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**Characterization of liver zonation-like transcriptomic patterns in HLCs derived
from hiPSCs in a microfluidic biochip environment**

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Abstract

The liver zonation is an important phenomenon characterized by a gradient of several functions along the liver acinus. However, this gradient remains difficult to reproduce in *in-vitro* conditions, making the obtention of an *in-vitro* method to recapitulate the liver zonation a challenging issue. In this study, we evaluated the spatial evolution of the transcriptome profile of human induced pluripotent stem cells (hiPSCs) differentiated toward hepatocytes-like cells (HLCs) phenotype in a microfluidic biochip environment. Cells collected at the inlet of the biochip, where the oxygen concentration is higher, were identified by the expression of genes involved in metabolic pathways related to cellular reorganization and cell proliferation. Cells collected in the middle and at the outlet of the biochips, where oxygen concentrations are lower, were characterized by the upregulation of genes involved in cellular detoxification processes (CYP450), PPAR signaling or arginine biosynthesis. In details, a subset of 16 transcription factors (TFs) was extracted and identified as upstream regulators to HNF1A and PPARA. These TFs are also known as regulators to target genes engaged in the Wnt/ β catenin pathway, in the TGF β /BMP/SMAD signaling, in the transition between Epithelial Mesenchymal Transition (EMT) and Mesenchymal Epithelial Transition (MET), in the homeostasis of lipids, bile acids and carbohydrates homeostasis, in drug metabolism, in the estrogen processing and in the oxidative stress response. Overall, the analysis allowed to confirm a partial zonation-like pattern in hiPSCs-derived HLCs in the microfluidic biochip environment. These results provide important insights into the reproduction of liver zonation *in-vitro* for a better understanding of the phenomenon.

Keywords: hiPSCs, HLCs, hepatocytes, liver zonation, microfluidic biochips, nanoCAGE

1. Introduction

The liver is the second largest organ in the human body and responsible for various vital functions. Due to its strategic position at the interface between the digestive system (i.e., intestines) and the rest of the body, it allows for efficient processing of absorbed nutrients (i.e., synthesis of serum proteins or intermediary metabolism of amino acids, lipids, and carbohydrates), and for the activation and detoxification of various compounds (e.g., xenobiotics and drugs). Since this has direct implications in both physiological and pharmaceutical processes, the liver thus appears as a critical organ for basic, translational and clinical research and has been the target for many modelling strategies ([Verneti *et al.*, 2017](#); [Forbes *et al.*, 2015](#); [LeCluyse *et al.*, 2012](#)).

The obtention of a realistic *in-vitro* model would allow for numerous applications in relation to the study of human hepatic functions, especially as an alternative to animal experiments. Further, it would enable the study of physio-pathological processes involved in various disorders, and to the conception of compounds of pharmacological interest. So far, physiological and pharmaceutical studies in the literature have been limited by the use animal and human primary hepatocytes. They have often been focused on the understanding of liver functions and diseases, as well as on the xenobiotic metabolism and toxicity assessments. It is known that standard two-dimensional static monocultures do not allow the maintenance of the histological and phenotypic characteristics of hepatocytes over a

long period of culture. However, efforts in the optimization of the culture methods have led to improvements of hepatic function regarding long-term maintenance and have allowed studying chronic effects *in-vitro* (Lauschke *et al.*, 2016; Khetani *et al.*, 2015; Bhatia *et al.*, 2014; LeCluyse *et al.*, 2012).

While *in-vitro* models cannot represent all the features of their *in-vivo* counterparts, and thus, exhibited significant limitations, their relative simplicity allows them to be attractive towards the study of specific parameters. Several sources of hepatic cells have been used, with each model having its own qualities: primary adult hepatocytes, oval cells (potential adult liver stem cells), mesenchymal stem cells, fetal liver progenitor cells, hiPSCs, and immortalized cell lines to name a few (Ware *et al.*, 2017; Zhang *et al.*, 2013). However, one of the main challenges remaining in tissue engineering consists of the organ-specific organization of the tissue to promote improved functionality.

The liver possesses a complex structure, composed of parenchymal cells (i.e., hepatocytes) and non-parenchymal cells (i.e., Kupffer cells, sinusoidal endothelial cells, stellate cells, periportal fibroblasts, and hepatic dendritic cells). Most of the volume and mass of the liver consists of hepatocytes, organized in lobules, representing individual functional units of the liver. Each lobule has a theoretical hexagonal structure, limited by portal triads and centered around the centrilobular vein. Along the porto-central axis of the liver lobules, the functional profile of hepatocytes can be divided into three categories. The periportal area, rich in oxygen, is specialized in glucose metabolism, and the perivenous area, poor in oxygen, is specialized in xenobiotic detoxification. Such observations imply that different pathways are dominant in the cells of each of these areas. While this might be true for certain function such as the synthesis of Albumin, which is found to take place

along the whole axis but to be higher in the periportal area, some functions are not subjected to zonation. For example, the synthesis of transthyretin and transferrin transporters is performed indifferently of zonation (Colnot *et al.*, 2011). Several hypotheses have been formed concerning functional zonation. The common point for these hypotheses is the existence of a concentration gradient of soluble compounds which allows the triggering of different signaling pathways (e.g., Wnt/ β -catenin or hedgehog pathways). As several of these pathways were reported to be modulated by the hypoxia signaling, oxygenation appears as a primary regulating factor of zonation (Kietzmann, 2017).

So far, standard culture protocols of cell lines have not permitted the reproduction of this gradient as the cells cultured together tend to adopt the same metabolic profile (LeCluyse *et al.*, 2012). Indeed, as cells grown on culture dishes or in fed-batch bioreactors are often subjected to the same stimulation over the culture area, the culture usually leads to the formation of homogeneous populations. In contrast, two-dimensional cultures of hepatic tissue in microfluidic chambers have exhibited potential zonation-like patterns (Tilles *et al.*, 2001; Allen *et al.*, 2004). While isolated primary cells present a better alternative in terms of functionality, typical isolation and purification processes usually destroy the zonal organization of the tissue. Alternatively, stem cells have appeared as a promising tool for *in-vitro* reproduction of *in-vivo* phenomena such as zonation. However, complete mature phenotypes are yet to be obtained, even though they appear as a vital option for a realistic representation of those phenomena. Thus, novel strategies for the establishment of an *in-vitro* hepatic tissue recapitulating the metabolic zonation are needed.

In addition, the work previously presented (Danoy *et al.*, 2019) has shown the possibility to extend hiPSCs differentiation protocols to microfluidic biochips which led to the formation of functional liver tissue. In this study, we present an extension of our previous work aiming to investigate liver zonation with hiPSCs-derived HLCs cultured in a microfluidic biochip. The response of the cells to an oxygen gradient in the biochip has been studied at the transcriptional level using RNA sequencing based on CAGE analysis (Plessy *et al.*, 2010; Poulain *et al.*, 2017). The presented results demonstrate the feasibility of this advanced hepatic model and further improve the understanding of the reproduction of metabolic zonation in an *in-vitro* microfluidic environment.

2. Material and methods

2.1 iPS cell source

The iPS cells used in this study (TKDN4-M clones) were provided from the Stem Cell Bank of the Institute of Medical Science of the University of Tokyo (established by Takayama and his colleagues using a retrovirus harbouring four reprogramming factors, *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*, Takahashi *et al.*, 2007).

2.2 Differentiation protocol

hiPSCs were cultured on standard Tissue Culture PolyStyrene (TCPS) dishes for the initial expansion as well as all differentiation steps. The dishes were coated with Matrigel (20mg/mL, Corning Matrigel hESC-Qualified Matrix) following the

manufacturer's recommendations. The exceeding amount of Matrigel was washed with Dulbecco Modified Eagle's Medium (DMEM) before further use for cell seeding. hiPSCs were seeded at a density of 1.00×10^5 cells/cm². In the maintenance and expansion phases, cells were cultured in mTeSR-1 medium (StemCell Technologies), supplemented with the RHO/ROCK pathway inhibitor Y-27632 (StemCell Technologies) for enhanced cells survival after thawing. The medium was then renewed every 24h with mTeSR-1. During this stage, the atmospheric conditions in the incubator were set at 37°C, 20% O₂, and 5%CO₂. Differentiation was then initiated as cell confluence reached 75% (Usually after 2 to 3 days of culture). Differentiation was performed following a four steps protocol (Labelled Step 0, Step 1, Step 2, Step 3 and Step 4). Details on the protocol as well as Media compositions and atmospheric conditions are recapitulated in Sup. Table S1 in Supp. File 1.

2.3. Protocol of transfer to microfluidic biochip

2.3.1 Microfluidic biochip design

Biochips were fabricated by replica molding in polydimethylsiloxane (PDMS). The molds were made from SU-8 photoresist using a double photolithography process. The culture chamber dimensions in the biochip were of 5 cm length, 1 cm width and 300 μm height. The design and fabrication process were fully detailed in our previous studies ([Danoy *et al.*, 2019](#)).

2.3.2 Culture in microfluidic biochip

Biochips were sterilized by autoclave after assembling and before further use. The inner surface of the biochip was coated with the Matrigel solution previously described overnight at 37°C. After a washing step with culture medium, hiPSCs were loaded into the biochips. Cells were detached at the end of step 3, inserted in the chip and culture for 48h under static conditions. The culture was then carried on for 5 additional days at a 10 μ L/min perfusion rate. Biochips were cultured under the same atmospheric conditions as Petri dishes. The perfusion circuit was composed of a peristaltic pump serially connected to a bubble trap and to the biochips. The perfusion pipes were in PTFE to reduce adsorption of growth factors. The total volume of the perfusion circuit was 2 mL.

2.3.3 Sampling toward zonation

At the end of the culture, the 5cm-long biochips were cut in 3 parts. The first part was 1cm long and labelled “Inlet sample” (n=3). The second part was labelled “Middle sample” and measured about 3cm (n=3). Finally, the third part was labelled “Outlet sample” and measured 1cm long (n=3). Chambers were cut open, and the cell layers were collected in Trizol reagent (Thermo Fisher Scientific) for mRNA extraction.

2.4 CAGE transcriptome profiling

NanoCAGE libraries were prepared and sequenced as previously described in [Poulain et al., 2017](#). Total RNA extraction, library preparation steps, and sequencing were performed as detailed in [Danoy et al., 2019](#).

2.5 CAGE data processing

NanoCAGE sequencing data were processed for CAGEscan analysis as detailed in (Lereau-Bernier *et al.*, 2018). Gene expression matrices were created using custom R scripts and the CAGEr package (Haberle *et al.*, 2015), then uploaded on the iDEP server (Ge, 2017) (<http://ge-lab.org:3838/idep/>) for differential gene expression (DGE) and Pathway Gene Set Enrichment Analysis (PGSEA). The ISMARA webserver (Balwierz, *et al.*, 2014) (<https://ismara.unibas.ch/mara/>) was used for Motif Activity Response Analysis (MARA) and the Ingenuity Pathway Analysis software (Qiagen) was used for pathway analysis.

2.6 RT-qPCR

RT-qPCR experiments were performed as previously described (Danoy *et al.*, 2019). Primer sequences selected for CDH2, SNAI2, GLUL, GCK, CYP3A4 and ACTB are shown in Table S2 of supplementary file 1. ACTB (β -Actin) was used as reference gene and undifferentiated hiPS cells as a reference sample for the normalization of gene expression data.

2.7 Immunostaining assays

Cells were fixed in 4% paraformaldehyde overnight at 4°C, washed and stored in PBS. Immunostaining was performed following the protocol previously described (Danoy *et al.*, 2019). The antibodies used were: Albumin (goat, A80-129A, Bethyl),

CYP3A4 (rabbit, ab135813, abcam), anti-goat Alexa Fluor 488 (donkey, ab150129, abcam), and anti-rabbit Alexa Fluor 568 (donkey, A10042, Thermofisher). All fluorescent images were taken using a confocal microscope (PowerIX70, Olympus).

2.8. Urea measurements

Urea levels in the culture medium were measured using GC/MS metabolomic processing. The protocol is detailed in section 3 of the [Supp. File 1](#).

2.9 Albumin measurements

The production of albumin was measured by ELISA sandwich assays following the protocol previously detailed ([Danoy *et al.*, 2019](#)).

2.10 Statistical analysis

Data are presented as the mean \pm standard error. Differences between groups were evaluated by ANOVA and then individually by Tukey HSD post-hoc test when the null hypothesis was rejected. Differences with $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were highlighted and considered statistically significant.

iDEP processing of nanoCAGE transcriptomic data was conducted with limma-voom ([Ritchie *et al.*, 2015](#); [Phipson *et al.*, 2016](#)) using a False Discovery Rate (FDR) cutoff of 0.4 for the DGE analysis (adjusted $p_value < 0.4$) and with a pathway significance cutoff of 0.25 for the PGSEA.

3. Results

3.1 Morphology and functional assays

After being allowed to adhere for 24h in the biochips, the cells were able to form a uniform monolayer (Fig. 1A). After 24h of perfusion, the tissue started to reorganize as part of the cells seemed to move toward the sides of the microchannels and were seen to adopt an elongated morphology (Fig. 1B). After 72h of perfusion, morphologies at the inlet and at the outlet started to differ noticeably. At the inlet, the tissue was dense and composed of several layers, including cells of cuboidal shape with a large nucleus (Fig. 1C). In this area, a network of cells of fibroblastic morphology could also be observed. At the outlet, the side of the channels exhibited a multilayer tissue while a monolayer of cuboidal cells was observed in the middle (Fig. 1D). After an additional 48h of perfusion, the differences between inlet and outlet seemed less pronounced and after a total of 144h of perfusion, no striking difference could be seen. In both cases, a dense 3D-multilayered tissue containing both hepatocytes-like cells and conjunctive-like cells was observed (Fig. 1E-F). For the purpose of comparison, a conjunctive tissue was not observed at both the start (Fig. 1G) and the end (Fig. 1H) of the culture in standard Petri dishes. In this culture condition, the formation of a tissue presenting

hepatic characteristics was observed and several 3D-like bud structures were found to be randomly distributed in the dishes (Fig. 1H).

Immunostaining of typical hepatic markers such as albumin and CYP3A4 confirmed the presence of hepatocyte-like cells in the biochips (Fig. 2A-B). Production of albumin by the cells was confirmed and measured to a level of 1000 ng/day/million cells (Fig. 2C). The metabolic ratio of urea was found to progressively increased over the culture time to finally reach a steady state production rate after 6 days (Fig. 2D). In addition, glycogen storage capacities were also confirmed in the cultures as illustrated by PAS staining (Fig. 2F-G).

3.2 NanoCAGE analysis

NanoCAGE transcriptomic data obtained from sequencing of biological replicates representative of three independent biochip experiments were used to compare the gene expression profiles of cell samples collected at the inlet, middle and outlet of the microfluidic device at the end of the experiment (after 6 days of culture). We added one technical replicate for each biochip compartment in the sequencing library, making a total of four replicates (3 biological replicates + 1 technical replicate) at each position of the chip (inlet, middle, outlet) in our data. Then, a gene expression matrix was created and processed online with iDEP version 8.1 (Ge, 2017) for transcriptome data analysis.

3.2.1 RNA expression at the inlet and at the center of the biochip.

In this experiment, data clustering did not lead to clearly discriminate between the transcriptome profiles of cell samples collected at the middle and at the outlet of the biochip (Fig. 3A). However, the expression profiles of inlet samples could be clearly discriminated from the other samples. Nevertheless, DGE analysis led to identifying a list of 214 genes differentially expressed between the cells collected at the inlet versus the cells collected from the center area of the biochip. Gene ontology analysis was further performed on this list of genes and returned GO biological processes, related to “cell adhesion”, “cell growth” and “epithelial migration” among the top annotations overrepresented at the inlet, whereas bile, steroid, small molecules, and protein metabolisms were highlighted in the middle of the biochips (Table 1). In addition, the GO molecular functions highlighted at the center of the biochips included “lipid binding”, whereas “cytoskeletal process” was featured at the inlet. In parallel, KEGG pathway related to PPAR signaling was overrepresented in cell samples collected at the middle of the chip, while “Hippo signaling” and “focal adhesion” were among the pathways upregulated at the inlet.

PGSEA was then performed with the entire set of genes. The heatmap of GO biological processes discriminating the inlet and the center of the chips revealed the upregulation of genes related to “cellular detoxification”, “regulation of lipid” and “response to hydrogen peroxide” in the cells from the middle area, while the “cell cycle”, “cyclin dependent kinase” and “haemopoietic progenitor cell differentiation” terms appeared overrepresented at the inlet (Fig. 3B). Moreover, the analysis performed with the GO molecular function geneset revealed that the terms “oxidoreductase activity” and “antioxidant response” were found to be increased in the cells from the center area, whereas the terms “microtubule” and “tubulin bindings” were representative of the situation at the inlet. Analysis with the KEGG geneset

showed that the terms “arginine biosynthesis”, “tyrosine metabolism” and “endocannabinoid signaling” were upregulated in the cells from the middle area of the chip (Fig. 3C; *Nb.* increasing the FDR value applied for the PGSEA to 0.4 led to extract several KEGG pathways related to drug metabolism upregulated in cells from the center area of the chip, including “CYP450”, “fatty acid synthesis”, “duct acids secretion”, and “PPAR signaling”, whereas “cell cycle”, “cell senescence”, and “Hippo signaling” were highlighted at the inlet, data not shown). Finally, analysis using the GO cellular component geneset revealed that “microtubules”, “cytoskeletons”, “nucleus” and “DNA” were among the terms overrepresented at the inlet while “peroxisomes”, “lipoproteins” and “apical parts of the cells” were the cellular compartments characterizing the cells from the middle area of the biochips (Fig. 3D). Details of the analysis as well as raw data are provided in Supp. File 2.

The list of 214 differentially expressed genes extracted by iDEP was further exploited for pathway analysis with the Ingenuity Pathway Analysis software (IPA). The analysis revealed “coagulation system”, “FXR/RXR activation”, “LXR/RXR activation” and “acute phase signaling” as among the top canonical pathways highlighted for the samples collected from the cells located at the middle of the device. Interestingly, HNF1A, IL6, PPARA, NKX2-8, and RORC were featured among the top upstream regulators. In addition, top molecular and cellular functions returned by the software included “cellular organization”, “cellular growth”, “cellular development”, “cellular movement” and “carbohydrates metabolism”. Among the top networks found to be activated in the cells collected from the middle area of the biochips, IPA identified “cell growth and proliferation” (*via* the ACTB, AMOT, APOB, FGA, FGG, GAPDH, GLIPR1, IGFBP5, RPS6KA1, SERPINA1, SPP1, TAGLN, TAGLN2, UBL3, and WWTR1 genes), “lipid metabolism”, “small molecules

biochemistry”, and “vitamin and mineral metabolism” (*via* the ACAA1, SULT2A1, PABP1, SERPINA1, HNMT, APOA2, TTR, AHSG, ATP8B1, and AFP genes). The top disease and function highlighted were related to “liver inflammation (APOB)”, “liver steatosis (APOB)”, “liver proliferation (PTN)”, “hyperproliferation (ACAA1, ACTB, ACTR3, AK1R1D1, AMDHD1, AMOT, ANXA3, APOB, ARHGAP29, CCDC80)”, and liver cholestasis (AKR1D1). A summary of the results obtained from IPA analysis is provided in [Supp. File 3](#).

Ultimately, MARA analysis was performed using nanoCAGE sequencing data collected for samples isolated at the inlet and at the middle of the microfluidic device. The top 10 TF motifs extracted by the analysis are provided in [Table 2](#). The major proteins binding to these motifs (with the corresponding gene having the strongest Pearson correlation coefficient for the motif identified) were found to be: PATZ1, MTF1, IKZF1, ESRA, SIN3A, GATA3, RCOR1, PML, E2F1, and NFIB. Especially, the motifs bound by PATZ1, IKZF1, ESRA, GATA3, RCOR1, and NFIB were found to be overactivated in the cells collected from the middle of the biochips, whereas the target motifs of MTF1, SIN3A, SP4, and E2F1 had higher activities in the cells at the inlet. Regarding the target genes of these TFs, it was found that they could be related to pathways in link with potential liver-related functions, summarized in [Table 2](#) (an exhaustive list of target genes for each TF and their different functions is provided in [Supp. File 4](#)). Among the possible roles assumed by these target genes, GO and REACTOME categories related to “carbohydrates and lipids metabolism”, “estrogen signaling”, “Wnt/ β catenin signaling”, “glutathione process”, “epithelial to mesenchymal or mesenchymal to epithelial transitions related signaling”, and “transcription factors” were identified. Based on those results, a network of interaction connecting the TFs and depending pathways (provided by ISMARA

analysis), was built as shown in Fig. 3E. Finally, the interaction networks of TF binding motifs for the potential upstream regulators identified by IPA analysis (HNF1A, PPARA, and STAT3) were identified (Fig. 4).

3.2.2 Characterization of the transcriptome of cells collected at the outlet of the biochips.

A comparison between the cells collected from the inlet and outlet of the biochips was performed. Pathway analysis led to extract upregulated GO biological processes in the cells collected from the outlet that were related to “drugs response”, “reactive oxygen processing”, and “zinc transport”, whereas the “cell cycle” and “cytoskeleton reorganization” annotations were highlighted in the cells collected from the inlet (Fig. 5A). Analysis using the KEGG pathway geneset illustrated the upregulation of drugs, CYP and arginine metabolisms; “PPAR signaling”, and several diseases (“measle” and “pathways in cancer”) in the cells collected from the outlet (Fig. 5B). These results appeared consistent with the results previously obtained from the comparison between the cells collected from the inlet and the center of the biochips, and further confirmed the trend observed for an increase in lipid, arginine and drug metabolisms inside the biochips toward the outlet. Additionally, ISMARA processing of the data collected from the samples harvested at the inlet and outlet of the biochips highlighted SIN3A (upregulated activity at the inlet), IKZB1, and PML (both with an upregulated activity at the outlet) as the top three TF motifs with high activities.

In a second step, a comparison between the cells collected from the center and the outlet of the biochips was performed. As shown before in the results obtained from hierarchical clustering (Fig. 3A), the differential gene expression analysis only identified 23 genes differentially expressed between the cells collected from the middle and the outlet of the biochips. No enriched gene set annotations could be identified from the DGE data, which suggests close gene expression patterns and cellular states in those two conditions. Nevertheless, pathway analysis led to the extraction of additional information such as “actin polymerization” (GO_biological process annotations) as well as the overrepresentation of disease-related pathways in the cells collected from the outlet such as “measles”, “toxoplasmosis”, and “phagosome” (KEGG pathway annotations), (Fig. 5C-D). IPA analysis was conducted with the short list of 23 genes and returned the “pentose phosphate pathway” as the main element in the top canonical pathway category, and ZNF143 was highlighted as a potential upstream regulator. Besides, “NRF2 mediated oxidative stress response” also appeared in the top tox list (a summary of the results obtained from the IPA analysis is provided in Supp. File 5).

Likewise, the MARA analysis using the data collected from the cells obtained from the middle area and the outlet revealed that the TF motifs bound by PATZ1, MTF1, TAF1, ESRA, YY1, TBL1XR1, SP4, ZEB1, ID4, and GATA3 were in the top 10 of the highly active motifs discriminated between those two conditions (Table 3) (an exhaustive list of target genes for each TF and their different functions is provided in Supp. File 6). The principal target genes of those TFs were found to be related to the mitochondria respiration and processing; the lipid and glucose metabolisms; the response to hypoxia, the Wnt signaling, the BMP signaling, the SMAD signaling, the tight junction regulation, the AP1 complex signaling (controlling

apoptosis, proliferation and differentiation), and the apoptosis (Table 3). Similarly, a network of interaction between the different TFs and their target pathways was constructed (Fig. 5E). Finally, the comparison of TF motifs activities over the length of the biochips (from the inlet, to the middle and the outlet) showed that the activity of motifs bound by PATZ1, GATA3 and ESRA reached a peak at the center of the biochip, while the MTF1 target motif reached a minimal activity in the same area.

3.3 RT-qPCR of zonation markers in different areas the biochip.

Based on the results obtained from the *in-silico* analysis of the transcriptomic data, the expression levels of several genes involved in substantial metabolic pathways and liver differentiation processes highlighted at the different locations of the biochip was investigated. For instance, it was found that GCK (glucokinase, carbohydrate metabolism), GLUL (glutamine synthetase, glutamine metabolism) and CYP3A4 tended to be overexpressed in the cells collected from the middle area and the outlet when compared to the cells collected from the inlet (but with a p_value around 0,1 for CYP3A4 due to variability between independent experiments). Although ARG1 is a periportal marker (arginase 1, metabolism of urea), overexpression of this gene in the cells collected from the inlet was not detected. As the process of transition between EMT and MET and as the TGF β /BMP/SMAD signaling were underlined in the analysis performed, the mRNA levels of several genes implicated in these pathways were measured. By doing so, CDH2, was found to be downregulated while SNAI2 to be upregulated in the cells collected from the inlet (Fig. 6). Finally, despite the fact that MARA highlighted some TFs such as PML, MTF1, RCOR1, PATZ1, HIF1A, and PPARA as potential upstream regulators, the

RT-qPCR analysis did not reveal different mRNA expression for these genes between the cells collected from the inlet, the middle area and the outlet of the biochips (data not shown).

Discussion

In this study, we cultured hiPSCs-derived HLCs in a microfluidic environment based on our previously developed protocol. (Danoy *et al.*, 2019) Since similar microfluidic setups have been shown to generate a gradient in oxygen based on cellular respiration (Matsumoto *et al.*, 2019) we investigated whether liver-specific patterning can be achieved in the biochip, acting as an acinar model *in-vitro*. Based on spatial transcriptomic analysis we considered a possible metabolic zonation of cells collected from different areas of the microfluidic channel.

First, we confirmed cellular identity of the tissue based on the production of typical liver markers. Cellular staining revealed that the tissue consists of Albumin and CYP3A4-positive cells (Fig. 2A, B), indicating highly differentiated hepatocyte-like cells. Consistent with previous results (Danoy *et al.*, 2019), a time-dependent increase in albumin production (Fig. 2C) highlights the advanced maturation during culture inside the biochip. Concurrently, urea production (Fig. 2D) was found to increase early during the maturation process and level off during the final days of the culture, indicating a metabolic stabilization towards the end of the maturation. To further identify cellular differentiation, we tested their ability to store glycogen, which has been used to discriminate hepatocytes from non-parenchymal cells (Raven *et al.*, 2017). In agreement with biomarker staining of Albumin and CYP3A4, we observed ubiquitous glycogen storage capabilities in the present tissue (Fig. 2E), further confirming cellular identity as hepatocyte-like cells. Based on this preliminary tissue

characterization, we then investigated the formation of zone-specific hepatic patterns by spatial transcriptomic analysis.

Due to their location near the inlet of the microfluidic channel, we hypothesized those cells to assume periportal functions, as they would be exposed to the highest levels of oxygen, nutrients, and shear stress. Indeed, gene ontology analysis of transcriptomic data revealed upregulation of distinct periportal pathways in this location. Interestingly, our analysis ([Table 1](#)) indicated an upregulation of Hippo signaling in this region. This pathway has only recently been implicated in the context of hepatic zonation ([Patel et al., 2017](#)), with its main functionality being related to liver regeneration. ([Manmadhan et al., 2019](#)) The central TF of the Hippo signaling pathway – YAP - is activated mostly in the periportal zone ([Patel et al., 2017](#)), both in hepatocytes and cholangiocytes. This pattern appears conclusive, as liver regeneration has been implicated to occur through bilateral transdifferentiation between hepatocytes and cholangiocytes ([Deng et al., 2018](#), [Schaub et al., 2018](#)). Upregulation of this pathway can further be explained by the highly mitogenic nature of stem cell-derived HLCs, indicated by gene ontology overrepresentation of cell growth pathways ([Fig.3B](#)).

Consistent with Hippo signaling, we also found upregulated TGF- β signaling ([Supp. File 4](#)) in the inlet region. Singular stimulation with TGF- β was found to allow transdifferentiation of hepatocytes towards cholangiocytes during liver regeneration. ([Schaub et al., 2018](#)) Interaction between TGF- β and Hippo signaling, however, was shown cause an EMT-like response in hepatocytes ([Oh et al., 2018](#)), enabling hepatocyte proliferation after partial hepatectomy. As we have confirmed cell types to be hepatocyte-like, this implies the upregulation to be caused by the latter pathway interaction, further indicating a highly regenerative periportal phenotype. With TGF- β

being an important pathway also in maintaining pluripotency in stem cells (Li, Wei & Ding, 2016), its implication in the present tissue was confirmed by the expression of maturation-dependent structural proteins. Here, N-Cadherin (CDH2) expression exhibited distinct downregulation (Fig. 5E) in the biochip. CDH2 expression is known to have lower expression levels in the periportal zone *in-vivo* through gradual patterning in the first postnatal week. (Doi et al., 2007) Additionally, TGF- β signaling is known to induce focal adhesion of cells by inducing various integrin molecules. (Tatler et al., 2016, Wang et al., 2016) Separately, integrins have been shown to be induced by hemodynamic shear stress, acting as a mechanosensor required for liver growth and regeneration. (Lorenz et al., 2018) Consequently, we found focal adhesion upregulated (Table 1) in our results, indicating the importance of multiple environmental factors combined for the development of zonal patterns. In this case, a link between Hippo signaling and shear stress could be implied in the context of the liver's unique capability of regeneration, based on their common modes of action.

In summary, transcriptomic analysis of the inlet region shows general upregulation of pathways involved in liver regeneration which match patterns found *in vivo*. Interestingly, interleukin-6 was found as a potential upstream regulator based on IPA (Suppl. File 3), which is consistent with processes such as liver regeneration, hepatocyte mitogen regulation, and hepatocyte homeostasis (Schmidt-Arras et al., 2016).

While cells near the inlet were characterized by their regenerative function based on interactions from Hippo and TGF- β signaling, as well as their respective downstream effects, targeted transcriptomics of the central region of the channel reveals well-established zonation patterns. Our results indicate upregulated pathways regarding cellular detoxification and xenobiotic metabolism (Fig. 3B), which

are generally known to have perivenous bias *in vivo*. (Kietzmann, 2017) These pathways are especially relevant in the context of using *in-vitro* hepatic cultures for drug development, as they are an integral part of toxicity assessment. While many different pathways are implicated in the regulation of xenobiotic degradation, many enzymes of interest, like cytochrome P450 enzymes (Gerbal-Chaloin et al., 2014), are affected by b-catenin signaling. In general, perivenous zonation has been shown to be influenced by the gradual activation of b-catenin through secretory Wnt proteins, or oxygen-dependent inactivation of phosphorylation processes. (Birchmeier et al., 2016, Gebhardt et al., 2010; Burke et al., 2006) Based on the assumption that cells in the central part of the biochip are influenced by this signaling pathway, we investigated other landmark genes that are known downstream targets. Expression of glutamine synthetase, which is only located near the central vein *in vivo*, was significantly upregulated (Fig. 6) in this location. This indicates active b-catenin signaling - a pathway that is often referred to as the master regulator of zonation (Burke et al., 2006) - in the differentiated tissue, further highlighting the successful induction of zonal patterns in the biochip.

Contrastingly, b-catenin was found to not alter homeostatic metabolic functions, which infers the need for other regulating pathways in this regard. (Behari et al., 2014) Generally, perivenous regions are specialized in, among others, pathways concerning glucose uptake, lipid metabolism, and bile acid metabolism. (Kietzmann, 2017) Based on our results, gene ontology analysis (Table 1) confirms spatial upregulation of these pathways in the biochip. PPAR signaling is considered to be the regulating pathway governing lipid metabolism. (Behari et al., 2014), bile acid and glucose homeostasis. (Li & Chiang, 2009) In agreement with these functions, pathway analysis further indicates both bile acid metabolism and

catabolism (Table 1) to be upregulated in the central cell population. Accordingly, increased gene expression of glucokinase (GCK, Fig.8) shows the spatial effect on cellular carbon metabolism as implied by PPAR signaling. (Kim et al., 2004) Conclusively, IPA identified STAT3, HNF1A and PPARA (Fig. 4, Suppl. File 3) to be the governing TFs in this region of the biochip. As highlighted above, this indicates the differential regulation of gene profiles in vitro in a way coherent with commonly described patterns in vivo. (Kietzmann, 2017)

Patterning in the central area of the biochip closely mimics perivenous phenotypes, instead of centrilobular hepatocytes. It appears that cells in this region have already sufficiently depleted oxygen levels supplied from the medium, generating perivenous oxygen tension and therefore enabling typical signaling cascades. Indeed, measurement of oxygen levels in a similar setup have shown to rapidly decrease oxygen levels. (Matsumoto et al., 2019) Hence, it is plausible to consider the central area in our experiment to have low, but physiological oxygen levels. Further analysis of cells from the outlet region of the biochip might therefore be expected to exhibit patterns of supraphysiological oxygen levels. Interestingly, many of the previously discussed pathways from the central region were also enriched in the outlet region (Fig. 5A), indicating that similar underlying mechanisms are involved in both groups. In contrast, as expected, hypoxic pathways were found to be enriched. Cytokine-mediated signaling pathways, as well as reactive oxygen species biosynthesis are upregulated, highlighting the influence of hypoxic stress due to oxygen depletion based on cellular respiration (Fig. 5B). Consequently, cell cycle pathways are downregulated in this region (Fig. 5B) which implies a hostile culture environment in this context.

Based on hierarchical clustering spatial transcriptomics clearly discriminated the inlet group from both central and outlet groups. (Fig. 3A) As previously discussed, this conclusion is supported by the underlying pathways governing the individual cellular regions according to zonal patterning. In order to identify the genes responsible for these patterns, we extracted differentially expressed, underlying TFs by utilizing ISMARA analysis.

Comparison of TFs between the inlet and central region of the biochip is generally in agreement with the previous discussion. (Fig. 3E) Inlet regions are showing enhanced motif activity regarding bile salt transports and EMT via MTF1, gluconeogenesis via PATZ1, and insulin reactome via SIN3A (Supp. File 4). Further, upregulation of PML indicates increased BMP signaling. In contrast, central cell population exhibit increased motif activity in typical perivenous pathways. Xenobiotic reactome exhibit potential enhancement via GATA3, downregulation of gluconeogenesis through PATZ1, and redox capabilities, as well as b-catenin functionality via RCOR1. Accordingly, comparison between central and outlet groups revealed a set of governing TFs, as summarized in Fig. 5E. Again, motif activity is found to be good agreement with previously discussed results. Noteworthy here is the upregulation of cellular stress reactions via AP1 (ID4 and MTF1) in the outlet region. Additionally, general downregulation of homeostatic functions is found at the outlet compared to the central part. Mitochondrial respiration motifs are enhanced through TAF1 and YY-1 in the center relative to the outlet of the biochip, highlighting the apparent hypoxic environment in this region.

While one of the central hypotheses regarding functional zonation, as it is observed along the porto-central axis of liver lobules, suggests that the oxygen gradient along the liver sinusoid could be the responsible extracellular stimulus

(Jungermann *et al.*, 1989; Gebhardt *et al.*, 1992), limitation of oxygen availability below a certain threshold can be expected to negatively affect cellular metabolism and thus functionality. As shown in the literature by both experimental data and numerical models, microfluidic devices can produce oxygen gradients along the cell culture chamber through the combination of the continuous injection and consumption of oxygen by the cells (Matsumoto *et al.*, 2019; Baudoin *et al.*, 2007; Allen *et al.*, 2004; Tilles *et al.*, 2001). Nevertheless, the exact geometry of the microfluidic channel requires special attention. Pericellular oxygen levels have been shown to quickly deplete, reaching values that do not promote healthy cell cultures due to the limitation of cellular respiration. (Matsumoto *et al.*, 2019) Therefore, a device shorter than the one presented might yield a higher spatial resolution regarding cellular patterns when sampled according to our classification.

Conclusion

In this work, hiPSCs were differentiated toward HLCs in a microfluidic biochip. Based on the expected formation of an oxygen gradient in this device, we aimed to characterize the induction of position-specific pathways in the tissue. After 7 days of culture in the biochip, the tissue formed presented an advanced zonation profile along the biochips as shown by the transcriptome analysis of the cells cultured at the inlet, in the central area and at the outlet of the biochip. The cells collected from the inlet were enriched in pathways relating to liver regeneration, showing upregulation of known zonal pathways, like Hippo and TGF- β signaling. Meanwhile, cells collected from the central area of the biochip were characterized by the upregulation of various pathways involved in cellular defense, including drug

metabolism and the homeostasis of glucose, and lipids. Our analysis showed that this upregulation was caused both by the “master regulator of zonation” (Burke *et al.*, 2006) b-catenin, as well as the PPAR pathway. Results were confirmed by RT-qPCR analysis in which the levels of mRNA of GCK and GLUL indicated a possible perivenous phenotype in the cells collected from the central area of the biochip. This study highlights the advantages of biochip technology and its uses in complex *in-vitro* modelling of situations, such as liver zonation, using hiPSCs-derived cells. Further studies with a precise and controlled environment, in terms of oxygen conditions, may allow a better understanding of this phenomenon and would present significant advances in the field of *in-vitro* modelling for the purpose of drug testing.

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Conflict of interest statement

The authors declare no conflict of interest.

Ethical issue

No ethic approval was required for the work presented in this study.

Figure legend

Figure 1: Cell morphologies observed in step 4 of the differentiation protocol: (A) after 24h of static culture in the biochip (B) after 24h of perfusion (C), after 72h of perfusion at the inlet and (D) in the middle area, and after 144h of perfusion (E) at the inlet and (F) in the middle area. Cell morphologies observed in step 4 of the differentiation protocol in Petri dishes on Day 1 (G) and Day 7 (H).

Figure 2: Immunostainings after 7 days of differentiation in step 4. Staining for Albumin (green, A) and CYP3A4 (red, B). Albumin (C) and Urea (D) productions measured in the cultures during step 4 of the differentiation protocol. PAS staining performed on samples at the end of the differentiation protocol (E-F).

Figure 3: Heat map clustering of the Inlet vs Middle area vs Outlet comparison (A). GO biological process (B), GO KEGG pathway (C), and GO cellular compartment (D) extracted from iDEP7 post treatment on the comparison between Inlet and Middle area. Interaction network connecting the different TF motifs highlighted by MARA analysis and their target pathways in the comparison between Inlet and Middle area (E).

Figure 4: Interaction network of key TF motifs identified by Ingenuity Pathway Analysis upon comparison of cells collected from the middle area and the inlet.

Figure 5: GO KEGG pathway (A) and GO biological process (B) extracted from iDEP7 post treatment on the comparison between Inlet and Middle area. GO KEGG pathway (C) and GO biological process (D) extracted from iDEP7 post treatment on the comparison between Middle area and Outlet. Interaction network connecting the different TF motifs highlighted by MARA analysis and their target pathways in the comparison between Middle area and Outlet (E).

Figure 6: Expression levels of selected genes measured by RT-qPCR on samples collected from the inlet, the middle area and the outlet of the biochip.

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