Efficient Generation of Functionally Relevant hPSC-Derived Hepatocytes and Liver Organoids for Hepatotoxicity and Liver Biology Modeling

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INTRODUCTION

Functionally relevant human hepatocyte culture systems are critical for drug safety and efficacy screening, cell therapy, as well as the study of liver biology and diseases, including nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Conventional systems for liver studies present challenges, including limited human relevance when using rodent models, rapid de-differentiation of primary human hepatocytes (PHHs) in culture, and a lack of metabolic maturity in immortalized cell lines, such as HepG2 and Huh7 cells. To address these challenges, we have developed the STEMdiff™ Hepatocyte Kit for the generation of a functionally relevant human pluripotent stem cell (hPSC)-derived hepatocyte model. This 3-stage serum-free differentiation protocol supports efficient and reproducible generation of hepatocyte-like cells (HLCs) over 21 days, through the patterning of human induced pluripotent (hiPS) or human embryonic (hES) stem cells to definitive endoderm (DE) cells, followed by specification to hepatic progenitor (HP) cells and finally, maturation to hepatocyte-like cells (HLCs). HLCs exhibit characteristic hepatocyte morphology, protein marker expression, and mature functionality, and both HP cells and HLCs can be used to establish hPSC-derived liver organoids using HepatiCult™ Organoid Growth Medium (OGM). These organoids can be expanded long term, cryopreserved, and further matured using HepatiCult™ Organoid Differentiation Medium (ODM). HLCs and hPSC-derived liver organoids differentiated in ODM also exhibit sensitivity to known hepatotoxic compounds such as ketoconazole, troglitazone, and rifampicin, demonstrating the utility of the STEMdiff™ Hepatocyte Kit workflow in liver modeling and drug screening applications.

METHODS

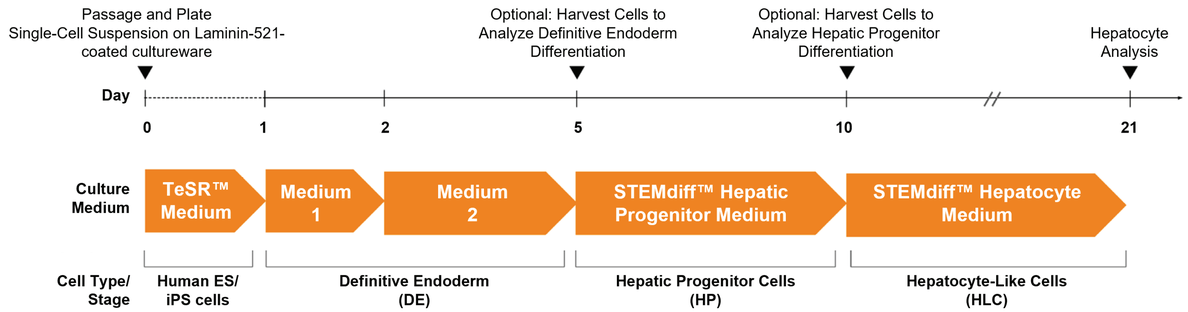

FIGURE 1. Differentiation Protocol for the Generation of hPSC-Derived Hepatocyte-Like Cells Using STEMdiff™ Hepatocyte Kit

On day 0, hPSCs maintained in mTeSR™1, mTeSR™ Plus, or TeSR™-AOF were dissociated into single-cell suspensions and seeded onto Laminin-521-coated 24- or 96-well tissue culture plates. Cells were maintained in the appropriate TeSR™ medium supplemented with 10 µM Y-27632 for the first 24 hours. Differentiation was initiated on day 1 by performing a full-medium change using STEMdiff™ Definitive Endoderm (DE) Medium 1, followed by three subsequent full-medium changes using STEMdiff™ DE Medium 2 on days 2, 3, and 4. On days 5, 6, 7, and 9, full-medium changes were performed using STEMdiff™ Hepatic Progenitor Medium. For the last 11 days of the differentiation, cells were cultured in STEMdiff™ Hepatocyte Medium, with full-medium changes performed every 2 days. Cells were harvested on days 5, 10, or 21 for downstream DE, HP, or HLC analyses respectively.

RESULTS

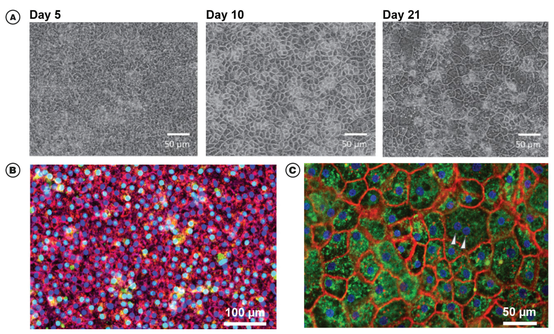

FIGURE 2. Differentiating cells underwent expected stage-specific changes in morphology and protein marker expression

(A) Representative bright-field images exhibit expected, stage-wise changes in morphology, with petal-like DE cell arrangement at day 5, typical hepatic polygonal morphology at day 10, and a decrease in the nucleus-to-cytoplasm ratio typical of hepatocytes at day 21. **(B)** HP cultures at day 10 exhibited uniform expression of fetal hepatocyte marker, AFP (red), hepatic transcription factor, HNF6 (green), and ductal progenitor marker, CK19 (magenta), as assessed by immunocytochemistry, suggesting a biphenotypic identity. **(C)** Similarly, HLC cultures at day 21 exhibited consistent and uniform expression of mature hepatocyte marker, ALB (green), and epithelial tight junction marker, EPCAM (red). Instances of bi-nucleation (white arrowheads), another characteristic feature of hepatocytes, were also observed.

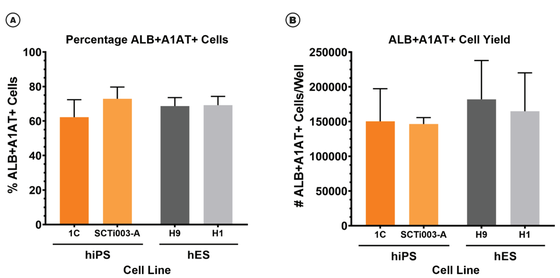

FIGURE 3. Hepatocyte-like cells were generated consistently and reproducibly across multiple hPSC lines

HLCs were generated using two hiPS (1C, SCTi003-A) and two hES (H9, H1) cell lines and then analyzed by flow cytometry at day 21 to assess expression of mature hepatocyte proteins, ALB and A1AT. Across all tested hiPS and hES cell lines, (A) 66 ± 8.1% of cells expressed both markers, yielding (B) 1.65 x 10^5 cells per well of a 24-well plate, on average (mean ± SD, n = 2 - 9).

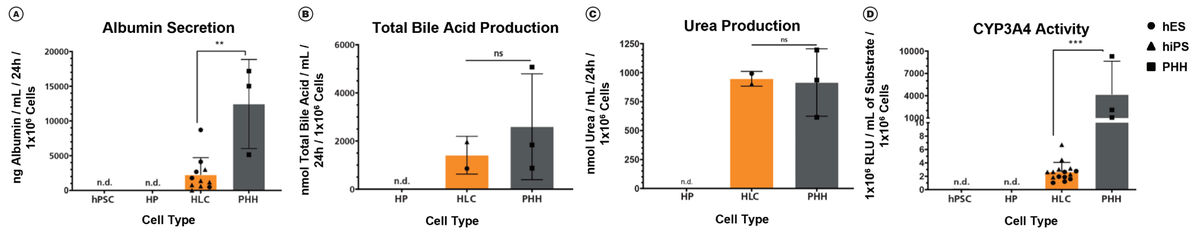

FIGURE 4. Hepatocyte-like cells exhibited mature hepatic functionality

Hepatic functionality was assessed in HPs and HLCs on day 10 and day 21, respectively. **(A)** Secreted albumin, **(B)** total bile acid, and **(C)** urea were only detected in the HLC and PHH cell culture supernatant samples. Similarly, **(D)** baseline CYP3A4 activity, in the absence of inducers or inhibitors, was only detected in the HLC and PHH cultures (n.d. = not detected; mean ± SD; n = 2 - 15). Ordinary one-way ANOVA used for statistical testing. ***p = 0.0007, **p = 0.0011, ns = not significant.

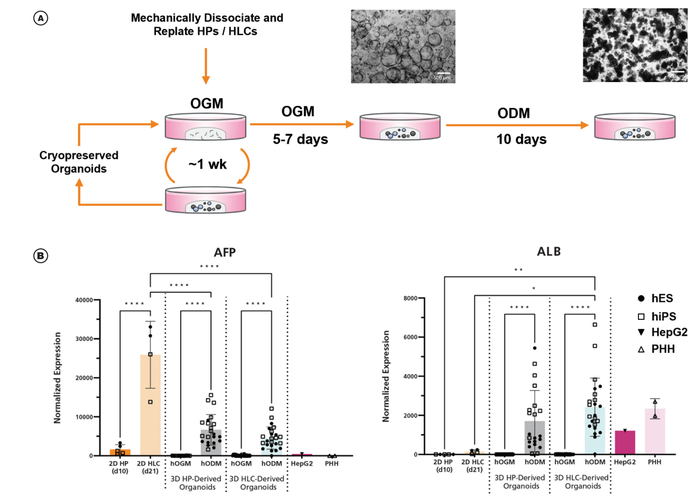

FIGURE 5. hPSC-derived liver organoids exhibited gene expression consistent with hepatic maturation when differentiated using HepatiCult™ ODM

(A) hPSC-derived liver organoids were established from HP cells and HLCs by mechanically dissociating the monolayer cultures, replating the cells into Corning® Matrigel® domes, and culturing in HepatiCult™ OGM. These organoids were serially passaged and further differentiated using HepatiCult™ ODM, following the protocols described in the HepatiCult™ Organoid Kit Technical Manual. **(B)** Gene expression levels for organoids expanded and differentiated across 10 passages were characterized by qPCR, and normalized to housekeeping gene, TBP. Relative to HLCs, fetal hepatocyte marker, AFP, was significantly downregulated and mature hepatic marker, ALB, was significantly upregulated in the HLC-derived differentiated organoids (hODM) (mean ± SD, n = 2). Ordinary one-way ANOVA used for statistical testing. ****p <0.0001, **p = 0.0035, *p = 0.0397.

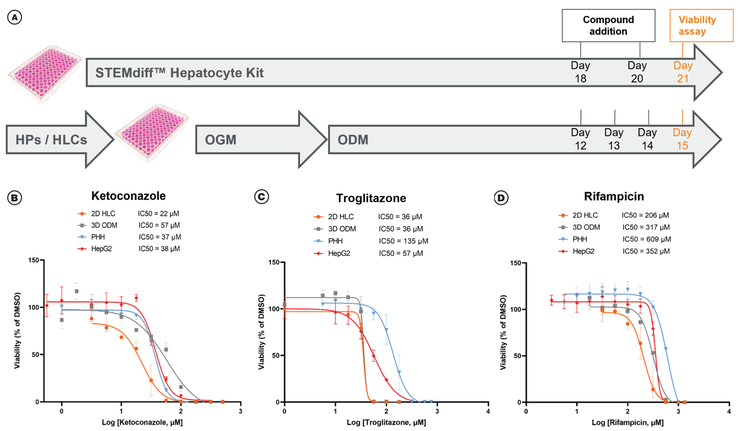

FIGURE 6. hPSC-derived hepatic cells and liver organoids are sensitive models of compound-induced hepatotoxicity

(A) 2D HLCs and HP- and HLC-derived differentiated organoids (3D ODM) were cultured in 96-well plates and treated with compounds or a 1% DMSO vehicle control diluted in culture media for the last 72 hours of the respective differentiation protocols. Viability was then assessed 24 hours after the last compound addition using the CellTiter-Glo® 3D Cell Viability Assay. Non-linear regression curves were plotted for **(B)** ketoconazole, **(C)** troglitazone, and **(D)** rifampicin, demonstrating a dose-dependent effect on cell viability, as well as increased sensitivity to these known hepatotoxic compounds in the 2D HLC and/or 3D ODM samples relative to PHHs and HepG2 cells.

Summary

- STEMdiff™ Hepatocyte Kit supports the efficient generation of hPSC-derived hepatocyte-like cells that exhibit mature hepatic phenotypes and functionality.
- Hepatic progenitor- and hepatocyte-like cell-derived liver organoids, established using HepatiCult™ Organoid Growth Medium, are compatible with long-term expansion, cryopreservation, and further differentiation to increase hepatic maturity.
- Hepatocyte-like cells and differentiated hPSC-derived liver organoids can be used for hepatotoxicity testing.