Differentiation of human induced pluripotent stem cells under flow conditions to mature hepatocytes for liver tissue engineering

Viktoriia Starokozhko\textsuperscript{2*}, Mette Hemmingsen\textsuperscript{1*}, Layla Larsen\textsuperscript{1}, Soumyaranjan Mohanty\textsuperscript{1}, Marjolijn Merema\textsuperscript{2}, Rodrigo Pimentel\textsuperscript{1}, Anders Wolff\textsuperscript{1}, Jenny Emnéus\textsuperscript{1}, Anders Aspegren\textsuperscript{3}, Geny Groothuis\textsuperscript{2#}, and Martin Dufva\textsuperscript{1#}

\textsuperscript{1} Department of Micro- and Nanotechnology, Technical University of Denmark, Denmark
\textsuperscript{2} Department of Pharmacy, University of Groningen, The Netherlands
\textsuperscript{3} Cellartis, Takara Bio Europe AB, Sweden

*Shared first author
#Shared last author

Submitted
Abstract

Hepatic differentiation of hiPSCs under flow conditions in a 3D scaffold is expected to be a major step forward for construction of bio artificial livers. Therefore, the aims of this study were to induce hepatic differentiation of hiPSCs under perfusion conditions and to perform functional comparisons with fresh human precision cut liver slices (hPCLS), an excellent benchmark for the human liver in vivo. The majority of the mRNA expression of CYP isoenzymes and transporters and the tested CYP activities, phase II metabolism, and albumin, urea and bile acid synthesis in the hiPSC derived cells reached values that overlap those of hPCLS, which indicates for the first time a high degree of hepatic differentiation. Flow based differentiation compared to static differentiation had strong positive effect on phase II metabolism, suppressed AFP expression but resulted in slightly lower activity of some of the phase I metabolism enzymes. Gene expression data indicates that hiPSCs differentiated into both hepatic and biliary directions. In conclusion, the hiPSC differentiated under flow conditions express a wide spectrum of liver functions at levels comparable to hPCLS and show excellent perspectives to be further developed to a bioartificial liver system used for toxicity testing or as a liver support device for patients in the clinic.

Keywords: hepatic differentiation, bioartificial liver, stem cells
Introduction

Even though fully matured primary human hepatocytes (PHH) exhibit all the specific liver functions, their limited availability and loss of liver specific functions during culturing *in vitro* are still the major limitations for their application in a bioartificial liver (BAL) (Ordovás *et al.*, 2013, Mizumoto *et al.*, 2012). Therefore, porcine primary hepatocytes and carcinoma cell lines (HepG2, HepG2/C3A and HepaRG) have been widely employed in liver engineering (Nibourg *et al.*, 2012, Palakkan *et al.*, 2013, Schwartz *et al.*, 2014). The drawbacks of these cells are however the risk of zoonotic diseases, immunological responses, tumor formation or poor liver specific functions compared to PHH (Nibourg *et al.*, 2012, Palakkan *et al.*, 2013).

Stem cells, especially human induced pluripotent stem cells (hiPSCs), have therefore received great attention during the past years for liver tissue engineering (Ordovás *et al.*, 2013, Nibourg *et al.*, 2012). hiPSCs represent a potentially unlimited cell source for a large-scale production of hepatocytes required for BAL development. Furthermore, the use of the patients’ own hiPSCs may allow for personalized treatment and thereby avoiding immunological reactions. Although hiPSC-derived hepatocyte-like cells have been shown to have certain liver-specific phenotypic characteristics and exhibit many of the liver specific functions (Song *et al.*, 2009, Chen *et al.*, 2012, Giesecke *et al.*, 2014, Si-Tayeb *et al.*, 2010), most of these functions are expressed at levels several magnitudes lower than in fresh liver tissue or freshly isolated human hepatocytes (Ulvestad *et al.*, 2013) suggesting that improvements in the differentiation protocols are still warranted.

In most of these studies the induced pluripotent stem cell-derived hepatocyte-like cells were obtained by maturation in 2D cultures, and the cells are loaded in a bioreactor only after maturation. Hepatic differentiation and maturation directly in the 3D bioreactor may offer great advantages such as overcoming the need to harvest the total amount of cells needed for the BAL from the 2D culture and loading in a 3D bioreactor. However, relatively few studies have investigated the hepatic differentiation of stem cells directly in a 3D perfusion bioreactor or BAL using embryonic stem cells (Sivertsson *et al.*, 2013, Miki *et al.*, 2011, Schmelzer *et al.*, 2010, Fonsato *et al.*, 2010, Pekor *et al.*, 2015, Wang *et al.*, 2012), and only two of them used hiPSCs (Lunie *et al.*, 2016, Giobbe *et al.*, 2015). Flow of the medium was shown to have beneficial effects on hepatic differentiation of ESC and fetal liver cells and to improve liver functions of ESC-derived hepatocytes (Schmelzer *et al.*, 2010, Pekor *et al.*, 2015, Lu *et al.*, 2010, Fonsato *et al.*, 2010, Pekor *et al.*, 2015, Wang *et al.*, 2012).
3D liver models in tissue engineering

Yu et al., 2014). Even simple recirculation of medium in a rotating bioreactor improved the function of the differentiated hepatocyte-like cells (Fonsato et al., 2010, Wang et al., 2012). The flow may not only physically influence the cells by creating flow forces, but it also may improve mass as well as gas transfer between the cells and the medium, and promote the removal of waste products (Pekor et al., 2015, Yu et al., 2014). However, in another study perfusion inhibited adipogenic differentiation of adipose derived stem cells possibly by washing away auto- or paracrine factors (Hemmingsen et al., 2013).

A limitation of many studies is the absence of a proper benchmark to evaluate whether the cells are fully differentiated with respect to the expression levels of liver specific markers and liver functions of the generated hepatocytes. For example, many studies have not used a benchmark at all while others have used PHH cultured in vitro for 2 days or more (Song et al., 2009, Iwamuro et al., 2012, Takayama et al., 2012). However, it is known that PHH cultured beyond 24-48 hours rapidly lose their phenotype and liver-specific functions, and using these cells as a benchmark results therefore in an overestimation of the maturation level of the stem cell derived hepatocytes (Ulvestad et al., 2013). By contrast, fresh human precision-cut liver slices (hPCLS) contain hepatocytes in their natural 3D tissue-matrix configuration, in contact to the other liver cell types, and retain expression as well as activity of phase I and phase II metabolic enzymes at levels comparable to the in vivo situation (Elferink et al., 2011, de Graaf et al., 2007). Therefore, hPCLS can be considered the gold standard for assessing the maturation of stem-cell derived hepatocytes into fully differentiated hepatocytes.

Here, we differentiate hiPSC-derived definitive endoderm (DE) cells into hepatocytes in situ in a perfusion bioreactor system. Hepatic differentiation and functionality of hiPSC-derived hepatocytes were assessed using fresh hPCLS as benchmark for ex vivo liver, and 2D static cultures were used to compare differentiation efficacy in 2D static and 3D flow systems.

Material and Methods

Differentiation of hiPSC-DE cells into hepatocytes under static conditions and flow conditions.

Human iPS-derived definitive endoderm (DE) cells (Cellartis® Definitive Endoderm ChiPSC18, Cat. No. Y10040) derived from human dermal fibroblasts, authenticated using STR and mycoplasma free according to qPCR (see further information about this cell line on http://www.clontech.com) were cultured and differentiated into hepatocytes for 25 days.
Differentiation of hiPSC to mature hepatocytes under flow conditions

... according to the suppliers’ recommendations in the Cellartis® Hepatocyte Differentiation Kit (Cat. No. Y30050), see Figure 1E. Briefly, the cell culture surface (cell culture plates or scaffold) was coated with Hepatocyte Coating (from Cellartis Hepatocyte Differentiation Kit, Cat. No. Y30050) at 37°C for 1-2 days and subsequently washed with Phosphate buffered saline solution (PBS, 10 mM Na phosphate in 0.9% NaCl, pH 7.4). DE cells were thawed and seeded in Hepatocyte Thawing and Seeding Medium at an initial density of 2.5 x 10^6 cells/scaffold and for the static references 1.5 x 10^5 cells/cm² in 24 well plate format (using polystyrene well plates or corresponding PDMS coated well plates, see below) in 1 ml of medium. The DE cells were differentiated in Hepatocyte Thawing and Seeding Medium for 2 days at 37°C, before changing to Hepatocyte Progenitor Medium for another 5 days of differentiation to hepatoblasts. The cells were then differentiated further in Hepatocyte Maturation Medium for 4 days to immature hepatocytes and finally matured in Hepatocyte Maintenance Medium for another 14 days of culture to mature hepatocytes. In the static cultures, the medium was exchanged every 2-3 days.

### Scaffolds fabrication and perfusion cell differentiation culture

Polydimethylsiloxane (PDMS) was chosen instead of hydrogels as scaffold material to due to its biocompatibility and structural stability enabling production of liter-sized scaffolds (Mohanty et al., 2015, 2016). Random porous scaffolds (Figure 1Ci, Cii) were fabricated from PDMS by using salt leaching techniques similar to that described previously (Yuen et al., 2011). Hexagonal combined structured/porous scaffolds (Figure 1Ciii, Civ) were made using a sacrificial mold with hexagonal pattern fabricated by 3D printing using commercially available water dissolvable polyvinyl alcohol (PVA) (MakerBot, USA) and packed with salt crystals as described in (Mohanty et al., 2015). The scaffolds were treated with oxygen plasma (125 W, 13.5 MHz, 50 sccm, and 40 millitorr) to render their surfaces hydrophilic and sterilized by autoclaving. They were coated with Hepatocyte coating (from Cellartis Hepatocyte Differentiation Kit, Cat. No. Y30050) by centrifugation at 300 x g for 5 minutes and then left overnight at 37°C. The scaffolds were subsequently washed with PBS centrifugation at 300 x g for 5 minutes and then left in a media at 37 °C for 2h prior to being used to experiments.

A self-sustained perfusion system with 16 parallel reactors was constructed (Figure 1A, B) holding PDMS scaffolds. The scaffold bioreactor array, glass vials, caps and PTFE tubing were sterilized by autoclaving before assembling in a laminar flow bench. 0.5 M NaOH was flushed throughout the system to ensure a sterile fluidic path. The system was...
subsequently flushed with sterile water and then with culture medium. Coated scaffolds were placed in cylindrical holes in a custom built tray. $2.5 \times 10^6$ freshly thawed DE cells in 30 μL of Hepatocyte Thawing and Seeding Medium was pipetted into each scaffold and cells were allowed to adhere for 3h at 37 °C under 95% air/5% CO$_2$. The seeding tray was inverted as well as placed vertically in four different positions to allow the cells to distribute throughout the scaffolds during the 3h. The scaffolds were then placed in the 4×4 bioreactor array of the fluidic platform, and media was perfused through the scaffolds at flow rates of either 1 or 5μL/min. The entire system was incubated at 37 °C under 95%air/5% CO$_2$. Cells were cultured and differentiated for 25 days.

**Human liver tissue**

Human liver material was obtained from liver tissue of 10 individual patients, remaining as surgical waste after reduced liver transplantation patients, from liver tissue donated after cardiac death but not suitable for transplantation due to the age or from patients undergoing hepatectomy for the removal of carcinoma. The experimental protocols were approved by the Medical Ethical Committee of the University Center Groningen.

hPCLS were prepared as described previously by de Graaf et al. (de Graaf *et al.*, 2010). The hPCLS were made about 200μm thick and had 5 mg wet weight. In order to remove cell debris and to restore function, hPCLS were pre-incubated in the incubator (Panasonic, USA) for 1 hour at 37º C in a 12-well plate filled with 1.3 ml of Williams’ Medium E (Gibco, USA) saturated with 80%O$_2$/5%CO$_2$ while gently shaking 90 cycles per minute.

*Static hPCLS culture.* After pre-incubation, slices were transferred individually to a 12-well plate filled with 1.3 ml of Hepatocyte Maintenance Medium (from Cellartis® Hepatocyte Diff Kit (Cat. No. Y30050) saturated with 80%O$_2$/5%CO$_2$ and supplemented with 50 μg/ml gentamycin (Invitrogen). Plates were gently shaking 90 cycles per minute in the incubator at 37ºC.

*hPCLS culture under flow condition.* After pre-incubation, slices were transferred individually into small micro-chambers of PDMS biochips. The fabrication process of the biochip, as well as a schematic view of the biochip set-up was extensively described before (van Midwoud *et al.*, 2010). Slices were embedded in Matrigel® (BD Biosciences, Bedford, MA, USA) as described previously and the biochips were perfused with 2 times diluted Hepatocyte Maintenance Medium from Cellartis® Hepatocyte Diff Kit supplemented with 50 μg/ml gentamycin at 10μl/min flow in a humidified incubation chamber saturated with a mixture of
95%O₂/5%CO₂ as described in detail before (van Midwoud et al., 2011b). Viability of hPCLS was assessed by analysis of ATP content and morphological examination after 0h and 24 h. More details are provided in Supplementary materials.

### Imaging and confocal microscopy

Phase contrast images of 2D flow cultures and fluorescence based imaging of the scaffolds were acquired by a Zeiss Axio Observer as described in details in Supplementary materials. Confocal acquisitions of the scaffolds were performed using a Zeiss LSM 700 module in the Axio Imager M2 upright microscope using a 40x/1.20 W Korr C-Apo objective. More details are provided in Supplementary materials.

### Functional characterization of hiPSC-derived hepatocytes and hPCLS

#### Phase I metabolism.
To test the activities of several different CYP isoenzymes, hPCLS and cells in perfused and static systems were exposed for 1-3 hours to a drug cocktail containing 10µM phenacetin (CYP1A), 10µM bupropion (CYP2B6), 50µM mephenytoin (CYP2C19), 10µM diclofenac (CYP2C9), 10µM bufuralol (CYP2D6) and 5µM midazolam (CYP3A) in Hepatocyte Maintenance Medium without phenol red and supplemented with 2 mM L-glutamine and antibiotics (50 µg/ml gentamycin for hPCLS and 0.1% penicilllin and streptavidin for cells. Medium was collected and stored at -80°C until further analysis. Metabolite concentrations were measured at Pharmacelsus (Germany) by LC/MS according to in house protocols. The metabolite production was normalized per milligram protein and per hour.

#### Phase II metabolism.
For Phase II metabolism studies, hPCLS and cells in perfused systems or in static condition were exposed to 100µM of 7-hydroxycoumarin (7-HC) (Sigma-Aldrich, St.Louis, MO, USA) for 1-3 hours. Medium was collected at outlet tubes or from the incubation medium and stored at -20°C until further analysis, using 7-HC, 7-HC-G and 7-HC-S as standards. The metabolite production was normalized per milligram protein and per hour.

### Gene expression

Total cellular RNA from cells or PCLS was purified by using the RNeasy Micro kit (Qiagen, 74004) or using Maxwell 16 LEV simplyRNA Tissue Kit (Promega, USA) respectively. The RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4374966) and quantitative real time PCR was
conducted using the TaqMan® Gene Expression Assays (Applied Biosystems 4331182). More details are provided in Supplementary Materials.

**Statistical analysis**

Four independent experiments were performed with 4 batches of DE cells from one donor, and hPCLS from 7 different human donors. Since the number of donor livers for slices and number of donors for stem cells are limited, and inter-individual variations are large in the human population, we conclude on the differentiation of the cells by comparing the range of expression or activity rather than the mean or median values.

Additional Methods can be found in Supplementary materials.

**Results**

**Differentiation of definitive endoderm cells into hepatocyte-like cells under 3D flow condition.**

Differentiation of hiPSC-derived DE cells into hepatocytes was performed in perfused 3D bioreactors with highly porous 3D PDMS scaffolds and for comparison in conventional 2D cultures in polystyrene (PS) wells or PDMS coated wells. Frozen DE cells were seeded at a density of $2.5 \times 10^6$ cells per scaffold and differentiated into hepatocytes as illustrated in Figure 1E. Differentiation under flow on 2D surface showed very good morphology of differentiated cells, provided that the amount of differentiation factors was reduced by 50%, since 100% differentiation factor concentration results in poor cell adhesion during flow (Supplementary Figure 1). Cells differentiated on PS and PDMS respectively under static conditions showed similar morphology (Supplementary Figure 2). It was not possible to obtain bright field images of the cells in the 3D scaffold due to the poor optical characteristics of the scaffold.

The cells adhered typically in clusters in the scaffolds (Figure 1Di, Dii) at a relatively low overall final cell density (200,000-300,000 cells/scaffold) as determined by visual inspection (Supplementary Figure 3) and measurement of protein content (Supplementary Figure 4) in the scaffold. As DE cells and differentiated hepatocytes adhere well to PDMS (Supplementary figure 3), the relatively low number of adhering cells to the scaffold is likely due to difficulties to seed the cells in the scaffolds although seeding was performed by
rotating the scaffolds in six different directions with 30 min incubation in each direction. Calculations indicate that a scaffold has a surface area of approximately 10 cm² (Supplementary Figure 5) and thus the cells in the 3D scaffold had an effective cell density of 20,000-30,000 cells/cm², which is lower than the corresponding 2D static cultures (usually 80,000-100,000 stem cell derived hepatocytes/cm², data not shown). However, as determined by visual inspection, the cells adhered typically in clusters, and therefore, the local cell densities were probably higher.

**Figure 1.** Differentiation of iPSC in scaffolds under perfusion. A. Design of the perfusion system. B. Image of assembled perfusion system driving 16-channels. C. PDMS scaffold with random pores (C.i and C.ii) and hexagonal channels (C.iii and C.iv). D.i, D.ii Confocal microscopy images of iPSCs derived hepatocyte like cells. Blue is nucleus (DAPI) and red is actin staining. E. Schematic overview of the experimental process of differentiation of DE cells to mature hepatocytes.

**Figure 2.** hPCLS at 0h (A) and 24h (B) of incubation (Haematoxylin & eosin staining). Scale bar: 200µm.
Differentiation of hiPSC-derived DE cells loaded in the scaffolds was performed at a flow rate of 1 μl/min (exchange rate every 50 minutes) and 5 μL/min (exchange rate every 10 minutes). It was calculated that in both flow regimens, shear forces were very low (1.1e-4 dyn/cm², or less, Supplementary Figure 6 and 7). Furthermore, the exchange rate of once per 10 minutes was shown to be compatible with differentiation into hepatocytes in 2D flow cultures (Supplementary Figure 1) and has previously shown to support differentiation of adipose derived stem cells into adipocytes using conditioned medium (Hemmingsen et al., 2013).

Comparison of gene expression of hepatocyte markers of human iPSC-derived hepatocytes and hPCLS

We investigated the hepatic differentiation of hiPSC-derived DE cells in the 3D scaffold at two different flow rates, 1 μL/min and 5 μL/min, and in two different scaffold designs (Figure 1C), and compared the results with cells differentiated under static 2D conditions in standard polystyrene (PS) well plates or in well plates coated with a PDMS layer. hPCLS were used as a benchmark for cells differentiated in the scaffold under perfusion. hPCLS were prepared from 10 individual livers of human donors, aged 20-73 year (60% female) as described (de Graaf et al., 2010). Due to the limited amount of liver material, not every test was performed on all donor livers. The morphological appearance and ATP content of the hPCLS after a preincubation of 1 hour to restore ATP levels (0h) and after 24h of incubation, indicated that the slices were viable. ATP levels were 9.7±1.3 pmol/μg protein and 8.24±0.76 pmol/μg protein at 0h and 24h respectively (mean±SEM). Morphology showed intact liver tissue at 0h and after 24 h of incubation (Figure 2).

The gene expression of the liver-specific genes of the cells differentiated under the conditions outlined above and the hPCLS is depicted in Figure 3. A summary based on classification of gene expression into broader groups is presented in Figure 4.

When comparing the differentiated cells in static cultures on PS with hPCLS, most of the CYP genes, the expression of the epithelial biliary cell markers (CK7, BGP) and the drug transporter ABCB1 (multidrug resistance protein, P-gp) in the cells was in the range of that seen in hPCLS. Large differences in gene expression were observed for the genes CAR, ALB and BSEP, which were clearly under expressed in differentiated cells compared to hPCLS. These three genes were, however, higher expressed in differentiated cells than in the DE cells (Figure 3 and 4). Furthermore, the differentiated cells showed higher expression of AFP and HNF4a than the hPCLS. The differentiated cells on PS therefore had a mixed phenotype,
where some genes suggest a partly to fully matured phenotype (HNF4a, CYP3A4, 3A5, 3A7 and 2B6 and P-gp), while others suggest a less mature phenotype (ALB, AFP, CAR, BSEP). In addition, maturation of a part of the cells into biliary epithelial cells is suggested by expression of CK7 and BGP.

Differentiation on the scaffold material PDMS in static cultures had only a minor impact on the gene expression compared to cells on PS; all genes showed overlapping expression in cells on PDMS and PS respectively (Figure 3 and 4).

**Figure 3.** Expression of different hepatic genes by DE cells cultured and differentiated under different conditions. Data are given for each individual sample to appreciate the variation within each condition and the overlap between the different conditions. Results are from four independent differentiation experiments and seven donors. Due to poor RNA yield, some genes where only analyzed in two (P-gp) or three (CK7, BSEP, BGP) of the cultures.

Flow modulated the gene expression of differentiated cells only to a small extent, with 5 µl/min performing slightly better than 1 µl/min for CYP 3A4 and 2B6. Flow also modulated the ALB and AFP expression; the ALB expression was suppressed by flow compared to the corresponding static cultures. The AFP expression was lowest in cells exposed to the 5 µl/min perfusion compared to static and perfusion with 1 µl/min, but did not result in the very low levels observed in hPCLS (Figure 3 and 4).
Figure 4. Summary of the comparison of the gene expression levels between the differentiated cells and the hPSC derived hepatocytes relative to the gene expression levels in hPCLS as follows: Black: all individual data of the cells are higher than those in hPCLS. Dark grey: the individual data of the cells are higher than or in the higher range of those of hPCLS. Middle grey: all data of the cells are in the same range as those of hPCLS. Light grey: the individual data of the cells are lower than or in the lower range of those of hPCLS. White: all individual data of the cells are lower than those of the hPCLS.

**Phase I metabolism**

Differentiated hiPSC-derived DE cells as well as hPCLS were exposed to the substrates under both static and flow conditions to account for possible effects of flow on metabolism. The metabolic activities in the hPCLS showed large inter-donor variations as expected, as inter-individual differences in drug metabolism are well described.

Overall the hPCLS showed similar metabolic activity when cultured under flow or in static conditions, although a lower metabolic activity was found for CYP3A and CYP2B6 activity under flow (Figure 5). These differences may be explained by binding of the lipophilic substrates midazolam and bupropion to the PDMS of the biochip (van Midwoud et al., 2011a), although it cannot be excluded that the perfusion conditions may have influenced the metabolic activity.

The hiPSC-derived hepatocytes differentiated under flow conditions as well as under static conditions showed overlapping activities of CYP3A, CYP1A, CYP2C9, CYP2D6 and CYP2C19 with the hPCLS (Figure 5). However, at a flow of 1 µl/min somewhat lower activities of CYP2C9 and CYP2C19 were found compared to a flow of 5 µl/min. CYP2B6 showed very low activities in cells compared to hPCLS irrespective of perfusion or static culture conditions. The overlap in activities of most of the CYP isoforms, with exception of CYP2B6, in the differentiated cells with those of hPCLS under flow, indicate a high degree of hepatocyte drug metabolic function in the differentiated cells.
Differentiation of hiPSC to mature hepatocytes under flow conditions

**Figure 5.** Phase I metabolite production of midazolam, phenacetin, diclofenac, bufuralol, bupropion and mephenytoin by hiPSC-derived hepatocytes and hPCLS cultured in static or under flow conditions. The individual values are expressed as pmol/min/mg protein. Results are from three independent differentiation experiments and seven donors.

Phase II metabolism.

Differentiated cells exhibited high uridine UDP-glucuronyltransferase (UGT) and sulfotransferase (SULT) activities when exposed to 7-hydroxycoumarin (7-HC) (Figure 6). Both phase II activities were higher in hiPSC-derived hepatocytes at a flow of 5 µl/min than at 1 µl/min. While the activities in cells cultured under static conditions were similar to those in hPCLS static cultures, the 7-HC-glucuronide (HC-G) production by cells cultured at 5µl/min flow in both hexagonal and random scaffolds was on average two-fold higher than in liver slices at flow conditions. In addition, the sulfation rate of 7-HC resulting in 7-hydroxycoumarin sulfate (HC-S) was 30-40 fold higher in cells differentiated under flow condition compared to differentiation under static condition and the hPCLS.
Figure 6. Production of 7-hydroxycoumarin glucuronide (HC-G) (left panel) and 7-hydroxycoumarine sulfate (HC-S) (right panel) from 7-hydroxycoumarin by hiPSC-derived hepatocytes and hPCLS cultured in static or under flow conditions. The individual values are expressed as pmol/h/mg protein. Results are from three independent differentiation experiments and seven donors).

Albumin and urea production

Albumin production by the hiPSC-derived hepatocytes was in the lower range of that of hPCLS (Figure 7A). No difference was observed between the two types of scaffolds at both flow rates or between static and perfusion cultures.

Figure 7. Albumin (A) and urea (B) production by hiPSC-derived hepatocytes and hPCLS cultured in static or under flow conditions. The individual data values are expressed as ng/h/mg protein for albumin and μg/h/mg protein for urea. Results are from three independent of differentiation experiments and seven donors.
Differentiation of hiPSC to mature hepatocytes under flow conditions

On average, the urea production by hiPSC-derived hepatocytes was below or in the lower range of that of hPCLS (0.06-7.6 μg/h/mg protein for hiPSC-derived hepatocytes and 1.6-11.9 μg/h/mg protein for hPCLS) (Figure 7B). Cells differentiated at 5μl/min flow and under static conditions tended to show higher urea synthesis (0.3-7.6 μg/h/mg protein) than those differentiated under 1μl/min flow (0.06-0.55 μg/h/mg protein).

Bile acid secretion

Bile acid secretion by the hiPSC-derived hepatocytes was at the same level of 25-30 pmol/h/mg protein in cells differentiated under static conditions for 22 days or 24 days as in hPCLS (Figure 8). We could not detect bile acids in the samples of the outflow medium obtained of the scaffolds, due to the high dilution of the excreted compounds, which is a consequence of the perfusion flow rate. The observed total bile acid secretion of 25-30 pmol/h/mg protein by differentiated cells or hPCLS would result in a concentration of about 20-100 pmol/ml at a flow rate of 1 and 5μl/min respectively, which is below the detection limit.

![Figure 8. Bile acid production by hiPSC-derived hepatocytes and human PCLS cultured in static conditions. Data are expressed as pmol/h/mg protein. Graph represents mean values ± SEM.](image)

Discussion

We have obtained highly differentiated hepatocytes. To assess their differentiation status, we compared the expression and function of the cells in this BAL model with fresh human liver slices that have in vivo like activities (de Graaf et al., 2007, de Graaf et al., 2010, van Midwoud et al., 2011a) and found as yet unprecedented liver functions in the
differentiated cells. Moreover, we found that DE cells can be successfully differentiated into hepatocyte-like cells in a 3D scaffold in a bioreactor under flow conditions, to a similar or only slightly better differentiation grade than under static 2D conditions, especially with respect to phase II sulfation activity and a lower AFP expression, which can make the production of a BAL easier and more effective in the future.

hiPSC derived cells differentiated under flow in a 3D bioreactor resulted in a BAL model with overlapping phase I metabolism (except for CYP2B6) and similar or higher phase II metabolism, compared to fresh human liver slices. Urea production was present in the hiPSC but was below or in the lower range of the hPCLS. However, the capacity of urea production in the BAL from ammonia from extrahepatic sources was not assessed since no ammonia was added to the medium and, further studies with exposure to extracellular ammonia are needed to show the ability of the cells to detoxify ammonia, which is important for patients with liver diseases where high concentrations of neurotoxic ammonia are detected. Bile acid production by hiPSC-derived hepatocytes was on the same level as in fresh tissue slices. The gene expression of P-gp in hiPSC-derived hepatocytes was shown to be higher than in hPCLS, which is remarkable as a 10-20 fold lower expression in differentiated hiPSC compared to human hepatocytes was found by Lu et al. (Lu et al., 2015). However, the gene expression of BSEP in the differentiated cells was lower than in the hPCLS. Similar to hPCLS, the hiPSC derived cells expressed both CK-7 and BGP indicating that the hiPSC derived cells are a mixture of both hepatocytes and biliary epithelial cells (BEC). This bipotent differentiation potential of iPSC-derived hepatic progenitor cells was also found previously (Yanagida et al., 2013). The albumin secretion of stem cell-derived hepatocytes achieved here is similar to hiPSC-derived hepatocytes (Gieseck et al., 2014) or 10-100 fold higher than in human ESC-derived hepatocytes (Miki et al., 2011) and 3-40 fold higher than mouse iPSC-derived hepatocytes (Iwamuro et al., 2012), but lower than in fresh tissue slices. Although the mRNA expression was high for HNF4a indicating hepatic differentiation and low for CYP3A7, which is a fetal enzyme with low expression in the adult liver, the relatively high expression of the fetal protein AFP indicates that maturation of the cells is not fully complete. This has also been observed by others (Chen et al., 2012, Gieseck et al., 2014, Iwamuro et al., 2012, Kim et al., 2015), and it needs to be addressed how relevant this is for the functioning of the BAL in patients who need liver support or for toxicity testing. Taken together, these results show overall that hiPSC differentiated under static conditions as well as under flow in a scaffold have liver functions close to those in fresh human liver tissue. The significant improvements with respect to liver functions of the differentiated cells presented
Differentiation of hiPSC to mature hepatocytes under flow conditions

here compared to other studies could be due to better differentiation protocols, resulting in a favorable balance of paracrine or autocrine factors affecting differentiation, whereas the difference between the cells differentiated under 2D static and 3D perfusion conditions could be ascribed to better nutrient delivery and waste removal.

Most studies have used PHH cultured in vitro for 1-3 days as benchmark for hepatic activity (Gieseck et al., 2014, Ulvestad et al., 2013, Lu et al., 2015, Takayama et al., 2012). Because PHH functions decrease rapidly and drastically (10-1000 fold after 48h culture) during in vitro culture (Ulvestad et al., 2013), using these PHH as standard tends to overestimate the metabolic function of hiPSC-derived hepatocytes. Therefore, the comparison of those data with our study is difficult. Moreover, comparison of the metabolic activity data between different studies is further hampered by the fact that the substrate concentrations and experimental conditions used are largely different. Fresh human PCLS, on the other hand, show similar metabolic activity as the fresh PHH and give a good representation of liver functions in vivo (Asplund et al., 2016). The only published study which used fresh hepatocytes as a control is the study of Ulvestad et al. (Ulvestad et al., 2013). Comparison of the results of that study with our data shows that the CYP3A, CYP2C9 and CYP1A activities of hiPSC-derived hepatocytes in our study were ten to several hundred fold higher than those of the iPSC-derived hepatocytes in the study of Ulvestad et al. We were the first to measure phase II metabolism in hiPSC and found that glucuronidation was comparable to PCLS but sulfation was remarkably higher after differentiation under flow, which requires further studies.

The gene expression of CYP-enzymes and their activity varied notably between donor livers, which is very well known in the human population, which is among others a result of polymorphisms and induction by environmental and physiological factors. For example, CYP1A2, CYP2D6, CYP2C9, CYP2C19, CYP2B6 and CYP3A4 are known to be important polymorphic and highly inducible enzymes in human (Zhou et al., 2009). With this in mind it is noteworthy that the gene expression and enzyme activities in hiPSC derived cells overlapped in most cases with a few discrepancies noted below. For example, the CYP3A5 gene was higher expressed in hiPSC-derived hepatocytes compared to hPCLS, whereas CYP3A4 gene expression only reached up to the lower range of human livers (Figure 3). However, as CYP3A4 and 3A5 have strongly overlapping specificities (Emoto and Iwasaki, 2006), it may explain why the total CYP3A metabolism of midazolam was similar in hiPSC-derived hepatocytes and hPCLS (Figure 5). Although, the gene expression of CYP2B6 in the differentiated cells was in the lower range of hPCLS possibly due to low CAR expression, the
activity of this enzyme was at least 10 times lower in hiPSC-derived hepatocytes than in hPCLS indicating a post transcriptional regulation. Future research will be focused to improve also the as yet under expressed CAR mediated pathway.

We found a limited influence of the flow rate on the hepatic differentiation of hiPSC in the BAL, as 5µL/min flow resulted in a somewhat better hepatocyte differentiation and maturation than the 1µL/min flow. This might be explained by the better nutrient and oxygen supply and removal of waste metabolites at the higher flow rate. Also the type of scaffold had no obvious impact on the differentiation.

In conclusion, most of the drug metabolism enzyme activities of the developed hiPSC-based BAL were in the same order of magnitude as in the fresh human tissue, which is an important achievement in liver tissue engineering and for future applications in drug metabolism and toxicity testing. A limitation of the present study is that besides hepatocytes and biliary epithelial cells, which were present in the developed BAL according to gene expression profiling, no non-parenchymal cells are present yet. Although no toxicity studies have been done yet, for future toxicity tests it is necessary to also add these other liver cell types to better represent the liver functions by the BAL. Moreover, future experiments with more donor individuals for both iPSC and PCLS will help to better estimate the variation in the population as well as the robustness of the differentiation protocol. Finally, future studies should show the BAL’s detoxification capacities for human serum.

Acknowledgements

We thank Pieter Oomen for his kind help with making the PDMS chips for hPCLS cultures. The authors thank Prof. Dr. Robert Porte and all the surgeons of the University Medical Center Groningen for providing the human liver tissue. The work was supported by the EU-funded project NanoBio4Trans (“A new nanotechnology-based paradigm for engineering vascularised liver tissue for transplantation”, Grant No: 304842).
Supplementary materials

Description of perfusion system

A schematic of the perfusion culture system and fluidic/air circuit is illustrated in Figure 1. The main components of the system include an array of 4×4 bioreactors for culture of cells, vials and vial trays for storage of culture media and waste and peristaltic pumps and motors for perfusion of media. All parts are secured onto a single platform. Four 8-channel micropumps (previously described (Skafte-Pedersen et al., 2009, Sabourin et al., 2013)) that generate pulsatile flow are included in the system, controlled by motors and controllers from the Lego Mindstorm (Lego, Billund, Denmark) kit. This allows culturing of cells at four different flow rates in a single experiment. The pumps allow flow rates of sub-µl/min to approximately 90 µl/min (Sabourin et al., 2013). The fluidic circuit is formed by the pumps, bioreactor array and media storage vials that are connected using polytetrafluoroethylene (PTFE) tubing (inner diameter of 0.8 mm) (BOLA 1818-10, Bohlender GmbH, Germany). Inlet and outlet vials are coupled with PTFE tubing and supplied with air supplemented with 5% CO2 through a sterile filter. To avoid the formation of gas bubbles in the microfluidic network, an overpressure of 30 kPa is put on the flow system during its operation. All components are mounted onto a base platform for portability and user-friendly handling. The entire system can be placed in an incubator for cell culture experiments.

Design of the bioreactor array

The bioreactor array allows culture of 16 cylindrical 3D constructs having thicknesses of 5 mm and diameters of 6 mm (Figure 1C). The bioreactors are designed with conical inlets and outlets for uniform delivery of media through the pores of a scaffold in the cylindrical cavity (Oikkels et al., 2010, Patrachari et al., 2012). Additionally, the conical inlet geometry gives rise to lower shear stress in the areas of the scaffold close to the inlet as assessed from a finite element study of the flow profile within two bioreactor designs as discussed in following sections. Silicone tubes (having inner diameter of 1.8 mm and 1 cm in height) are press fit to the ports of the bioreactor (inner diameter of 1 mm and outer diameter of 2 mm) and serve as connectors between the bioreactor and the rest of the perfusion network.

The 4×4 bioreactor array was implemented in an easily exchangeable single device. The upper plate of the device (having dimensions of 100 x 100 x 5 mm³) incorporates the outlet ports for waste removal from each bioreactor. The lower plate (with dimensions of 100
x 100 x 10 mm$^3$) incorporates 4×4 cylindrical chambers (each with a diameter of 6 mm and height of 5 mm) for housing scaffolds and inlet ports for perfusion of media into the chambers. The two parts of the array are secured together using screws with a custom designed polydimethylsiloxane (PDMS) gasket (having a thickness of 1 mm) placed between them in order to ensure a tight seal and form a leak proof system. The gasket was designed such that it incorporated a raised lip (0.5 mm high) around each bioreactor array.

**Fabrication**

The two parts of the bioreactor array were fabricated by micromilling the required features into polycarbonate substrates. The gasket was fabricated by moulding of PDMS in a custom milled polycarbonate mould. Vial trays capable of housing 32 vials (16 for holding cell culture media and 16 for storage of the waste media), were fabricated in 5 mm sheets of polymethylmethacrylate (PMMA) using a CO$_2$ laser cutter (Epilog Mini 18 Laser, CO 80403, USA).

**Viability determination of hPCLS**

ATP content of hPCLS was assessed according to the manufacturer’s protocol of the ATP Bioluminescence Assay Kit CLS II (Roche, Mannheim, Germany) in a black 96-well plate in the Lucyl luminometer (Anthos, Durham, NC) using a standard ATP calibration curve. The ATP content was normalized for protein content of the hPCLS as described below. Morphology was assessed on 4 μm sections of formaldehyde fixed, paraffin-embedded slices, stained with hematoxylin and eosin according to the described protocol (de Graaf et al., 2010).

**Protein Content of hPCLS and iPSC-derived hepatocytes in the scaffold**

The pellet left from homogenized ATP samples was used to determine the protein content of hPCLS using the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany) as described before using bovine serum albumin for the standard curve (Starokozhko et al., 2015). Protein content of cells in the scaffold was measured according to the manufacturer instructions of Pierce® BCA Protein Assay Kit (cat. no. 23227) after protein extraction by an over-night incubation of the scaffold in 0.2 M NaOH. More information is found in Supplementary Figure 4.
Gene expression analysis

Total cellular RNA was purified by using the RNeasy Micro kit (Qiagen, 74004). Differentiated cells were lysed directly in the scaffold in the bioreactor using the lysis buffer provided in the Qiagen RNeasy Micro kit. The lysate was collected in microtubes and purified according to manufacturer’s instructions (Qiagen, 12/2007). Total RNA from the hPCLS was isolated using Maxwell 16 LEV simplyRNA Tissue Kit (Promega, USA). The RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4374966) according to the manufacturer’s instructions (06/2010). Quantitative real time PCR was conducted using the TaqMan® Gene Expression Assays (Applied Biosystems 4331182), ALB (albumin) ID: Hs00910225_m1, AFP (alpha-fetoprotein) ID: Hs00173490_m1, CYP2B6 ID: Hs04183483_g1, CYP3A4 ID: Hs00604506_m1, CYP3A5 ID: Hs00241417_m1, CYP3A7 ID: Hs00426361_m1, HNF4A (hepatocyte nuclear factor-4-alpha) ID: Hs00230853_m1, NR1I3 (CAR, constitutive androstane receptor) ID: Hs00231959_m1, ABCB11 (BSEP, Bile Salt Export Pump) ID: Hs00184824_m1, ABCB1 (P-gp, permeability glycoprotein) ID: Hs00184500_m1, and KRT7 (cytokeratin-7) ID: Hs00559840_m1, TaqMan® Gene Expression Master mix (Applied Biosystems, 4370048) and RNase-free water according to the manufacturer’s instructions (Applied Biosystems 11/2010). The respective \( C_t \) values obtained after analysis in a Chroma4 real time PCR machine (MJ Research, the program run at 50°C for 2 minutes, 95 °C for 10 minutes and 40 cycles of 15 sec at 95 °C and 1 minute at 60 °C) were normalized to the \( C_t \) value of CREBBP (CREB-binding protein) ID: Hs00231733_m1. CREBBP has been shown to be a good candidate to use for normalization since the gene expression does not change significantly during differentiation (Synnergren et al., 2007). The gene expression is presented as data points using the formula: \( 2^{-\Delta C_t} \).

Imaging

Phase contrast images of 2D flow cultures were acquired by a Zeiss Axio Observer. Z1 microscope equipped with a 10x/0.3 Plan-Neofluar objective, and a Zeiss Axiocam MRm B/W camera. A scan of each cell culture chamber was recorded with an exposure time of 5msec. All images were acquired with a z-stack of 5 image planes (6 µm between each image plane). The images were processed by applying the AxioVision Extended Focus module on the z-stacks to obtain the best focused image, stitching the individual images together and finally converting the stitched images to one image.
Fluorescence based imaging of a cross section of the scaffold was carried out at day 22 of the cell culture differentiation. Scaffolds were sectioned longitudinally (along the axis of flow). Each scaffold was stained with either Höechst for showing cell distribution or Calcein AM for live cell imaging. Non-fluorescent Calcein AM is converted to highly fluorescent Calcein by intracellular esterase activity and stains viable cells green. Cells were imaged using an inverted microscope (Zeiss Axio Observer) using the appropriate excitation lights and filters.

**Confocal microscopy**

Scaffolds were sectioned longitudinally (along the axis of flow) on day 22 of the cell culture differentiation. Each sample was fixed in 3% paraformaldehyde (in PBS) for 10 minutes and permeabilized with Triton X-100 for 5 minutes. Subsequently, they were stained for 30 minutes with either Höechst 33342 (Invitrogen) for labeling the cell nuclei as well as Phalloidin (F432, Invitrogen) for labeling the F-actin. Confocal acquisitions were performed using a Zeiss LSM 700 module in the Axio Imager M2 upright microscope using a 40x/1.20 W Korr C-Apo objective. The confocal settings were as follows, section thickness 0.8 µm, pixel dwell 0.79 µs, pixel size 145 nm, optimal Z section number determined by the confocal software. To eliminate any possible cross-talk between channels, images were collected with a sequential scan, using the following laser lines and mirror settings: 488(30%) 495-560nm; 555(30%) 605-700nm.

**Albumin synthesis**

Albumin production was measured using the Human Albumin ELISA kit (Bethyl Laboratories, Montgomery, USA) according to the supplier’s recommendations. In brief, medium was collected from the well plate (static conditions) after 24 of incubation or at the outlet tubes (perfused systems) for 24 hours from both differentiated cell and hPCLS cultures and stored at -20°C until analysis. Samples were diluted if necessary and human albumin was used to prepare a calibration curve. The amount of albumin was calculated based on a standard curve generated as a 4-parameter curve fit. Values are expressed as ng albumin produced per hour, per milligram total protein.

**Bile acid production**

Total bile acid (TBA) content was measured using the Total Bile Acid kit (Diazyme Laboratories, Poway, CA, USA) in medium after 24 h incubation (static cultures) or 24 h
perfusion (perfused cells and slices). 1ml of medium was concentrated 10 times using the CentriVap Benchtop Vacuum Concentrator at 35°C (Labconco, Kansas City, MO, USA). TBA content was determined according to the manufacturer’s protocol of the TBA kit with a few modifications. Conjugated cholic acid (50µM) was used as a calibrator. Measurement was performed at 37°C in a 96-well plate in the Synergy HT plate reader (BioTek, Winooski, VT, USA). The absorbance was read at 405nm at 5 and 30 min. The TBA production is expressed as median with interquartile range.

**Urea synthesis**

Urea concentrations in the medium were measured using the Urea Assay Kit (Abnova, Taiwan). Medium samples from differentiated cells and PCLS flow and static cultures were concentrated 10 times before measurement using the CentriVap Benchtop Vacuum Concentrator at 35°C. Urea content was determined according to the manufacturer’s protocol of the Urea Assay kit with a few modifications. Accordingly, 25µL of samples were added to each well and incubated for 30 min at room temperature with a reagent mix. The absorbance was read at 430nm and urea levels calculated based on standard curve of urea standard provided with the kit and expressed as µg urea produced per h, per mg total protein. Data are expressed as individual values and the median is indicated.

**Supplementary Figure 1. Effect of concentration of signaling factors on cell morphology at flow conditions.** Left panels: low magnifications. Right panels high magnifications. DE cells were cultured and differentiated at 2D flow conditions in a chamber having dimensions of 1.5 mm (w) x 6 mm (l) x 0.5 mm (h). The concentration of signaling factors was either the normal used and optimized for static culture conditions or ½ the normal concentration but otherwise a full base medium. The cells were perfused at two different flow rates, 250 nL/min or 500 nL/min, corresponding to an exchange of the medium in the entire chamber every 20 and 10 minutes. Phase contrast images acquired at day 25. The shown images are a representative area of one chamber out of 4 chambers for each condition. A better cell attachment and less dead/ floating cells were observed at perfusion with medium supplemented with half of the normal concentration of signaling factors compared to perfusion with medium supplemented with the normal concentration of signaling factors.
Supplementary Figure 2. Cell morphology of differentiating cells at static conditions. Phase contrast images were acquired at day 9, 15, and 19. The cell morphology was very similar between the PDMS scaffold material and the PS conventional surface substrate, although some larger cells were observed on PDMS.

Supplementary figure 3. Microscopy imaging of hiPSC-derived DE cells cultured and hepatic differentiated inside a porous scaffold at perfusion conditions: Image at day 22 after cell seeding of the middle of a cross-sectioned scaffold. A) Scan of entire cross-sectioned scaffold showing cell distribution. Höechst stained cell nuclei in blue color. B) Close-up view of cell distribution with Höechst stained cell nuclei. The homogeneous blue fields in the image are likely not cells but reflections within the scaffold. The fluorescence image is merged with a phase contrast image of the scaffold. C) Calcein-AM live-stained cells in green.
Supplementary figure 4. Determination of protein content in the BAL. DE cells were loaded into the BAL as described in material and methods. Three replicates of the four investigated BALs (two different scaffold designs and two different flow conditions), were prepared and cultured for one week. Scaffolds with cells were perfused with Dulbecco’s Phosphate Buffered Saline with MgCl₂ and CaCl₂ (Sigma D8662) for 30 min to remove protein containing medium and then transferred to an 1.5 mL Eppendorf tube with 0.5 mL 0.2M NaOH. To enhance distribution of NaOH within the scaffold, the tubes were vortexed for 30 seconds three times with 10 minutes incubation between each vortex. The tubes were incubated over night at 4°C. To enhance the release of cell material from the scaffold into the NaOH, the tube was again vortexed for 3 X 30 seconds with 10 minutes incubation between each vortex, and then centrifuged at 500 x g for 5 minutes. The supernatant was diluted 1:1 with milliQ water to 0.1M NaOH. The protein content was measured by the use of the Pierce® BCA Protein Assay Kit (cat. no. 23227) according to the supplier’s microplate procedure. The absorbance was read at 570 nm and protein calculated based on a standard curve for bovine serum albumin. The results showed that the variance between the same type of scaffold was limited. If it is assumed that 0.1 mg protein corresponds to 100.000 cells (Anders Aspegren, personal observations), each BAL contains about 200.000-270.000 cells. This corresponds well with theoretical calculations that there can be a maximum of about 600,000-1,000,000 cells per BAL (see Supplementary figure 6).
**Supplementary figure 5.** Theoretical calculations of the surface area of a scaffold. Two estimates were made: 

**Estimate 1** was based on an idealized network of cubes that are connected and then surrounded by PDMS. The calculation involved trying to find out how many salt cubes that can fit into the volume of the scaffold and then calculate the surface area by taking the number of salt particles multiplied by the total surface of one salt particle. The side of a salt cube is approximately 0.35 mm as determined by scanning electron microscopy investigations (Canali et al., 2015). The distance to the next salt particle is estimated to be 0.1 mm (can also be larger) meaning that a salt crystal takes up about 0.45*0.45*0.45= 0.091125 mm$^3$ including the surrounding PDMS. The cylinder volume (Figure 1C) is $r^2\pi h=3^2*3*\pi*5=141mm^3$. 141/0.091125= 1556 particles. The surface area of a salt particle is 0.35*0.35*6=0.735 mm$^2$. 1556*0.735= 11.39 cm$^2$. However because the salt crystals need to touch each other in order to form a network, some of the area is lost. In the idealised situation, each cube looses about 1.5 sides in surface area as it shares that area with other salt crystals. Therefore the area is estimated to be 11.39*9/12=8.5 cm$^2$. In **Estimate 2** we used the measured porosity (determined to be 65%) (Canali et al., 2015) of a random scaffold as input parameter and calculated the number of salt molecules that could fit into pores with a total volume of 0.65 multiplied by the scaffold volume: $r^2\pi h*0.65=3^2*3*\pi*5*0.65=92$ mm$^3$. Volume of salt particle is 0.35*0.35*0.35mm$^3$=0.042875mm$^3$. Number of salt particles in scaffold are 92/0.042875=2145 particles. Number of particles multiplied by the surface of each salt cube $(6*0.35*0.35mm^2=0.735mm^2)$ = total surface volume: 1577 mm$^2$=15.8 cm$^2$. However, just as in the case above, some of the sides of the cubes are shared between each sugar cube. Using the estimate above, it is suggested that the surface area is 15.8 cm$^2$*9/12= 11.85 cm$^2$. In this case, the surface area is estimated to be about 12 cm$^2$, which is close to **Estimate 1** of 11.39 cm$^2$. For simplicity we estimate the surface of a scaffold to 10 cm$^2$ (1000 mm$^2$).

The number of cells fitting the scaffold is proportional to the surface of one side of the cuboidal cell. A 20 µm x 20 µm cell surface will result in a theoretical cell limit of 1000/(0.02*0.02)=2.5 million cells per scaffold. This is given that the cell surface area is the same in the 3D scaffold as on the 2D batch culture (compare figure 1D and suplementary figure 3). Typically 80,000-100,000 iPSC-differentiated hepatocytes can be harvested per cm$^2$ cell culture dish indicating that the maximal total cell number in the scaffold is between 800,000-1,000,000 cells.
Chapter III

**Supplementary figure 6.** Evaluation of shear stress acting on the 3D scaffold at the microscale

Velocity profile (a) and pressure gradient (b) within the bioreactor as well as in the 3D scaffold. Different COMSOL simulations were performed to calculate the shear stress that cells sense within the 3D scaffold. The first analysis was made at the macro scale, evaluating the velocity field and pressure gradient in the reactor as well as in the 3D porous scaffold.

The domain region outside the scaffold was modeled by using the incompressible Navier-Stokes equation, which describes how the velocity, pressure, temperature and density of a moving fluid are related and include the effects of viscosity on the flow.

\[
\rho \frac{\partial u}{\partial t} - \eta \nabla^2 u + \rho (u \cdot \nabla)u + \nabla p = F \tag{1}
\]

\[
\nabla \cdot u = 0 \tag{2}
\]

where \(\rho\) is fluid density (kg\(\cdot\)m\(^{-3}\)), \(u\) is the fluid velocity (m\(\cdot\)s\(^{-1}\)), \(\eta\) is viscosity (Pa\(\cdot\)s), \(p\) is the pressure (Pa), \(F\) the volume force (N), and \(\nabla\) the standard del (nabla) operator.

The region containing the porous scaffold was modeled by using the Darcy-Brinkman equation, assuming that the scaffold has uniform microarchitecture and cylindrical pores.

\[
\mu \nabla^2 u_s - \frac{\mu}{\kappa} u_s = \nabla \tag{3}
\]

\[
\nabla u_s = 0 \tag{4}
\]

where \(\mu\) is the effective viscosity of the porous medium (Pa\(\cdot\)s), \(u_s\) is the fluid velocity (m\(\cdot\)s\(^{-1}\)), \(p\) is the fluid pressure (Pa) and \(\kappa\) is the permeability of the porous scaffold (m\(^2\)).

A flow rate of 3.3 \(\mu\)l/min was applied to the inlet, the boundary condition at the outlet was set at zero pressure, and the no slip boundary conditions were used along the walls of the model.
Supplementary figure 7.

Velocity profile (a) and shear stress (b) profile inside of a single channel having a pore diameter of 200μm within the 3D porous scaffold. The model shows that with a pore diameter of 200μm the shear stress acting on the wall is $1.2 \times 10^{-5} N \cdot m^{-2}$, which is beyond the shear stress limit that leads hepatocytes to death. To evaluate the magnitude of the shear stress acting on the walls of the porous channels within the 3D scaffold, an analysis at the micro-scale was assessed.

If we considered the scaffold as a cylindrical structure with interconnected channels with different pores diameters, the flow rate for a single channel is described by the Fanning equation:

$$Q_l = \frac{\pi D_i^4 \Delta P}{128\mu L}$$  \hspace{1cm} (5)

where $\mu$ is the medium viscosity and $L$ and $D_i$ are the channel length (that is the scaffold height), and the channel diameter respectively and $\Delta P$ is the total pressure drop. The total pressure drop was evaluated in the analysis at the macro-scale, and the mean pore diameter was evaluated experimentally and it was found to be 200μm (Mohanty et al, 2016) The single channel was modeled with the equation (1) and (2), by using as input the flow rate calculated with the equation (5). For a channel having the diameter of 200μm, the shear stress acting on the walls, which correspond to the domain where the cells formed focal adhesions, is approximately $1.2 \times 10^{-5} N \cdot m^{-2}$.
Differentiation of hiPSC to mature hepatocytes under flow conditions

References


3D liver models in tissue engineering


van Midwoud PM, Merema MT, Verpoorte E, Groothuis GM. 2011a, Microfluidics enables small-scale tissue-based drug metabolism studies with scarce human tissue, Journal of Laboratory Automation, 16: 468-76.


Part B

3D liver models in toxicology