Research Article

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Phenotypic and functional analyses show stem cell-derived hepatocyte-like cells better mimic fetal rather than adult hepatocytes

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Background & Aims: Hepatocyte-like cells (HLCs), differentiated from pluripotent stem cells by the use of soluble factors, can model human liver function and toxicity. However, at present HLC maturity and whether any deficit represents a true fetal state or aberrant differentiation is unclear and compounded by comparison to potentially deteriorated adult hepatocytes. Therefore, we generated HLCs from multiple lineages, using two different protocols, for direct comparison with fresh fetal and adult hepatocytes.

Methods: Protocols were developed for robust differentiation. Multiple transcript, protein and functional analyses compared HLCs to fresh human fetal and adult hepatocytes.

Results: HLCs were comparable to those of other laboratories by multiple parameters. Transcriptional changes during differentiation mimicked human embryogenesis and showed more similarity to pericentral than periportal hepatocytes. Unbiased proteomics demonstrated greater proximity to liver than 30 other human organs or tissues. However, by comparison to fresh material, HLC maturity was proven by transcript, protein and function to be fetal-like and short of the adult phenotype. The expression of 81% phase 1 enzymes in HLCs was significantly upregulated and half were statistically not different from fetal hepatocytes. HLCs secreted albumin and metabolized testosterone (CYP3A) and dextrorphan (CYP2D6) like fetal hepatocytes. In seven bespoke tests, devised by principal components analysis to distinguish fetal from adult hepatocytes, HLCs from two different source laboratories consistently demonstrated fetal characteristics.

Conclusions: HLCs from different sources are broadly comparable with unbiased proteomic evidence for faithful differentiation down the liver lineage. This current phenotype mimics human fetal rather than adult hepatocytes.

Keywords: Human embryonic stem cell; Embryo; Hepatic; Liver; Hepatotoxicity.
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Abbreviations: HLC, hepatocyte-like cell; PSC, pluripotent stem cell; ESC, embryonic stem cell; Wnt, wingless-related integration site; DE, definitive endoderm; FGF, fibroblast growth factor; BMP, bone morphogenetic protein; HGF, hepatocyte growth factor; DEX, dexamethasone; OSM, oncostatin M; AFP, alpha-fetoprotein; CYP, cytochrome P450; MEF, mouse embryonic fibroblast; IPSC, induced pluripotent stem cell; iTRAQ, isobaric tagging for relative and absolute quantification; GSTp, glutathione S-transferase π.

Introduction

Hepatocyte-like cells (HLCs), differentiated from pluripotent stem cells (PSCs), offer promise as *in vitro* models of human liver development, function, and toxicity [1,2]. Most protocols have attempted mimicry of embryogenesis through the addition of soluble factors to the media. Activin A [3–13], alone or together with Wingless-related integration site (Wnt) 3A [7–9,14,15], promotes definitive endoderm (DE)-like differentiation. Fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) family

members encourage hepatic differentiation [5,6,8,9,11–13]; and hepatocyte growth factor (HGF), the synthetic glucocorticoid, dexamethasone (DEX), and oncostatin M (OSM) support increased maturity [3,4,6,8–14,16]. However, fully mature hepatocytes have not been produced, which raises two unanswered questions: are cells aberrant because human liver development has not been followed with adequate specificity; or, if the lineage is correct, are HLCs actually 'stuck' in a fetal-like state? Assessment of the latter is problematic for two largely unaddressed reasons. HLC maturity is over-estimated if compared to sub-optimal adult hepatocytes. Thawed cells taken into culture are challenging to maintain [17]; in a well-controlled example, over 90% of the cytochrome P450 (CYP) 3A activity was lost in cryopreserved cells compared to freshly plated cells [18]. This illustrates the risk of over-interpreting the HLC phenotype if compared against dedifferentiated controls. Secondly, fresh fetal hepatocyte controls have been lacking when assessing HLC function. This risks misunderstanding as we have recently shown human fetal hepatocytes possess proteins, such as CYP3A4, commonly interpreted as adult markers [19].

To address these persisting questions about the differentiation and maturity of HLCs, we implemented a protocol with sufficient commonality to allow comparison with multiple previous reports. We analysed a wide range of human ESC lines, derived under different conditions alongside H9 cells, the most popular line for generating HLCs [3,7,9,10,12,14,18,20]. HLCs were assessed by proteome analysis and in a series of assays against fresh human fetal and adult hepatocytes. We also included cells differentiated by a second protocol in an extended array of new tests for differentiation status, devised by unbiased proteomics and principal components analysis that distinguish fetal from fresh adult and dedifferentiated adult hepatocyte phenotypes [19].

Materials and methods

Human tissue and cells, and their culture

Human embryonic stem cell (ESC) lines were obtained with consent either directly from the derivation laboratory or the UK Stem Cell Bank. Cells were maintained on inactivated mouse embryonic fibroblast (MEF) cells [21]. The differentiation protocol (Fig. 1) was commenced 3–4 days post passage onto fresh MEFs using Wnt3a (R&D Systems, UK) and Activin A (Peprotech, UK), diluted in RPMI media (Sigma-Aldrich, UK); followed by BMP2, OSM, FGF2, HGF (all R&D Systems) and DEX (Sigma-Aldrich, UK), diluted in Hepatocyte Culture Medium (HCM) (Lonza, UK). Information on the human fetal and adult hepatocyte controls can be found in the Supplementary Materials and methods. Human induced pluripotent stem cells (IPSCs) were developed and differentiated as previously reported [6,22].

Immunoblotting, immunofluorescence, cell sorting and cell proliferation and apoptosis studies

Immunoblotting and immunofluorescence were conducted as previously reported (Supplementary Table 1) [19,23]. Fluorescent activated cell sorting (FACS), cell proliferation and apoptosis are described in Supplementary Materials and methods.

Protein isolation and proteomic analysis

Protein isolation from whole cell extracts and labelling for isobaric tagging for relative and absolute quantification (iTRAQ) proteomics was described by Rowe *et al.* [19]. Quantitation of proteins was relative to a common reference preparation included in each run across different experiments. Protein identification and interrogation are described in Supplementary Materials and methods.

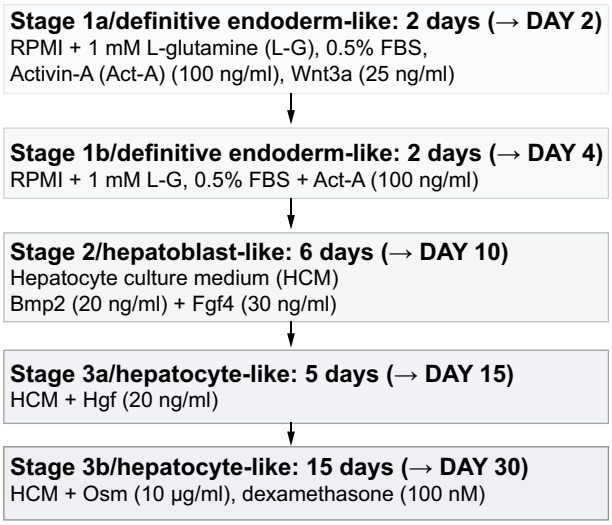

Fig. 1. The three-stage differentiation protocol. RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum.

Phenotypic analysis

Gene expression analysis by RNA sequencing (RNA-seq) and quantitative PCR is described in Supplementary Materials and methods. Albumin and urea secretion into the media was measured using a human albumin ELISA kit and the QuantiChrom™ urea assay kit (both from Bethyl Laboratories). Comparisons with fetal and adult hepatocyte data used the unpaired two-tailed Student's *t* test. CYP3A activity was assessed in duplicate by incubation with P450-Glo™ CYP3A4 assay reagent (Luciferin-PFBE; Promega Ltd). For CYP analysis by mass spectrometry, cells were incubated with 1 mM testosterone or 1 mM dextromethorphan (Sigma, UK) in HCM. Conditioned medium was collected and diluted 1:1 in 0.5 μM phenacetin (Sigma) stop solution in methanol. CYP activity was calculated per min incubation. Alcohol dehydrogenase activity of cell lysates was assessed using a detection kit following the manufacturer's instructions (Abcam, UK). Results were standardized to the amount of protein measured by Bradford assay.

Results

Differentiation of human ESCs to HLCs

Based on previous studies [3–16], iteration of a 3-stage protocol (Fig. 1) was devised to differentiate a range of human ESC lines, derived under diverse conditions to HLCs. During stage 1, Brachyury protein was increased by Activin A on day 2–3, at and after which FOXA2, GATA4 and SOX17 increased (Fig. 2A). However, the low serum caused significant cell death, which was prevented by Wnt3A (25 ng/ml) for the first two days of culture [7–9,14,15], leading to robust detection of the three nuclear transcription factors by day 4 (Fig. 2B). FOXA2, SOX17, and GATA4 were detected in >50% of cells for each ESC line, indicating a shared but variable propensity for DE-like differentiation. More homogeneous differentiation was observed in H9 cells (77–98% of cells positive for FOXA2, SOX17, and GATA4) and HUES7 cells (84–96% cells positive for the three transcription factors) (Fig. 2C and Supplementary Fig. 1A). At the end of stage 2 (hepatoblast-like cells), 91% of HUES7 and 98% of H9 derivatives contained AFP, of which at least two-thirds clearly dual-stained for nuclear HNF4α (Supplementary Fig. 1B and C; only robust HNF4α staining was counted most likely underestimating the entire population of $HNF4\alpha^{+}/AFP^{+}$ cells). Approximately 25% of these AFP^{+} cells were