# Efficient Differentiation of Homogeneous Functional Hepatocytes from Human-Induced Pluripotent Stem Cells

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## Abstract

Human induced pluripotent stem cells (iPSCs) hold tremendous promise for regenerative medicine, disease modeling, drug screening, and toxicology studies. Directed differentiation of iPSCs into hepatocytes could afford unlimited supply of liver cells for various liver-based applications. A variety of protocols have been established during the past decade for the differentiation of iPSCs into hepatocytes through recapitulating major signaling pathways involved in the different stages of embryonic hepatogenesis, using either growth factors or small molecules. Although successful derivation of hepatocyte-like cells (HLCs) has been achieved through those protocols, the differentiation efficiency is mostly not ideal, and the cells at the end of the differentiation always showed a heterogeneous population. In the current study, four previously published hepatocyte differentiation protocols were tested and compared, and based on the results an improved protocol was established that enables highly efficient and reproducible differentiation of iPSCs into homogeneous functional HLCs. The protocol started with single-cell culture (instead of colonies) of iPSCs, and employed both growth factors and small molecules for the differentiation. Compared to the existing protocols, the new protocol yielded a differentiated HLC population that was more homogeneous and with a morphology more closely resembling that of primary human hepatocytes (PHHs). The final population of cells not only expressed specific hepatic markers at both the transcriptional and the protein levels, but also possessed key hepatic functions, including serum protein (albumin, fibronectin, and alpha-1 antitrypsin) secretion, urea synthesis, glycogen storage, and more importantly for toxicology applications, cytochrome P450 activity. Therefore, the method presented here would be a valuable tool for highly efficient generation of homogeneous functional hepatocytes from human iPSCs, and the resultant HLCs could be potentially useful as an *in vitro* model for predictive toxicology.

## Methods

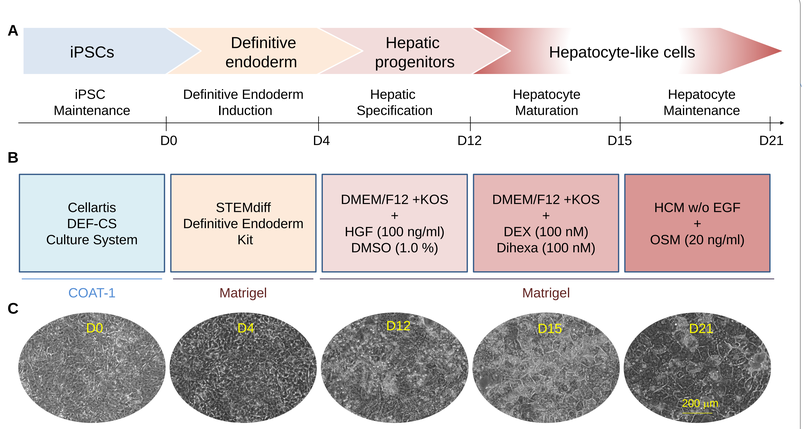


**Figure 1.** Schematic illustration of HLC differentiation from human iPSCs. (A) Cell states, sequential steps, and timeline of the differentiation process. (B) Media, growth factors, and small molecules used at each stage of the differentiation. (C) Representative phase-contrast micrographs of iPSCs (D0), definitive endoderm (DE) cells (D4), hepatic progenitors (D12), immature HLCs (D15), and mature HLCs (D21).

## Results

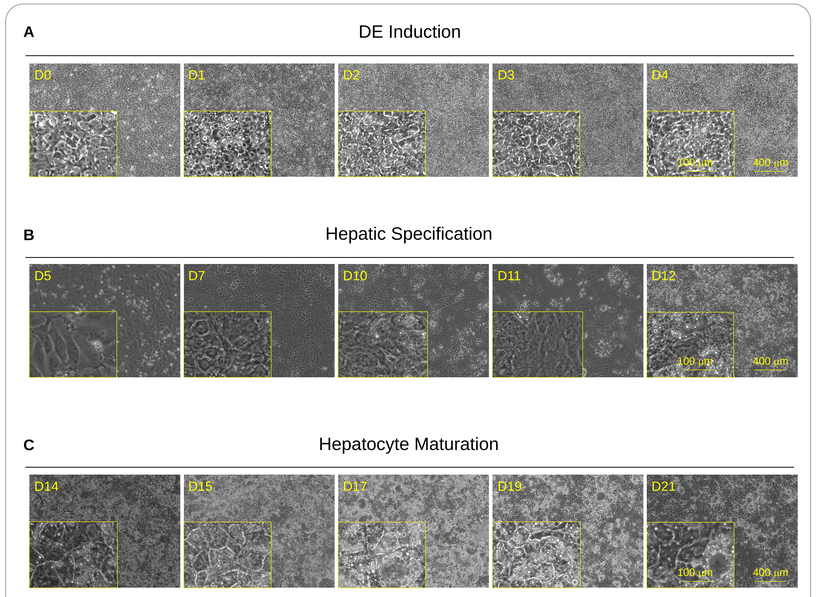


**Figure 2.** Sequential cell morphological changes during the process of HLC differentiation, shown by representative phase-contrast micrographs during (A) DE induction; (B) hepatic specification; and (C) hepatocyte maturation.

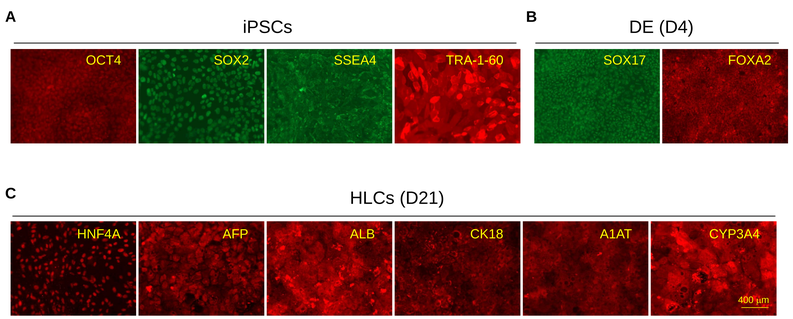


**Figure 3.** Cells generated at major stages of the differentiation expressed characteristic stage-specific protein markers, as demonstrated by immunofluorescence staining of OCT4, SOX2, SSEA4 and TRA-1-60 for iPSCs (A), SOX17 and FOXA2 for DE cells (B), and HNF4A, AFP, ALB, CK18, A1AT, and CYP3A4 for HLCs (C).

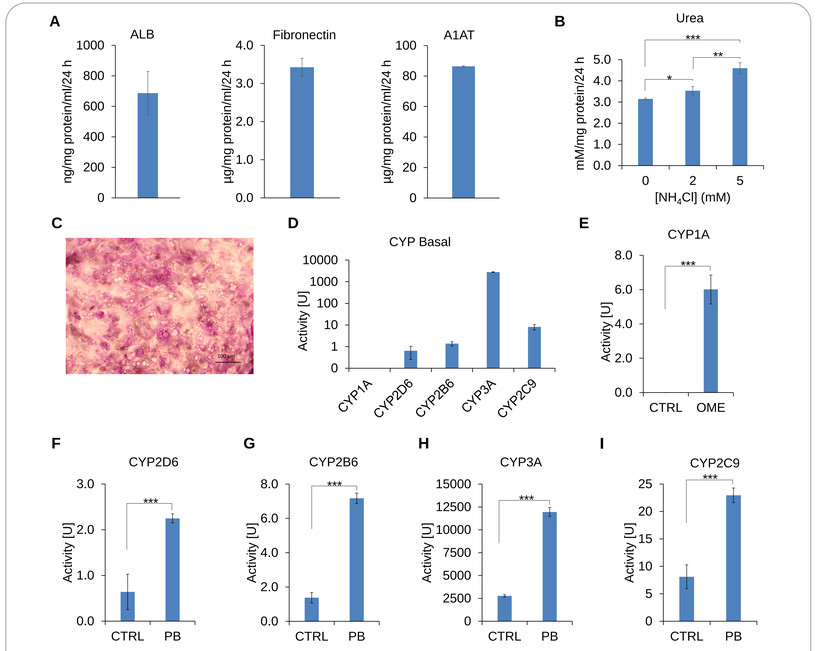


**Figure 4.** Functional characterizations of HLCs. (A) Secretion of serum proteins ALB, fibronectin, and A1AT. (B) Urea synthesis. (C) PAS staining showing glycogen storage. (D) CYP basal activity. (E–I) Induction of CYP1A, CYP2D6, CYP2B6, CYP3A, and CYP2C9, respectively, after treatment with omeprazole (OME) or phenobarbital (PB). For CYP activity, 1 unit (U) = 1 pmol metabolite/mg protein/h, and the metabolites are acetaminophen, 1'-hydroxy bufuralol, hydroxy bupropion, 1'-hydroxy midazolam, and 4'-hydroxy diclofenac for CYP1A, CYP2D6, CYP2B6, CYP3A, and CYP2C9, respectively. All values are presented as mean ± standard deviation. n = 3 for serum protein secretion and CYP activity, and n = 4 for urea synthesis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by unpaired two-tailed t-test.

## Conclusion

- A step-wise improved method has been established that enables efficient and reproducible differentiation of human iPSCs into homogeneous functional HLCs.
- The use of STEMdiff Definitive Endoderm kit, combined with Cellartis DEF-CS single-cell culture system, provides a robust induction of homogeneous DE cells.
- Single cell dissociation and re-plating of DE cells before initiating hepatic specification allows a homogeneous starting population for hepatocyte differentiation.
- DMSO and HGF are able to guide highly efficient hepatic specification. Further treatment with DEX, dihexa, and OSM promotes cell maturation into functional HLCs.
- HLCs generated using this method displayed CYP activities inducible by two well-known hepatotoxic compounds OME and PB, suggesting the potential usefulness of these cells in toxicity testing and assessment.

Regulatory Impact Statement – This work supports the US FDA in developing predictive stem cell-based screening assays for hazard assessment of chemicals of concern in its regulated products.