

Towards human insulin resistance in vitro disease models using 3D-cultured MSC-derived hepatocyte-like cells

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Abstract: Insulin resistance affects a substantial proportion of the world population and is closely related to obesity and type II diabetes mellitus, all of which are associated with high clinical and economic burden and mortality risk. The liver has a central role in metabolic homeostasis, by controlling the energy metabolism pathways in response to hormonal stimuli. Therefore, the use of *in vitro* disease liver models is needed for studying these pathologies. The main goal of this work is to improve current culture protocols to obtain an insulin resistance model. In this work, mesenchymal stem cells were differentiated into hepatocyte-like cells (HLCs) and maintained in culture as self-assembled spheroids. Importantly, given that current protocols include high concentrations of glucose, insulin and dexamethasone that interfere with the cells' energy metabolism, the aim was to reduce the concentrations of these molecules in the culture medium to more physiologic values (5 mM, 1 nM and 100 nM, respectively) and to subject cells to insulin and glucagon stimuli to validate the model in terms of energy metabolism responses. HLC spheroid cultures were successfully adapted to more physiologic media, maintaining spheroid size and morphology. Expression of hepatic-specific genes (*ALB*, *HNF4A*, *CYP3A4*, and *CK-18*), proteins ALB and HNF4A and urea production were maintained in the more physiologic media. HLC spheroids expressed periportal and perivenous markers simultaneously, both at the gene and protein level, with higher accumulation of periportal markers at the periphery of the spheroids. HLCs cultured in the more physiologic medium also showed enhanced response to insulin and glucagon when compared to the less physiologic medium and were able to respond to fatty acid incubation with intracellular accumulation of triglycerides, and therefore, HLC spheroids cultured in more physiologic media are better suited to study hepatic energy metabolism. This work constitutes a small step towards the development of a reliable insulin resistance model.

Keywords: Hepatocyte-like cells; Spheroid cultures; Insulin resistance; Human neonatal mesenchymal stem cells; Metabolism.

I. INTRODUCTION

Insulin resistance affects between 15.5 and 46.5 % of adults worldwide¹. It occurs when normal levels of insulin cannot trigger a normal response in target organs such as the liver, muscle, and adipose tissue, affecting the metabolic homeostasis of the body². Due to impaired insulin signaling, glucose production by the liver is no longer suppressed and there is increased lipid production in the liver and triacylglycerol (TAG) hydrolysis in the adipose tissue, resulting in hyperglycemia and excess fatty acids in circulation and in the liver. To compensate the loss of signaling, the pancreas produces higher amounts of insulin, but, eventually, β cells may become worn out and thus unable to produce sufficient amounts of insulin, leading to the development of type II diabetes.

The liver has a central role in the development of insulin resistance and, more generally, in the metabolic homeostasis of the body. It is responsible for processing carbohydrates, lipids, and proteins, synthesizing bile acids and metabolizing drugs and toxins, with most of these functions being performed by hepatocytes, the liver's parenchymal cells³.

The liver's metabolic functions are tightly regulated by hormones insulin and glucagon, which correspond to two opposing states, respectively: the fed state, characterized by high circulating levels of glucose and fatty acids, and the fasting or starvation state, when glucose and fatty acid levels are lower, and the body needs to resort to energy storages to maintain blood sugar levels and meet energy demands.

Glucose entering hepatocytes after a meal is phosphorylated into glucose 6-phosphate (G6P), so that it does not leave the cell. In the fed state, G6P can be used to produce glycogen, through glycogenesis, or free fatty acids, to be stored as TAG, and amino acids. Alternatively, G6P can be transformed into pyruvate to produce energy via glycolysis. Glycolysis is the main pathway used by the liver to produce ATP in the postprandial period, when glucose levels are high, because insulin promotes the synthesis of key glycolysis enzymes⁴. Pyruvate from glycolysis can then be converted into acetyl coenzyme A (CoA), which will enter the tricarboxylic acid cycle and be used to produce energy through oxidative phosphorylation. The presence of insulin leads to increased conversion of pyruvate into acetyl-CoA, through inhibition of pyruvate dehydrogenase kinase 4 (PDK4) expression, because PDK4 inhibits acetyl-CoA formation⁴.

In the fasting state, glucagon is released and cyclic AMP levels within hepatocytes rise. In these conditions, peroxisome proliferator-activated receptor- γ coactivator 1- α (PGC-1 α) is induced and leads to the expression of genes involved in glycogenolysis and gluconeogenesis. G6P molecules obtained from these processes are dephosphorylated by glucose 6-

phosphatase (G6Pase) into glucose, to be released into circulation and used by other tissues⁵. Conversely, the synthesis of enzymes involved in glycolysis is inhibited by glucagon, leading to decreased consumption of glucose to form pyruvate.

The liver also plays a part in the regulation of fatty acid metabolism. The *de novo* synthesis of fatty acids, a process requiring high amounts of energy, occurs in postprandial periods, when energy availability is higher. Fatty acids are esterified to a molecule of glycerol in the liver, to form TAG. Because the majority of TAG molecules are stored in the adipose tissue, with only a small amount being stored in lipid droplets within hepatocytes, TAG molecules produced in the liver are packaged into very low-density lipoproteins (VLDL) and released into circulation, to be used by peripheral tissues. These pathways are induced in the presence of insulin.

Upon fasting or starvation, the glucagon-mediated rise in cAMP leads to decreased fatty acid synthesis in hepatocytes. In adipose tissue, glucagon leads to increased TAG hydrolysis and thus higher levels of FFAs in circulation, which, together with glucagon, induce the peroxisome proliferator-activated receptor isotype α (PPAR- α), the main regulator of β oxidation⁶. This nuclear receptor promotes the transcription of rate-limiting enzymes in β oxidation, such as the liver isoform of carnitine palmitoyltransferase I (CPT1A), involved in the transport of fatty acids out of the mitochondria, where they are produced, or acyl CoA oxidase 1 (ACOX1), involved in the oxidation of very long chain fatty acids.

Bile acids are essential for the digestion and absorption of lipids and regulate cholesterol homeostasis. Bile acid synthesis occurs in the liver and is regulated through a negative feedback mechanism, in which bile acids activate the farnesoid X receptor (FXR), a nuclear receptor that regulates gene transcription and, in particular, CYP enzyme cholesterol 7 α -hydroxylase (CYP7A1), the only rate-limiting enzyme in this pathway. The presence of insulin leads to decreased FXR expression, and thus promotes bile acid synthesis⁷. Conversely, glucagon inhibits CYP7A1, resulting in a decrease in bile acid production in fasting conditions⁸.

All the previously described metabolic functions of the liver are performed by hepatocytes. These cells are arranged in structural units, the lobules, which are hexagonal in shape and contain a branch of the portal vein, a branch of the hepatic artery and a bile duct at each corner and a central vein around which hepatocytes are radially arranged. Blood flows from the periphery of the lobule into the central vein and oxygen, hormones and nutrients are gradually absorbed by hepatocytes, creating gradients within the lobule, and resulting in hepatocytes performing different functions depending on their location within the lobule, a phenomenon known as ‘metabolic zonation’. In the outer or periportal (PP) zone of the lobule, hepatocytes perform functions typically requiring higher amounts of oxygen or nutrients, such as gluconeogenesis, β -oxidation or albumin synthesis. Functions such as glycolysis, lipogenesis and TAG synthesis are performed by hepatocytes near the central vein, known as perivenous (PV) hepatocytes^{9,10}. The Wnt/ β -catenin pathway is described to play a major role in the regulation of metabolic zonation, particularly in the PV region: blocking this pathway results in downregulated PV gene expression and a reduction in the PV area of the lobule¹¹. More recently, Cheng *et al.* proposed that glucagon also regulates hepatic zonation by opposing the effects of the Wnt/ β -catenin pathway¹². In their study, glucagon-null mice showed altered gene expression patterns, with a significant reduction of the PP zone and the expression of its characteristic markers, and the opposite effect on the PV zone.

Better understanding organ functions and disease mechanisms through the use of *in vitro* models is essential to find novel therapeutic solutions. Regarding hepatic *in vitro* models, several approaches have been explored, with different cell sources and culture systems: primary human hepatocytes (PHHs) are the ‘gold standard’ regarding *in vitro* liver models, but their availability is scarce and they tend to rapidly dedifferentiate in culture, losing their phenotype and eventually dying¹³; immortalized hepatic cell lines can be used as an alternative, but these cells express lower levels of key hepatic transcription factors and drug-metabolizing enzymes that do not accurately reproduce physiologic conditions^{14–16}. More recently, human stem cells have been used to obtain hepatocyte-like cells (HLCs). Our group has previously developed a protocol for hepatic differentiation of human neonatal mesenchymal stem cells (hnMSCs) that allows for the obtention of HLCs able to express hepatic markers and perform hepatic functions for more than 2 weeks in culture^{17,18}. This protocol has also been adapted to three-dimensional (3D) cultures, because these allow for a better recreation of the liver microenvironment, with the promotion of cell-cell and cell-matrix interactions. In fact, 3D cultures have been shown to improve phenotype and function in both PHHs and HLCs^{18,19}.

The protocols currently used for obtaining HLCs rely on high concentrations of glucose, dexamethasone, and insulin in the culture medium. The addition of these compounds results in improved hepatic growth and differentiation, but their use in concentrations much higher than the ones found *in vivo*, such as those used in current protocols, may lead to imbalances in the energy metabolism of cultured cells, and thus render them inappropriate for studying metabolic diseases such as insulin resistance^{20–22}. In this work, the effect of lowering glucose, insulin, and dexamethasone concentrations in the culture medium was tested on HLC spheroid cultures, to determine if their differentiated phenotype and metabolic capabilities, in terms of energy metabolism responses to hormonal stimuli, are affected. For a better understanding of HLCs’ phenotype, the PP/PV phenotype of HLC spheroids was also studied. Finally, we tried to determine HLCs’ response to incubation with fatty acids, mimicking current western diets.

II. METHODS

II.1. Reagents

Culture media, supplements and reagents were purchased from Sigma-Aldrich, unless stated otherwise. Fetal bovine serum (FBS), trypsin-EDTA and Insulin-transferrin-selenium (ITS) were purchased from Gibco/Life Technologies. Penicillin-streptomycin (PenStrep) was purchased from Lonza. FGF-2, HGF, FGF-4, OSM and dexamethasone were purchased from Peprotech. OA was purchased from Merck. Rat-tail collagen was produced in house according to Rajan *et al.*²³.

II.2. Human neonatal MSC culture

hnMSCs were isolated from umbilical cords as described by Miranda *et al.*²⁴ and Santos *et al.*²⁵, and expanded as undifferentiated cells in alpha modified Eagle's medium (α -MEM) supplemented with 10 % FBS (growing medium) and passaged every 2-4 days, when 70-80 % confluence was reached. All cell cultures were maintained at 37 °C in a humidified atmosphere with 5 % CO₂. Cell viability assessment was performed using the trypan blue exclusion method.

II.3. Hepatocyte differentiation of hnMSCs

The hepatocyte differentiation followed a three-step protocol, according to Cipriano *et al.*^{17,18}. hnMSCs were initially seeded in a 0.2 mg/mL rat-tail collagen-coated surface at a density of 1.5×10^4 cells/cm² in growing medium, reaching a cell confluency of 90 % in 24 h. Iscove's modified Dulbecco's medium (IMDM) supplemented with 1 % PenStrep and 0.1% amphotericin B was used as basal medium (BM) for the differentiation. In the first step cells were maintained for 48 h in BM supplemented with 2 % FBS, 10 ng/mL of EGF and 4 ng/mL of FGF-2. In the second step cells were maintained for 10 days in BM supplemented with 20 ng/mL of HGF, 10 ng/mL of FGF-4, 4 ng/mL of FGF-2, 0.61 g/L of nicotinamide and 1 % ITS. At day 10 of the differentiation 1 % DMSO was added. For the third step (after day 13) cells were maintained in standard high-glucose differentiation medium (Diff), which consists in BM supplemented with 8 ng/mL of OSM, 1 μ M of dexamethasone, 1 % DMSO and 1 % ITS. At day 17 the cells were trypsinized and re-inoculated in ultra-low attachment (ULA) plates (Corning, Inc.), to promote 3D spheroid formation, or rat-tail collagen-coated 2D culture plates, as stated below. 5 % FBS and 20 μ M of 5-AZA were added to Diff on this day. From day 21 onwards the cells were maintained in one of four media studied with varying concentrations of insulin, dexamethasone and glucose: high- and low-glucose differentiation media (Diff and Diff -glu, respectively) and high- and low-glucose physiologic media (Physiol +glu and Physiol, respectively). All media were supplemented with 1 % PenStrep, 0.1 % amphotericin B, 8 ng/mL of OSM and 1 % DMSO. Both differentiation media (Diff and Diff -glu) were supplemented with 1 % ITS and 1 μ M dexamethasone, while both physiologic media were supplemented with 1 nM insulin and 100 nM dexamethasone²⁶. High-glucose media (Diff and Physiol +glu) contained 25 mM of glucose and IMDM was used as the basal medium and low-glucose media (Diff -glu and Physiol) contained 5 mM of glucose and Dulbecco's modified Eagle's medium (DMEM) was used as the basal medium.

II.3.1. Spheroid culture

At day 17 the cells were trypsinized and re-inoculated in 6-well ULA plates with a cell density of 5×10^5 cells/mL in 1 mL of medium per well, to promote 3D spheroid formation. Cells were suspended in Diff supplemented with 5 % FBS and 20 μ M of 5-AZA. Six hours after inoculation the cells were pipetted up and down to prevent the formation of large aggregates. After 24 h, 1 mL of Diff was added to each well. At day 21 the culture medium was changed to one of the four options tested: Diff, Diff -glu, Physiol +glu or Physiol. Spheroids were maintained in this medium until day 34, with 50 % medium changes every 3-4 days.

II.3.2. 2D culture

At day 17 the cells were trypsinized and re-inoculated in 24-well collagen-coated 2D culture plates with a cell density of 2×10^4 cells/cm². Cells were suspended in Diff supplemented with 5 % FBS and 20 μ M of 5-AZA. After 24 h, the medium was exchanged to Diff, thus removing the FBS and 5-AZA. At day 21 the culture medium was changed to one of the four options tested: Diff, Diff -glu, Physiol +glu or Physiol. Cells were maintained in this medium until day 34, with medium changes every 3-4 days.

II.4. HLC spheroids visualization and measurement

Spheroids were visualized and photographed using an inverted microscope with contrast phase (Motic AE2000). The average diameter of spheroids was calculated, from a minimum of 20 spheroids in each day and replicate, by calculating

the geometric mean of three diameter measurements per spheroid, according to: average diameter = $(d1 \times d2 \times d3)^{1/3}$. Images were acquired with moticam 2500 and spheroid diameters were measured using the ImageJ software.

II.5. Response to insulin and glucagon

At day 34 HLCs were subjected to insulin and glucagon stimuli to study the effect of these conditions on the cells' energy metabolism. For the insulin stimulus assay, the cells were initially incubated for two hours in starvation medium (SM): DMEM supplemented with 1 % of PenStrep, 0.1 % of amphotericin B, 1 % of DMSO, 8 ng/mL of OSM, 0.2 % of bovine serum albumin (BSA) and 4 mM glutamine, followed by incubation for 8 h in SM alone (control) or in SM with 80 nM insulin, as described by Correia *et al.*²⁷. For the glucagon stimulus, cells were incubated for 8 h in SM (control) or SM with 100 nM of glucagon. At the end of the assay cells were resuspended in TRIzol and stored at -80 °C.

II.6. Urea quantification

The rate of urea production was quantified from cell culture supernatants using a quantitative colorimetric urea quantification kit (QuantiChrom™ Urea Assay Kit, BioAssay Systems), according to the manufacturer's instructions. Absorbance was measured at 520 nm in a microplate reader (SPECTROstar Omega, BMG Labtech). Results are expressed as rate of production: $\mu\text{g}/(10^6 \text{ cells.h})$.

II.7. Gene expression analysis

Total RNA of spheroid samples with $0.5\text{-}1.5 \times 10^6$ cells was isolated using TRIzol (Life Technologies) and extracted according to the manufacturer's instructions. RNA concentration was determined by measuring absorbance at 260 nm. cDNA was synthesized from 1 μg of RNA using NZY First-Strand cDNA Synthesis Kit (NZYTech), according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using PowerUp SYBR Green Master Mix (Life Technologies) for a final reaction volume of 15 μL , using 2 μL of template cDNA and 0.333 μM of forward and reverse primers. Reaction was performed on QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems), consisting of 2 minutes at 50 °C, 10 minutes at 95 °C, 40 cycles of 15 seconds at 95 °C followed by 1 minute at 60 °C. Finally, a dissociation step was added to determine the melting temperature of a single target sequence as a measure of quality and specificity. For quantification, the comparative Ct ($2^{-\Delta\Delta\text{Ct}}$) method was used, with normalization to the reference gene β -actin. Results are presented relative to undifferentiated hnMSCs, for hepatic-specific gene expression measurement, or relative to controls, for insulin and glucagon stimuli assays.

II.8. Oil red O staining

Oil red O staining was used to stain lipid droplets in 2D-cultured cells incubated from day 21 onwards with concentrations of 400 μM , 300 μM , 200 μM or 100 μM of either palmitic acid (PA) or oleic acid (OA) in a 10 % BSA solution, as well as both fatty acids together in concentrations of 200 μM , 150 μM , 100 μM or 50 μM each. Incubation with BSA was used as control. On day 34, cells were fixed in a 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) solution for 10 minutes at room temperature (RT). Cells were then washed with PBS and incubated with 1.2 mg/mL oil red O and 60 % (v/v) isopropanol solution for 10 minutes at RT. Cells were again washed with PBS and placed in water for visualization using an inverted microscope with contrast phase (Motic AE2000).

II.9. Immunocytochemistry

Spheroids were collected, centrifuged, and the culture medium removed. Fixation was performed overnight with a 4 % PFA and 4 % sucrose in PBS solution. Spheroids were embedded in a 2 % agarose solution, followed by dehydration in increasing ethanol concentrations (70 % EtOH overnight and 80 % EtOH, 90 % EtOH and 100 % EtOH for 1 hour each) and clearing with xylene for 1 hour. Agarose plugs were embedded in liquid paraffin overnight to form paraffin blocks. 5 μm sections were cut using a microtome. Deparaffinization was performed overnight in xylene, followed by rehydration in decreasing ethanol concentrations (100 % EtOH and 96 % EtOH for 10 minutes each, followed by 80 % and 70 % EtOH for 5 minutes each). Samples were incubated for 10 minutes in a 0.3 % triton in PBS solution for permeabilization, washed with PBS, incubated for 1 h in a blocking solution with 2.5 % BSA (w/v) and 2 % FBS (v/v) in PBS and with the primary antibodies overnight at 4 °C. Primary antibodies used were GLS2 (rabbit polyclonal IgG; Abcam), GS (mouse monoclonal IgG; Santa Cruz Biotechnology), ALB (rabbit polyclonal IgG; Santa Cruz Biotechnology) and HNF4A (mouse monoclonal IgG; Perseus Proteomics Inc.). Incubation with secondary antibodies (mentioned below) was performed for 1 h at RT prior to incubation with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 5 minutes. Finally, Aqua-Poly/Mount coverslipping medium (Polyscience Europe) was applied. Samples were observed on a fluorescence microscope (Zeiss Axio Scope.A1) coupled with a camera (AxioCam HRm) for image acquisition, using the ZEN Lite 2012 software.

Excitation/emission wavelengths of 590/617 nm (goat anti-rabbit Alexa Fluor 594; Life Technologies), 495/519 nm (donkey anti-mouse Alexa Fluor 488; Life Technologies) and 358/461 nm (DAPI) were used for signal detection.

II.10. Statistical analysis

Results are given as mean \pm standard deviation (SD) of independent measurements or assays (n=2 for gene expression and urea measurement in response to insulin, glucagon and fasting stimuli and n=3 for remaining assays). Statistical analysis was performed using GraphPad Prism 7. To analyze differences between groups, two-way ANOVA was used. $p < 0.05$ was considered statistically significant.

III. RESULTS AND DISCUSSION

III.1. HLC spheroid cultures were successfully adapted to more physiologic concentrations of glucose, insulin, and dexamethasone

HLC spheroids were able to be maintained in culture for two weeks, from days 21 to 34, in the four media. Spheroids cultured in the four conditions maintained similar morphology and appearance, with smooth edges, throughout the two weeks in culture (Figure 1). Spheroid size was also maintained between days 21 and 34 in all culture media (Figure 1). The average spheroid diameter at day 21 was $133.9 \pm 14.2 \mu\text{m}$, indicating the absence of necrotic cores²⁸.

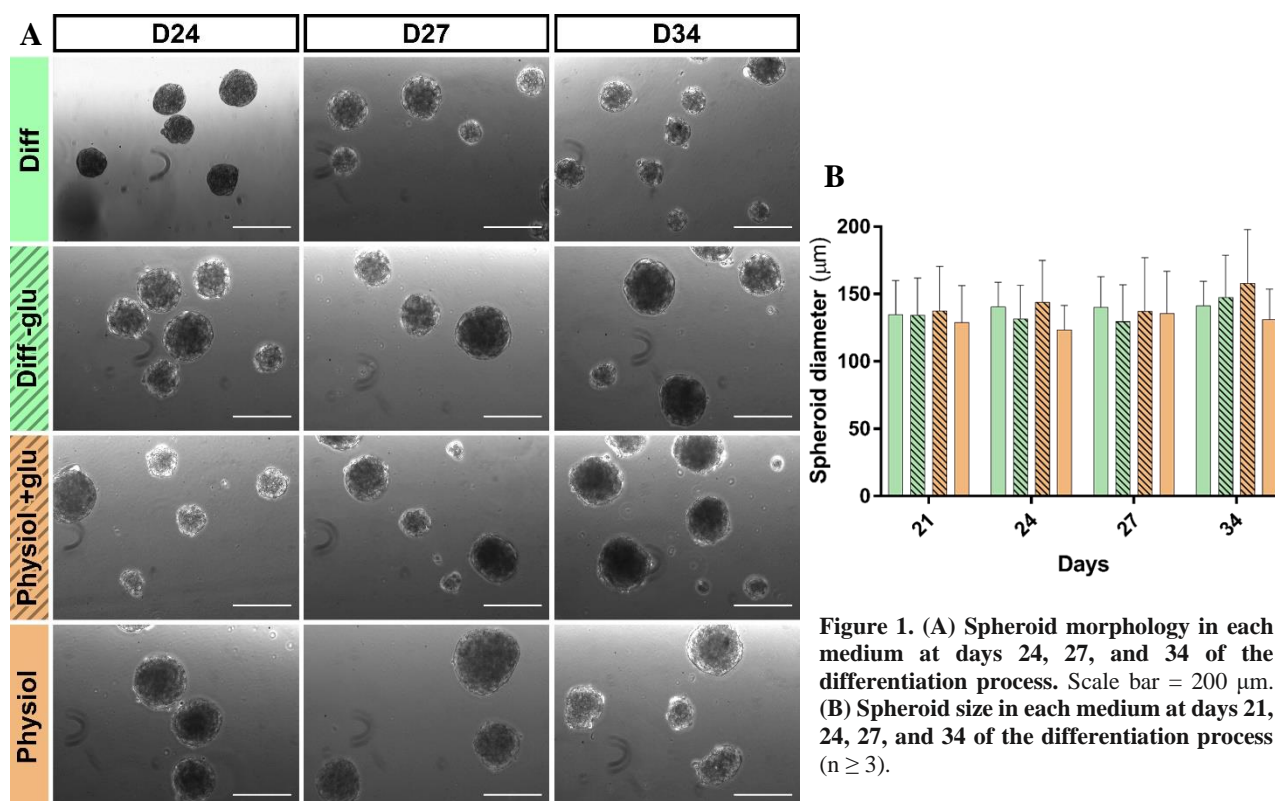
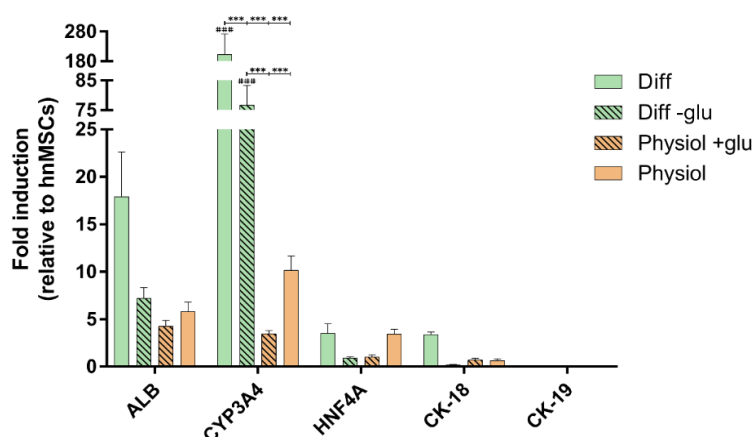


Figure 1. (A) Spheroid morphology in each medium at days 24, 27, and 34 of the differentiation process. Scale bar = 200 μm . (B) Spheroid size in each medium at days 21, 24, 27, and 34 of the differentiation process (n \geq 3).

III.2. Low concentrations of glucose, insulin, and dexamethasone enable the maintenance of HLC spheroids' hepatic phenotype

Expression levels of hepatic genes were measured at day 34 in the four media, to evaluate the influence of glucose, insulin and dexamethasone on the differentiation and maturation of spheroids (Figure 2). The expression of hepatic genes *ALB*, *HNF4A* and *CK-18* was maintained in all media. *CK-19*, a marker of biliary lineages, was absent in all media, indicating a commitment to the hepatic lineage during the differentiation process. *CYP3A4* expression levels in the most physiologic medium were 10-fold higher than those of undifferentiated hnMSCs. Spheroids cultured in both differentiation media, with high insulin and dexamethasone concentrations, presented significantly higher *CYP3A4* expression, which is consistent with the powerful CYP inducing capabilities of these compounds. Therefore, HLC spheroids are able to express *CYP3A4* at a basal level when cultured with low concentrations of insulin and dexamethasone, and at much higher levels upon induction with higher concentrations of these molecules.

Figure 2. Gene expression analysis of hepatic markers of HLC spheroids at day 34. The graph represents the fold-induction of genes expressed by HLCs relative to undifferentiated hnMSCs. Data is normalized to the reference gene β -actin ($n = 3$). *, **, *** Significantly differs from other culture media formulations with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. #, ##, ### Significantly induced or repressed expression with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.



These studies were also performed in 2D-cultured HLCs, a simpler, more extensively used and characterized model. The *CYP3A4* expression in 2D-cultured HLCs is higher than that of 3D-cultured HLCs for all media, and *ALB* expression was higher in Physiol in 2D when compared to 3D (Figure 3). No other significant differences between the two culture systems were found, and thus spheroid cultures seem to adapt to more physiologic media as well as 2D cultures.

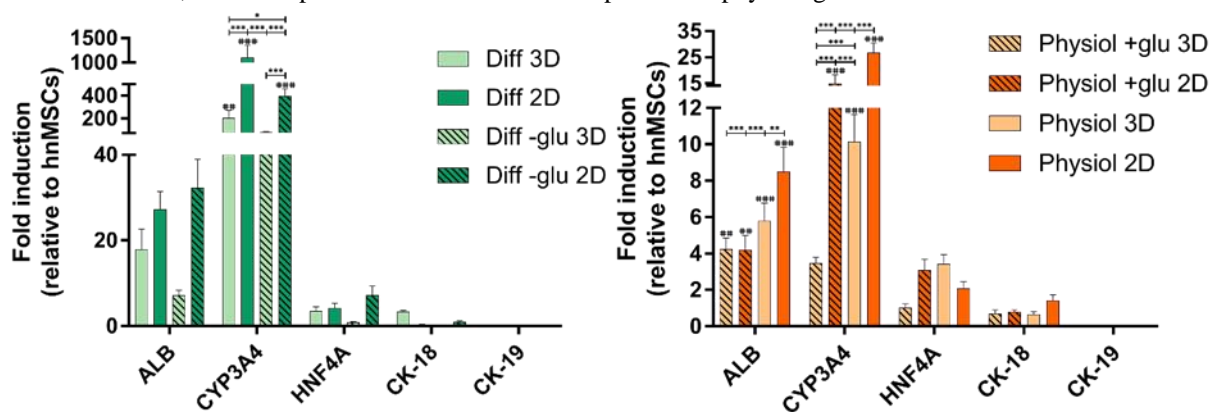


Figure 3. Comparison of gene expression analysis of hepatic markers on 2D- and 3D-cultured HLCs at day 34. The graph represents the fold-induction of genes expressed by HLCs relative to undifferentiated hnMSCs. Data is normalized to the reference gene β -actin ($n \geq 3$). *, **, *** Significantly differs from other media formulations or culture system with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. #, ##, ### Significantly induced or repressed expression with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

The presence of *ALB* and *HNF4A* was also evaluated at the protein level in spheroids cultured in Diff and Physiol, through immunocytochemistry/immunofluorescence. HLCs within the spheroids expressed *ALB* and *HNF4A* in both Diff and Physiol (Figure 6 C, D, O, P), which is in accordance with the gene expression results, and further demonstrates the maintenance of hepatic phenotype up to day 34 in more physiologic HLC cultures.

Urea production, a very important function of hepatocytes, commonly used to assess the function of in vitro hepatocyte cultures, was measured at days 27 and 34 in the four media. Urea production was significantly higher than that of undifferentiated hnMSCs in all media on both days and moderately increased in high-glucose media when compared with low-glucose media, particularly between Diff and Diff -glu at day 27 (Figure 4). Dexamethasone and insulin are reported to have opposite actions on urea synthesis in hepatocytes, with dexamethasone inducing urea production and insulin decreasing it²⁹. Simultaneously, glucose has an inhibitory effect on urea synthesis, by inhibiting glucagon and also via a direct effect of the carbohydrate itself³⁰. However, ureagenesis and amino acid catabolism have been reported to be increased in patients with metabolic imbalances such as in poorly controlled type II diabetes mellitus³¹, a state characterized

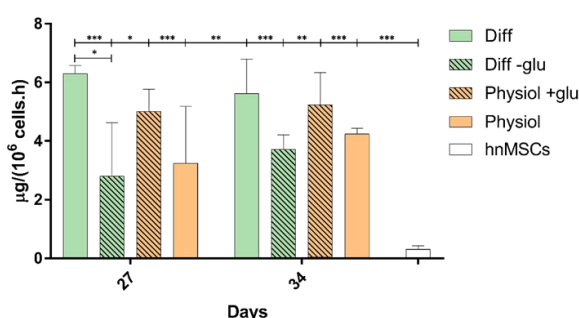


Figure 4. Urea production of HLC spheroids cultured in Diff, Diff -glu, Physiol +glu and Physiol at days 27 and 34 and of undifferentiated hnMSCs. Data is presented as mean \pm SD ($n \geq 3$).

by hyperglycemia and hyperinsulinemia, analogous to that of Diff, and thus the trend for higher urea production in high-glucose media may indicate that cells cultured in these conditions could be presenting some characteristics of type II diabetes.

The previous results show that lowering the concentration of glucose, insulin, and dexamethasone in the culture medium does not affect the phenotype and function of HLCs, and thus, Physiol, the most physiologically relevant medium, was chosen to proceed with further studies on the energy metabolism and phenotype of HLC spheroids, and Diff, the previously validated culture medium, was used as a comparison.

III.3. HLC spheroids cultured in more physiologic conditions show enhanced response to insulin and glucagon

Until recently, most hepatic *in vitro* models focused on biotransformation, using insulin and dexamethasone in high concentrations, to induce hepatic differentiation and the expression of biotransformation enzymes. However, Kato *et al.* found that culturing primary rat hepatocytes in high concentrations of insulin led to a marked decrease in insulin receptor expression and impaired functions such as glycogen synthesis and lipogenesis³².

To study whether cells cultured in Diff, a medium rich in glucose, insulin, and dexamethasone, presented impaired energy metabolism, and whether a more physiologic culture medium would improve the cells' response, HLC spheroids cultured in Diff and Physiol were subjected to insulin and glucagon stimuli and the expression of genes involved in the energy metabolism was measured.

HLC spheroids cultured in Physiol showed a much more physiologic response to insulin than those cultured in Diff (Figure 5 A). All genes were significantly more inhibited by insulin in Physiol when compared to Diff, indicating that there was increased glycolysis, fatty acid synthesis, and bile acid synthesis, and decreased gluconeogenesis and fatty acid β oxidation in Physiol-cultured HLCs in response to this hormone. In Diff, only *PDK4* and *G6PASE* expression levels were reduced by incubation with insulin. Upon incubation with glucagon, almost all genes were significantly more upregulated in Physiol than in Diff (Figure 5 B). Particularly, *PDK4* and *CPT1A* were significantly upregulated in the presence of glucagon in spheroids cultured in Physiol. *G6PASE* and *PGC1A* expression, however, were inhibited in the presence of glucagon in both media, contrary to what was expected. The fact that some genes only respond partially to glucagon could be due to the incubation period used. In fact, Lv *et al.* found that *G6PASE* expression levels in PHHs were highest only 2 hours after incubation with glucagon, and, thus, studying gene expression at different time points may be useful for a better understanding of the effects of this hormone on some metabolic pathways in HLCs³³.

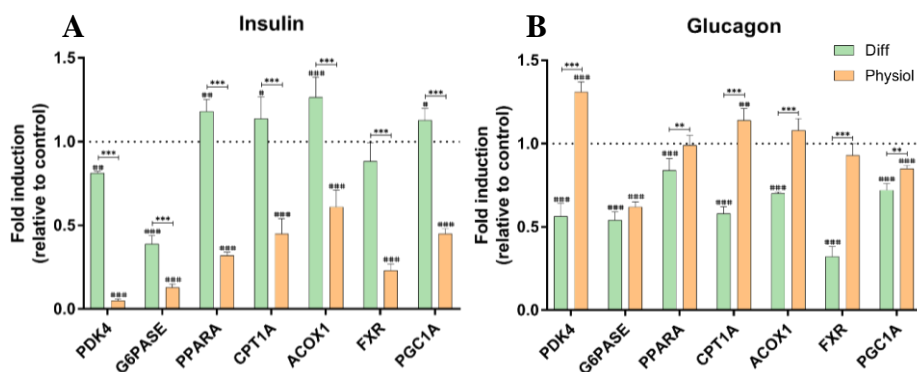


Figure 5. Gene expression analysis of energy metabolism genes in response to (A) insulin and (B) glucagon. Data is normalized to the reference gene β -actin and presented relative to starvation medium ($n = 2$). *, **, *** Significantly differs from other culture medium formulation with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. #, ##, ### Significantly induced or repressed expression with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

III.4. Insulin and glucagon regulate the expression of periportal and perivenous markers in HLC spheroids

The localization within the spheroids of the two landmark proteins of the PP and PV areas, GLS2 and GS, respectively, was studied, to try to determine if there was an expression pattern that could be correlated with the gradients formed within the spheroids, as well as the influence of hormonal stimuli on their expression. For this purpose, HLC spheroids cultured in Diff and Physiol were incubated at day 34 with insulin, glucagon, or in the absence of hormonal stimuli and were collected for immunocytochemistry/immunofluorescence for GLS2 and GS detection. The presence of ALB and HNF4A was also studied, to check for maintenance of hepatic phenotype.

Overall, GLS2 and GS were detected in all conditions and throughout most of the cells in the aggregates (A-X), suggesting that HLCs within the spheroids present an intermediate phenotype, with both PP and PV characteristics. ALB and HNF4A were also detected in all spheroids and conditions. No noticeable differences in expression patterns were found between spheroids incubated with insulin and glucagon. Even though all proteins were present throughout all the aggregates, in many samples there was a higher intensity of GLS2 or ALB at the periphery of the spheroids when compared to the central region (Figure 6 C, G, K, M, O, Q, S) and this pattern did not seem to depend on glucagon or insulin incubation, and thus may be attributable to the higher presence of nutrients and oxygen in the outer region of the spheroids.

Indeed, in the liver, GS is expressed in the PV region, exclusively in 1 to 3 layers of hepatocytes closest to the hepatic veins³⁴, while GLS2 and ALB are found in the PP zone, where oxygen and nutrient availability is higher³⁵. In our study, there seemed to be some zonation of GLS2 and ALB, but the PV marker GS and the liver-enriched transcription factor HNF4A did not present clear expression patterns. Previous studies report that HNF4A is indeed found throughout the whole lobule of the mouse liver in a homogeneous fashion³⁶.

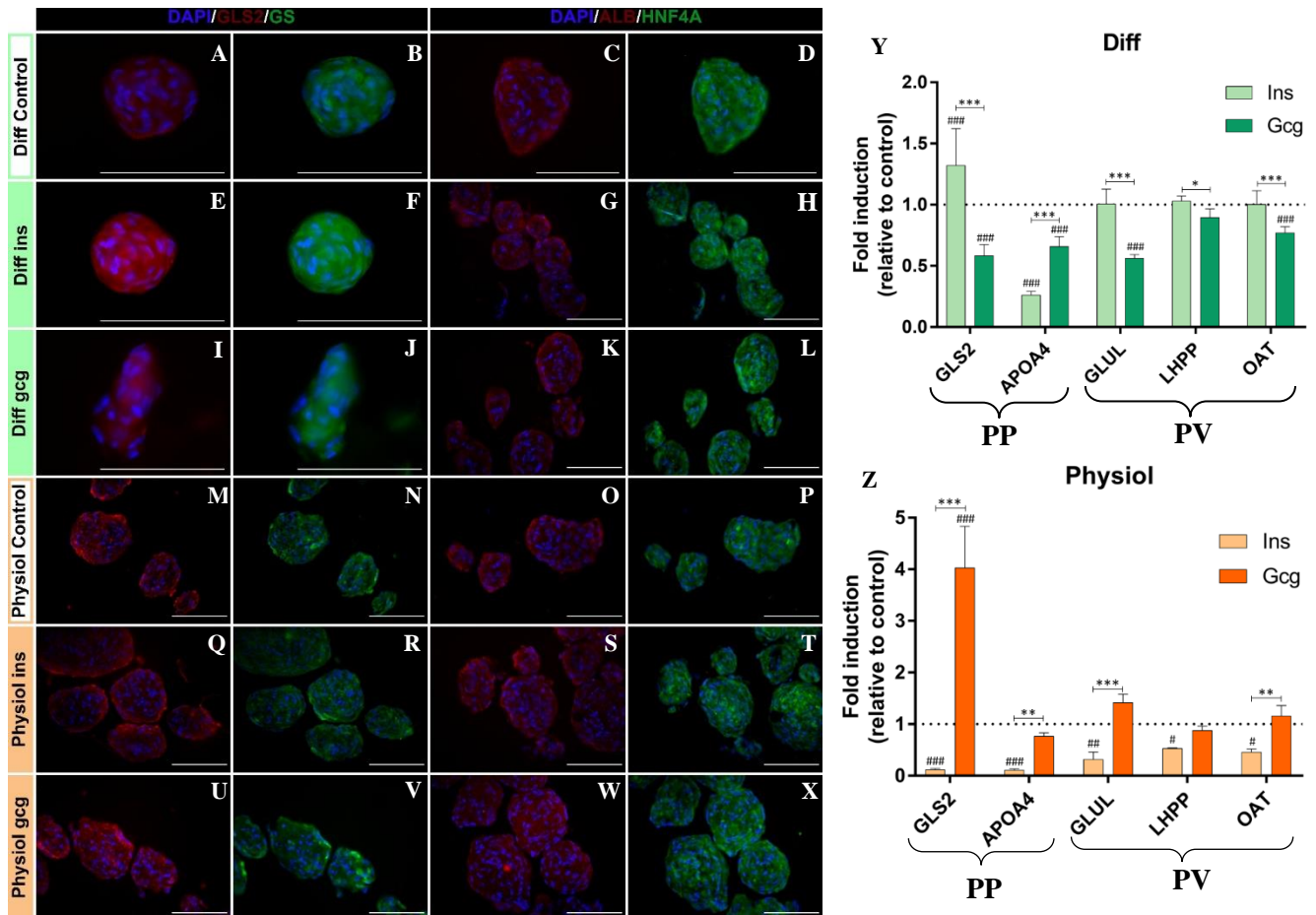


Figure 6. Hepatic zonation within HLC spheroids: (A-X) Immunocytochemical staining of HLC spheroids cultured in Diff and Physiol, incubated with insulin (ins) and glucagon (gcg) (day 34). Cells incubated in SM were used as controls. Scale bar = 100 μm. (Y, Z) Gene expression analysis of PP and PV genes in response to insulin and glucagon stimuli. Data is normalized to the reference gene β -actin and presented relative to starvation medium (n = 2). *, **, * Significantly differs from other culture medium formulation with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. #, ##, ### Significantly induced or repressed expression with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.**

The influence of hormones on the expression of PP and PV genes was studied through qRT-PCR after 8-hours incubation with insulin or glucagon. The genes chosen to represent the PP zone were the PP marker *GLS2* and apolipoprotein A-IV (*APOA4*). For the PV zone, the *GLUL* was chosen, as well as phospholysine phosphohistidine inorganic pyrophosphate phosphatase (*LHPP*) and ornithine aminotransferase (*OAT*). These genes are regulated by the Wnt/ β -catenin pathway and were found by Cheng et al. to be differentially expressed upon glucagon modulation, and thus present themselves as interesting targets for the study of hormonal influence on metabolic zonation¹².

Upon incubation with insulin, all PP and PV genes were significantly downregulated in Physiol (Figure 6 Z). However, this effect was slightly more pronounced for *GLS2* and *APOA4*. Both genes are characteristic of the PP zone and *APOA4* has been described to be inhibited by insulin. Because spheroids cultured in Diff are maintained in extremely high concentrations of insulin from day 21 onwards, they may be unable to appropriately respond to insulin stimuli, as demonstrated in the previous chapter, which may explain the lack of responsiveness to hormones in this condition. In spheroids cultured in Physiol, glucagon incubation led to a drastic increase in *GLS2* expression. *GLS2* is stimulated by glucagon, through cAMP, and thus is expected to be induced by the presence of this hormone³⁷, as is the case in this work. The fact that there were no changes in the expression levels of several genes that have previously been shown, by Cheng et al., to be regulated by this hormone¹² possibly indicates that the incubation time used may not have been sufficient to induce meaningful changes in their expression. However, Cheng et al. used mouse hepatocytes in their study, and thus interspecies differences may also play a role in the different gene expression patterns observed in human HLCs.

III.5. Oleic acid counteracts palmitic acid-induced lipid accumulation in 2D-cultured HLCs

Modern western diets are characterized by excessive nutrient intake and, in particular, an excess in fatty acid consumption³⁸. PA and OA are the most common fatty acids in western diets and in the livers of patients with steatosis, also known as fatty liver^{39,40}. Having demonstrated that Physiol is more appropriate for studies on energy metabolism than Diff, it was chosen for further studying HLCs' metabolic capabilities, namely, the effect of incubating HLCs with fatty acids in the accumulation of lipids within the cells. For this purpose, HLCs were cultured in 2D monolayer, an extensively used system that is easier to handle, and incubated from day 21 onward with OA and PA, either alone or in a 1:1 ratio, in concentrations ranging from 50 μ M to 400 μ M, similar to those found on the blood of patients with hepatic steatosis⁴⁰. Lipid droplets inside the cells were visualized via oil red O staining.

Both PA and OA incubation resulted in increased fat accumulation in a dose-dependent manner (Figure 7). However, the effect of PA was substantially more intense than that of OA. PA and OA combined led to more intense staining than OA alone but less intense than PA alone. HLCs are, therefore, responsive to high levels of fatty acids in the culture, mimicking intracellular fat accumulation in hepatocytes in vivo with high-fat diet^{41,42}.

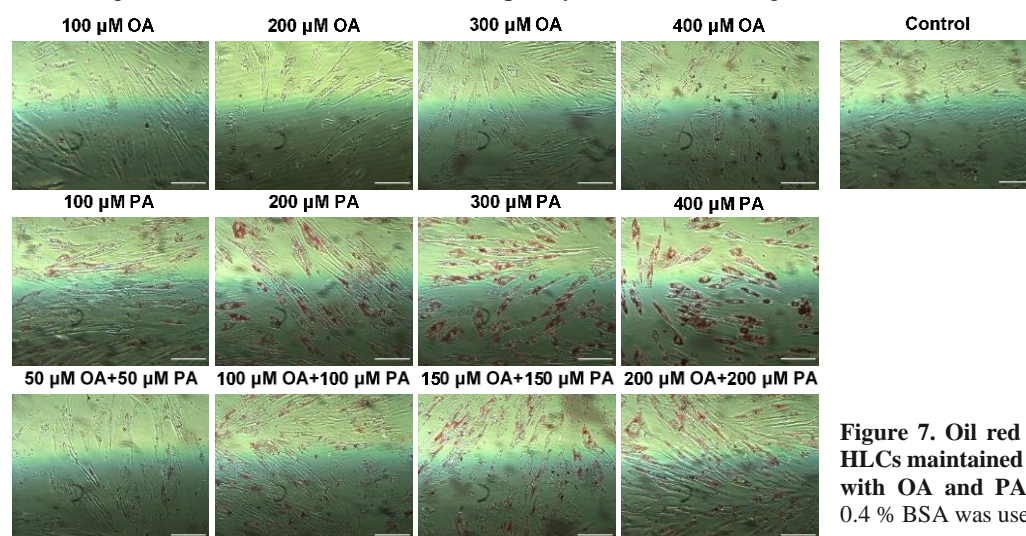


Figure 7. Oil red O staining of 2D-cultured HLCs maintained in Physiol upon incubation with OA and PA (day 34). Incubation with 0.4 % BSA was used as control. Scale bar = 200

IV. CONCLUSIONS

In this work it was possible to show that high concentrations of glucose, insulin, and dexamethasone at the maturation stage of hepatic differentiation are not needed to sustain HLC spheroids' differentiated phenotype and functionality. Furthermore, HLC spheroids cultured in more physiologic medium present a much more physiologic response to insulin and glucagon in terms of energy metabolism gene expression than those cultured in a less physiologic medium and, therefore, are more suitable to be used in studies regarding the energy metabolism. Additionally, HLC spheroids present both periportal and perivenous markers, with expression of periportal markers at the periphery of the aggregates, indicating that there could be some zonation within the spheroids. Finally, HLCs cultured in more physiologic medium are able to respond to oleic and palmitic acid incubation with dose-dependent intracellular lipid accumulation, and there seems to be a protective effect of oleic acid against the more detrimental effects of palmitic acid.

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