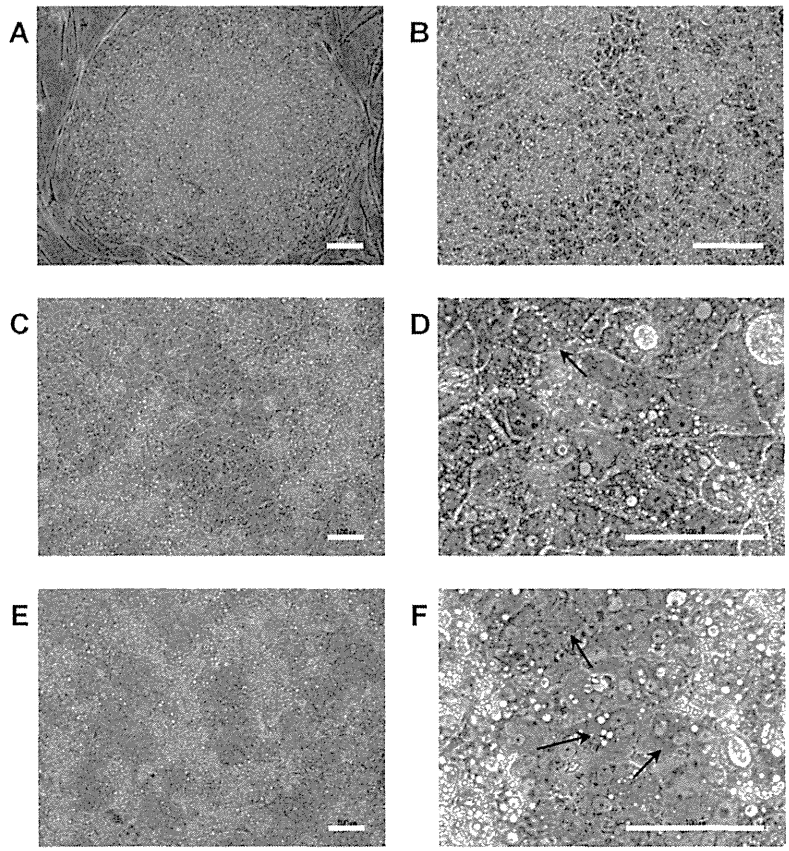


Figure 3. Morphological changes observed in hiPS cells during hepatic differentiation. The images show morphological changes in human iPS (hiPS) cells (Windy). (A) Undifferentiated hiPS cells; (B) hepatic progenitor cell-like cells after 12 days of differentiation; (C, D) hepatocyte-like cells after 25 days of differentiation in the absence of valproic acid (VPA); and (E, F) hepatocyte-like cells after 25 days of differentiation in the presence of 2-mM VPA for 168 h from day 12. Arrows indicate binuclear cells. Scale bar, 100 μm.
doi:10.1371/journal.pone.0104010.g003

Expression of drug-metabolizing enzymes in differentiated cells

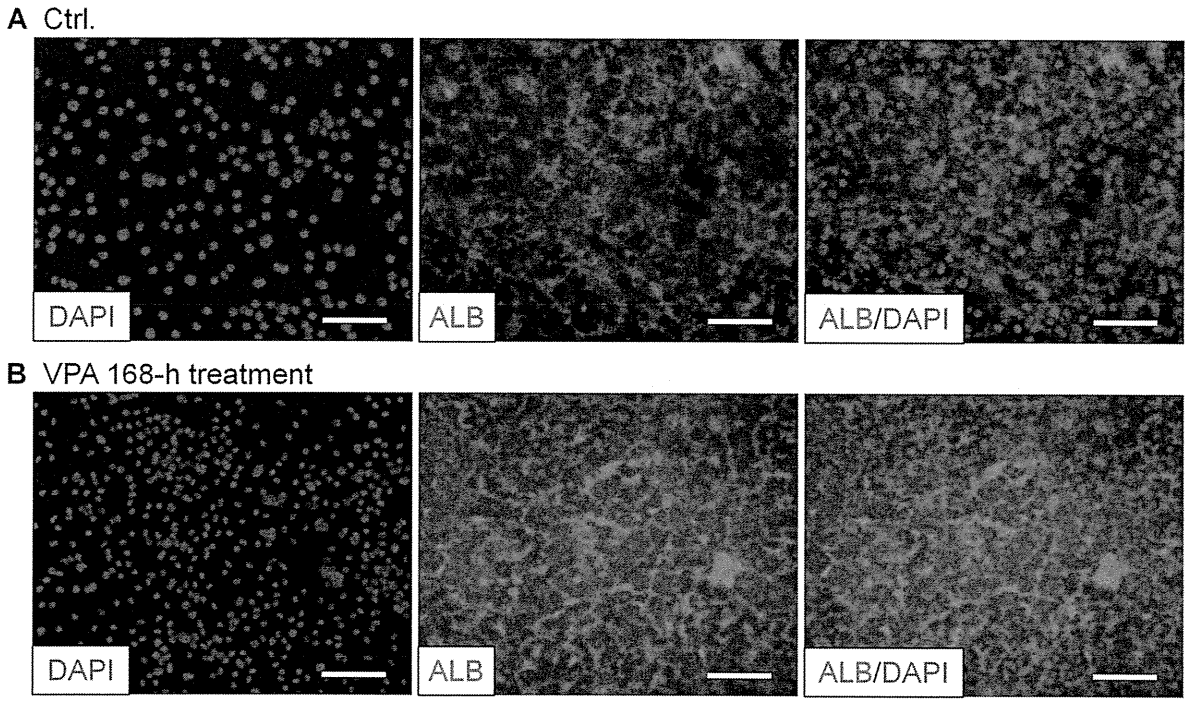
From results of the mRNA expression analysis and immunofluorescence staining of ALB, it was suggested that the 168-h VPA treatment efficiently promoted hepatic differentiation from hiPS cell-derived HPCs. To confirm the hepatic functions of these cells, we investigated the expression of drug-metabolizing enzymes under these conditions. After 25 days of differentiation, mRNAs encoding major drug-metabolizing enzymes were detected. In particular, CYP2C9, CYP2C19, CYP3A4, and UDP-glucuronosyltransferase (UGT) 1A1 mRNAs significantly increased after the VPA treatment; in contrast, the levels of these mRNAs were low compared with those of HPHs 48 h (Fig. 4C). Furthermore, we successfully detected drug-metabolizing enzyme activities in cells that were differentiated from hiPS cells. The metabolites generated by CYP1A1/2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, UGT, and sulfotransferase was detected in the cells (Fig. 5). The CYP2C9 and CYP3A4/5 metabolites significantly increased after the 168-h VPA treatment.

Effects of HDAC inhibitors during hepatic differentiation from hiPS cells

VPA has various pharmacological actions, including the inhibition of GABA transaminase and HDAC and the blockage of ion channels. Thus, using specific inhibitors, we investigated which of these actions was involved in hepatic differentiation. ALB expression is an indicator of hepatic differentiation, because ALB is synthesized in the liver. ALB mRNA level was unaffected by treatment with GABA transaminase inhibitors and ion channel blockers for 168 h from day 12 (Fig. 6A). In contrast, treatment with all HDAC inhibitors (except NCC149) significantly increased ALB mRNA expression. Furthermore, a dramatic suppression of HDAC activity during the VPA treatment was confirmed (Fig. 6B). The ALB mRNA was expressed in multiple hiPS cell lines, and its expression after the 168-h VPA treatment was significantly higher than that detected in the VPA-untreated groups (Fig. 6C). Taken together, these data demonstrate the versatility of VPA in hepatic differentiation by acting via HDAC inhibition.

Discussion

This study presented a new culture protocol that provides effective differentiation from hiPS cells into hepatocytes. The


A Ctrl.
DAPI
ALB
ALB/DAPI
B VPA 168-h treatment
DAPI
ALB
ALB/DAPI

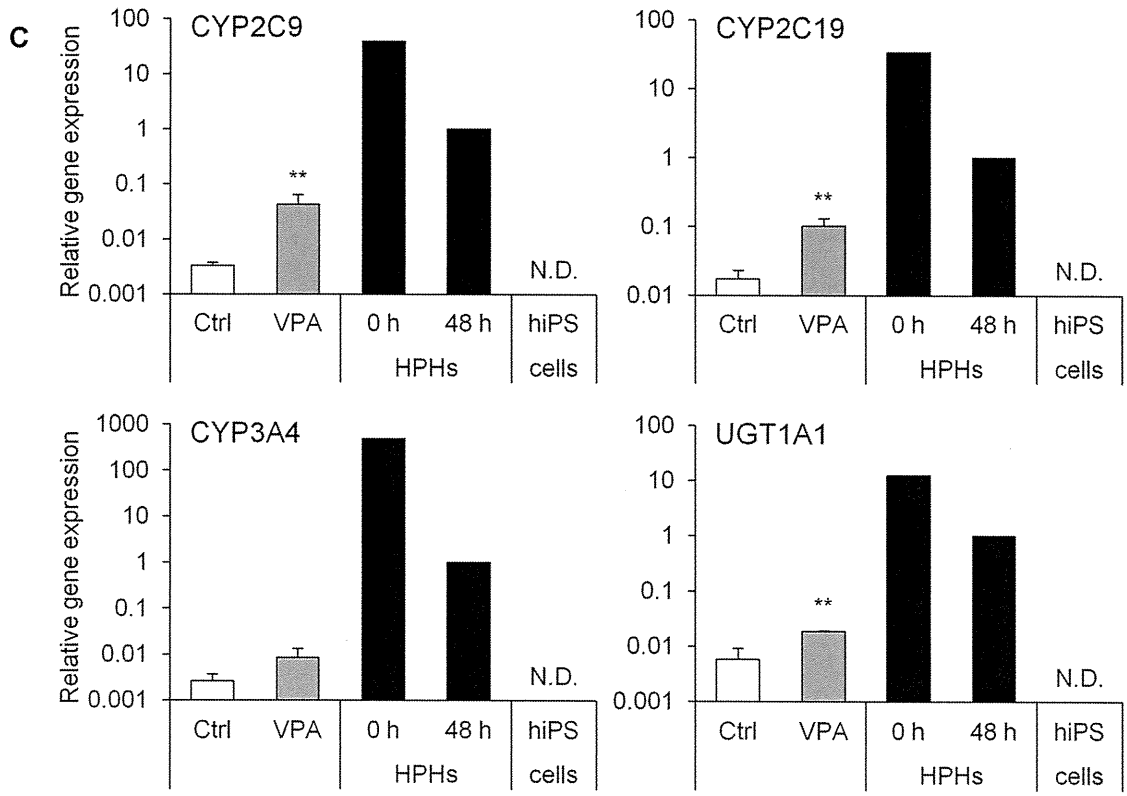

C
CYP2C9
CYP2C19
CYP3A4
UGT1A1
Relative gene expression
100
10
1
0.1
0.01
0.001
1000
**
N.D.
Ctrl
VPA
0 h
48 h
HPHs
hiPS cells

Figure 4. Immunofluorescence staining of ALB and effects of VPA on mRNAs encoding drug-metabolizing enzymes. Human iPS (hiPS) cells (Windy) were differentiated into hepatocytes. Valproic acid (VPA) was added to the medium for 168 h from day 12. (A, B) hiPS cell-derived hepatocyte-like cells in the absence of VPA (A) and hiPS cell-derived hepatocyte-like cells treated with VPA (B) were stained for ALB (red). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue); (C) Cryopreserved human primary hepatocytes (HPHs) were cultured for 0 (just after thawing) and 48 h. Each bar represents the mean ± standard deviation ($n = 3$). The graph represents gene expression relative to that detected in human hepatocytes cultured for 48 h. Levels of statistical significance compared with VPA-untreated hepatocyte-like cells [control (Ctrl)]: $^{**}P<0.01$. CYP, cytochrome P450; UGT, UDP-glucuronosyltransferase; N.D., not detected.
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differentiated cells expressed hepatocyte markers and exhibited drug-metabolizing enzyme activities. These observations indicate that hiPS cells differentiated into functional hepatocyte-like cells. Furthermore, these differentiation characteristics were significantly enhanced by the administration of VPA during the final step of HPCs maturation.

Hepatic differentiation from hiPS cells exhibited differing patterns after various VPA treatment times. ALB, PXR, and TAT mRNAs increased after the 72- and 168-h VPA treatments, suggesting that VPA promotes differentiation of hiPS cell-derived HPCs into hepatocytes. However, these mRNA expression levels were decreased after the 312-h VPA treatment compared with the 168-h VPA treatment. Vasculature-like structures were observed in differentiated cells after the 312-h VPA treatment; these cells expressed the lymphatic endothelial marker gene (Fig. S1). These results suggest that VPA promotes hepatic differentiation from hiPS cell-derived HPCs in a time-dependent manner.

The inducibility of the CYP3A4 mRNA by RIF also depended on the VPA treatment times. Because RIF is a ligand of PXR, this observation may reflect PXR expression, which was low in cells that received the 312-h VPA treatments. Thus, we assumed that the 312-h VPA treatment inhibited hepatic differentiation because of a toxic effect. VPA is metabolized by CYP and the metabolites exhibit hepatotoxicity [24]. Accordingly, the 168-h VPA treatment would promote hepatic differentiation, whereas hepatotoxicity would be present after the longer VPA treatment. Thus, the duration of the VPA treatment appears to control the specificity of hepatic differentiation from hiPS cell-derived HPCs, and the present data indicate that the 168-h VPA treatment was optimal.

The liver plays a key role in drug metabolism, and it expresses phase I enzymes, such as various CYP isoforms [25], and phase II conjugating enzymes, such as UGT and sulfotransferase. Previous studies on hepatic differentiation reported that drug-metabolizing enzyme activity was determined by measuring chemiluminescence and fluorescence using P450-Glo (Promega) and ethoxyresorufin [7,20]. A few studies measured drug-metabolizing enzyme activity using specific substrates [8]. To evaluate metabolic functions, we incubated the cells that were differentiated from hiPS cells in a medium containing substrates of drug-metabolizing enzymes. The metabolites of these substrates were detected in the supernatant after incubation; the observation that the hepatocyte-like cells generated metabolites from probe substrates was valuable. These results suggest that the present hiPS cell-derived hepatocyte-like cells have appropriate drug-metabolizing enzyme activities. VPA is known as an inhibitor of CYP2C9, CYP2C19, and CYP3A4 [26], but not as an inducer of CYPs such as RIF [27]. The activities of CYP2C9 and CYP3A4/5, however, were significantly increased by the 168-h VPA treatment. Importantly, in the present experiment, the hepatocyte-like cells used in the 168-h VPA treatment groups were cultured without VPA for the final 6 days. Accordingly, the increases in hepatic marker genes and drug-metabolizing enzyme activities observed after the 168-h VPA treatment suggest that VPA promotes hepatic differentiation.

The high reliability of ALB as a marker of hepatocyte differentiation is consistent with the fact that this protein is synthesized in the liver. In the present study, hiPS cell-derived hepatocyte-like cells expressed the ALB mRNA, which was markedly induced by the VPA treatment. In immunofluorescence experiments, ALB protein expression was also detected in almost all VPA-treated cells. In addition, the effects of VPA on ALB mRNA expression were observed in multiple differentiated hiPS cell lines, further indicating that VPA is a useful agent for generating hiPS cell-derived hepatocytes.

Previous studies demonstrated that VPA inhibits HDAC and GABA transaminase and blocks ion channels [14,15]. Among various specific small-molecule inhibitors of HDAC and GABA transaminase and various ion channel blockers that were used during hepatic differentiation, only HDAC inhibitors functioned as effective differentiation agents. In fact, we showed that VPA inhibited HDAC activity during treatment. HDAC include various isoforms [17]. In particular, differentiation-promoting effects of the HDAC3 inhibitor T247 [22] were lower than those of other HDAC inhibitors, and the HDAC8-selective inhibitor NCC149 [23] had no effect on hepatic differentiation. In contrast, the inhibitors of HDAC1, 2, and 3, which are classified into class I HDAC [28], such as VPA, NaB, TSA, vorinostat, and MS-275, had strong effects on hepatic differentiation. Hence, the inhibition of HDAC1 and HDAC2 may promote hepatic differentiation from hiPS cell-derived HPCs. Previous studies showed that HDAC inhibitors affect DNA binding of transcriptional factors that are involved in cell growth and differentiation [14]. Although the precise mechanisms that the inhibition of HDAC promotes hepatic differentiation are unclear, the expression of genes involved in hepatic differentiation may be increased by the inhibition of HDAC in hiPS cell-derived HPCs. Ware *et al.* reported that HDAC inhibitors promote self-renewal of mouse/human ES cells, and differentiation into retinal neurons from butyrate-treated ES cells was delayed [29]. Thus, HDAC inhibitors may interfere with differentiation of ES/iPS cells. However, these HDAC inhibitors were used at low concentrations for promoting self-renewal, and at high concentrations for inducing differentiation. Moreover, they administered the inhibitors to undifferentiated ES cells. In our study, we administered VPA to HPCs. Thus, the use and purpose of HDAC inhibitors were different between our study and previous studies. We believed that VPA would have various effects depending on the cell-differentiation state or its concentration.

Dong *et al.* reported that human bone marrow stromal stem cells differentiated into hepatocyte-like cells by pretreatment with VPA [19]. In addition, undifferentiated mouse ES cells treated with VPA and without leukemia inhibitory factor, maintained the undifferentiated state, during initiation of the hepatic differentiation process [20]. Yamashita *et al.* reported that the HDAC inhibitor TSA suppressed cell growth and promoted differentiation by regulating the cell cycle in HepG2 cells (human hepatocyte carcinoma cells) [30]. Taken together, these reports suggest that HDAC inhibitory effect or cell-cycle arrest effect of VPA or TSA facilitated hepatic differentiation. However, other HDAC inhibitors remain uninvestigated. Furthermore, whether HDAC inhibitors affect the maturation process during differentiation from hiPS cells into hepatocytes remains unknown. The current findings revealed novel effects of VPA on hepatic differentiation from hiPS

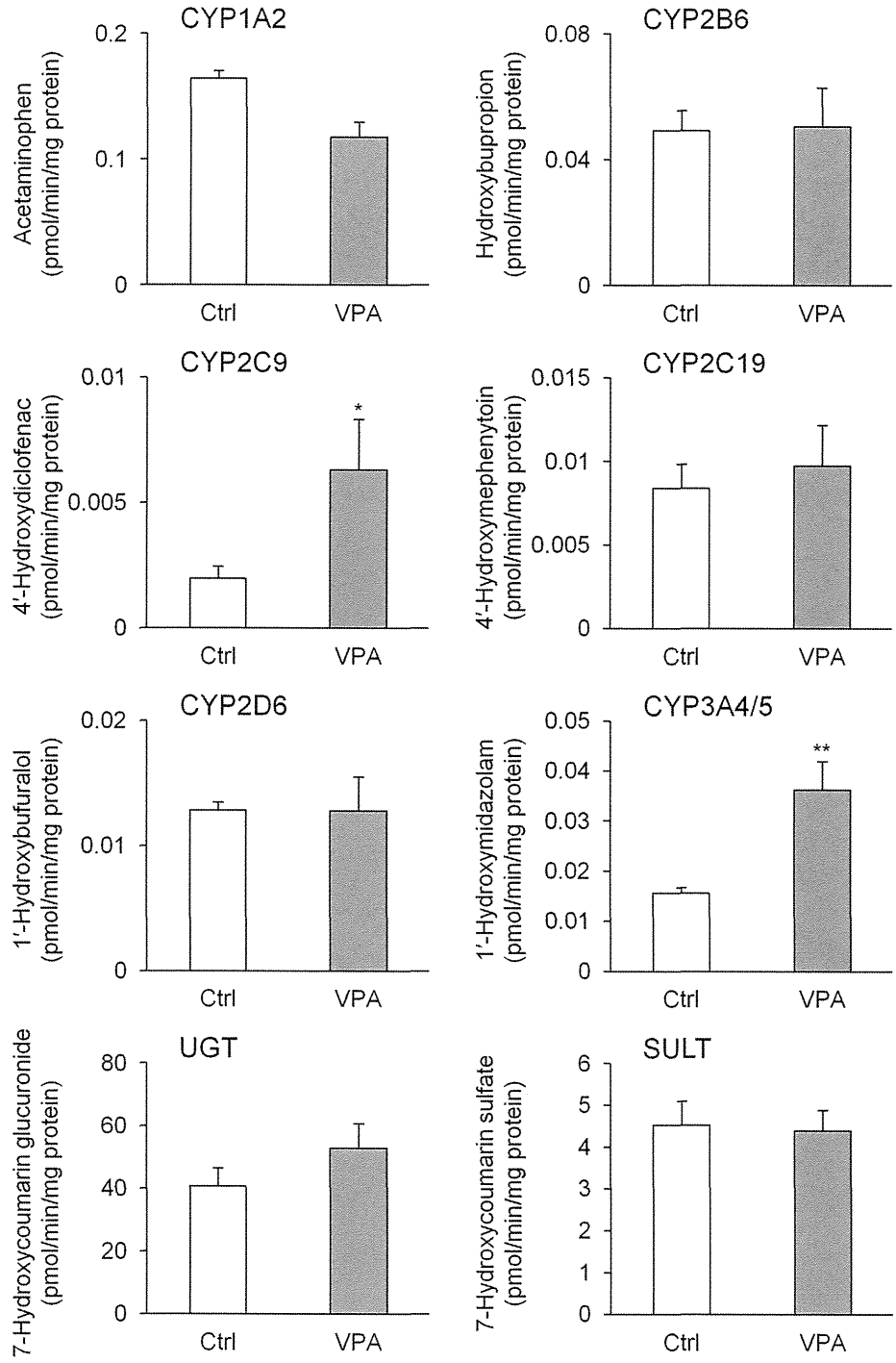

Figure 5. Drug-metabolizing enzyme activities in hepatocyte-like cells differentiated from hiPS cells using VPA. Human iPS (hiPS) cells (Windy) were differentiated into hepatocytes. Valproic acid (VPA) was added to medium for 168 h from day 12. Acetaminophen, hydroxybupropion, 4′-hydroxydiclofenac, 4′-hydroxymephenytoin, 1′-hydroxybufuralol, 1′-hydroxymidazolam, 7-hydroxycoumarin glucuronide, and 7-hydroxycoumarin sulfate were biotransformed from phenacetin, bupropion, diclofenac, (*S*)-mephenytoin, bufuralol, midazolam, 7-hydroxycoumarin, and 7-hydroxycoumarin by cytochrome P450 (CYP) 1A1/2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, UDP-glucuronosyltransferase (UGT), and sulfotransferase (SULT), respectively. Each bar represents the mean ± standard deviation ($n=3$). Levels of statistical significance compared with VPA-untreated hepatocyte-like cells [control (Ctrl)]: $^{*}P<0.05$ and $^{**}P<0.01$.
doi:10.1371/journal.pone.0104010.g005

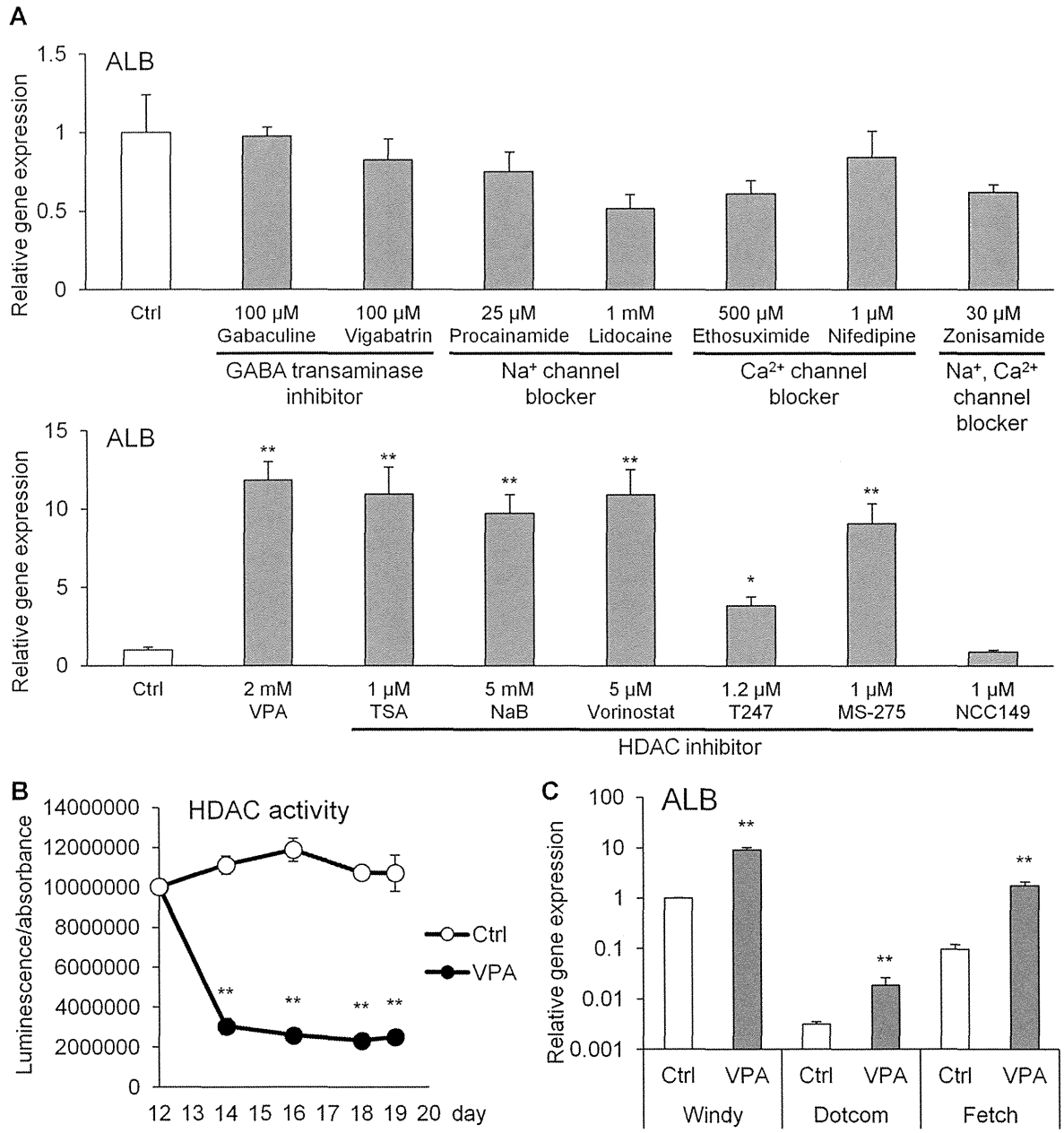

Figure 6. Effects of small-molecule compounds on hepatic differentiation from hiPS cells. All compounds were added to the medium for 168 h from day 12. (A) The albumin (ALB) mRNA expression level was analyzed in hepatocyte-like cells differentiated from hiPS cells (Windy). Each bar represents the mean ± standard deviation ($n=3$). The graph represents gene expression relative to that detected in compound-untreated hepatocyte-like cells [control (Ctrl)]. Levels of statistical significance compared with Ctrl: $^{*}P<0.05$ and $^{**}P<0.01$. (B) Time-dependent changes in HDAC activity in differentiating human iPS (hiPS) cells (Windy). Symbols represent the mean ± standard deviation ($n=4$). Levels of statistical significance compared with Ctrl: $^{**}P<0.01$. (C) The ALB mRNA expression level was analyzed in hepatocyte-like cells differentiated from three hiPS cell lines (Windy, Dotcom, and Fetch). Each bar represents the mean ± standard deviation ($n=3$). The graph represents gene expression relative to that detected in VPA-untreated hepatocyte-like cells differentiated from Windy. Levels of statistical significance in each cell line compared with each Ctrl, respectively: $^{**}P<0.01$. The abbreviations used are: NaB, sodium butyrate; TSA, trichostatin A.
doi:10.1371/journal.pone.0104010.g006

cells. The VPA-induced hepatic differentiation from hiPS cell-derived HPCs depended on the treatment period. The action of VPA was observed in multiple hiPS cell lines. The HDAC-inhibitory effect promoted hepatic differentiation from hiPS cells.

In conclusion, the present study demonstrated that VPA, a small-molecule compound, promoted hepatic differentiation from hiPS cells primarily by inhibiting HDAC. This new differentiation method using small-molecule compounds, which are convenient

and inexpensive, would be valuable for large-scale production of functional hepatocyte-like cells differentiated from hiPS cells, because the method is simple and there is no contamination with exogenous viruses or cells. The hiPS cell-derived hepatocyte-like cells may be useful for drug development studies and liver transplantation.

Supporting Information

Figure S1 Effects of VPA on FLT4 expression. Fms-related tyrosine kinase 4 (FLT4) is known to lymphatic endothelial marker. Human induced pluripotent stem cells (Windy) were differentiated into hepatocytes. Valproic acid (VPA) was added to the medium for 72 h from day 18 (72 h), 168 h from day 12 (168 h), or 312 h from day 12 (312 h) at a final concentration of 2 mM. Each bar represents the mean ± standard deviation (n = 3). The graph represents gene expression relative to that in VPA-untreated hepatocyte-like cells [control (Ctrl)].
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: YK TI K. Nagata KK SO K. Nakamura TM. Performed the experiments: YK SY KM RO MS TN. Analyzed the data: YK SY KM RO MS TN. Contributed reagents/materials/analysis tools: TS NM. Wrote the paper: YK TI K. Nagata KK TS NM K. Nakamura TM.

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Drug Metab. Pharmacokinet. 29 (5): 407–413 (2014).

Regular Article

Selective Culture Method for Hepatocyte-like Cells Differentiated from Human Induced Pluripotent Stem Cells

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Full text and Supplementary materials of this paper are available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: This study aimed to establish culture conditions which are able to give the differentiation of induced pluripotent (iPS) cells to hepatocytes. To this end, we examined the usefulness of a culture medium containing the components involved in the intermediary metabolism in the liver. More specifically, we examined the effect of the "modified L-15 medium" containing galactose, phenylalanine and ornitine, but deprived of glucose, tyrosine, arginine and pyruvic acid. The medium was altered according to changes in the expression of enzymes that participate in liver-specific pathways. After 25 days of differentiation, the differentiated cells expressed hepatocyte markers and drug-metabolizing enzymes. These expression levels were increased using modified L-15 medium. The survival of human fetal liver cells and the death of human fibroblasts were observed during culture in modified L-15 medium. Most of the cells that differentiated from human iPS cells using modified L-15 medium were stained by anti-human albumin antibody. These results suggest that iPS cells can be converted to high purity-differentiated hepatocytes by cultivating them in modified L-15 medium.

Keywords: induced pluripotent stem cells; differentiation; hepatocytes; selection medium; cytochrome P450; energy sources

Introduction

Human induced pluripotent stem (iPS) cells have been generated directly from human fibroblast cells by inducing their expression of defined reprogramming factors (OCT3/4, SOX2, KLF4, and c-MYC).[1] Recent studies have shown that iPS cells are comparable to embryonic stem (ES) cells because they exhibit the potential for multilineage differentiation and intensive *in vitro* proliferation. The utilization of human iPS cells is anticipated in a variety of applications, including drug development studies involving the prediction of hepatic drug metabolism and liver toxicity. Furthermore, human iPS cell-derived hepatocytes may represent a source for cell transplantation to treat severe liver diseases in the future. Previous studies have already reported hepatocyte differentiation from human iPS cells using cytokines, such as growth factors, transcription factor overexpression by virus vectors, and co-culture with other cells.[2–8] However, it is difficult to obtain large numbers of highly pure human iPS cell-

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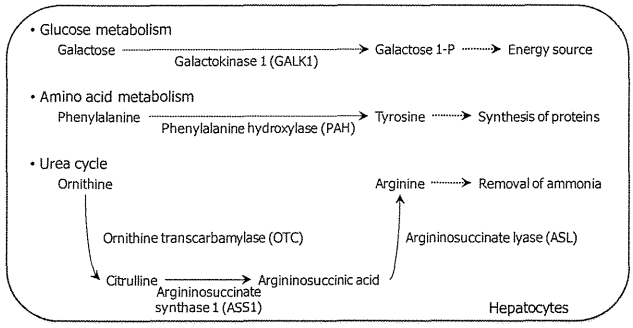

Fig. 1. Hepatocyte-specific metabolic pathways
Hepatocytes express galactokinase 1 (GALK1), phenylalanine hydroxylase (PAH), ornithine transcarbamylase (OTC), argininosuccinate synthase 1 (ASS1), and argininosuccinate lyase (ASL). Galactose is metabolized into galactose 1-phosphate by GALK1. Finally, galactose 1-phosphate is transformed into glucose 6-phosphate, and undergoes glycolysis for energy generation. Phenylalanine is metabolized into tyrosine by PAH. Ornithine is transformed into arginine by OTC, ASS1, and ASL. Glucose, tyrosine, and arginine are essential substrates for cell survival. Hepatocytes can utilize substrates by metabolizing galactose, phenylalanine, and ornithine to galactose 1-phosphate, tyrosine, and arginine, respectively, if the essential substrates are not present.

derived hepatocytes both simply and inexpensively using these methods.

Cells have many metabolic pathways and can produce essential nutrients autonomously. Tohyama *et al.* cultured human ES/iPS cell-derived cardiomyocytes in a glucose-depleted culture medium containing abundant lactate and found that only cardiomyocytes survived.[9] Using this approach, human ES/iPS cell-derived cardiomyocytes that did not form tumors after transplantation were obtained with a purity of up to 99%. Hepatocytes also have specific metabolic pathways, which involving glucose and amino acid metabolism, and the urea cycle (**Fig. 1**). Therefore, it is possible that only hepatocytes can survive in a medium that contains the energy sources metabolized by these pathways. Galactokinase 1 (GALK1), which is involved in the conversion of galactose to glucose, is included because glucose is an essential energy source for cells. GALK1 is highly expressed in the liver and kidney.[10,11] Phenylalanine hydroxylase (PAH), which participates in the conversion of phenylalanine to tyrosine, is included because tyrosine is decomposed to acetoacetate and fumarate, which then enters the citric acid cycle and generates energy.[12] Furthermore, tyrosine is used by cells to synthesize proteins and it participates in signal transduction processes such as tyrosine kinase signaling, which functions as an on/off switch for many cellular functions. PAH activity is found in the liver and kidney.[12] Ornithine transcarbamylase (OTC), argininosuccinate synthase 1 (ASS1), and argininosuccinate lyase (ASL), which are involved in the conversion of ornithine to arginine in the urea cycle, must be included because arginine plays an important role in cell division, wound healing, and removal of ammonia, which is toxic to the human body.[13]

It was previously reported that a medium lacking glucose, tyrosine, and arginine, but including galactose, phenylalanine, and ornithine, enriched the hepatoblast-like cells that differentiated from mouse ES cells.[14] A similar medium reportedly eliminated undifferentiated human iPS cells.[15] However, a simple and safe selective culture method for human iPS cell-derived hepatocytes is not available. In the present study, we focused on liver-specific metabolic pathways and attempted to selectively culture hepatocytes differentiated from human iPS cells using a medium containing energy sources metabolized by liver-specific energy metabolic enzymes.

Materials and Methods

Materials: Activin A and hepatocyte growth factor (HGF) were purchased from PeproTech Inc. (Rocky Hill, NJ). Fetal bovine serum (FBS) was purchased from Biowest SAS (Nuaillé, France). Accutase™ was purchased from MS TechnoSystems (Osaka, Japan). Oncostatin M (OSM) and Y-27632 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BD Matrigel™ Matrix Growth Factor Reduced (Matrigel™) was purchased from BD Biosciences (Bedford, MA). Mouse monoclonal anti-human albumin (ALB) antibody was purchased from Abcam (Cambridge, UK). KnockOut™ serum replacement (KSR), KnockOut™ Dulbecco's modified Eagle's medium (KO-DMEM), and Alexa Fluor® 568 goat anti-mouse IgG were purchased from Invitrogen Life Technologies Co. (Carlsbad, CA). Human fetal liver total RNA from a 38-week-old male donor and human adult liver total RNA from a 64-year-old male donor were purchased from BioChain Institute Inc. (Newark, CA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo Laboratories (Kumamoto, Japan). Cosmedium 004 (Cosmedium) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). L-15 medium E, which does not contain glucose, tyrosine, arginine, serum, or pyruvic acid, was provided by Cosmo Bio Co., Ltd. All other reagents were of the highest quality available.

Cell culture: Cryopreserved human primary hepatocytes (lot. HPCH10/0910463; 10 donors aged 32–76 years) were obtained from XenoTech, LLC (Lenexa, KS) and thawed using thawing medium without additives (Cat. No. MIL261; Biopredic International, Rennes, France), according to the manufacturer's instructions. Cells were plated on collagen I-coated plates in basal hepatic cell medium (Cat. No. MIL600; Biopredic International) containing additives for hepatocyte seeding medium (Cat. No. ADD221; Biopredic International) for 12 h. The medium was then replaced with basal hepatic cell medium containing additives for hepatocyte culture (Cat. No. ADD222; Biopredic International), and the cells were cultured for 36 h.

The human iPS cell line (Windy), derived from human embryonic lung fibroblast cell line MRC-5, was kindly provided by Umezawa *et al.* of the National Institute for Child Health and Development, Tokyo, Japan. Undifferentiated human iPS cells were cultured based on a previously reported method.[16]

HepG2 cells (Cell No: RCB0523; human hepatocyte carcinoma cells) and HFL-III (Cell No: RCB1886; human fibroblasts derived from the embryonic lung) cells were obtained from the Riken BRC through the National Bio-Resource Project of the MEXT, Japan. These cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, and 1% minimal essential medium with non-essential amino acids (MEM NEAA). Human fetal liver (hFL) cells were obtained from the Applied Cell Biology Research Institute (Kirkland, WA) and were cultured in Williams' medium E containing 10% FBS, 2 mM L-glutamine, and 1% MEM NEAA.

Hepatocyte selection medium: We produced a hepatocyte selection medium and a control medium based on L-15 medium E. L-15 medium E was produced from Leibovitz's L-15 medium by removing glucose, tyrosine, arginine, serum, and pyruvic acid. Modified L-15 medium, *i.e.*, the hepatocyte selection medium, contained galactose (100, 200, 450, or 900 mg/L) with either FBS

(1, 2, 5, or 10%) or 10% KSR in L-15 medium E. The L-15 control medium consisted of 900 mg/L glucose, 300 mg/L tyrosine, and 500 mg/L arginine, with either 10% FBS or 10% KSR in L-15 medium E. The main differences between these media and Leibovitz's L-15 medium are summarized in **Table 1**.

Differentiation of human iPS cells into hepatocytes: Human iPS cells were cultured in Roswell Park Memorial Institute (RPMI) + GlutaMAX™ medium containing 0.5% FBS and 100 ng/mL activin A for 3 days. The culture medium was then replaced with RPMI + GlutaMAX™ medium containing 2% KSR and 100 ng/mL activin A. After 2 days in this medium, the cells were dissociated using Accutase™, seeded on 24-well plates coated with a thin layer of Matrigel™, and cultured in KO-DMEM containing 20% KSR, 1% dimethyl sulfoxide (DMSO), 1% GlutaMax™, 1% MEM NEAA, and 0.1 mM 2-mercaptoethanol for 7 days. Subsequently, the cells were cultured in Cosmedium containing 10 ng/mL HGF, 20 ng/mL OSM, and 100 nM dexamethasone (DEX) for 10 days. Finally, the cells were cultured in Cosmedium alone for 3 days (**Fig. 2A**). This method was defined as the conventional method. L-15 control medium and modified L-15 medium were used in the differentiation process, instead of the media described above. Treatment with DMSO, HGF, OSM, and DEX proceeded for the same length of time as in the conventional method. Morphologic changes were observed by phase contrast microscopy (ECLIPSE TS100, Nikon Instruments Inc., Tokyo, Japan).

Table 1. Main differences from Leibovitz's L-15 medium

Medium	Difference
L-15 medium E[a]	Pyr (−), Gal (−), Tyr (−), Arg (−)
L-15 control medium	Pyr (−), Gal (−), Glu (+), serum (10% FBS),
Modified L-15 medium	Pyr (−), Gal (100–900 mg/L), Tyr (−), Arg (−), serum (1–10% FBS or 10% KSR)

[a]L-15 medium E is the basal medium for L-15 control medium and modified L-15 medium. Pyr, pyruvic acid; Gal, galactose; Tyr, tyrosine; Arg, arginine; Glu, glucose; FBS, fetal bovine serum; KSR, KnockOut™ serum replacement.

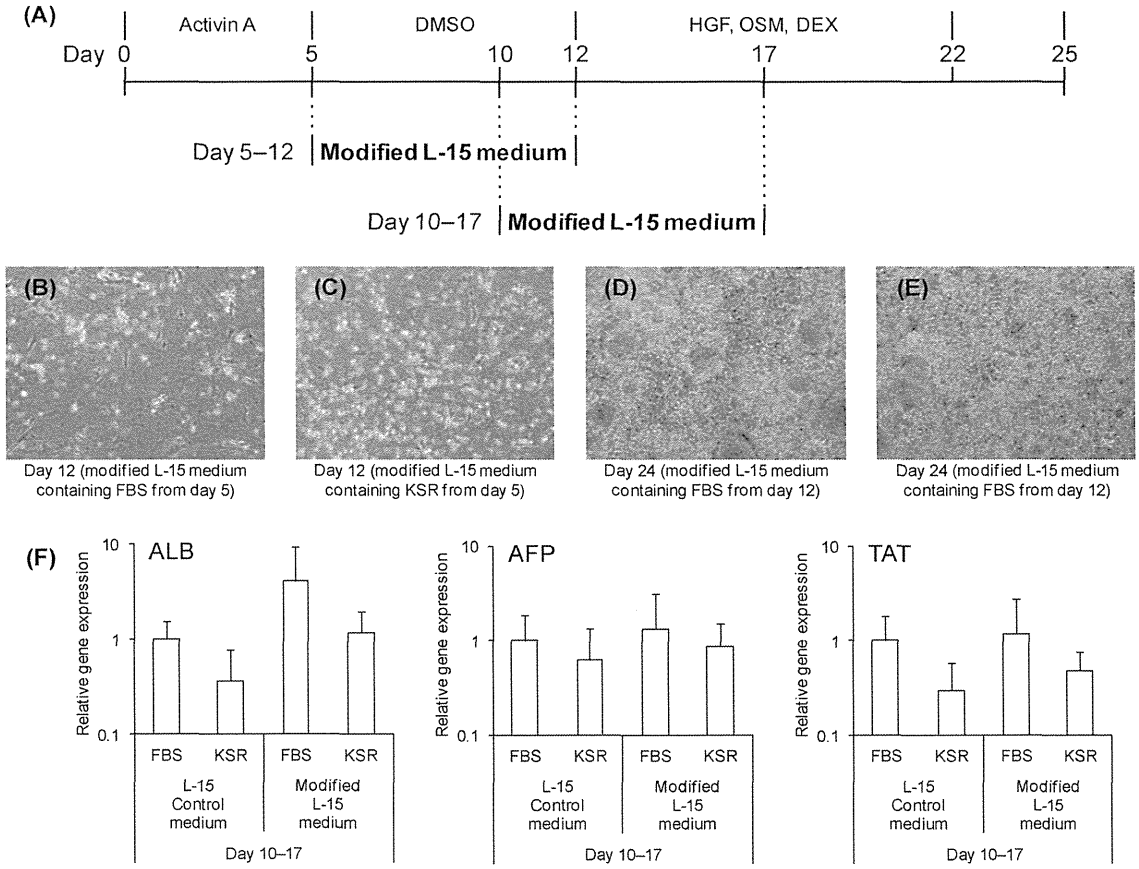

Fig. 2. Morphologic changes and mRNA expression in differentiated human induced pluripotent stem (iPS) cells
(A) Human iPS cells were differentiated into endodermal cells using 100 ng/mL activin A for 5 days, and then into hepatic progenitor cells using 1% dimethyl sulfoxide (DMSO) for 7 days. Finally, the hepatic progenitor cells were matured using 10 ng/mL hepatocyte growth factor (HGF), 20 ng/mL oncostatin M (OSM), and 100 nM dexamethasone (DEX) for 10 days. For the last 3 days, the cells were cultured in Cosmedium 004 alone. These factors were used in all conditions. The effect of modified L-15 medium on differentiation was examined using the medium for 7 days from day 5 or 10 instead of the media used in the conventional method. (B–E) Morphologic changes in differentiated human iPS cells under the following conditions: B, cultivation with modified L-15 medium containing 900 mg/L galactose and 10% fetal bovine serum (FBS) for 7 days from day 5 at day 12; C, cultivation with modified L-15 medium containing 900 mg/L galactose and 10% KnockOut™ serum replacement (KSR) for 7 days from day 5 at day 12; D, cultivation with modified L-15 medium containing 900 mg/L galactose and 10% FBS for 7 days from day 12 at day 24; E, cultivation with modified L-15 medium containing 900 mg/L galactose and 10% KSR for 7 days from day 12 at day 24. (F) Human iPS cells were cultured in modified L-15 medium for 7 days from day 10. L-15 control medium was used for the same period as the modified L-15 medium. L-15 control medium contained either 10% FBS or 10% KSR. Modified L-15 medium contained 900 mg/L galactose and either 10% FBS or 10% KSR. Each bar represents the mean ± S.D. ($n = 3$). The values represent relative gene expression levels where the level in the group of differentiated human iPS cells using L-15 control medium containing FBS for 7 days from day 10 was taken as 1. ALB, albumin; AFP, α-fetoprotein; TAT, tyrosine aminotransferase.

Table 2. Sequences of the primers used for real-time RT-PCR analysis

Gene names	Forward primer sequences (5′–3′)	Reverse primer sequences (5′–3′)
ALB	GAGCTTTTTGAGCAGCTTGG	GGTTCAGGACCACGGATAGA
AFP	AGCTTGGTGGTGGATGAAAC	TCTGCAATGACAGCCTCAAG
TAT	ATCTCTGTTATGGGGCGTTG	TGATGACCACTCGGATGAAA
PXR	AGGATGGCAGTGTCTGGAAC	AGGGAGATCTGGTCCTCGAT
GALK1	AAGTGGCCACGTACACCTTC	GATGAACTGGTCCATGATGC
PAH	TGTCCATGAGCTTTCACGAG	TTAAAACCAGGGTGGTCAGC
OTC	AACAGGCTTTGCACTTCTGG	TCGAGCCAATACTGCATCTG
ASS1	TGAAGGTGACCAACGTCAAG	TCTCCACGATGTCAATACGG
ASL	GCACCAAGGAATTCAGCTTC	CTGTCGGGGTTTTTCTTCTG
CYP3A4	CTGTGTGTTTCCAAGAGAAGTTAC	TGCATCAATTTCCTCCTGCAG
CYP3A5	CTCTCTGTTTCCAAAAGATACC	TGAAGATTATTGACTGGGCTG
CYP3A7	AGATTTAATCCATTAGATCCATTCG	AGGCGACCTTCTTTTATCTG
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG

ALB, albumin; AFP, α-fetoprotein; TAT, tyrosine aminotransferase; PXR, pregnane X receptor; GALK1, galactokinase 1; PAH, phenylalanine hydroxylase; OTC, ornithine transcarbamylase; ASS1, argininosuccinate synthase 1; ASL, argininosuccinate lyase; CYP, cytochrome P450; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

RNA extraction and reverse transcription (RT) reaction: After differentiation, the total RNA was extracted from cells using an RNeasy® Mini Kit (Qiagen NV, Venlo, Limburg, Netherlands) according to the manufacturer's instructions. The first-strand cDNA was generated from 0.5 μg of total RNA. The RT reaction was performed using a PrimeScript™ RT Reagent Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions.

Real-time RT-polymerase chain reaction (PCR) analysis: To determine the expression levels, mRNA samples were analyzed using SYBR Green real-time quantitative RT-PCR. The mRNA levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. All of the PCR procedures were performed using an ABI 7300 Fast Real-Time PCR System with SDS software version 1.4 (Applied Biosystems, Inc., Foster City, CA). The PCR was performed using SYBR Premix EX Taq™ II (Takara Bio Inc.) according to the manufacturer's instructions. The primers used are summarized in **Table 2**.

Cell growth assay: HepG2 cells, HFL-III cells, and hFL cells were seeded in 96-well tissue culture plates at 2×10^4 cells/well with DMEM or Williams' medium E-based culture medium. At 24 h after seeding, the medium was replaced with culture medium, L-15 control medium, or modified L-15 medium containing various concentrations of FBS and galactose. Cell numbers were determined 3 or 7 days after cultivation using the MTT assay. MTT was added to yield a final concentration of 500 μg/mL in the culture medium and cells were incubated for 4 h at 37°C. After incubation, the medium was removed and precipitated formazan was dissolved in DMSO. The absorbance of each sample was measured using a Multiskan FC (Thermo Fisher Scientific Inc., Waltham, MA) at 570 nm.

Immunofluorescence staining: After the differentiation of human iPS cells into hepatocytes, the cells were fixed for 20 min at room temperature in 4% paraformaldehyde and permeabilized in methanol for 5 min at −30°C. After blocking with phosphate-buffered saline containing 2% skim milk for 30 min at room temperature, the cells were incubated with a 1:200 dilution of mouse monoclonal anti-human ALB antibody for 60 min at room temperature, followed by incubation with a 1:500 dilution of Alexa Fluor® 568 goat anti-mouse IgG for 60 min at room temperature. Finally, the cells were incubated with 1 μg/mL 4′,6-diamidino-2-phenylindole for 5 min at room temperature and visualized using an ECLIPSE Ni microscope (Nikon Instruments Inc.).

Results

Expression of hepatic marker genes and enzymes of energy metabolism: The effect of modified L-15 medium on hepatic differentiation of human iPS cells was evaluated using the medium for 7 days from day 5 or 10 (**Fig. 2A**). We also compared whether FBS or KSR was a suitable serum. Most cells died when differentiated cells were cultured in modified L-15 medium for 7 days from day 5 (**Figs. 2B** and **2C**). In contrast, cells proliferated further and differentiated when they were cultured in modified L-15 medium for 7 days from day 10 (**Figs. 2D** and **2E**). The mRNAs of hepatic marker genes, such as ALB, α-fetoprotein (AFP), and tyrosine aminotransferase were expressed in the differentiated cells (**Fig. 2F**). The expression levels of these markers were higher in the FBS group than in the KSR group. In particular, the ALB mRNA expression level was increased by approximately 3.5-fold when using modified L-15 medium containing FBS compared with modified L-15 medium containing KSR. However, differences of AFP and TAT mRNA expression were not observed between L-15 control medium and modified L-15 medium. In order to determine the optimal timing for switching to the modified L-15 medium, human iPS cells were differentiated into hepatocytes under the conventional method. During hepatic differentiation, enzymes that participate in liver-specific energy metabolic pathways exhibited time-dependent gene expression (**Fig. 3**). The expression levels of GALK1, PAH, and OTC gradually increased from day 5, reaching a plateau on day 10. ASS1 and ASL mRNAs were also expressed, but they slightly varied throughout the differentiation process.

Optimization of modified L-15 medium for hepatocytes derived from human iPS cells: We investigated the effect of an additional period with energy sources and the effect of serum on the time-dependent expression of the enzymes involved with energy metabolism. **Supplementary Figure S1A** shows the additional period with energy sources during the hepatic differentiation of human iPS cells. Differentiated cells expressed hepatic marker genes and the drug-metabolizing enzyme cytochrome P450 (CYP) 3A4 (**Supplementary Fig. S1B**). The expression levels were highest in the S2 group, which was differentiated with modified L-15 medium containing FBS and galactose instead of glucose

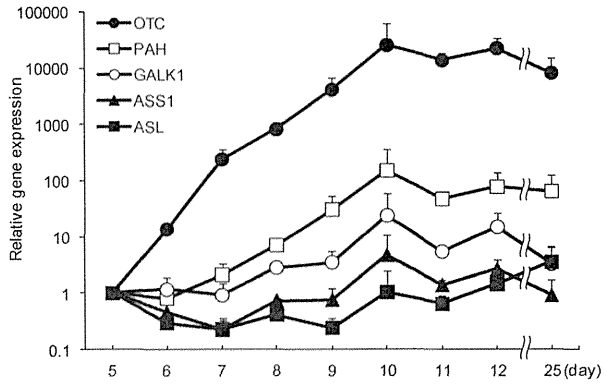

Fig. 3. mRNA expression levels of enzymes of energy metabolism
Differentiated cells were analyzed to determine any time-dependent changes in the mRNA expression levels of enzymes that participate in liver-specific energy metabolism pathways. Human induced pluripotent tem (iPS) cells were differentiated using the conventional method. Each symbol represents the mean ± S.D. [$n = 3$, except for part of the ornithine transcarbamylase (OTC) expression]. The values represent relative gene expression levels, where the level in the group of differentiated human iPS cells at day 5 was taken as 1. PAH, phenylalanine hydroxylase; GALK1, galactokinase 1; ASS1, argininosuccinate synthase 1; ASL, argininosuccinate lyase.

from day 9, and lacking tyrosine from day 9 and arginine from day 10.

Hepatocytes can convert galactose into glucose and release it extracellularly. In addition, glucose is present in FBS as blood sugar. Therefore, we examined the effects of galactose and the FBS concentration to prevent the utilization of glucose. When HepG2, hFL, and HFL-III cells were cultured for 3 days, the viability of HFL-III cells tended to be lower than that of HepG2 and hFL cells (**Supplementary Fig. S2A**). The growth of HFL-III cells was dramatically suppressed by culture in modified L-15 medium for 7 days, but not sufficiently after 3 days (**Supplementary Fig. S2B**). The cell survival rate was highest among hFL cells, followed by HepG2 cells, and lowest in HFL-III cells. The results of the MTT assay on day 7 showed that the cell growth of each cell line decreased in an FBS concentration-dependent manner, whereas that of hFL cells decreased in a galactose concentration-dependent manner when FBS concentration was low.

Four conditions (1 or 2% FBS with 450 or 900 mg/L galactose) were selected on the basis of our investigation of the effect of galactose and FBS concentration, and human iPS cells were differentiated into hepatocytes using these conditions. Modified L-15 medium was used at period S2 (**Fig. 4A**). Differentiated cells expressed ALB, AFP, pregnane X receptor (PXR), CYP3A4, CYP3A5, and CYP3A7 mRNAs (**Fig. 4B**). The expression levels of ALB, CYP3A4, and CYP3A5 mRNAs in both the 1% FBS with 900 mg/L galactose and 2% FBS with 450 mg/L galactose groups increased 1.6-, 6-, and 3-fold, respectively, compared with the L-15 control medium group. Compared with the conventional method of differentiation using Cosmedium, the expression levels of ALB and CYP3A4 mRNAs in both groups increased 5- and 9-fold, respectively. Binuclear cells, which have the typical hepatocyte morphology, were observed among the differentiated cells. Most cells of the differentiated from human iPS cells cultured in modified L-15 medium containing 1% FBS with 900 mg/L galactose or 2% FBS with 450 mg/L galactose were stained with anti-ALB antibody (**Fig. 4C**).

Discussion

To facilitate drug development and regenerative medicine, the establishment of a methodology which allows us to differentiate iPS cells to hepatocytes is eagerly anticipated. In order to achieve this objective, we examined here the effect of culture medium components on the differentiation, focusing on liver-specific intermediary metabolism. Hepatocytes express specific enzymes, including GALK1, PAH, OTC, ASS1, and ASL, involved in the metabolism and synthesis of energy sources. In the differentiation process, differentiated human iPS cells were killed by cultivation in modified L-15 medium from day 5 and cultivation could not be continued. On the other hand, cell survival was observed after cultivation in modified L-15 medium from day 10. This suggests that only differentiated hepatocyte-like cells can survive in medium containing energy sources involved in liver-specific metabolic pathways. Hepatocyte-like cells expressed GALK1, PAH and OTC, and their expression gradually increased and attained a plateau level from day 10 onward. These results suggest that the optimal timing for the introduction of galactose and removal of tyrosine and arginine is around day 10. When we undertook a detailed examination of the appropriate conditions, the expression levels of hepatic marker genes and drug-metabolizing enzymes were highest in the S2 group. It also appears that FBS is more effective than KSR for differentiating iPS cells to hepatocytes.

Hepatocytes can release glucose generated intracellularly into the extracellular environment. In the present study, galactose-derived glucose may be released extracellularly by excessive galactose addition. Glucose was also included in modified L-15 medium containing 10% FBS at a concentration of approximately 100 mg/L in the present study. Cells differentiated from human iPS cells, excluding hepatocyte-like cells, may be able to utilize this glucose. Therefore, we cultured HepG2, hFL, and HFL-III cells in modified L-15 medium containing galactose and FBS at various concentrations. Consequently, the cell growth and survival of HFL-III cells were inhibited. This suggests that HFL-III cells cannot metabolize the galactose, phenylalanine, or ornithine present in modified L-15 medium to energy sources because the cells are fibroblasts. HepG2 cells also exhibited suppressed growth and survival compared with hFL cells. According to the Reference Database for Expression Analysis of the Laboratory for Systems Biology and Medicine, the expression levels of GALK1, PAH, and OTC are low in HepG2 cells, indicating that HepG2 cells cannot utilize the energy sources present in modified L-15 medium. hFL cells were used as a model of immature hepatocytes. The survival rate of hFL cells was the highest of all the cell lines. However, hFL cell proliferation was also suppressed by decreasing galactose concentration at low FBS concentration. In terms of FBS concentration, 10% FBS was insufficient to suppress the survival of HepG2 cells. This suggests that modified L-15 medium containing a low FBS concentration (1% or 2%) and a high galactose concentration (450 or 900 mg/L) would be appropriate for the selection of hepatocytes.

After 25 days of differentiation, differentiated hepatocyte-like cells cultured in the modified L-15 medium expressed ALB, PXR, and CYP3A4 at higher levels than those cultured in L-15 control medium. In particular, combinations of FBS and galactose, including 1% FBS with 900 mg/L galactose or 2% FBS with 450 mg/L galactose, were more effective in increasing the mRNA expression level of CYP3A4, which is a major drug-metabolizing enzyme in

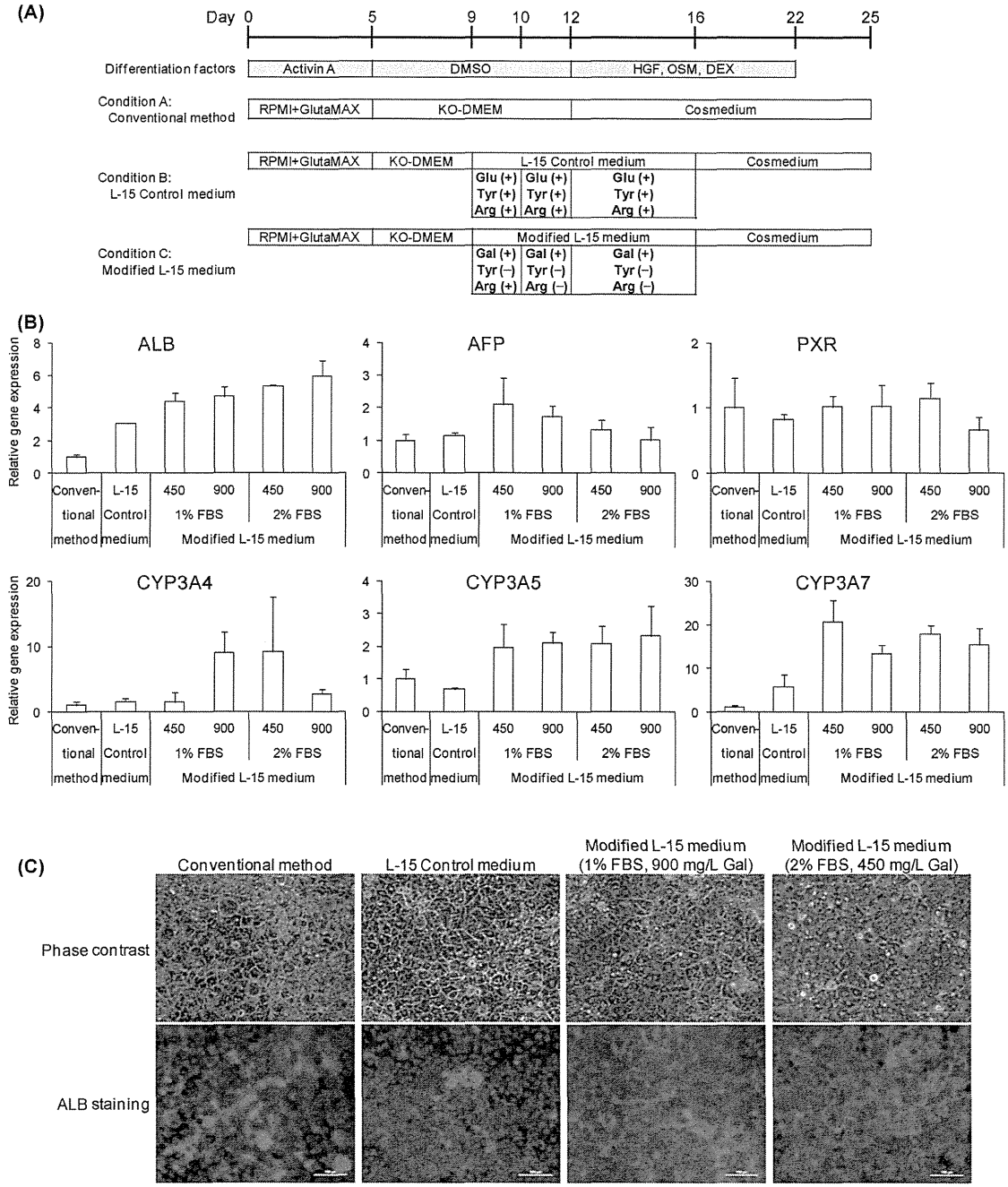

Fig. 4. Effects of fetal bovine serum (FBS) and galactose on differentiated human induced pluripotent stem (iPS) cells
Human iPS cells were differentiated using the conventional method, L-15 control medium, or modified L-15 medium containing fetal bovine serum (FBS, 1 or 2%) and galactose (Gal, 450 or 900 mg/L) from days 9–16. L-15 control medium and modified L-15 medium were used in the differentiation process, instead of the media described above. Bold text indicates key components used in the present study. Arginine (Arg) was added to modified L-15 medium for 1 day from day 9. Treatment with dimethyl sulfoxide (DMSO), hepatocyte growth factor (HGF), oncostatin M (OSM), and dexamethasone (DEX) proceeded for the same length of time as in the conventional method. (A) Schematic of the protocol used for the differentiation of human iPS cells into hepatocytes. Modified L-15 medium was prepared by adding or removing each of the energy sources, which was then replaced. (B) Expression of hepatic marker genes and drug-metabolizing enzymes. Each bar represents the mean ± S.D. ($n = 3$). The values represent relative gene expression levels where the level in the group of differentiated human iPS cells using the conventional method was taken as 1. 450 and 900 refer to galactose concentrations of 450 and 900 mg/L, respectively. (C) Morphologic changes (upper) and immunofluorescence staining of albumin (ALB, lower) in differentiated human iPS cells. Cells were stained with anti-ALB antibody (red). Nuclei were counterstained with 1 μg/mL 4′,6-diamidino-2-phenylindole (blue). AFP, α-fetoprotein; PXR, pregnane X receptor; Cosmedium, Cosmedium 004; CYP, cytochrome P450; Glu, glucose; KO-DMEM, KnockOut™ Dulbecco's modified Eagle's medium; RPMI, Roswell Park Memorial Institute; Tyr, tyrosine.

the adult human liver[17,18] that is important in pharmacokinetic studies. Under these conditions, some differentiated cells exhibited a binuclear morphology and most of the differentiated cells were stained with anti-ALB antibody. ALB is a major human plasma protein that is produced by hepatocytes; therefore, ALB expression is believed to be an indicator of differentiation into hepatocytes.

A previous study has shown that a hepatocyte selection medium lacking glucose and arginine but containing galactose and ornithine eliminates undifferentiated human iPS cells, and this medium contributes to a successful culturing of primary human hepatocytes without any damage.[15] Furthermore, a similar medium enriched hepatoblast-like cells differentiated from mouse ES cells *via* embryoid body formation.[14] However, embryoid body formations tend to differentiate spontaneously into the multiple cell lineages included in endodermal cells. The direct differentiation method used in the present study would also be suitable for endodermal cells using activin A[19] to more efficiently differentiate hepatocytes from human iPS cells. We also demonstrated that optimization of the selection conditions was necessary for human iPS cells in contrast to mouse ES cells. Based on our results, we propose that human iPS cells that differentiated into non-liver cells were eliminated and only hepatocytes were cultured in modified L-15 medium. In addition, we showed that modified L-15 medium containing appropriate concentrations of FBS and galactose selected only differentiated hepatocyte-like cells.

The hepatocyte-like cells prepared in this study were considered to be close to fetal cells, because they expressed AFP and CYP3A7, which are an index of immature hepatocytes and a fetus-specific CYP, respectively.[20–22] However, our method can isolate hepatocyte- or hepatoblast-like cells in a safe, simple, and inexpensive manner. Takebe *et al.* reported that functional human liver was generated *via* the transplantation of liver buds created from human iPS cells *in vitro*.[7] Therefore, the cells obtained using our method may represent a source of cells for liver transplantation.

In conclusion, we have provided valuable information on the selection of human iPS cell-derived hepatocyte-like cells using modified L-15 medium in which FBS and galactose concentrations were adjusted during the appropriate period of hepatocyte differentiation. We anticipate that these differentiated hepatocytes will be utilized in drug development studies, including pharmacokinetic and toxicologic analyses, and regenerative medicine.

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Regular Article

An Efficient Method for Differentiation of Human Induced Pluripotent Stem Cells into Hepatocyte-like Cells Retaining Drug Metabolizing Activity

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Summary: The use of human induced pluripotent stem (iPS) cells would be of great value for a variety of applications involving drug development studies. Several reports have been published on the differentiation of human iPS cells into hepatocyte-like cells; however, the cells were insufficient for application in drug metabolism studies. In this study, we aimed to establish effective methods for differentiation of human iPS cells into hepatocytes. Two human iPS cell lines were differentiated by addition of activin A, dimethyl sulfoxide, hepatocyte growth factor, oncostatin M, and dexamethasone. The differentiated cells expressed hepatocyte markers and drug-metabolizing enzymes, revealing that the human iPS cells were differentiated into hepatocyte-like cells. Expression of CYP3A4 and UGT1A1 mRNAs increased with treatment with typical inducers of the enzymes, and the response of the cells against the inducers was similar to that of human hepatocytes. Furthermore, the drug-metabolizing activity of CYP3A4, as monitored by testosterone 6β-hydroxylase activity, was elevated by these inducers. In conclusion, we established methods for differentiation of hepatocyte-like cells expressing drug metabolizing activity from human iPS cells. The hepatocyte-like cells derived from human iPS cells will be useful for drug metabolism studies.

Keywords: iPS; differentiation; hepatocyte; drug-metabolizing enzyme; CYP

Introduction

Induced pluripotent stem (iPS) cells have been generated directly from fibroblast cells by the expression of defined reprogramming factors (OCT3/4, SOX2, KLF4, and c-MYC).[1–4] Recent studies have shown that iPS cells are comparable to embryonic stem (ES) cells including multilineage differentiation potential and intensive proliferation *in vitro*.[5] iPS cell-derived

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hepatocytes could provide a valuable model system for novel pharmaceutical drug discovery assays, investigation of drug metabolism, and prediction of liver toxicity.[5,6] However, efficient generation of highly differentiated hepatocytes from iPS cells has not yet been established without transduction of transcription factors in addition to treatment with optimal growth factors.

Cytochrome P450 (CYP) proteins display differential expression levels according to the developmental stages of the liver. CYP3A7 is a major enzyme in the human fetal and newborn liver,[7,8] whereas CYP3A4 is expressed throughout development and finally accounts for about 30% of the amount of total CYP in the human adult liver.[9,10] The human fetal and adult liver expression levels of CYP3A enzymes are increased by exposure to a variety of drugs,[11–13] which in turn leads to accelerated metabolism and concomitant use of the drugs. Several studies have examined the differentiation of ES or iPS cells into hepatocyte-like cells,[14–21] and these cells expressed hepatic markers such as albumin (ALB), α-1 antitrypsin, and hepatocyte nuclear factor (HNF) 4α. However, the activities of drug-metabolizing enzymes have not been detected in the ES or iPS cell-derived hepatocytes using simple methods. Thus, the ES or iPS cell-derived hepatocytes do not entirely recapitulate mature liver function.

We recently established an efficient method to differentiate ES cells into hepatocyte-like cells.[22,23] In the present study, iPS cell-derived hepatocyte-like cells exhibited a sequential pattern of expression of hepatic markers and drug-metabolizing enzymes. Furthermore, drug-metabolizing activity was induced by treatment with drugs, similar to what is observed in human liver and hepatocytes.

Materials and Methods

Materials: Umezawa *et al.* established 2 human iPS cell lines (Fetch, NIHS0604 and Lollipop, JCRB1336), derived from the embryonic human lung fibroblast cell line MCR-5. HepG2 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Mouse embryonic fibroblasts (MEF) were obtained from Oriental Yeast (Tokyo, Japan). BD Matrigel Matrix Growth Factor Reduced (Matrigel) was obtained from BD Biosciences (Bedford, MA). Dulbecco's modified Eagle's medium (DMEM), and DMEM and Ham's nutrient mixture F-12 (DMEM/F12) were obtained from Sigma-Aldrich (St. Louis, MO). KnockOut Serum Replacement (KSR), KnockOut DMEM (KO-DMEM), Roswell Park Memorial Institute (RPMI) + GlutaMax medium, GlutaMax, minimal essential medium nonessential amino acids (MEM NEAA), and SuperScript III were obtained from Invitrogen Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Hyclone (Waltham, MA). Basic fibroblast growth factor (bFGF) and activin A were obtained from PeproTech (Rocky Hill, NJ). Modified Lanford medium was obtained from Charles River (Tokyo, Japan). Accutase was obtained from MS TechnoSystems (Osaka, Japan). Hepatocyte growth factor (HGF) was obtained from R&D Systems (Minneapolis, MN). RNeasy Mini Kit was obtained from Qiagen (Valencia, CA). Oncostatin M (OSM), dexamethasone (DEX), omeprazole (OME), rifampicin (RIF), dimethyl sulfoxide (DMSO), and human normal adult liver total RNA, derived from a 64-year-old male donor, were obtained from Wako Pure Chemicals (Osaka, Japan). SYBR Green real-time polymerase chain reaction (PCR) Master Mix was obtained from Takara Bio (Otsu, Japan). Collagen Type I (collagen I)-coated microplate was obtained from Asahi Glass (Chiba, Japan). Dissociation solution for human ES/iPS cells was obtained from ReproCELL Incorporated (Tokyo, Japan). All other reagents used were of the highest quality available.

Human iPS cell culture: Undifferentiated human iPS cells were maintained on a feeder layer of mitomycin C-treated MEF on a gelatin-coated dish in a human iPS medium at 37°C in humidified air with 5% CO_2. The human iPS medium consisted of DMEM/F12 supplemented with 20% KSR, 2 mM L-glutamine, 1% MEM NEAA, 0.1 mM 2-mercaptoethanol, and 5 ng/mL bFGF. The medium was changed daily. Human iPS cell colonies composed of closely packed cells were split approximately every 3 to 4 days by incubation in a dissociation solution for 5 min at 37°C and passaged onto new mitomycin C-treated MEF.

Differentiation of human iPS cells into hepatocyte-like cells: Differentiation was initiated when the human iPS cells reached a confluence level of approximately 70%. The human iPS cells were cultured with RPMI + GlutaMax medium containing 0.5% inactivated FBS and 100 ng/mL activin A. Three days later, the culture medium was switched to the RPMI + GlutaMax medium containing 2% KSR and 100 ng/mL activin A for 2 days. After induction of differentiation, the cells were dissociated by the addition of Accutase for 5 min at 37°C and passaged onto 24-well plates coated with collagen I or a thin layer Matrigel (dilute Matrigel 1:30 with cold human iPS medium) and cultured in KO-DMEM containing 20% KSR, 1% GlutaMax, 1% MEM NEAA, 0.1 mM 2-mercaptoethanol, and 1% DMSO for 7 days. The cells were subsequently cultured in modified Lanford medium containing 10 ng/mL HGF, 20 ng/mL OSM, and 100 nM DEX for 9 days. The cells were then cultured in modified Lanford medium for 4 days (**Fig. 1**).

Drug treatments: To clarify the effects of inducers on the expression of CYPs, the cells were treated with 40 μM RIF, 50 μM OME, or 100 μM DEX as described previously[22–25] for the final 72 h of the differentiation protocol. The compounds were dissolved in DMSO, which was added to the culture medium at a final concentration of 0.1%.

RNA extraction and reverse transcription reaction: An RNeasy Mini Kit was used to extract total RNA from the cells according to the manufacturer's instructions. First-strand cDNA was generated from 2–4 μg of total RNA. Reverse transcription (RT) reaction was performed using SuperScript III, according to the manufacturer's instructions.

Real-time RT-PCR analysis: For determination of expression levels, mRNAs were analyzed using SYBR Green real-time quantitative RT-PCR. Relative mRNA expression levels in each sample were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels. All PCR procedures were performed using the ABI 7300 Fast System SDS software version 1.3.1 (Applied Biosystems, Foster City, CA), according to each

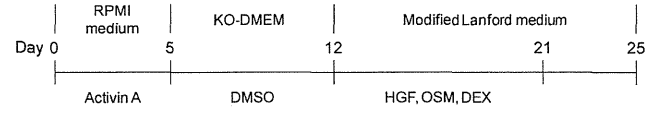

Fig. 1. Differentiation into hepatocytes from 2 human iPS cell lines
Two human iPS cell lines (Fetch and Lollipop) were differentiated into endoderm cells by addition of 100 ng/mL activin A for 5 days, and then into hepatocytes by the addition of 1% DMSO for 7 days. The hepatocytes were then matured by the addition of 10 ng/mL HGF, 20 ng/mL OSM, and 10^{-7} M DEX for 9 days. For the final 4 days, the cells were cultured in modified Lanford medium alone, without HGF, OSM, or DEX.

Table 1. Sequences of primers for real-time RT-PCR analysis

Gene names	Forward primer sequences (5′- -3′)	Reverse primer sequences (5′- -3′)
HNF4α	GAGCTGCAGATCGATGACAA	TACTGGCGGTCGTTGATGTA
ALB	GAGCTTTTTGAGCAGCTTGG	GGTTCAGGACCACGGATAGA
AFP	AGCTTGGTGGTGGATGAAAC	TCTGCAATGACAGCCTCAAG
CYP1A2	CTTTGACAAGAACAGTGTCCG	AGTGTCCAGCTCCTTCTGGAT
CYP1B1	ACCAGGTATCCTGATGTGCAGAC	AGGTGTTGGCAGTGGTGGCATGAG
CYP2C9	GAACACCAAGAATCGATGGACA	TCAGCAGGAGAAGGAGAGCATA
CYP3A4	CTGTGTGTTTCCAAGAGAAGTTAC	TGCATCAATTTCCTCCTGCAG
CYP3A5	CTCTCTGTTTCCAAAAGATACC	TGAAGATTATTGACTGGGCTG
CYP3A7	AGATTTAATCCATTAGATCCATTCG	AGGCGACCTTCTTTTATCTG
UGT1A1	CAGCAGAGGGGACATGAAAT	ACGCTGCAGGAAAGAATCAT
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG

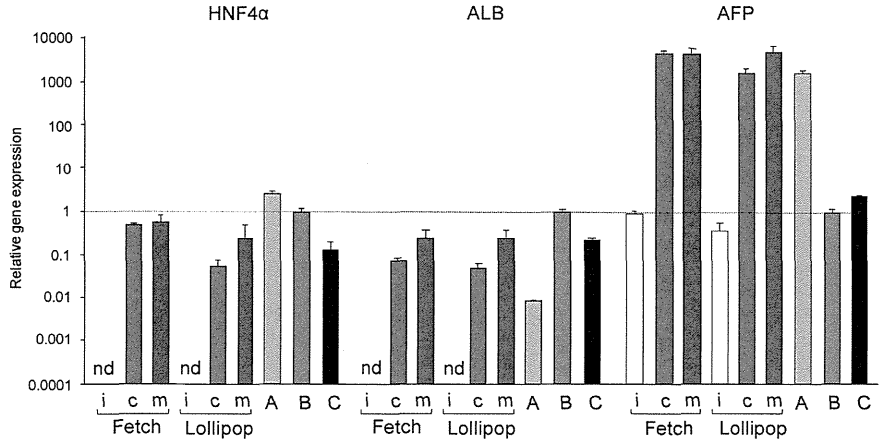

Fig. 2. Expression levels of liver marker protein mRNAs
The expression levels of HNF4α, ALB, and AFP mRNAs in undifferentiated human iPS cells (i) and hepatocyte-like cells differentiated from two human iPS cell lines (Fetch and Lollipop) were analyzed using real-time PCR. Collagen I (c) or Matrigel (m) was used for the differentiation as the extracellular matrix. A, B, and C represent HepG2 cells, human adult liver, and hepatocytes, respectively, as positive controls. Each bar represents the mean ± SD from triplicate experiments. Values were normalized to the level of GAPDH mRNA. The graph represents the relative gene expression level when the level in the liver was taken as 1. nd, not detected.

manufacturer's instructions. PCR was performed using diluted cDNA template in a 25-μL reaction mixture containing 0.3 μM of each primer and 12.5 μL of SYBR Green real-time PCR Master Mix. The primers used are summarized in **Table 1**.

Assay for testosterone 6β-hydroxylase activity: Human iPS cell-derived hepatocyte-like cells were cultured with modified Lanford medium containing 100 μM testosterone for 6 h at 37°C. After incubation, the reaction medium was collected into the tubes with 1.25 mL of ethyl acetate, and 10 μL of 1 μM ethoxyresorufin was added to the medium as an internal standard. The tube was vortexed and centrifuged. Organic phase aliquots (1 mL) were transferred to microcentrifuge tubes. Ethyl acetate was evaporated under nitrogen gas, and the samples were dissolved with 100 μL of a mixture of 10 mM ammonium acetate (98%) and methanol (2%) containing 0.1% formic acid. Metabolites were analyzed using liquid chromatography coupled with tandem mass spectrometry according to the method reported previously.[26]

cDNA microarray analysis: An RNeasy Mini column (Qiagen) was used to extract and purify RNA (10 μg). The RNA was labeled using a SuperScript Indirect cDNA Labeling Kit (Invitrogen) and Cy™3 or Cy™5 Mono-Reactive Dye (GE Healthcare, Little Chalfont, UK). The dye-coupled cDNAs were purified using a MiniElute PCR purification kit (Qiagen) and hybridized to an Agilent 44 K human 60-mer oligo microarray (Agilent Technologies, Santa Clara, CA) according to Agilent instructions. An Agilent microarray scanner (Agilent Technologies) was used to wash, dry, and scan the slides. A Genespring GX software package (Agilent Technologies) was used to process and analyze the data. Ingenuity IPA software (Ingenuity Systems Redwood City, CA) was used to perform pathway analysis.

Results

Expression of hepatic marker gene: In the present study, we succeeded in differentiating human iPS cells into hepatocyte-like cells that exhibited drug metabolizing activity. Activin A was used to differentiate two human iPS cell lines, Fetch and Lollipop, into endoderm cells.[27] The endoderm cells were then induced to hepatocytes by the addition of DMSO.[28] The hepatocytes were subsequently matured by the addition of HGF, OSM, and DEX (**Fig. 1**).[29–31] Collagen I or Matrigel was used as extracellular matrix in the hepatic differentiation of the human iPS cells. The expression of mRNAs encoding typical hepatic marker proteins in the cells differentiated from both human iPS cell lines was compared with those in HepG2 cells, human adult liver, and primary hepatocytes (**Fig. 2**). HNF4α, ALB, and α-fetoprotein (AFP) mRNAs were detected in the cells differentiated from human iPS cells. The expression levels of these mRNAs in the cells differentiated from the two iPS cell lines were similar and the cells cultured on Matrigel were relatively higher than those cultured on collagen I. The expression levels of HNF4α and ALB mRNAs

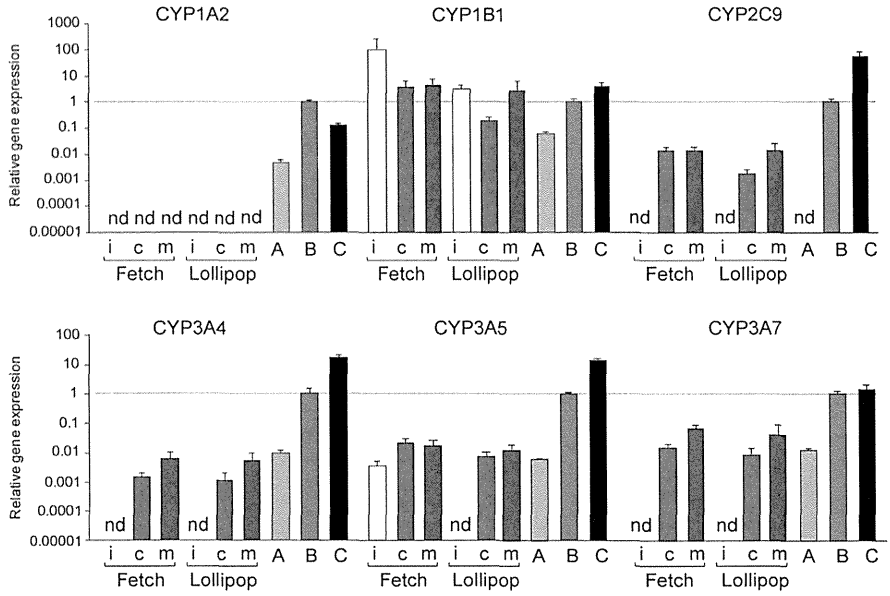

Fig. 3. Expression levels of CYP mRNAs
The expression levels of CYP1A2, CYP1B1, CYP2C9, CYP3A4, CYP3A5, and CYP3A7 mRNAs in undifferentiated human iPS cells (i) and hepatocyte-like cells differentiated from 2 human iPS cell lines (Fetch and Lollipop) were analyzed using real-time PCR. Collagen I (c) or Matrigel (m) was used for the differentiation as the extracellular matrix. A, B, and C represent HepG2 cells, human adult liver, and hepatocytes, respectively, as positive controls. Each bar represents the mean ± SD from triplicate experiments. Values were normalized to the levels of GAPDH mRNA. The graphs represent the relative gene expression level when the level in the liver was taken as 1. nd, not detected.

in the cells differentiated from iPS cells were similar to those in hepatocytes. However, the expression of AFP mRNA, a marker protein for an immature liver,[32] in the cells was almost identical to that of the HepG2 cells, and was markedly higher than the expression in human adult liver and hepatocytes.

Expression of CYP and UGT gene: Figure 3 shows the expression of CYP mRNAs in the cells differentiated into hepatocytes from iPS cells. The majority of the CYPs involved in drug metabolism display tissue-specific expression in the liver and small intestine.[33] Of these, the mRNA encoding CYP1A2, a liver-specific CYP,[34] was not detected in the cells differentiated into hepatocytes. On the other hand, three CYP3A mRNAs were detected in the cells, but their expression levels were approximately 100–1,000 times lower in the cells differentiated into hepatocytes than in liver and hepatocytes, and were similar to that in HepG2 cells. It is of interest that CYP2C9 mRNA was detected in the cells differentiated into hepatocytes but not in HepG2 cells, although the expression level was lower than that of the liver and hepatocytes. The expression of CYP1B1 mRNA, which is not a liver-specific CYP, was observed in all cells used in this experiment at similar levels. The expression profiles for these CYPs were not largely different between the two cell lines; however, the expression levels of CYP mRNAs tended to be higher in the cells cultured on Matrigel than in those cultured on collagen I. To determine the expression of phase I and II enzymes, the differentiated cells were compared with adult or fetal hepatocytes using microarray analysis. The expression levels of mRNAs encoding the phase II enzymes, uridine diphosphate glucuronosyltransferase (UGT) 2A3, UGT2B4, and UGT2B7, were high in the differentiated cells. In contrast, the expression levels of majority of the phase I enzymes in the differentiated cells were lower than those in adult hepatocytes (**Fig. 4**).

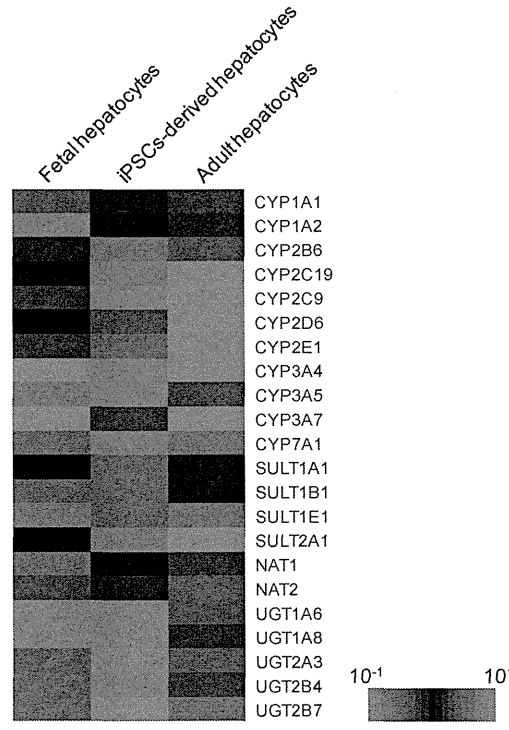

Fig. 4. Microarray analysis of phase I and II enzymes
Human iPS cells (Fetch) were differentiated into hepatocyte-like cells. After differentiation, total mRNA was extracted from the cells. The expression levels of phase I and II enzymes were analyzed by microarray analysis as described in Materials and Methods. The expression levels of fetal liver cells, hepatocyte-like cells differentiated from human iPS cells, and adult hepatocytes are presented in the left, center, and right columns, respectively.

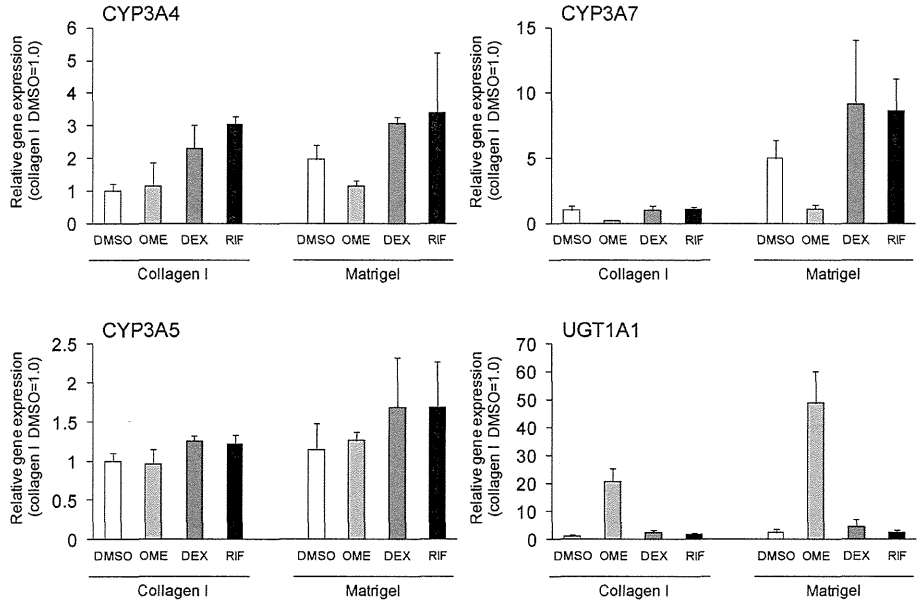

Fig. 5. Effects of drugs on expression of CYP3A enzymes and UGT1A1 mRNAs in the hepatocyte-like cells
The cells differentiated from human iPS cells (Fetch) were treated with OME, DEX, and RIF for 72 h. The total mRNA was extracted from the cells. The expression of CYP3A and UGT1A1 mRNAs were analyzed by microarray analysis as described in Materials and Methods. Each bar represents the mean ± SD from triplicate experiments. Values were normalized to the levels of GAPDH mRNA. The graphs represent the relative gene expression level when the levels in the hepatocyte-like cells using collagen I and DMSO were assigned a value of 1.

Inducibility of CYP3A enzymes and drug metabolizing activity: A specific property of some drug-metabolizing enzymes is the induction of their expression by treatment with drugs. **Figure 5** presents the effects of inducers on expression of CYP3A enzymes and UGT1A1. In the cells differentiated into hepatocytes from human iPS cells on collagen I, expression of CYP3A4 mRNA increased 2.5- and 3-fold with treatment with DEX and RIF, respectively, although expressions of CYP3A5 and CYP3A7 mRNAs were not markedly increased. When the human iPS cells were cultured on Matrigel, marked induction of CYP3A enzymes was not observed. On the other hand, UGT1A1 mRNA was strongly induced by treatment with OME in the cells differentiated into hepatocytes. The induced level was higher in the cells cultured on Matrigel (50-fold induction) than on collagen I (22-fold induction). UGT1A1 is a typical drug-metabolizing enzyme that is strongly induced by polycyclic aromatic hydrocarbons and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) through activation of aryl hydrocarbon receptor (AhR).[35] OME is also known as an AhR activator.[36]

Based on the induction of CYP3A4 mRNA expression, we measured testosterone 6β-hydroxylase activity, which is catalyzed by CYP3A enzymes. As shown in **Figure 6**, testosterone 6β-hydroxylase activity was induced 2.3- and 2.7-fold by treatment with DEX and RIF, respectively, in the cells cultured on collagen I. The activity in the cells cultured on Matrigel was approximately 2.5-fold higher than that of the cells cultured on collagen I in the control (DMSO) and was markedly induced by treatment with RIF.

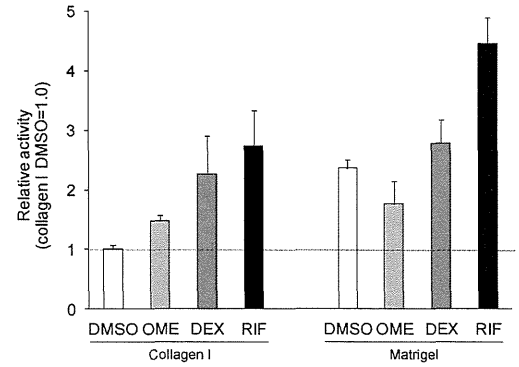

Fig. 6. Effects of drugs on testosterone 6β-hydroxylase activity in hepatocyte-like cells
The cells differentiated from human iPS cells (Fetch) were treated with OME, DEX, and RIF for 72 h and then cultured with the medium containing testosterone for 6 h. 6β-Hydroxytestosterone was analyzed using liquid chromatography coupled with tandem mass spectrometry as described in Materials and Methods. Each bar represents the mean ± SD from triplicate experiments. The graphs represent the relative activity ratios when the value in the hepatocyte-like cells using collagen I and DMSO were assigned a value of 1.

Discussion

In the present study, we established methods for the differentiation of human iPS cells into functional hepatocytes as determined by constitutive and induced expression of CYP3A4 mRNA. Several groups have previously reported the differentiation of human iPS cells into hepatocytes. Song *et al.* reported that human iPS cells can be induced to differentiate into hepatocyte-like cells by using six growth factors and supplements.[17] In addition, Si-Tayeb *et al.* reported that human iPS cells can be induced to efficiently differentiate into hepatocytes by using five growth factors and supplements under hypoxic conditions.[15] Another study by Takayama *et al.* described the use of a highly efficient method for the generation of functional hepatocytes from human iPS cells by sequential transduction of the sex determining region Y box 17 (SOX17), hematopoietically expressed homeobox (HHEX) and HNF4α or forkhead box A2 (FOXA2), and

HNF1α.[18,19] Hepatic differentiation methods (*e.g.*, 3D coculture[20] and hollow fiber[21]) have also been used. However, highly efficient methods are complicated and extremely expensive. Because of the facile approaches that utilized only humoral factors, the responsiveness including CYP activity of differentiated human iPS cells-derived hepatocytes to inducers was unclear.

In the present study, a three-step protocol using three growth factors and two small-molecule compounds was used to differentiate human iPS cells into hepatocytes. The human iPS cells were differentiated into endoderm cells by the addition of 100 ng/mL activin A[27] and were subsequently differentiated into hepatocytes by the addition of 1% DMSO.[28] The hepatocytes were matured by the addition of 10 ng/mL HGF,[28] 20 ng/mL OSM,[28] and 10^{-7} M DEX.[30] Activin A is necessary for the differentiation of human iPS cells into endoderm cells.[27] Few studies have used DMSO, bone morphogenetic protein 4 (BMP4), FGF2, FGF7 or FGF10 for differentiation of human iPS cells into hepatic progenitor cells from human stem cells.[15,17,28] In addition, we used these single factors alone or in combinations, but no increase in differentiation efficiency was observed (data not shown). Further, we selected DMSO, which is structurally stable and inexpensive compared with recombinant proteins such as the cytokines described above. DMSO has been known to maintain the functions of adult hepatocytes *in vitro*[37] and was used to differentiate HepaRG cells.[38] The hepatic progenitor cells were matured by addition of HGF, OSM, and DEX, necessary for maturation of hepatocytes.[29–31]

After 25 days of differentiation, the mRNAs encoding hepatocyte markers, such as ALB, AFP, and drug-metabolizing enzymes, were expressed in the cells differentiated from human iPS cells; the expression of these mRNA were increased using Matrigel. In a previous report, Matrigel led to high expression of liver-enriched transcription factors such as HNF4α[39] and laminin, a main component of Matrigel, increased ALB mRNA level and ALB secretion.[40] The result in the present study is consistent with these findings. The expression level of CYP2C9 mRNA in the cells differentiated from human iPS cells was higher than that in HepG2 cells, although lower than that in hepatocytes. This result suggested that the cells are similar to hepatocytes rather than HepG2 cells, a hepatocellular carcinoma cell line. In addition, mRNA expression levels of CYP3A4 and UGT1A1 in the cells were increased by each inducer and presented responsiveness similar to that of the hepatocytes. These results indicate that human iPS cells were differentiated into hepatocyte-like cells under the present conditions. Furthermore, the cells showed testosterone 6β-hydroxylase activity that was mediated by CYP3A4, and the activity increased with addition of RIF or DEX. CYP3As, in particular CYP3A4, are strongly induced by treatment with RIF and DEX in livers.[41] These data indicated that functional hepatocyte-like cells had been differentiated from human iPS cells. Induction of the *CYP3A4* and *UGT1A1* genes is mediated through the activation of pregnane X receptor (PXR)/constitutive androstane receptor (CAR) and AhR, respectively.[42] AhR and CAR were clearly detected, but PXR was not detected in the hepatocyte-like cells (data not shown). Thus, the CYP3A4 induction observed in this study may have been dependent on CAR and glucocorticoid receptor activation by RIF and DEX, respectively. Furthermore, induction of mRNAs encoding CYP1A2 as well as CYP1A1 was observed in hepatocyte-like cells treated with TCDD, although CYP1A2 mRNA was not detected in untreated hepatocyte-like cells (data not shown). The expression level of CYP3A7 mRNA was reduced when the cells were treated with OME. However, the reason for this remains unknown. Krusekopf *et al.* reported that CYP3A4 in HepG2 cells was induced by OME, whereas CYP3A5, CYP3A7, and CYP3A43 were unaffected or even slightly downregulated by OME.[43] They suggested that the expression of CYP3A4 is differently regulated from that of CYP3A5, CYP3A7, and CYP3A43 depending on the inducer. Expression of the CYP3A subfamily might be differently regulated by OME.

In fetal liver, it is known that the expression level of CYPs is extremely low compared with that in a mature liver.[44] In the hepatocyte-like cells differentiated from human iPS cells, the expression levels of HNF6 and CCAAT-enhancer-binding protein alpha (CEBPα), transcriptional factors involved in the differentiation into mature liver cells, were low (data not shown). From the results of microarrays, the expression levels of phase II enzymes were relatively high in the differentiated cells. In contrast, the expression levels of the phase I enzymes were low, which demonstrated that the human iPS cell-derived hepatocyte-like cells were still immature. These results may suggest that the hepatocyte-like cells from human iPS cells were differentiated at least in part to fetal liver-like cells. Therefore, the differentiated cells may be useful for evaluation in a human fetal liver model.

Multiple iPS cell lines were differentiated into hepatocytes using this method, although there were differences in the differentiation ability. There is a variation of differentiation propensity among multiple human ES/iPS cell lines[45,46] because of the method of stem cell generation, culture conditions, and genetic backgrounds of the donor cells. In future, it is necessary to optimize a differentiation method adapted to each cell line, and to select a cell line that is easy to differentiate into cells of interest.

In conclusion, the three-step protocol and the limited number of differentiation factors used in this study constituted an efficient method for differentiation of human iPS cells into hepatocyte-like cells that showed drug metabolizing activity and induction of CYP3A4 by RIF. However, the expression levels of drug-metabolizing enzymes were very low compared with those in mature livers. Further studies are needed to increase the expression of drug-metabolizing enzymes produced under the conditions of our method.

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