

可能性のほかに、ホストとなる（患者）肝臓にがん細胞が存在したときに MS 細胞の特徴である **trophic effect**（サイトカインなどを放出することにより周辺の細胞に栄養を供給する効果）が、がん細胞の転移および浸潤能を高める危険性についても詳細に検討する必要がある。

ヒト初代培養肝細胞と同程度の機能を有した多能性幹細胞由来の肝細胞への分化誘導法が確立されれば、多能性幹細胞由来の肝細胞は肝疾患患者への細胞移植治療の新しい細胞源として利用されることが期待される。ヒト ES 細胞、iPS 細胞、MS 細胞を含めた多能性幹細胞から分化誘導した肝細胞を利用する細胞移植治療の早期実現のためには、よりいっそうの基礎研究が必要であるとともに、基礎研究者・臨床医・政府（規制当局）が密接に連携して取り組むことが必要であろう。

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Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1 α transduction

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Background & Aims: Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) can be utilized as a tool for screening for hepatotoxicity in the early phase of pharmaceutical development. We have recently reported that hepatic differentiation is promoted by sequential transduction of SOX17, HEX, and HNF4 α into hESC- or hiPSC-derived cells, but further maturation of hepatocyte-like cells is required for widespread use of drug screening.

Methods: To screen for hepatic differentiation-promoting factors, we tested the seven candidate genes related to liver development.

Results: The combination of two transcription factors, FOXA2 and HNF1 α , promoted efficient hepatic differentiation from hESCs and hiPSCs. The expression profile of hepatocyte-related genes (such as genes encoding cytochrome P450 enzymes, conjugating enzymes, hepatic transporters, and hepatic nuclear receptors) achieved with FOXA2 and HNF1 α transduction was comparable to that obtained in primary human hepatocytes. The hepatocyte-like cells generated by FOXA2 and HNF1 α transduction exerted various hepatocyte functions including albumin and urea secretion, and the uptake of indocyanine green and low density lipoprotein. Moreover, these cells had the capacity to metabolize all nine tested drugs and were successfully employed to evaluate drug-induced cytotoxicity.

Conclusions: Our method employing the transduction of FOXA2 and HNF1 α represents a useful tool for the efficient generation of metabolically functional hepatocytes from hESCs and hiPSCs, and the screening of drug-induced cytotoxicity.

Keywords: FOXA2; HNF1 α ; Hepatocytes; Adenovirus; Drug screening; Drug metabolism; hESCs; hiPSCs.

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Introduction

Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) [1] or human induced pluripotent stem cells (hiPSCs) [2] have more advantages than primary human hepatocytes (PHs) for drug screening. While application of PHs in drug screening has been hindered by lack of cellular growth, loss of function, and de-differentiation *in vitro* [3], hESC- or hiPSC-derived hepatocyte-like cells (hESC-hepa or hiPSC-hepa, respectively) have potential to solve these problems.

Hepatic differentiation from hESCs and hiPSCs can be divided into four stages: definitive endoderm (DE) differentiation, hepatic commitment, hepatic expansion, and hepatic maturation. Various growth factors are required to mimic liver development [4] and to promote hepatic differentiation. Previously, we showed that transduction of transcription factors in addition to treatment with optimal growth factors was effective to enhance hepatic differentiation [5–7]. An almost homogeneous hepatocyte population was obtained by sequential transduction of SOX17, HEX, and HNF4 α into hESC- or hiPSCs-derived cells [7]. However, further maturation of the hESC-hepa and hiPSC-hepa is required for widespread use of drug screening because the drug metabolism capacity of these cells was not sufficient.

In some previous reports, hESC-hepa and hiPSC-hepa have been characterized for their hepatocyte functions in numerous ways, including functional assessment such as glycogen storage and low density lipoprotein (LDL) uptake [7]. To make a more precise judgment as to whether hESC-hepa and hiPSC-hepa can be applied to drug screening, it is more important to assess cytochrome P450 (CYP) induction potency and drug metabolism capacity rather than general hepatocyte function. Although Duan *et al.* have examined the drug metabolism capacity of hESC-hepa, drug metabolites were measured at 24 or 48 h [8]. To precisely



estimate the drug metabolism capacity, the amount of metabolites must be measured during the time when production of metabolites is linearly detected (generally before 24 h). To the best of our knowledge, there have been few reports that have examined various drugs metabolism capacity of hESC-hepa and hiPSC-hepa in detail.

In the present study, seven candidate genes (*FOXA2*, *HEX*, *HNF1 α* , *HNF1 β* , *HNF4 α* , *HNF6*, and *SOX17*) were transduced into each stage of hepatic differentiation from hESCs by using an adenovirus (Ad) vector to screen for hepatic differentiation-promoting factors. Then, hepatocyte-related gene expression profiles and hepatocyte functions in hESC-hepa and hiPSC-hepa generated by the optimized protocol, were examined to investigate whether these cells have PHs characteristics. We used nine drugs, which are metabolized by various CYP enzymes and UDP-glucuronosyltransferases (UGTs), to determine whether the hESC-hepa and hiPSC-hepa have drug metabolism capacity. Furthermore, hESC-hepa and hiPSC-hepa were examined to determine whether these cells may be applied to evaluate drug-induced cytotoxicity.

Materials and methods

In vitro differentiation

Before the initiation of cellular differentiation, the medium of hESCs and hiPSCs was exchanged for a defined serum-free medium, hESF9, and cultured as previously reported [9]. The differentiation protocol for the induction of DE cells, hepatoblasts, and hepatocytes was based on our previous report with some modifications [5,6]. Briefly, in mesendoderm differentiation, hESCs and hiPSCs were dissociated into single cells by using Accutase (Millipore) and cultured for 2 days on Matrigel (BD biosciences) in differentiation hESF-DIF medium which contains 100 ng/ml Activin A (R&D Systems) and 10 ng/ml bFGF (hESF-DIF medium, Cell Science & Technology Institute; differentiation hESF-DIF medium was supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ M 2-mercaptoethanol, 10 μ M ethanolamine, 10 μ M sodium selenite, and 0.5 mg/ml bovine serum albumin, all from Sigma). To generate DE cells, mesendoderm cells were transduced with 3000 VP/cell of Ad-*FOXA2* for 1.5 h on day 2 and cultured until day 6 on Matrigel in differentiation hESF-DIF medium supplemented with 100 ng/ml Activin A and 10 ng/ml bFGF. For induction of hepatoblasts, the DE cells were transduced with each 1500 VP/cell of Ad-*FOXA2* and Ad-*HNF1 α* for 1.5 h on day 6 and cultured for 3 days on Matrigel in hepatocyte culture medium (HCM, Lonza) supplemented with 30 ng/ml bone morphogenetic protein 4 (BMP4, R&D Systems) and 20 ng/ml FGF4 (R&D Systems). In hepatic expansion, the hepatoblasts were transduced with each 1500 VP/cell of Ad-*FOXA2* and Ad-*HNF1 α* for 1.5 h on day 9 and cultured for 3 days on Matrigel in HCM supplemented with 10 ng/ml hepatocyte growth factor (HGF), 10 ng/ml FGF1, 10 ng/ml FGF4, and 10 ng/ml FGF10 (all from R&D Systems). In hepatic maturation, cells were cultured for 8 days on Matrigel in L15 medium (Invitrogen) supplemented with 8.3% tryptose phosphate broth (BD biosciences), 10% FBS (Vita), 10 μ M hydrocortisone 21-hemisuccinate (Sigma), 1 μ M insulin, 25 mM NaHCO₃ (Wako), 20 ng/ml HGF, 20 ng/ml Oncostatin M (OsM, R&D systems), and 10⁻⁶ M Dexamethasone (DEX, Sigma).

Results

Recently, we showed that the sequential transduction of SOX17, *HEX*, and *HNF4 α* into hESC-derived mesendoderm, DE, and hepatoblasts, respectively, leads to efficient generation of the hESC-hepa [5–7]. In the present study, to further improve the differentiation efficiency towards hepatocytes, we screened for hepatic differentiation-promoting transcription factors. Seven candidate genes involved in liver development were selected. We then examined the function of the hESC-hepa and hiPSC-hepa

generated by the optimized protocol for pharmaceutical use in detail.

Efficient hepatic differentiation by Ad-FOXA2 and Ad-HNF1 α transduction

To perform efficient DE differentiation, T-positive hESC-derived mesendoderm cells (day 2) (Supplementary Fig. 1) were transduced with Ad vector expressing various transcription factors (Ad-*FOXA2*, Ad-*HEX*, Ad-*HNF1 α* , Ad-*HNF1 β* , Ad-*HNF4 α* , Ad-*HNF6*, and Ad-*SOX17* were used in this study). We ascertained the expression of *FOXA2*, *HEX*, *HNF1 α* , *HNF1 β* , *HNF4 α* , *HNF6*, or *SOX17* in Ad-*FOXA2*-, Ad-*HEX*-, Ad-*HNF1 α* -, Ad-*HNF1 β* -, Ad-*HNF4 α* -, Ad-*HNF6*-, or Ad-*SOX17*-transduced cells, respectively (Supplementary Fig. 2). We also verified that there was no cytotoxicity of the cells transduced with Ad vector until the total amount of Ad vector reached 12,000 VP/cell (Supplementary Fig. 3). Each transcription factor was expressed in hESC-derived mesendoderm cells on day 2 by using Ad vector, and the efficiency of DE differentiation was examined (Fig. 1A). The DE differentiation efficiency based on CXCR4-positive cells was the highest when Ad-*SOX17* or Ad-*FOXA2* were transduced (Fig. 1B). To investigate the difference between Ad-*FOXA2*-transduced cells and Ad-*SOX17*-transduced cells, gene expression levels of markers of undifferentiated cells, mesendoderm cells, DE cells, and extraembryonic endoderm cells were examined (Fig. 1C). The expression levels of extraembryonic endoderm markers of Ad-*SOX17*-transduced cells were higher than those of Ad-*FOXA2*-transduced cells. Therefore, we concluded that *FOXA2* transduction is suitable for use in selective DE differentiation.

To promote hepatic commitment, various transcription factors were transduced into DE cells and the resulting phenotypes were examined on day 9 (Fig. 1D). Nearly 100% of the population of Ad-*FOXA2*-transduced cells and Ad-*HNF1 α* -transduced cells was α -fetoprotein (AFP)-positive (Fig. 1E). We expected that hepatic commitment would be further accelerated by combining *FOXA2* and *HNF1 α* transduction. The DE cells were transduced with both Ad-*FOXA2* and Ad-*HNF1 α* , and then the gene expression levels of *CYP3A7* [10], which is a marker of fetal hepatocytes, were evaluated (Fig. 1F). When both Ad-*FOXA2* and Ad-*HNF1 α* were transduced into DE cells, the promotion of hepatic commitment was greater than in Ad-*FOXA2*-transduced cells or Ad-*HNF1 α* -transduced cells.

To promote hepatic expansion and maturation, we transduced various transcription factors into hepatoblasts on day 9 and 12 and the resulting phenotypes were examined on day 20 (Fig. 1G). We ascertained that the hepatoblast population was efficiently expanded by addition of HGF, FGF1, FGF4, and FGF10 (Supplementary Fig. 4). The hepatic differentiation efficiency based on asialoglycoprotein receptor 1 (ASGR1)-positive cells was measured on day 20, demonstrating that *FOXA2*, *HNF1 α* , and *HNF4 α* transduction could promote efficient hepatic maturation (Fig. 1H). To investigate the phenotypic difference between Ad-*FOXA2*-, Ad-*HNF1 α* -, and Ad-*HNF4 α* -transduced cells, gene expression levels of early hepatic markers, mature hepatic markers, and biliary markers were examined (Fig. 1I). Gene expression levels of mature hepatic markers were up-regulated by *FOXA2*, *HNF1 α* , or *HNF4 α* transduction. *FOXA2* transduction strongly upregulated gene expression levels of both early hepatic markers and mature hepatic markers, while *HNF1 α* or *HNF4 α* transduc-

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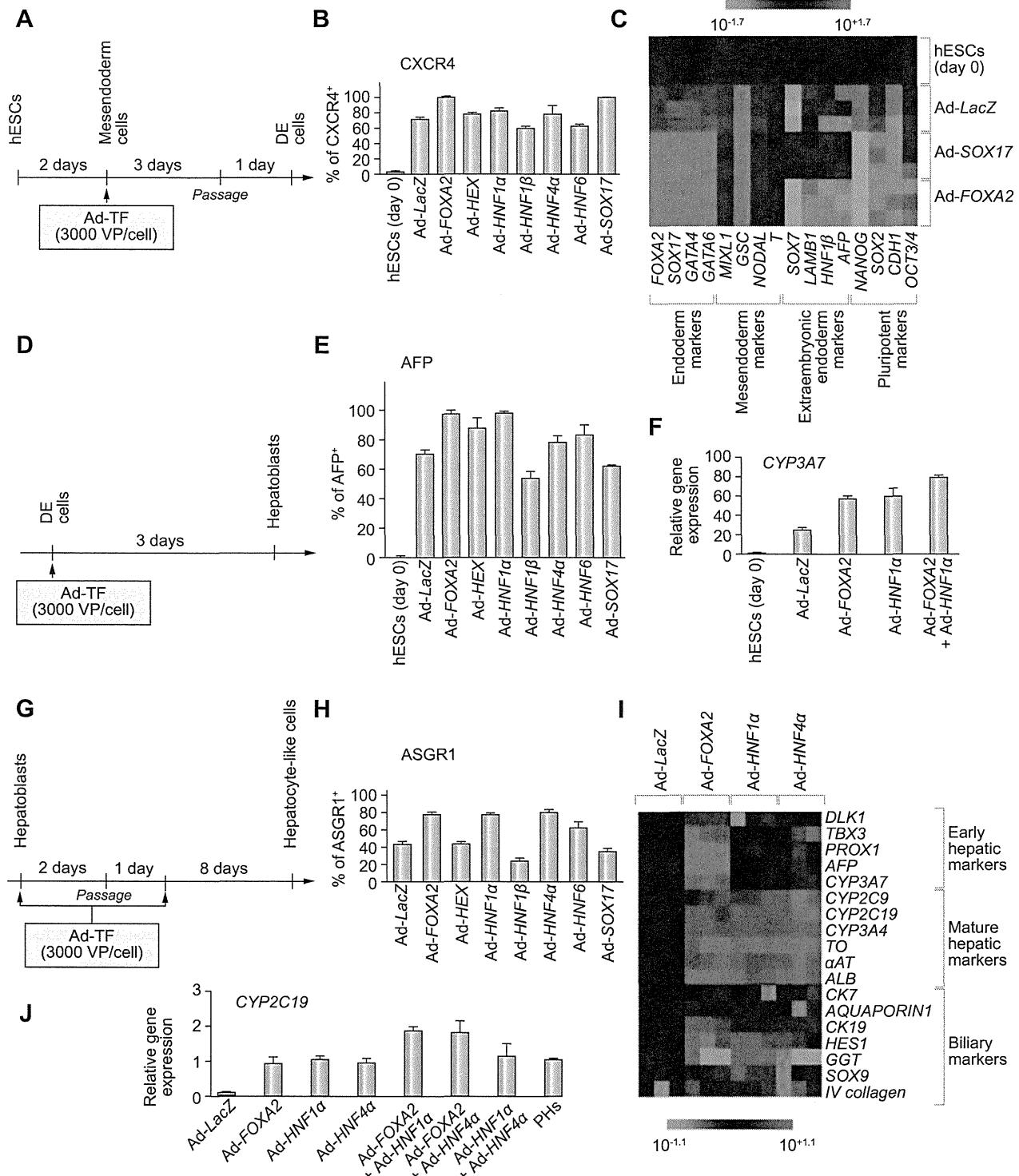
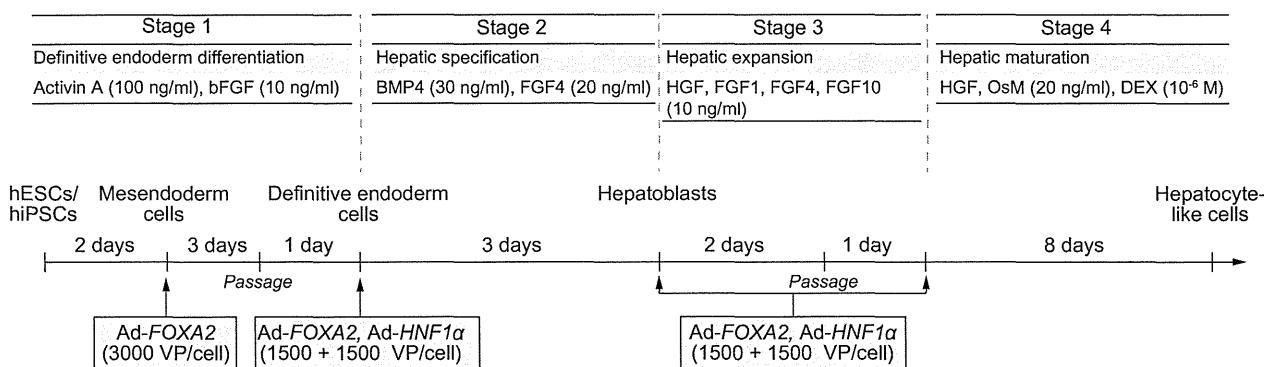
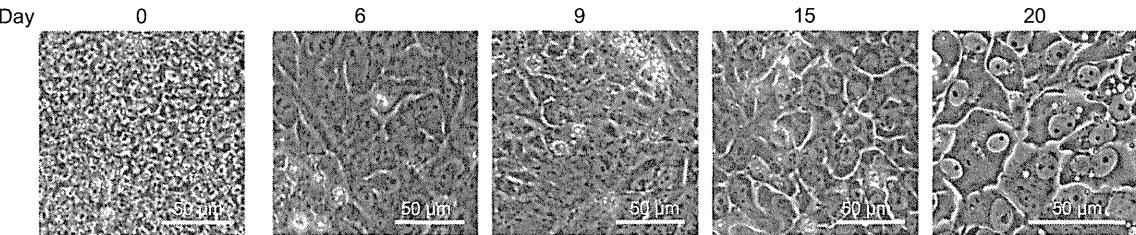


Fig. 1. Efficient hepatic differentiation from hESCs by FOXA2 and HNF1 α transduction. (A) The schematic protocol describes the strategy for DE differentiation from hESCs (H9). Mesendoderm cells (day 2) were transduced with 3000 VP/cell of transcription factor (TF)-expressing Ad vector (Ad-TF) for 1.5 h and cultured as described in Fig. 2A. (B) On day 5, the efficiency of DE differentiation was measured by estimating the percentage of CXCR4-positive cells using FACS analysis. (C) The gene expression profiles were examined on day 9. (D) Schematic protocol describing the strategy for hepatoblast differentiation from DE. DE cells (day 6) were transduced with 3000 VP/cell of Ad-TF for 1.5 h and cultured as described in Fig. 2A. (E) On day 9, the efficiency of hepatoblast differentiation was measured by estimating the percentage of AFP-positive

A



B



C

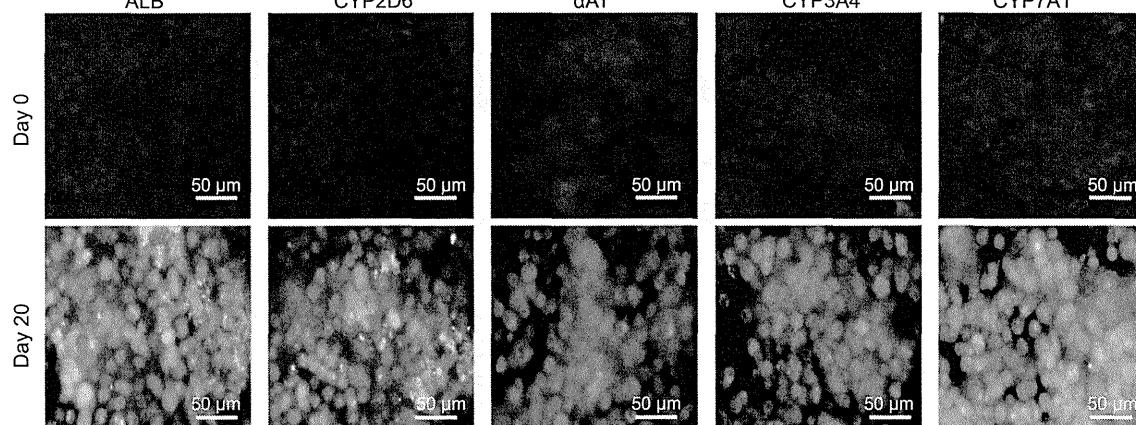


Fig. 2. Hepatic differentiation of hESCs and hiPSCs by FOXA2 and HNF1 α transduction. (A) The differentiation procedure of hESCs and hiPSCs into hepatocytes via DE cells and hepatoblasts is schematically shown. Details of the hepatic differentiation procedure are described in Materials and methods. (B) Sequential morphological changes (day 0–20) of hESCs (H9) differentiated into hepatocytes are shown. (C) The expression of the hepatocyte markers (ALB, CYP2D6, α AT, CYP3A4, and CYP7A1, all green) was examined by immunohistochemistry on day 0 and 20. Nuclei were counterstained with DAPI (blue).

tion did not up-regulate the gene expression levels of early hepatic markers. Next, multiple transduction of transcription factors was performed to promote further hepatic maturation. The combination of Ad-FOXA2 and Ad-HNF1 α transduction and the com-

bination of Ad-FOXA2 and Ad-HNF4 α transduction result in the most efficient hepatic maturation, judged from the gene expression levels of CYP2C19 (Fig. 1J). This may happen because the mixture of immature hepatocytes and mature hepatocytes coor-

cells using FACS analysis. (F) The gene expression level of CYP3A7 was measured by real-time RT-PCR on day 9. On the y axis, the gene expression level of CYP3A7 in hESCs (day 0) was taken as 1.0. (G) The schematic protocol describes the strategy for hepatic differentiation from hepatoblasts. Hepatoblasts (day 9) were transduced with 3000 VP/cell of Ad-TF for 1.5 h and cultured as described in Fig. 2A. (H) On day 20, the efficiency of hepatic differentiation was measured by estimating the percentage of ASGR1-positive cells using FACS analysis. The detail results of FACS analysis are shown in Supplementary Table 1. (I) Gene expression profiles were examined on day 20. (J) Hepatoblasts (day 9) were transduced with 3000 VP/cell of Ad-TFs (in the case of combination transduction of two types of Ad vector, 1500 VP/cell of each Ad-TF was transduced) for 1.5 h and cultured. Gene expression levels of CYP2C19 were measured by real-time RT-PCR on day 20. On the y axis, the gene expression level of CYP2C19 in PHs, which were cultured for 48 h after the cells were plated, was taken as 1.0. All data are represented as mean \pm SD (n = 3).

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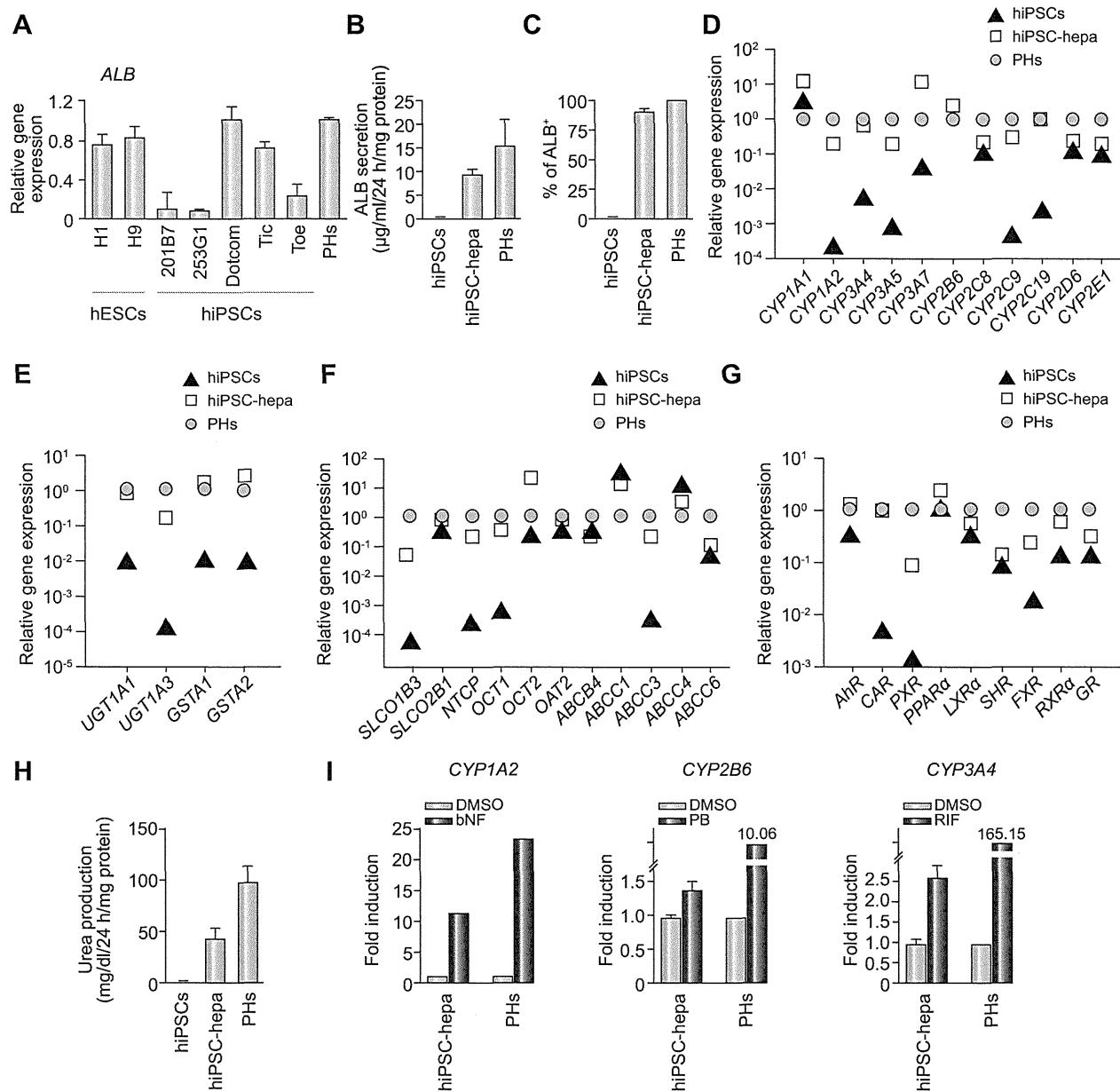


Fig. 3. The hepatic characterization of hiPSC-hepa. hESCs (H1 and H9) and hiPSCs (201B7, 253G1, Dotcom, Tic, and Toe) were differentiated into hepatocyte-like cells as described in Fig. 2A. (A) On day 20, the gene expression level of *ALB* was examined by real-time RT-PCR. On the y axis, the gene expression level of *ALB* in PHs, which were cultured for 48 h after cells were plated, was taken as 1.0. (B–I) hiPSCs (Dotcom) were differentiated into hepatocyte-like cells as described in Fig. 2A. (B) The amount of *ALB* secretion was examined by ELISA in hiPSCs, hiPSC-hepa, and PHs. (C) hiPSCs, hiPSC-hepa, and PHs were subjected to immunostaining with anti-*ALB* antibodies, and then the percentage of *ALB*-positive cells was examined by flow cytometry. (D–G) The gene expression levels of CYP enzymes (D), conjugating enzymes (E), hepatic transporters (F), and hepatic nuclear receptors (G) were examined by real-time RT-PCR in hiPSCs, hiPSC-hepa, and PHs. On the y axis, the expression level of PHs is indicated. (H) The amount of urea secretion was examined in hiPSCs, hiPSC-hepa, and PHs. (I) Induction of *CYP1A2*, *2B6*, or *3A4* by DMSO or inducer (bNF, PB, or RIF) of hiPSC-hepa and PHs, cultured for 48 h after the cells were plated, was examined. On the y axis, the gene expression levels of *CYP1A2*, *2B6*, or *3A4* in DMSO-treated cells, which were cultured for 48 h, were taken as 1.0. All data are represented as mean \pm SD ($n = 3$). All data are represented as mean \pm SD ($n = 3$).

dinately works to induce hepatocyte functions. Taken together, efficient hepatic differentiation could be promoted by using the combination of FOXA2 and HNF1 α transduction at the optimal stage of differentiation (Fig. 2A). At the stage of hepatic expansion and maturation, Ad-HNF4 α can be substituted for Ad-HNF1 α (Fig. 1J). Interestingly, cell growth was delayed by FOXA2 and

HNF4 α transduction (Supplementary Fig. 5). This delay in cell proliferation might be due to promoted maturation by FOXA2 and HNF1 α transduction. As the hepatic differentiation proceeds, the morphology of hESCs gradually changed into a typical hepatocyte morphology, with distinct round nuclei and a polygonal shape (Fig. 2B), and the expression levels of hepatic markers

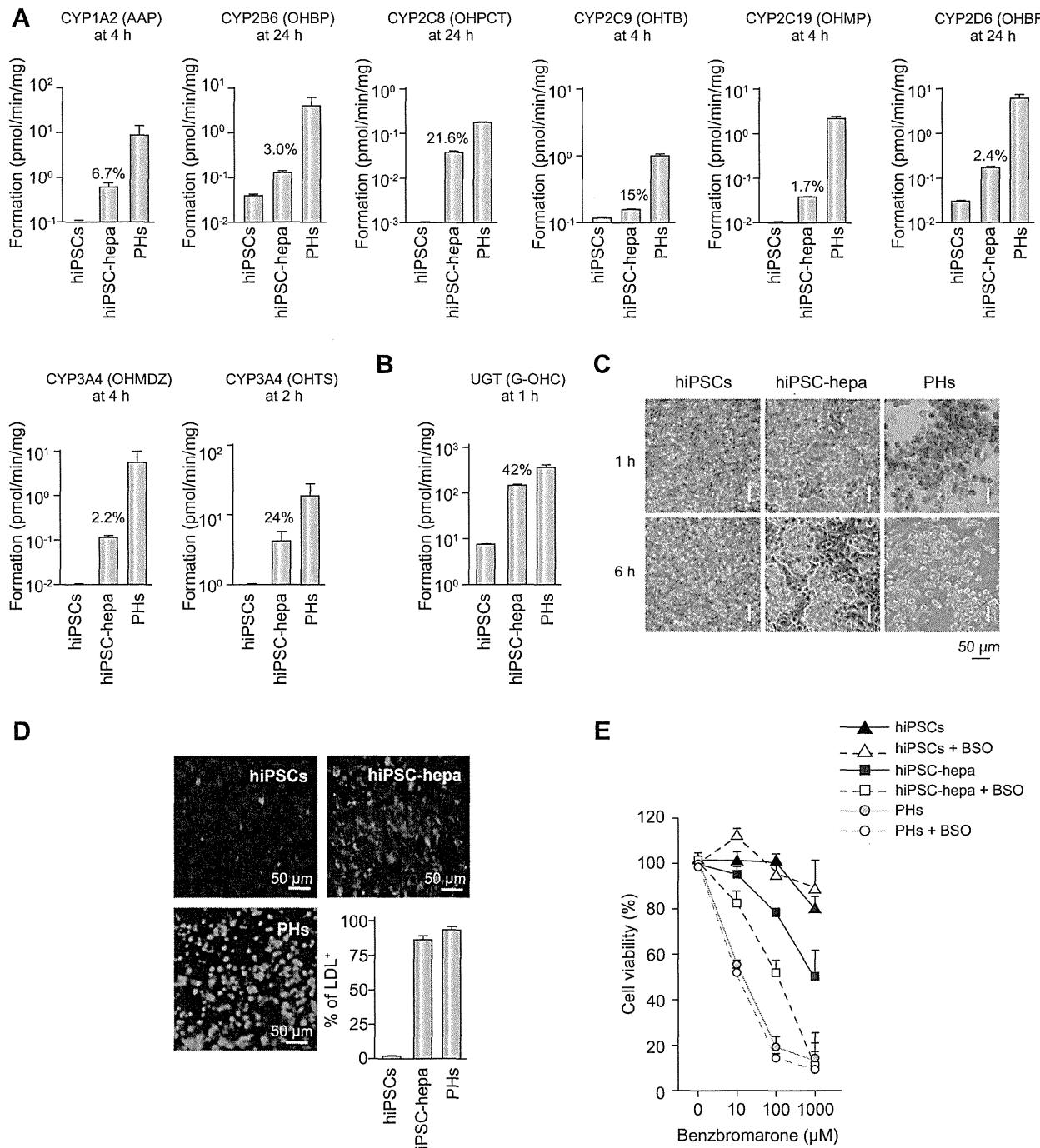


Fig. 4. Evaluation of the drug metabolism capacity and hepatic transporter activity of hiPSC-hepa. hiPSCs (Dotcom) were differentiated into hepatocytes as described in Fig. 2A. (A and B) Quantitation of metabolites in hiPSCs, hiPSC-hepa, and PHs, which were cultured for 48 h after the cells were plated, was examined by treating nine substrates (Phenacetin, Bupropion, Paclitaxel, Tolbutamide, S-mephenytoin, Bufuralol, Midazolam, Testosterone, and Hydroxyl coumarin; these compounds are substrates for CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A4, 3A4 and UGT (B), respectively), and then supernatants were collected at the indicated time. The quantity of metabolites (Acetaminophen [AAP], Hydroxybupropion [OHBP], 6 α -hydroxypaclitaxel [OHPCT], Hydroxytolbutamide [OHTB], 4 β -hydroxymephenytoin [OHMP], 1 β -hydroxybufuralol [OHBF], 1 β -hydroxymidazolam [OHMDZ], 6 β -hydroxytestosterone [OHTS], 7-Hydroxycoumarin glucuronide [G-OHC], respectively) was measured by LC-MS/MS. The ratios of the activity levels in hiPSC-hepa to the activity levels in PHs rate are indicated in the graph. (C) hiPSCs, hiPSC-hepa, and PHs were examined for their ability to take up ICG (top) and release it 6 h thereafter (bottom). (D) hiPSCs, hiPSC-hepa, and PHs were cultured with medium containing Alexa-Flour 488-labeled LDL (green) for 1 h, and immunohistochemistry was performed. Nuclei were counterstained with DAPI (blue). The percentage of LDL-positive cells was also measured by FACS analysis. (E)

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(ALB, CYP2D6, alpha-1-antitrypsin [α AT], CYP3A4, and CYP7A1) increased (Fig. 2C). Hepatic gene expression levels (Supplementary Fig. 6A), amount of ALB secretion (Supplementary Fig. 6B), and CYP2C9 activity level (Supplementary Fig. 6C) of Ad-FOXA2- and Ad-HNF1 α -transduced cells were significantly higher than those of Ad-SOX17-, Ad-HEX-, and Ad-HNF4 α -transduced cells. These results indicated that FOXA2 and HNF1 α transduction promotes more efficiently hepatic differentiation than SOX17, HEX, and HNF4 α transduction.

Characterization of the hESC-hepa/hiPSC-hepa

As we have previously reported [6], hepatic differentiation efficiency differs among hESC/hiPSC lines. Therefore, it is necessary to select a hESC/hiPSC line that is suitable for hepatic maturation in the case of medical applications such as drug screening. In the present study, two hESC lines and five hiPSCs lines were differentiated into hepatocyte-like cells, and then their gene expression levels of ALB (Fig. 3A) and CYP3A4 (Supplementary Fig. 7A), and their CYP3A4 activities (Supplementary Fig. 7B) were compared. These data suggest that the iPSC line, Dotcom [11,12], was the most suitable for hepatocyte maturation. To examine whether the iPSC (Dotcom)-hepa has enough hepatic functions as compared with PHs, the amount of albumin (ALB) secretion (Fig. 3B) and the percentage of ALB-positive cells (Fig. 3C) were measured on day 20. The amount of ALB secretion in hiPSC-hepa was similar to that in PHs and the percentage of ALB-positive cells was approximately 90% in iPSC-hepa. We also confirmed that the gene expression levels of CYP enzymes (Fig. 3D), conjugating enzymes (Fig. 3E), hepatic transporters (Fig. 3F), and hepatic nuclear receptors (Fig. 3G) in hiPSC-hepa were similar to those of PHs, although some of them were still lower than those of PHs. Because the gene expression level of the fetal CYP isoform, CYP3A7, in hiPSC-hepa was higher than that of PHs, mature hepatocytes and hepatic precursors were still mixed. We have previously confirmed that Ad vector-mediated gene expression in the hepatoblasts (day 9) continued until day 14 and almost disappeared on day 18 [7]. Therefore, the hepatocyte-related genes expressed in hiPSC-hepa are not directly regulated by exogenous FOXA2 or HNF1 α . Taken together, endogenous hepatocyte-related genes in hiPSC-hepa should have been upregulated by FOXA2 and HNF1 α transduction.

To further confirm that hiPSC-hepa have sufficient levels of hepatocyte functions, we evaluated the ability of urea secretion (Fig. 3H) and glycogen storage (Supplementary Fig. 8). The amount of urea secretion in hiPSC-hepa was about half of that in PHs. HiPSC-hepa exhibited abundant storage of glycogen. Because CYP1A2, 2B6, and 3A4 are involved in the metabolism of a significant proportion of the currently available commercial drugs, we tested the induction of CYP1A2, 2B6, and 3A4 by chemical stimulation (Fig. 3I). CYP1A2, 2B6, and 3A4 are induced by β -naphthoflavone [bNF], phenobarbital [PB], or rifampicin [RIF], respectively. Although undifferentiated hiPSCs did not respond to either bNF, PB, or RIF (data not shown), hiPSC-hepa produced

more metabolites in response to chemical stimulation, suggesting that inducible CYP enzymes were detectable in hiPSC-hepa (Fig. 3I). However, the induction potency of CYP1A2, 2B6, and 3A4 in hiPSC-hepa were lower than that in PHs.

Drug metabolism capacity and hepatic transporter activity of hiPSC-hepa

Because metabolism and detoxification in the liver are mainly executed by CYP enzymes, conjugating enzymes, and hepatic transporters, it is important to assess the function of these enzymes and transporters in hiPSC-hepa. Among the various enzymes in liver, CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4, UGT are the important phase I and II enzymes responsible for metabolism. Nine substrates, Phenacetin, Bupropion, Paclitaxel, Tolbutamide, S-mephenytoin, Bufuralol, Midazolam, Testosterone, and Hydroxyl coumarin, which are the substrates of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A4 (Fig. 4A), and UGT (Fig. 4B), respectively, were used to estimate the drug metabolism capacity of hiPSC-hepa compared with that of PHs. To precisely estimate the drug metabolism capacity, the amounts of metabolites were measured during the phase when production of metabolites was linear (Supplementary Fig. 9). These results indicated that our hiPSC-hepa have the capacity to metabolize these nine substrates, although the activity levels were lower than those of PHs. The hepatic functions of hiPSC-hepa were further evaluated by examining the ability to uptake Indocyanine Green (ICG) and LDL (Fig. 4C and D, respectively). In addition to PHs, hiPSC-hepa had the ability to uptake ICG and to excrete ICG in a culture without ICG for 6 h (Fig. 4C), and to uptake LDL (Fig. 4D). These results suggest that hiPSC-hepa have enough CYP enzyme activity, conjugating enzyme activity, and hepatic transporter activity to metabolize various drugs.

To examine whether our hiPSC-hepa could be used to predict metabolism-mediated toxicity, hiPSC-hepa were incubated with Benzbromarone, which is known to generate toxic metabolites, and then cell viability was measured (Fig. 4E). Cell viability of hiPSC-hepa was decreased depending on the concentration of Benzbromarone. However, cell viability of hiPSC-hepa was much higher than that of PHs. To detect drug-induced cytotoxicity with high sensitivity in hiPSC-hepa, these cells were treated with Buthionine-SR-sulfoximine (BSO), which depletes cellular GST, and result in a decrease of cell viability of hiPSC-hepa as compared with that of non-treated cells (Fig. 4E). These results indicated that hiPSC-hepa would be more useful in drug screening under a condition of knockdown of conjugating enzyme activity.

Discussion

The establishment of an efficient hepatic differentiation technology from hESCs and hiPSCs would be important for the application of hESC-hepa and hiPSC-hepa to drug toxicity screening. Although we have previously reported that sequential transduc-

The cell viability of hiPSCs, hiPSC-hepa, PHs, and their BSO-treated cells (0.4 mM BSO was pre-treated for 24 h) was assessed by Alamar Blue assay after 48-hr exposure to different concentrations of benzbromarone. The cell viability is expressed as a percentage of that in cells treated only with solvent. All data are represented as mean \pm SD (n = 3).

tion of SOX17, HEX, and HNF4 α into hESC-derived cells could promote efficient hepatic differentiation [7], further hepatic maturation of the hESC-hepa and hiPSC-hepa was needed for this application. To further improve the differentiation efficiency of every step of hepatic differentiation (hESC to DE cells, DE cells to hepatoblasts, and hepatoblasts to hESC-hepa), we initially performed a screening of transcription factors. In the stage of DE differentiation, FOXA2 transduction could promote the most efficient DE differentiation (Fig. 1C). In the stage of hepatic commitment, expansion, and maturation, the combination of FOXA2 and HNF1 α transduction strongly promoted hepatic commitment and maturation (Fig. 1F and J), although in the stage of hepatic expansion and maturation, HNF4 α transduction was as efficient as that of HNF1 α (Fig. 1J). Since HNF1 α is one of the target genes of HNF4 α [13], the signaling through HNF4 α to HNF1 α would be important for efficient hepatic expansion and maturation. Considering these results together, we ascertained a pair of two transcription factors, FOXA2 and HNF1 α , that could promote efficient hepatic differentiation from hESCs. In embryogenesis, the expression of FOXA2 and HNF1 α is initially detected in DE or hepatoblasts, respectively and the expression levels of both FOXA2 and HNF1 α are elevated as the liver develops [14,15]. Therefore, our hepatic differentiation technology, which employs FOXA2 and HNF1 α transduction, might mimic the gene expression pattern during embryogenesis.

We found that the gene expression levels of CYP enzymes, conjugating enzymes, hepatic transporters, and hepatic nuclear receptors were upregulated by FOXA2 and HNF1 α transduction (Fig. 3D–G). In contrast to the high expression levels of hepatocyte-related genes, CYP induction potency and the drug metabolism capacity of our hiPSC-hepa were lower than those of PHS (Figs. 3I and 4A and B). One of the possible reasons for the difference between gene expression levels of CYP enzymes and CYP induction activity might be that there were insufficient expression levels of hepatic nuclear receptors (such as PXR, SHR, and FXR) in hiPSC-hepa (Fig. 3G). Because many CYPs require high expression levels of hepatic nuclear receptor for efficient drug metabolism [16], transduction of these hepatic nuclear receptor genes in hiPSC-hepa or development of a differentiation method that induces high expression of these nuclear receptors might improve the drug metabolic capacity. Another explanation for the low CYP activities in hiPSC-hepa, maybe that hiPSCs were established from an individual with low CYP activities; in fact, it is known that large individual differences in CYP activities are observed among individuals. It might be important to use a hiPSC line established from a person with high CYP activities. It is essential to investigate the reasons behind this significant discordance, an issue that our group is currently planning to study.

In summary, our method, consisting of sequential FOXA2 and HNF1 α transduction along with the addition of adequate soluble factors at each step of differentiation, is a valuable tool for the efficient generation of functional hepatocytes derived from hESCs and hiPSCs. The hiPSC-hepa exhibited a number of hepatocyte functions (such as ALB secretion, uptake of LDL or ICG, glycogen storage, and drug metabolism capacity). In addition, the hiPSC-hepa were successfully applied to the evaluation of drug-induced cytotoxicity. Therefore, the hESC-hepa and hiPSC-hepa might be used for drug screening in early phases of pharmaceutical development.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2012.04.038>.

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Supplemental figure 1

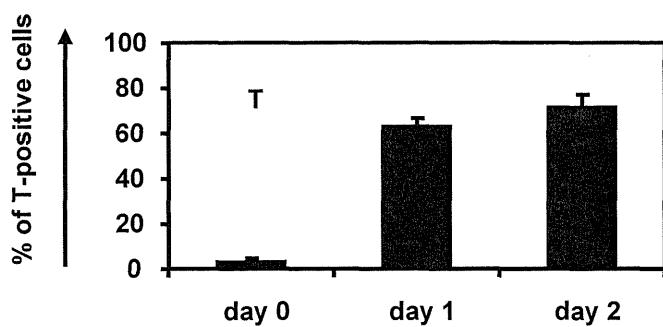


Fig. S1 The formation of mesendoderm cells from hESCs

hESCs (H9) were differentiated as described in **Figure 2A** and subjected to immunostaining with anti-T antibodies on day 0, 1, or 2. The percentage of antigen-positive cells was measured by FACS analysis. All data are represented as means \pm SD ($n=3$).

Supplemental figure 2

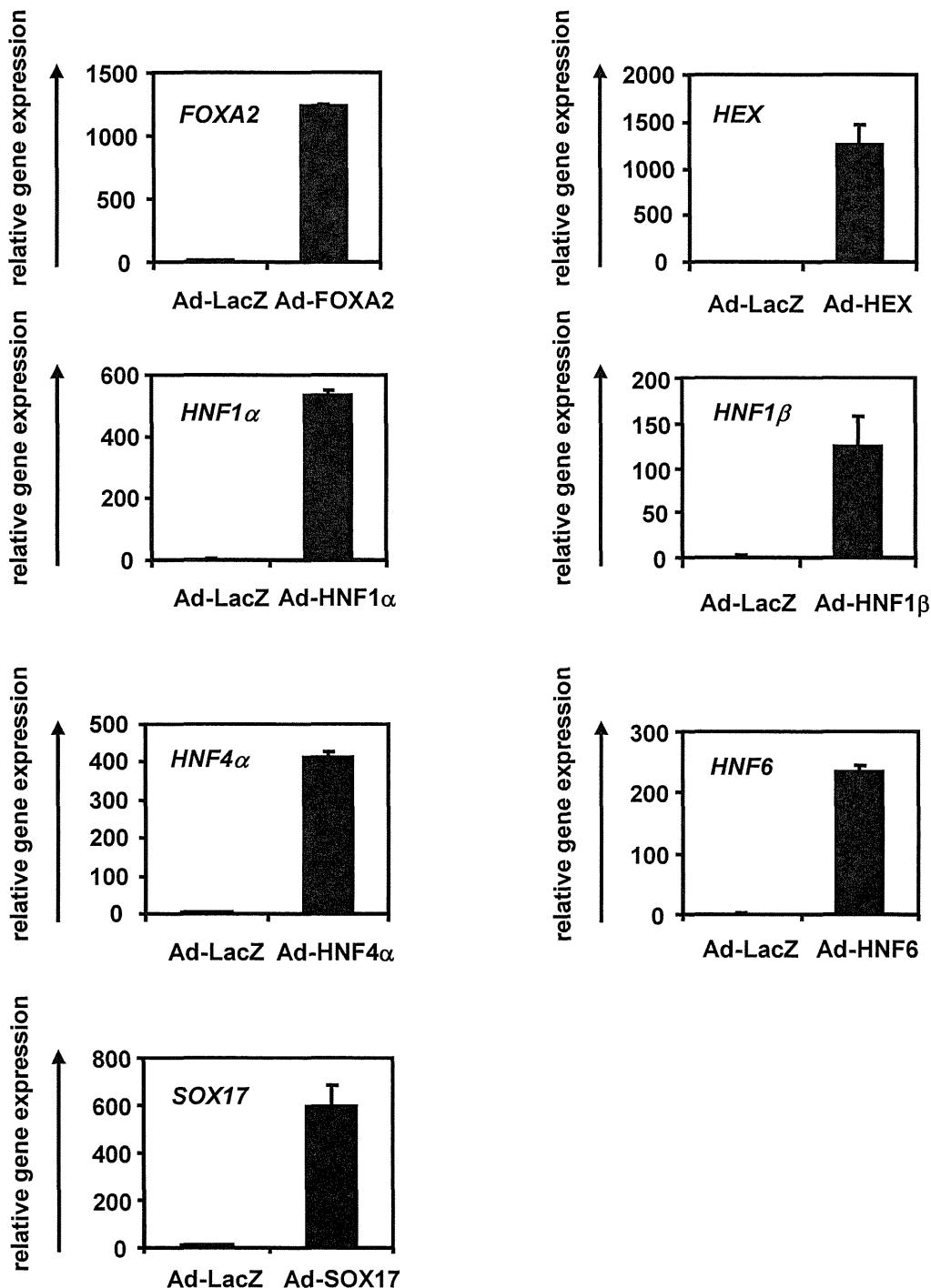


Fig. S2 Overexpression of FOXA2, HNF1 α , HNF4 α , SOX17, HEX, HNF1 β , or HNF6 mRNA in mesendoderm cells by Ad-FOXA2, Ad-HNF1 α , Ad-HNF4 α , Ad-SOX17, Ad-HEX, Ad-HNF1 β , or Ad-HNF6 transduction, respectively

hESCs (H9) were differentiated into mesendoderm cells (day 2) as described in **Figure 2A** and were transduced with 3,000 VP/cells of Ad-FOXA2, Ad-HNF1 α , Ad-HNF4 α , Ad-SOX17, Ad-HEX, Ad-HNF1 β , or Ad-HNF6 for 1.5 hr. On day 4, real-time RT-PCR analysis of FOXA2, HNF1 α , HNF4 α , SOX17, HEX, HNF1 β , or HNF6 expression was performed in Ad-FOXA2-, Ad-HNF1 α -, Ad-HNF4 α -, Ad-SOX17-, Ad-HEX-, Ad-HNF1 β -, or Ad-HNF6-transduced cells, respectively. On the y axis, the gene expression levels of FOXA2, HNF1 α , HNF4 α , SOX17, HEX, HNF1 β , or HNF6 in Ad-LacZ-transduced cells on day 4 were taken as 1.0. All data are represented as means \pm SD (n=3).

Supplemental figure 3

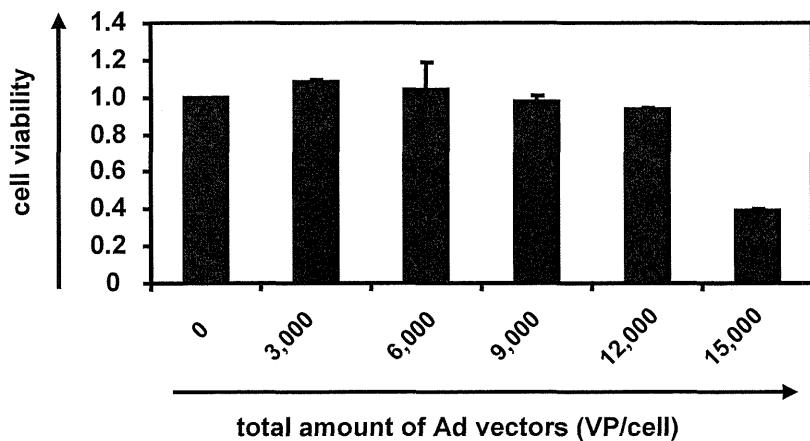
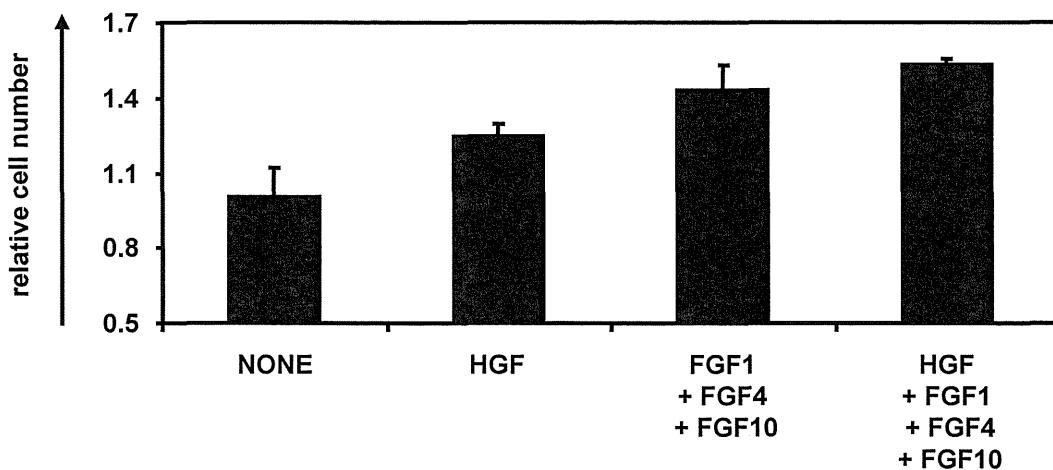


Fig. S3 Optimization of the amount of Ad vectors to transduce

hESC (H9)-derived cells were transduced with 750, 1,500, 2250, 3,000, or 3,750 VP/cell of Ad-LacZ for 1.5 hr on day 2, 6, 9, and 12, and then cultured as described in **Figure 2A**. On day 20, the cell viability was evaluated with Alamar Blue assay. The horizontal axis represents the total amount of Ad vector (3,000, 6,000, 9,000, 12,000, or 15,000 VP/cell, respectively). On the y axis, the level of non-transduced cells was defined as 1.0. All data are represented as means \pm SD ($n=3$).

Supplemental figure 4

A



B

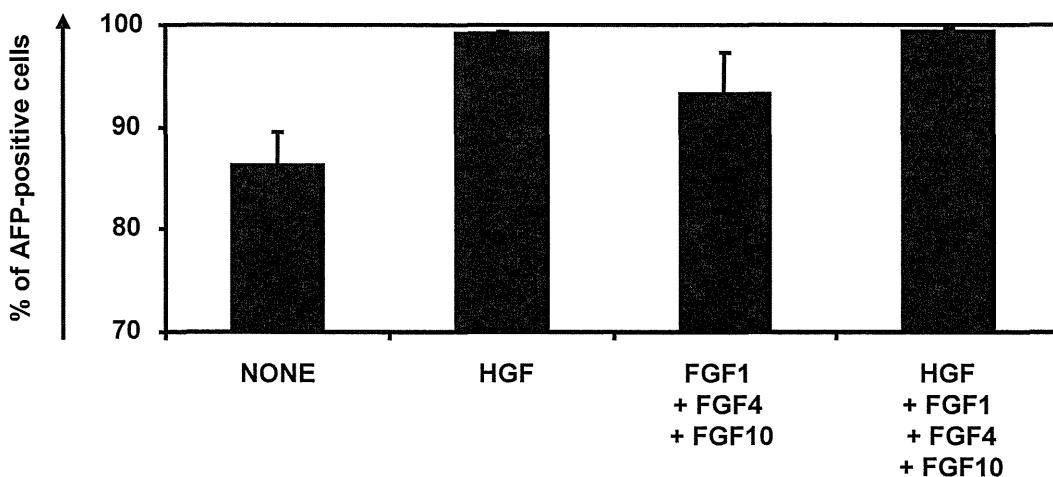


Fig. S4 Expansion of the hepatoblast population by HGF, FGF1, FGF4, and HGF stimulation
 hESCs (H9) were differentiated into hepatoblasts as described in **Figure 2A**. The hepatoblasts (day 9) were cultured with the HCM without additional growth factors (NONE), the HCM containing HGF, the HCM containing FGF1 + FGF4 + FGF10, or the HCM containing HGF + FGF1 + FGF4 + FGF10. The concentration of the growth factors used in this experiment was 10 ng/ml. (A) After the hepatoblasts (day 9) were cultured with the medium containing various growth factors (no additional growth factors, addition of HGF, addition of FGF1 + FGF4 + FGF10, or addition of HGF + FGF1 + FGF4 + FGF10) for 3 days, the number of the cells was counted on day 12. The cell number of untreated population was taken as 1.0. (B) On day 12, the cells were subjected to immunostaining with anti-AFP antibodies. The percentage of antigen-positive cells was measured by FACS analysis. All data are represented as means \pm SD ($n=3$). The results showed that addition of HGF, FGF1, FGF4, and FGF10 increased the number of the hepatoblasts.

Supplemental figure 5

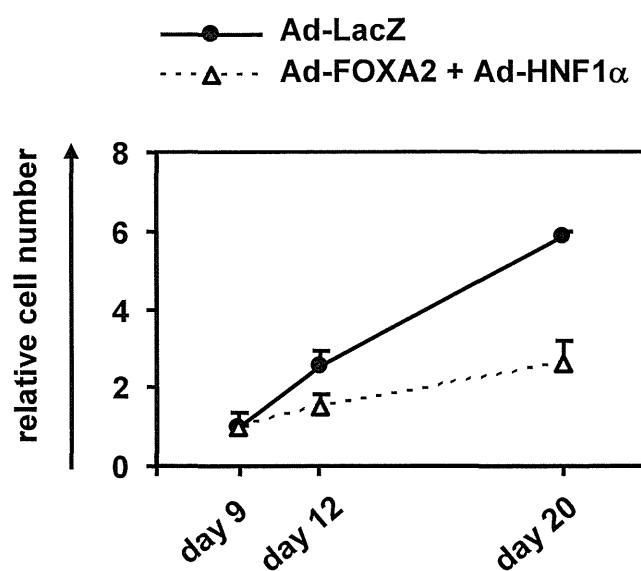


Fig. S5 Arrest of hepatoblast proliferation by FOXA2 and HNF1 α transduction.

hESCs were differentiated into the hepatoblasts (day 9) according to the protocol described in **Figure 2A**, and then transduced with 3,000 VP/cell of Ad-LacZ or 1,500 VP/cell of each Ad-FOXA2 and Ad-HNF1 α for 1.5 hr on day 9 and 12 and cultured until day 20 according to the protocol described in Figure 2A. The cells were not passaged on day 11. The cell number was counted on day 9, 12, and 20 of differentiation. The cell number on day 9 was taken as 1.0. All data are represented as means \pm SD ($n = 3$).

Supplemental figure 6

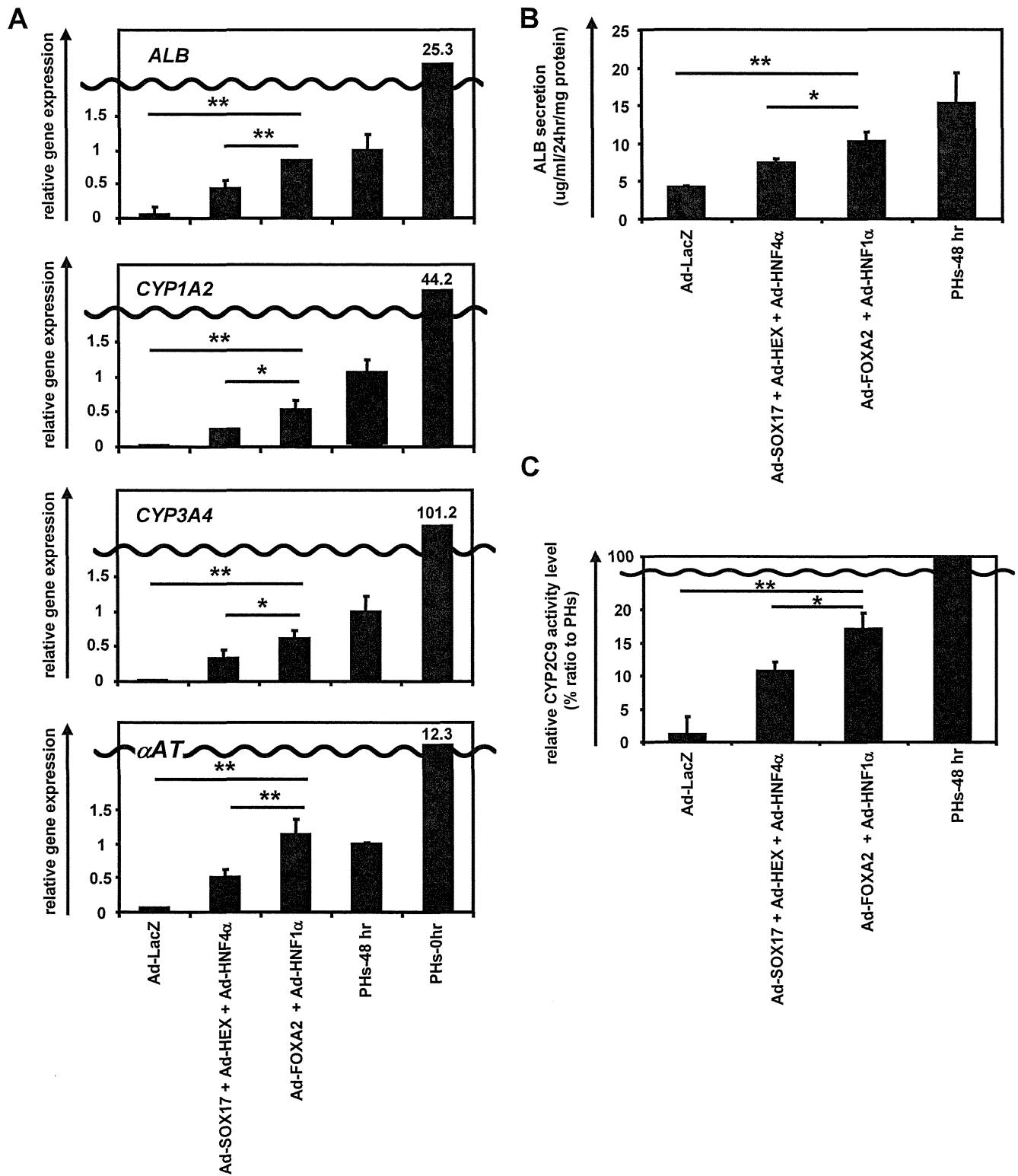
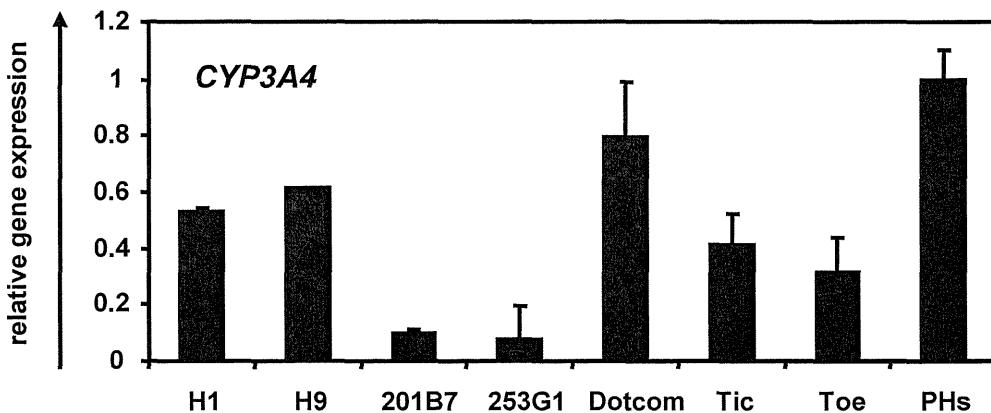


Fig. S6 FOXA2 and HNF1 α transduction promote more efficient hepatic differentiation as compared with SOX17, HEX, and HNF4 α transduction

hiPSCs (Dotcom) were differentiated into hepatocytes as described in Figure 2A. (A) On day 20, the gene expression levels of *ALB*, *CYP1A2*, *CYP3A4*, and α *AT* were examined by real-time RT-PCR in Ad-LacZ-transduced cells (Ad-LacZ), Ad-SOX17-, Ad-HEX-, and Ad-HNF4 α -transduced cells (Ad-SOX17 + Ad-HEX + Ad-HNF4 α), Ad-FOXA2- and Ad-HNF1 α -transduced cells (Ad-FOXA2 + Ad-HNF1 α), PHs cultured for 48 hr after plated (PHs-48hr), and PHs collected immediately after thawing (PHs-0hr). On the y axis, the gene expression levels of *ALB*, *CYP1A2*, *CYP3A4*, and α *AT* in PH-48hr were taken as 1.0. (B) The amount of ALB secretion was examined by ELISA in Ad-LacZ, Ad-SOX17 + Ad-HEX + Ad-HNF4 α , Ad-FOXA2 + Ad-HNF1 α , PHs-48hr, and PH-0hr. (C) The CYP2C9 activity level was examined in Ad-LacZ, Ad-SOX17 + Ad-HEX + Ad-HNF4 α , Ad-FOXA2 + Ad-HNF1 α , PHs-48hr, and PH-0hr. On the y axis, the CYP2C9 activity levels in PH-48hr were taken as 1.0. All data are represented as means \pm SD ($n=3$).

Supplemental figure 7

A



B

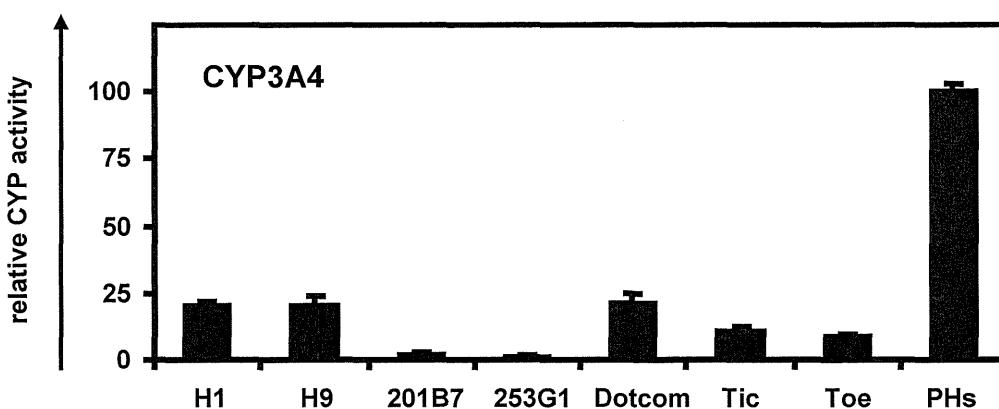


Fig. S7 Comparison of the hepatic differentiation capacity of various hESC and hiPSC lines

hESCs (H1 and H9) and hiPSCs (201B7, 253G1, Dotcom, Tic, and Toe) were differentiated into hepatocyte-like cells as described in **Figure 2A**. (A) On day 20, the gene expression level of *CYP3A4* was examined by real-time RT-PCR. On the y axis, the gene expression level of *CYP3A4* in PHs, which were cultured for 48 hr after the cells were plated, was taken as 1.0. (B) On day 20, the *CYP3A4* activity level was examined by using a P450-GloTM *CYP3A4* Assay Kit. On the y axis, the *CYP3A4* activity levels in PHs were taken as 100. All data are represented as means \pm SD ($n=3$).

Supplemental figure 8

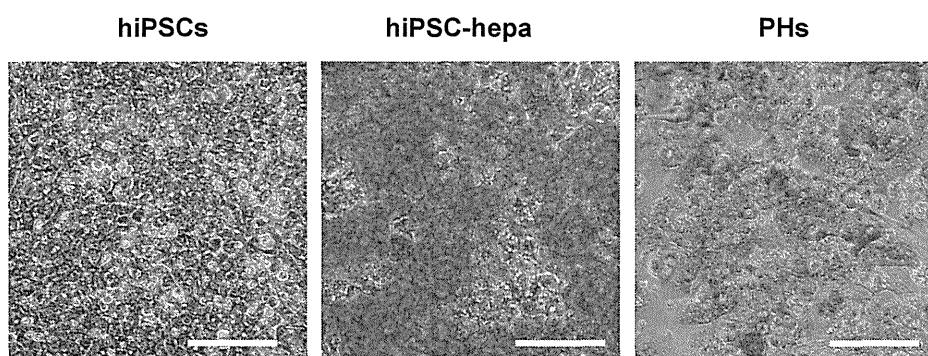


Fig. S8 Storage of glycogen in hiPSC-hepa

hiPSCs (Dotcom) were differentiated into hepatocytes as described in **Figure 2A**. Glycogen storage of hiPSCs, hiPSC-hepa, and PHs, which were cultured for 48 hr after the cells were plated, was assessed by Periodic Acid-Schiff (PAS) staining. PAS staining was performed on day 20 of differentiation. Glycogen storage is indicated by pink or dark red-purple cytoplasms. The scale bars represent 50 μ m.

Supplemental figure 9

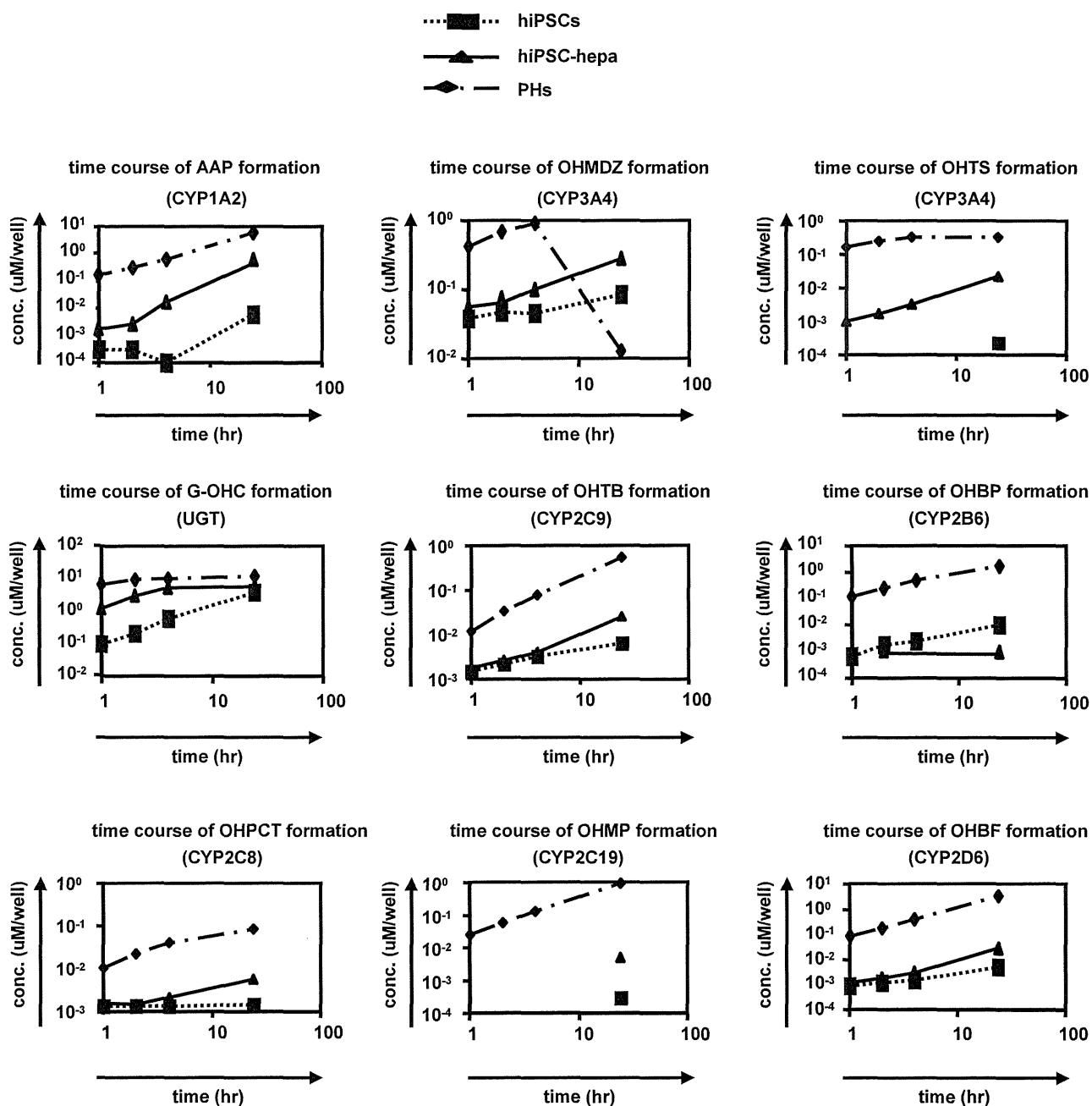


Fig. S9 The time course of metabolites formation in hiPSCs, hiPSC-hepa, or PHs

hiPSCs (Dotcom) were differentiated into hepatocytes as described in **Figure 2A**. Quantitation of metabolites in hiPSCs, hiPSC-hepa, and PHs treated with nine substrates (Phenacetin [PHE], Bupropion [BP], Paclitaxel [PCT], Tolbutamide [TB], S-mephenytoin [MP], Bufuralol [BF], Midazolam [MDZ], Testosterone [TS], and Hydroxyl coumarin [OHC]) was performed. Supernatants were collected at 1, 2, 4, or 24 hr after incubation with each substrate, which were the probes for CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A4 and UGT, respectively. The quantity of metabolites (Acetaminophen [AAP], Hydroxybupropion [OHBP], 6 α -hydroxypaclitaxel [OHPCT], Hydroxytolbutamide [OHTB], 4'-hydroxymephenytoin [OHMP], 1'-hydroxybufuralol [OHBF], 1'-hydroxymidazolam [OHMDZ], 6 β -hydroxytestosterone [OHTS], 7-Hydroxycoumarin glucuronide [G-OHC], respectively) was measured by LC-MS/MS. The substrates and that metabolites used in this study are summarized in **Table S5**. All data are represented as means \pm SD ($n=3$).