RAPID COMMUNICATION

Generation of Functional Human Hepatic Endoderm from Human Induced Pluripotent Stem Cells

Gareth J. Sullivan,[1] David C. Hay,[1] In-Hyun Park,[2] Judy Fletcher,[1] Zara Hannoun,[1] Catherine M. Payne,[1] Donna Dalgetty,[1] James R. Black,[1] James A. Ross,[1] Kay Samuel,[1] Gang Wang,[1] George Q. Daley,[2] Je-Hyuk Lee,[3] George M. Church,[3] Stuart J. Forbes,[1] John P. Iredale,[1] and Ian Wilmut[1]

With the advent of induced pluripotent stem cell (iPSC) technology, it is now feasible to generate iPSCs with a defined genotype or disease state. When coupled with direct differentiation to a defined lineage, such as hepatic endoderm (HE), iPSCs would revolutionize the way we study human liver biology and generate efficient "off the shelf" models of human liver disease. Here, we show the "proof of concept" that iPSC lines representing both male and female sexes and two ethnic origins can be differentiated to HE at efficiencies of between 70%-90%, using a method mimicking physiological relevant condition. The iPSC-derived HE exhibited hepatic morphology and expressed the hepatic markers albumin and E-cadherin, as assessed by immunohistochemistry. They also expressed alpha-fetoprotein, hepatocyte nuclear factor-4a, and a metabolic marker, cytochrome P450 7A1 (Cyp7A1), demonstrating a definitive endodermal lineage differentiation. Furthermore, iPSC-derived hepatocytes produced and secreted the plasma proteins, fibrinogen, fibronectin, transthyretin, and alpha-fetoprotein, an essential feature for functional HE. Additionally iPSC-derived HE supported both CYP1A2 and CYP3A4 metabolism, which is essential for drug and toxicology testing. *Conclusion:* This work is first to demonstrate the efficient generation of hepatic endodermal lineage from human iPSCs that exhibits key attributes of hepatocytes, and the potential application of iPSC-derived HE in studying human liver biology. In particular, iPSCs from individuals representing highly polymorphic variants in metabolic genes and different ethnic groups will provide pharmaceutical development and toxicology studies a unique opportunity to revolutionize predictive drug toxicology assays and allow the creation of *in vitro* hepatic disease models. (HEPATOLOGY 2010;51:329-335.)

Human induced pluripotent stem cells (iPSCs) are reprogrammed mature somatic fibroblasts which represent a pluripotent cell population able to generate all primary cell types *in vitro*.[1-3] The ability to derive iPSCs from an indefinite range of genotypes makes them an attractive resource on which to model liver function reflecting the complexity of polygenic influences on metabolism *in vitro*. Another facet of iPSC technology is the ability to study the impact of gene polymorphisms in a native chromatin setting and model gene interactions with precision. Therefore iPSC-derived models hold great potential to develop a detailed understanding of human liver disease and metabolism including drug toxicity (for a review, see Dalgetty et al.[4]). Any methods which might

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HE, hepatic endoderm; hESC, human embryonic stem cell; iPSC, induced pluripotent stem cell; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; PHH, primary human hepatocyte;

From the [1]Medical Research Council (MRC) Centre for Regenerative Medicine, University of Edinburgh, Edinburgh, UK; [2] Harvard Stem Cell Institute, Division of Pediatric Hematology/Oncology, Cambridge, MA; and [3]Department of Genetics, Harvard Medical School, Boston, MA.

Received July 31, 2009; accepted September 21, 2009.

Address reprint requests to: Gareth J. Sullivan or David C. Hay, MRC Centre for Regenerative Medicine, Chancellor's Building, 49, Little France Crescent, Edinburgh, UK EH16 4SB. E-mail: (G.J.S.) gsulliva@staffmail.ed.ac.uk; (D.C.H.) davehay@talktalk.net; fax: +44 (0) 131 242 6629.

Address reprint requests to: In-Hyun Park, Harvard Stem Cell Institute, Division of Pediatric Hematology/Oncology, Cambridge, MA. E-mail: InHyun.Park@childrens.harvard.edu; fax: 617-730-0222.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23335

Potential conflict of interest: Dr. Daley is a consultant for and advises MPM Capital, Pierian, Epizyme, and Solasia.

Additional Supporting Information may be found in the online version of this article.

streamline and standardize the process of drug and toxicology testing, which currently relies on primary human hepatocytes (PHHs), would represent a significant development. Therefore, an iPSC resource representative of polymorphic variants and ethnic groups, unhindered by quality and supply, would revolutionize predictive drug toxicology assays and have an effect on drug attrition.

Presently, PHHs are the gold standard cell type used in predictive drug toxicology. Unfortunately, PHHs are a scarce, heterogeneous, and expensive resource which function only short-term *in vitro*. The generation of hepatic endoderm (HE) from iPSCs has the potential to fulfill the major challenge to acquire the reliable and clonal source of functional human hepatocyte cells for biotechnology purposes. To date, efficient models of deriving HE from iPSCs have not been described or developed. Capitalizing on our recent investigations that human embryonic stem cells (hESCs) can be stimulated to form HE,[5] we have developed a parallel methodology for iPSCs; here, we describe the generation of functional HE from multiple human iPSC lines that can potentially model human drug metabolism.

Materials and Methods

Generation of Human iPSCs. iPSCs from diabetic North American Indian (JDM-iPS1) and female Caucasian (PGP9-iPS1) were reported previously.[6,7] Fibroblasts (1×10^5) of normal male Caucasian (American Type Culture Collection; CRL-2465) were plated in one well of a six-well plate and infected with four individual retroviruses, each containing a single reprogramming factor (Oct4 [octamer transcription factor 4], Sox2 [SRY-related HMG box 2], Klf4 [Kruppel-like factor 4], and c-MYC), was used at a multiplicity of infection of 10.[1] After 3 days of infection, cells were split into 10-cm plates preseeded with irradiated mouse embryonic fibroblasts (MEFs) and cultured under hESC culture medium conditions until colonies appeared. Colonies were picked, replated onto irradiated MEFs, and expanded for characterization.

Cell Culture and Differentiation. iPS cell colonies were maintained in hESC medium (80% knockout/Dulbecco's modified Eagle medium [KO/DMEM], 20% KO serum replacement [SR], 10 ng/mL basic fibroblast growth factor, 1 mM L-glutamine, 100 mM nonessential amino acids, 100 mM 2-mercaptoethanol, 50 U/mL penicillin, and 50 mg/mL streptomycin [Invitrogen]) on an irradiated mouse embryonic feeder layer (CF-1, VHbio). Before HE differentiation, iPSCs were cultured on Matrigel (BD Biosciences). The iPSCs were differentiated to hepatocyte-like cells using activin A and Wnt3a (R&D Systems) on Matrigel (BD Biosciences). Although the differentiation protocol was similar to that of Hay et al.,[5] one major modification was required in order to generate human HE from human iPSCs. In brief, after iPSCs were passaged onto Matrigel and cultured in MEF-conditioned medium until a confluence of 50%-70% was attained, MEF-conditioned medium was then replaced with Roswell Park Memorial Institute/B27, and iPSCs were treated with activin A and Wnt3a for 3 days and required a further 2-day incubation in activin A (100 ng/mL) alone before HE was specified using established conditions as follows: Cells were cultured in SR/DMSO (KO/DMEM containing 20% SR, 1 mM glutamine, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 1% dimethyl sulfoxide [DMSO]). The final maturation step involved culturing the cells in L-15 medium which was supplemented with 8.3% fetal bovine serum, 8.3% tryptose phosphate broth, 10 μM hydrocortisone 21-hemisuccinate, 1 μM insulin, 2 mM glutamine, with 10 ng/mL hepatocyte growth factor and 20 ng/mL oncostatin M.[5] For further information, see Supporting Fig. 2.

Flow Cytometry. Cells were resuspended at 1×10^7 cells/mL in fluorescence-activated cell sorting/phosphate-buffered saline (FACS-PBS) (PBS supplemented with 0.1% bovine serum albumin and 0.1% sodium azide). Aliquots of 1×10^6 cells were incubated for 40 minutes at 4°C with optimum concentration (determined by titration), of primary antibody to stage-specific embryonic antigen-1 (SSEA1) (mouse immunoglobulin M [IgM]), SSEA4 (mouse IgG_3) (Developmental Studies Hybridoma Bank, Iowa City, IA), TRA-1-60 (anti-mouse IgM; Chemicon), and epithelial cell adhesion molecule–allophycocyanin (CD326, Biolegend). Cells were washed twice to remove unbound antibody and resuspended in 100 μL FACS-PBS. Binding of primary antibody was detected using optimum concentration (determined by titration) of appropriate isotype-specific fluorochrome-labeled secondary antibody or avidin:anti-mouse IgM-phycoerythrin (PE) and anti-rat IgM-PE and anti-mouse IgG_3–fluorescein isothiocyanate (Jackson Laboratory) and streptavidin (Caltag Medsystems). After incubation for 40 minutes at 4°C, cells were washed twice and finally resuspended in 250 μL FACS-PBS. Unstained cells and cells labeled with secondary antibody alone were included as controls. Dead and apoptotic cells and debris were excluded from analysis using an electronic "live" gate on forward scatter and side scatter parameters. Data for up to 25,000 "live" events were acquired for each sample using a FACSCalibur cytometer equipped with 488 nm and 633 nm lasers and analyzed using CellQuest software (Becton Dickinson).

Reverse Transcription Polymerase Chain Reaction. RNA was isolated using RNeasy kit (Qiagen) following manufacturer's instruction and DNA was removed by the treatment with deoxyribonuclease (Qiagen). Complementary DNA was synthesized using 2 μg total RNA with reverse transcriptase (Roche Diagnostics) in a 20-25 μL volume. Polymerase chain reaction (PCR) was carried out as described.[1,5] Primer sequences and PCR conditions are provided in Supporting Table 1. The quantitative PCR analysis was carried out as described in Hay et al.[5]

Assay for Teratoma Formation. For teratoma formation, cells were harvested, washed once with DMEM/F12, and mixed with Matrigel (BD Biosciences) and collagen.[8,9] Cells (1×10^6) were injected intramuscularly into immune-compromised NSG (NOD [nonobese diabetic] SCID [severe combined immunodeficient] gamma) mice. Teratomas formed within 6-8 weeks, and paraffin sections were stained with Masson's trichromatin for all histological determinations.

Immunocytochemistry. Cells were fixed with chilled methanol (−20°C) for 10 minutes, washed with PBS, and blocked with 10% goat serum and 0.02%-0.1% Triton X-100 for 1 hour. The cells were then incubated with primary antibody at the appropriate dilution at 4°C overnight. Secondary antibody was applied for 30 minutes after washing with PBS. The cells were finally mounted with Mowiol (Calbiochem) and then visualized and captured using a Leica DM IRB microscope.

Enzyme-Linked Immunosorbent Assay. Enzyme-linked immunosorbent assays (ELISAs) were carried out as previously described.[5]

Cytochrome P450 Assay. CYP1A2 and CYP3A4 activity was assessed using the pGlo kit (catalog numbers V8771, V8901; Promega) according to manufacturer's instruction for nonlytic CYP450 activity estimation. CYP activities are expressed as relative light units (RLU/mL) per of media, normalized against percentage of hepatocyte-like cells.

Results

We have previously demonstrated successful generation of iPSCs that are capable of self-renewal and

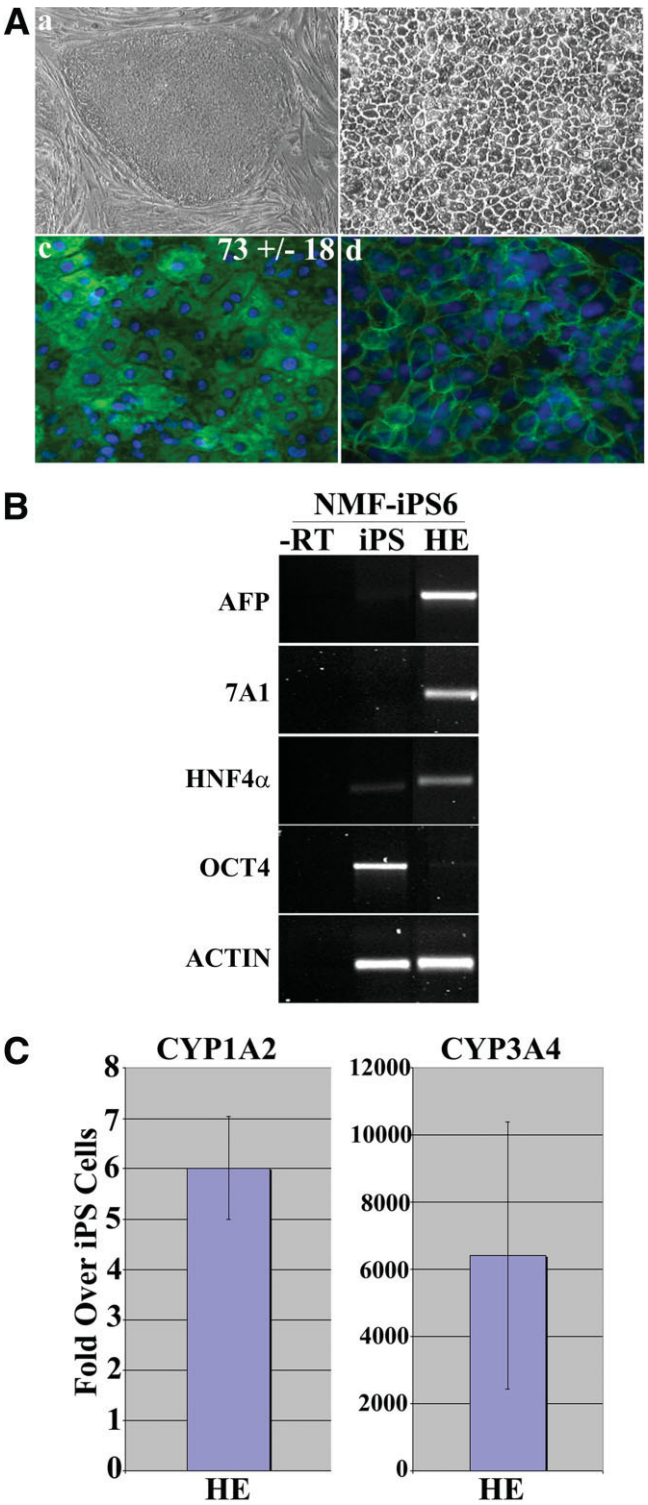

Fig. 1. Derivation of hepatic endoderm from induced pluripotent stem cells (iPSCs). (A) (panel a) Phase contrast microscopy demonstrating the typical iPSC colony morphology and (panel b) iPSC-derived hepatic endoderm (HE) following 14 days in the differentiation procedure (magnification ×20). (panel c) iPSC-derived HE stains positive for albumin at day 14 in the differentiation procedure. The numbers represent the efficiency of the procedure ± standard error. (panel d) iPSC-derived HE stains positive for E-cadherin at day 14 in the differentiation procedure. (B) Reverse transcription PCR gene expression of iPSCs and iPSC-derived HE. iPSC-derived HE express the markers alpha-fetoprotein, CYP7A1, and hepatocyte nuclear factor-4alpha, but not OCT-4. The iPSCs strongly express OCT4, in addition HNF4 alpha is present potentially due to spontaneous differentiation found in iPSC culture. PCR reactions were controlled using a reverse transcription minus (RT−) control and a beta-actin control. Details of primers and cycles can be found in the Supporting Methods section. (C) Quantitative PCR assessment of CYP1A2 and CYP3A4 expressed as fold over iPSC.

differentiation by retroviral transduction of four transcription factors.[1] As iPSCs colonies appeared, they were manually disaggregated and plated onto a feeder layer and sequentially passaged (Supporting Fig. 1).[6,7] The derived iPSC lines were characterized using a number of stem cell criteria: cell morphology; stem cell gene expression; stem cell surface expression of SSEA3, SSEA4, and Tra-1-60; and absence of SSEA1 and teratoma formation *in vivo* (Supporting Fig. 1).[6,7] By applying the method we had used for differentiating hESCs[5], we attempted to generate hepatic endodermal lineage from human iPSCs. We initially focused our efforts on an iPSC line derived from normal adult Caucasian male, NMF-iPS6 (Fig. 1A, panel a). NMF-iPS6 cells were differentiated toward hepatic endoderm via physiologically relevant conditions; treatment with Wnt3a/activin A, activin A, followed by DMSO and a final maturation step with hepatic growth factor and oncostatin M (Fig. 1A, panel b).[5] Differentiation of iPSCs into hepatic endoderm was associated with a dramatic change in cellular morphology similar to hepatocyte differentiation. Hepatic phenotype was assessed by the albumin production (Fig. 1A, panel c) and E-cadherin (Fig. 1A, panel d) confirmed by immunofluorescence. We observed an efficiency of HE generation of between 70%-90%, as assessed by albumin-positive cells (Fig. 1A, panel c). HE derived from the male Caucasian iPSCs (NMF-iPS6) expressed a number of key hepatic transcripts as assessed by reverse transcription PCR, namely alpha-fetoprotein and hepatocyte nuclear factor-4. In addition, we observed the expression of the endodermal markers Sox17 and cysteine-X-cysteine receptor-4 (CXCR4)[10] at day 5 in the procedure (data not shown) and CYP7A1 (Fig. 1), which demonstrates both a definitive endoderm origin and importantly is not derived from yolk sac.[11] Additionally, upon differentiation, the pluripotency marker OCT4 which is expressed in iPS cells became downregulated (Fig. 1B). One of the immediate potential applications of iPSC-derived HE is human drug toxicity assessment, and therefore we investigated the expression of two key adult cytochrome P450s: CYP1A2 and CYP3A4. Both enzymes were induced in HE cells compared with undifferentiated iPSCs, with a ~sixfold increase in CYP1A2 and ~6000-fold increase in CYP3A4 levels (Fig. 1C).

In addition to the male Caucasian NMF-iPS cell line, we also applied the HE differentiation protocol to iPSCs derived from a diabetic North American Indian (JDM-iPS1) and a female Caucasian (PGP9f-iPS1) (Fig. 2A, panels a and b). Both iPSC lines differentiated into HE with similar efficiencies as male Caucasian NMF-iPS6 cell line. HE differentiation was assessed by cell morphology and albumin staining (Fig. 2A, panels c, d, and e). When we analyzed hepatic gene expression in the iPSC-derived HE, both lines exhibited similar gene expression patterns

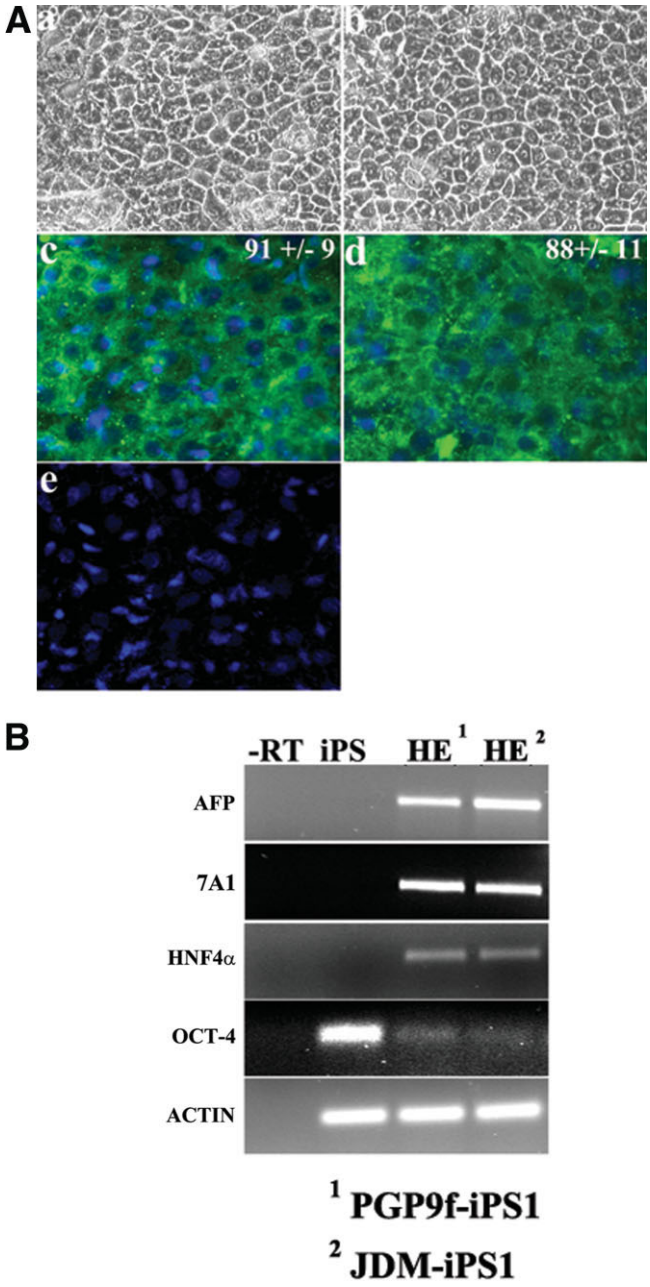

Fig. 2. Derivation of hepatic endoderm (HE) from two other induced pluripotent stem cell (iPSC) lines. (A) (panels a,b) Phase contrast microscopy demonstrating the typical iPSC HE morphology following 14 days in the differentiation procedure (magnification ×20). (panels c and d) iPSC-derived HE stains positive for albumin at day 14 in the differentiation procedure. The numbers represent the efficiency of the procedure ± standard error. (B) Reverse transcription PCR gene expression of iPSCs and iPSC-derived HE. iPSC-derived HE express the markers alpha-fetoprotein and hepatocyte nuclear factor-4alpha, but not OCT-4. The iPSCs strongly express OCT4. PCR reactions were controlled using a reverse transcription minus (RT−) control and a beta-actin control. Details of primers and cycles can be found in the Supporting Methods section.

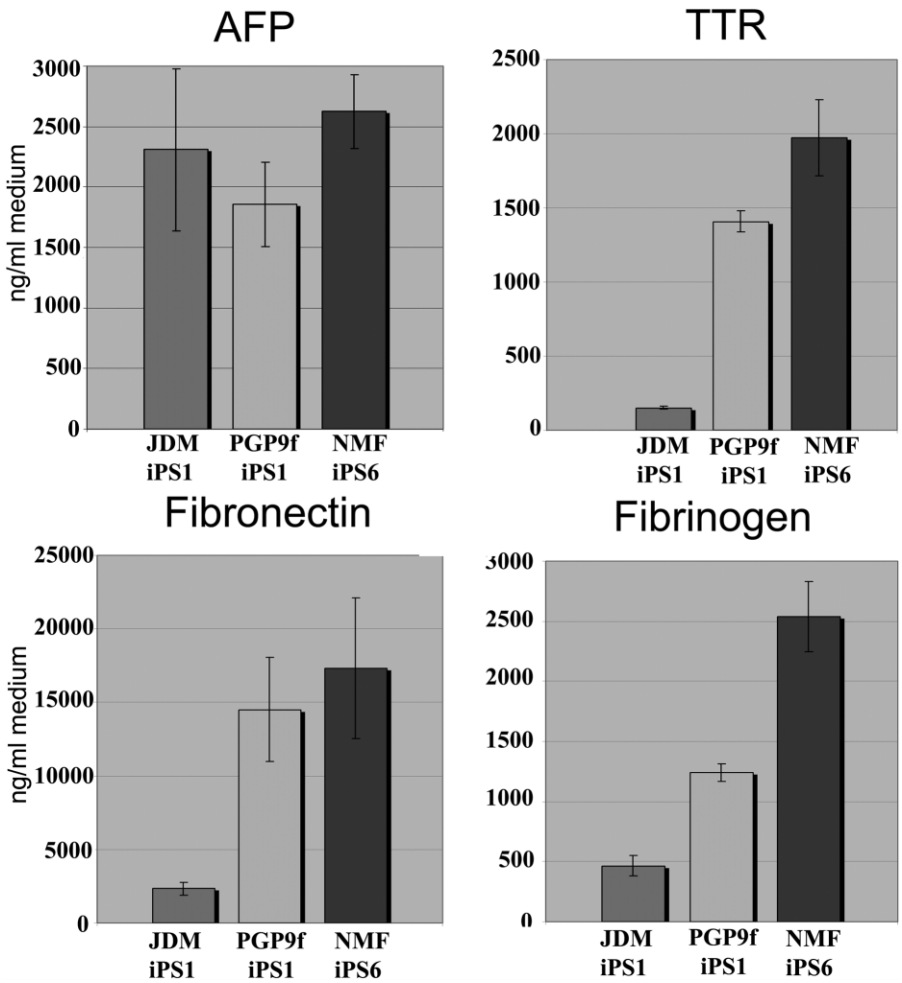

Fig. 3. Induced pluripotent stem cell (iPSC)-derived hepatic endoderm (HE) produces hepatic-specific serum proteins. iPSC-derived HE was maintained in 1 mL of hepatocyte culture medium for 24 hours. The following morning, culture supernatants were harvested and serum protein production was measured by ELISA and quoted as nanograms per milliliter of tissue culture medium per 24 hours. Note that background has been removed from the presented data. Each line is represented as a different colored bar: JDM-iPS1 is presented by the first bar in all graphs, PGP9ff-iPS1 is represented by the middle bar in each graph, and NMF-iPS6 is represented by the last bar in each graph. In all lines, the serum protein tested was detected. Fibrinogen, transthyretin (TTR), fibronectin, and alpha-fetoprotein (AFP) (n = 6).

as that observed from NMF-iPS6 cells, indicating hepatic identity (Fig. 2B).

Although iPSC-derived HE expressed a number of liver genes, we were also keen to assess their liver-specific function in culture. An important functional marker for HE is the production and export of serum proteins. We assessed iPSC-HE production of these key serum proteins and measured their levels by ELISA (Fig. 3). In all lines tested, we detected substantial amounts of alpha-fetoprotein, transthyretin, fibronectin, and fibrinogen at levels equivalent to those reported for HE derived from hESCs.[5]

In order to further functionally validate, iPSC-derived HE was assessed for its metabolic ability. The cytochrome P450 enzymes are critical in drug metabolism, and CYP1A2 and CYP3A4 are key enzymes. The function of these CYP450 components were examined, and importantly, all lines exhibited CYP1A2 and CYP3A4 activity as assessed by the generation of a luminescent metabolite (Fig. 4). CYP1A2 metabolism was similar between lines PGP9f-iPS1 and NMF-iPS6, but was higher in line JDM-iPS1, whereas we observed only slight variation with CYP3A4 metabolism in all three lines tested.

Discussion

Here, we demonstrate for the first time the derivation of HE from human iPSCs of both sexes and two ethnicities. The iPSC-derived HE was functionally equivalent to hESC-derived HE, and interestingly, all iPSC lines tested so far showed higher efficiency to form functional HE. The generic ability of iPSCs to form HE in response to our model[5] has not been observed with hESCs in deriving efficient levels of HE. Therefore, one could speculate that this is due to the consistent manner in which the iPSCs were reprogrammed and may play an important role in their developmental potential. It also suggests that iPSCs may prove a more valuable and uniform starting material for derivation of HE, than are hESCs, which show dramatic line-to-line variability in susceptibility to individual lineage differentiation.

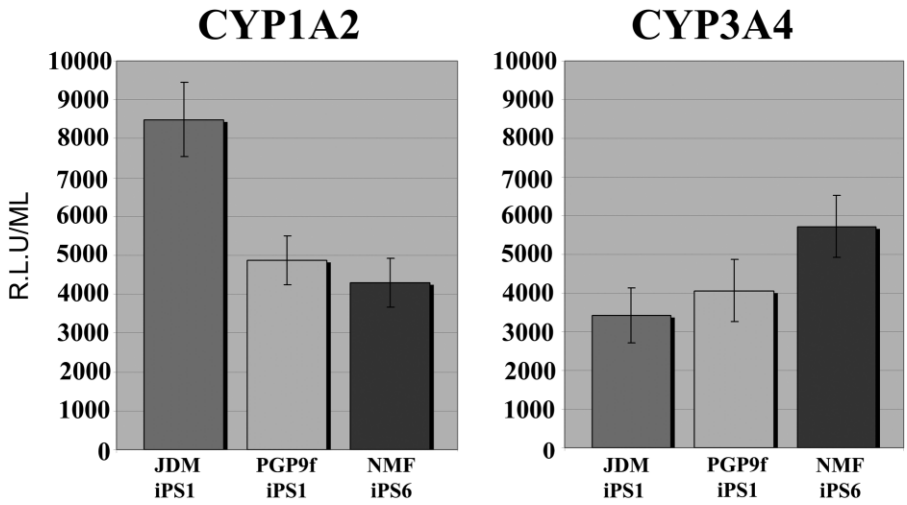

Fig. 4. Induced pluripotent stem cell (iPSC)-derived hepatic endoderm (HE) displays cytochrome P450 metabolism. iPSC-derived HE were incubated with hepatocyte culture medium supplemented with 50 μM CYP3A4 or CYP1A2 pGlo substrates (Promega) as per manufacturer's instructions. At 4 hours after treatment, 50 μL of culture medium was removed and read on a luminometer (POLARstar optima). CYP1A2 and CYP3A4 activity is expressed as relative light units (RLU) per milliliter of tissue culture medium (n = 6). JDM-iPS1 is represented by the first bar in all graphs, PGP9f-iPS1 is represented by the middle bar in each graph, and NMF-iPS6 is represented by the last bar in each graph.

Such a resource has the ability to revolutionize the manner in which we define drug metabolism, and model liver disease and human liver development. Because iPSC-derived HE can be differentiated *in vitro*, an unlimited supply of ethically and genetically diverse HE models can be obtained. This will become a powerful resource allowing the study of ethnic/polymorphic variation on xenobiotic metabolism involving poor metabolizers (e.g., CYP2C9/warfarin) and disease genotypes (e.g., alpha-1-antitrypsin). In addition, the ability to model liver development *in vitro* will allow the development of novel biomarkers for both disease and the identification of stage-specific markers during the differentiation process.[12]

An iPSC library could be developed through identification and reprogramming of human fibroblasts displaying metabolically different features for key polymorphisms. Presently, the ability to model the human liver and disease using hESCs or PHHs is limited by the number of stem cell lines available and the ability to produce functional HE from individual ESC lines. Therefore, the application of iPSC HE technology will bypass the issues associated with hESCs and PHHs.

In conclusion, our studies provide a proof of concept that multiple iPSC lines can be efficiently differentiated to functioning HE. In addition, our study provides a novel approach that overcomes the current limitations associated with PHHs and hESCs. We predict that this technology will be applicable to iPSC lines derived from healthy and diseased patients from different ethnic backgrounds, allowing the creation of a library. The development of such a resource is essential in the identification and testing of new medicines and the modeling of disease.

Acknowledgment: We thank Dr. Val Wilson for the analysis of the teratoma data. Antibodies used for flow cytometry were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. I.W. was supported by Scottish Funding Council. D.C.H. was supported by a RCUK Fellowship and J.P.I. is supported by an MRC programme grant.

References

1. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 2008;451:141-146.
2. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-872.
3. Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, et al. Human induced pluripotent stem cells free of vector and transgene sequences. Science 2007;318:1917-1920.
4. Dalgetty DM, Medine CN, Iredale JP, Hay DC. Progress and fuure challenges in stem cell-derived liver technologies. Am J Physiol Gastrointest Liver Physiol 2009;297:G241-G248.
5. Hay DC, Fletcher J, Payne C, Terrace JD, Gallagher RC, Snoeys J, et al. Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. Proc Natl Acad Sci U S A 2008; 105:12301-12306.
6. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, et al. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol 2009;27:361-368.

7. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, et al. Disease-specific induced pluripotent stem cells. Cell 2008;134:877-886.
8. Prokhorova TA, Harkness LM, Frandsen U, Ditzel N, Burns JS, Schroeder HD, et al. Teratoma formation by human embryonic stem cells is site-dependent and enhanced by the presence of Matrigel. Stem Cells Dev 2009;18:47-54.
9. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 1982;21:6188-6193.
10. Yasunaga M, Tada S, Torikai-Nishikawa S, Nakano Y, Okada M, Jakt LM, et al. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. Nat Biotechnol 2005;23:1542-1550.
11. Asahina K, Fujimori H, Shimizu-Saito K, Kumashiro Y, Okamura K, Tanaka Y, et al. Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells. Genes Cells 2004;9:1297-1308.
12. Gadue P, Gouon-Evans V, Cheng X, Wandzioch E, Zaret KS, Grompe M, et al. The generation of monoclonal antibodies specific for cell surface molecules expressed on early mouse endoderm. Stem Cells 2009;27:2103-2113.