Donor–dependent and other non-defined factors have greater influence on the hepatic phenotype than the starting cell type in induced pluripotent stem cell derived hepatocyte-like cells

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Running title: iPSC-HLCs: Does the starting cell type matter?

Title: Donor-dependent and other non-defined factors have greater influence on the hepatic phenotype than the starting cell type in induced pluripotent stem cell derived hepatocyte-like cells.

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Abstract

Drug-induced liver injury (DILI) is the greatest cause of post-marketing drug withdrawal; therefore, substantial resources are directed towards triaging potentially dangerous new compounds at all stages of drug development. One of the major factors preventing effective screening of new compounds is the lack of a predictive *in vitro* model of hepatotoxicity. Primary human hepatocytes offer a metabolically-relevant model for which the molecular initiating events of hepatotoxicity can be examined; however, these cells vary greatly between donors and dedifferentiate rapidly in culture. iPSC-derived hepatocyte-like cells offer a reproducible, physiologically-relevant and genotypically-normal model cell; however, current differentiation protocols produce hepatocyte-like cells with a relatively immature phenotype. During the reprogramming of somatic cells, the epigenome undergoes dramatic changes; however, this 'resetting' is a gradual process, resulting in an altered differentiation propensity, skewed towards the lineage of origin, particularly in early passage cultures. We therefore performed a comparison of human hepatocyte- and dermal fibroblast-derived iPSCs, assessing the impact of epigenetic memory at all stages of hepatocyte-like cell differentiation. These results provide the first isogenic assessment of the starting cell type in human iPSC-derived hepatocyte-like cells. Despite a trend towards improvement in hepatic phenotype in albumin secretion and gene expression, few significant differences in hepatic differentiation capacity were found between hepatocyte and fibroblast-derived iPSCs. We conclude that the donor and inter-clonal differences have a greater influence on the hepatocyte phenotypic maturity than the starting cell type. Therefore, it is not necessary to use human hepatocytes for generating iPSC-derived hepatocyte-like cells.

Introduction

Drug induced liver injury (DILI) confers major clinical burden onto healthcare providers[1, 2] and is the most common cause of post-marketing drug withdrawal[3], yet a method to accurately predict a toxic liability or model the mechanisms which cause DILI remains elusive. This is in part due to the lack of a translatable *in vitro* model which reliably triages potentially dangerous compounds during drug development or allows for mechanistic analyses. The gold standard for investigating DILI is the primary human hepatocyte (PHH) due to their metabolic competence; however, PHH dedifferentiate rapidly in culture and vary greatly between donors[4]. Conversely, hepatic cell lines are scalable and readily available but only represent a single (abnormal) genotype and lack metabolic relevance[5]. Therefore, an expandable, metabolically-relevant, reproducible, population-representative and physiologically/genotypically normal source of cells would be an invaluable resource for investigating and predicting DILI.

One such resource which has the potential to address the role of hepatocytes in hepatotoxicity is induced pluripotent stem cells (iPSC)-derived hepatocyte-like cells (HLCs). Consequently, the generation of HLCs has been an area of intense research since the first differentiation protocols were described in 2004 by Lavon *et al*[6]. In the intervening years, incremental improvements have been seen in both efficiency of differentiation and phenotype maturity. Despite these improvements, the hepatic phenotype still remains closer to a foetal or neonatal expression profile rather than an adult phenotype[7-9]. This has led researchers to look elsewhere for improvements in the differentiation of stem cells towards HLCs, including the use of different extracellular substrates[10], 3D culture systems[11], small molecules[12] and products of the microbiome[13].

One further area of interest has been to investigate the cell of origin. Previous work has shown that during the reprogramming process, the hypermethylation of the genes associated with the somatic cell phenotype occurs at a relatively late stage of induced pluripotency[14]. Consequently, induced pluripotent stem cells (iPSCs) maintain a memory of the cell from which they were derived[15]. This phenomenon, termed epigenetic memory, was first described by two separate studies in 2010 [16, 17] demonstrating that the starting cell type can influence the differentiation propensity of the iPSC towards the lineage of origin. This was subsequently found to be true of other cell types, such as pancreatic β cells, which demonstrated increased PDX1 and insulin gene expression in spontaneously differentiated iPSCs-derived from β cells when compared to non-β cell-derived iPSCs and ESCs [18].

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Epigenetic memory has also been investigated using hepatocytes (table 1); however, no study has provided definitive proof of whether the starting cell type significantly impacts upon the hepatic differentiation capacity of iPSCs. Genotype-controlled experiments in mice have shown a transient advantage in HLC gene expression of albumin and CK18 in hepatic-derived iPSCs compared to MEF-derived iPSCs [19]. However, this advantage was greater when iPSCs were derived from hepatoblasts than mature hepatocytes and dissipated with time in culture. Liu *et al.*, published the first human comparison in 2011[20], demonstrating no significant advantage in hepatic phenotype when PHH-derived iPSCs were compared to iPSCs derived from other cell types derived from different donors, as measured by albumin secretion, CYP3A activity assays; however, a similar but distinct global epigenetic profile was reported [20].

More recently, Takayama et al. corroborated the report from Lee et al.,[19] in human cells, demonstrating a small but significant advantage when hepatocytes were compared to other cell types; however, the cells were sourced from different donors and the advantage was again found to dissipate with time in culture[21]. The importance of donor-dependent genetic differences was further highlighted by Kajiwara *et al.,* who showed that donor differences were the largest determinant of hepatocyte-like cell quality, rather than the cell of origin, although this study did not use PHH-derived iPSCs[22].

Therefore, the investigation of epigenetic memory in hepatocyte-like cells can only be truly assessed using a comparison of human genotype-controlled iPSCs derived from hepatocytes and non-hepatocytes isolated from the same donor. Herein, we describe the comparison of isogenic PHH- and dermal fibroblast derived iPSCs and the hepatic differentiation potential of these cells.

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Methods

Primary human hepatocyte and dermal fibroblast isolation and culture

Liver and skin resections were received as surgical waste (Aintree hospital, Liverpool, United Kingdom) with full patient consent and ethical approval from the relevant institutional review boards (National Research Ethics Service REC ref: 11/NW/0327). Primary human hepatocytes were isolated using a modified version of the previously described 2-step collagenase method as previously described[23].

Human dermal fibroblasts (HDFs) were isolated using a previously published protocol[24]. Skin biopsies were cut using scalpel and forceps into approximately 1cm^2 pieces and placed into a 6 well plate (3 pieces/well). Two drops of Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO) supplemented with 10% (v/v) foetal bovine serum (FBS; Life technologies, Carlsbad, CA), 2mM L-glutamine (Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin (Sigma-Aldrich; HDF media) were placed on top of each segment and left to attach overnight. Drops of HDF media were replaced daily to keep the pieces submerged. After 7 days, 1ml of media was added/well and changed every 48 hours. After 2 weeks HDF outgrowths were observable. Once confluent, HDFs were detached with 0.05% trypsin (Sigma Aldrich) and expanded for reprogramming and cryopreservation.

Sendai virus reprogramming of PHH and HDFs

PHH were plated on embryonic stem cell (ESC)-qualified Matrigel (Corning, Corning, NY) at a density of $1x10^5 – 5x10^5$ cells/well in a 6 well plate. Cells were cultured in Williams E medium supplemented with 2.5% (v/v) FBS (Life technologies), 50ng/ml hepatocyte growth factor (HGF; Promokine, Heidelberg, Germany), 50ng/ml epidermal growth factor (EGF, Life technologies), 15mM HEPES buffer (Sigma-Aldrich), 100nM dexamethasone (Sigma-Aldrich), 0.2% (v/v) insulin-transferrin-selenium (Life technologies) and 0.5% (v/v) penicillin/streptomycin (PHH reprogramming media). Following 3 days of culture, Sendai viruses containing Oct4, Sox2, Klf4 and c-Myc transcription factors (Life technologies) were added at multiplicities of infection (MOI) 3-10. After 24 hours, media was replaced with non-virus containing PHH reprogramming media and culture continued for a further 2 days. Subsequently, cells were then cultured in Essential 6 media (Life technologies) containing basic fibroblast growth factor (bFGF; 100ng/ml; Life technologies) and 0.5% (v/v) penicillin/streptomycin for 25-30 days, replacing the media every 24 hours.

HDFs were plated in a 6 well plate at $5x10^6$ cells/well or $3x10^5$ cells/ well, respectively. After 48 hours of culture, cells were transduced with Sendai viruses containing Oct4, Sox2, Klf4 and c-Myc at MOI 3 or 5. After 24 hours, media was replaced and changed every 48 hours for 7 days. On day 7 of

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reprogramming, cells were trypsinised (0.05% trypsin) and re-plated onto 10cm^2 dishes coated with mouse embryonic fibroblasts (MEFs; Globalstem, Rockville, MD) or 6 well plates coated with Matrigel. Cells were plated in HDF media which was replaced with either DMEM/F12 media supplemented with 20% (v/v) KnockOut serum replacement media, 1x non-essential amino acids, 50μM 2-mercaptoethanol (Life technologies) and 0.5% (v/v) penicillin/streptomycin on MEF cultures or Essential 6 media supplemented 100ng/ml basic-FGF (Life technologies) for Matrigel cultures. Media was changed daily and colonies formed from day 21 onwards.

iPSCs were maintained on either MEF-coated plates in DMEM/F12 media supplemented with 20% (v/v) KnockOut serum replacement media, 1x non-essential amino acids, 50μM 2-mercaptoethanol and 0.5% (v/v) penicillin/streptomycin or on Matrigel in Essential 8 medium. All early passage comparison assays were performed using <p10 (donors 1 and 2) or <p15 (donor 3) iPSCs. Characterisation was performed using iPSCs >p15 and late passage analysis was conducted using iPSCs >p30.

Differentiation to hepatocyte-like cells

Cells were transferred to Matrigel culture 1:1 from MEF culture and cultured in Essential 8 medium. Upon confluence, cells were washed once with Dulbecco's phosphate buffered saline (DPBS) and disassociated with Accutase at 37°C for ~5 minutes. Cells were then centrifuged at 200 x g for 5 minutes and re-suspended in RPMI media supplemented with 1x B27 and 10μM ROCK inhibitor (Merck Millipore, Billerica, MA). Cells were then counted and plated at 1.5x10^5 cells/cm^2 on Matrigel coated 24 well plates in RPMI media (Life technologies) supplemented with 1x B27 (Life technologies), 0.5% (v/v) penicillin/streptomycin, 10μM ROCK inhibitor (Merck Millipore), 100ng/ml Activin A and 50ng/ml Wnt3a (R&D Systems, Minneapolis, MN). Following overnight plating, cell media was replaced daily with RPMI media containing 1x B27, 0.5% (v/v) penicillin/streptomycin, 100ng/ml Activin A and 50ng/ml Wnt3a. After 3 days, Wnt3a was omitted from the media for a further 2 days. At day 5, media was replaced with KnockOut DMEM media containing 20% (v/v) KnockOut serum, 1mM L-glutamine, 0.5% (v/v) penicillin/streptomycin, 1x non-essential amino acids, 100μM 2-mercaptoethanol and 1% (v/v) DMSO. Media was changed every 48 hours for 7 days. At day 12, media was replaced with HepatoZyme culture media (Life Technologies) supplemented with 2mM L-glutamine, 0.5% (v/v) penicillin/streptomycin, 20ng/ml HGF, 20ng/ml OSM (Promokine) and 100nM dexamethasone. At day 22, cells were lysed for HLC comparisons. Samples were also taken at definitive endoderm (day 5) and hepatic endoderm (day 12) stages.

Spontaneous differentiation assays

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Cells were disassociated using gentle cell disassociation reagent (Stem Cell Technologies; Vancouver, British Columbia, Canada) and scraped in to DMEM/F12 media supplemented with 20% (v/v) KnockOut serum, 1x non-essential amino acids, 100µM 2-mercaptoethanol, 0.5% (v/v) penicillin/streptomycin and 10µM ROCK inhibitor. MEFs were removed by gravitational separation and cells plated in 12-well non-tissue culture treated plates (Corning) in triplicate (1:1 ratio). Media was changed every 48 hours without ROCK inhibitor. For gene expression comparisons, cells were cultured for 16 days before lysing in QIAzol (QIAgen). For characterisation experiments, cells were cultured for 7 days, before transfer to attachment factor-coated 48 well tissue-culture treated plates for re-attachment. Cells were cultured for a further 7 days, before fixing with 4% (v/v) PFA for immunofluorescence assessment.

Pyrosequencing

DNA was extracted using the QIAamp DNA mini kit according to the manufacturer's instructions (QIAgen). 250ng DNA/sample was then bisulphite converted using the EZ DNA Methylation-Gold kit (Zymo, Irvine, CA) according to the manufacturer's protocol. Genes bearing CpG islands within their promoter region were ascertained using the NCBI gene information and the online tool CpG island searcher (http://cpgislands.usc.edu/). PCR and pyrosequencing primers (sequencing, biotinylated and non-biotinylated, table S2) were designed using the Pyromark Assay Design 2.0 software (QIAgen, Manchester, UK) and purchased from Eurofins (Eurofins, Luxembourg). PCR products were generated from the bisulphite-converted samples for all primer sets using optimised conditions. Single-strand pyrosequencing templates were generated from PCR product following binding to streptavidin beads and subsequent washes with 70% ethanol, 0.2M NaOH and 10mM Tris-Acetate pH 7.5. Pyrosequencing was undertaken on a PyroMark Q96 ID instrument (QIAgen).

Immunofluorescence

Cells were fixed with 4% (w/v) paraformaldehyde (Sigma Aldrich) for 15 minutes and subsequently washed three times with DPBS buffered with $MgCl_2$ and $CaCl_2$ (DPBS+; Life technologies). Fixed cells were blocked with DPBS+ supplemented with 10% (v/v) donkey serum (Sigma Aldrich) and 0.01% (v/v) Triton X-100 (Sigma Aldrich) for 30 minutes. Primary antibodies were diluted (table 2.2) in DPBS+ containing 1% (v/v) donkey serum 0.01% (v/v) Triton X-100 and incubated overnight at 4°C. Cells were washed three times with DPBS+ and alexafluor secondary antibody diluted 1:750 in DPBS+ supplemented with 1% (v/v) donkey serum with 0.01% (v/v) triton X-100 added and incubated at room temperature for 2 hours. Cells were washed three times and Hoechst stain (1mg/ml; Sigma Aldrich) added at a 1:7500 dilution in DPBS+ for 10 minutes. Cells washed three

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times and imaged using the Axio Observer Z1 fluorescence microscope with the AxioCam MR digital camera. Images processed using Zen lite software (Carl Zeiss).

For albumin staining, 10x blocking buffer (Abcam, Cambridge, United Kingdom) was used to replace donkey serum. Staining with Tra-1-60 and SSEA-4 conjugated antibodies was performed without triton X-100 and with a single 90 minute incubation at room temperature following blocking with 10% (v/v) donkey serum in DPBS+.

qRT-PCR analysis of gene expression

Primers for use in quantitative reverse transcription polymerase chain reaction (qRT-PCR) gene expression studies were designed using the NCBI Primer BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer quality was assessed using OligoCalc and purchased from Eurofins (Table S3). Samples were collected in QIAzol (QIAgen), and extracted using the miRNeasy extraction kit according to the manufacturer's protocol (QIAgen) Extracted RNA content was measured using the NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA was reverse transcribed to complementary DNA (cDNA) using the ImProm-II reverse transcription kit (Promega).

Due to low RNA yield following extraction, whole transcriptome amplification was performed on selected samples using the QuantiTect Whole Transcriptome Kit (Qiagen), following the manufacturer's protocol. Briefly, 100ng of starting RNA from each sample was mixed with reverse transcriptase kit component and incubated at 37°C for 30 minutes and 95°C for 5 minutes. Ligation mix was then added to each samples and incubated for 2 hours at 22°C. Finally, the amplification mix was added and incubated for 8 hours at 30°C and 5 minutes at 95°C. Temperature controlled steps were carried out using the GeneAmp PCR system 9700 thermal cycler system (Applied Biosystem, Waltham, MA) and amplification confirmed using the NanoDrop spectrophotometer.

Gene expression analysis was performed using the SYBRGreen JumpStart Taq ReadyMix (Sigma Aldrich). Briefly, 10-100μg of cDNA was amplified by qRT-PCR as follows: 95°C for 10 minutes, then 40 cycles for 95°C 15 seconds and 60°C for 60 seconds using the ViiA7 qRT-PCR machine (Applied Biosystems). Results were calculated using the C_T values generated, normalised against GAPDH or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase (SDHA)[25] and calculated relative to a calibrator sample (e.g. PHH) using $2^{-\Delta\Delta CT}$ method [26]. Sendai virus expression analysis of iPSCs was conducted using the TaqMan iPSC Sendai Detection Kit (Life Technologies) according the manufacturer's instructions. Results were normalised using GAPDH and calculated relative to the reprogramming plate using $2^{-\Delta\Delta CT}$ method.

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Metabolism studies

Cells were incubated for 15 minutes at 37°C (5% CO_2) in standard culture media with a final substrate cocktail concentration of 1mM testosterone (CYP3A4) and 0.25mM dextromethorphan (CYP2D6) (Sigma Aldrich) in MeOH or H_2O, respectively (Thermo Fisher Scientific). 0.5μM Phenacetin (Sigma Aldrich) in 100% MeOH was then added to the incubation media (1:1 v/v) as a stop solution and an internal standard for LC-MS-MS analysis. The media containing the respective metabolites, 6β-OH-testosterone and dextrorphan, was then filtered using 96-well filter plates (Merck Millipore) and analysed by LC-MS-MS. Results were normalised by protein content of the well following quantification by Bradford assay.

Albumin ELISA

The albumin concentration was measured by ELISA following the manufacturer's protocol (Bethyl Laboratories, Montgomery, TX) protocol. Briefly, 96-well high-affinity binding plates (Thermo Fisher Scientific) were coated with coating antibody in coating buffer for 1 hour and subsequently blocked with 1% (w/v) bovine serum albumin (BSA; Sigma Aldrich) for 30 minutes. Cell supernatants were added to wells at appropriate dilutions and incubated at room temperature for 1 hour. Horseradish peroxidase-conjugated detection antibody was then added to each well at a 1:50,000 concentration. Following a 1 hour incubation, 3,3',5,5'-Tetramethylbenzidine substrate solution was added, incubated for 20 minutes and the reaction stopped with 0.18M sulphuric acid (Thermo Fisher Scientific). Plates were read at 450nm using a MRX[e] Revelation plate reader (Dynex Technologies, Chantilly, VA).

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Results

Generation of iPSCs derived from PHH and HDFs of multiple donors.

To assess the differences between hepatocyte-like cells differentiated from PHH- and HDF-derived iPSCs, a reprogramming technique capable of inducing pluripotency in both cell types was required. Reprogramming of PHH has been reported as relatively inefficient compared to more traditionally used cell types, such as HDFs[27]. Therefore, a technique capable of reprogramming PHH was first optimised.

PHH are known not to respond well to trypsinisation and re-plating, therefore it was hypothesised that developing a protocol which did not require re-plating would be the most successful route. Furthermore, given that the cell cycle is a vital component of reprogramming[28], the media composition described by Liu et al.,[29] which contains high levels of known mitogenic factors HGF and EGF, was used (Figure S1a).

Using these conditions, PHH were transduced with the OSKM-containing Sendai viruses at MOI 5 and 10 and examined during the reprogramming period for colony formation (Figure S2a). At day 20, the first colonies were noted with similar, but incomplete iPSC-like morphology. These colonies appeared to be heterogeneous and underwent growth arrest during the culture period (Figure S2a). At day 24, one colony was noted which exhibited the hallmark features of an iPSC colony (Figure S2b). After tracking the colony over several days, the colony remained homogenous and continued to expand. This colony was manually picked for expansion and fully characterised.

Using this protocol, iPSCs were successfully generated from hepatocyte cultures from three separate donors (Figure 1 and Table S4). Subsequent to successful PHH-reprograming, the dermal fibroblasts of the corresponding donors were also reprogrammed using a well-established dermal fibroblast protocol (Figure S1b). Pluripotency was assessed using a panel of embryonic stem cell-enriched markers: Oct4, Sox2, Nanog, Tra-1-60 and SSEA-4 (Figure S3). All lines examined demonstrated expression of each of the markers, indicating that they had established a pluripotent expression profile.

The functional differentiation capacity of these lines was also assessed using embryoid body (EB) assays. This assay encourages cellular differentiation and, if truly pluripotent, should result in differentiation towards cell types from all three germ layers: ectoderm, mesoderm and endoderm. The presence of each of these germ layers within the differentiated cell population was investigated by immunofluorescence, using the markers TUJ1 (Neuron-specific class III beta-tubulin; ectoderm), α-SMA (alpha smooth muscle actin; mesoderm) and AFP (Alpha-Fetoprotein; endoderm) (Figure S4).

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All of the iPSC lines generated stained successfully for these markers, indicating that all selected lines were functionally pluripotent.

Donor-dependent differences in PHH- and HDF-derived iPSCs gene expression profile.

To fully compare the iPSCs generated from PHH and HDFs, comparisons were required at all stages of culture and differentiation. Therefore, the expression of important pluripotency-associated genes at the iPSC-stage was compared. Across these markers, Oct4 and Nanog showed similar levels of expression between both cell types of origin. Sox2 expression was significantly greater in the PHH-derived lines for donor 3; however, this was not true of donors 1 and 2 (Figure 2a). Furthermore, analysis of Wnt3, which has been proposed as an iPSC marker of definitive endoderm potential (i.e. high Wnt3 expression correlates with high purity D.E formation)[30], again showed no significant differences between PHH and HDF-derived iPSCs; however, a donor-dependent difference was observed (Figure S5).

HNF4α promoter analysis in PHH- and HDF-derived iPSCs demonstrates a small but consistent cell-type specific difference in methylation which decreases with time in culture.

As the inherent memory of iPSCs is reported to be a consequence of incomplete epigenetic reprogramming, the potential differences seen between PHH- and HDF-derived lines at the iPSC stage were investigated. In this context, we assessed the methylation status of key hepatic genes: HNF4α and FOXA2, since their epigenetic status would likely influence the differentiation status of the HLCs. During the process of reprogramming, the methylation of HNF4α and FOXA2 promoter regions in both PHH and HDFs was significantly increased to similar levels between PHH and HDF-derived clones (Figure 2b). We assessed global methylation utilising an assay for LINE1 (Long interspersed nuclear elements retrotransposable element 1) which is repeated throughout the genome and therefore often used as a surrogate marker of whole genome methylation status[31]. Our results indicated very similar global methylation indexes between PHH- and HDF-derived iPSCs (65.35% and 65.43%, respectively; Figure 2b).

Notwithstanding the global methylation similarity, across the five CpG sites examined within the HNF4α promoter region, each site was consistently ~1% less methylated in the PHH-derived lines when compared to the HDF-derived lines (Figure 2c). Across all lines and CpG sites investigated, 1.22% less methylation was observed in PHH-derived samples; however, this trend was found to decrease with time in culture, with a difference of 0.42% methylation between late passage PHH-derived and HDF-derived iPSCs (Figure 2d). No conserved methylation differences were found

between PHH and HDF-derived iPSCs in the FOXA2 promoter; however, FOXA2 methylation was found to increase (1.22%) between early and late passage iPSCs.

Spontaneous differentiation of PHH- and HDF-derived iPSCs reveals the greatest discriminator between the iPSCs is the donor, rather than the starting cell type.

To investigate if the cell-type of origin influences the inherent differentiation propensity of iPSCs, the hepatic/endoderm-enriched gene expression of embryoid bodies (EBs) was compared. Using a previously published method for comparing iPSCs of different origins[18], EBs were generated and cultured in suspension for 16 days. The expression of known endoderm and hepatic markers was then compared.

The results presented in figure 3 suggested a very slight trend towards the enhancement of hepatic-associated gene expression in PHH-derived EBs in five of the genes tested, i.e. Albumin, AFP, SOX17, FOXA2 and CXCR4. However, GATA4 and HNF4α showed a trend towards higher expression in the HDF-derived clones. Despite these trends, only Sox17 showed significantly greater expression in PHH-derived iPSCs, although this difference was only observed in one of the three donors. Taken together there was very little difference in the spontaneous differentiation propensity of PHH and HDF-derived iPSCs across the investigated hepatic and endodermal-associated genes. Indeed, the greatest determinant of gene expression appeared to be the donor, rather than the starting cell type.

Donor- and clonal-dependent variation are a greater influence on the gene expression of hepatocyte-like cells than the starting cell type.

The differentiation of iPSCs towards hepatocyte-like cells was achieved using a protocol adapted from previous publications[22, 32](Figure S6a) and successful differentiation was confirmed by immunofluorescence at each stage: definitive endoderm (D.E; Sox17), hepatic endoderm (H.E; HNF4α) and HLCs (Albumin) (Figure S6b).

To fully evaluate the differentiation procedure and to assess any inherent advantage as a result of the different starting cell types, we compared the gene expression at each stage of differentiation. Figure S7a demonstrates a comparison of several key markers of definitive endoderm. The results followed a similar pattern to previous analysis, demonstrating no significant differences between starting cell types when the gene expression of FOXA2, SOX17 and GATA4 was compared. Interestingly, both GATA4 and FOXA2, showed a larger dependence on the donor of origin than starting cell type.

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Similar analysis was also undertaken for the hepatic endoderm stage of differentiation, using AFP and HNF4α as stage-specific markers. Despite a trend towards enhanced levels of both markers in the PHH-derived iPSCs, due to inter-clonal variation, only HNF4α in donor 2 demonstrated significantly enhanced gene expression in PHH-derived hepatic endoderm (Figure S7b).

Final stage HLCs were then compared using a panel of mature and immature hepatic markers alongside functional assays with the individual donor's PHH used as the gold standard comparator (Figure 4, Figure 5 and S8).

Gene expression analysis of these markers demonstrated no significant differences between the lines generated from different cell types in any of the donors. Despite this, some genes did show a trend towards improved gene expression in the PHH-derived clones; for example, in all three donors, greater expression of HNF4α and CYP2D6 is found in PHH-derived HLCs when compared to the corresponding donor's HDF-derived HLCs. Conversely, the other markers lack conformity, with the greater expression of the marker gene differing between PHH and HDF-derived HLCs across the assessed donors (Figure 4c).

Functional comparisons show no significant difference between PHH- and HDF-derived hepatocyte-like cells.

To assess the functional capacity of the HLCs, albumin secretion was compared at day 22 of differentiation. The data demonstrated a non-significant trend pointing towards PHH-derived clones having slightly higher, but non-significantly different, albumin secretion in two of the three donors (Figure 5a). When examined together, the difference between PHH- and HDF-derived HLC albumin secretion was not statistically significant (Figure 5b).

CYP3A and CYP2D6 activity was also assessed using probe substrates testosterone and dextromethorphan, respectively (Figure 5c and d). Again, no significant differences were found between the HLCs derived from the different starting cell types.

Discussion

In this study, we have investigated the hepatocyte differentiation potential of PHH- and HDF-derived iPSCs derived from the same donor in a multi-faceted panel of assays, to give a thorough comparison of the hepatic phenotype.

Our main hypothesis, that the starting cell type influences the differentiation capacity of the cell, was driven by the reported epigenetic memory which remains from the somatic cell following reprogramming[16, 17]. Our results suggested a small but statistically non-significant epigenetic memory may be found in the developmentally important liver-enriched transcription factor HNF4α promoter region in reprogrammed cells derived from human hepatocytes[34, 35]. This is in contrast to 25% difference in the methylation of the PDX1 gene promoter region reported between beta-pancreatic cell-derived iPSC and non-beta-pancreatic cell-derived iPSCs[18]. Throughout the analysis, a general trend towards a slight, non-significant advantage, to starting with primary human hepatocytes, when compared to dermal fibroblasts, was also noted. This was most evident with HNF4α expression at the hepatic endoderm stage of differentiation. Therefore, the slightly enhanced phenotype in PHH-derived iPSCs at the HLC stage may be driven by the small differences in the methylation patterns of HNF4α reported in this study and other hepatic-associated genes. Moreover, the lack of a clear enhancement in cell differentiation, as described in pancreatic beta cell-derived iPSCs[18], may be a consequence of the relatively small differences in the epigenetic profile of PHH- and HDF-derived iPSCs. Our findings that the noted differences in HNF4α methylation decrease with time in culture are in keeping with previous literature, which has demonstrated that the distinct epigenetic patterns and enhanced phenotype in a given cell type-derived iPSC are lost following repeat passaging [16, 17, 19, 21].

Throughout this study, few significant differences were found between the PHH- and HDF-derived-iPSCs. Recent work has suggested that the major determinant in hepatocyte-like cell differentiation phenotype is the donor[22]. This was found to be true for the gene expression analysis of the iPSC, definitive endoderm and embryoid body samples. The gene expression profile of HLCs has been reported to be influenced by the genotype of the corresponding donor PHH[21, 36]. Therefore, to reduce genotype-derived donor variation and allow for a more representative comparison of the starting cell type, HLC samples were compared to the corresponding donor's PHH. Despite this analytical approach, no significant differences were found between PHH- and HDF-derived HLCs.

Whilst the donor appears to be a major determinant of phenotype, other non-defined factors, not related to starting cell type or donor, also appear to influence the hepatic differentiation capacity of

iPSCs. This inter-clonal variation contributed to the lack of significant findings between starting cell types. For example, albumin secretion was found to be generally higher in PHH-derived clones; however, when all lines were compared, no significant differences were seen between PHH- and HDF-derived lines. This was in part due to the donor 1 HDF-derived line, Liv4FA, which displayed much greater albumin secretion and gene expression than all other lines. If Liv4FA is not included in the analysis, there is significantly greater HLC albumin secretion in lines derived from PHH. Thus, our results suggest that both donor- and clone-dependent variation has greater influence on HLC phenotype than the starting cell type. Further work is therefore required to define the differences between iPSC clones which infer the capacity for hepatic differentiation.

Previous work has described the reprogramming of human hepatocytes as being relatively inefficient compared to HDFs and that it was only achievable with cells derived from relatively young donors [27]. In contrast, we were able to generate clones from donors which were comparatively old, i.e. 63 and 66 years old. Interestingly, despite using only four clones for the analysis, we were able to generate 10 clones from donor 3 (27 years old). These data point towards an enhancement of hepatocyte reprogramming efficiency in younger donors. The effect of age has been discussed in depth in other starting cell types; with multiple studies in mice showing a decline in reprogramming efficiency with age[37]. However, the effect of age on reprogramming efficiency in human cells is more difficult to assess due to the unknown influence of the donor's genetic background. As a consequence the effect of age is under debate; Sommers et al., reported no significant correlation between age and efficiency[38], whereas, Trockovic et al., did find a correlation, in a P21-dependent manner[39].

The isolation of PHH is relatively well-established; however, the lack of a *bona fide* cell surface marker and the sensitivity of the cells during the isolation process mean that, to the best of our knowledge, a 100% homogenous population of hepatocytes is not currently achievable. Therefore, there remains a possibility that not all of the iPSC clones derived from the PHH-enriched population were derived from hepatocytes. However, if PHH-derived clones were to be used in future experiments, the inability to guarantee a homogenous starting population of hepatocytes will likely remain; therefore, this work is also representative of the practical limitations associated with PHH as a starting cell source.

It must also be acknowledged that the slight enhancements seen in PHH-derived clones may well be limited by the restricted differentiation maturity which is currently achievable in simple 2D HLC culture systems, as used in this study. In line with this, our PHH-derived iPSCs do appear to show a significant trend (Figure S6b) towards enhancement of phenotype at the hepatic endoderm stage which is carried through to our HLC analysis. Previous work in mice has shown that the greatest

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advantage in HLC differentiation was achieved by reprogramming hepatoblasts, rather than adult hepatocytes[19]. This may be a consequence of the relatively immature phenotype of HLCs. Therefore, future investigations into the starting cell type would require the development of protocols which produce a more mature phenotype; however, this in itself remains a major bottleneck in the field of HLCs [7]. Moreover, through the inclusion of spontaneous differentiation assays and methylation analysis alongside our HLC comparisons, we have demonstrated that the starting cell type is unlikely to be of significant influence, even when protocols which allow for enhanced differentiation are developed.

Taken together, the lack of any major advantage to PHH-derived iPSCs is of significance to the field of iPSC-derived HLCs, suggesting that any trend towards enhanced expression in PHH-derived clones is not greater than the inter-clone and donor variation derived from other non-defined influencing factors. Furthermore, the access and phenotypic range of available PHH samples is very restricted in comparison to the easily accessible skin or blood samples used in the majority of current studies[24, 40]. Therefore, the similarity between the cell types for all tested parameters is reassuring for current and future studies, particularly those which require specific genotypic backgrounds, i.e. for disease studies, or a large phenotypic range for population-representative studies.

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References

1. Sgro C, Clinard F, Ouazir K, et al. Incidence of drug-induced hepatic injuries: A French population-based study. Hepatology. 2002;36:451-455.
2. Meier Y, Cavallaro M, Roos M, et al. Incidence of drug-induced liver injury in medical inpatients. European journal of clinical pharmacology. 2005;61:135-143.
3. Wilke RA, Lin DW, Roden DM, et al. Identifying genetic risk factors for serious adverse drug reactions: Current progress and challenges. Nature Reviews Drug Discovery. 2007;6:904-916.
4. Fraczek J, Bolleyn J, Vanhaecke T, et al. Primary hepatocyte cultures for pharmaco-toxicological studies: at the busy crossroad of various anti-dedifferentiation strategies. Arch Toxicol. 2013;87:577-610.
5. Castell JV, Jover R, Martinez-Jimenez CP, et al. Hepatocyte cell lines: their use, scope and limitations in drug metabolism studies. Expert opinion on drug metabolism & toxicology. 2006;2:183-212.
6. Lavon N, Yanuka O, Benvenisty N. Differentiation and isolation of hepatic-like cells from human embryonic stem cells. Differentiation; research in biological diversity. 2004;72:230-238.
7. Kia R, Sison RL, Heslop J, et al. Stem cell-derived hepatocytes as a predictive model for drug-induced liver injury: are we there yet? British journal of clinical pharmacology. 2013;75:885-896.
8. Baxter M, Withey S, Harrison S, et al. Phenotypic and functional analyses show stem cell-derived hepatocyte-like cells better mimic fetal rather than adult hepatocytes. Journal of hepatology. 2015;62:581-589.
9. Lu J, Einhorn S, Venkatarangan L, et al. Morphological and Functional Characterization and Assessment of iPSC-derived Hepatocytes for In Vitro Toxicity Testing. Toxicological Sciences. 2015.
10. Cameron K, Tan R, Schmidt-Heck W, et al. Recombinant Laminins Drive the Differentiation and Self-Organization of hESC-Derived Hepatocytes. Stem Cell Reports.
11. Subramanian K, Owens DJ, Raju R, et al. Spheroid culture for enhanced differentiation of human embryonic stem cells to hepatocyte-like cells. Stem cells and development. 2014;23:124-131.
12. Shan J, Schwartz RE, Ross NT, et al. Identification of small molecules for human hepatocyte expansion and iPS differentiation. Nat Chem Biol. 2013;9:514-520.
13. Avior Y, Levy G, Zimerman M, et al. Microbial-derived lithocholic acid and vitamin K2 drive the metabolic maturation of pluripotent stem cells-derived and fetal hepatocytes. Hepatology. 2015;62:265-278.
14. Koche RP, Smith ZD, Adli M, et al. Reprogramming Factor Expression Initiates Widespread Targeted Chromatin Remodeling. Cell Stem Cell. 2011;8:96-105.
15. Lister R, Pelizzola M, Kida YS, et al. Corrigendum: Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature. 2014;514:126-126.
16. Kim K, Doi A, Wen B, et al. Epigenetic memory in induced pluripotent stem cells. Nature. 2010;467:285-290.
17. Polo JM, Liu S, Figueroa ME, et al. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nature biotechnology. 2010;28:848-855.
18. Bar-Nur O, Russ HA, Efrat S, et al. Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. Cell Stem Cell. 2011;9:17-23.
19. Lee SB, Seo D, Choi D, et al. Contribution of hepatic lineage stage-specific donor memory to the differential potential of induced mouse pluripotent stem cells (iPSC). Stem cells (Dayton, Ohio). 2012;30:997-1007.

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20. Liu H, Kim Y, Sharkis S, et al. In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. Science translational medicine. 2011;3:82ra39.

21. Takayama K, Morisaki Y, Kuno S, et al. Prediction of interindividual differences in hepatic functions and drug sensitivity by using human iPS-derived hepatocytes. Proceedings of the National Academy of Sciences of the United States of America. 2014;111:16772-16777.

22. Kajiwara M, Aoi T, Okita K, et al. Donor-dependent variations in hepatic differentiation from human-induced pluripotent stem cells. Proc Natl Acad Sci USA. 2012;109:12538-12543.

23. Kia R, Kelly L, Sison-Young RLC, et al. MicroRNA-122: A Novel Hepatocyte-Enriched in vitro Marker of Drug-Induced Cellular Toxicity. Toxicological Sciences. 2015;144:173-185.

24. Aasen T, Belmonte JCI. Isolation and cultivation of human keratinocytes from skin or plucked hair for the generation of induced pluripotent stem cells. Nat. Protocols. 2010;5:371-382.

25. Veazey KJ, Golding MC. Selection of Stable Reference Genes for Quantitative RT-PCR Comparisons of Mouse Embryonic and Extra-Embryonic Stem Cells. PLoS ONE. 2011;6:e27592.

26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.). 2001;25:402-408.

27. Hansel MC, Gramignoli R, Blake W, et al. Increased reprogramming of human fetal hepatocytes compared with adult hepatocytes in feeder-free conditions. Cell transplantation. 2014;23:27-38.

28. Xu Y, Wei X, Wang M, et al. Proliferation Rate of Somatic Cells Affects Reprogramming Efficiency. Journal of Biological Chemistry. 2013;288:9767-9778.

29. Liu H, Ye Z, Kim Y, et al. Generation of endoderm-derived human induced pluripotent stem cells from primary hepatocytes. Hepatology. 2010;51:1810-1819.

30. Jiang W, Zhang D, Bursac N, et al. WNT3 Is a Biomarker Capable of Predicting the Definitive Endoderm Differentiation Potential of hESCs. Stem Cell Reports. 2013;1:46-52.

31. Daskalos A, Nikolaidis G, Xinarianos G, et al. Hypomethylation of retrotransposable elements correlates with genomic instability in non-small cell lung cancer. International journal of cancer. Journal international du cancer. 2009;124:81-87.

32. Sullivan GJ, Hay DC, Park IH, et al. Generation of functional human hepatic endoderm from human induced pluripotent stem cells. Hepatology. 2010;51:329-335.

33. Williams JA, Ring BJ, Cantrell VE, et al. Comparative Metabolic Capabilities of CYP3A4, CYP3A5, and CYP3A7. Drug Metabolism and Disposition. 2002;30:883-891.

34. Torres-Padilla ME, Fougere-Deschatrette C, Weiss MC. Expression of HNF4alpha isoforms in mouse liver development is regulated by sequential promoter usage and constitutive 3' end splicing. Mechanisms of development. 2001;109:183-193.

35. Deng XG, Qiu RL, Wu YH, et al. Overexpression of miR-122 promotes the hepatic differentiation and maturation of mouse ESCs through a miR-122/FoxA1/HNF4a-positive feedback loop. Liver international : official journal of the International Association for the Study of the Liver. 2014;34:281-295.

36. Asplund A, Pradip A, van Giezen M, et al. One Standardized Differentiation Procedure Robustly Generates Homogenous Hepatocyte Cultures Displaying Metabolic Diversity from a Large Panel of Human Pluripotent Stem Cells. Stem Cell Rev and Rep. 2015:1-15.

37. Mahmoudi S, Brunet A. Aging and reprogramming: a two-way street. Current opinion in cell biology. 2012;24:744-756.

38. Sommer CA, Stadtfeld M, Murphy GJ, et al. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. Stem cells (Dayton, Ohio). 2009;27:543-549.

39. Trokovic R, Weltner J, Noisa P, et al. Combined negative effect of donor age and time in culture on the reprogramming efficiency into induced pluripotent stem cells. Stem Cell Research. 2015;15:254-262.

40. Agu Chukwuma A, Soares Filipa AC, Alderton A, et al. Successful Generation of Human Induced Pluripotent Stem Cell Lines from Blood Samples Held at Room Temperature for up to 48 hr. *Stem Cell Reports*. 2015.

Disclosure of Potential Conflicts of Interest

JM is an employee of AstraZeneca and holds shares in the company. No further conflicts of interest to declare.
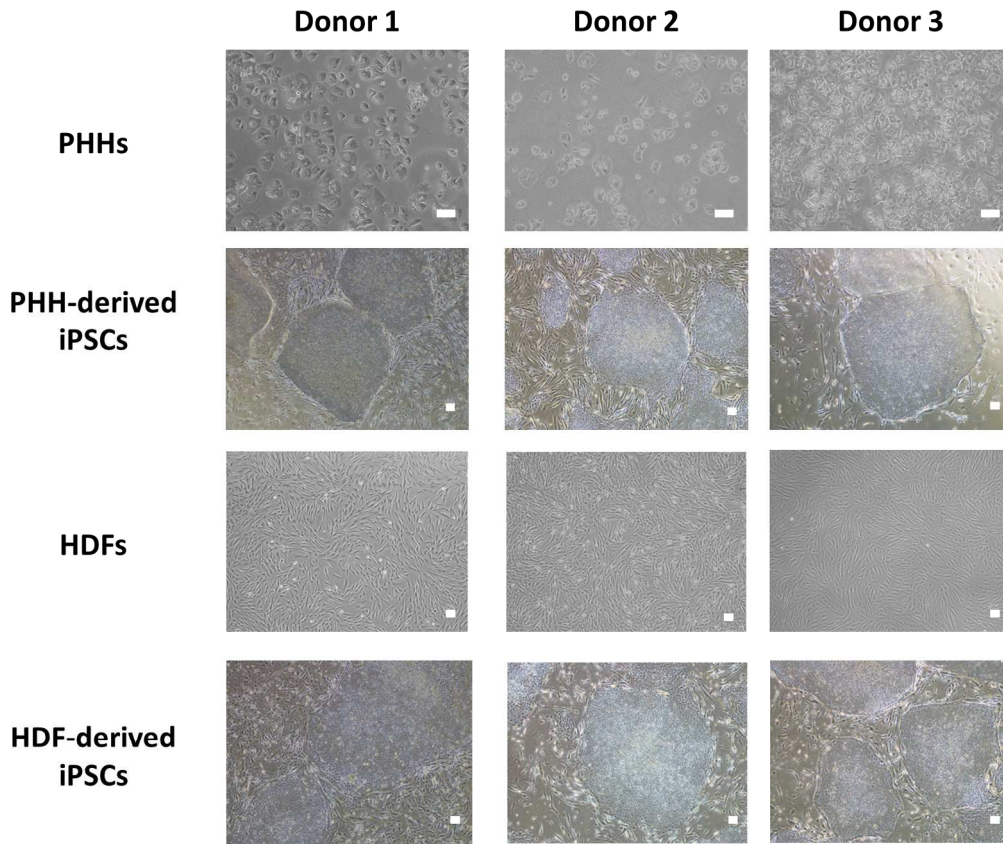
Figure legends

Figure 1: Isolation and reprogramming of somatic cells. The morphology of PHH and HDFs from each donor prior to reprogramming and selected examples of the iPSCs which were generated from each of these starting cell types/donors. PHH: Magnification: x100, scale bar: 100µm; iPSCs and HDFs: magnification x40, scale bar: 100µm.
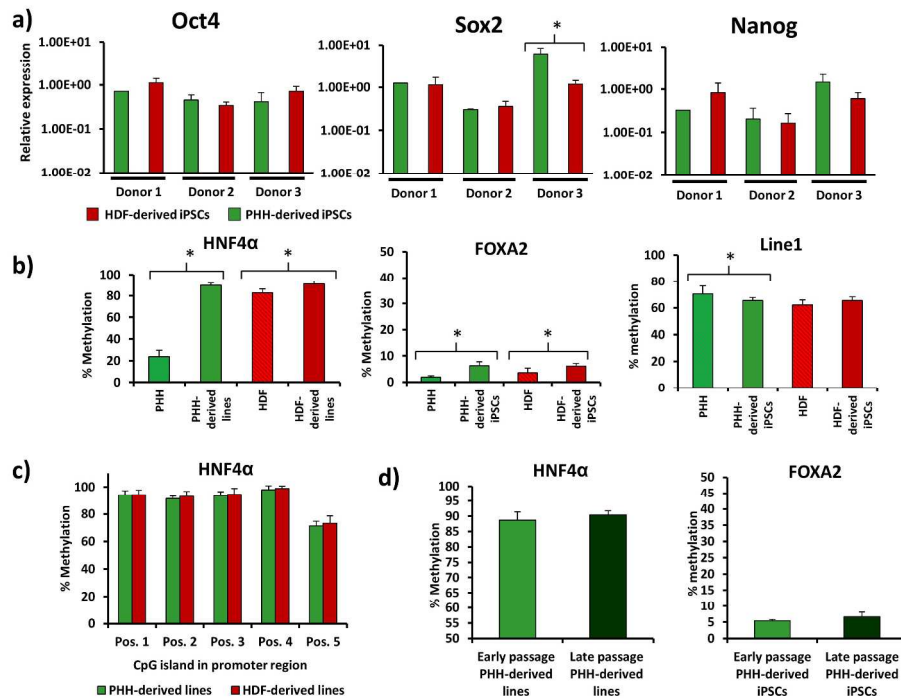
Figure 2: Comparison of iPSC gene expression by qRT-PCR and methylation status by pyrosequencing. **a)** Nanog, Sox2 and Oct4 gene expression determined by qRT-PCR and presented as $2^{-\Delta\Delta CT}$ relative to hESC comparator and normalised with GAPDH. (*) denotes p>0.05 unpaired T test. **b)** The changes in methylation status of HNF4α, FOXA2 and Line1 in PHH (48hrs culture) and HDFs pre- and post-reprogramming. (*) denotes p>0.05 one-way ANOVA. **c)** the difference in HNF4α methylation status of the PHH- and HDF-derived iPSCs across the 5 CpG sites located in the analysed sequence. **d)** The methylation status of HNF4α and FOXA2 which occur following repeat passaging of the cells during standard culture procedures.

Figure 3: Comparison of endoderm and hepatic associated gene expression in PHH- and HDF derived embryoid bodies using qRT-PCR. All genes shown as $2^{-\Delta\Delta CT}$ relative to a hESC-derived EB comparator and normalised to GAPDH and Succinate dehydrogenase. Error bars represent standard deviation between the means of each PHH-/HDF-derived iPSC line which were derived from three individual differentiation cultures. Each sample tested by qRT-PCR was loaded in duplicate. (*) denotes p>0.05 unpaired T test.

Figure 4: Comparison of hepatocyte-like cell gene expression measured by qRT-PCR. Gene expression of key hepatic genes obtained by q-PCR analysis. Results normalized using GAPDH and Succinate dehydrogenase and displayed as $2^{-\Delta\Delta CT}$ relative to PHH of the corresponding donor. ★N.B. Donor 1 shown relative to isogenic plated PHH; donor 2 and 3 shown relative to non-plated isogenic PHH. Error bars represent standard deviation between the means of each PHH-/HDF-derived iPSC line which were derived from three individual differentiation cultures. Each sample tested by qRT-PCR was loaded in duplicate. (*) denotes p>0.05 unpaired T test.

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Figure 5: Functional comparison of hepatocyte-like cells. **a)** albumin secretion in PHH and PHH- and HDF-derived HLCs at the final stage of differentiation measured by ELISA and normalised using total protein content of corresponding well. (*) denotes $p > 0.001$ one-way ANOVA. Error bars: SD. **b)** Albumin secretion comparison of PHH and HDF-derived HLCs from all three donors *$p > 0.05$ unpaired T test. **c)** and **d)** CYP activity as detected using LC-MS-MS method analysing the turnover of probe substrates and quantification of metabolites for c) 6β-OH-testosterone (CYP3A) and d) Dextrorphan (CYP2D6). * denotes $p > 0.05$ one-way ANOVA. Error bars: SD.

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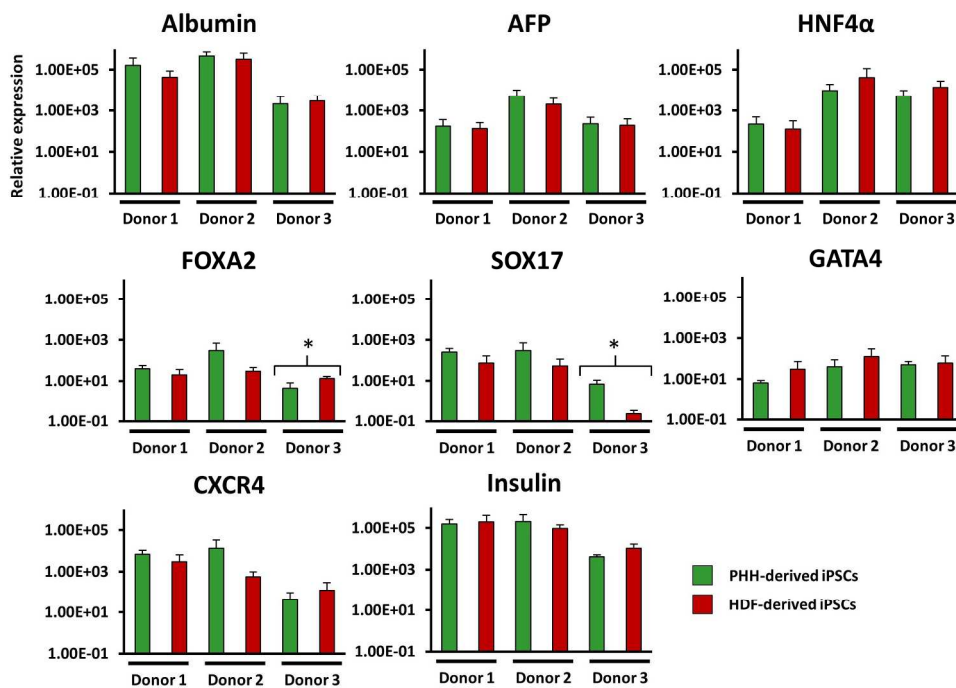
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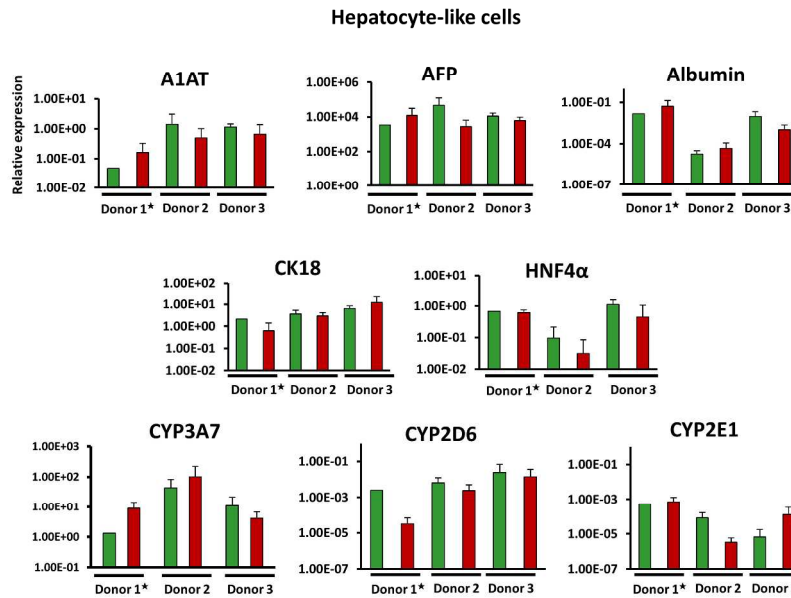
Hepatocyte-like cells

Comparison of hepatocyte-like cell gene expression measured by qRT-PCR. Gene expression of key hepatic genes obtained by q-PCR analysis. Results normalized using GAPDH and Succinate dehydrogenase and displayed as 2-ΔΔCT relative to PHH of the corresponding donor. ★N.B. Donor 1 shown relative to isogenic plated PHH; donor 2 and 3 shown relative to non-plated isogenic PHH. Error bars represent standard deviation between the means of each PHH-/HDF-derived iPSC line which were derived from three individual differentiation cultures. Each sample tested by qRT-PCR was loaded in duplicate. (*) denotes p>0.05 unpaired T test.

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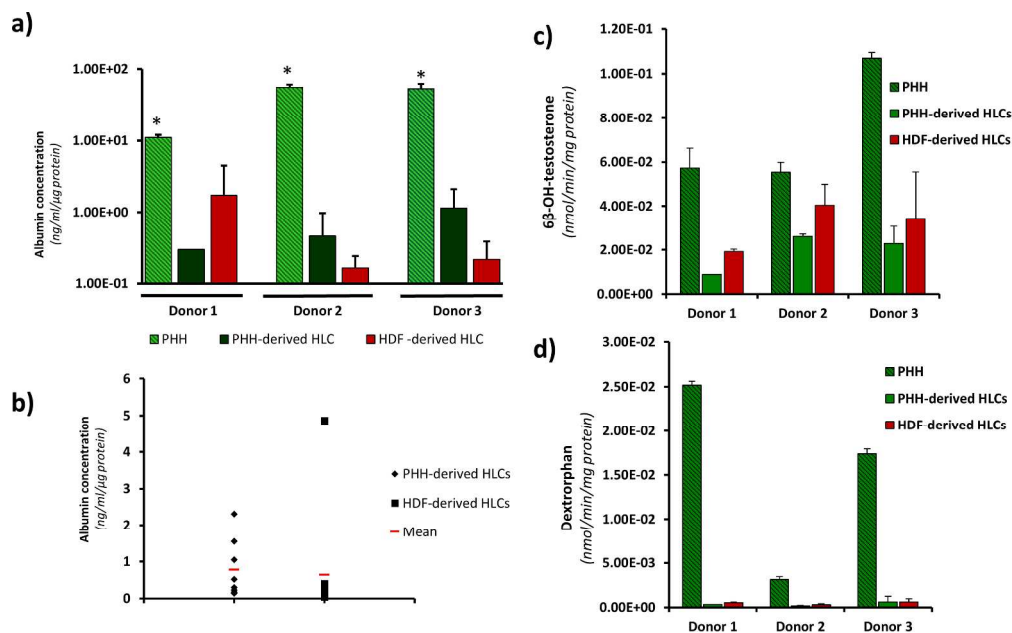
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Authors	Species	Starting cell types	Assays used	Improved phenotype?
(Liu et al., 2011)	Human	Hepatocytes Fibroblasts Keratinocytes BM-MSCs	Albumin secretion CYP3A4 activity	Yes, but non-significant
(Lee et al., 2012)	Mouse	Mouse ESCs Fibroblasts Hepatoblasts Hepatocytes	Albumin secretion HLC gene expression	Yes, but transient
(Kajiwara et al., 2012)	Human	PBMCs Fibroblasts	Albumin/Urea secretion	No, donor dependent
(Takayama et al., 2014)	Human	Hepatocytes Fibroblasts PBMCs HUVECs	Albumin secretion CYP3A4 activity TAT expression	Yes, but transient

Table S1: Summary of the previously published investigations of starting cell type for hepatocyte-like cell differentiation

	Name	Sequence 5'-3'	Modification
➘	HNF4ameth-F	TGGGTGATTAGAAGAATTAATAAG	
↩°	HNF4ameth-Rb	CAACACAACCACCAAAAAC	5' biotin
➡	HNF4ameth-S	TGATTAGAAGAATTAATAAGATA	
➘	FOXA2meth-F	TGTGATTGAAAAGTAATTTTGAA	
↩°	FOXA2meth-Rb	CAAACAACCCCTCTAACAAC	5' biotin
➡	FOXA2meth-S	GAAAAGTAATTTTGAAATA	

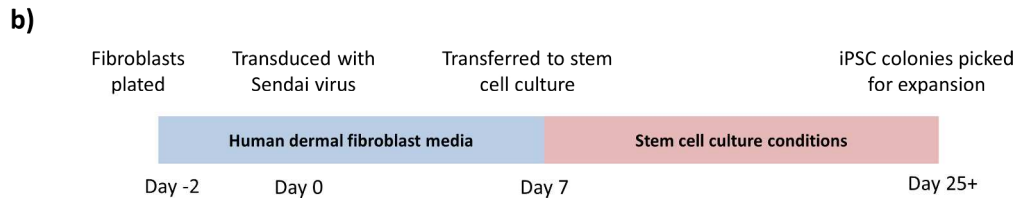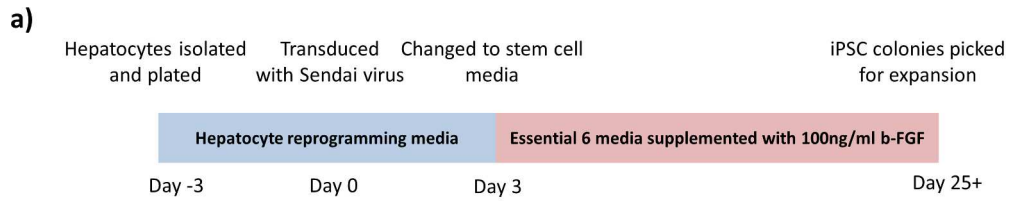**Table S2**: Primer sequences used for pyrosequencing analysis.

➡ : Sequencing primer; ➘: Forward primer; ↩: Reverse primer; ↩°/➘° : Biotinylated primer.

Primer		Sequence (5' to 3')
ALB	F	CCTGTTGCCAAAGCTCGATG
	R	ATCTCCATGGCAGCATTCGC
A1AT	F	TCCGATAACTGGGGTGACCT
	R	AGACGGCATTGTCGATTCACT
CYP1A2	F	AGCACCTGCCTCTACAGTTGG
	R	TGGTGGACTTTTCAGGCCTTT
CYP3A4	F	TGTGCCTGAGAACACCAGAG
	R	GTGGTGGAAATAGTCCCGTG
CYP3A7	F	AGACGGGCTTCATCCAATGTG
	R	ATGGTGCTAACTGGGGGTGG
CYP2E1	F	ACCCTGAGATCGAAGAGAAGC
	R	AAATGGTGTCTCGGGTTGCT
CYP2D6	F	TTCCCAAGGGGTGTTCCTGG
	R	TCACGGCTTTGTCCAAGAGA
CK18	F	ACATCCGGGCCCAATATGAC
	R	GGTGCTCTCCTCAATCTGCT
GAPDH	F	CTATAAATTGAGCCCGCAGCC
	R	GCCCAATACGACCAAATCCGT
HNF4α	F	GTTGACGATGGGCAATGACAC
	R	TCTTTGTCCACCACGCACTG
AFP	F	GCGGCCTCTTCCAGAAACTA
	R	AATAATGTCAGCCGCTCCCT
SOX17	F	GGATACGCCAGTGACGACCA
	R	GACTTGCCCAGCATCTTGCTC
FOXA2	F	ATTGCTGGTCGTTTGTTGAGG
	R	TTCATGCCGTTCATCCCCAG
GATA4	F	CGACACCCCAATCTCGATATG
	R	GTTGCACAGATAGTGACCCGT
CXCR4	F	GAAACCCTCAGCGTCTCAGT
	R	AGTAGTGGGCTAAGGGCACA
WNT3	F	GAGCCCAGAGATGTGTACTGC
	R	CTTCTAATGGAGCCCCACCT
PDX1	F	CAAAGCTCACGCGTGGAAAG
	R	TTTTTCCACTTCATGCGGCG
ISL1 (Insulin)	F	ACAAGCAGCCGGAGAAGAC
	R	TGGATATTAGTTTTGTCATTGGGCT
SDHA	F	TGGTTGTCTTTGGTCGGG
	R	GCGTTTGGTTTAATTGGAGGG
OCT4	F	AGACCATCTGCCGCTTTGAG
	R	GCAAGGGCCGCAGCTT
SOX2	F	AACCAGCGCATGGACAGTTAC
	R	TGGTCCTGCATCATGCTGTAG
NANOG	F	GGCTCTGTTTTGCTATATCCCCTAA
	R	CATTACGATGCAGCAAATACGAGA

Table S3: Primer sequences used for qRT-PCR analysis.

	DONOR 1	DONOR 2	DONOR 3
Age at surgery	66	63	27
BMI	27.9	31.2	32.1
Sex	Male	Female	Male
Diagnosis	Colorectal carcinoma with liver metastases	Colorectal carcinoma with liver metastases	Focal nodular hyperplasia
Co-morbidity	Type II diabetes mellitus	Hypertension Chronic liver disease Type II diabetes mellitus	None
No. of PHH-derived clones	1	5	10

Table S4: Details of the donors from which the cells used for reprogramming were derived, including key parameters such as age, co-morbidities and the number of iPSC clones derived from each donor PHH.

a)

| Hepatocytes isolated and plated | Transduced with Sendai virus | Changed to stem cell media | | iPSC colonies picked for expansion |

| Hepatocyte reprogramming media | Essential 6 media supplemented with 100ng/ml b-FGF |

| Day -3 | Day 0 | Day 3 | | Day 25+ |

b)

| Fibroblasts plated | Transduced with Sendai virus | Transferred to stem cell culture | | iPSC colonies picked for expansion |

| Human dermal fibroblast media | Stem cell culture conditions |

| Day -2 | Day 0 | Day 7 | | Day 25+ |

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a)
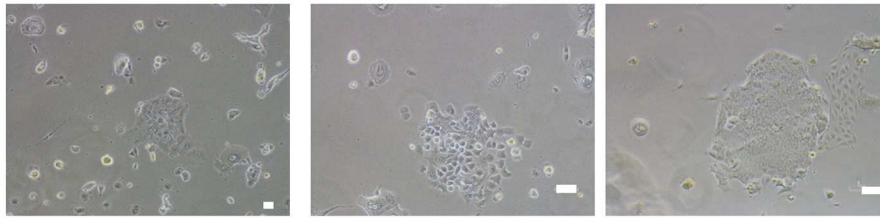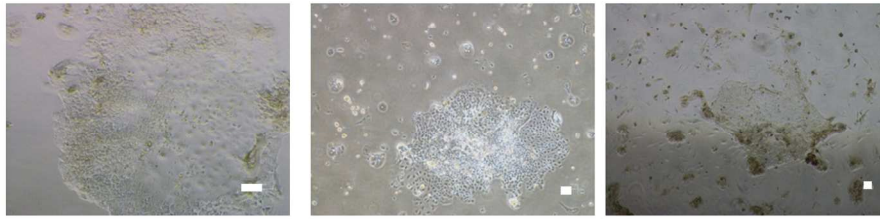
Day 5	Day 6	Day 11
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Day 20	Day 20	Day 20

b)
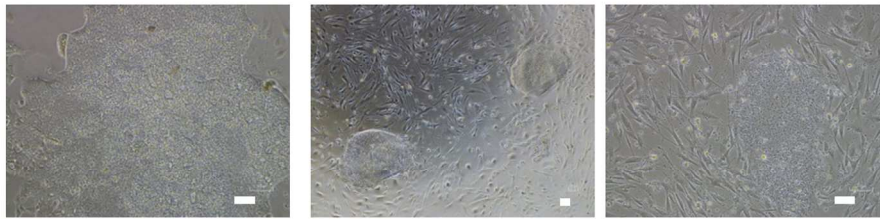
| Day 24 - Liv4HJ | Liv4HJ – P1 | Liv4HJ – P2 |

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Definitive endoderm

a)

SOX17

FOXA2

GATA4

b)   **Hepatic endoderm**

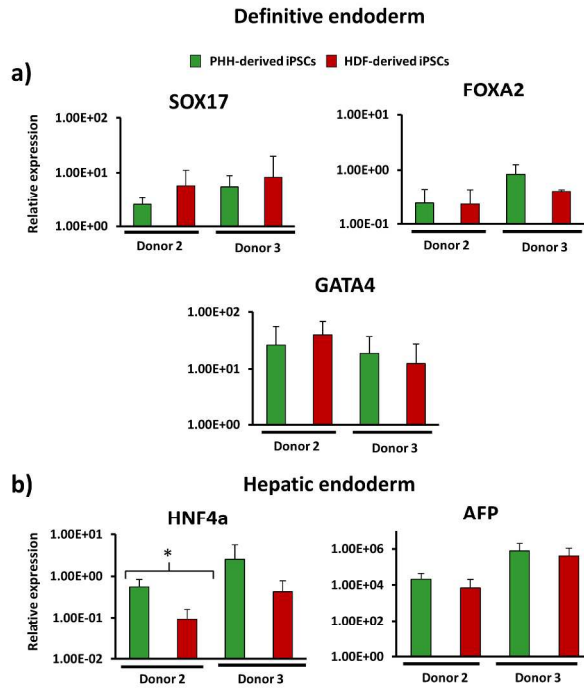HNF4a   AFP
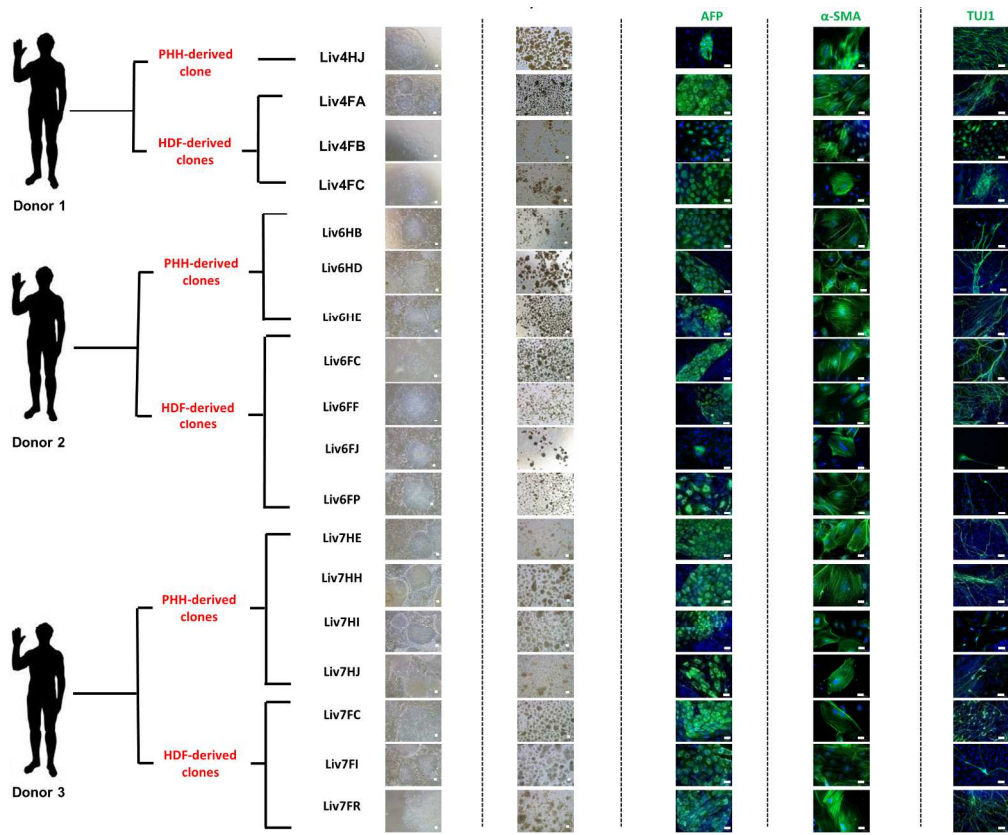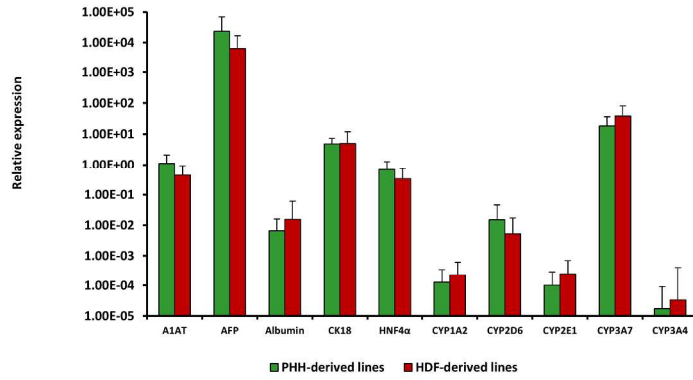
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Supplementary figure legends

Figure S1: Protocols for the reprogramming of somatic cells. a) PHH and b) HDF reprogramming protocols. Schematic diagram of the protocols used to reprogram each cell type. PHH: Primary human hepatocytes; HDFs: Human dermal fibroblasts; b-FGF: basic fibroblast growth factor

Figure S2: Reprogramming of PHH to IPSCs. **a)** The morphological changes during reprogramming and the different cell types which are generated during this period. **b)** Successfully generated iPSC clone with correct morphology and growth characteristics before and after manual picking and passaging. Magnification: x100, scale bar: 100μm; x40, scale bar: 100μm.

Figure S3: Generation and characterization of each iPSC line confirmed by morphology and immunofluorescence. Morphology of each line is shown next to the immunofluorescence images of nuclear pluripotency markers OCT4, SOX2 and Nanog and surface markers Tra-1-60 and SSEA-4. Immunofluorescence images: x200 magnification, 50μm scale bar. Light microscopy images: x100 magnification, 100μm scale bar.

Figure S4: Functional pluripotency characterization using embryoid body assay confirmed by immunofluorescence**.** Embryoid bodies generated from each iPSC line and stained for markers of the three germ layers associated with pluripotency: Endoderm (AFP), Mesoderm (α-SMA) and Ectoderm (Tuj1). Immunofluorescence images: x200 magnification, 50μm scale bar. Light microscopy images: x100 magnification, 100μm scale bar.

Figure S5: Wnt3 gene expression determined by q-PCR and presented as $2^{-\Delta\Delta CT}$ relative to hESC comparator and normalized with GAPDH. Error bars represent standard deviation between the means of each PHH-/HDF-derived iPSC line which were derived from three individual differentiation cultures. Each sample tested by qRT-PCR was loaded in duplicate.

Figure S6: Differentiation of all lines to hepatocyte-like cells and confirmation by immunofluorescence. **a)** A schematic diagram of the protocol used for the differentiation experiments, including the growth factors/small molecules used at each stage of differentiation. **b)** Immunofluorescence analysis of iPSCs during each stage of differentiation: Definitive endoderm (Sox17), Hepatic endoderm (HNF4α) and hepatocyte-like cells (albumin). Immunofluorescence images: x200 magnification, 50μm scale bar. Light microscopy images: x100 magnification, 100μm scale bar.

Figure S7: Comparison of gene expression during hepatocyte-like cell differentiation measured by qRT-PCR. **a)** Definitive endoderm associated genes at day 5 of differentiation. All genes shown as $2^{-\Delta\Delta CT}$ relative to a hESC-derived control and normalized using GAPDH and Succinate dehydrogenase gene expression. Error bars represent standard deviation between the means of each PHH-/HDF-derived iPSC line which were derived from three individual differentiation cultures. Each sample tested by qRT-PCR was loaded in duplicate. **b)** Hepatic endoderm associated genes at day 12 of differentiation. N.B. donor 1 D.E and H.E samples were not generated during the low passage differentiation experiments and therefore are not included in this analysis.

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Figure S8: Combined gene expression of hepatocyte-like cells derived from PHH and HDF-derived lines. Gene expression of key hepatic genes obtained by q-PCR analysis and displayed as $2^{-\Delta\Delta CT}$ relative to PHH of the corresponding donor. N.B. CYP3A4 was not detected in donor 1 and 2 HLCs, results shown are derived from donor 3 only. Results normalized using GAPDH and Succinate dehydrogenase. Error bars represent standard deviation between the means of each PHH-/HDF-derived iPSC line which were derived from three individual differentiation cultures. Each sample tested by qRT-PCR was loaded in duplicate.