for the printing (**Figure 2**). In comparison to previously published oxygen plasma technique [17] the islands were less uniform, with some holes in the center that did not affect cell seeding (**Figure 3**). With the collagen pattern an average diameter of 498.78 μm and an average diameter of 475.43 μm with fibronectin were quantified using ImageJ (**Table I**), which is similar to the ~ 500 μm diameter of each pillar on the PDMS stamp used. A statistical analysis was performed, showing that the average diameter obtained with the fibronectin stamping was significantly lower with respect to the average diameter revealed with the collagen pattern from the plasma technique. Given the range of 125600 μm^2 – 282600 μm^2 that correspond to islands with 400 and 600 μm in diameter, the software was able to detect 76 circular domains of collagen and 46 of fibronectin. Moreover, a good center-to-center island spacing was obtained during the protein transfer, with 1209.64 and 1188.12 μm for collagen and fibronectin patterns, respectively (**Table I**); none of the technique was significantly different from the collagen plasma control. All these numbers were close to 1200 μm spacing of the pillars on the stamp itself.

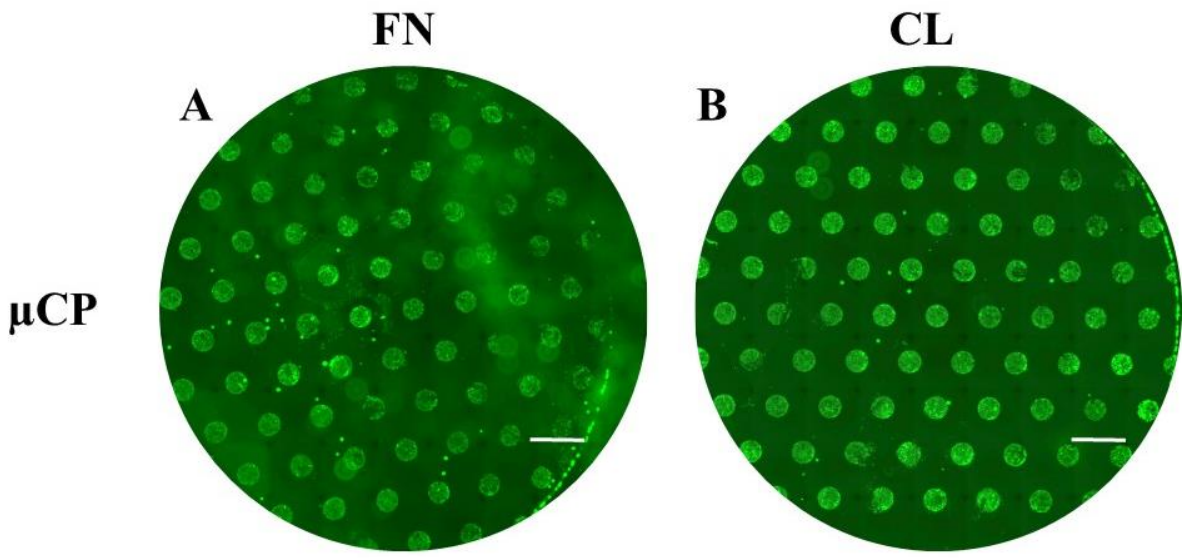

Figure 2. Morphology of the circular domains obtained on glass coverslips by microcontact printing (µCP) of ECM proteins. (A) µCP of human fibronectin (FN) diluted in 1X PBS at a concentration of 50 µg/mL. (B) µCP of rat tail collagen I (CL) diluted in ddH2O at a concentration of 50 µg/mL. All scale bars are 1200 µm.

TABLE I

ANALYSIS OF ECM ISLANDS COMPARING THE OXYGEN PLASMA AND THE MICROCONTACT PRINTING TECHNIQUES.

Substrate	**ECM**	**Island diameter**	**Island spacing**
Plastic - plasma	Fibronectin	498.41±24.9 N=65	1194.62±30.3 N=10 random
Plastic - plasma	Collagen	499.87±29.6 N=87	1199.41±28.1 N=10 random
Glass Coverslip - stamping	Fibronectin	475.43±38.1 **** N=46	1188.12±16.1 N=10 random
Glass Coverslip - stamping	Collagen	498.78±33.3 N=76	1209.64±24.4 N=10 random

Average island diameter and average islands spacing is shown for each technique.
**** $P < 0.0001$.

1.3.2 Cell morphology and viability on printed glass coverslips and polyacrylamide gels

HepG2 and PHHs were successfully patterned on ECM-printed glass coverslips and the live/dead assays performed on HepG2s showed that cells were attached and viable on collagen islands (**Figure 3A and 3B**). PHHs patterning on fibronectin needed more time than on collagen but led to the same positive results with respect to viability (**Figure 3C and 3D**). The hepatocytes attached in every island even if at the fluorescein level we observed different intensity among islands, suggesting variability in the amount of protein transferred per island. Nonetheless, any differences in amount of protein transfer did not significantly affect cell adhesion and spreading.

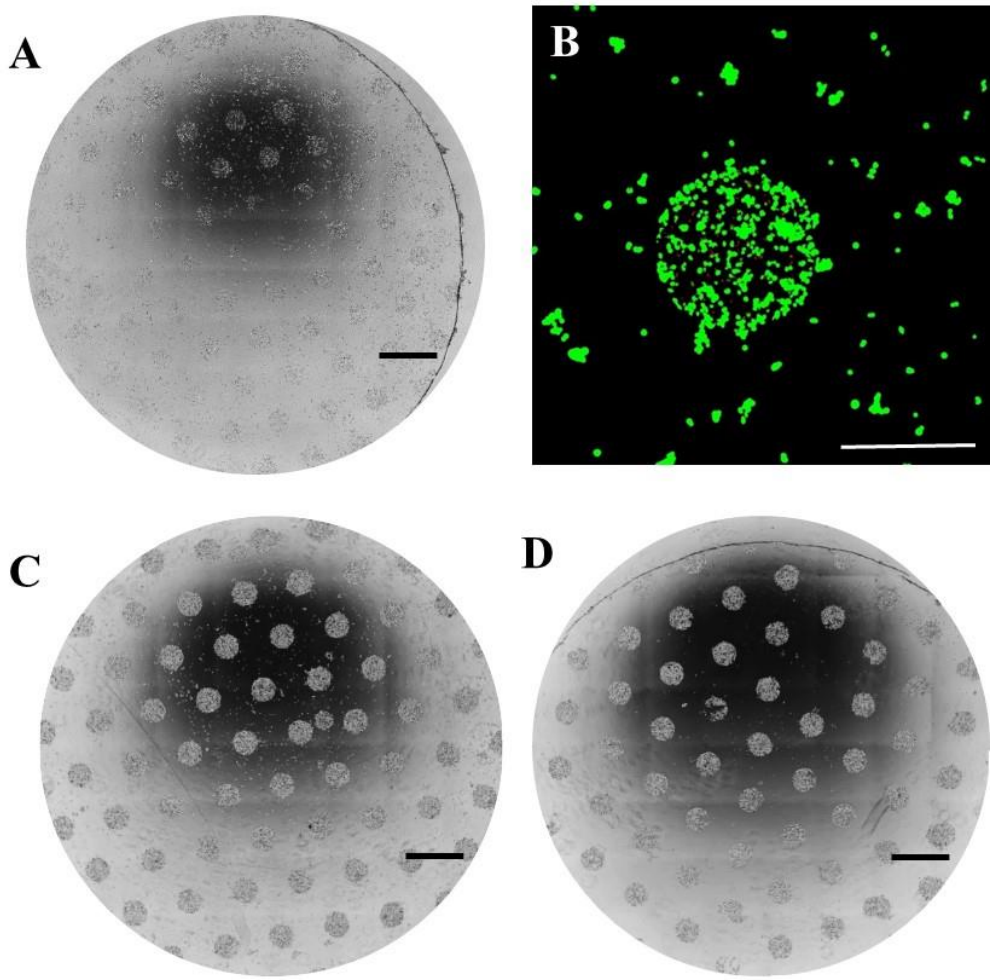

Figure 3. Hepatocytes seeded on ECM domains on glass coverslips. (A) HepG2 on CL islands. Scale bar = 1200 µm. (B) Live-dead assay performed on HepG2. Scale bar = 500 µm. (C) PHHs on CL islands. Scale bar = 1200 µm. (D) PHHs on FN islands. Scale bar = 1200 µm.

Moreover, HepG2 were successfully seeded on PA gels of 25 kPa stiffness patterned with 100 µg/mL rat tail collagen I (**Figure 4**). The cells spread a bit outside the islands too, which may be due to excess collagen transfer; nonetheless, the circular geometry of the protein domains was maintained. As it is shown in **Figure 4B**, HepG2s were able to be fully seeded on the islands

obtained with a 50 g weight on the stamp, while they were not able to properly attach on the islands obtained without the use of that weight (**Figure 4C**). For the latter, the collagen was not properly transferred from the stamp to the gel surface, which led to poor cell attachment in certain parts of the patterned domains.

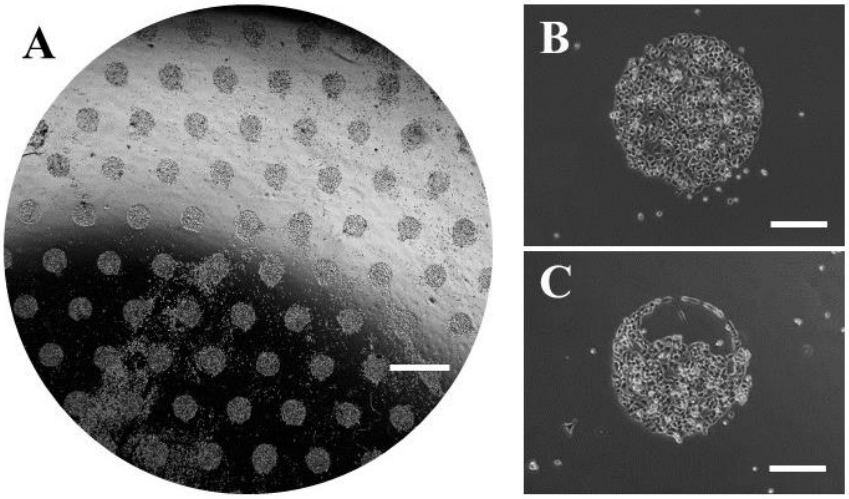

Figure 4. HepG2 seeded on 100 μg/mL rat tail collagen I domains on 25 kPa PA gels. (A) Scale bar = 1200 μm. HepG2 attached on protein domains obtained with the use of 50 g weight on the PDMS stamp (B) and on protein domains obtained without the use of a weight (C). Scale bars = 200 μm.

1.3.3 Morphology and function of MPCCs on printed glass coverslips

3T3-J2 fibroblasts could be co-cultured with PHHs (MPCCs) on both collagen and fibronectin patterns on glass coverslips (**Figure 5**). On both patterns, hepatocytes could maintain prototypical hepatic morphology and pattern fidelity for at least two weeks and both co-culture formats displayed lipid accumulation and bile canaliculi formation, as it is characteristic of MPCCs from previously published literature [20].

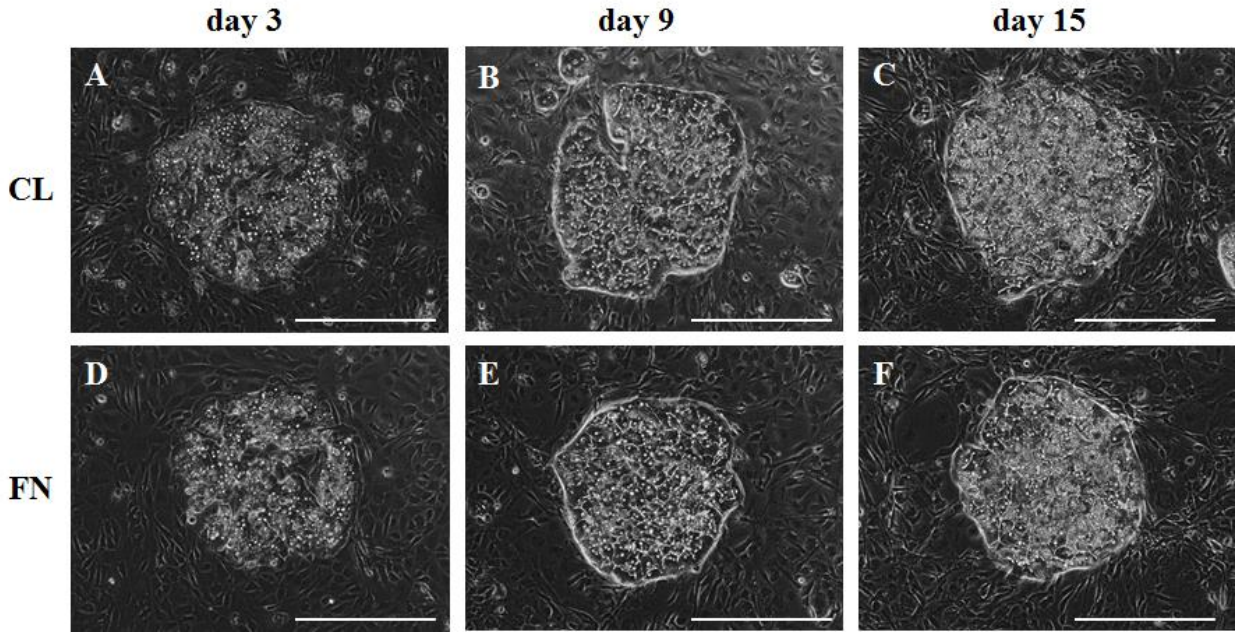

Figure 5. MPCC morphology on glass coverslips patterned with collagen (A-C) and fibronectin (D-F). All scale bars are 500 μm.

At the functional level, to verify that our stamped pattern was as good as oxygen plasma pattern to maintain liver cell functions, albumin and urea secretions and CYP450 enzyme activity were tested and the functions of MPCCs established on collagen plasma patterns were used as control. As shown in **Figure 6A**, albumin secretion increased after the third day of culture and was then approximately constant between day 7 and day 15 of culture. Albumin secretion levels in stamped conditions were typically lower than the plasma pattern control (no statistical significance), albeit kinetic trends across the conditions were similar. All graphs were normalized on a per cell basis because on the glass coverslips the patterned islands where less than what it can be obtained with the plasma ablation technique. We counted the number of islands obtained with both techniques and an average of ~ 80 islands with microcontact printing and ~ 90 islands with oxygen plasma was estimated, giving ~ 26,000 and 30,000 PHHs, respectively. Regarding urea secretions, MPCCs on both stamped and plasma patterns had a similar constant secretion for two weeks (**Figure 6B**). The above discussed results suggest that MPCCs could be established on both collagen and fibronectin stamped in a precise way on glass coverslips and that these patterns could maintain hepatocyte functions for up to two weeks, but not at the same magnitude as the plasma technique. These results support the use of the microcontact printing technique using different ECM proteins for the purposes of in vitro liver tissue modeling.

For CYP450 enzyme activities (**Figure 6C and 6D**), both CL stamping and CL plasma conditions had similar CYP2A6 activities at both day 7 and day 15 of culture; FN stamping condition for this enzyme was lower the first week but then it increased during the second week of culture. Regarding CYP3A4, we observed significantly lower activities of hepatocytes cultured on the stamped patterns compared to the plasma control at the end of the two weeks ($P < 0.0001$), while after the first week just the fibronectin stamping condition led to significantly

lower hepatocytes CYP3A4 activity ($P < 0.05$) compared to the plasma control. Even during the CYP450 data analysis we normalized the results on a per cell basis, considering 1 million cells in our cultures. These results suggest that the stamped patterns could maintain and support PHH functions to a certain extent, but not to the same extent as the plasma pattern, at least for albumin secretion and CYP3A4 activity.

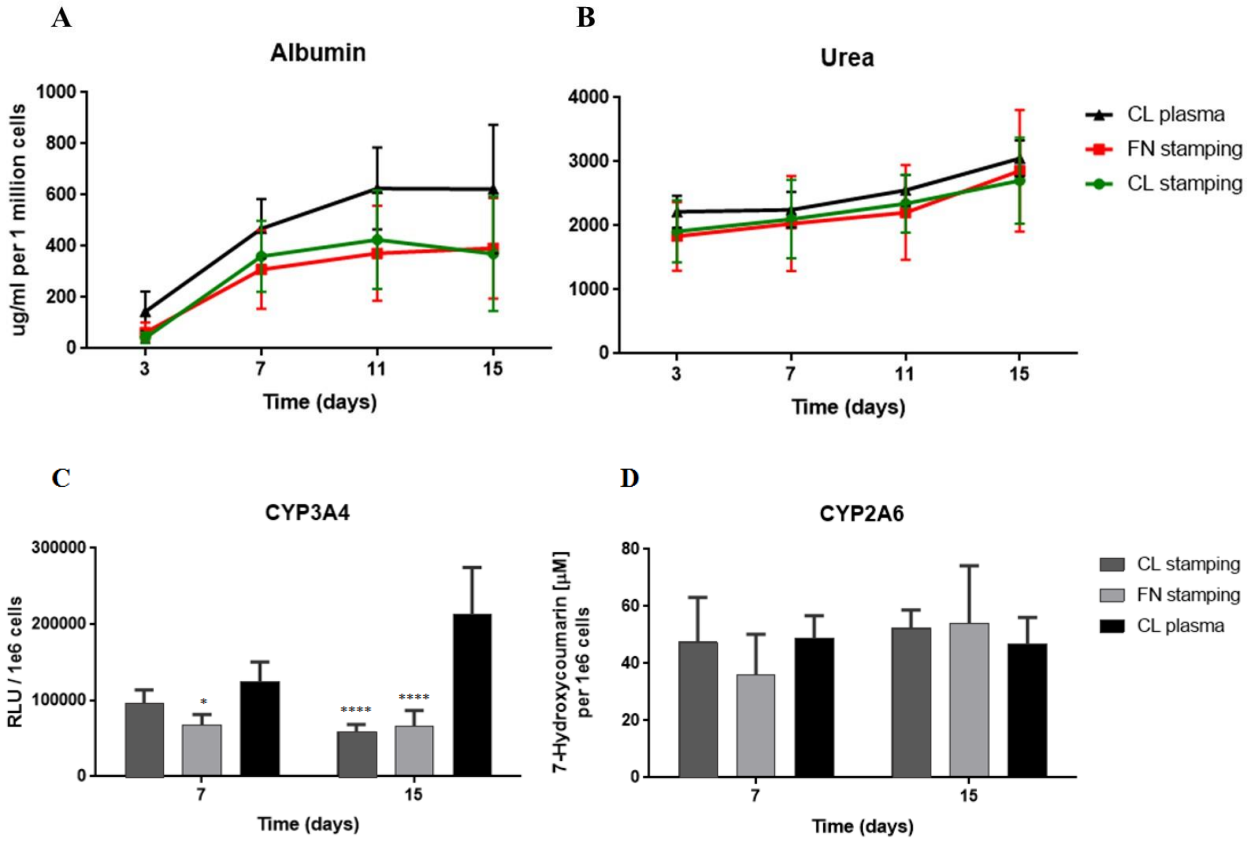

Figure 6. Functions of MPCCs on either printed or plasma-treated domains. Albumin and urea secretion, and CYP450 enzyme activity of MPCCs established onto collagen and fibronectin domains stamped on glass coverslips and on collagen islands obtained with the plasma ablation technique during two weeks of culture. (A) Albumin secretion; (B) urea secretion; (C) CYP3A4 activity; (D) CYP2A6 activity. Statistical analysis was performed relative to the plasma condition. * $P < 0.05$, **** $P < 0.0001$.

1.4 DISCUSSION

In this study, we optimized the microcontact printing technique in order to establish an *in vitro* liver model for drug screening and investigating liver related pathways on different ECM protein patterns, such as collagen I and fibronectin and on different substrates, such as glass and polyacrylamide. This technique can be used to observe cell morphology and functions on different protein patterns. The results of different protein patterns showed that rat tail collagen I and human fibronectin can be patterned on glass coverslips maintaining the pillar geometry of the PDMS stamp. Moreover, collagen can be stamped on polyacrylamide gels of 25 kPa stiffness. In particular, this value corresponds to cirrhotic liver [7], but is still much lower than the stiffness of commonly used glass and plastic (~ GPa). In the future, this technique will be used to establish liver models on different surfaces (e.g. softer gels such as 4 kPa and 0.2 kPa that correspond to fibrotic and healthy liver stiffnesses [7]) for long-term studies.

The optimization of all the parameters assessed in our protocol was done gradually, considering few variables in each attempt. For instance, in order to decide the correct amount of protein concentration we started with rat tail collagen I of 1 mg/mL diluted in ddH2O. This is a high concentration but we wanted to be sure that the collagen was transferred while using the fluorescein in imaging. The second attempt was done using 100 µg/mL collagen solution but visually the pattern was not so uniform as with the higher concentration but the cells attached regardless, so we decided to lower the concentration further down to 50 µg/mL that led to fair pattern and good cell attachment. Reducing the concentration of the solution is important in order to save protein as our protocol is designed to be used with different ECM proteins that may be not abundantly available and/or are very expensive to source. Another variable of interest was the cell culture surface on which the protein solution was stamped. The first attempt was done on

the 24 well tissue culture polystyrene plates, but we were not able to reproducibly stamp proteins onto this surface across the entire well. Such an issue may be due to the polystyrene plate itself since it is made with injection molding that can lead to irregular surfaces not conducive to stamping. On the other hand, glass coverslips are manufactured to be flatter and more regular in surface morphology than injection molded polystyrene. The size of the glass coverslips was chosen to be able to place them into the wells of the 24-well plate to enable multi-well screening applications. Another parameter taken into account was the inking method. The first attempts with stamping on tissue culture plates were done by pipetting the collagen solution of 200 μL directly onto the features of the PDMS stamps and waiting 20 minutes for the solution to absorb. The degree of hydrophilicity or hydrophobicity of the stamp where the protein solution is temporarily patterned depends on the nature of the ECM solution itself. It is obvious that there needs to be a sort of binding between the stamp and the protein. This binding have to be firm enough such that the solution can be disconnected from the stamp, and that the pattern is stable on the final receiving surface. After protein absorption on the pillar surfaces, the stamp was positioned with the coated features against the glass surface and after one minute the pattern was properly transferred. This is the procedure mostly adopted by researchers [12, 22]; however, the pattern obtained with this method was not very satisfactory in our case. In particular, we observed a non-uniform transferred pattern between adjacent coverslips and in some cases also non-uniformity in the islands of the same coverslip. Such a result could be due to the variable drying rates of the collagen solution adhered to different parts of the PDMS stamp. Thus, a different method was pursued using a cotton swab inked with the protein solution. In 10 minutes, all the inking, drying and printing steps were done, while with the pipetting of the solution, more than 30 minutes were needed; thus, we cut down the time for experimentation via our use of the

cotton swabs. With the swabbing a good collagen pattern was achieved while with the fibronectin solution we preferred to stay with the pipetting method modifying the inking and stamping time, 5 minutes and 2 minutes respectively, as a better pattern was achieved with this technique. Regarding the oxygen plasma and its ability to confer hydrophilicity to the surfaces, we tried to apply it also to the PDMS stamps but we observed that the protein transfer was worse, perhaps because the plasma confers too much hydrophilicity to the stamps and they retain some solution that was not transferred onto the coverslip surface. By keeping the stamps hydrophobic a better transfer was achieved because the solution was more likely accepted by the hydrophilic coverslips. Regarding the drying time, compressed air drying, room temperature drying and hot plate drying were tried and the best result was reached using compressed air directly on the inked stamps. When drying at room temperature, more time was needed for the protein to dry up, and the transfer was not as satisfactory as when compressed air was used. Moreover, by the use of compressed air, more time was saved because just 20 - 40 seconds were needed to completely dry the stamps.

As shown in **Figure 2,** there is a detectable difference in FN and CL islands pattern. By giving a range of island area measurements to the software ImageJ, we obtained back the number of areas present in the figure that were contained in that range, corresponding to the number of circular domains present in the well represented in the figure. The software detected 46 islands for fibronectin coating and 76 for collagen (**Table I**). This difference is due to the fact that some islands did not maintain a perfect circular geometry and moreover some non-ECM fluorescent spots were present in the well at the moment of the capture of the image that affected the quantification analysis. However, the direct manual count of patterned islands is comparable among the two proteins. This count showed that on the coverslips we can obtain ~ 80 domains,

while it was previously shown that with the oxygen plasma ~ 90 islands can be patterned in each well of a 24-well polystyrene plate [23, 24]; such is due to the differences in the overall stamp diameter.

Two different liver cells were used in this study. HepG2 is a well differentiated human hepatocellular carcinoma (HCC) cell line which was isolated from the HCC of a 15-year-old Caucasian American. This cell line is widely used in hepatotoxicity research [25, 26] and we utilized it here for morphological and viability studies. The presence of fluorescein bound to the collagen did not affect cell attachment; however, more cell spreading outside the collagen doamins was observed in the presence of fluorescein relative to the unlabeled collagen control. HepG2 did not attach to fibronectin-coated surfaces (data not shown), thereby necessitating the use of PHHs for further studies since PHHs are known to adhere to adsorbed fibronectin in previous studies [27]. PHHs adhered onto both fluorescent and non-fluorescent circular domains showing satisfactory micropatterned islands (**Figure 3**). Further analysis regarding the quantification of the amount of protein transferred will be necessary in the future, because in one of our experiments we didn't get cell attachment on the glass coverslips even in presence of the pattern. We hypothesize that this phenomenon could be due to the low protein amount that was actually transferred even if at the fluorescein level the coverslips were similar to each other.

MPCCs established on the collagen domains obtained with the oxygen plasma ablation technique were shown to improve CYP3A4 enzyme activity (**Figure 6C**) and albumin secretion (**Figure 6A**) over two weeks as compared to the stamping technique, while CYP2A6 (**Figure 6D**) and urea synthesis (**Figure 6B**) showed similar trends of activity among the conditions. The data were normalized on a per cell basis because a different number of islands and subsequent number of seeded hepatocytes were obtained with the two patterning techniques. Even after

normalization by cell number, albumin secretion and CYP3A4 were higher in the plasma patterned MPCCs. However, because urea synthesis and CYP2A6 activity were comparable, we could have over-estimated the number of hepatocytes actually seeded onto the CL and FN stamped glass coverslips. As discussed earlier, some islands were not fully filled by PHHs because of the low protein amount that was transferred and this could have led to an even lower number of PHHs adhered. Moreover, at the edges of the glass coverslips we obtained a non-uniform pattern, caused by islands, or part of them, filled by PHHs that de-differentiated over the course of a few days because they were not properly surrounded by fibroblasts which are needed to support hepato-specific functions. In the future, we plan to design a more thoroughly investigation into the actual number of functional hepatocytes in each condition (e.g. cell viability analysis).

Polyacrylamide patterns were analyzed just by cell seeding since fluorescein was absorbed by the gel, thereby leading to high background fluorescence. In particular, HepG2 were patterned onto 25 kPa PA gel. PA gels have been the gold standard for studying cell behavior and response to structures with elastic modulus from 1 to 100 kPa [10, 28] and this range is close to the stiffnesses of many soft tissues. In the field of regenerative medicine, it is important to study cells' behavior *in vitro* on these soft substrates in order to analyze the physiologically relevant behavior that cells would display *in vivo*. Conventional micro-contact printing has some limitations when dealing with soft substrates because sagging of the substrate and difficulty in removing the stamp will cause nonconformance in the pattern and moreover the possible disruption of the pattern itself [12]. In addition, patterning on PA gels is challenging because of its hydrogel nature and the aqueous environment, so, in the general field of cell biology, even though there is much enthusiasm for patterning on soft substrates, it is still a current engineering

challenge [13]. In this study, however, we successfully stamped proteins onto a PA gel since it was covalently bonded to a plastic backing of a plate by the manufacturer.

In conclusion, we optimized a microcontact printing protocol to pattern different ECM proteins onto glass coverslips and PA gel of 25 kPa to establish liver cell attachment and liver cell culture models. In particular, MPCCs of PHHs and 3T3-J2 fibroblasts were established on collagen and fibronectin patterned coverslips. PHH functions in these models showed similar trends to the oxygen plasma model, especially for urea synthesis and CYP2A6 activity. In the future, we aim to establish the MPCC model onto softer PA surfaces to determine hepatocyte phenotype as a function of varying stiffnesses; such as inquiry can aid in the engineering of models of normal liver physiology and liver diseases, especially fibrosis and cirrhosis. Ultimately, as we show here, microcontact printing is a promising technique to control cell-cell and cell-ECM interactions for liver cell cultures adhered to substrates of varying stiffness.

CHAPTER 2. DETECTION OF ENDOTHELIAL PROTEIN MARKERS IN HUMAN LIVER CULTURE PLATFORMS

2.1 INTRODUCTION

2.1.1 Importance of liver sinusoidal endothelial cells in liver

In order to develop an engineered multicellular liver model that has benefit in the pharmaceutical industry, it is important to understand how the cells interact with each other *in vitro* and how they enhance or inhibit their respective functions relative to the complex physiology of the liver. In order to establish a valid model, cells need to be clearly identified and characterized in culture as they can change phenotype or express different markers depending on the interactions with other cell types [29]. Primary human liver sinusoidal endothelial cells (LSECs) make up ~ 70% of all the non-parenchymal cell (NPC) types present in the liver [30]. The space of Disse, an extracellular matrix proteins layer, separates LSECs and hepatocytes within the hepatic sinusoid (**Figure 1**). LSECs contain fenestrae that create a discontinuous endothelial membrane and are important in liver physiology for the exchange of nutrients and other molecules between the blood and the hepatocyte layer. LSEC fenestrae dimensions range between 50 and 150 nm [31]. Fenestrae number and diameter varies under physiological and pathological conditions [31]. LSECs are positioned at the interface between the sinusoidal side, where they are exposed to the mixture of arterial blood and the portal blood coming from the gut and the pancreas, and the luminal side, where they interact with hepatic stellate cells and regulate the functional maintenance and regeneration of hepatocytes through paracrine action [32].

LSECs thus enable a permeable barrier allowing exchange of molecules to hepatocytes and stellate cells. Crosstalk between LSECs and hepatocytes (paracrine interactions and contact signaling) is thus essential for maintaining endothelial cell growth and differentiation [33] as it is known that when cultured alone, both hepatocytes and LSECs lose their phenotypic function in a few days [32].

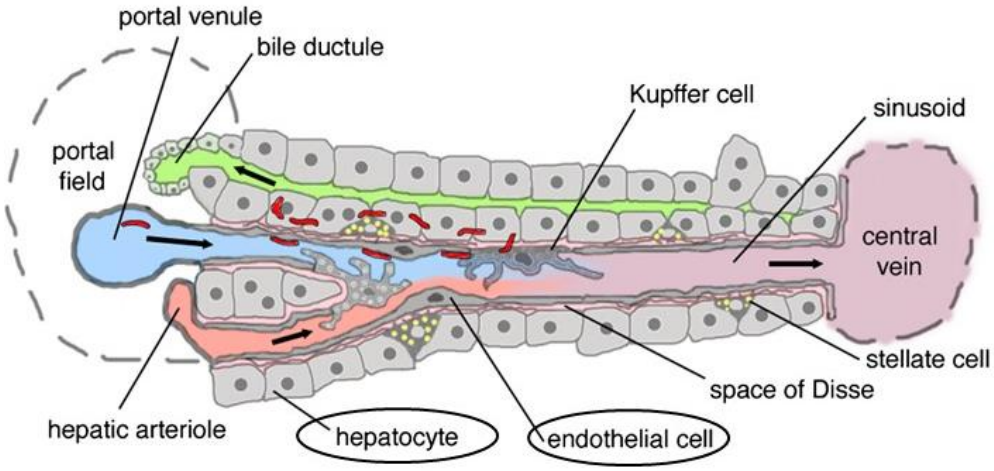

Figure 7. Liver sinusoid showing the spatial location of hepatocytes and endothelial cells in the liver lobule. From [70].

2.1.2 Research on liver sinusoidal endothelial cells characterization

The identification of the numerous fenestrations contained by LSECs is the most optimal way to assess their true phenotype but this is only possible via electron microscopy and its use is impractical for routine phenotyping of high-throughput systems. Markers that have been utilized to recognize LSECs include FcγIIb2 [33], CD32b (also called anti-SE-1) [32], stabilin-2 [34, 35, 36], L-SIGN [36, 37], LYVE-1 [38, 39, 40], CD31 [41, 42], CD54 [43], Factor VIII [44 - 47], and vWF [48]. The presence of many of these markers in LSECs remains controversial [48]. The dissimilarities between *in vivo* and *in vitro* observations, in addition to differences among species, need to be further understood. In fact, one important LSEC-specific marker is CD31 (cluster of differentiation 31 or PECAM-1) that is a glycoprotein present at high levels on different types of endothelial cells [41, 42]. The expression on endothelial cells is localized at junctions between adjacent cells. CD31 is known to have various roles in vascular biology and to be located intracellularly in rat LSECs right after the establishment of cultures, but later it is expressed on the membrane surface [49, 50]. However, LSECs share many markers with other cell types [48]; for instance, CD31 is also expressed by leukocytes and platelets [36, 51]. An interesting marker is the antigen recognized by the SE-1 antibody [52] that was shown to correlate with the presence of fenestrations on rat LSECs [32]; however, it is not clear if this marker is also expressed in other types of endothelial cells outside of the liver. To date, no single marker has been identified that is uniquely expressed by LSECs both *in vitro* and *in vivo* in humans; thus, it is necessary to measure multiple markers to monitor their presence in *in vitro* LSEC cultures [48]. In addition, the need for clear identification is even more important when LSECs are cultured with other cell types, and culture platforms that involve more than one cell type are necessary for applications in drug screening and regenerative medicine [29].

In the literature, several studies that involve LSECs co-cultures have been performed [53] but many utilize non-human derived cells, which are known to be significantly different than their human counterparts in metabolic and other phenotypic functions [54, 55, 56]. Recently, Ware B. et al showed that at the gene expression level, when LSECs are tri-cultured with 3T3-J2 murine embryonic fibroblasts and primary human hepatocytes (PHHs), the LSECs display an increased expression of CD31 and Factor VIII at the mRNA transcript level over three weeks, relative to co-cultures containing LSECs and PHHs or LSECs monocultures [43]. However, while pioneering, this study lacked phenotypic (functional) characterization of the endothelial cells; it is known that gene expression does not always correlate to functional outcomes [57].

2.1.3 Purpose of the study

In this work, we characterized LSECs phenotype in mono, co- and tri-cultures at the functional level. Phenotypic characterization of Factor VIII and immunostaining of CD31 and SE-1 markers were performed over a 2-week period among the various culture formats. Adopting the tri-culture models established in [43] and the data reported in that study, we hypothesized that LSECs function could be enhanced while cultured with PHHs and fibroblasts at the functional level. A general issue of using primary LSECs is their limited availability; thus, we also investigated the phenotype of more readily available human umbilical vein endothelial cells (HUVECs) in the same platforms used for LSECs cultures towards determining if HUVECs can become LSEC-like cells when cultured with PHHs and fibroblasts.

2.2 MATERIALS AND METHODS

2.2.1 Creation and maintenance of cultures

Primary Human Liver Sinusoidal Endothelial Cells (LSECs) and Human Umbilical Vein Endothelial Cells (HUVECs) were selected as human endothelial cell types to use in the experiments. HUVECs were selected as the non-liver control. Primary endothelial cells were cultured at 37ºC, 10%CO2 in EGM-2 BulletKit medium (Lonza, Walkersville, MD) on tissue culture polystyrene coated with 2 µg/cm^2 fibronectin (Corning Life Science, Manassas, VA). The endothelial cells were treated with 0.05% (m/vol) Trypsin-EDTA for 5 minutes at 37ºC to release cells into suspension, centrifuged, re-suspended in fresh culture medium supplemented with human vascular endothelial growth factor (VEGF, Thermo Fisher Scientific, Waltham, MA) and seeded in co-culture with PHHs at a density of 0.3e6 cells/mL.

In order to micropattern PHHs on collagen coated domains of 500 µm in diameter and 1200 µm center to center spacing, rat tail collagen I (Corning) was lithographically patterned using an oxygen plasma technique in each well of a 24 well format plate [58]. Briefly, collagen was diluted in ddH2O at 25 µg/mL concentration and 300 µL/well of this solution was incubated in wells at 37ºC for 2 hours. Then, after a double rinsing of ddH2O and subsequent drying, a polydimethylsiloxane (PDMS) master mold was placed in the plate to protect regions of the collagen-coated surface from oxygen plasma such that once the mold is removed, the protected protein is maintained, obtaining ~ 90 micropatterned collagen domains for cell attachment [68, 69]. PHHs from donor HUM4055A (54-year-old white female who died of stroke) were seeded at a density of 0.66e6 cells/mL and they selectively attached to the collagen domains. The overnight medium used was supplemented with 2 µg/cm^2 fibronectin to coat the remaining

surface area of the well not used by PHHs to enable attachment of the other NPC types (3T3-J2 murine embryonic fibroblasts, LSECs and HUVECs) the following day. The NPC types, after trypsinization and centrifugation, were re-suspended in culture medium containing 40 ng/mL recombinant VEGF in a high-glucose Dulbecco's modified eagle medium base (Corning) and were subsequently seeded at a density of 0.3e6 cells/mL. In particular, micropatterned co-cultures (MPCCs) and tri-cultures (MPTCs) were obtained by seeding different cell types (**Figure 2**). To create coplanar micropattened tri-cultures, ~ 45,000 endothelial cells (LSECs or HUVECs) were mixed in a 50:50 ratios with a suspension of ~ 45,000 3T3-J2 fibroblasts and this mixture was then seeded in each well of a 24 well plate. Culture medium was collected and changed every 2 days (300 µL/well) on co-cultures and tri-cultures for two weeks.

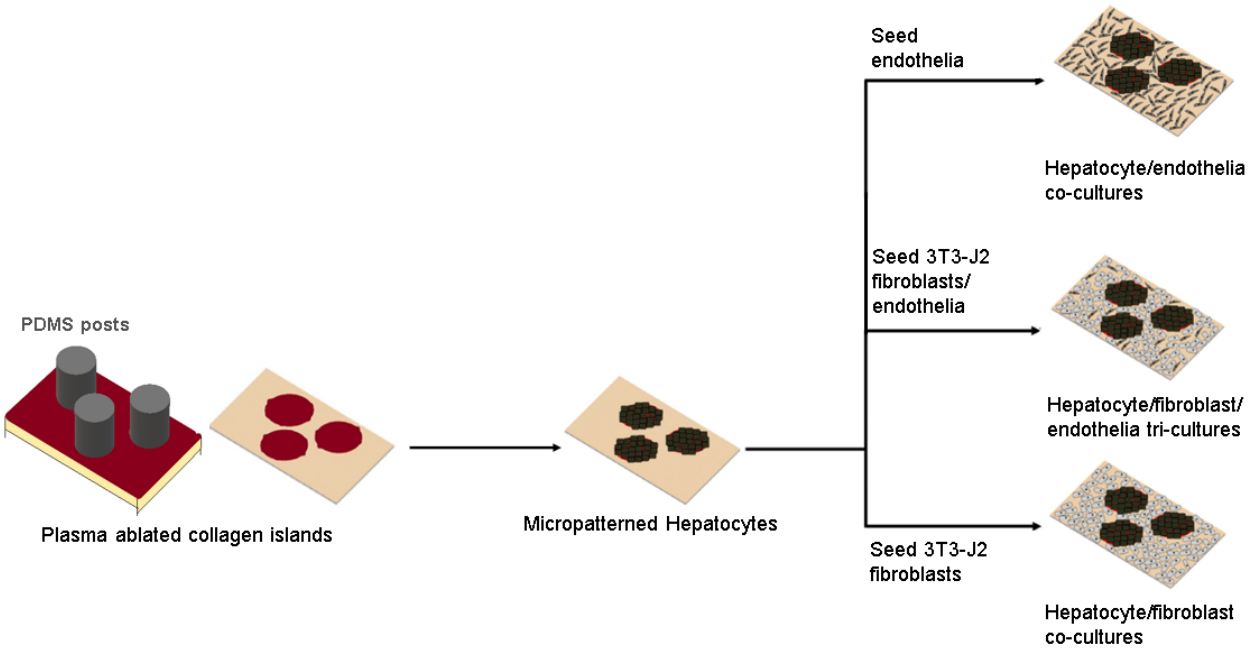

Figure 8. Schematic of co-cultures and tri-cultures creation. PHHs are attached on collagen islands and cultured along with fibroblasts and/or endothelial cells in the surrounding areas with a 50:50 ratio of fibroblasts:endothelia in tri-cultures. From [43].

Experiments were done with both growth-arrested or proliferative 3T3-J2 fibroblasts to evaluate their role in the cultures. To growth arrest the fibroblasts, 60 µL of Mitomycin C (Sigma) was mixed in 30 mL of fibroblast medium and this mixture was added to a T150 flask (Corning Life Sciences) containing the adherent fibroblasts. The flask was then placed in the incubator at 37°C for 4 hours. After this time, the flask was rinsed three times with 1X phosphate buffered saline (PBS, Corning Life Sciences) to remove any excess mitomycin and 30 mL of fibroblast medium (without mitomycin C) was added to the flask.

2.2.2 Assessment of cell morphology and endothelial markers

Cell morphology was assessed and monitored using an EVOS FL microscope (Thermo Fisher Scientific) with 4x, 10x and 20x phase contrast objectives. Endothelial characterization was performed by CD31 (PECAM-1) (Cell Signaling Technology, Danvers, MA) and SE-1 (Novus Biologicals, Littleton, CO) immunofluorescent staining.

Immunofluorescent staining was done by fixing the cells with 4% paraformaldehyde (PFA) solution in 1X PBS for 15 minutes at room temperature, followed by three rinses with 1X PBS. Then, the fixed cultures were blocked with 2% BSA + 0.03% Triton-X-100 in 1X PBS for 45 minutes at 37 ºC. After the blocking step, cultures were placed in the incubator at 37ºC with 2% BSA + 0.03% Triton-X-100 + 1:50 ratio (10 µg/mL) mouse anti-human CD31 antibody or 5µg/mL of mouse anti-human SE-1 antibody for 2 hours followed by three rinses with 1X PBS. Next, cultures were incubated at 37ºC for 1 hour with 2% BSA + 0.03% Triton-X-100 + 1:50 ratio (20 µg/mL) of donkey anti-mouse secondary antibody linked to a NorthernLights™ red fluorescent dye (R&D Systems). Immunofluorescent signal was assessed using the RFP (red

fluorescent protein) channel of an EVOS FL microscope (Thermo Fisher Scientific). Culture supernatants were collected every two days for two weeks and human Factor VIII expression was assessed with a sandwich enzyme linked immunosorbent assay (ELISA) kit per manufacturer instructions (Aviva System Biology).

2.2.4 Data analysis

Each experiment was carried out in four replicates for each condition in order to stain two wells and have lysates from the other two wells. Data processing and visualization were performed using Microsoft Excel and GraphPad Prism (La Jolla, CA). Error bars on graphs represent standard deviation for each condition. Statistical significance was determined using two-way analysis of variance (ANOVA) with a Dunnett's post-hoc test.

2.3 RESULTS

2.3.1 LSECs and HUVECs morphology over culture

Both primary LSECs and HUVECs displayed similar morphologic characteristics over a 15 days' culture period (**Figure 3**). At day 15, HUVECs showed a more elongated shape and started to patch together, leaving some holes between cells.

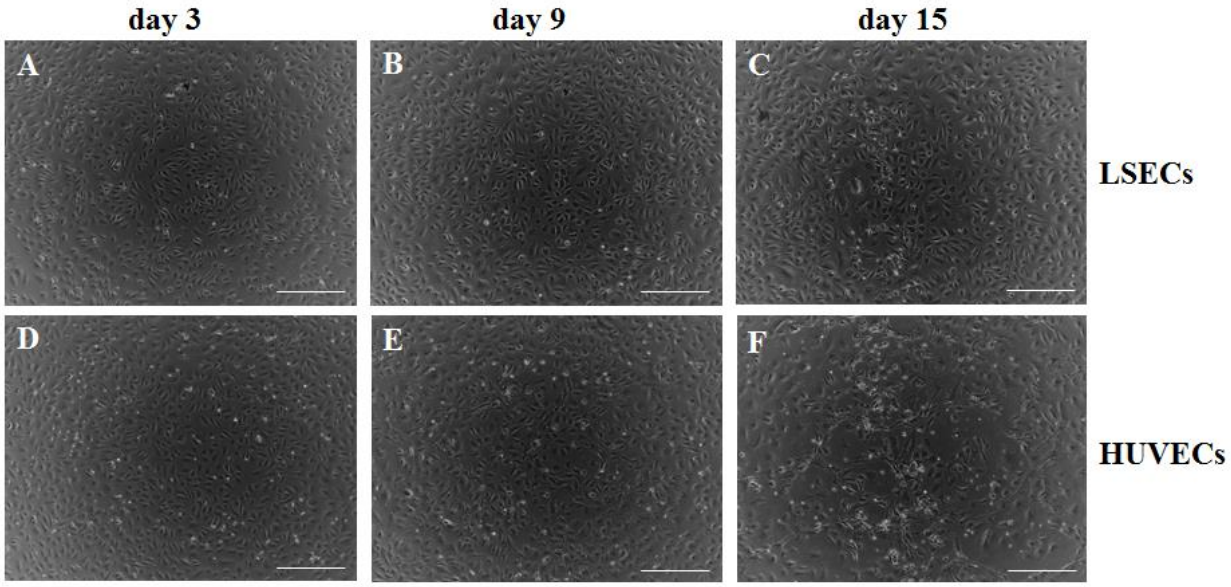

Figure 9. Two-weeks pure cultures of LSECs and HUVECs. Liver sinusoidal endothelial cells (LSECs, A-C) and human umbilical vein endothelial cells (HUVECs, D-F) over 2 weeks in mono-cultures. All the scale bars are 1 mm.

2.3.2 CD31 and SE-1 immunofluorescent staining of pure LSECs and HUVECs

CD31 protein staining was comparable in both LSEC and HUVEC monocultures and this result is supported by data previously found at the gene expression level [43]. In contrast, SE-1 expression was greater in LSECs (**Figure 4**). To assess this, we tried two different kind of dilutions of the primary antibody (10 and 5 µg/mL) based on vendor data and, interestingly, we obtained more signal from the lowest dilution. In particular, **Figure 4** shows the expression obtained with 5 µg/mL dilution from the staining performed on primary human LSECs and primary HUVECs fixed after 3 days of culture.

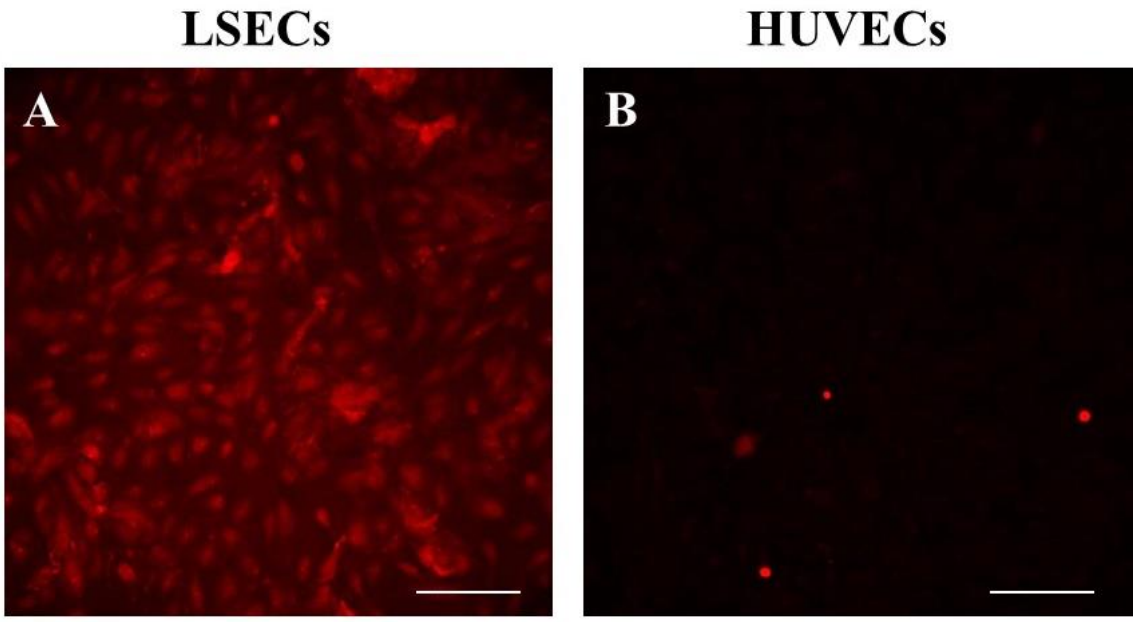

Figure 10. Sinusoidal endothelial (SE-1) marker immunostaining in LSECs (A) and HUVECs (B) at day 3 of culture. All scale bars are 200 µm.

2.3.3 Morphology of hepatocyte/endothelia and hepatocyte/fibroblast co-cultures relative to tri-cultures and monocultures

Micropatterned co-cultures of primary human hepatocytes and endothelial cells (**Figure 5J, 5K, 5M, 5N**) were compared to co-cultures of PHHs and fibroblasts (**Figure 5I, 5L**). Moreover, micropatterned tri-cultures with both endothelial cell types (**Figure 5A, 5B, 5E, 5F**) were compared to co-cultures of fibroblasts and endothelia (**Figure 5C, 5D, 5G, 5H**) to understand the role of 3T3-J2s in influencing endothelial marker expression. Both micropatterned endothelial co-cultures (LSEC-MPCCs or L-MPCC and HUVEC-MPCCs or H-MPCC) displayed a loss of prototypical hepatocyte morphology over two weeks compared to micropatterned co-culture of PHHs/fibroblasts (MPCC). In particular, PHHs lost their polygonal shape, visible bile canaliculi and multinucleation in endothelial co-cultures while in MPCC hepatocytes maintained these morphological markers. In tri-cultures, islands morphology was better maintained over two weeks compared to PHHs/endothelia co-cultures. This was probably due to the 3T3-J2s in the tri-cultures, since it is known that these fibroblasts help stabilize the PHH phenotype over several weeks [59]. Regarding NPCs co-cultures (**Figure 5C, 5D, 5G, 5H**), we showed that fibroblasts play an important role in the cultures because as it is shown in **Figure 5G and 5H** compared to **Figure 5C and 5D**, 3T3-J2s at day 15 inhibited endothelial growth due to a reduction in the available surface area due to the presence of both cell types.

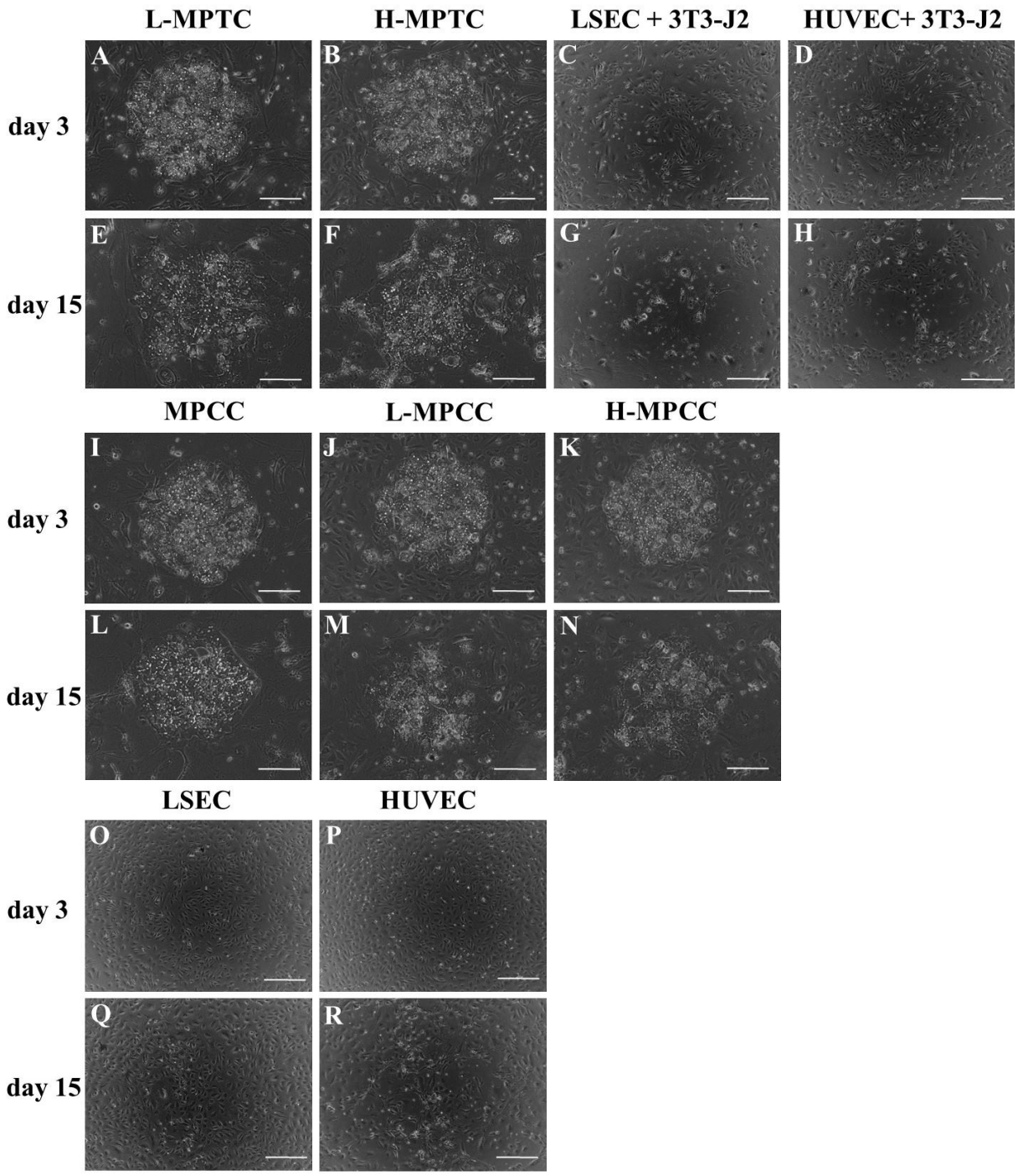

Figure 11. Morphology of tri-cultures and co-cultures at day 3 and 15 of culture. (A, B, E, F) Tri-cultures of PHH/endothelia/3T3-J2 (L-MPTC and H-MPTC, scale bars = 200 µm) and (J, K, M, N) co-cultures of PHH/endothelia (L-MPCC and H-MPCC, scale bars = 200 µm), (I, L) PHH/3T3-J2 (MPCC, scale bars = 200 µm), (C, D, G, H) endothelia/3T3-J2 (LSEC + 3T3-J2 and HUVEC + 3T3-J2; scale bars = 1 mm) and (O, Q, scale bars = 1 mm) LSEC and (P, R, scale bars = 1 mm) HUVEC at day 3 and day 15 of culture.

2.3.4 Marker expression in hepatocyte/endothelia and hepatocyte/fibroblast co-cultures relative to tri-cultures and monocultures

We performed immunofluorescent staining to assess the expression of CD31 and SE-1 molecular markers at day 15 on fixed MPTCs, MPCCs, random co-cultures and endothelial monocultures. CD31 expression was comparable between LSECs and HUVECs (**Figure 6L and 6M**), and between LSEC + 3T3-J2 and HUVEC + 3T3-J2 (**Figure 6C and 6D**). In both tri-cultures and co-cultures involving PHHs, CD31 expression was low as very few endothelial cells were present in the culture at the day of the fixing. We found CD31 presence also in the MPCC condition even if the signal was low, potentially either due to cross-reactivity of the antibody with mouse fibroblast proteins or contamination of the PHH lot with endothelial cells from the native liver. SE-1 expression was much higher in LSEC monoculture (**Figure 6Q**) compared to HUVEC (**Figure 6R**) and this result is consistent with what is shown in **Figure 4**. But we could not detect any signal from the other conditions, with LSEC + 3T3-J2 (**Figure 6G**) and H-MPCC (**Figure 6P**) being exceptions. It is likely that both hepatocytes and fibroblasts play important roles in regulating endothelial CD31 and SE-1 expression.

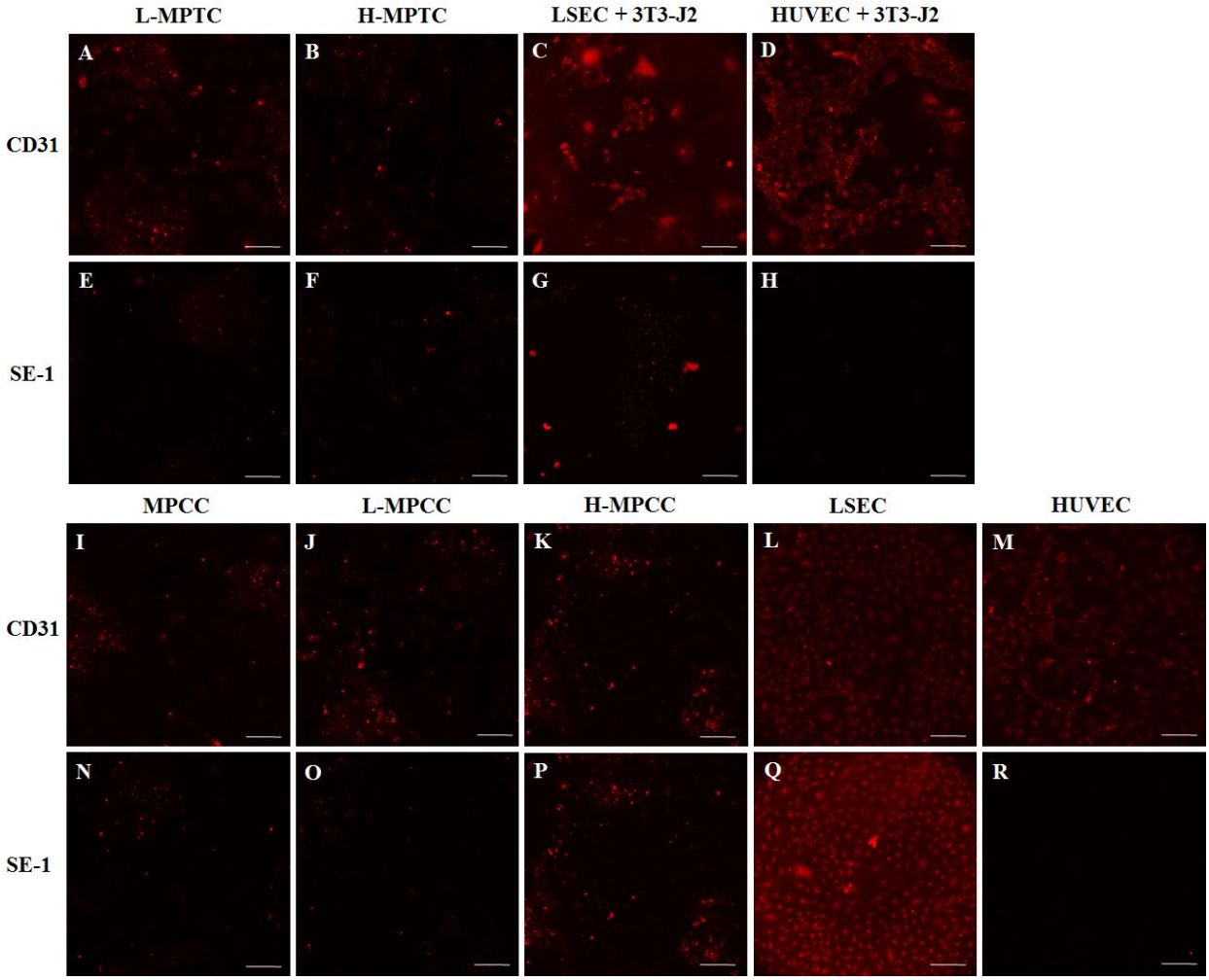

Figure 12. Immunofluorescent staining of CD31 and SE-1 on the different conditions fixed at day 15 of culture. (A, E) L-MPTC = PHH/3T3-J2/LSEC; (B, F) H-MPTC = PHH/3T3-J2/HUVEC; (C, G) LSEC + 3T3-J2; (D, H) HUVEC + 3T3-J2; (I, N) MPCC = PHH/3T3-J2; (J, O) L-MPCC = PHH/LSEC; (K, P) H-MPCC = PHH/ HUVEC; (L, Q) LSEC; (M, R) HUVEC. All scale bars are 150 µm.

Factor VIII production was null in PHH/3T3-J2 co-cultures as we expected as it is an endothelial cell marker [44, 45, 46, 47]. Factor VIII showed increased expression in LSEC and HUVEC monocultures over two weeks (**Figure 7**). This is likely due to endothelial proliferation

during the culture period, thereby increasing factor VIII production in the media supernatants. In both co-cultures and tri-cultures containing PHHs, it was significantly lower relative to pure LSEC at day 3 of culture and subsequently decreased over time, resulting in no detectable levels after a week of culture in the tri-cultures and some detectable production in the co-cultures. At day 3 factor VIII production by LSECs was significantly higher relative to all the other conditions with L-MPCC condition being an exception. Regarding pure LSECs, there was a relevant increase in the production of the marker after 9 days with a decreasing trend the second week, but still significantly higher than all other conditions with exception of pure HUVEC cultures. From a general perspective every condition downregulated factor VIII relative to pure LSECs and HUVECs. Moreover, the functional data in co-cultures correlated with the morphological observations (**Figure 5G and 5H**) in that we observed decreased endothelial presence over time.

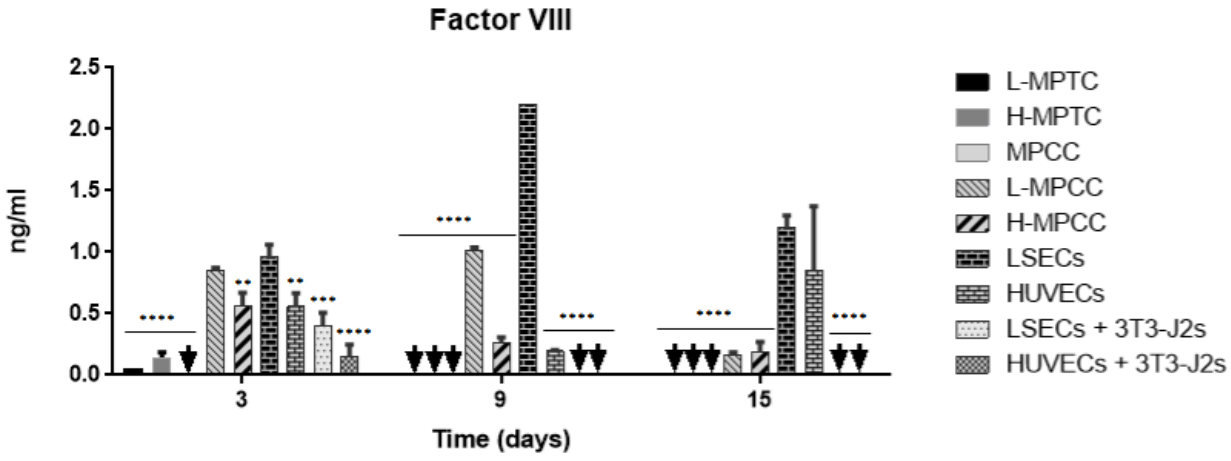

Figure 13. Factor VIII expression in tri-, co- and monocultures over two weeks. Significance relative to LSECs condition: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

2.4 DISCUSSION

In this study, we assessed LSEC phenotype in mono-, co- and tri-cultures. In particular, we investigated the expression of Factor VIII, a critical coagulation cofactor, CD31 and SE-1 markers. Because of LSECs' limited availability, we wanted to investigate the effects of HUVECs in our liver models. In particular, we wanted to see if HUVECs could become LSEC-like cells when cultured with PHHs and fibroblasts. HUVECs are an abundant and available cell type used for many studies [60] because of straight-forward isolation techniques and low cost [61]. Collagen micropatterning was used to control hepatocyte homotypic interactions in order to isolate this effect from the interactions between hepatocytes and endothelial cells [62]. Fibronectin was used to coat culture wells because it was shown previously to enable LSEC attachment [31]. Multi-cellular cultures are important to understand the mechanism that are involved in physiology and pathology [29].

CD31 expression was comparable between both LSEC and HUVEC monocultures fixed at day 3 of culture (data not shown) and at day 15 (**Figure 6L and 6M**). This is consistent with previous published data [41, 63], as CD31 is typically used as a generic endothelial marker. SE-1 expression was much higher in LSEC monocultures compared to HUVEC monocultures fixed both at day 3 (**Figure 4**) and day 15 (**Figure 6Q and 6R**). This is consistent with previous published data where SE-1 was used to assess rat LSECs phenotype as it correlates with the presence of fenestrations [32]; however, our is the first study that shows SE-1 expression in human LSECs. In contrast to the above-mentioned study by March S. et al using rat LSECs, the SE-1 expression in human LSECs did not decrease over time, which suggests potential species-specific differences. Moreover, March S. et al found that when LSECs are cultured with PHHs and 3T3-J2s, they expressed both SE-1 antigen and factor VIII for more days compared to pure

cultures [32] which is in contrast with our findings. Again, this can be due to differences among species, confirming the fact that animal models are not able to predict reliable human outcomes. Before running the experiment, we first hypothesized that at the functional level LSECs function could be enhanced while cultured with PHHs and fibroblasts as this fact has been already shown at the gene expression level [43] but in our case it is possible that especially in tri-cultures the presence of fibroblasts, while inhibiting LSEC proliferation, also inhibit LSEC marker expression or maybe the expression is sensitive to the passage number of cultured cells. But, interestingly we found that there was some expression of this marker in H-MPCC. It is plausible that when cultured with PHHs, HUVECs can become more LSEC-like cells and start expressing different markers because of the liver environment. Moreover, we observed both CD31 and SE-1 antigens' presence in 3T3-J2 monocultures fixed at day 3 (data not shown). Thus, SE-1 may not be a suitable marker for characterizing the LSEC phenotype in cultures containing 3T3-J2 fibroblasts [58], which are necessary to induce PHH functions over several weeks [64] via production of molecules present in the liver [65, 66]. On the other hand, PHHs monocultures or with endothelial cells (LSECs or HUVECs) are known to rapidly lose phenotypic functions [43].

LSECs have been shown to be the only source of Factor VIII in the adult liver [45]. HUVECs are normally used for the study of the function and pathology of endothelial cells such as angiogenesis, but their factor VIII production is controversial as in the literature it has been reported to be both present [46] and absent [44] in the supernatant of cultured HUVECs. In our repeated experiments, we detected both the presence and absence of factor VIII from HUVEC monocultures, which could be due to their plasticity in expressing this factor as a function of passage and/or culture variations; such an outcome needs to be further investigated in the future with more detailed molecular studies. The umbilical cord contains one vein, the umbilical vein,

that continues towards the transverse fissure of the liver, where it splits into two. One branch connects with the hepatic portal vein which transports blood into the liver [67]. The fact that the umbilical vein is directly connected to the liver may lead to HUVEC plasticity in variable production of factor VIII. Our results showed that both LSECs and HUVECs in tri-culture and co-culture formats produced similar decreasing trends over time regarding factor VIII expression. Moreover, factor VIII production was null in MPCC (PHH/fibroblast co-cultures) showing that 3T3-J2s are negative for this marker. These results suggest that HUVEC can be used as a first approximation when modeling interactions between PHHs and endothelial cells.

In conclusion, we showed that SE-1 marker can be used to distinguish LSECs phenotype from HUVECs (over CD31, which was present in both cell types) but only in pure mono-cultures; in co-cultures containing 3T3-J2 fibroblasts, cross-reactivity with fibroblasts was observed. Pre-labeling the endothelial cells with a fluorescent dye in the future may allow identification of cells that are positive for both the pre-label and SE-1 markers. Additionally, co-cultures and tri-cultures had nearly undetectable levels of SE-1 with H-MPCCs being the sole exception, which requires further studies to determine whether the HUVECs can express an LSEC-like phenotype upon co-culture with PHHs. In contrast to SE-1, the fibroblasts did not produce factor VIII; however, factor VIII production declined to negligible levels in both co-cultures and tri-cultures (but not in pure endothelial monocultures), which necessitates further investigations into the effects of PHHs and fibroblasts on the endothelial growth and expression of phenotypic markers. Our above-mentioned results were in contrast to previous results in similar rat liver model systems [32], which suggests that species-specific differences are important considerations when designing studies using liver models. Ultimately, our investigations into LSEC versus HUVEC protein marker expression in different culture formats

provide insights into how multiple cell types needed for human liver models reciprocally affect each other, which constitutes important foundational knowledge towards building multicellular liver models for drug screening and regenerative medicine.

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