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ORIGINAL ARTICLE

Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells

Zhihua Song[1, 2], Jun Cai[1], Yanxia Liu[1], Dongxin Zhao[1], Jun Yong[1, 2], Shuguang Duo[1], Xijun Song[1], Yushan Guo[1, 2], Yang Zhao[1], Han Qin[1, 2], Xiaolei Yin[1, 2], Chen Wu[1, 2], Jie Che[1], Shichun Lu[3], Mingxiao Ding[1], Hongkui Deng[1, 2]

[1]The MOE Key Laboratory of Cell Proliferation and Differentiation, Department of cell biology, College of Life Sciences, Box 38, Peking University, Beijing 100871, China; [2]Laboratory of Chemical Genomics, Shenzhen Graduate School of Peking University, Shenzhen 518055, China; [3]Beijing YouAn Hospital, Capital Medical University, Beijing 100069, China

Human induced pluripotent stem (iPS) cells are similar to embryonic stem (ES) cells, and can proliferate intensively and differentiate into a variety of cell types. However, the hepatic differentiation of human iPS cells has not yet been reported. In this report, human iPS cells were induced to differentiate into hepatic cells by a stepwise protocol. The expression of liver cell markers and liver-related functions of the human iPS cell-derived cells were monitored and compared with that of differentiated human ES cells and primary human hepatocytes. Approximately 60% of the differentiated human iPS cells at day 7 expressed hepatic markers alpha fetoprotein and Alb. The differentiated cells at day 21 exhibited liver cell functions including albumin Asecretion, glycogen synthesis, urea production and inducible cytochrome P450 activity. The expression of hepatic markers and liver-related functions of the iPS cell-derived hepatic cells were comparable to that of the human ES cell-derived hepatic cells. These results show that human iPS cells, which are similar to human ES cells, can be efficiently induced to differentiate into hepatocyte-like cells.

***Keywords*:** induced pluripotent stem cells, iPS, differentiation, hepatic cells, embryonic stem cells
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Introduction

Hepatocyte transplantation restores damaged liver function in host animals and serves as a promising way to replace orthotopic liver transplantation for the treatment of a wide range of liver diseases [1]. However, sources of donor human hepatocytes are limited, and patients who receive a liver transplant require immune suppressive drugs. Embryonic stem (ES) cells essentially proliferate infinitely *in vitro* and maintain the ability to differentiate into a variety of tissue cells, including hepatocytes. Thus, ES cells can serve as an inexhaustible cell source for hepatocyte transplantation [2].

Induced pluripotent stem (iPS) cells were generated directly from fibroblast cells as the result of the introduction of four factors [3-7], and shared many characteristics with ES cells, including multi-lineage differentiation potential and intensive proliferation *in vitro*. In addition, the establishment of human iPS cells is ethically acceptable and does not require oocytes. Thus, through human iPS cell differentiation, genetically identical somatic cells could be generated for the treatment of a wide variety of diseases. The human iPS cells were shown to be able to differentiate into neural cells [8-11], osteogenic cells

Correspondence: Hongkui Deng
Fax: +86-010-6275-6954
E-mail: hongkui_deng@p ku.edu.cn

Abbreviations: iPS cells (induced pluripotent stem cells); ES cells (embryonic stem cells); MEF (mouse embryonic fibroblast); HCM (Hepatocyte Culture Medium); FGF4 (fibroblast growth factor 4); BMP2 (bone morphogenetic protein 2); HGF (hepatocyte growth factor); KGF/FGF7 (keratinocyte growth factor/fibroblast growth factor 7); OCT4 (octamer-binding factor 4); KLF4 (Krüppel-like factor4); UTF1 (undifferentiated embryonic cell transcription factor 1); PBS (phosphate-buffered saline); SOX (SRY-box containing gene); FoxA2 (forkhead box A2); CK (cytokeratin); AFP (alpha fetoprotein); ALB (albumin); AAT (alpha 1-antitrypsin); Cyp (cytochrome P450); PEPCK (phosphoenolpyruvate carboxykinase); HNF4α (hepatocyte nuclear factor 4α); HNF6 (hepatocyte nuclear factor 6); CEBPα (CCAAT enhancer binding protein alpha); TDO2 (tryptophan 2,3-dioxygenase); RT-PCR (reverse transcription PCR); PAS (Periodic Acid-Schiff)

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[12], cardiac cells [13], adipogenic cells [14], pancreatic cells [15, 16], vascular cells [17], hematopoietic [12, 18] and endothelial cells [18]. However, the directed differentiation of human iPS cells into hepatic cells has not yet been reported.

We have recently developed an efficient method to establish human iPS cell lines with a new set of four transcription factors [19], and we have previously established a protocol to induce the efficient hepatic differentiation of human ES cells [20]. In this study, human iPS cell lines were induced to differentiate into hepatic cells with a modified protocol. The differentiated iPS cells expressed hepatic markers and possessed human hepatocyte functions. By monitoring the expression of liver cell markers at different differentiation stages, we found that human iPS cells can be directly induced to differentiate into hepatocyte-like cells with the very similar differentiation process to that of human ES cells. Our study shows that human iPS cells could potentially be a promising cell source for liver disease-related researches.

Results

Characterization of human iPS cells

In this study, we utilized human iPS cell lines 3U1 and 3U2 as two representative cell lines from a panel of human iPS cell lines that were generated in our laboratory. These two cell lines were established by transducing the genes *Oct-4*, *Sox2*, *Klf4* and *Utf1* into adult human fibroblasts [19]. To check the stemness of the iPS cell lines, the expression of core transcription factors and surface markers including octamer-binding factor 4 (OCT4), NANOG, SRY-box containing gene (SOX)2, SSEA4, TRA1-60 and TRA1-81 was tested by reverse transcription PCR (RT-PCR) or immunofluorescence assay, and the results showed that the expression pattern of these stem cell markers in iPS cells is similar to that of the H1 hES cells (Supplementary information, Figure S1).

Efficient hepatic differentiation of human iPS cells

Here, we modified our previous protocol [20] and developed an improved differentiation method for hES cells and human iPS cells, as shown in Figure 1A. This new hepatic differentiation protocol was composed of four stages: endoderm induction, hepatic specification, hepatoblast expansion and hepatic maturation. 3U1 and 3U2 iPS cells were able to differentiate into hepatic cells in comparison to a well-established human ES cell line H1, which was able to be induced efficiently to differentiate into hepatic lineage cells [20]. During the differentiation,

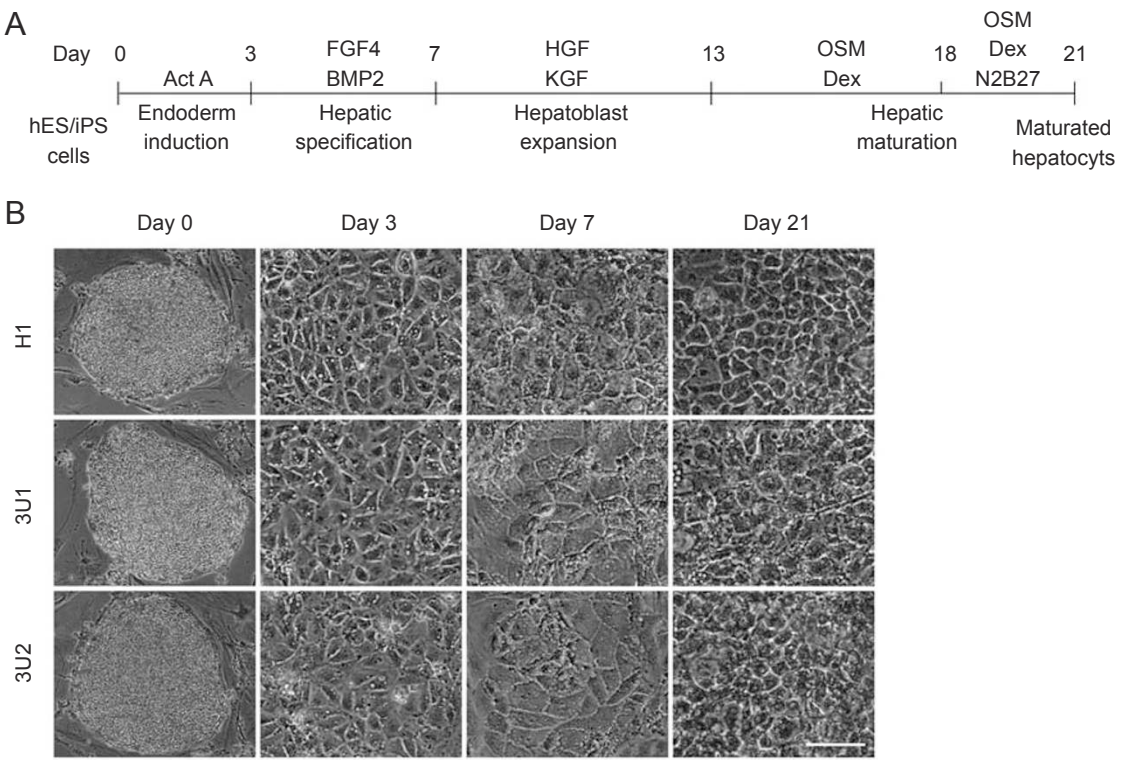

Figure 1 The hepatic differentiation of human iPS cells and ES cells. **(A)** A flow chart showing the stepwise differentiation protocol. **(B)** Morphological changes of human ES cells and iPS cells at different stages of differentiation. Scale bar = 100 μm.

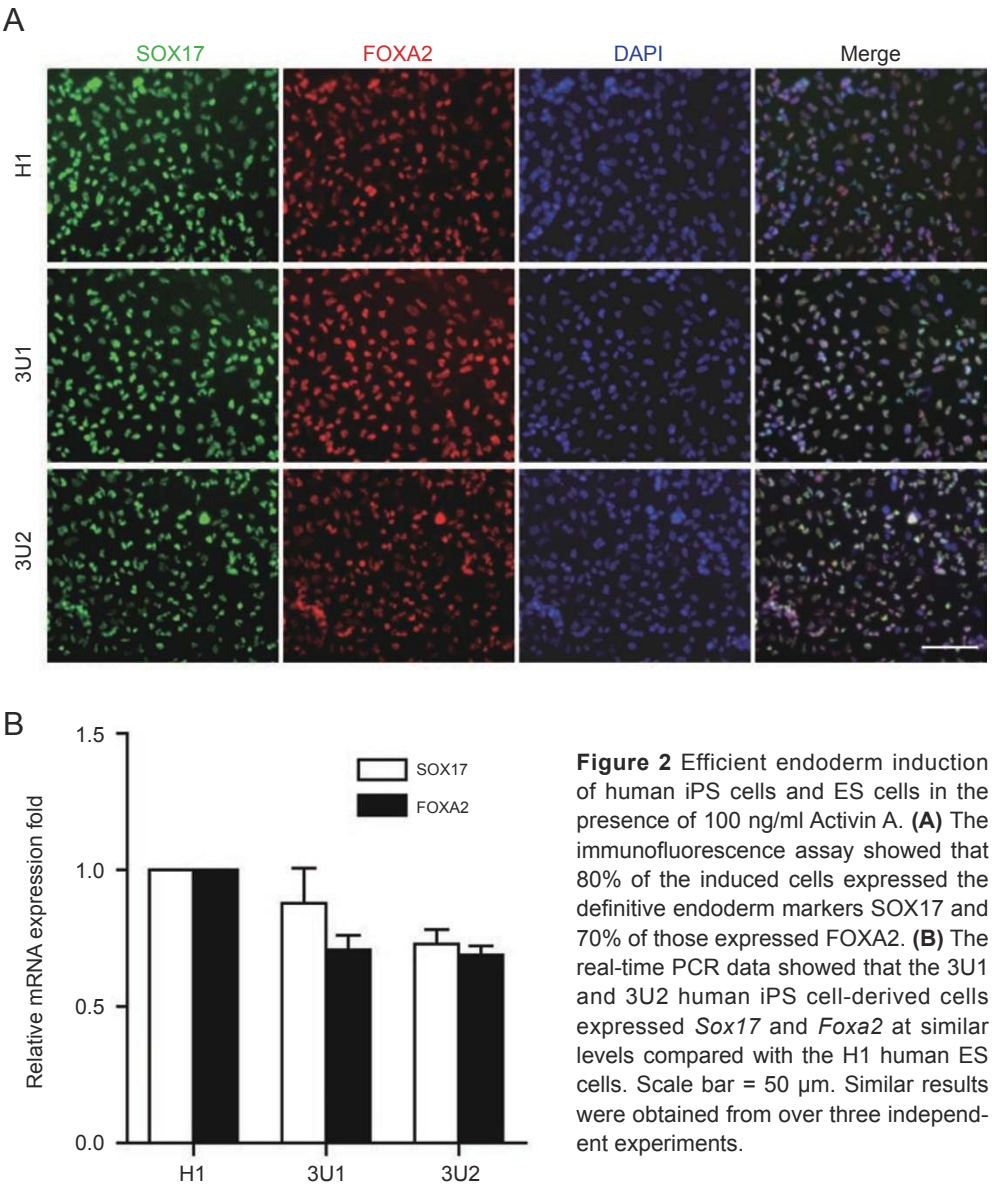

Figure 2 Efficient endoderm induction of human iPS cells and ES cells in the presence of 100 ng/ml Activin A. **(A)** The immunofluorescence assay showed that 80% of the induced cells expressed the definitive endoderm markers SOX17 and 70% of those expressed FOXA2. **(B)** The real-time PCR data showed that the 3U1 and 3U2 human iPS cell-derived cells expressed *Sox17* and *Foxa2* at similar levels compared with the H1 human ES cells. Scale bar = 50 μm. Similar results were obtained from over three independent experiments.

the cell morphology change of 3U1 and 3U2 human iPS cells resembled that of the H1 hES cells at each differentiation stage (Figure 1B).

In the first stage, Activin A efficiently induced the endoderm differentiation of both human iPS cell lines 3U1 and 3U2, and the hES cell line H1. After 3 days of Activin A treatment, approximately 80% of the cells in culture expressed the endoderm marker SOX17, 70% of the cells in culture expressed another endoderm marker forkhead box A2 (FOXA2), and about 50-60% of the total cells in culture were double positive (Figure 2A), but no cell was found to express the extraembryonic endoderm cell markers SOX7 or CDX2 (data not shown), as detected by immune staining of the differentiated cells. The expression of endoderm genes in the differentiated cells was also checked by real-time PCR, and the results showed that human iPS cell-derived cells express endoderm markers *SOX17* and *FOXA2* at similar levels compared with H1 hES cell-derived cells (Figure 2B), while at day 0, neither hES cells H1 nor iPS cells 3U1 and 3U2 expressed SOX17 or FOXA2 (Supplementary information, Figure S2A and S2B). Similar results were obtained from over three independent experiments. These results indicate that human iPS cells could be efficiently induced to differentiate into endoderm cells by Activin A.

To induce hepatic specification from endoderm cells, fibroblast growth factor 4 (FGF4) and bone morphogenetic protein 2 (BMP2) were added to the culture. After

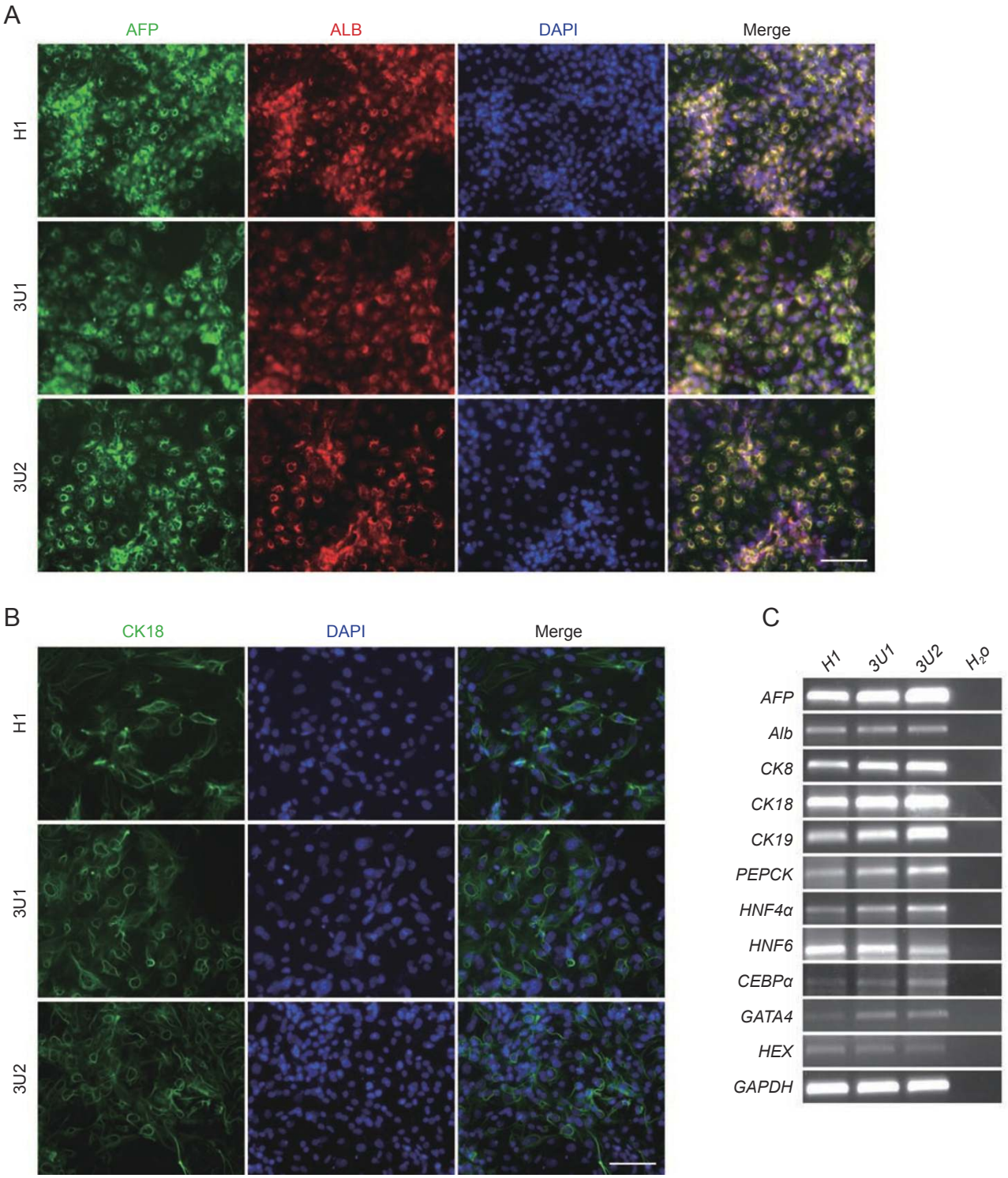

Figure 3 Hepatic specification of human iPS cell- and ES cell-derived endoderm cells after 4 days of treatment with FGF and BMP. **(A**, **B)** At day 7, the differentiated cells expressed the hepatic cell markers AFP, ALB and CK18. **(C)** *AFP*, *Alb*, *CK8*, *CK18, CK19* and *PEPCK* mRNA were expressed at this stage and the liver-enriched transcription factors *HNF4α*, *HNF6*, *CEBPα*, *GATA4* and *HEX* were activated. H_2O was used as a negative control. Scale bar = 100 μm. Similar results were obtained in at least three independent experiments.

4 days of treatment, the cells were tested for the expression of hepatic markers and liver-enriched transcription factors by immunostaining or RT-PCR. For both iPS cell and hES cell differentiation, approximately 60% of the cells in culture expressed the hepatic cell markers alpha fetoprotein (AFP), ALB (albumin) (Figure 3A) and more differentiated cells expressed cytokeratin (CK)18 (Figure 3B), as detected by immunofluorescence. We checked the expression of AFP, ALB and CK18 at day 0 in the undifferentiated iPS cells and found negative staining (Supplementary information, Figure S2). The differentiated cells also expressed hepatic genes including *AFP*,

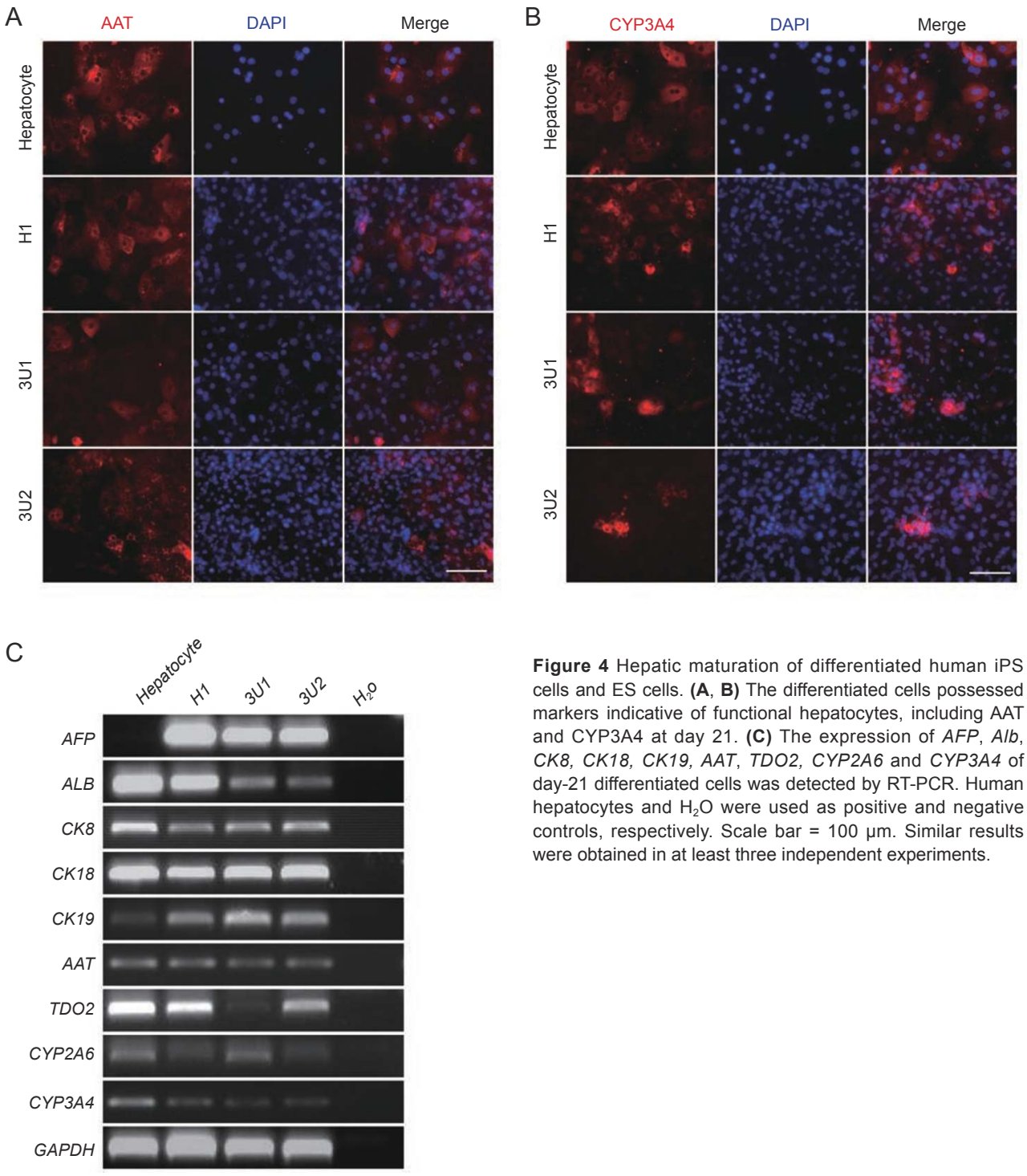

Figure 4 Hepatic maturation of differentiated human iPS cells and ES cells. **(A**, **B)** The differentiated cells possessed markers indicative of functional hepatocytes, including AAT and CYP3A4 at day 21. **(C)** The expression of *AFP*, *Alb*, *CK8, CK18, CK19, AAT*, *TDO2, CYP2A6* and *CYP3A4* of day-21 differentiated cells was detected by RT-PCR. Human hepatocytes and H_2O were used as positive and negative controls, respectively. Scale bar = 100 μm. Similar results were obtained in at least three independent experiments.

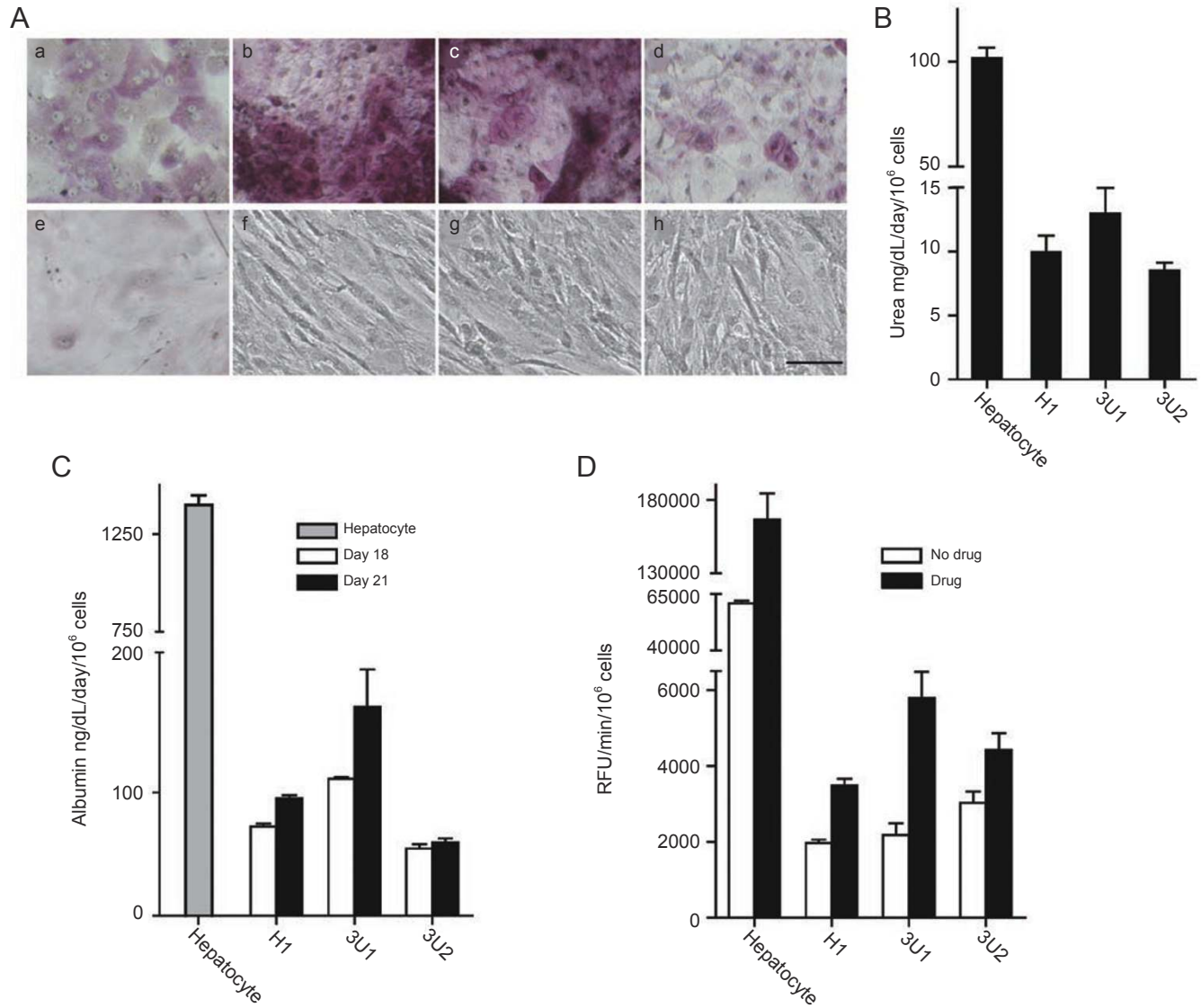

Figure 5 Functional testing of the maturated hepatic cells derived from human iPS cells and ES cells. **(A)** Glycogen synthesis was tested by PAS staining at day 21. a and e, human hepatocytes and feeder cells, respectively; b, c, d and f, g, h represent H1 ES cell-, 3U1 and 3U2 iPS cell derivatives with or without growth factors, respectively. Scale bar = 100 μm. **(B)** Urea productions by H1-, 3U1- and 3U2-derived cells. **(C)** Albumin secretion of differentiated cells from H1 ES cell-, 3U1 and 3U2 iPS cells. **(D)** The differentiated human iPS cells and hES cells exhibited cytochrome P450 isozyme activities after phenobarbital sodium induction. Human hepatocytes were used as a positive control.

ALB, *CK8*, *CK18*, *CK19* and *PEPCK* and the liver-enriched transcription factors *HNF4α*, *HNF6*, *CEBPα*, *GATA4* and *HEX* at the mRNA level, as detected by RT-PCR (Figure 3C), while these genes were not expressed in the undifferentiated iPS cells and hES cells (data not shown). These results indicated that human iPS cells could be efficiently induced into hepatic lineage at this stage. Similar results were obtained in at least three independent experiments.

To expand these early hepatic cells, hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) were then added to the culture medium during the third stage. We tested for the expression of Ki67, a marker of proliferating cells, at day 13 in the differentiated H1 hES cells and 3U1 and 3U2 iPS cells. As shown in Supplementary information, Figure S3, compared to the control group that did not have KGF treatment, the addition of KGF led to more Ki67-positive cells in culture, and consequently more cells were double positive for AFP and Ki67. Similar results were obtained in at least three independent

experiments.

At the end of the final differentiation stage, we tested the expression of hepatic markers in the differentiated 3U1 and 3U2 human iPS cells. A majority of the day-21 cells expressed hepatic markers, such as AFP and Alb (data not shown). Furthermore, at day 21, some of the differentiated 3U1 and 3U2 human iPS cells were stained positive for alpha 1-antitrypsin (AAT) (Figure 4A) and CYP3A4 (Figure 4B), a pattern similar to H1 hES cell derivatives and human hepatocytes. The RT-PCR data showed that the hepatic functional genes, including *Alb*, *AAT*, *TDO2*, *CYP2A6* and *CYP3A4*, as well as other liver-enriched genes including *AFP*, *CK8*, *CK18* and *CK19* were expressed (Figure 4C). Similar results were obtained in at least three independent experiments.

Evaluation of the maturated hepatic cells derived from human iPS cells

We performed several experiments to further test liver cell-specific functions in the differentiated human iPS cells. At day 21, we assayed glycogen storage by Periodic Acid-Schiff (PAS) staining in the hepatic cells derived from the 3U1 and 3U2 human iPS cells with amylase pre-treatment. Similar to H1 hES cell-derived cells and human hepatocytes, many of the differentiated 3U1 and 3U2 human iPS cells were stained by PAS (Figure 5A). The differentiated cells of both H1 hES cells and 3U1 and 3U2 iPS cells without growth factor treatment showed fibroblast-like morphology, and were hardly PAS positive (Figure 5A, f, g and h). In addition, the mouse embryonic fibroblast (MEF) feeder cells were also PAS negative (Figure 5A, e).

Urea synthesis of the differentiated cells was tested with a urea nitrogen determination system. At the end of the hepatic maturation stage, urea could be detected in the culture medium supernatant of the day 21 differentiated 3U1 and 3U2 human iPS cells. The production levels were similar to that of hES cell derivatives (Figure 5B). We performed this assay thrice with four parallel samples in each experiment, and similar results were obtained. Human hepatocytes were used here as a positive control.

To detect albumin secretion, we collected culture medium supernatant samples and measured albumin levels with an ELISA assay. Hepatic cells derived from 3U1 and 3U2 human iPS cells secreted albumin at the similar level to hES cell derivatives both at days 18 and 21 (Figure 5C). The culture medium of undifferentiated iPS cells and hES cells including MEF feeder had a blank readout in the ELISA assay test. We repeated this assay thrice with four parallel samples each time, and obtained similar results. Human hepatocytes were used as a positive control.

The inducible P450 activity of the differentiated cells was also tested. As shown in Figure 5D, the differentiated human iPS cells exhibited cytochrome P450 isozyme activities after phenobarbital sodium induction, and the pattern of response to the drug induction was similar to hES cell-differentiated cells (Figure 5D). We performed this assay thrice with two parallel samples with or without drug induction in each experiment, and similar results were obtained. Human hepatocytes were used here as a positive control. Altogether, these functional results indicate that the differentiated human iPS cells are similar to human ES cell derivatives, and that the induced hepatocyte-like cells from human iPS cells possess some characteristics of adult hepatocytes.

Discussion

Here, we show that human iPS cells can be directly induced to differentiate into hepatocyte-like cells using a stepwise differentiation method. The hepatic differentiation efficiency of human iPS cells is comparable to that of the H1 human ES cell line. Our results showed that during human iPS cell differentiation, the process of endoderm cell induction and hepatocyte marker expression was very similar to that of differentiated human ES cells. The functions and hepatic marker expression of the human iPS cell-derived hepatic cells were similar to that of the hepatic cells differentiated from human ES cells (Figures 4, 5 and Supplementary information, Table S1). To our knowledge, this is the first time that human iPS cells are shown to be able to be directly differentiated into hepatic cells. The iPS cell technology allows for the generation of patient-specific iPS cells. Therefore, by comparing the hepatic differentiation process of iPS cells from liver disease patients and that of normal iPS cells, the *in vitro* differentiation of human iPS cells could serve as a model to study the mechanisms of human liver disease development.

Some previous reports studying iPS cell differentiation showed that the gene expression patterns differ between the cells differentiated from iPS and ES cells [14, 21]. However, the iPS cells used in these studies were established in the presence of c-Myc. Because c-Myc is essential for the self-renewal and differentiation of ES cells [22, 23], the reactivation of c-Myc may interfere with the differentiation of iPS cells. To build a disease model with iPS cells, it is preferable to use iPS cell lines established without c-Myc. In the future, it would be better to use human iPS cell lines established by non-integrative methods [24-28].

An important potential use of human iPS cell-derived

hepatic cells is for drug development. Most drugs rely on liver P450 activity for detoxification, which cannot be tested in animal liver cells due to species differences [29]. CYP3A4 is involved in the metabolism of a large range of xenobiotics. In this study, we tested the expression of CYP in the differentiated cells and found that the human iPS cell- and ES cell-derived hepatic cells expressed Cyp3A4, as detected by RT-PCR (Figure 4C) and immune staining (Figure 4B). These cells also possessed inducible P450 2B4 activity (Figure 5D), suggesting that human iPS cell-derived hepatic cells possess liver cell functions in certain extent and may also be used as a potential cell source for the generation of hepatocytes for drug metabolism tests. Because human iPS cells can be established from different populations, human iPS cell-derived hepatic cells could potentially be used to study the differences in drug metabolism among populations.

In this study, we compared human ES cell- and iPS cell-derived hepatic cells with human hepatocytes in liver-enriched gene expression and liver-specific functions (Figures 4, 5 and Supplementary information, Table S1). Considering the urea synthesis and albumin secretion, the production levels of either hES cell- or iPS cell-derived hepatic cells were approximately 10% of those of human hepatocytes (Figure 5B and 5C). The cytochrome P450 isozyme activities of the differentiated hES and iPS cells were nearly 30-fold lower than those of human hepatocytes either at basal level or after drug induction (Figure 5D). In addition, the liver-enriched genes including *AAT*, *TDO2*, *CYP2A6* and *CYP3A4* examined in the differentiated hES and iPS cells were less expressed compared with human hepatocytes (Figure 4C). These results suggest that the differentiated human iPS cells and ES cells need to be further improved at the final maturation stage.

Materials and Methods

Culture and differentiation of the human iPS cells and ES cells into definitive endoderm

The human ES cell line H1 was obtained from the WiCell research institute (Madison, WI, USA). The passage number of the H1 cells used here ranged from 33 to 48. Human iPS cell lines 3U1 and 3U2 were established by transducing human fibroblasts with lentivirus containing *Oct-4*, *Sox2*, *Klf4* and *Utf1*, as previously described [19]. The passage number of the 3U1 and 3U2 cells used here ranged from 4 to 19. Human ES cells and iPS cells were cultured in DMEM/F12 with 20% knockout serum replacement, 1 mM/l glutamax, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 100 U penicillin/streptomycin (all from Invitrogen/Gibco, Grand Island, NY, USA), and 10 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA) on a mitomycin-C-treated MEF feeder layer in a standard gas atmosphere of humid air with 5% CO_2.

For endoderm induction, human ES cells and iPS cells were incubated for 24 h with RPMI 1640 medium (Invitrogen/Gibco, Rockville, MD, USA), supplemented with 0.5 mg/ml albumin fraction V (Sigma-Aldrich, St Louis, MO, USA) and 100 ng/ml Activin A (Peprotech). On the following 2 days, 0.1 and 1% insulin-transferrin-selenium (Invitrogen/Gibco) was added to this medium, respectively.

Hepatic differentiation of human iPS cells and ES cells

Following Activin A treatment, the differentiated human iPS cells and hES cells were cultured in Hepatocyte Culture Medium (HCM) (Cambrex, Baltimore, MD, USA) containing 30 ng/ml FGF4 and 20 ng/ml BMP2 (both from Peprotech) for 4 days. Then, the differentiated cells were incubated in HCM containing 20 ng/ml HGF and 20 ng/ml KGF (both from Peprotech) for 6 days, in HCM containing 10 ng/ml oncostatin-M (R&D, Minneapolis, MN, USA) plus 0.1 μM dexamethasone (Sigma-Aldrich) for 5 days, and in DMEM containing N2, B27, 1 mM/l glutamax, 1% nonessential amino acids and 0.1 mM β-mercaptoethanol (all from Invitrogen/Gibco) for another 3 days.

Human hepatocytes isolation and culture

Primary human hepatocytes were isolated from donor livers that were not used for liver transplantation following informed consent, by a two-step EDTA/collagenase perfusion and then cryopreserved [30]. Cell viability was more than 80% as determined by trypan blue exclusion. After thawing, human hepatocytes were cultured in matrigel (BD, NJ, USA) coated dishes with HCM. The medium was refreshed everyday.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde (Dingguo, Beijing, China) for 15 min, blocked and permeabilized with 10% normal goat or rabbit serum (Zsbio, Beijing, China) and 0.2% tritonX-100 (Fisher, UK) in phosphate-buffered saline (PBS) at room temperature for 45 min. Then the cells were incubated with primary antibody overnight at 4 °C. For surface markers staining, the permeabilization step was omitted. Cells were washed with PBS between each step. The primary antibodies against human rabbit anti Oct4 (Abcam, La Jolla, CA, USA), goat anti Nanog (R&D), mouse anti SSEA4, mouse anti TRA-1-60, mouse anti TRA-1-81 (Santa Cruz, CA), rabbit anti Ki67 (Invitrogen), goat anti SOX17 (R&D), rabbit anti FOXA2 (Upstate), mouse anti CK18 and mouse anti AFP (Invitrogen) were diluted at 1:200, the antibody against human rabbit anti ALB (DAKO, Glostrup, Denmark) was diluted at 1:500, and the antibodies against human rabbit anti AAT (Invitrogen) and rabbit anti CYP3A4 (AbD Serotec, Oxford, UK) were diluted at 1:200. After five washes with PBS, FITC-conjugated or TRITC-conjugated secondary antibody (Invitrogen) was diluted at 1:200 and applied to the cells at 4 °C overnight. Then, 1 μg/ml 4,6-diamidino-2-phenylindole (Roche, Germany) was used to stain the cell nuclei. Also, corresponding isotype antibody or the normal serum from the same species with the primary antibody was used as negative control.

RT-PCR and real-time PCR analysis of gene expression

Reverse transcription PCR and real-time PCR were performed as described previously [20]. Total RNA was isolated from cells using TRIzol Reagent (Invitrogen) and reverse-transcribed using a reverse transcription system (Promega, Madison, WI, USA) ac-

cording to the manufacturer's protocol. Prior to reverse transcription, the RNA samples were treated with RNase-free DNase I (NEB, Ipswich, MA, USA) to remove the trace DNA contamination.

PAS stain for glycogen

The PAS staining system was purchased from Sigma-Aldrich. Cells in culture dishes were fixed in 4% paraformaldehyde and were treated with 5 g/l amylase (Sigma) for 15 min at 37 °C as described [31]. The further assay was performed according to the manufacturer's instructions.

Urea nitrogen kinetic quantitative determination

The urea nitrogen determination system was purchased from STANBIO (Boerne, TX, USA). Cells were trypsinized and counted with a hemocytometer. The sample supernatants were stored at −20 °C and the assay was performed according to the manufacturer's instructions. We measured the absorbance using a Synergy HT Multi-Detection Microplate Reader from BioTek with Gen5 software. The urea production was normalized to the total cell numbers.

Albumin secretion ELISA assay

The human albumin content in the supernatant was determined by the Human Albumin ELISA Quantitation kit (Bethyl Laboratory, Montgomery, TX, USA) according to the manufacturer's instructions. Cells were trypsinized and counted with a hemocytometer. The albumin secretion was normalized to the total cell numbers.

Cytochrome P450 activity assay

The cytochrome P450 2B fluorescent detection kit was purchased from Sigma-Aldrich. For the cytochrome P450 activity assay, 200 μg/ml phenobarbital sodium was added to the differentiated human iPS and ES cells during the last 3 days and human hepatocytes for 3 days. The medium was refreshed everyday. The samples were homogenized with an Ultrasonic crusher (Scientz, Ningbo, China) and the assay was performed according to the manufacturer's instructions. We measured the fluorescence with the BioTek Multi-Detection Microplate Reader, using Gen5 software. The maximum kinetic reaction velocity was normalized to the total cell numbers.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)