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

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Preface

The work presented in this thesis was performed at the Chemical Biology and Toxicology group, Instituto de Investigação do Medicamento (iMed), Faculty of Pharmacy, University of Lisbon (Lisbon, Portugal), during the period February 2020-January 2021, under the supervision of Prof. Joana Miranda. The thesis was co-supervised at Instituto Superior Técnico by Prof. Cláudia Lobato da Silva.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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Resumo

A resistência à insulina afeta uma parte substancial da população mundial e está ligada à obesidade e diabetes mellitus tipo II, todas elas associadas a elevados encargos clínicos, económicos, e risco de mortalidade. O fígado tem um papel central na homeostase metabólica, através do controlo das vias do metabolismo energético em resposta a estímulos hormonais. Assim, é necessária a utilização de modelos hepáticos de doença *in vitro* para estudar estas patologias.

O objetivo principal deste trabalho é melhorar os atuais protocolos de cultura de forma a obter um modelo de resistência à insulina. Células estaminais mesenquimais foram diferenciadas em células tipo-hepatócito (CTH) e mantidas em cultura como esferoides formados por autoagregação. Dado que os atuais protocolos de cultura incluem altas concentrações de glucose, insulina e dexametasona, que poderão interferir com o metabolismo energético das células, o objetivo foi reduzir estas concentrações no meio de cultura para valores mais fisiológicos (5 mM, 1 nM e 100 nM, respetivamente) e expor as células a estímulos de insulina e glucagon para validar o modelo em termos das respostas do metabolismo energético.

As culturas tridimensionais de CTH foram adaptadas com sucesso para meios mais fisiológicos, tendo-se mantido o tamanho e morfologia dos esferoides. A expressão de genes específicos hepáticos (*ALB*, *HNF4A*, *CYP3A4*, e *CK-18*), proteínas ALB e HNF4A e produção de ureia foram mantidas nos meios mais fisiológicos. Os esferoides de CTH apresentaram expressão de marcadores periportais e perivenosos simultaneamente, tanto ao nível génico como proteico, com maior acumulação de marcadores periportais na periferia dos esferoides. CTH cultivadas em meio mais fisiológico apresentaram melhorias na resposta à insulina e glucagon quando comparadas com o meio menos fisiológico e responderam à incubação com ácidos gordos através de acumulação intracelular de triglicéridos.

Esferoides de CTH cultivados em meios mais fisiológicos são mais adequados para o estudo do metabolismo energético. As condições experimentais devem ser melhoradas para melhor compreender o papel das hormonas na zonação metabólica nos esferoides. Este trabalho constitui um passo no sentido de desenvolver um modelo fidedigno de resistência à insulina.

Palavras-chave: Células tipo-hepatócito; Esferoides; Resistência à insulina; Células estaminais mesenquimais neonatais humanas; Metabolismo

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 could 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.

HLC spheroids cultured in more physiologic media are better suited to study hepatic energy metabolism. Experimental conditions must be improved to better understand the role of hormones in metabolic zonation within the spheroids. This work constitutes a step towards developing a reliable insulin resistance model.

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

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List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
5-AZA	5-azacytidine
α-MEM	Alpha modified Eagle's medium
ABC	ATP-binding cassette family
ACC	Acetyl-coenzyme A carboxylase
ACOX1	Acyl coenzyme A oxidase 1
AFP	α-fetoprotein
ALB	Albumin
APOA4	Apolipoprotein A-IV
BM	Basal medium
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BSEP	Bile salt export pump
cAMP	Cyclic AMP
CK-18	Cytokeratin-18
CK-19	Cytokeratin-19
CoA	Coenzyme A
CPT	Carnitine palmitoyltransferase
CREB	cAMP-response element-binding protein
CYP	Cytochrome P450 superfamily
CYP7A1	Cytochrome P450 enzyme cholesterol 7α-hydroxylase
DAPI	4’,6-diamidino-2-phenylindole
Diff	Differentiation medium
Diff -glu	Low-glucose differentiation medium
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor

ER	Endoplasmic reticulum
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FFA	Free fatty acid
FGF	Fibroblast growth factor
FXR	Farnesoid X receptor
G6P	Glucose 6-phosphate
G6Pase	Glucose 6-phosphatase
GCK	Glucokinase
GI	Gastrointestinal
GLS2	Glutaminase 2
GLUL	Glutamine synthetase (gene)
GS	Glutamine synthetase (protein)
HepaRG	Human hepatoma cell line
HepG2	Human hepatoma cell line
HGF	Hepatocyte growth factor
HHEX	Hematopoietically-expressed homebox protein
HLC	Hepatocyte-like cell
HNF4α	Hepatocyte nuclear factor-4α
hnMSC	Human neonatal mesenchymal stem cell
IMDM	Iscove's modified Dulbecco's medium
iPSC	Induced pluripotent stem cell
Irs	Insulin receptor substrate
ITS	Insulin-transferrin-selenium
LHPP	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase
MSC	Mesenchymal stem cell
NADPH	Nicotinamide adenine dinucleotide phosphate
OA	Oleic acid
OSM	Oncostatin M
PA	Palmitic acid
PBS	Phosphate-buffered saline
PDH	Pyruvate dehydrogenase
PDK4	Pyruvate dehydrogenase kinase 4

PenStrep	Penicillin-streptomycin
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate-carboxykinase
Physiol	Physiologic medium
Physiol +glu	High-glucose physiologic medium
OAT	Ornithine aminotransferase
OATP	Organic anion transporting polypeptide
PFA	Paraformaldehyde
PFK-1	Phosphofructokinase-1
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1-α
PHH	Primary human hepatocyte
PK	Pyruvate kinase
PKA	Protein kinase A
PP	Periportal
PPAR-α	Peroxisome proliferator-activated receptor isotype α
PV	Perivenous
qRT-PCR	Quantitative real-time polymerase chain reaction
SHP	Short heterodimer partner
SM	Starvation medium
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TGFβ	Transforming growth factor β
UCM-MSC	Umbilical cord matrix-derived MSC
ULA	Ultra-low attachment
VLDL	Very low-density lipoprotein

Introduction

1) The Liver

1.1) Anatomy and Function

The liver is, apart from skin, the largest organ in the human body and it is vital for the normal functioning of the human organism, due to its involvement in several physiologic processes. This organ is a key player in the energy metabolism, through processing of carbohydrates, lipids, and proteins, to build up energy reserves or promote their utilization, as needed. The synthesis of bile acids, essential for cholesterol metabolism and for fat absorption, and the synthesis of most of the proteins secreted in the blood stream are also performed by the liver[1]. Because it is particularly exposed to foreign chemicals, the liver also plays a major role in the biotransformation of drugs and toxins, as well as steroids, and hormones.

In terms of structure, the liver is irrigated by the portal vein, which carries blood from the spleen and gastrointestinal (GI) tract and accounts for 70 % of hepatic blood flow, and the hepatic artery, which carries most of the oxygen reaching the liver[2]. It contains five different cell types, as well as a complex and diverse extracellular matrix (ECM) to support them. Hepatocytes are the liver's parenchymal cells, accounting for 60-65 % of the total number of cells and around 80 % of total liver volume[2]. These are polygonal epithelial, sometimes binucleated cells, rich in mitochondria to support their very active metabolism, and have large amounts of endoplasmic reticulum (ER), which is where drug metabolization and protein synthesis occur[3]. Hepatocytes are responsible for most of the major hepatic functions, such as metabolism regulation, detoxification, and protein synthesis, rendering them an essential role for disease modeling, toxicology assessment and drug screening *in vitro*. The non-parenchymal cell types present in the liver include specialized endothelial cells, which line the liver's vascular channels, named sinusoids, and produce and secrete a number of substances, such as cytokines, ECM components, and growth factors; Kupffer cells, which are the liver-resident macrophages, are located within the sinusoids and have great phagocytic capacity, being involved in protection against bacterial infection; Ito or stellate cells are fat-storing cells involved in vitamin A storage and hepatic fibrosis processes; and cholangiocytes are the epithelial cells that line the lumen of bile ducts[2].

Within the liver, hepatocytes are arranged into functional structural units – the lobules (Figure 1). These are hexagonal in shape, containing at each corner a branch of the portal vein, a branch of the hepatic artery and a bile duct, which constitute the portal triad. The hepatocytes in the lobule are radially arranged around a central vein. Between the radial columns of hepatocytes are the sinusoids. Blood flows in the sinusoids from the portal vein and hepatic artery, at the periphery of the lobule, into the central vein. There is usually only one layer of hepatocytes between sinusoids, and there are large gaps

between the endothelial cells lining the sinusoids, maximizing the contact area between the hepatocytes and the blood, and facilitating substance exchange. Between the hepatocytes and the endothelial cells that line the sinusoids there is a space called space of Disse, which contains extracellular fluid, and through which substance exchanges are performed[3].

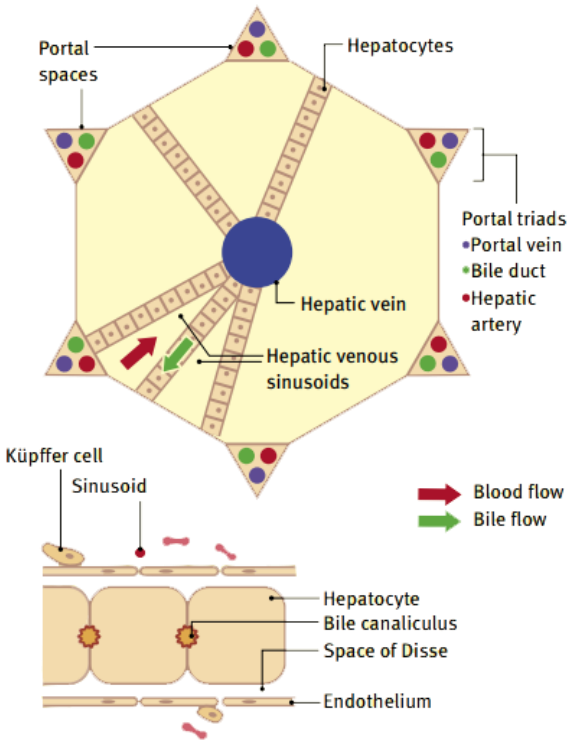

Figure 1. Hepatic lobule and sinusoid. The lobules are hexagonal in shape, containing at each corner a branch of the portal vein, a branch of the hepatic artery and a bile duct, which constitute the portal triad. The hepatocytes in the lobule are radially arranged around a central vein and between the radial columns of hepatocytes are the sinusoids. Blood flows in the sinusoids from the portal vein and hepatic artery, at the periphery of the lobule, into the central vein. Between the hepatocytes and the endothelial cells that line the sinusoids there is a space called space of Disse, which contains extracellular fluid, and through which substance exchanges are performed. Image adapted from Campbell (2006)[3].

Due to the lobular arrangement of hepatocytes in the liver and the inward direction of blood flow within the lobule, there is a concentration gradient of oxygen, nutrients, and waste products across the lobule: peripheral cells, located near the portal triad, are exposed to higher concentrations of oxygen and nutrients, while those closer to the central vein are exposed to lower amounts of oxygen and nutrients and to higher levels of metabolic byproducts. This leads to differences in the phenotype of hepatocytes depending on their location in the lobule, a phenomenon known as ‘metabolic zonation’ (Figure 2). Even though these differences are gradual, two main zones can be considered: the periportal (PP) zone, which includes hepatocytes located closer to the portal triad, and the pericentral or perivenous (PV) zone, comprised of hepatocytes nearer to the central vein. Metabolic functions with higher oxygen or nutrient requirements, such as gluconeogenesis and β oxidation, are more predominant in the PP zone, where oxygen and nutrients are more abundant, while metabolic pathways such as glycolysis and lipogenesis are more present in PV hepatocytes[4,5]. Both albumin production and ureagenesis are also predominantly located in the PP region[6,7]. Using single-cell RNA sequencing of mouse hepatocytes, Halpern *et al*. found that, in fact, around 50 % of hepatic genes are zonated[8].

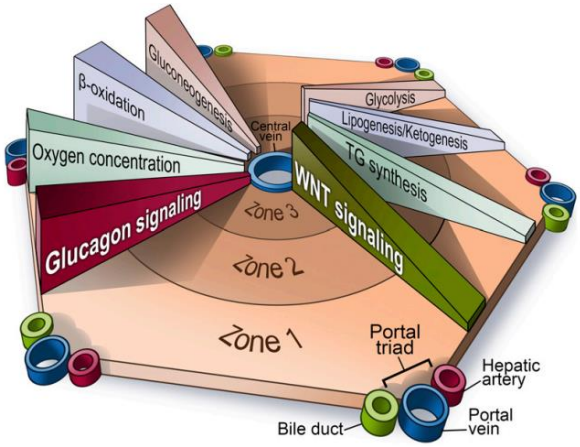

Figure 2. Hepatic lobule zonation. Hepatocyte functions vary within the lobule, with gluconeogenesis and β oxidation more predominant in the PP area and TAG synthesis, lipogenesis and glycolysis characteristic of the PV zone. Glucagon signaling acts predominantly on PP hepatocytes and opposes Wnt signaling acting in the PV zone. Image from Kusminski *et al*. (2018)[9].

These processes are controlled by complex mechanisms not yet fully understood. However, it is widely described in the literature that the Wnt/β-catenin pathway plays a major role in their regulation. Benhamouche *et al*. showed that β-catenin is present only in PV hepatocytes of mouse livers and its expression is complementary to that of its negative regulator *Apc*, which is present in PP hepatocytes[10]. Furthermore, in the absence of the *Apc* gene there was an expansion of the PV zone, with the whole lobule expressing markers of PV hepatocytes. On the other hand, blocking Wnt/β-catenin signaling resulted in opposite effects: a decrease in PV gene expression and increased area stained by PP markers, further reinforcing the pivotal role of these pathways in regulating hepatic zonation.

The ECM of the liver is essential for the maintenance of the cells' phenotype. The parenchymal ECM, located in the space of Disse, consists of fibronectin and collagen type I, with collagen type III in smaller amounts. The basement membrane, present around the blood vessels, is composed of laminin, collagen type IV and perlecan[7].

1.2) Hepatic drug metabolism and transport

The abundant blood supply to the liver comes mainly from the portal vein but also from the hepatic artery. Since the portal vein drains blood from the GI tract, many substances, namely drugs, that are ingested are absorbed there and directly transported to the liver, the primary site of drug metabolization. The concentration of a drug that reaches the systemic circulation depends on how much of it is absorbed in the GI tract, metabolized in the liver and excreted. This is the first-pass effect, in which the liver has a critical role, and protects the organism from potential toxic effects of drugs[11].

Exogenous substances that reach the liver can be water-soluble or fat-soluble. The first enter the cells via carrier proteins, while the latter, which are larger in number, are able to easily and more quickly penetrate the cell membranes, due to their lipophilic nature[2]. These lipophilic compounds cannot be

directly excreted, since they will be reabsorbed from urine in the kidneys, so they must be made water-soluble (hydrophilic)[2] through a process called biotransformation, which is divided in two phases.

Phase I reactions (functionalization reactions) involve the activation or insertion of reactive groups, such as -OH, -NH_2, -SH and -COOH, through oxidation, reduction or hydrolysis reactions[12]. These groups are hydrophilic/polar, and their presence will slightly increase the molecule's water solubility. The main reactions in this phase are catalyzed by the cytochrome P450 (CYP) enzyme family[11]. These enzymes are monooxygenases that have a very wide range of substrates, being able to metabolize both endogenous and exogenous substances. Their expression is highest in hepatocytes, but they exist in most tissues. In particular, CYP3A4 is the predominant form of CYP enzymes in the adult liver, accounting for 30-40 % of its CYP enzyme content and was estimated to be involved in the metabolization of a large portion of marketed drugs[13–15]. After solubilization by CYP enzymes, foreign compounds can be excreted through the kidneys, or they can proceed to phase II of the biotransformation process.

Phase II reactions (conjugation reactions) involve the conjugation of a molecule with an endogenous substance (*e.g.,* glucuronide, sulphate, or acetyl groups), producing highly hydrophilic products that are able to be excreted in the urine or bile. These reactions are catalyzed by transferases, such as the uridine diphosphate-glucoronosyltransferases[11].

For the biotransformation process to occur, transport of substances and their metabolites in and out of the cells through transporter proteins is necessary. Influx transporters, such as the organic anion transporting polypeptides (OATPs), the organic anion transporter family or the organic cation transporter family, are involved in the uptake of molecules and are expressed in the basolateral/sinusoidal membranes, in a prime location to receive compounds coming through the systemic circulation. The four OATP family members expressed in the liver, OATP1B1, OATP1B3, OATP2B1 and OATP1A2, are responsible for transporting bilirubin, bile acids, steroids, and multiple drugs[16]. Drugs transported by organic anion transporters include diuretics, antibiotics and antivirals, while organic cation transporters typically transport anesthetics, antiallergics and antihypertensives, among others[7]. The transport of substances out of the cells is mediated by energy-dependent transporter proteins belonging to the ATP-binding cassette (ABC) transporter family of efflux proteins or by the organic solute transporter proteins, involved in the transport of sterols[12]. These transporters are located both in basolateral/sinusoidal membranes and canalicular/apical membranes of hepatocytes, allowing for excretion into the circulation and the bile, respectively. The ABC family has seven subfamilies and includes a large number of transporters with different expression patterns and substrate affinities[17]. Its members, the multidrug resistance-associated proteins (MRP) 3 and 4, are responsible for drug efflux into blood, while bile acid efflux transporters include MRP2 and the multidrug resistance protein 1[7].

1.3) Hepatic Metabolic Functions and Metabolism Regulation

The liver plays a key role in the energy metabolism and is essential for the maintenance of physiologic levels of glucose in circulation. When this metabolism is somehow disrupted it can lead to the development of insulin resistance, diabetes and nonalcoholic fatty liver diseases[18–20].

The energy metabolism in the liver is mostly dependent on the action of two hormones, insulin and glucagon, for its normal functioning. Insulin is produced by the β cells in the pancreas and secreted in response to elevated levels of glucose, amino acids and GI hormones in the blood, which occurs after feeding. The presence of insulin leads to a buildup of energy reserves, by promoting glycogen and triacylglycerol (TAG) synthesis and inhibiting their degradation, as well as promoting protein synthesis (Figure 3). Conversely, glucagon, a hormone secreted by pancreatic α cells, is released when the blood concentration of glucose or amino acids is low, in fasting or starvation conditions. Glucagon acts by making the energy reserves available, promoting the breakdown of stored glycogen, stimulating gluconeogenesis and consequent glucose release into the blood stream as well as TAG secretion and amino acid uptake by the liver[21] (Figure 3).

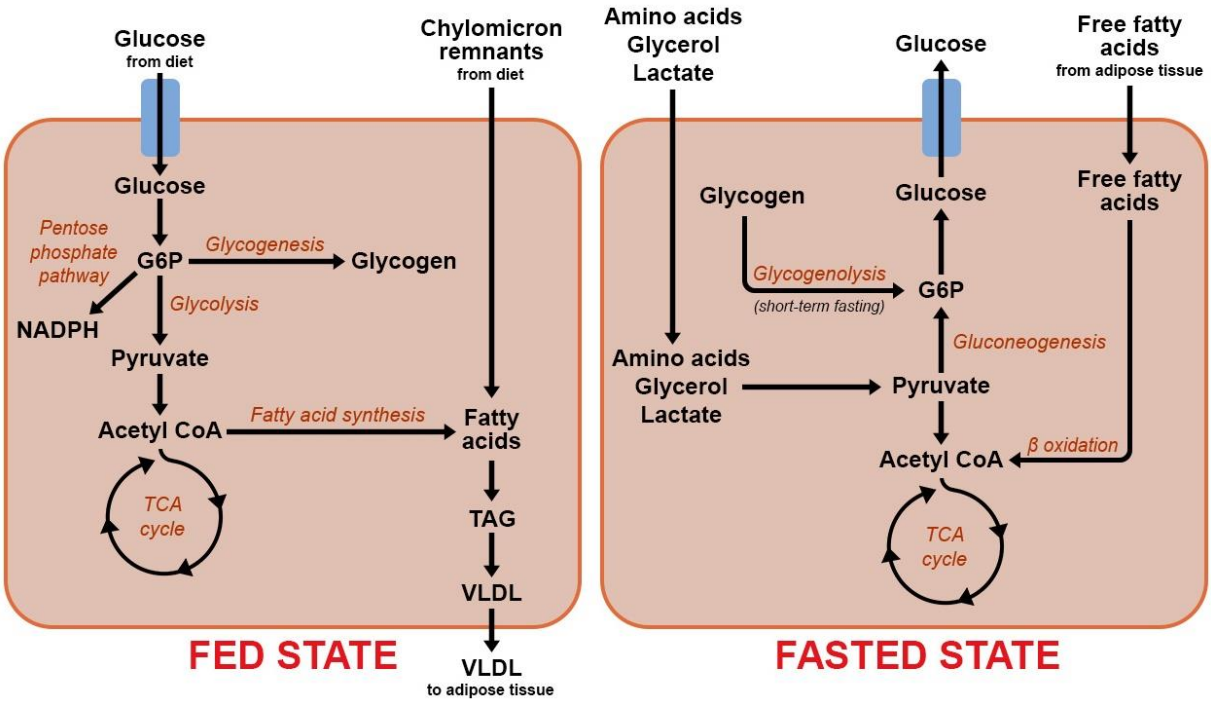

Figure 3. Liver metabolism in the fed and fasted states. In the fed state, insulin promotes a buildup of energy reserves, with an increase in glycogenesis, and TAG synthesis. In the fasted state, glucagon promotes the breakdown of stored energy, with increased glycogenolysis and β oxidation, as well as increased production of glucose through gluconeogenesis.

The distribution of insulin and glucagon across the lobule is not homogeneous: in postprandial periods, the concentration of glucagon falls by around 50 % from the PP to the PV zone, as it is extracted by hepatocytes, while the concentration of insulin only decreases by 15 %[22]. Thus, the concentration of glucagon is higher in the PP zone, while insulin is higher in the PV zone. More recently, Cheng *et al*. demonstrated that glucagon also contributes to liver zonation by opposing the effects of the Wnt/β-catenin pathway[23]. They found that glucagon-null mice had altered gene expression patterns, more

evidently in the PP region. The area in which glutaminase 2 (GLS2), a classical marker for the PP zone, appeared was significantly reduced in the absence of glucagon, while glutamine synthetase (protein: GS; gene: *GLUL*), a marker of the PV zone, was present in an increased number of hepatocytes further away from the central vein. These results reveal that glucagon counteracts the action of the Wnt/β-catenin pathway in the metabolic zonation of the liver.

1.3.1) Glucose Metabolism

The glucose metabolism in the liver is a very complex network, regulated by the presence of hormones, such as insulin and glucagon (

Figure 4), and by the availability of substrates (*e.g.* glucose)[21].

After a meal, the glucose molecules in the portal bloodstream enter the hepatocytes through the glucose transporters present in these cells. Because phosphorylated sugars cannot go through the cell membrane, once the glucose molecules are inside the hepatocytes, they can be entrapped by being phosphorylated into glucose 6-phosphate (G6P) by the glucokinase (GCK) enzyme, which, due to its high K_m, acts only when the intracellular concentration of glucose is high[21]. In the fed state, G6P can be used to produce glycogen, through glycogenesis, as a way to store glucose; it can enter the phosphate pentose pathway and generate nicotinamide adenine dinucleotide phosphate (NADPH), which is used in many pathways, including the synthesis of fatty acids and drug metabolization; it can be transformed into pyruvate through glycolysis and then used to produce energy via the tricarboxylic acid (TCA) cycle and the electron transport chain. Glycolysis is the main pathway used by the liver to produce ATP in the postprandial period, when glucose levels are high[24]. The three limiting steps of the glycolysis pathway are catalyzed by three enzymes: GCK, phosphofructokinase-1 (PFK-1) and pyruvate kinase (PK) (Figure 4). Secretion of insulin in the fed state leads to an increase in gene transcription and synthesis of these enzymes, which, in turn, leads to the metabolization of glucose into pyruvate[21]. Pyruvate conversion to acetyl coenzyme A (CoA) is also regulated by insulin. The presence of insulin leads to decreased expression of pyruvate dehydrogenase kinase 4 (PDK4), an enzyme that phosphorylates and inhibits pyruvate dehydrogenase (PDH), which catabolizes the conversion of pyruvate into acetyl-CoA (Figure 4). Thus, insulin leads to the metabolization of pyruvate into acetyl-CoA[21,24].

In contrast, in the fasting state, when circulating levels of glucose are low, glucagon is released and the liver acts to increase glucose levels in the blood, since it is the source of fuel for many of our organs. This is particularly relevant because the brain resorts almost exclusively to glucose as fuel and severe prolonged hypoglycemia could lead to brain damage, coma and, eventually, death[25]. In short periods of fasting, glucagon induces breaking down of glycogen stores through glycogenolysis to obtain G6P that is used mainly to maintain blood glucose levels within a physiologic range (4 to 7.8 mM), in between meals[21,26].

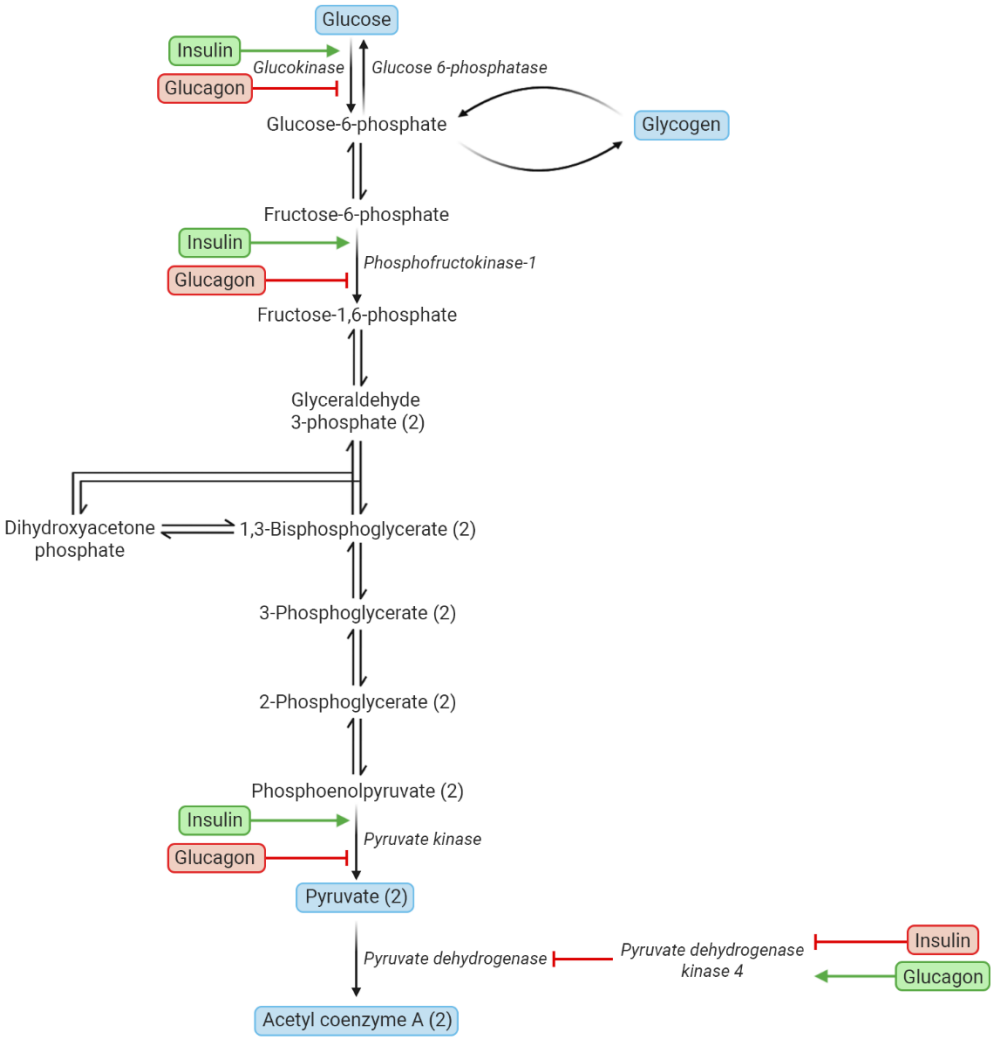

Figure 4. Glucose metabolism and the effects of insulin and glucagon on its rate-limiting steps. Insulin and glucagon act on enzymes glucokinase, phosphofructokinase-1 and pyruvate kinase, leading to their induction or repression, respectively. Conversion of pyruvate into acetyl coenzyme A is also hormonally regulated.

For longer fasting periods, glycogen reserves become depleted and the liver needs to synthesize glucose from lactate (released into the blood stream by muscle after exercise), pyruvate, glycerol (from the hydrolysis of TAG in adipose tissue) or α-ketoacids (resulting from the metabolization of glucogenic amino acids), in a process called gluconeogenesis[21]. G6P molecules from glycogenolysis and gluconeogenesis are dephosphorylated into glucose by glucose 6-phosphatase (G6Pase) to be released into circulation[27].

Glucagon increases levels of cyclic AMP (cAMP) inside the cell, which, through the action of cAMP-dependent protein kinase A (PKA), leads to phosphorylation and activation of cAMP-response element-binding protein (CREB). CREB is a transcription factor that stimulates the expression of gluconeogenic genes, such as phosphoenolpyruvate (PEP)-carboxykinase (PEPCK), the enzyme that catabolizes the conversion of oxaloacetate into PEP, and G6Pase by inducing the expression of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α)[28]. Hepatocyte nuclear factor-4α (HNF4α), a hepatic nuclear receptor, is coactivated by PGC-1α, which increases in fasting states, and binds to promoter regions of gluconeogenesis genes. Conversely, the synthesis of GCK, PFK-1 and PK is inhibited by

glucagon, resulting in a decrease in the consumption of glucose to form pyruvate[21].Thus, the presence of glucagon under fasting conditions favors the gluconeogenic pathway.

1.3.2) Fatty Acid Metabolism

In the fed state, when the levels of carbohydrates are high, the liver can use them to produce fatty acids. Fats ingested in the diet are broken down into free fatty acids (FFAs) and transported through the blood stream associated with serum albumin, a protein secreted by the liver. The liver uses fatty acids to produce TAG molecules as a way to store energy[21].

The *de novo* synthesis of fatty acids occurs in postprandial periods, when ATP availability is higher, since it requires a large amount of energy. This process occurs in the cytosol and consists in the repeated incorporation of carbons from acetyl-CoA, using ATP and NADPH, to form a carbon chain. In the rate-limiting regulated step of fatty acid synthesis, acetyl-CoA is carboxylated into malonyl-CoA by acetyl-CoA carboxylase (ACC). The presence of insulin leads to ACC activation and fatty acid synthesis. After obtaining malonyl-CoA, the multifunctional enzyme fatty acid synthase catalyzes the remaining reactions of fatty acid synthesis, progressively elongating the carbon chain, until a length of 16 carbons is reached. The end product of the fatty acid synthesis pathway is a fully saturated molecule of palmitate[21].

In the liver, the main location of TAG synthesis, three fatty acids are esterified to a molecule of glycerol to form TAG. Because of their high hydrophobicity, TAG molecules form lipidic droplets that are stored in the cytosol. In normal conditions, the majority of the body's TAG is stored in adipose tissue, with only a little amount being stored in the liver. In diseased states, such as in non-alcoholic fatty liver disease, there is an increase in the hepatic accumulation of these molecules, which is associated with other health problems, including diabetes and insulin resistance, and may lead to hepatic fibrosis and increased mortality risk[29]. TAG molecules from the liver can also be packaged into very low-density lipoproteins (VLDL) and released into circulation, to provide an energy source for peripheral tissues[21].

In a fasting state, the rise of glucagon levels leads to an increase in the intracellular levels of cAMP in the liver, and PKA activates AMP-activated protein kinase, which phosphorylates and inactivates ACC[30]. Thus, in fasting periods, fatty acid synthesis is downregulated. On the other hand, there is a rise in circulating levels of FFAs due to TAG hydrolysis in the adipose tissue which, together with glucagon, induce the peroxisome proliferator-activated receptor isotype α (PPAR-α), the main regulator of β oxidation[31]. β oxidation occurs in mitochondria and peroxisomes and involves progressively shortening the fatty acid chain to obtain acetyl CoA subunits that can be converted to ketone bodies or enter the TCA cycle. While short fatty acids can directly cross the mitochondrial membrane, long-chain fatty acids can only enter the mitochondria, where β oxidation occurs, with the help of carnitine palmitoyltransferase (CPT) I and II, localized in the outer and inner membrane of the mitochondria, respectively[21]. This transport system is known as the carnitine shuttle and its rate-limiting step involves CPT1A, the liver isoform of CPT1. On the other hand, very-long-chain fatty acids are first oxidized and shortened in

peroxisomes, and only then undergo mitochondrial oxidation. The first step of peroxisomal β oxidation is a dehydrogenation step catalyzed by the rate-limiting enzyme acyl CoA oxidase 1 (ACOX1). PPAR-α is a transcriptional regulator of many of the enzymes in these pathways, including ACOX1 and CPT1A[32].

1.3.3) Bile Acid Metabolism

Bile acids are essential for the digestion and absorption of lipids and regulate cholesterol homeostasis. Cholesterol is a precursor of steroid hormones and vitamin D, as well as a structural component of cell membranes. However, when cholesterol is in excess, the liver is responsible for converting it into bile acids, which are then stored as bile in the gallbladder, until their release to the GI tract after the ingestion of food. Most are reabsorbed in the intestine and return to the liver via the portal vein. This process constitutes the enterohepatic cycle, which allows for the recovery of around 95 % of bile acids in the pool[33].

To compensate for the small amount that is lost in the feces the liver performs *de novo* bile acid synthesis[21]. The synthesis of bile acids contributes to convert cholesterol into a more water-soluble molecule and starts with a hydroxylation by CYP enzyme cholesterol 7α-hydroxylase (CYP7A1), the only rate-limiting enzyme in this pathway. Bile acid synthesis is regulated by a negative feedback mechanism: bile acids activate the farnesoid X receptor (FXR), a nuclear receptor that regulates gene transcription and is involved in the maintenance of metabolic homeostasis. Once active, FXR leads to an inhibition of CYP7A1 gene expression. This effect may be achieved via the nuclear receptor short heterodimer partner (SHP), which is induced by FXR and inhibits CYP7A1 expression. In fact, SHP knockout mice show an increase in CYP7A1 expression and, consequently, in bile acid synthesis[34]. FXR also induces the bile salt export pump (BSEP) located in hepatocyte membranes, thus promoting the secretion of bile acids[35]. In addition, FXR plays a role in lipid metabolism through induction of PPAR-α and control of the expression of genes that regulate these pathways[36,37].

The presence of high concentrations of insulin was shown to result in a decrease in FXR expression in rat hepatocytes and thus promoting bile acid synthesis[38]. However, high amounts of glucose induced FXR expression and this effect was not prevented by insulin. Song and Chiang found that glucagon inhibited CYP7A1 expression through PKA phosphorylation of HNF4α, in a mechanism independent from the FXR/SHP pathway[39].

2) Insulin Resistance

Obesity and diabetes prevalence increased greatly in the past years, with an estimated 422 million people worldwide with diabetes in 2014, most of which have type II diabetes, and 13 % of adults estimated to be obese in 2016[40,41]. These conditions are associated with a higher mortality risk and a high economic burden[42]. Both obesity and type II diabetes are associated with insulin resistance, as a precursor condition, which is defined as a condition in which normal levels of insulin cannot trigger the expected response in target tissues (skeletal muscle, adipose tissue and liver), affecting the metabolic homeostasis of the body[43].

Current western diets are characterized by high nutrient intake and high fatty acid content, with lower ratios of health-promoting unsaturated fatty acids to more detrimental saturated fatty acids[44]. Excessive ingestion of lipids and carbohydrates leads to metabolic changes that cause insulin resistance. The most common saturated and unsaturated fatty acids in western diets and in the livers of patients with hepatic steatosis (also known as fatty liver) are palmitic acid (PA) and oleic acid (OA), respectively[45,46], with PA having been associated with the development of insulin resistance in hepatocytes[47,48].

The effects of insulin resistance depend on the tissue and its functions (Figure 5). In the muscle, insulin resistance causes diminished glycogen synthesis and glucose uptake, while in adipose tissue, lipolysis and FFA release increase and uptake of triglycerides from VLDL decreases[43]. Hepatic insulin resistance is characterized by an inability to suppress hepatic glucose production through increased gluconeogenesis and decreased glycogen synthesis, leading to hyperglycemia. At the same time, lipogenesis is still activated by insulin and there is an increase in the FFA flux coming from the adipose tissue, resulting in an accumulation of TAG in the liver. VLDL production is increased to secrete the excess TAG, leading to hypertriglyceridemia, which will result in worsened insulin resistance in the muscle and adipose tissue[49]. This paradox of the inability of insulin to suppress gluconeogenesis and simultaneously being able to activate lipogenesis is called ‘selective insulin resistance’ and has been attributed by some authors to metabolic zonation. Kubota *et al.* showed that the expression of insulin receptor substrates 1 and 2 (Irs1 and Irs2, respectively) was differently affected by hyperinsulinemia[50]. While the expression of Irs2, located across the whole lobule, is suppressed by insulin, Irs1 levels are not affected by the presence of this hormone. Because Irs1 is mainly located in the PV zone, the primary site of lipogenesis, these results may explain the development of selective insulin resistance and why, in this state, lipogenesis is not impaired. Furthermore, because PP hepatocytes increase the FFA uptake, PV hepatocytes are exposed to lower FFA levels and thus may be able to better maintain their insulin responsiveness and the resulting lipogenic metabolism[9].

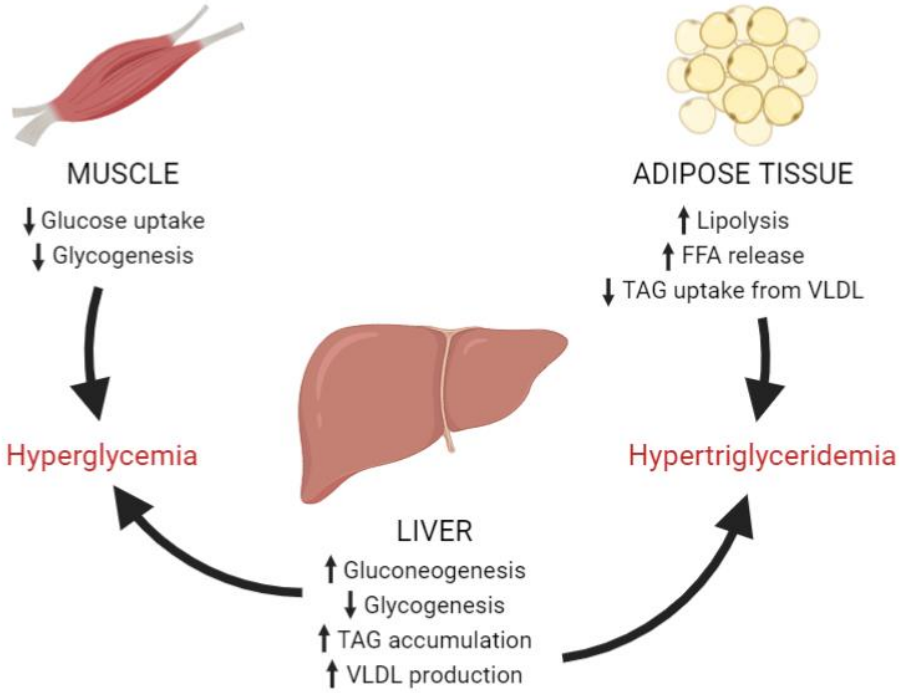

Figure 5. Insulin resistance. Loss of insulin signaling results in decreased glucose uptake by the muscles, decreased glycogenesis in the muscle and liver, and increased gluconeogenesis in the liver, leading to increased glucose levels in the blood. Simultaneously, lipolysis and FFA release by the adipose tissue cause an increase in TAG accumulation in the liver. Hepatic VLDL production is increased to secrete the excess TAG, resulting in hypertriglyceridemia.

As a consequence of the aforementioned metabolic alterations, and over some time, compensatory hyperinsulinemia develops, with increased insulin secretion to compensate for its reduced effect[51]. Eventually, pancreatic β cells may become worn out and be unable to produce sufficient amounts of insulin, leading to more severe hyperglycemia and, ultimately, type II diabetes may be diagnosed.

As such, the liver has a central role in the development of insulin resistance: while the deletion of hepatic insulin receptors in mice leads to insulin resistance and glucose intolerance[52], skeletal muscle-specific deletion of insulin receptors results in disrupted fatty acid metabolism, but no alterations in glucose or insulin levels are evident[53]. Nevertheless, the molecular mechanisms behind insulin resistance are not entirely understood, but it is thought that defects in signaling pathways, particularly the phosphatidylinositol-3-kinase pathway involved in metabolic regulation, may be the main cause[51]. Therefore, the need for *in vitro* liver models enabling the study of the mechanisms responsible for insulin resistance is increasing.

3) *In Vitro* Liver Models

The great burden of liver diseases and its major role in drug metabolization makes the liver a very interesting candidate for *in vitro* modeling. *In vitro* models allow us to investigate in detail the function of organs and the mechanisms of diseases otherwise difficult or impossible to study *in vivo*, as well as evaluating drug effects and toxicity, while avoiding the disadvantages of using animals, such as ethical and economic issues and unreliable results due to interspecies differences[7]. The establishment of reliable cell-based *in vitro* liver models is of great importance for further research on liver pathologies and for drug development. However, several components must be considered, such as the cell source, the composition of the culture medium and the culture system. All of these parameters influence the success of the model and their optimization is critical to achieve accurate results. The extreme complexity of hepatic tissue, with its elaborate three-dimensional (3D) structure, intricate zonation and blood flow patterns, and the numerous metabolic functions performed by hepatocytes, also contribute to the challenge of modeling the liver. The development of *in vitro* hepatic models capable of reproducing these functions will contribute to more reliable toxicological studies and ultimately aid in the drug development process, as well as provide a platform to better understand hepatic function and disease.

The 'gold standard' cells used for *in vitro* hepatic models are primary human hepatocytes (PHHs). PHHs are one of the most used models because, since these cells are directly isolated from the human liver, they represent with a high degree of fidelity the *in vivo* phenotype, when compared to other cell sources[54]. However, there are still some limitations associated with the use of these cells as *in vitro* models of the liver. Their availability is limited and the isolation process is extremely complex and often results in poor cell viability or low cell yields[55]. Another major drawback of the use of PHHs is the loss of function in culture. PHHs in culture start to dedifferentiate, i.e. gradually lose their function and polarization, and are only able to be maintained in culture for a few days[7,56]. Although hepatocytes from non-human animal sources can be considered an alternative, in terms of availability, the ethical concerns associated with their use and the species differences constitute barriers to their routine use as hepatic models.

Immortalized hepatic cell lines such as HepG2 or HepaRG are regarded as an alternative to PHHs in terms of availability, cost and ability to proliferate[57]. However, these cells may present genetic and metabolic abnormalities, because of their cancerous origin[7,58]. Furthermore, some of these cell lines have been shown to have downregulated expression of enzymes involved in urea formation[59] and drug-metabolizing enzymes and transporters[60–62] when compared to PHHs, which hinders their potential application in drug toxicity studies.

4) Alternative Models to Primary Human Hepatocytes

As mentioned above, even though PHHs are the obvious choice to model the liver *in vitro*, as they are the native hepatic cells, their low availability, difficult isolation, and tendency to dedifferentiate after short times in culture constitute major drawbacks for their utilization. This has driven scientists to find suitable alternative cell sources and culture systems to reproduce hepatic tissue. Stem cells have emerged as a promising alternative cell source to obtain viable hepatocytes, while more complex culture systems, such as 3D cultures, are gaining increasing attention as a strategy to better reproduce physiologic interactions between cells and induce/maintain a differentiated phenotype.

4.1) Stem Cell-Derived Hepatocytes

Stem cells show great promise for biomedical applications such as regenerative medicine, toxicological studies, and disease modeling. These cells are able to self-renew indefinitely without significant changes to their characteristics and to differentiate into one or more types of functionally mature cells.

4.1.1) Definition and Classification of Stem Cells

Stem cells are classified, according to their differentiation potential, into totipotent, pluripotent, multipotent or unipotent stem cells, or, according to their origin, into embryonic, fetal, or adult stem cells. The zygote and the cells resulting from its first few divisions are totipotent, with the potential to give rise to all the tissues in the human body, as well as extraembryonic tissues, such as the placenta. The cells from the inner cell mass of the blastocyst, embryonic stem cells (ESCs), are pluripotent and able to differentiate into cells from all three germ layers – endoderm, mesoderm, and ectoderm – but not into extraembryonic tissues. Most adult stem cells are multipotent and have the ability to differentiate into a few specialized cell types present in a given organ or tissue. Finally, unipotent stem cells, such as germline stem cells, can only give rise to one cell type along a single lineage. The pluripotency of stem cells is governed by certain factors, of which Oct4, Sox2, Nanog and Klf4 are examples[63]. In 2006, Yamanaka was able to reprogram fully differentiated fibroblasts into pluripotent stem cells similar to ESCs by culturing them with a combination of four factors, Oct3/4, Sox2, c-Myc, and Klf4, under ESC culture conditions, creating the first induced pluripotent stem cells (iPSCs)[64]. Another class of stem cells are the mesenchymal stem cells (MSCs) that have emerged as an alternative to pluripotent stem cells due to their ease of isolation and their high expansion potential.

MSCs are multipotent adult stem cells of mesodermal origin. They were first discovered and isolated from bone marrow by Friedenstein *et al*.[65], but since then have been found in many tissues, such as adipose tissue, umbilical cord, synovial fluid, fetal liver, dental tissue, among others[66]. However, bone marrow, being the first tissue in which MSCs were discovered, is still commonly regarded as the ‘gold

standard', to which other MSC sources are usually compared. According to the International Society for Cellular Therapy, the minimal criteria for the definition of human MSCs involve plastic adherence in standard culture conditions, expression of CD105, CD73 and CD90, lack of expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR and, finally, ability to differentiate into chondroblasts, adipocytes and osteoblasts *in vitro*[67]. In addition to differentiating into these three cell types, MSCs have been shown to be able to differentiate into other cells from all three germ layers, such as cardiomyocytes from the mesoderm, neurons from the ectoderm and hepatocytes and pancreatic cells from the endoderm[66]. The differentiation potential of MSCs and thus their clinical applicability depend, however, on their origin. Due to their unique properties, MSCs have an immense potential for cell therapy and tissue regeneration.

4.1.2) Stem Cells as Hepatocyte Sources

Stem cells have emerged as an alternative source of hepatocytes for *in vitro* liver models. It is nowadays possible to obtain hepatocyte-like cells (HLCs) from ESCs[68], iPSCs[69] and MSCs from several sources[70–72]. However, despite their high proliferation rate, the use of ESCs raises ethical concerns due to their embryonic origin. While iPSCs solve the ethical problems associated with ESCs and can be derived from the patient, allowing personalized *in vitro* disease modeling, the efficiency of the reprogramming process must still be improved[73]. Moreover, there can be some variability in HLCs derived from iPSCs, due to reprogramming differences and epigenetic memory[74,75]. Alternatively, MSCs, despite being less commonly used for hepatic differentiation, do not raise safety or ethical concerns, and are more easily obtained than most of the previously discussed stem cell types.

Indeed, hepatic differentiation has been achieved with MSCs derived from many sources, namely bone marrow[70], adipose tissue[76], umbilical cord blood[70], placenta[77], amniotic epithelium[78], amniotic fluid[79,80], endometrium[81], dental pulp[82] and umbilical cord matrix[83,84]. However, to date, no study comparing the hepatic differentiation efficiency of all these sources has been done, but it has been suggested that MSCs derived from human neonatal tissues (hnMSCs) have a better hepatic differentiation potential than those of adult tissues. Lee *et al*. compared the hepatic differentiation potential of MSCs from several neonatal tissues, the bone barrow, and adipose tissue, having found that hepatocyte growth factor (HGF) expression was significantly higher in HLCs derived from all neonatal tissues, and tyrosine aminotransferase (TAT), a marker of late hepatocyte lineage, was only present in hnMSCs[85]. Yu *et al*. compared the differentiation potential of bone marrow-derived MSCs with umbilical cord matrix-derived MSCs (UCM-MSCs), having found that the latter showed higher expression of the hepatic-specific markers albumin (ALB), CYP3A4, TAT, G6P, and α1 antitrypsin, at both gene and protein levels, and showed higher albumin and urea production capabilities[86]. Moreover, hnMSCs present other advantages over the other MSCs sources: according to studies by Cipriano *et al.* and De Kock *et al.*, hnMSCs express endoderm markers and early liver-specific markers and liver progenitor markers[71,87]; while obtaining adult tissues for MSC isolation usually involves an invasive procedure, neonatal tissues such as the umbilical cord and placenta are considered medical waste and usually yield higher cell

numbers[88]; and UCM-MSCs have been shown to be more proliferative than MSCs from other sources[86,89].

Thus, UCM-MSCs present as a promising source of HLCs for *in vitro* studies. However, obtaining HLCs with a fully differentiated phenotype, similar to that of PHHs, has been challenging. In fact, HLCs seem to have, in most cases, a somewhat immature phenotype, with fetal-like hepatocyte characteristics, which appears to be a common issue with stem cell-derived HLCs. Thus, finding culture conditions, *e.g.,* medium composition and culture configuration, that allow us to obtain a more mature phenotype is of great importance.

4.1.3) Hepatocyte Differentiation Strategies

The process of differentiating stem cells into hepatocytes *in vitro* usually seeks to mimic what happens during the embryonic development of the liver. Liver development can be divided into four stages: i) endoderm induction; ii) foregut and hepatic competence induction; iii) hepatoblast and liver bud formation and iv) differentiation of the hepatoblast into hepatocyte (Figure 6). This process requires a series of complex interactions with signaling molecules, the ECM and other cells and is extremely difficult to reproduce *in vitro*. In order to do this, specific molecules, such as cytokines, growth factors and epigenetic modifiers, are added to the culture medium, to provide signals for commitment into one or other cell fate at particular stages of differentiation.

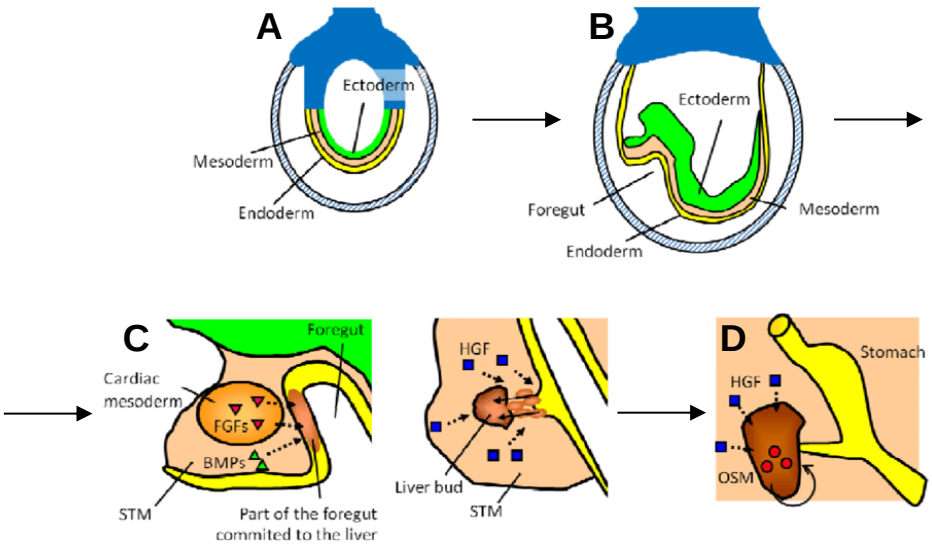

Figure 6. Liver development. A) The three germ layers – ectoderm, mesoderm, and endoderm – are formed; B) The primitive gut tube starts to develop from the endoderm and is patterned into foregut, midgut and hindgut; C) The liver bud is generated from part of the foregut; D) Hepatoblasts differentiate into hepatocytes to form the liver. Image adapted from Katsuda *et al.* (2012)[90].

During the early stages of embryonic development, the primitive streak starts to emerge, and the process of gastrulation begins. Cells migrate through the primitive streak and eventually originate the three primary germ layers. The definitive endoderm germ layer, which emerges from the anterior end of the primitive streak around the third week of gestation, will give rise to cell types from the respiratory

and GI tracts and associated organs[91]. After gastrulation, the primitive gut tube starts to develop from the endoderm and signals from adjacent mesoderm cells induce the tube's patterning into hindgut, midgut, and foregut, with the latter giving rise to the liver.

Liver development is controlled by signaling pathways, the most important of which are controlled by transforming growth factor β (TGFβ), Wnt, fibroblast growth factor (FGF), Notch and bone morphogenetic protein (BMP) ligands in a time-dependent way[91]. Nodal ligands belong to the TGFβ family of transcription factors. The Nodal signaling pathway provides the necessary cues for the beginning of endoderm and mesoderm development, as well as being needed for gastrulation and gut tube patterning[92]. Nodal signaling operates in a dose-dependent manner, with high levels leading to endoderm development and low levels resulting in mesoderm formation[93]. Nodal regulates the expression of transcription factors such as Gata5, Sox17 and Foxa2, which are responsible for directing cell fate towards endoderm and promote the distinction between endoderm and mesoderm. Wnt/β-catenin signaling, together with Nodal and FGF, promotes patterning of the gut tube and induces the expression of the hematopoietically-expressed homebox (*HHEX*) gene, Sox2 and Foxa2 in the anterior portion of the endoderm[91,92]. These transcription factors will be essential for foregut formation. The addition of epidermal growth factor (EGF) and FGF to the culture medium in an initial phase of hepatic differentiation of stem cells induces the expression of *HHEX* and promotes endoderm commitment and foregut induction[71].

After foregut endoderm is formed, it starts to divide into organ-specific domains. FGF-2 induces differentiation into different organs in a dose-dependent manner, with low to intermediate levels leading to ALB expression[94]. BMP signaling from the septum transversum mesenchyme is also needed to induce the expression of hepatic genes and to exclude a pancreatic fate[95]. These pathways act together to induce hepatic specification, but the exact way in which they do so is still not completely understood.

Hepatoblasts are bipotential liver precursor cells that express genes associated with biliary lineages (*e.g.* cytokeratin-19 (CK-19)), fetal liver (*e.g.* α-fetoprotein (AFP)) and mature hepatocytes (*e.g.* ALB, cytokeratin-18 (CK-18) and HNF4α), and thus can give rise to both hepatocytes and cholangiocytes. The same pathways that had a role in earlier development are also involved in hepatoblast proliferation and differentiation. In particular, the expression of β-catenin, together with HGF and FGF, leads to liver enlargement and hepatoblast proliferation. At this point, due to the presence of specific transcription factors (*e.g.* HHEX and Gata4), the hepatoblasts migrate to the stroma and the liver bud starts to form[92]. The growth and differentiation of this structure is, once more, regulated by FGF, BMP, Wnt and HGF, as well as hepatic transcription factors. Notch mediates the differentiation of hepatoblasts located near the portal vein mesenchyme into cholangiocytes, while the remainder give rise to hepatocytes. Hepatic gene expression *in vitro* can be achieved either by co-culturing with cardiac tissue or adding FGF-2[91]. In experimental settings, HGF and FGF are usually added at this stage. Nicotinamide has also been shown to have potential for hepatogenic induction[96]. Oncostatin M (OSM), a cytokine produced by hematopoietic cells in the fetal liver, promotes the differentiation of hepatoblasts into hepatocytes and favors the expression of adult hepatocyte genes, but requires the presence of glucocorticoids such as dexamethasone[97]. These two compounds are used to induce hepatocyte differentiation *in vitro*.

In addition to cytokines and growth factors, other approaches to differentiate stem cells into HLCs have been explored, including the use of epigenetic modifiers as well as other small molecules[71,98–102]. Stem cell pluripotency and differentiation are also regulated by epigenetic events, such as DNA methylation and histone modifications (*e.g.*, histone acetylation), through which gene expression can be regulated. The enzymes that catalyze DNA methylation are DNA methyltransferases, by adding a methyl group to cytosine residues, resulting in repression of gene transcription. Nucleoside analogs, such as 5-azacytidine (5-AZA), inhibit DNA methyltransferases, resulting in a selective increase in gene expression[103]. Dimethyl sulfoxide (DMSO) is also a molecule with innumerous applications that exerts effects on cell metabolism, growth and apoptosis, possibly due to its reported effects on epigenetics and, particularly, DNA methylation[104]. Several authors have reported that the use of DMSO and/or 5-AZA in combination with growth factors results in improved differentiation and HLC phenotype[71,98–100].

Insulin and dexamethasone are proven to promote hepatic differentiation *in vitro*, and insulin and glucose also improve hepatocyte viability, and thus, these molecules are extensively used in HLC differentiation protocols[105]. However, the current differentiation protocols used in our laboratory include glucose concentrations that are approximately 5x higher than those observed *in vivo* and insulin concentrations that are more than 1000x higher than the physiologic ones. Furthermore, dexamethasone is an exogenous glucocorticoid that is not found in the human body. Because excess glucose, insulin and glucocorticoids are shown to lead to impaired glucose metabolism, their use in high concentrations may not be suitable for deriving HLCs for studies on the energy metabolism, and more physiologic media need to be developed for this purpose[106,107].

4.2) 3D liver models

Another important parameter to consider in order to obtain a mature phenotype and a physiologically relevant model is the tissue structure. Indeed, several culture systems can be used to mimic the liver. Conventional two-dimensional (2D) cultures consist in a monolayer of cells that grow attached to a surface. While these types of cultures are easy to manipulate and have a relatively low cost, the culture configuration fails to recreate the liver microenvironment, namely cell-cell, cell-matrix interactions and overall tissue architecture; blood flow; and oxygen and nutrient gradients[108]. This results in impaired cell function and leads to hepatocyte dedifferentiation. Thus, the use of 3D culture configurations as a strategy to improve hepatocyte phenotype and function has been gaining increasing attention, showing promising results[109].

Hepatocyte or HLC spheroids can be obtained through scaffold-based methods or scaffold-free methods, *e.g.* by using hanging drop platforms or by culturing the cells in low-attachment surfaces[7]. In these low-adherence conditions, the cells tend to naturally self-assemble, forming spheroid aggregates. This type of 3D culture has been shown to improve the phenotype of primary human hepatocytes when compared with 2D cultures. A multicenter study comparing 2D sandwich with 3D spheroid PHH cultures found that 3D cultures presented higher expression of phase I and phase II enzyme genes (including

CYP1A2, *2E1*, *3A43*, *2D6*, *2A6*, *2B6*, and *3A7*, *UGT1A1* and *1A3*), higher CYP enzyme activities (CYP1A2, 2C8, 2C9, 2D6, 3A4), and increased sensitivity to hepatotoxic compounds[110]. Vorrink *et al*. compared 3D spheroid cultures of PHHs with conventional monolayer cultures, having found that the expression of phase I and II drug-metabolizing enzyme genes (*CYP2C8*, *2C9*, *1A2*, *2D6*, *3A4*, *UGT1A1* and *2B15*), several drug or bile salt transporter genes (*ABCC2*, *ABCC3*, *ABCB11* and *SLCO1B1*), and other characteristic hepatic genes (*ALB*, *PXR* and *HNF4A*) was higher in spheroids during the whole 21-day culture period[111]. Spheroid cultures also presented higher CYP1A2, 3A4, 2C8, 2D6 and 2C9 activities for 21 days. These results are probably due to the preservation of the 3D architecture of the cells, cell polarization, the promotion of cell-cell contacts and the presence of ECM molecules, secreted by the cells, surrounding the aggregates, providing them cues that are important for their function[108].

This configuration has also been used to derive HLCs from stem cells in studies using ESCs, iPSCs or MSCs. The main results of several studies comparing stem cell-derived HLCs cultured in 2D monolayers and 3D spheroids are summarized in Table 1.

Table 1. Main results of studies comparing 2D monolayer and 3D spheroid differentiation of human stem cells into HLCs.

Stem cell type	Origin	Culture method	Results (3D vs. 2D)	References
ESCs	ICM of the blastocyst	Self-aggregating spheroids	Higher expression of energy metabolism-related genes (G6Pase catalytic subunit and lipid droplet-related *HSD17B13*), *CYP1A1*, and members of the solute carrier family (including phosphate, pyruvate, lactate, glutamate and xenobiotics transporters). Increased *CYP3A4* and *CYP1A2* induction by xenobiotics. Lower expression of *AFP* and *ALB*.	Sengupta *et al*. (2014)[112]
ESCs	ICM of the blastocyst	Self-aggregating spheroids	Higher expression of *ALB*, *AFP*, *HNF4A*, *UGT1A1* and arginase 1 (*ARG1*), sustained for 35 days. Higher expression of PEPCK and glycoprotein metabolism protein ASGPR-1. Increased albumin secretion, sustained for 32 days.	Subramanian *et al*. (2014)[68]
ESCs	ICM of the blastocyst	Spheroids in AlgiMatrix scaffolds	Higher expression of *ALB*, *CYP3A4*, and *CYP7A1*. Increased CYP3A4 activity. Increased urea secretion.	Ramasamy *et al*. (2013)[113]

Stem cell type	Origin	Culture method	Results (3D vs. 2D)	References
ESCs	ICM of the blastocyst	Spheroids in a nanopillar plate	Higher expression of *ALB*, CYP genes, *UGT1A1*, *UGT1A3*, hepatic transporters (including bile acid, drug, phospholipid, hormone, and bilirubin transporters), *PXR* and *PPARA*. Increased albumin and urea secretion.	Takayama *et al*. (2013)[114]
iPSCs	Fetal lung fibroblast	Spheroids in a nanopillar plate	Increased CYP2C9 and CYP3A4 activity and induction. Susceptibility to a vast array of hepatotoxic drugs.	Takayama *et al*. (2013)[114]
iPSCs	Fibroblast	Self-aggregating spheroids	Similar glucose consumption and lactate production. Lower expression of *NANOG*, *SOX17* (endoderm development) and *AFP*. Similar *ALB*, *CYP3A4* and *HNF4A* expression. Increased urea secretion.	Meier *et al*. (2017)[115]
iPSCs	Fibroblast	Self-aggregating spheroids	Higher expression of ALB and CYP3A4. Lower expression of HNF4A, CK-19 and AFP. Increased albumin secretion.	Torizal *et al*. (2019)[116]
MSCs	Umbilical cord matrix	Self-aggregating spheroids	Increased glucose consumption and urea production. Similar lactate and albumin production. Increased CYP1A1, 1A2, 2B6, 2C9, and 3A4 activity and induction. Increased bupropion conversion and sensitivity to diclofenac.	Cipriano *et al*. (2017)[117]
MSCs	Umbilical cord	Spheroids in a polymer scaffold	Increased secretion of albumin and urea. Higher expression and activity of CYP1A2, 2E1, 2C9, and 3A4. Increased sensitivity to acetaminophen and ethanol.	Chitrangi *et al*. (2017)[118]
MSCs	Bone marrow	Spheroids in a nanofibrous scaffold	Higher expression of *AFP* and *HNF4A* during maturation phase and of *ALB*, *HNF4A* and *CK-18* after the differentiation process. Increased albumin secretion.	Bishi *et al*. (2013)[119]

Although the use of 3D spheroid cultures for maturation of MSC-derived HLCs is still a rather unexplored area, with few studies on the subject, similarly to 3D-cultured PHHs, most authors report enhanced

expression of some hepatic genes, such as CYP genes, increased CYP activity, higher sensitivity to hepatotoxic substances, such as acetaminophen or diclofenac, and often improved albumin or urea secretion, when compared to 2D cultures (Table 1)[117–119]. These findings contribute to the growing body of evidence supporting the benefits of 3D models and their potential for drug screening and toxicology applications. However, in the majority of studies, when comparing PHHs and 3D-cultured HLCs, the latter still often show lower expression of hepatic genes and sometimes poorer function[112,114,115,117].

Most studies on 3D liver models focus on toxicology applications, but there has been increasing interest in applying these models to *in vitro* modeling of diseases related with the energy metabolism. There are studies on 3D liver models of other metabolic diseases of the liver, such as hepatic steatosis and non-alcoholic fatty liver disease, but very few focusing specifically on insulin resistance. Kozyra *et al*. developed a 3D model of steatosis and insulin resistance using PHHs, through continued exposure to high concentrations of insulin, FFAs and monosaccharides[120]. Spheroids cultured in these conditions developed steatosis and showed signs of insulin resistance, namely, increased PEPCK, PDK4 and G6Pase expression. Steatosis was reversed after FFA deprivation or exposure to antisteatotic compounds, highlighting the potential of these models to study steatosis, insulin resistance and related pathologies, as well as potential therapeutic options. However, to our knowledge, there are no studies on this topic using MSC-derived HLCs.

Motivation and Aims

Modern western diets and sedentary lifestyles, highly present in our societies, are closely related with the development of insulin resistance[121]. The latter is a risk factor for metabolic diseases such as type II diabetes and hypertension, which are associated with higher mortality risk and a high economic burden[19,42].

The liver has a major role in the regulation of whole-body energy homeostasis. In healthy individuals, it maintains blood glucose levels within physiologic ranges by promoting storage or synthesis of glucose and fatty acids, in response to hormones insulin and glucagon. Thus, the liver is a key player in the development of insulin resistance. When hepatocytes become less sensitive to insulin, they are no longer able to adapt their response, and an imbalance arises, with increased gluconeogenesis, TAG synthesis and VLDL secretion, accompanied by decreased glycogen synthesis. Hepatocytes, being responsible for most hepatic functions, are a fundamental part of this process. Thus, to further understand the mechanisms of insulin resistance and to contribute to the establishment of appropriate therapeutic options, it is necessary to develop reliable *in vitro* models that faithfully reproduce healthy and insulin resistant hepatocytes, with a particular focus on the energy metabolism functions, which are not yet adequately explored.

In our lab, a hepatic differentiation protocol for deriving hepatocyte like cells (HLCs) from hnMSCs has been previously developed[71]. This differentiation protocol has subsequently been successfully adapted to 3D culture conditions[117]. Therefore, taking advantage of this knowledge, the main goal of this work is to bring this model forward and to contribute to the development of an *in vitro* hepatic insulin resistance model. For this purpose, the following objectives were defined:

1) Adapt 3D hnMSC-derived HLC cultures to more physiologic culture media, with lower concentrations of glucose, insulin, and dexamethasone;
2) Evaluate the phenotype of HLCs cultured in more physiologic culture media;
3) Study the influence of insulin and glucagon in the modulation of HLCs' phenotype;
4) Study the role of insulin and glucagon in 3D-cultured HLCs' energy metabolism;
5) Assess the response of HLCs to incubation with fatty acids.

Materials and Methods

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*.[122]

2) Human Neonatal Mesenchymal Stem Cell Culture

hnMSCs were isolated from umbilical cords as described by Miranda *et al*.[88] and Santos *et al*.[123], 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 % CO_2. Cell viability assessment was performed using the trypan blue exclusion method.

3) Hepatocyte Differentiation of hnMSCs

The hepatocyte differentiation followed a three-step protocol, according to Cipriano *et al*.[71,117] (Figure 7). hnMSCs were initially seeded in a 0.2 mg/mL rat-tail collagen-coated surface at a density of 1.5×10^4 cells/cm^2 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, with a medium change after 3 days, at day 6. 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 μM 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 μM 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) (

Figure **8**). 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 µM dexamethasone, while both physiologic media were supplemented with 1 nM insulin and 100 nM dexamethasone[124]. 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.

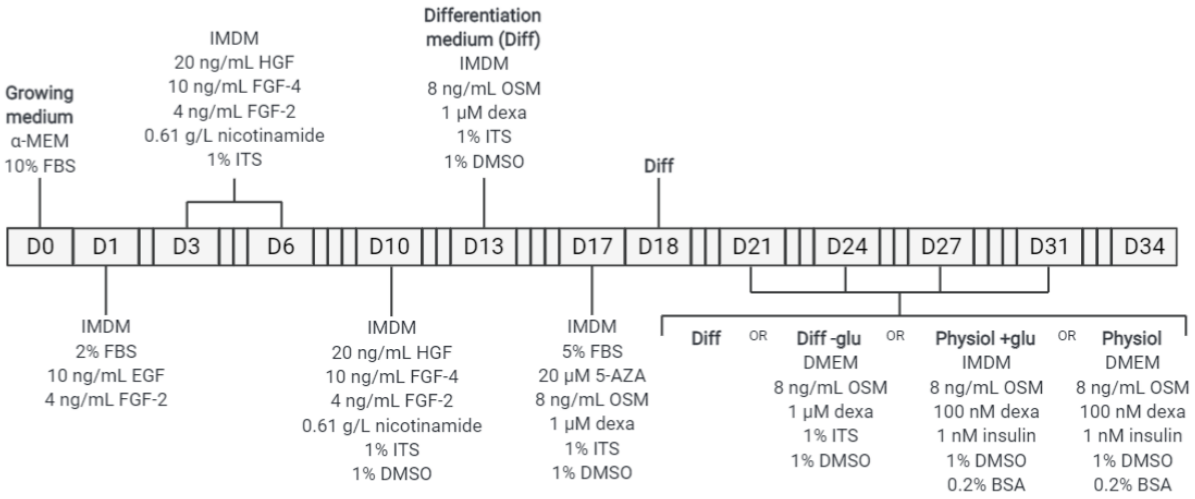

Figure 7. Hepatocyte differentiation protocol. All media from D1 forward contain 1 % of PenStrep and 0.1 % of amphotericin B.

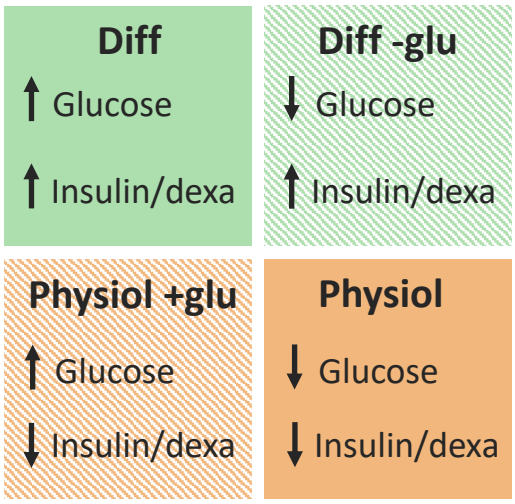

Figure 8. Culture media formulations used for HLC spheroid maintenance from day 21 forward.

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 µM 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 each medium until day 34, with 50 % medium changes every 3-4 days.

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/cm^2. Cells were suspended in Diff supplemented with 5 % FBS and 20 µM 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 each medium until day 34, with medium changes every 3-4 days.

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.

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*.[125]. For the glucagon stimulus, cells were incubated for 8 h in SM (control) or SM with 100 nM of glucagon (Figure 9). At the end of the assay cells were resuspended in TRIzol and stored at −80 ºC until proceeding with RNA isolation and gene expression analyses.

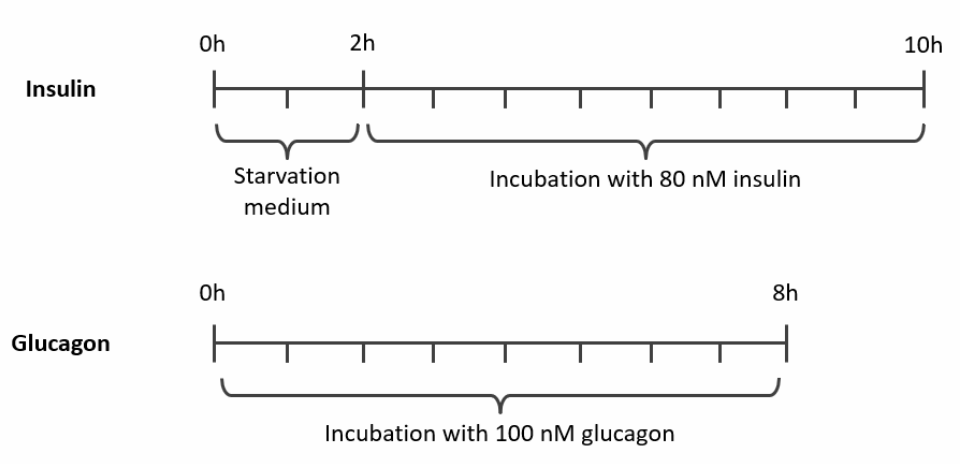

Figure 9. Insulin and glucagon stimuli assays.

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: µg/(10^6 cells.h).

7) Gene Expression Analysis

Total RNA of spheroid samples with 0.5-1.5 × 10^6 cells was isolated using TRIzol (Life Technologies) and extracted according to the manufacturer's instructions. RNA concentration was determined by measuring the 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. Primer sequences are provided in the Annexes. Reaction was performed on QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems) and consisted of a two-minute uracil-DNA glycosylase activation step at 50 °C and a denaturation step at 95 °C for 10 minutes, followed by 40 cycles of a denaturation step of 15 seconds at 95 °C and annealing/extension at 60 °C for 1 minute. 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 ($2^{-\Delta\Delta Ct}$) method was used, with gene expression normalized to the reference gene β-actin. Results are either presented

relative to undifferentiated hnMSCs, for hepatic-specific gene expression measurement, or relative to controls, for insulin and glucagon stimuli assays.

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 PA or 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 alone was used as control. On day 34, cells were fixed in a 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) solution for 10 mins at room temperature. Cells were then washed with PBS and incubated with 1.2 mg/mL oil red O and 60 % (v/v) isopropanol in water solution for 10 minutes at room temperature. Cells were again washed with PBS and placed in water for visualization using an inverted microscope with contrast phase (Motic AE2000).

9) Immunocytochemistry

Spheroids were collected, centrifuged, and the culture medium was removed. Fixation was performed overnight with a 4 % PFA and 4 % sucrose in PBS solution. Afterwards, 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 then embedded in liquid paraffin overnight and paraffin blocks were formed. 5 µm sections were cut by resorting to 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, followed by washing with PBS and 1h-incubation in a blocking solution with 2.5 % BSA (w/v) and 2 % FBS (v/v) in PBS and incubation with the primary antibodies overnight at 4 °C. The 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 the secondary antibodies (mentioned below) was performed for 1 h at room temperature prior to incubation with 4’,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 5 minutes for nuclei staining. 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 for image acquisition (AxioCam HRm), 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.

10) Statistical Analysis

Results are given as mean ± 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* values < 0.05 were considered statistically significant.

Results and Discussion

1) HLC spheroids were successfully adapted to more physiologic concentrations of glucose, insulin, and dexamethasone

A differentiation protocol to derive HLCs from hnMSCs as well as its adaptation to 3D spheroid culture conditions has previously been optimized by our group[71,117]. It includes a 3-step procedure that mimics liver embryogenesis, with commitment, differentiation, and maturation stages. In particular, for 3D conditions, there is a re-inoculation step at day 17, at which point cells are inoculated in ULA plates with low culture medium volume, to promote aggregation. During the following 24h the cells self-aggregate and form clusters, and eventually compact into spheroids with smooth edges. This differentiation protocol utilizes Diff medium, which includes glucose, insulin, and dexamethasone in high concentrations: 25 mM of glucose, 1.72 µM of insulin and 1 µM of dexamethasone. Even though insulin and dexamethasone are reported to induce hepatocyte differentiation and maturation, the values at which they are used in Diff are not representative of normal physiology[96,97,126,127]. In fact, high levels of glucose and glucocorticoids are reported to cause insulin resistance in hepatocytes, and thus, to be able to use these cells for energy metabolism studies, there was a need to adapt this protocol[106,128]. Insulin and dexamethasone concentrations were lowered, based on values used by Estall *et al*. for the culture of primary rat hepatocytes, to more physiologic values and a physiologic medium, Physiol, was created, with 1 nM insulin and 100 nM dexamethasone[124]. Low-glucose (-glu) and high-glucose (+glu) versions of Diff and Physiol, respectively, were also evaluated, with 25 mM and 5 mM of glucose, giving rise to the four media studied (Diff, Diff -glu, Physiol +glu and Physiol). These formulations were introduced in cultures at day 21 of the differentiation process.

Our results show that HLC spheroids were able to be maintained in culture for two weeks, from days 21 to 34, in the four media. During this process, spheroids cultured in the four conditions were similar in morphology and appearance, maintaining cohesion and smooth edges in every culture media studied, throughout the two weeks (Figure 10).

The size of the spheroids was measured immediately after changing the culture medium to one of the four media studied, at day 21, and then at days 24, 27, and 34. The size of the spheroids was maintained during the whole period in culture (Figure 11), similarly to previous studies on the same cells, and no significant differences were observed between the four media[117]. The average diameter at day 21, after spheroids are fully formed, was 133.9 ± 14.2 µm (mean ± SD), meaning that the presence of necrotic cores was avoided[129].

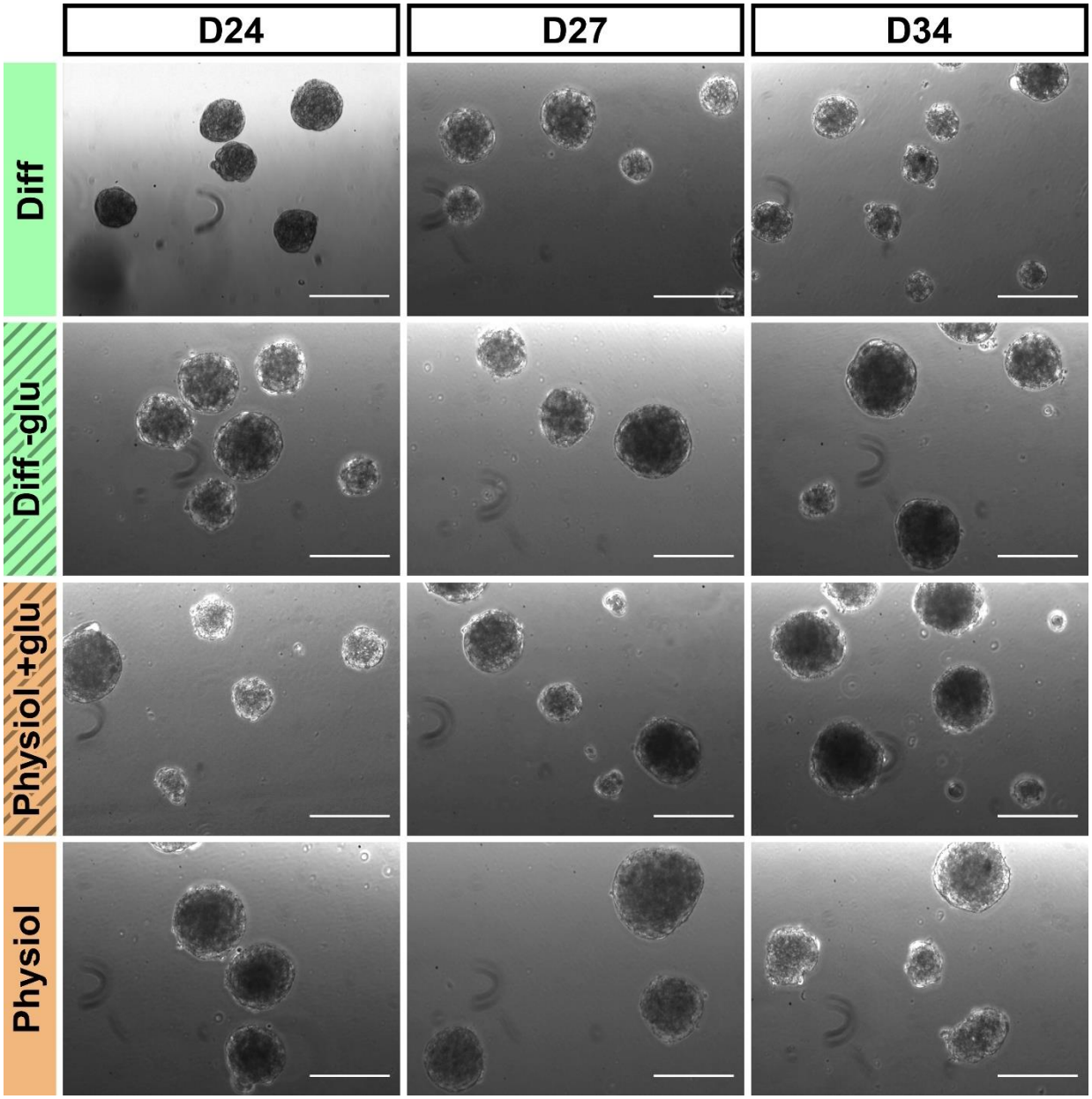

Figure 10. Spheroid morphology during the differentiation process, at days 24, 27 and 34, in Diff, Diff -glu, Physiol +glu and Physiol. Scale bar = 200 µm.

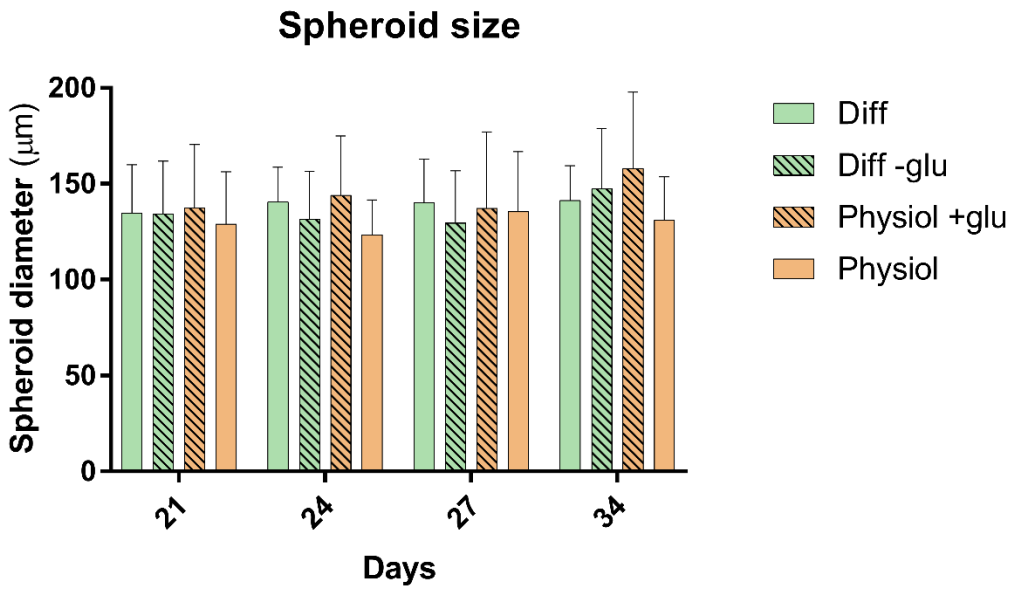

Figure 11. Spheroid diameter at days 21, 24, 27 and 34 of the differentiation process in each culture medium (n ≥ 3).

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

The influence of glucose, insulin, and dexamethasone concentrations on the differentiation and maturation of the spheroids was evaluated by measuring the expression of hepatic-specific genes (*ALB*, *CYP3A4*, *HNF4A*, *CK-18*, and the biliary marker *CK-19*) relative to undifferentiated hnMSCs, at day 34 of HLC culture (Figure 12), as well as urea production at days 27 and 34 of HLC culture (Figure 14). For this purpose, HLCs were maintained for 2 weeks as 2D monolayer or 3D spheroids in one of four media: high glucose, insulin, and dexamethasone levels (Diff); low glucose and high insulin and dexamethasone levels (Diff -glu); high glucose and low insulin and dexamethasone levels (Physiol +glu); and, finally, the most physiologically relevant medium, with low glucose, insulin, and dexamethasone levels (Physiol).

Overall, the expression of *ALB*, *HNF4A* and *CK-18* was not affected by the reduction in glucose, dexamethasone, and insulin concentrations in the culture medium (Figure 12).

ALB was overexpressed in spheroids cultured in all media by more than 4-fold and its expression was not significantly affected by decreasing glucose, insulin, and dexamethasone concentrations in culture media.

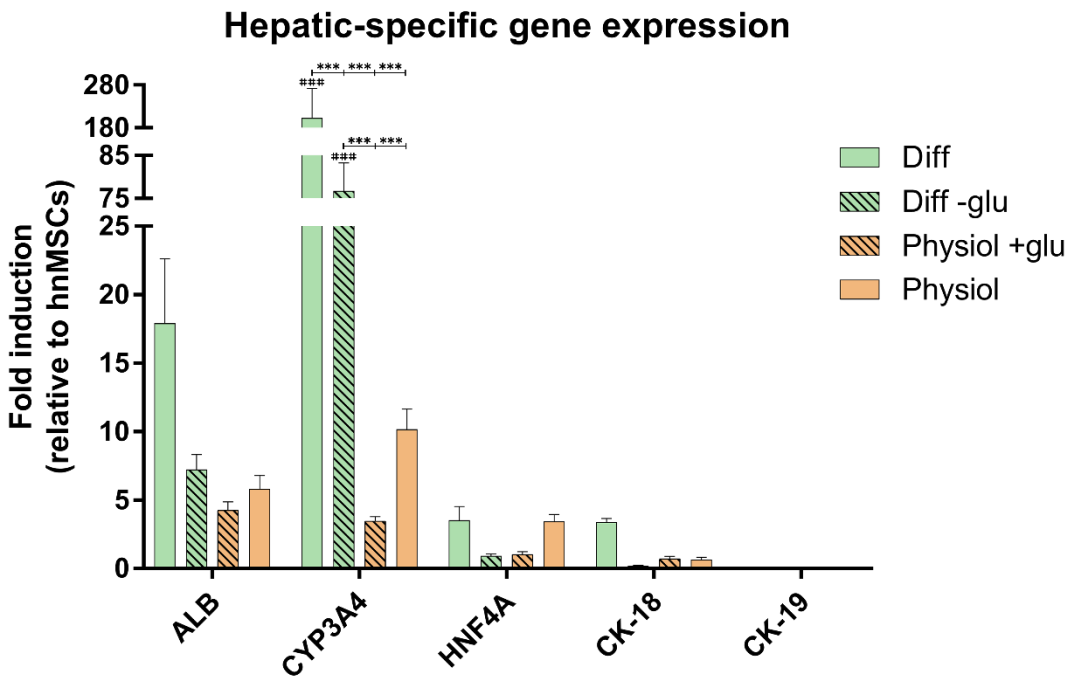

Figure 12. Gene expression analysis of hepatic markers of HLC spheroids at day 34. The graph represents the fold-induction of genes expressed by HLCs relative to undifferentiated hnMSCs. Data is normalized to the reference gene β-actin (n = 3). *, **, *** Significantly differs from other culture media 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.

HNF4A is a nuclear receptor present in hepatocytes, and the most abundant DNA-binding protein in these cells, and therefore usually accepted as a hepatic specific marker[130]. Herein, the *HNF4A* expression levels were ~3.5-fold higher in both Diff and Physiol when compared to undifferentiated hnMSCs, while no changes were observed in Diff -glu and Physiol +glu.

While hepatoblasts express both *CK-18* and *CK-19*, differentiation into hepatocytes involves a gradual decrease in *CK-19* expression, with mature hepatocytes expressing only *CK-18*[131]. Herein, *CK-18* expression was maintained in all media, with a slightly higher expression in Diff, which may be partially explained by the presence of high concentrations of dexamethasone, known to induce cytokeratin expression[132]. *CK-19* under-expression at day 34 in HLC spheroids seems to indicate that there was a commitment into hepatic lineages, with only residual levels of biliary markers.

CYP3A4 expression, however, was significantly higher in cells cultured with high levels of insulin and dexamethasone (Diff and Diff -glu) when compared to media with more physiologic values of these molecules. Indeed, incubation with insulin has been shown to lead to increased expression of *CYP3A* in rat hepatocytes[133]. Dexamethasone is also a known inducer of *CYP3A4* expression[134]. Pascussi *et al*. showed that dexamethasone incubation in ranges that include the concentrations used in our protocol induced *CYP3A4* expression in HepG2 in a biphasic manner, with moderate induction at nanomolar concentrations, such as those used in Physiol, and a much more substantial induction at higher concentrations[135], similarly to what was found in HLCs cultured in differentiation media and physiologic media. Therefore, 3D-cultured HLCs express *CYP3A4* at a basal level when cultured with low levels of insulin and dexamethasone, but the expression of this gene is inducible by culturing these cells in higher

concentrations of these molecules. Furthermore, dexamethasone influences drug metabolizing enzyme expression in different ways, inducing the synthesis of some, such as CYP3A4, and inhibiting others[136], and, thus, its use may mask the results of toxicological studies. Because HLCs cultured in Physiol +glu and Physiol are exposed to lower levels of dexamethasone but are still able to express *CYP3A4*, this model also shows potential for use in toxicological applications.

These studies have also been 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 13). 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.

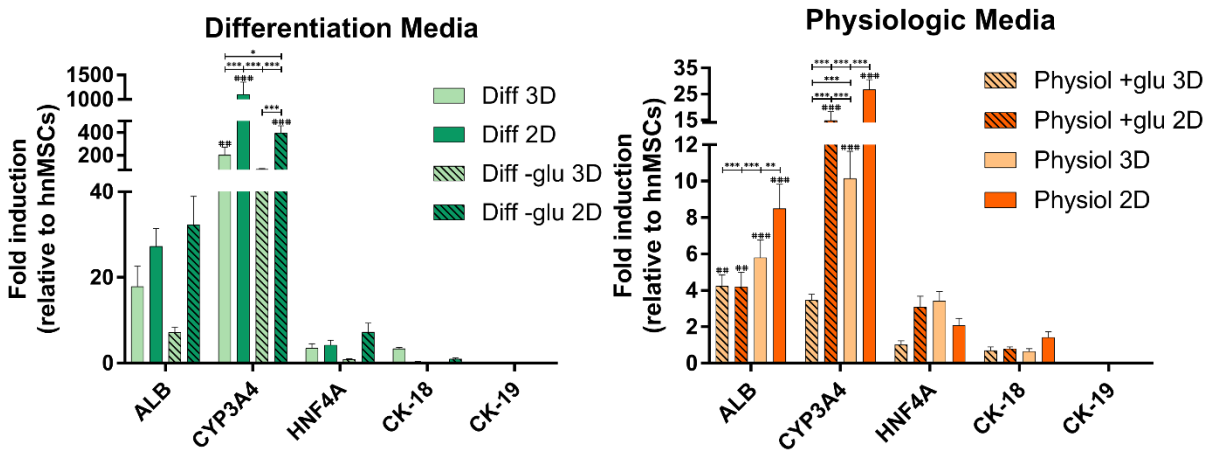

Figure 13. Comparison of gene expression analysis of hepatic markers on 2D- and 3D-cultured HLCs at day 34. The graph represents the fold-induction of genes expressed by HLCs relative to undifferentiated hnMSCs. Data is normalized to the reference gene β-actin (n ≥ 3). *, **, *** Significantly differs 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.

Cipriano *et al*. have previously compared the expression of these genes in 2D and 3D HLC cultures in Diff using the same cells as in this work, having found that *ALB* expression levels were similar in both cultures and *HNF4A* and *CK-18* were decreased in 3D cultures, although non-significantly, while *CYP3A4* expression levels were slightly increased in 3D spheroid cultures[117]. Other studies comparing the hepatic differentiation of hMSCs into HLCs using spheroids and 2D cultures report increased *ALB*, *HNF4A* and *CYP3A4* expression in 3D cultures[118,119].

To verify if the preserved expression of hepatic genes in HLCs cultured in more physiologic medium was also maintained at the protein level, the localization of hepatic proteins ALB and HNF4A in HLC spheroids cultured in Diff and Physiol was studied using immunocytochemistry/immunofluorescence (Figure 17 C, D, O, P). It was possible to verify that HLCs within the spheroids expressed ALB and HNF4A in both Diff and Physiol, 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 is a very important function of hepatocytes, and it is commonly used to assess the function of *in vitro* hepatocyte cultures regarding their ammonia detoxification ability. To determine if

medium composition affected HLCs' function and, in particular, the capacity to convert ammonia into urea, production of urea at days 27 and 34 was quantified from culture supernatants in all 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 the low-glucose ones, particularly between Diff and Diff -glu at day 27 (Figure 14).

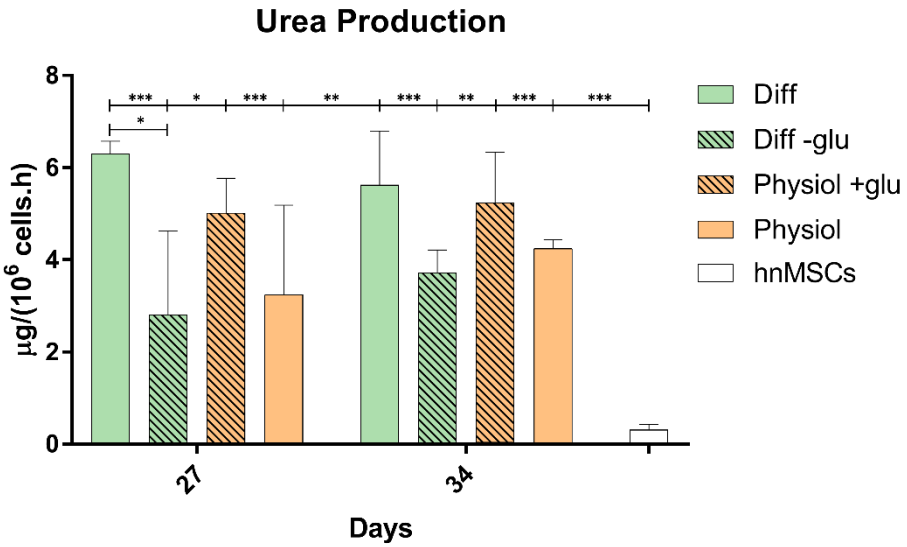

Figure 14. Urea production of HLC spheroids cultured in Diff, Diff -glu, Physiol +glu and Physiol at days 27 and 34 and of undifferentiated hnMSCs (n ≥ 3).

Dexamethasone and insulin are reported to have opposite actions on urea synthesis in hepatocytes, with dexamethasone inducing urea production and insulin decreasing it. Husson *et al*. reported that fetal rat hepatocytes cultured with dexamethasone showed increased activity of urea-cycle enzymes, but the addition of insulin partially reverted this effect[137]. In healthy humans, glucose has an inhibitory effect on urea synthesis, by inhibiting glucagon and also via a direct effect of the carbohydrate itself[138]. 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 mellitus[139], a state characterized by hyperglycemia and hyperinsulinemia, analogous to that of Diff. Given this complexity, it is not entirely clear what the net result of changing glucose, insulin and dexamethasone concentrations on cultured cells is or which players dominate the effect on urea synthesis. However, the trend for higher urea production in high-glucose media may indicate that cells cultured in high glucose concentrations could be presenting some characteristics of type II diabetes.

When comparing 2D cultures with 3D cultures, urea production was higher in spheroids than in 2D cultures in Diff and Physiol +glu in both days, in accordance with the literature[113,117,118] (Figure 15). At day 27 of culture, spheroids in Physiol displayed lower urea production than the 2D counterpart. However, at day 34, this trend was reversed. In general, spheroids present equal or improved function when compared to monolayer cultures, independently of culture medium.

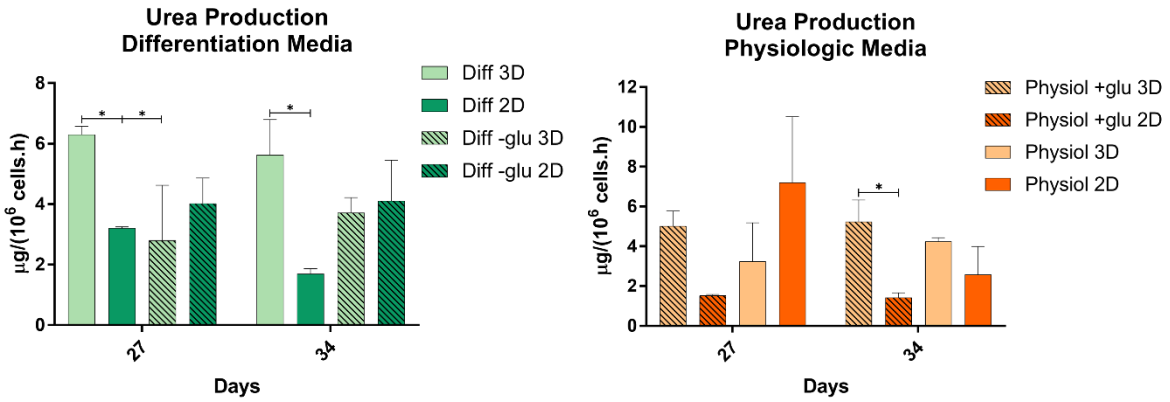

Figure 15. Comparison of urea production at days 27 and 34 of 3D and 2D cultures of HLCs, cultured in Diff, Diff -glu, Physiol +glu and Physiol (n = 3). *, **, *** Significantly differs from other media formulations or culture system with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

In summary, the results presented in this chapter show that HLCs cultured in lower glucose, dexamethasone, and insulin concentrations, i.e., in more physiologic media, are able to sustain their hepatic phenotype through the maintenance of the expression of key hepatic genes, such as *ALB*, *HNF4A* and drug-metabolizing enzyme *CYP3A4*. HLC spheroids cultured in a more physiologic medium also present hepatic markers ALB and HNF4A, at the protein level, and have preserved urea production capabilities. 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.

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

A major function of hepatocytes is the regulation of the energy metabolism in response to insulin and glucagon levels in the blood. Hepatocytes are sensitive to the levels of these hormones and their metabolism is adjusted accordingly, with insulin promoting the accumulation of energy reserves after a meal and glucagon leading to their breakdown. These functions, however, are not commonly studied *in vitro*, with most models focusing on biotransformation and, therefore, using high concentrations of insulin and dexamethasone in cultures, due to their ability to promote hepatic differentiation and induce the expression of enzymes involved in the biotransformation process. However, the use of these molecules in high concentrations does not allow the study of energy metabolism regulation, and thus, it is necessary to develop *in vitro* hepatic models that use more physiologic concentrations of these molecules.

Kato *et al*. found that insulin receptors were decreased by 50 % and functional responses, such as glycogen synthesis and lipogenesis, were markedly reduced in primary rat hepatocytes cultured with 100 nM of insulin, a value that is much lower than the concentration of insulin in Diff[140]. Given that fasting insulin levels in the blood are even lower, ranging from 0.018 nM to 0.09 nM[43], it is expected that HLCs cultured in Diff, with 1.72 µM of insulin, also show some degree of insulin resistance and are not as responsive to this hormone as healthy hepatocytes. As such, there was the need to decrease insulin concentration in the last step of our protocol to a more physiologically representative value, 1 nM, as used by Estall *et al*.[124].

To study the regulation of energy metabolism in HLCs and the effect of the culture medium composition in this process, the most physiologic medium, Physiol, was studied and compared with Diff, which has previously been validated for energy metabolism studies[71,117]. 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, specifically, genes involved in glycolysis and lipogenesis (*PDK4*), gluconeogenesis (*G6PASE*), fatty acid metabolism (*PPARA*, *CPT1A*, *ACOX1*), bile acid metabolism (*FXR*), and mitochondrial biogenesis and function (*PGC1A*). The expression was compared with spheroids cultured in the same conditions but without insulin and glucagon. All genes studied are normally downregulated by insulin and upregulated by glucagon *in vivo*.

HLC spheroids cultured in Physiol showed a much more physiologic response to insulin than those cultured in Diff (Figure 16 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 16 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 HLCs[141].

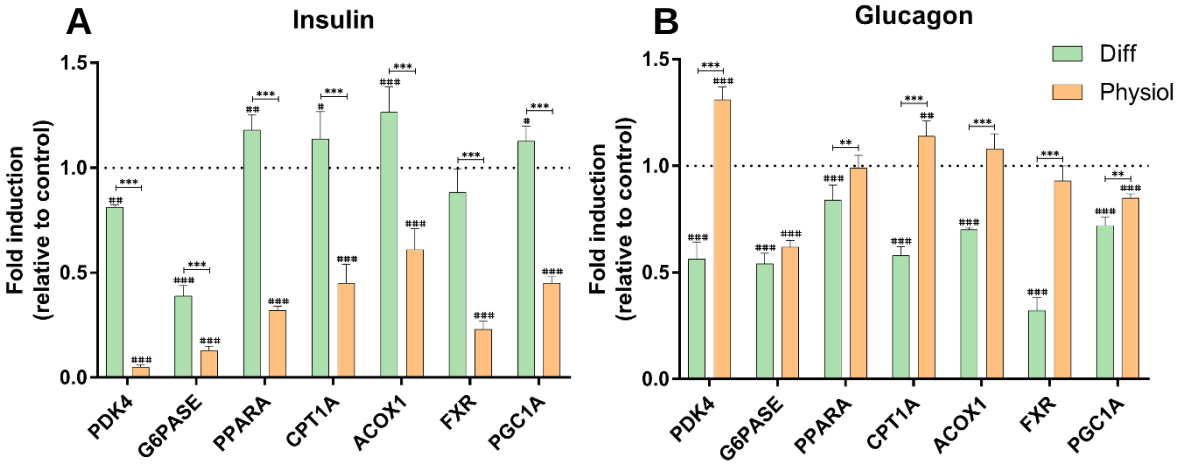

Figure 16. Gene expression analysis of energy metabolism genes in response to insulin (A) and glucagon (B) stimuli. Data is normalized to the reference gene β-actin and presented relative to starvation medium (n = 2). *, **, *** Significantly differs from other culture medium formulation with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. #, ##, ### Significantly induced or repressed expression with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Overall, HLC spheroids cultured in Physiol were able to respond appropriately to insulin stimulus, through a decrease in expression levels of all genes involved in the energy metabolism, indicating that there was a shift towards a state that mimics postprandial conditions *in vivo*. This trend was not observed in spheroids cultured in Diff. Regarding incubation with glucagon, HLC spheroids cultured in Diff showed a non-physiologic response, with decreased expression of all genes analyzed, while spheroids cultured in Physiol responded through increased expression of *PDK4*, *CPT1A* and *ACOX1*. Therefore, culturing cells in high concentrations of glucose, insulin, and dexamethasone significantly affects their ability to respond to hormonal stimuli, while the use of more physiologic concentrations of these molecules results in enhanced response to insulin and glucagon and, generally, a more physiologic response in terms of the energy metabolism gene expression.

4) Insulin and glucagon modulate the expression of periportal and perivenous markers in HLC spheroids

Hepatocyte zonation is governed by factors carried by the blood, such as oxygen, hormones, and substrates. The unidirectional flow across the lobule and its sequential interaction with hepatocytes leads to the formation of gradients across the lobule, which are important for the regulation of the zonated phenotype[142]. The concentration of glucagon, as well as oxygen and nutrients, is higher in the PP zone, while insulin is present in the PV zone at higher concentrations than glucagon, due to differences in their uptake by hepatocytes along the sinusoids[22]. Thus, oxygen- or energy-demanding pathways, including gluconeogenesis, β oxidation, and ureagenesis are located mainly in the PP zone. Conversely, TAG synthesis and glycolysis are performed by PV hepatocytes.

Due to the 3D conformation of spheroids, oxygen, hormone, and nutrient gradients are also created within the aggregates, which could mimic the gradients created across the lobule *in vivo*. Thus, the study of metabolic zonation within HLC spheroids and its modulation by external stimuli presents itself as an interesting topic. The precise role of insulin on hepatic zonation is not yet clear. However, since it was recently discovered by Cheng *et al*. that glucagon has a role on zonation, and these two hormones usually have opposite roles on the control of metabolic processes, we decided to study the effect of both insulin and glucagon on the zonated phenotype of 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 (ins), glucagon (gcg), or in the absence of hormonal stimuli (controls) 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 (Figure 17). This suggests that HLCs within the spheroids cultured in Diff and Physiol 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 17 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 many mammal livers, including those of humans, GS is expressed in the PV region, exclusively in 1 to 3 layers of hepatocytes closest to the hepatic veins[143], while GLS2 and ALB are found in the PP zone, where oxygen and nutrient availability is higher[144]. 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 fashion, being involved in the suppression of the expression of GS and other PV proteins in PP hepatocytes, and possibly in the stimulation of GS expression in the PV zone[145]. Brosch *et al.* suggest that this differential PP and PV action of the homogeneously expressed HNF4A may be due to epigenetic events such as DNA methylation[146].

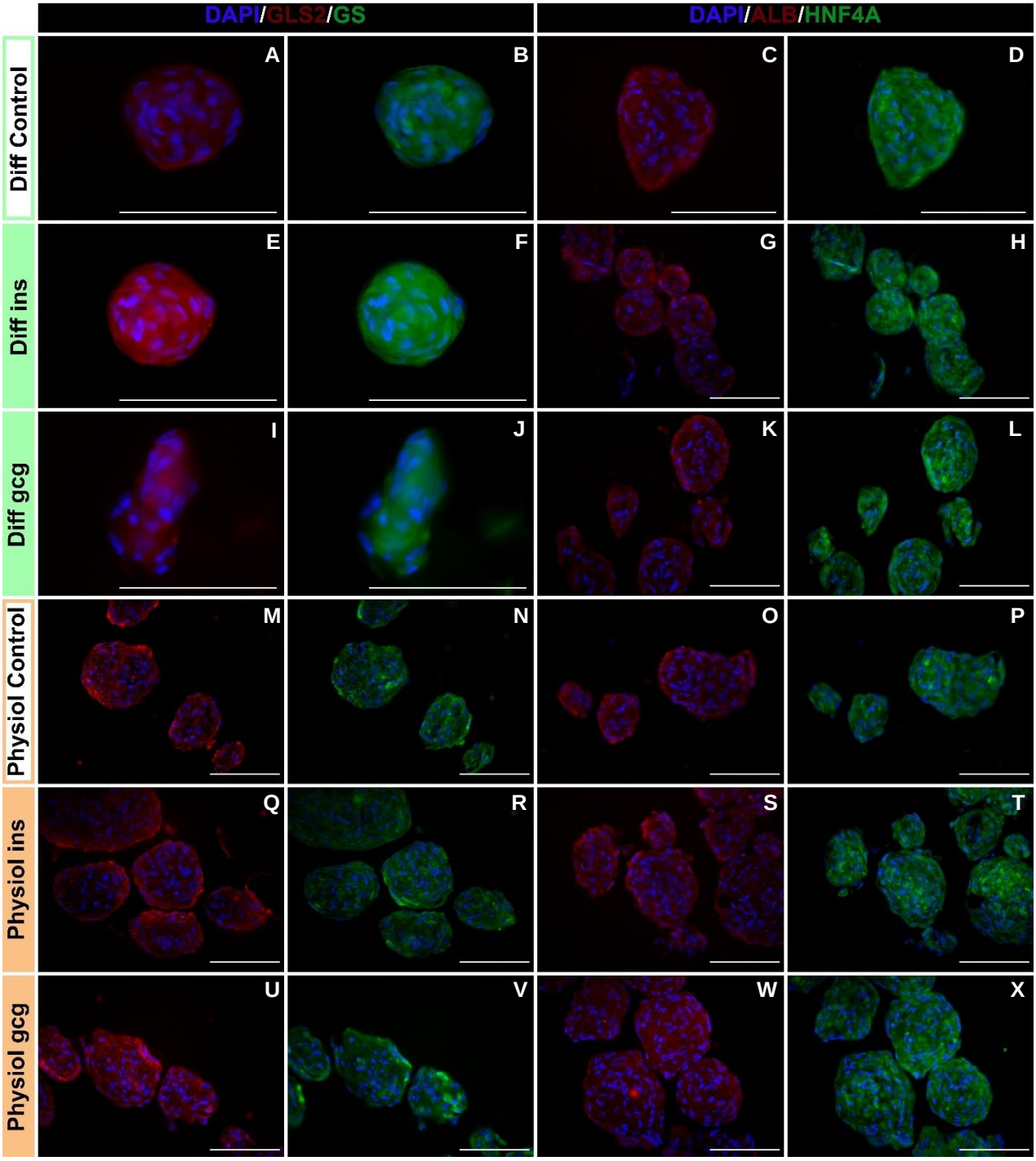

Figure 17. Immunocytochemical staining of HLC spheroids cultured in Diff and Physiol, incubated with insulin (ins) and glucagon (gcg) (day 34). Cells incubated in SM were used as controls. Scale bar = 100 µm.

To further understand the phenotype of 3D spheroid HLC cultures in terms of PP and PV functions, and whether hormonal signals influence this phenotype, spheroids cultured in Diff and Physiol were incubated on day 34 with insulin or glucagon for 8 hours and the expression levels of PP- and PV-specific genes were analyzed through qRT-PCR. The genes chosen to represent the PP zone were the PP marker *GLS2* and apolipoprotein A-IV (*APOA4*). For the PV zone, the PV marker *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 zonation[23].

Upon incubation with insulin, all PP and PV genes were significantly downregulated in Physiol (Figure 18). However, this effect was slightly more pronounced for *GLS2*, involved in the hydrolysis of glutamine into glutamate and ammonia, and *APOA4*, a gene involved in fatty acid metabolism, having a role in chylomicrons and lipoprotein secretion and catabolism. Both genes are characteristic of the PP zone and *APOA4* has been described to be inhibited by insulin. In spheroids cultured in Diff, insulin incubation led to overexpression of *GLS2*, downregulation of *APOA4*, and no changes in PV gene levels. 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 in this condition.

Upon incubation with glucagon, no significant differences were found on the expression levels of zonated genes of HLCs cultured in Diff (Figure 18). These data reinforce, once again, that culturing cells with high concentrations of insulin, dexamethasone and glucose impairs their ability to respond to hormonal stimuli and to modulate their phenotype accordingly. 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 hormone[147], as is the case in this work. No changes were found in *GLUL* expression in Physiol-cultured spheroids. Even though Cheng *et al*. found that glucagon deficient mice had increased levels of *GLUL*, this increase was much lower than that of *GLS2*. Considering the duration of incubation in our work, it is not surprising that no changes in the expression of this gene were found. Furthermore, according to some authors, *GLUL* appears to have very stable expression patterns and therefore may not be easily influenced by external factors such as hormonal stimuli[142]. Regarding *APOA4*, *LHPP* and *OAT*, Cheng *et al*. found that the expression of these genes was affected by glucagon, namely that glucagon deficient mice had increased levels of *APOA4* by approximately 1-fold and decreased *LHPP* and *OAT* levels by approximately 3- and 4-fold, respectively, when compared to wild type mice[23]. 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 hormone[23] possibly indicates, once more, 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.

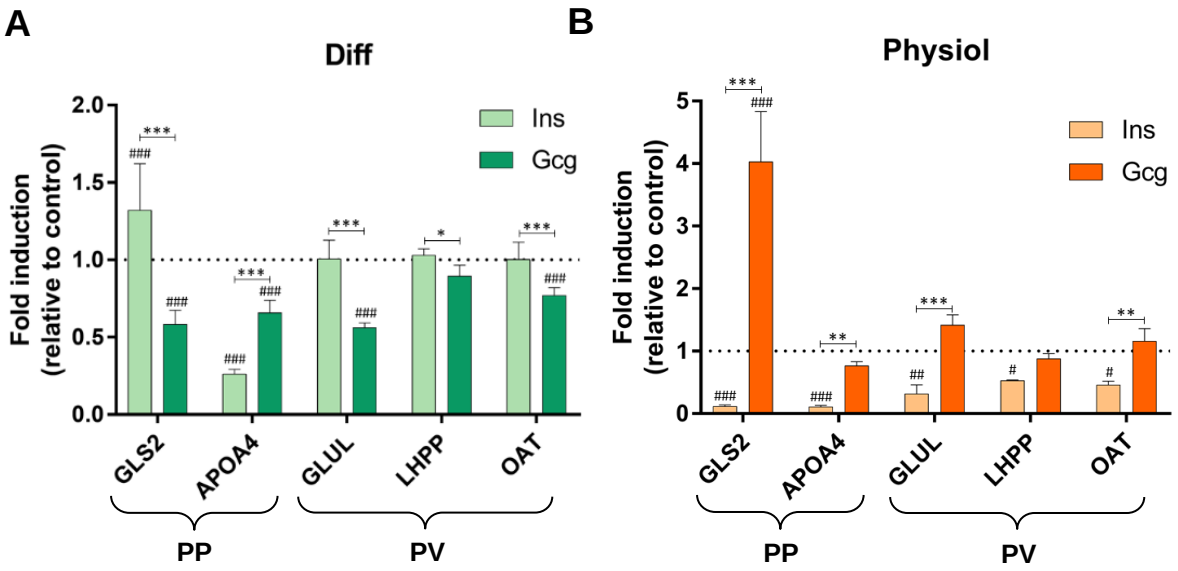

Figure 18. Gene expression analysis of PP and PV genes of HLC spheroids cultured in Diff (A) and Physiol (B) in response to insulin and glucagon stimuli. Data is normalized to the reference gene β-actin and presented relative to starvation medium (n = 2). *, **, *** Significantly differs from other culture 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.

HLC spheroids express both PP and PV genes, with *GLS2* being, overall, the most responsive gene, significantly repressed by insulin and induced by glucagon, as expected, since it is a PP gene previously shown to be regulated by glucagon[23]. Spheroids cultured in lower concentrations of glucose, insulin and dexamethasone were more sensitive to hormonal stimuli, as seen in the previous chapter. Insulin and glucagon incubation lead to some changes in the expression of zonated genes, indicating a possible role of these hormones in liver zonation, but further studies with optimized conditions are needed, to better ascertain their contribution for hepatic zonation.

In addition, to further understand if HLC spheroids cultured in Diff and Physiol responded to hormonal stimuli in terms of their function, urea production was measured upon incubation with insulin and glucagon for 8 h at day 34. Glucagon induced no alterations on ureagenesis on spheroids cultured in Diff and Physiol, but the latter showed increased urea production in response to insulin (Figure 19). As previously described, insulin leads to decreased synthesis of urea[137]. Conversely, glucagon induces urea cycle enzymes, resulting in increased ureagenesis[148,149]. These patterns were not present in HLC spheroids. These results are, however, preliminary, and further experiments and optimization of the incubation time and hormone concentrations may be necessary to better assess if HLC spheroids are able to adapt their function in response to hormonal stimuli.

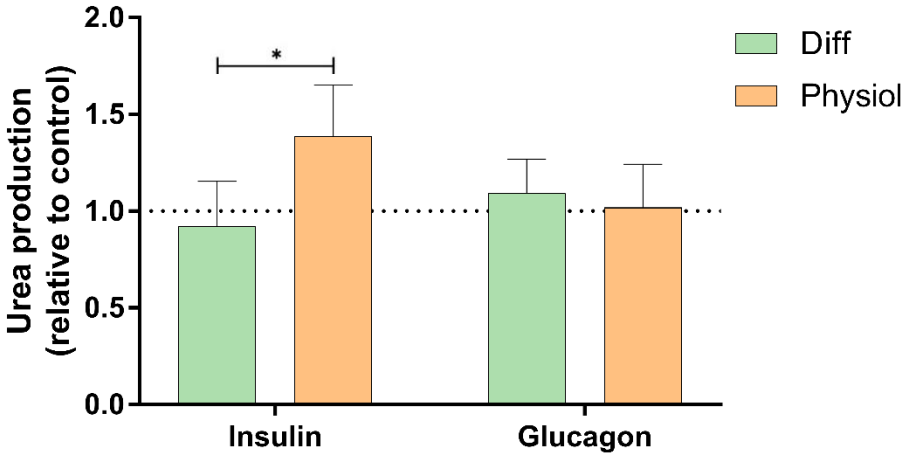

Figure 19. Urea production in response to insulin and glucagon stimuli at day 34 of HLC spheroids cultured in Diff and Physiol. Urea production capability is presented relative to starvation medium (n = 2).

Halpern *et al*. found that Wnt signals from endothelial cells surrounding the central veins regulated the expression of around 29 % of zonated hepatic genes[8]. Several genes were also found to be regulated by oxygen, a factor believed to be another major player in hepatic zonation. *In vivo*, oxygen tension falls by around 50 % from the PP zone to the PV zone of the lobule[22,142]. Studies on oxygen mass transfer within hepatocyte spheroids show that this may not be the case for spheroids cultured in the laboratory, which may have much lower differences in oxygen tension between the exterior layer of hepatocytes and those in the center of the spheroids[129].

Factors affecting hepatic zonation seem to act hierarchically, i.e., morphogen gradients, such as Wnt, establish a basic stable zonation pattern, while more fluctuating factors, such as nutrient or hormone gradients, contribute to a more dynamic zonation pattern[4,150]. Glucagon levels decrease by around 50 % along the sinusoids, while insulin decreases by 15 % in postprandial periods and 50 % in the remaining times, resulting in an increase in the insulin/glucagon ratio from the PP area to the PV area after a meal[4]. The zonated phenotype is regulated by the concentrations of all these factors within the lobule, through complex interactions[142], and, because these conditions are extremely hard to recreate *in vitro*, it is especially difficult to reproduce hepatocyte zonation in the lab. The system used in this work is extremely simple when compared to the complexity of the hepatic tissue, and thus it is expected that not all aspects will correspond to those found *in vivo*.

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 consumption[44]. 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[46,151].

Having demonstrated that Physiol is more appropriate for studies on energy metabolism than Diff, since spheroids cultured in this medium show an enhanced and more physiologic response to insulin and glucagon, Physiol 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 µM to 400 µM, similar to those found on the blood of patients with hepatic steatosis[151]. Lipid droplets inside the cells were visualized via oil red O staining.

Both PA and OA incubation resulted in increased fat accumulation, with higher number and size of lipid droplets inside the HLCs, in a manner that was proportional to fatty acid concentration in the culture medium (Figure 20). 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. 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[152,153].

In vitro studies on this subject also report a dose-dependent lipidic accumulation inside hepatocytes after OA and PA incubation[154–156]. There are different reports on whether PA or OA cause higher intracellular accumulation of TAGs, but the different concentrations, incubation times, and cell types used may explain the differences observed. Zeng *et al*. report increased lipid accumulation in the OA+PA group but higher cytotoxicity when HepG2 cells were incubated with PA, an effect that was proportional to the incubation time and concentration[154]. OA showed a protective effect against PA-induced lipotoxicity, with restoration of cell viability when PA and OA were added to the culture medium simultaneously. A similar effect was observed by Moravcová *et al*. in primary rat hepatocytes, accompanied by analogous changes in metabolic functions such as albumin production[156]. In HLCs, 200 µM of PA alone seems to result in a higher number of lipid droplets than 200 µM of PA combined with 200 µM of OA (Figure 20), which may also represent this protective effect of OA. OA is a monounsaturated fatty acid (C18:1) and PA is a saturated fatty acid (C16:0). This difference is crucial for their biological effects. The fact that OA is more easily converted into TAGs than PA could be a cause for the different effects of these fatty acids, however, higher TAG levels do not always correlate with lower cytotoxicity[155,157]. The mechanisms surrounding fatty acid mediated cytotoxicity are not yet completely clear, and it has also been suggested that different fatty acids could have different capabilities in terms of the activation of protective pathways, leading to different responses[157].

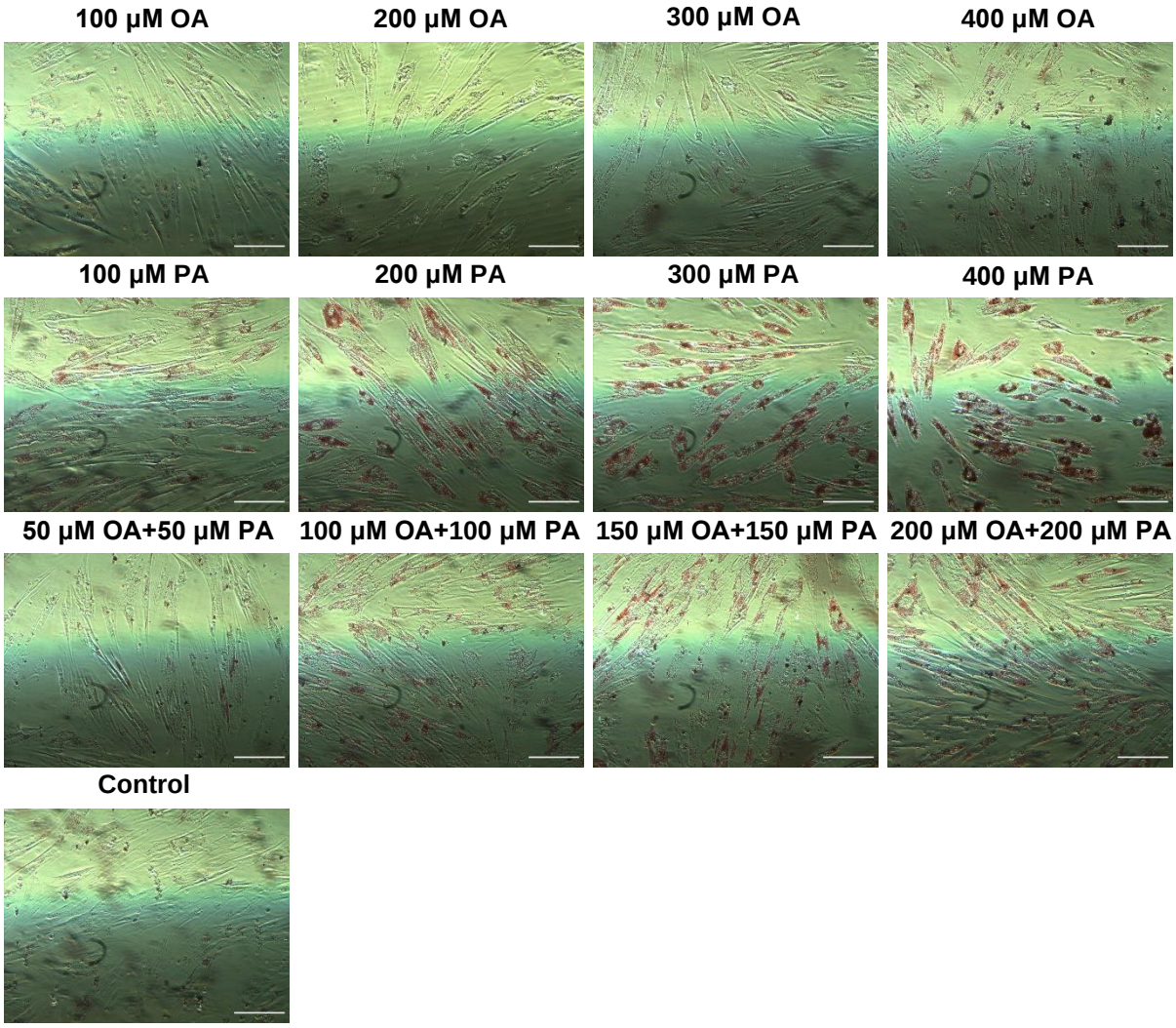

Figure 20. Oil red O staining of 2D-cultured HLCs maintained in Physiol upon incubation with OA and PA (day 34). Incubation with 0.4 % BSA was used as control. Scale bar = 200 µm.

Concluding Remarks and Future Prospects

The work developed in this thesis showed that it is possible to adapt 3D hnMSC-derived HLC cultures from a previously validated non-physiologic culture medium, proven to induce hepatic differentiation and maintain a mature hepatic phenotype, with stable and inducible biotransformation activity, to more physiologic culture media, with lower concentrations of glucose, insulin and dexamethasone, to enable the study of the energy metabolism, with the aggregates maintaining their characteristic size and morphology. Additionally, spheroids cultured in more physiologic media maintained the expression of hepatic markers and presented similar urea production capabilities.

3D-cultured HLCs expressed both PP and PV genes, being capable of performing both PP and PV functions. Gene expression patterns did not seem to be greatly influenced by short-term hormonal stimuli, applied for 8 h, particularly in spheroids cultured in high glucose, insulin and dexamethasone concentrations, in which there was almost no induction or repression of gene expression by insulin and glucagon. In spheroids cultured in more physiologic medium, gene expression was more responsive to hormones, especially PP gene *GLS2*, the only gene to significantly respond to both insulin and glucagon. Characteristic PP and PV markers were present throughout the aggregates, indicating the existence of an intermediate phenotype, with simultaneous PP and PV features. There was, however, zonation of PP markers, which were more present near the periphery of the spheroids, where oxygen, nutrient and hormone concentrations are higher, similarly to the PP zone. These patterns, however, did not seem to be influenced by the presence of insulin or glucagon, contrarily to gene expression. Because only short-term hormonal stimuli were tested, in the future it would be interesting to study hormonal influence during longer periods and their effects on metabolic zonation, as well as the effects of other factors that drive zonation.

To better understand the pathophysiology of insulin resistance and other associated diseases, it is important to obtain HLCs that are metabolically competent and able to regulate the energy metabolism similarly to hepatocytes *in vivo*. Thus, the expression of several genes involved in the energy metabolism in response to the metabolism-regulating hormones insulin and glucagon was studied. Spheroids cultured in the more physiologic medium showed significantly enhanced and more physiologic response to hormonal stimuli, in several metabolic pathways, including glucose metabolism, fatty acid metabolism, and bile acid metabolism, when compared to spheroids cultured in the previously defined medium. Therefore, the hypothesis that lower concentrations of glucose, insulin, and dexamethasone would result in a more physiologic response seems to be validated, and thus Physiol is a good candidate for HLC culture when the goal is studying the energy metabolism.

Finally, 2D-cultured HLCs were incubated with varying concentrations of saturated and unsaturated fatty acids common in modern western diets, resulting in an intracellular accumulation of lipids, with a more

pronounced effect upon incubation with PA, a saturated fatty acid, than the unsaturated OA. Interestingly, PA and OA combined led to less intracellular accumulation of lipids than PA alone. Thus, OA seems to have a protective effect against PA-induced steatosis in HLCs. To further understand the cells' response to fatty acids, the expression of key metabolic genes should be analyzed throughout the incubation period. Furthermore, these experiments have only been performed in 2D cultures and the response of 3D-cultured HLCs to fatty acid incubation should also be characterized, in order to develop a 3D insulin resistance model.

To conclude, with this work we have shown that it is possible to culture stem cell-derived HLC spheroids in more physiologic media without losing their phenotype and function. In fact, these cells are more responsive to phenotype modulation by hormones and are capable of mimicking a steatotic state in response to fatty acids in the culture medium. This work is a starting point towards the development of a hepatic insulin resistance model that can help to better understand this condition and eventually aid in the development of novel therapeutic options.

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Annexes

1) Primers

Table 2. Nucleotidic sequence of primers used for qRT-PCR.

Gene name	Forward sequence	Reverse sequence
B-ACTIN	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
ALB	TGCTTGAATGTGCTGATGACAGGG	AAGGCAAGTCAGCAGGCATCTCATC
CYP3A4	ATTCAGCAAGAAGAACAAGGACA	TGGTGTTCTCAGGCACAGAT
HNF4A	ATTGACAACCTGTTGCAGGA	CGTTGGTTCCCATATGTTCC
CK-18	TGGTACTCTCCTCAATCTGCTG	CTCTGGATTGACTGTGGAAGT
CK-19	ATGGCCGAGCAGAACCGGAA	CCATGAGCCGCTGGTACTCC
PDK4	TCTGAGGCTGATGACTGGTG	GGAGGAAACAAGGGTTCACA
G6PASE	CAGAGCAATCACCACCAAGC	ACATTCATTCCTTCCTCCATCC
PPARA	CTGTCATTCAAGCCCATCTTC	TTATTTGCCACAACCCTTCC
CPT1A	TCCAGTTGGCTTATCGTGGTG	TCCAGAGTCCGATTGATTTTTGC
ACOX1	ACTCGCAGCCAGCGTTATG	AGGGTCAGCGATGCCAAAC
FXR	AGAACCTGGAAGTGGAACC	CTCTGCTACCTCAGTTTCTCC
PGC1A	GCTGAAGAGGCAAGAGACAGA	AAGCACACACACCACACACA
GLUL	AAGAGTTGCCTGAGTGGAATTTC	AGCTTGTTAGGGTCCTTACGG
GLS2	GCCTGGGTGATTTGCTCTTTT	CCTTTAGTGCAGTGGTGAACTT
APOA4	CTCAAGGGACGCCTTACGC	GTCCTGAGCATAGGGAGCCA
LHPP	CTGTGTGGTAATTGCAGACGC	TAGTAACGCCCTTTTCCCAGT
OAT	GTGGGGCTATACCGTGAAGG	TGGTCCAAAACCATCGTAACTG