hiPSC-derived hepatocytes closely mimic the lipid profile of primary hepatocytes: A future personalised cell model for studying the lipid metabolism of the liver

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Funding information
Finnish Cardiovascular Foundation; Instrumentariumin Tiedesäätiö; FP7 Health, Grant/Award Numbers: F2-2013-602222, 2012-3057392

Abstract
Hepatocyte-like cells (HLCs) differentiated from human-induced pluripotent stem cells offer an alternative platform to primary human hepatocytes (PHHs) for studying the lipid metabolism of the liver. However, despite their great potential, the lipid profile of HLCs has not yet been characterized. Here, we comprehensively studied the lipid profile and fatty acid (FA) metabolism of HLCs and compared them with the current standard hepatocyte models: HepG2 cells and PHHs. We differentiated HLCs by five commonly used methods from three cell lines and thoroughly characterized them by gene and protein expression. HLCs generated by each method were assessed for their functionality and the ability to synthesize, elongate, and desaturate FAs. In addition, lipid and FA profiles of HLCs were investigated by both mass spectrometry and gas chromatography and then compared with the profiles of PHHs and HepG2 cells. HLCs resembled PHHs by expressing hepatic markers: secreting albumin, lipoprotein particles, and urea, and demonstrating similarities in their lipid and FA profile. Unlike HepG2 cells, HLCs contained low levels of lysophospholipids similar to the content of PHHs. Furthermore, HLCs were able to efficiently use the exogenous FAs available in their medium and simultaneously modify simple lipids into more complex ones to fulfill their needs. In addition, we propose that increasing the polyunsaturated FA supply of the culture medium may positively affect the lipid profile and functionality of HLCs. In conclusion, our data showed that HLCs provide a functional and relevant model to investigate human lipid homeostasis at both molecular and cellular levels.

Abbreviations: APOA1, apolipoprotein A-I gene; APOB, apolipoprotein B gene; ASGR, asialoglycoprotein receptor; BMP4, bone morphogenic protein 4; CE, cholesteryl ester; Cer, ceramide; DAG, diacylglycerol; DE, definitive endoderm; EGF, epidermal growth factor; ELOVL, fatty acid elongase; FA, fatty acid; FADS, fatty acid desaturase; FASN, fatty acid synthase; Gb3, globotriaosylceramide; GCK, Glucokinase; Glc/GalCer, glucosyl/galactosylceramide; GSL, glycosphingolipid; HGF, hepatocyte growth factor; hiPSC, human induced pluripotent stem cell; HLC, hepatocyte-like cell; HLR, Human Liver Total RNA; LacCer, lactosylceramide; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPL, lysophospholipid; M1, Method 1; M2, Method 2; M3, Method 3; M4, Method 4; M5, Method 5; MUFAs, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PHH, primary human hepatocyte; PI, phosphatidylinositol; PL, phospholipid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SL, sphingolipid; SM, sphingomyelin; TAG, triacylglycerol; UGCG, UDP-glucose ceramide glucosyltransferase.
KEYWORDS
fatty acid (FA), gas chromatography, hepatocyte-like cell (HLC), HepG2, human-induced pluripotent stem cell (hiPSC), lipidomics, mass spectrometry (MS), primary human hepatocyte (PHH)

1 | INTRODUCTION

The liver plays an important role in the regulation of many physiological functions of the body, including lipid and carbohydrate metabolism, glycolytic/urea metabolism, plasma protein synthesis, and the detoxification of a wide variety of molecules (Si-Tayeb et al., 2010). Hepatocytes, which comprise about 70% of the liver’s mass, originate from the anterior portion of the definitive endoderm (DE), one of the three embryonic layers (Blouin, Bolender, & Weibel, 1977). Hepatocytes handle many crucial metabolic functions of the liver, including the synthesis of lipoproteins, triacylglycerols (TAGs), cholesterol, and phospholipids (PLs; Gordillo, Evans, & Gouon-Evans, 2015).

Primary human hepatocytes (PHHs) are currently considered the “gold standard” in cell modeling for studying, for example, liver physiology, toxicity, and lipid homeostasis. Typically, PHHs are obtained from cadaveric donors, but they are scarce and functionally heterogeneous, and it is hard to maintain them in a culture. When cultured, PHHs lose functionality relatively fast, and their liver-specific features progressively deteriorate, which particularly hampers long-term studies (Elaut et al., 2006; Godoy et al., 2013). To address these limitations, various human hepatoma cell lines, including HepG2 and Huh7, have been used due to their ease of handling, unlimited life span, and stable phenotype. Nevertheless, they do not faithfully mirror the metabolic activities of healthy liver cells. In fact, the expression levels and profiles of genes involved in liver-specific functions are poorly presented in these systems (Olsavsky et al., 2007). As a result, hepatoma cell lines have failed to predict the numerous adverse hepatotoxic side effects of new drugs (Castell, Jover, Martínez-Jiménez, & Gómez-Lechón, 2006). Alternatively, animal models, such as rats and mice, or animal primary hepatocytes have been widely used to study lipid metabolism and lipoprotein production in the liver (Kvilekval, Lin, Cheng, & Abumrad, 1994). However, the information gained from murine cells is not fully translatable to humans, and there are significant differences in lipid metabolism between the species. Animal models are also expensive and unsuitable for large-scale screening. Furthermore, due to growing ethical concerns, there is an urgent need to reduce the use of rodents and other animal models in research.

Human-induced pluripotent stem cells (hiPSCs) provide an unlimited supply of tissue-specific differentiated cell types for disease modeling and cell therapy. Hepatocytes differentiated from pluripotent stem cells circumvent the problem of the limited availability of cells faced when working with PHHs. They show very similar characteristics to PHHs; for instance, they secrete albumin as well as urea, and to some extent express drug transporters and cytochrome P450 enzymes (Kia et al., 2012). Unlike rodent hepatocytes and some human hepatoma cells, these hepatocyte-like cells (HLCs) are sensitive to hepatitis C virus infection and support viral replication similar to PHHs (Schwartz et al., 2012). Patient-specific iPSC lines provide us with an advantageous system of HLCs from patients of different genetic backgrounds, a prerequisite for the development of personalised medicine. The generation of hiPSC-derived HLCs (hiPSC-HLCs) in large quantities enables their use in modeling inborn errors of hepatic metabolism, understanding the molecular basis of liver cell differentiation, studying disease mechanisms, and facilitating drug discovery and safety. HLCs have already proven to be of great value in developing novel therapeutics (Medine et al., 2013; Szkolnicka et al., 2014) and identifying the noncoding micro-RNAs regulating human liver damage (Szkolnicka et al., 2016; Yang et al., 2016). HLCs have also been successfully used as in vitro cell culture systems, for example, to recapitulate the pathophysiology of familial hypercholesterolemia (Cayo et al., 2012) and human cholesterol homeostasis (Krueger et al., 2013). Lipid defects are central to the pathogenesis of many common diseases, such as atherosclerosis (Meikle et al., 2011; Stübiger et al., 2012) and nonalcoholic fatty liver disease (Ruhanen et al., 2017; Younossi et al., 2016). Therefore, hiPSC-HLCs offer a great platform for investigating the basic mechanisms of lipid metabolism and its dysregulation in a patient-specific manner. However, to date, no detailed studies have yet been performed on the lipid profile and fatty acid (FA) metabolism of HLCs. To better use HLCs as a cell model to study the role of molecular lipids in liver diseases, it is essential to know their lipid profile in relation to actual human adult liver tissue and to the currently used cell models, namely PHHs and hepatoma cell lines.

Hepatic differentiation methods have greatly improved over the last decade, enabling the efficient generation of high quality HLCs from pluripotent stem cells (Cameron et al., 2015; Hannan, Segeritz, Touboul, & Vallier, 2013; Hay et al., 2008; Kajiwara et al., 2012; Si-Tayeb et al., 2010). Nevertheless, the experimental details and some differentiation factors differ between the current protocols, which might affect the phenotype of the HLCs. To reproduce the physiological conditions of the human liver, it is crucial to generate functional HLCs that are as similar as possible to PHHs. In this study, we describe five protocols for generating HLCs from hiPSCs and comprehensively compare the morphology, genetics, biochemistry, and functional traits of the HLCs produced. In addition, we compare the HLCs with the two most common cell models currently used—PHHs and HepG2 cells—as well as human liver tissue. Most importantly, the lipid profiles of HLCs, PHHs, and HepG2 cells are analyzed by both mass spectrometry (MS) and gas chromatography, and the similarities and main differences between the cell types are discussed in light of selected key genes involved in FA metabolism. Finally, the lipid profile of HLCs is fully characterized, and the potential and
limitations of HLCs as a model for studying lipid metabolism are evaluated.

2 | MATERIAL AND METHODS

2.1 | Ethical issues

The study and patient recruitment have been approved by the Ethics Committee of Tampere University Hospital (approval number: R12123). All participants providing skin biopsies were adults more than 18 years old who had signed an informed consent form after receiving both oral and written descriptions of the study.

2.2 | hiPSC reprogramming and cell culture

Three hiPSC lines (UTA.10100.EURCAs, UTA.11104.EURCAs, and UTA.11304.EURCCs) were generated directly from the fibroblasts of three individuals. Pluripotency was induced with the Sendai reprogramming kit (OCT4, SOX2, KLF4, C-MYC; CytoTune; Life Technologies, USA) based on the protocol described by Ohnuki, Takahashi, Yamanaka, (2009) and Takahashi & Yamanaka, (2006). hiPSCs were then maintained as described before (Kiamehr et al., 2017). Details of the hiPSC cell culture are also described in the Supporting Information.

2.3 | Hepatic differentiation

In all methods except Method 4 (M4), hiPSCs were transferred from mouse embryonic fibroblasts to Geltrex™ (USA; Cat: A14133-01, 1:50 dilution), kept in mTeSR1™ medium, and adapted to the changed culture conditions for a few passages before commencing the differentiation. In M4, hiPSCs were adapted on Laminin 521 (BioLamina, Lot: 80104) first, and the differentiation was completed on Laminin mix 111/521-coated plates (3:1 ratio, 10 µg/ml). The hiPSCs used in all methods were at passage 20 or higher before commencing the differentiation. Three lines were differentiated using each method, except Method 5 (M5), which was applied to two cell lines. Figure 1 shows a schematic view of all five methods used in the study. Details of the five differentiation methods are described in detail in the Supporting Information.

2.4 | PHHs and HepG2 cells

Cryopreserved PHHs (Cat. No. HMCPIS, Lot. HU8210, USA) were purchased from Gibco®, and hepatocellular carcinoma cells (HepG2, ATCC-HB-8065, Lot. No. 59947519) were purchased from ATCC®. Both cells were plated according to the manufacturer’s instructions. PHHs were cultured in William’s E medium (A1217601, Gibco, USA) supplemented with cocktail B (Gibco, CM 4000) and dexamethasone, whereas the HepG2 cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% FBS.

| Method | Description | Plate Coating | Media | Duration |
|--------|-------------|---------------|-------|----------|
| M1     | hiPSC on Geltrex for 3-4 days | - | Act. A, CHIR 99021, NaB | 2 days |
| M2     | hiPSC on Geltrex for 3-4 days | - | Act. A, NaB | 3-4 days |
| M3     | Seeding single iPSCs on Geltrex for 1 day | - | Act. A, Wnt3, NaB | 3-5 days |
| M4     | Seeding single iPSCs on Laminin 521/111 for 1 day | - | Act. A, Wnt3, ROCK | 1 day |
| M5     | iPSCs on Geltrex for 24 hrs | STEMdiff™ DE Kit for 4 days | Act. A, Wnt3, NaB | 3-5 days |

FIGURE 1 A schematic representation of the five hepatic differentiation protocols used for generating hepatocyte-like cells (HLCs). Differentiation in Method 1 and Method 2 (M1 and M2) were started with hiPSC colonies, in Method 3 and Method 4 (M3 and M4) with dissociated iPSC single cells, and in Method 5 (M5) with 24 hrs post-cultured hiPSCs. Plates in Method 4 (M4) were coated with a mix of Laminin 111/521 (3:1 ratio) instead of the Geltrex™ used in the remainder of the methods. hiPSC: human-induced pluripotent stem cell [Color figure can be viewed at wileyonlinelibrary.com]

2.5 | RT-polymerase chain reaction

RNA extraction and polymerase chain reaction (PCR) for pluripotency markers (OCT4, NANOG, SOX2, and SSEA4) were performed as published before (Manzini, Viiri, Marttila, & Aalto-Setälä, 2015).

2.6 | Quantitative PCR (qPCR) analysis

RNA samples were collected at the hiPSC, DE, and HLC stages, and RNA was extracted using an RNaseasy kit (Qiagen, Germany, Cat. No. 74106). Complementary DNA (cDNA) was generated using a high capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions in the presence of an RNase inhibitor. cDNA was multiplied either by the Power SYBR Green PCR Master Mix (Life Technology, Cat. No. 1408470, Austin, TX) and gene-specific primers (OCT4, SOX17, FOXA2, AFP, ALB) or by the TaqMan Universal Master Mix (Applied Biosystems. 4304437, Austin, TX) and gene-specific TaqMan probes (APOA1, APOB, FADS1, FADS2, ELOVL2, ELOVL5, FASN) using the BioRad CFX384 Real-Time PCR Detection System. Values were normalized to GAPDH, which was used as an endogenous control, and relative quantification was performed.
calculated by the ΔΔCT method (Livak & Schmittgen, 2001). PHH was used as the reference sample.

First Choice® Human Liver Total RNA (hLTR, Cat. No. AM7960), purchased from Ambion®, was used as an extra control. The results from qPCR were compared for the HLCs and the three reference samples (PHHs, hLTR, and HepG2 cells).

2.7 | Immunostaining

Cells were fixed, stained, and visualized as described before (Kiamehr et al., 2017). Details are also provided in the Supporting Information. The percentage of ALB-positive cells and binuclear HLCs were calculated manually by counting HLCs in 3–5 stained areas and calculating the average.

2.8 | Low-density lipoprotein uptake

The ability of the cells to uptake low-density lipoprotein (LDL) was evaluated by incubating the HLCs with labeled LDL (Cell-based assay kit, Cayman, USA, Cat. No. 10011125) for 4 hr, after which the cells were imaged by fluorescent microscopy.

2.9 | FACS analysis

To analyze the number of CXCR4-positive cells, endodermal cells were detached with Versene (Gibco®, UK), suspended in 3%–5% bovine serum albumin buffer, stained with a PE-conjugated CXCR4 antibody (R&D Systems FAB173P, Minneapolis, 10 µl for 10^6 cells) for 15 min at RT, washed three times, and analyzed using the Accuri™ C6 device (BD Biosciences).

2.10 | Albumin, urea, and TAG secretion

At the late stage of hepatic differentiation, HLCs were evaluated for their functionality. The albumin, urea, and TAG content of the conditioned medium were determined, respectively, with the Human Albumin ELISA Quantitation kit (Bethyl Laboratory), the QuantiChrom™ Urea Assay Kit (BioAssay Systems, USA), and the Triglyceride Quantification Kit (BioVision Inc., Cat. No. K622-100, USA) according to the manufacturers’ instructions. The values were normalized to cell numbers. The results were then compared with the data from the PHHs and HepG2 cells, which were cultured in parallel with the HLCs.

2.11 | Lipid mass spectrometry

2.11.1 | Lipid sample preparation and extraction

Lipids (sphingolipids [SL], cholesterol, glycerolipids, and glycerophospholipids) were extracted from the HLCs by Hamilton Robotics AB and studied by shotgun lipidomics. The detailed procedure is described by Kiamehr et al., (2017).

2.11.2 | Mass spectrometric analyses and data processing

In the shotgun lipidomics (cholesterol ester [CE], diacylglycerol [DAG], sphingomyelin [SM], lysophosphatidylcholine [LPC], lysophosphatidylethanolamine [LPE], lysophosphatidylserine, lysophosphatidylglycerol, and lysophosphatidylinositol [LPI]), lipid extracts were analyzed on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500) equipped with a robotic nanoflow ion source (NanoMate, Advion Biosciences Inc., Ithaca, NY) as described by Heiskanen, Suoniemi, Ta, Tarasov, & Ekroos, (2013). Molecular lipids were analyzed in positive ion mode using lipid class-specific precursor ion or neutral loss scans (Ekroos, Chernushevich, Simons, & Shevchenko, 2002; Ekroos et al., 2003).

Sphingolipids (ceramide [Cer], glucosyl/galactosylceramide [Glc/GalCer], lactosylceramide [LaCer], and globotriaosylceramide [Gb3]) and molecular PLs (phosphatidylcholines [PC], phosphatidylethanolamines [PE], and phosphatidylinositols [PI]) were analyzed with a targeted approach using ultra-high-pressure liquid chromatography-mass spectrometry (UHPLC-MS; Merrill, Sullards, Allegood, Kelly, & Wang, 2005). An analytical Acquity BEH C18, 2.1 × 50 mm column with a particle size of 1.7 µm (Waters, Milford, MA) heated to 60°C was used. Mobile phases consisted of 10 mM ammonium acetate in water with 0.1% formic acid (solvent A) and 10 mM ammonium acetate in acetonitrile:s/isopropanol (4:3, v/v) containing 0.1% formic acid (solvent B). The flow rate was set to 500 µl/min. Sphingolipids were separated with a 15 min linear gradient from 75% B to 100% B, while molecular PLs were analyzed using a 10 min gradient from 75% B to 80% B. Both sphingolipids and PLs were analyzed on a hybrid triple quadrupole/linear ion trap mass spectrometer (5500 QTRAP) equipped with an UHPLC system (CTC HTC PAL autosampler and Rheos Allegro pump or Shimadzu Nexera X2) using a multiple reaction monitoring based method in positive ion mode for sphingolipids and negative ion mode for molecular PLs. Curtain gas was set at 25; the ion spray voltage was set at 5000 V in positive ion mode and to 25; the ion spray voltage was set at 4500 V in negative ion mode, and the ion source was heated to 400°C in positive mode and to 300°C in negative ion mode. The collision energy was optimized for each lipid class. Identified lipids were quantified by normalizing against their respective internal standard (Eising et al., 2006) and total protein concentrations in the cell sample. Total protein concentrations were determined using the Micro BCA™ Protein Assay Kit (Thermo Scientific Pierce Protein Research Products) according to the manufacturer’s instructions. Data processing was performed by MultiQuant, LipidView (AB Sciex) software and SAS.

2.12 | FA gas chromatography

To confirm our observations from the MS analysis of molecular lipids and to investigate the effect of the medium on the FA profile of the cells, the FA composition of HLCs, PHHs, and HepG2 cells and their media was analyzed by gas chromatography as described in detail by Kiamehr et al., (2017). Briefly, the acyl chains in the cell pellet
lipids or the lipid residues of the nitrogen-dried media were converted to FA methyl esters (FAMEs) in a transesterification reaction with 1% methanolic H$_2$SO$_4$. The quantitative analysis of the FAMEs, which were extracted into hexane, was performed using a Shimadzu GC-2010 Plus gas chromatograph with a flame-ionization detector, and the FAME structures were identified by Shimadzu GCMSQP2010 Ultra with a mass selective detector. In both systems, the components of the FAME mixtures were separated in ZB detector, and the FAME structures were identified by Shimadzu Shimadzu GC capillary columns (30 m, 0.25 mm ID, 0.25 µm film; Phenomenex). The calculations of the FA compositions and concentrations followed standard procedures (Kiamehr et al., 2017), and the FAs were calculated from the methyl end (e.g., 22:6n-3).

2.13 Statistical analysis

GraphPad Prism version 5.02 software was used for the data analysis. Data are presented as means ± standard deviation with n representing the number of independent experiments. The results were compared using one-way analysis of variance, followed by Bonferroni's multiple-comparison test. A p value < 0.05 was considered statistically significant.

3 RESULTS

3.1 Cell morphology during hepatic differentiation

In all methods, dramatic morphological changes were observed, particularly during the first few days of DE differentiation. In all methods except M5, migrating DE cells possessed a spiky morphology, whereas in M5, migrating cells were instead more round or square-shaped (Supporting Information Figure S1a). In addition, the amount of cell death in M5 was considerably lower than in the other methods. No morphological differences were observed between the DE cells treated with CHIR 99021 (Method 1, M1) or Wnt3 (Methods 2-4, M2-4). Initiating differentiation with single cells in M3 and M4 did not yield a higher efficiency of DE formation compared with M1 and M2, which were started with colonies (Supporting Information Figures S1a and S3). However, we did observe cells with a DE morphology appearing one or even two days earlier in methods initiated with single cells compared with methods initiated with colonies (Supporting Information Figure S1a). At the hepatic specification stage, cells treated with basic fibroblast growth factor, bone morphogenetic protein 4 (BMP4), and hepatocyte growth factor (HGF; M1) clearly had a different morphology compared with the cells treated with DMSO (M2–5; Supporting Information Figure S1b), which might imply different pathways toward hepatoblasts in those protocols.

Binucleation is a feature of adult hepatocytes and generally considered a sign of terminal differentiation (Miyaoaka & Miyajima, 2013). We found 29% of the PHHs and on average 10% of the HLCs to be binuclear (Figures 1b and 2a). The HLCs differentiated by M4 showed the closest binuclearity (16%) to the PHHs. No individual cell line was shown to be more potent in generating binuclear cells.

3.2 Characterization of DE and HLCs at the protein level

hiPSCs expressed OCT4 protein, which was lost during the DE stage, while the expression of DE markers SOX17 and FOXA2 was upregulated (Supporting Information Figure S2a). The efficiency of the DE differentiation, estimated by measuring the CXCR4 expression by flow cytometry, did not differ across M1, M2, and M3 (Supporting Information Figure S2b). In M4 and M5, the amount of CXCR4-positive cells was lower than in the other methods.

The immature hepatic marker AFP was expressed in hepatic progenitor cells and remained expressed until the later stages in all five methods (Figure 2a). In addition, ALB, LDL receptor (LDL-R), and asialoglycoprotein receptor (ASGR) were all expressed in mature HLCs (Figure 2a and Supporting Information Figure S5). The average of the ALB-positive cells in M1 to M5 was 9.8%, 16.6%, 20.8%, 37.7%, and 31.5%, respectively, and the percentage in M4 was significantly higher than in M1, M2, and M3, but not in M5 (Figure 2b). More than 90% of M2- to M5-HLCs were positive for ASGR (data not shown).

3.3 Gene regulation

As expected, OCT4 was highly expressed at the iPSC stage, whereas at the DE stage, OCT4 was dramatically downregulated in most of the cell lines, and SOX17 and FOXA2 were highly expressed (Supporting Information Figure S6). Further differentiation toward HLCs resulted in significant downregulation of SOX17, whereas FOXA2 remained upregulated during the rest of the differentiation and maturation of the HLCs (Figure 2c and Supporting Information Figure S7). The HLCs expressed SOX17 at the same levels as hLTR. The level of FOXA2 in the HLCs was comparable to those in the reference samples PHH, hLTR, and HepG2. AFP was upregulated during the early and late hepatic differentiation stages, indicating the immature characteristic of the HLCs. The expression of AFP in the M2-HLCs was statistically significantly higher than in the M1-HLCs. ALB was dramatically upregulated in mature HLCs, up to $2 \times 10^5$-fold compared with that in the hiPSCs, and its levels remained close but below those found in the PHHs. The expression of ALB in the M5-HLCs was significantly higher than in the M1-HLCs ($p < 0.01$) and M3-HLCs ($p < 0.05$). The levels of ALB expression were comparable between the HepG2 and the PHHs; however, ALB expression was about 19-fold higher in the hLTR when compared with the PHHs.

3.4 Hepatic maturation and functionality

The liver is responsible for producing serum albumin. Therefore, we evaluated the ability of HLCs to secrete albumin into the conditioned medium. All HLCs were able to secrete albumin (Figure 2d). Albumin secretion by UTA.11304 differentiated by M3 was about fourfold
FIGURE 2  Continued.
higher compared with the same cell line differentiated by M1 or M2, and twofold higher compared with M4 (data not shown), but no other statistically significant differences were observed between the differentiation protocols. However, PHHs secreted significantly larger amounts of albumin than the HLCs or even the HepG2 cells.

The level of secreted urea by the cell lines differentiated by M3 and M4 was considerably higher compared with same lines differentiated by M1, M2, and M5 and relatively closer to the amount of urea secreted by the PHHs (Figure 2d). In fact, the level of urea secreted by the HLCs differentiated by M1, M2, and M5 was comparable to the HepG2 cells, which secreted 6.5-fold less urea than the PHHs.

TAG secretion in the cell lines differentiated by M3, M4, and M5 was at similar levels as in the PHHs (Figure 2d). However, cell lines differentiated by M1 and M2 secreted, on average, 5.9- and 3.8-fold more TAG than the PHHs. We were not able to detect secreted TAG in the HepG2 culture medium due to medium interference.

Apolipoprotein A-I (APOA1) encodes for apoA, which is the main protein component of high-density lipoproteins (HDL). The product of apolipoprotein B (APOB) is the main protein component of very-low-density lipoprotein (VLDL) and LDL. The expression of both APOA1 and APOB in the HLCs was comparable to the HepG2 cells, PHHs, and hLTR, which further indicated the functionality of the HLCs (Figure 2c). Interestingly, both APOA1 and APOB were expressed almost 5.5-fold more in hLTR compared with the PHHs.

All the cell lines were able to uptake the labeled LDL from the culture medium (Supporting Information Figure S5). This was confirmed by staining the LDL-R by monoclonal antibody (Supporting Information Figure S4).

### 3.5 Lipid profiles of HLCs differentiated by different methods

The HLCs differentiated by M3, M4, and M5 showed superior functionality as for TAG and urea secretion when compared with M1 and M2. In addition, the M5-HLCs expressed higher ALB compared with the HLCs differentiated by M1 and M3. Therefore, we selected the HLCs differentiated by M3, M4, and M5, analyzed their lipid profile by MS, and compared their lipid profile. In addition, the lipid contents of their unconditioned media were studied to investigate the influence of culture media lipids and their FAs on the cells.

Overall, 15 major lipid classes—including CE, DAG, PC, LPC, PI, LPI, PE, LPE, SM, Cer, LacCer, Glc/GalCer, and Gb3—were investigated, and altogether more than 150 molecular species were detected and studied (Supporting Information Tables S2 and S3).

The lipid profile of the HLCs differentiated by M3, M4, and M5 closely resembled each other (Figures ). In fact, only the levels of three PC species (PC 16:1–20:4, PC 17:0–18:1, and PC 17:0–20:4) were slightly, but statistically significantly, lower in M4 (Figure 3, marked by blue arrows) compared with the other methods. No other significant differences were found in any of the molecular species between the methods. When comparing the total levels of lipid classes, only Cer was found at significantly lower concentrations in the cells produced by M5 compared with those produced by M4 (Figure 5b). However, at the molecular species level, none of the Cer species differed significantly between M4 and M5 (Figure 5a and Supporting Information Table S2).

### 3.6 Lipid profiles of HLCs, PHHs, and HepG2 cells

Next, we compare the lipidomes of the HLCs with the PHHs and HepG2 cells and describe our findings for each lipid class separately. In addition, we highlight the differences and similarities between the PHHs and HepG2 cells.

#### 3.6.1 Phospholipids

In the HLCs, the molecular species of PC, PE, and PI containing saturated FAs (SFAs) and monounsaturated FAs (MUFAs) were mostly similar to those in the PHHs, with the exceptions of PC 18:1/18:1 (p < 0.05), PC 17:0–18:1 (p < 0.001), and PE 18:0–18:1 (p < 0.05), which were statistically significantly higher in the HLCs (Figure 3, marked by red arrows). When the PHHs were compared with the HepG2 cells, a large number of species showed statistically significant differences, particularly the species containing 14:0, 16:0, and 18:1 FAs. In PC and PE, the molecular species containing polyunsaturated FAs (PUFAs) were present in significantly higher concentrations in the PHHs compared with both the HLCs and HepG2 cells. This difference was especially pronounced for the species containing an SFA coupled with FA 18:2 or its derivative 20:4 (e.g., 16:0–18:2, 18:0–18:2, 16:0–18:2, and 18:0–20:4). The HepG2 cells, however, contained more of the species where an MUFA was

**FIGURE 2** Characterization and functionality of hiPSC-derived hepatocyte-like cells (hiPSC-HLCs) differentiated from three cell lines by five methods and their comparison to primary human hepatocytes (PHHs), HepG2 cells, and human liver total RNA (hLTR). (a) Immunostaining of cells for AFP (red) and ALB (green). Nuclei are stained with DAPI (blue). The red inset on the lower right shows the comparison of the morphology and binuclearity (red arrows) of M5-HLCs and PHHs. The scale bar represents 200 μm for the HLCs and 100 μm for the PHHs and HepG2 cells. (b) The graph on the left shows the average percentage of binuclear HLCs in each method and their comparison to PHHs. The graph on the right shows the average percentage of ALB-positive HLCs in each method. Each bar represents the mean ± SD of the manual counts from the immunostaining image analysis of at least five areas. (c) Real-time qPCR analysis of the SOX17, FOXA2, AFP, ALB, APOA1, and APOB genes at the hiPSC and hepatic stage and their comparison to the reference samples. Each sample was run in triplicate and the bars represent the mean ± SD of three biological replicates from three individual cell lines. The gene expression data were normalized to the housekeeping gene GAPDH and are presented relative to the PHHs. (d) Biochemical analysis of the conditioned media from the HLCs for albumin, urea, and triacylglycerol. Values are normalized as 1 million cells per 24 hr. The bars represent the mean ± SD of three biological replicates of the three cell lines. *p < 0.05, **p < 0.01, ***p < 0.001. DAPI: 6-diamidino-2-phenylindole; n.s.: not significant; qPCR: quantitative PCR; SD: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]
coupled to a PUFA (or another MUFA). The total levels of PC and PI classes were similar among all three compared cell types. Total PE was, however, detected at lower levels in the HLCs compared with the PHHs (statistically significant in M4 and M5 vs the PHHs). This was mostly due to the higher levels of PE 18:0–18:2 and PE 18:0–20:4 in the PHHs. The concentration of PC, PE, and PI in the HLC and PHH media was either zero or negligible, while the HepG2 medium contained high amounts of PC (12.9 µM) but only minimal amounts of PE and PI (Supporting Information Figure S7 and Table S3). The profile and concentration of molecular lysophospholipids (LPLs) such as LPC, LPE, and LPI in the HLCs were very close to those of the PHHs (Figure 3a). Consequently, the total LPL levels of the HLCs and PHHs were also similar (Figure 3b). The HepG2 cells, however, contained considerably higher levels of LPL as total levels and strikingly high levels of the molecular lyso-species, with the 18:1 acyl residue in each LPL class (Figure 3 and Supporting Information Table S2).

3.6.2 Neutral lipids

CE concentration was the highest in the HepG2 cells, intermediate in the HLCs, and the lowest in the PHHs. The CE species profile of the HLCs and HepG2 cells was different because 16 CE species showed statistically significantly higher concentrations in the HepG2 cells
When interpreting these differences, the effect of medium
CE concentration was considered. The HepG2 medium contained
154 µM of CE, whereas the CE concentrations were negligible in the
HLC and PHH media (Supporting Information Figure S7 and Table S3).

Similar to PLs, the HLCs contained higher levels of DAG species
with FA 18:1 when compared with the PHHs. However, the level of
major DAG species 16:0–18:1 detected in the HLCs was clearly
closer to that detected in the PHHs compared with what was found
in the HepG2 cells. The PHHs, on the other hand, contained higher
levels of DAG species with FA 18:2 (the minor species DAG 18:
1–18:2 being an exception with its equally low levels in the HLCs,
PHHs, and HepG2 cells). As also observed in PC, the DAG species
with relatively short chain FAs (e.g., 14:0, 16:0, and 16:1) were
detected at higher concentrations in the HepG2 cells than in the
HLCs and PHHs (Figure 4A).

3.6.3 | Sphingolipids

Despite higher total SM concentration, HLCs mimicked the SM
profile of PHHs and HepG2 cells, except that SM d18:1/15:0 and SM
d18:1/16:1 were present at statistically significantly higher concen-
trations in the HepG2 cells (Figure 5a). Both the HLCs and HepG2
cells contained significantly higher levels of SM d18:1/16:0 compared
with the PHHs. The level of SM in the HLC and PHH media was
undetectable, whereas the HepG2 medium contained 6.5 µM of SM
(Supporting Information Figure S7 and Table S3).

In terms of the overall SL profile, the HepG2 cells situated
between the PHHs and HLCs. In fact, lipid class data showed that the
HLCs contained lower levels of Cer but higher levels of LacCer, Glc/
GalCer, and Gb3 (members of glycosphingolipid [GSL] family)
compared with the PHHs (Figure 5b). Closer examination showed
that Cers and, particularly, the saturated species with long- and very-
long-chain FAs (e.g., Cer d18:0/22:0 and Cer d18:1/24:0) were higher
in the PHHs (Figure 5a), in fact, the Cer species profile of the HLCs
resembled more that of the HepG2 cells. On the other hand, the
HLCs contained more GSLs—especially the species d18:1/16:0,
d18:1/24:0, and d18:1/24:1—and the differences were the most
pronounced in the Glc/GalCer class. The HepG2 medium contained
trace amounts of SLs, and the concentration of SLs in the HLC and
PHH media was either negligible or undetectable (Supporting
Information Figure S7 and Table S3).

3.7 | FA analysis

The HLCs contained 15 mol% PUFAs versus 29 mol% and 10.5 mol%
in the PHHs and HepG2 cells, respectively (Figure 6a). The FA
18:2n-6 was, in proportion, the highest PUFA detected in the PHHs (14 mol%); it was also the highest PUFA detected in the PHH medium (89 mol%; Figure 6b and Supporting Information Table S3). Both the HLCs and HepG2 cells had a low supply of 18:2n-6 in their media and, hence, lower relative levels of 18:2n-6 in their cellular FA profile. Interestingly, the HLCs seemed to be able to compensate for the shortage of supply better than the HepG2 cells, and the levels of 20:4n-6, a bioactive metabolite of 18:2n-6, were only 1.4-fold lower in the HLCs compared with the PHHs, indicating an active biosynthesis of 20:4n-6 from its precursor in the HLCs.

The total levels of MUFAs in the HLCs (43 mol%) were higher than in the PHHs (25 mol%), but they were still lower than in the HepG2 cells (52 mol%) (Figure 6a). FA 18:1n-9 (with its 31 mol% of all FAs) was the major MUFA in the HLCs, a reflection of the high concentration of 18:1n-9 in their medium. FA 16:1n-7 (an SCD Δ9-desaturation product of 16:0) had the lowest values in the HLCs (0.6-fold the PHH level) and the highest values in the HepG2 cells (1.4-fold the PHH level). Consequently, the relative concentration of 18:1n-7 (an fatty acid elongase (ELOVL)5/6 chain elongation product of 16:1n-7) was the highest in the HepG2 cells (14 mol%) and lower in HLCs and PHHs (4 mol% and 2 mol%, respectively). This implies that the high 18:1n-7 is a specific feature of HepG2 cells, marking a clear distinction from the HLCs and PHHs, which appeared similar in having 18:1n-9 as their main MUFA component.

The total SFAs in the HLCs, PHHs, and HepG2 cells were 40 mol%, 46 mol%, and 37 mol%, respectively (Figure 6a). Relative to the PHHs, the lower SFAs in the HLCs were attributable to the slightly
FIGURE 6  Fatty acid (FA) analysis (mole% in total FAs) of the M3-HLCs, PHHs, and HepG2 cells versus their media. (a) Total proportions of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and dimethyl acetals (DMAs, derived from plasmalogen PL alkenyl chains) in the studied cell types and their media. The ratio of total unsaturated FAs (UNFAs) to total SFAs is shown on the top bar. (b) Mirrored bar chart presenting the FA profiles in the M3-HLCs, PHHs, and HepG2 cells (red bars) in comparison to their media (blue bars). Each bar represents the mol% of the detected FAs. Error bars in the HLCs are calculated from the mean ± standard deviation of three biological replicates. (c) Schematic image of the metabolic pathways of SFAs, MUFAs, and PUFAs, including the responsible genes (FASN, SCD, ELOVL, FADS). The figure is adapted from the work by Glück et al., (2016). HLC: hepatocyte-like cell; PHH: primary human hepatocyte; PL: phospholipid [Color figure can be viewed at wileyonlinelibrary.com]
lower proportion of all major individual FAs (14:0, 16:0, and 18:0). The lower amount of total SFAs in the HepG2 cells, however, was mainly due to the considerably lower percentage of 18:0 compared with the PHHs (2.2-fold less).

3.8 | Gene expression of key enzymes in FA metabolism

To further investigate the metabolism of FAs in the HLCs, PHHs, and HepG2 cells, we studied the expression of five key genes involved in the synthesis pathways of FA by qPCR. Lipogenesis is highly affected by glucose homeostasis in the liver. Therefore, the expression of Glucokinase (encoded by GCK), the key enzyme involved in glycolysis, was also studied. The results revealed that FA synthase (FASN), a key gene in de novo FA synthesis, was expressed in the HLCs mostly at similar levels as in the PHHs (Figure 7a). The FASN expression level of the HepG2 cells was higher than that of the PHHs. The expression of GCK in HLCs was at similar levels as in the PHHs (Figure 7b). Comparing the methods, M3-HLCs and M4-HLCs expressed GCK at statistically significantly higher levels than M1-HLCs. FA desaturase 1 (FADS1) was expressed at a statistically significantly lower level in all the HLCs ($p < 0.001$) when compared with the PHHs, but at similar levels as in hLTR and the HepG2 cells. In the HepG2 cells, hLTR, and almost half of the HLCs, FADS2 was expressed at lower levels than in the PHHs. In the other half of the studied HLCs, the expression of FADS2 was similar to that in the PHHs (Figure 7a). In addition to the FADSs, FA elongases ELOVL2 and ELOVL5, working in sequence with the desaturases, are essential for the metabolism of long-chain and highly unsaturated FAs (Figure 6c). ELOVL2 was expressed in most of the HLC lines at similar levels to the PHHs, but some lines produced by M2, M3, and M5 showed statistically significantly higher expression levels (Figure 7a). The expression of ELOVL2 was statistically significantly higher in the HepG2 cells than in the PHHs. ELOVL5, on the other hand, was expressed at roughly similar levels in all analyzed cell types (PHHs, HepG2 cells, hLTR, and HLCs).

**FIGURE 7** Expression levels of genes involved in the metabolism of fatty acids (FAs) (a) and glucose (b). (a) Real-time qPCR analysis of ELOVL2, ELOVL5, FADS1, FADS2, and FASN in the HLCs (differentiated by five methods), PHHs, HepG2 cells, and human liver total RNA (hLTR). (b) Real-time qPCR analysis of GCK in HLCs (differentiated by five methods), PHHs, HepG2 cells, hiPSCs, and human liver total RNA (hLTR). The expression of each gene was normalized to the endogenous control gene GAPDH and is presented relative to the PHHs. Each sample was run in triplicate, and the bars in the HLCs represent the mean ± standard deviation of three biological replicates. PHHs were used as reference group to calculate the statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. HLC: hepatocyte-like cell; PHH: primary human hepatocyte; qPCR: quantitative PCR.
4 | DISCUSSION

hiPSC-HLCs are a promising cell culture platform for studying lipid homeostasis and its aberrations related to metabolic syndromes manifested, for example, in atherosclerosis and fatty liver disease. In the current study, we successfully generated functional HLCs from hiPSCs using five hepatic differentiation protocols. We then compared these HLCs comprehensively for gene and protein expression, lipid composition, and functional traits. Similar comparisons were also made with the two common hepatic cell models, PHHs and HepG2 cells. All five differentiation protocols produced HLCs capable of expressing ALB, uptaking LDL, and secreting albumin, urea, and TAG. The lipid profiles of the HLCs, PHHs, and HepG2 cells were analyzed by both MS and gas chromatography. In addition, the effects of FA supply from the culture media on the cells’ lipid profile were examined. In this study, we performed, to our knowledge, the most thorough characterization of the metabolic traits of HLCs and investigated their potential as a new cell model for lipid studies in parallel with PHHs and HepG2 cells.

4.1 | Hepatic differentiation

Most protocols for differentiating HLCs from pluripotent stem cells follow a three-step differentiation through the DE phase, continuing to the hepatoblast stage, and finally to mature HLCs. Various growth factors and cytokines known to be necessary for liver development are used through these procedures (Cameron et al., 2015; Chen et al., 2012; Gerbal-Chaloin et al., 2014; Hannan et al., 2013; Hu & Li, 2015; Mallanna & Duncan, 2013; Schwartz, Fleming, Khetani, & Bhatia, 2014; Si-Tayeb et al., 2010; Sullivan et al., 2010; Takayama et al., 2012). We observed that at the DE stage, the initial density of the hiPSCs played a more critical role than the type of DE inducer (e.g., CHIR 99021 or Wnt3). In fact, high initial cell density in M1, M2, and M5 leads to remaining undifferentiated colonies, whereas in M3 and M4, this results in the formation of new dense and undifferentiated areas. On the other hand, very low initial cell density could lead to highly sparse cells and consequently unsuccessful differentiation. Hence, it is fundamental to find the optimal cell density for each cell line in each method.

At the hepatoblast stage, cells in M1 were supplemented with FGF2 and BMP4, mimicking the stimuli present during liver development (Rasmussen, 2015). However, in other methods, DMSO was used instead of FGF2 and BMP4. DMSO has histone deacetylase inhibitor activity and significantly increases the expression levels of BMP2 and BMP4 while decreasing the expression levels of stem cell markers, such as Oct4 (Behbahan et al., 2011; Choi et al., 2015; Cysz, Minger, & Thomas, 2015; Santos, Figueira-Coelho, Martins-Silva, & Saldanha, 2003). At Day 10, the cells in M1 had clear borders; they had also formed sinusoidal canaliculi-like structures (Supporting Information Figure S1b). However, at the end of the hepatoblast stage and the beginning of the maturation stage, the cells in M2, M3, M4, and M5 also eventually gained sinusoidal canaliculi-like structures (Supporting Information Figure S3). In addition, small lipid droplets were clearly visible in the cells in the culture medium containing DMSO, which could be due to excess of FA supplies in that medium (Kiamehr et al., 2017).

For the maturation of hepatocytes, HGF and oncostatin M were used. Epidermal growth factor (EGF) was present only in M3, M4, and M5. It has been shown that HGF and EGF—two tyrosine kinase receptor ligands—together decrease the expression of several genes involved in the metabolism of FAs (Michalopoulos, Bowen, Mulè, & Luo, 2003). In our study, EGF seemed to affect urea and TAG secretion but not the albumin secretion of the HLCs. In fact, M3-, M4-, and M5-HLCs secreted both urea and TAG similarly to the PHHs, but clearly different from the levels secreted by the M1- and M2-HLCs (Figure 2D).

It has been proposed that the unnatural microenvironment provided by current culture systems is partly responsible for the immature features of HLCs (Godoy et al., 2015), and that coating the culture plates with a mixture of Laminin 521/111 could further promote HLC maturation (Cameron et al., 2015). Hence, in M4 we replaced the Geltrex coating with Laminin 521/111. Even though we did not observe this coating to improve functionality, it increased the number of ALB-positive cells by 82% and the binucularity of HLCs by 65% compared with those in M3. In the adult liver, 15%–30% of hepatocytes are binuclear, and binucleation is usually considered a sign of terminal differentiation in hepatocytes, even though binuclear hepatocytes are still able to divide (Miyaoka & Miyajima, 2013). One study suggested that polyploid cells are more resistant to stressful conditions (Anatskaya & Vinogradov, 2007). However, the effect of size and number of nuclei on the hepatocyte function is an intriguing question yet to be answered.

It is well established that the levels of lipoproteins in the serum have a clear correlation with the risk of atherosclerosis (Moss, 1991). It has been shown that HLCs produce, secrete, and uptake cholesterol in vitro and that they robustly express apoprotein genes, for example, APOA1, APOA2, APOB, APOC, and APOE (Rasmussen, 2015). HLCs also respond to statin treatment in vitro by reducing the amount of secreted cholesterol (Krueger et al., 2013). Here, we confirmed that HLCs are capable of uptaking labeled LDL (Supporting Information Figure S5) as well as expressing APOA1 and APOB at similar levels compared with PHHs (Figure 2c), which is in line with findings in other studies (Godoy et al., 2015). APOA1 and APOB gene expression levels were studied as a surrogate for estimating the HDL and LDL production levels of HLCs.

Overall, the HLCs differentiated by M3, M4, and M5 showed superior characteristics for studying the liver function and lipid metabolism compared with the HLCs differentiated by M1 and M2. The M3-, M4-, and M5-HLCs secreted TAG at similar levels as the PHHs. In addition, the M3- and M4-HLCs secreted urea at levels closer to the PHHs when compared with the HLCs differentiated by other methods. M4 and M5 produced the most albumin-positive HLCs, and the M4-HLCs showed the highest binucularity compared with the HLCs differentiated by the other methods. Furthermore, we observed that the cell lines differentiated by M5 showed the least variation. This could be attributed to the application of the STEMdiff™ DE kit at the DE stage, which resulted in uniform DE differentiation. Because the DE stage is the key phase in hepatic differentiation, evenly differentiated
DE cells could result in more uniform hepatic differentiation, reducing possible variation between cell lines. Nevertheless, it should be considered that the Laminin coating applied in M4 and the STEMdiff™ DE kit used in M5 adds to the overall costs of the differentiation and makes these methods more expensive than, for example, M3.

4.2 Lipid profiling

PUFAs and their bioactive derivatives play important roles during cell proliferation and differentiation (Bieberich, 2012; Kim, Kim, Kim, Kim, & Han, 2009). It has previously been shown that the addition of certain FAs, such as docosahexaenoic acid (22:6 n-3) or eicosapentaenoic acid (20:5 n-3), to the media could promote neuronal differentiation in neuronal stem/progenitor cells through the regulation of the cell cycle (Katakura et al., 2013). Furthermore, a definite mixture of FAs induces adipocyte-like differentiation of osteosarcoma cells through the activation of peroxisome proliferator-activated receptors (Diascro et al., 1998). It is known that the endogenous capacity of cultured cells to modify PUFAs for their needs can partially compensate for suboptimal supply from the microenvironment and culture medium (Zhang, Kothapalli, & Brenna, 2016). A simplified schematic figure of the metabolic pathways of SFAs, MUFAs, and PUFAs and the genes involved is presented in Figure 6c. PHHs possess an excellent capacity to metabolize diet-derived PUFAs for their needs that found in PHHs. The elevated cellular contents of 16:1n-7 prevented them from producing any significant amounts of n-9 PUFAs. PUFAs have been shown to be important in the maturation and functionality of HLCs (Kiamehr et al., 2017).

In deficient states, endogenous synthesis of the 20:3n-9 from 18:1n-9 occurs (Kamada et al., 1999). It is of note that the HLCs and HepG2 cells both contained 2–3 mol% of n-9 PUFAs (20:2n-9 + 20:3n-9), which means that the cells suffered from essential PUFA deficiency. The large supply of 18:2n-6 in the PHH medium, however, prevented them from producing any significant amounts of n-9 PUFAs. PUFAs have been shown to be important in the maturation and functionality of HLCs (Kiamehr et al., 2017).

Therefore, we propose that providing HLCs with a PUFA-rich medium may help to produce HLCs with a lipid profile even closer to PHHs. A high supply of PUFA to PHHs inhibits their endogenous MUFAs synthesis from SFAs (Ntambi, 1999). Likewise, in our study, the total MUFA in the PHHs remained low compared to the HLCs and HepG2 cells. The lower the PUFA totals, the higher the MUFA totals were at the expense of the SFAs.

Hepatocytes can uptake FAs from the cell culture medium. Simultaneously, hepatocytes are able to produce FAs by hydrolyzing glucose to pyruvate, which links glycolysis to lipogenesis where pyruvate is used to synthesize FAs through de novo lipogenesis (Rui, 2014). Glucokinase (encoded by GCK gene) is central to the glucose homeostasis and is the key enzyme involved in the first step of glycolysis. Changes in hepatocyte GCK expression represent an adaptive action to impaired glucose/lipid metabolism to maintain glucose homeostasis (Massa, Gagliardino, & Francini, 2011). In our study, there were no significant differences in the expression of GCK between the HLCs and the PHHs. However, the HepG2 cells expressed GCK at statistically significantly lower levels than the PHHs, which is in line with previous findings at protein level (Wiśniewski, Vildhede, Norén, & Artursson, 2016). FASN is a major determinant of the capacity of a tissue to synthesize FAs de novo, and palmitate (16:0) is its primary product (Figure 6c; Jensen-Urstad & Semenkovich, 2012). Again, most HLC lines expressed FASN equally to the PHHs, whereas the HepG2 cells had clearly higher expression levels.

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4.3 FAs mirrored in molecular species

We observed that the PL species profile of cells was highly affected by the culture medium. The PHHs’ lipid profile reflected the high
medium supply of 18:2n-6, which after uptake was metabolized to
20:4n-6 and incorporated into the PLs. Consequently, the PLs
composed of 16:0–18:2, 18:0–18:2, 16:0–20:4, and 18:0–20:4
were abundant in the PHHs. The much lower supply of 18:2n-6 to the
HLCs and HepG2 cells kept the levels of the above-mentioned PL
species low. However, the HLCs could still actively synthesize 20:4
and couple this PUFA with SFAs and MUFAs. To compensate for the
overall PUFA deficiency, the HepG2 cells produced more MUFAs
(observed in high levels of 18:1n-7), which is reflected in species such
as 16:0–18:1 and 18:1/18:1. The HLCs instead compensated for the
PUFA shortage by uptaking 18:1n-9, which was abundant in their
medium.

In general, the HLCs and HepG2 cells contained lower concen-
trations of Cer and higher concentrations of LacCer, Glc/GalCer, and
Gb3 when compared with the PHHs. Cer synthesis is a complex
process orchestrated by six CerSs (CerS1–CerS6), each of which
synthesizes Cers with distinct FA chain lengths (Cingolani, Futerman,
& Casas, 2016). Cer has important structural and signaling roles
(Gault, Obeid, & Hannun, 2010). It has been shown that at time of
cellular stress, the generation of C16:0-ceramide via CerS5 is
proapoptotic, whereas synthesis of C24:0-ceramides via CerS2 is
prosurvival (Mesicek et al., 2010), and the balance between these
long- and very-long-chain Cers is critical for cellular homeostasis. In
our study, similar levels of d18:1/16:0 were observed in all three cell
types, but the PHHs had significantly higher levels of d18:1/24:0
(Figure 5a). Transferring the PHHs from a physiological environment
to an artificial 2D system could be stressful for them. Therefore, it is
intriguing to speculate whether the survival or antiapoptosis
mechanism is turned on in PHHs. On the other hand, HepG2 cells
might suppress the apoptotic Cer signal by converting Cer to
complex GSLs, leading to lowered levels of Cer in HepG2 cells.
Overall, the interpretation of the observed Cer profile of the studied
cell types is very complicated and demands further studies.

The UDP-glucose ceramide glucosyltransferase (UGCG) gene
encodes the enzyme UDP-glucose Cer glucosyltransferase, which
catalyzes the first glycosylation step in the biosynthesis of GSLs. The
product of this reaction is glucosylceramide, which is the core
structure of many GSLs, including Glc/GalCer (Tokuda et al., 2013).
We have previously reported a higher expression of UGCG in HLCs
compared with PHHs (Kiamehr et al., 2017), which correlates well
with our lipidomics findings of high Glc/GalCer in HLCs. In the C24
species of every sphingolipid class, the ratio of the 24:0 species to the
24:1 species was higher in the PHHs than in the HLCs or HepG2 cells.
This may reflect the dominance of 24:1n-9 over 24:0 in both the HLC
and HepG2 media and the reversed ratio of these trace MUFAs in the
PHH medium.

The PHHs were cultured in a low FA medium. As a result, the
PHHs contained low levels of CE in their cellular lipid profile. Unlike
the PHH cells, the HepG2 cells—which were grown in FA-rich
medium—accumulated a large amount of the neutral storage lipids
CE and TAG, the latter being represented via its hydrolysis product
DAG. As is normal for cultured cells, the highest CE species was 18:1,
followed by 16:0 and 16:1, and there were also traces of various
other species. In line with the CE profile, the HepG2 cells contained
large amounts of DAG, chiefly 16:0–18:1 and 18:1/18:1. The HLCs
also showed an accumulation of neutral lipids (although milder than
in the HepG2 cells), which is likely due to their treatment with an FA-
rich medium at the hepatoblast stage (Day 6 to Day 12; Kiamehr
et al., 2017). Therefore, our work reveals the need to revise the
generally overlooked FA content of culture media and to tailor it
according to the HLCs’ needs. In addition, we propose that increasing
the supply of PUFA in the culture medium may positively affect the
lipid profile and functionality of the HLCs.

To our knowledge, this is the first comparative study that
considers the effects of various stimuli from different protocols on
the differentiation and phenotype of HLCs. We are also the first to
show the detailed lipid profile of HLCs and to evaluate their ability to
metabolize FAs. Taken together, HLCs differentiated from hiPSCs
provide the advantage of direct studies of cellular and molecular
mechanisms that regulate lipid homeostasis in the liver. They also
facilitate a novel platform for the discovery, optimization, and study
of the modes of action of molecules modulating key lipids. This
platform would allow the functional assessment of the impact of
genetic variations in developing metabolic diseases, such as athero-
sclerosis.

5 | CONCLUSIONS

HLCs mimicked the lipid profile of PHHs very well and, thus, showed
to be a better liver cell model than HepG2 cells, especially in terms of
their low LPL content. HLCs were capable of metabolizing FAs to
produce C20–22 highly unsaturated FAs to fulfill the complex
biological functions mediated by those FAs. By improving the PUFA
supply in the medium, we can produce HLCs, which can serve as a
powerful and functional cell model to study patient-specific
mechanisms in lipid aberrations leading to pathological states, such as
atherosclerosis or fatty liver disease.

ACKNOWLEDGEMENTS

This research received funding from the European Commission’s
Seventh Framework Programme [FP7–2007–2013] under two grant
agreements, HEALTH.2012–3057392 “Personalized diagnostics and
treatment for high risk coronary artery disease” (RiskyCAD), and
HEALTH-F2–2013-602222 “Targeting novel lipid pathways for
treatment of cardiovascular disease” (Athero-Flux). Research funding
was also received from Instrumentarium Science Foundation
(Instrumentariumin Tiedesaatio) and the Finnish Cardiovascular
Foundation. The authors would like to thank Markus Haponen,
Henna Lappi, and Merja Lehtinen for their technical support. Jyrki
Siivola, Maki Kotaka, Mauro Scaravilli, Ebrahim Afyounian, and Eric
Dufour are acknowledged for their valuable help and advice in
performing the experiments and analyzing the results. The authors
would like to thank Professor Kenji Osafune for providing the great
opportunity to visit his laboratory in CiRA, Kyoto University, Japan.
The authors gratefully acknowledge the Tampere facility of iPS Cells and Flow Cytometry for their services.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this study.

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How to cite this article: Kiamehr M, Alexanova A, Viiri LE, et al. hiPSC-derived hepatocytes closely mimic the lipid profile of primary hepatocytes: A future personalised cell model for studying the lipid metabolism of the liver. J Cell Physiol. 2019;234:3744–3761. https://doi.org/10.1002/jcp.27131