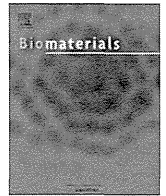


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3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing

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ABSTRACT

Although it is expected that hepatocyte-like cells differentiated from human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells will be utilized in drug toxicity testing, the actual applicability of hepatocyte-like cells in this context has not been well examined so far. To generate mature hepatocyte-like cells that would be applicable for drug toxicity testing, we established a hepatocyte differentiation method that employs not only stage-specific transient overexpression of hepatocyte-related transcription factors but also a three-dimensional spheroid culture system using a Nanopillar Plate. We succeeded in establishing protocol that could generate more matured hepatocyte-like cells than our previous protocol. In addition, our hepatocyte-like cells could sensitively predict drug-induced hepatotoxicity, including reactive metabolite-mediated toxicity. In conclusion, our hepatocyte-like cells differentiated from human ES cells or iPS cells have potential to be applied in drug toxicity testing.

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1. Introduction

Hepatocyte-like cells that are generated from human embryonic stem cells (hESCs) [1] or human induced pluripotent stem cells (hiPSCs) [2] are expected to be used in drug screening instead of primary (or cryopreserved) human hepatocytes (PHs). We recently demonstrated that stage-specific transient transduction of transcription factors, in addition to treatment with optimal growth factors and cytokines, is useful for promoting hepatic differentiation [3–6]. The hepatocyte-like cells, which have many hepatocyte characteristics (the abilities to uptake low-density lipoprotein and Indocyanine green, store glycogen, and synthesize urea) and drug metabolism capacity, were generated from hESCs/hiPSCs by

combinational transduction of FOXA2 and HNF1 α [6]. However, further maturation of the hepatocyte-like cells is required because their hepatic characteristics, such as drug metabolism capacity, are lower than those of PHs [6].

To promote further maturation of the hepatocyte-like cells, we subjected them to three-dimensional (3D) spheroid cultures. It is known that various 3D culture conditions (such as Algimatrix scaffolds [7], cell sheet technology [8], galactose-carrying substrata [9], and basement membrane substratum [10]) are useful for the maturation of the hepatocyte-like cells. Nanopillar Plate technology [11] used in the present study makes it easy to control the configuration of the spheroids. The Nanopillar Plate has an arrayed μ -scale hole structure at the bottom of each well, and nanopillars were aligned further at the bottom of the respective holes. The seeded cells evenly drop into the holes, then migrate and aggregate on top surface of the nanopillars, thus likely to form the uniform spheroids in each hole. Not only 3D spheroid cultures [12] but also Matrigel overlay cultures [13] are useful for maintaining the hepatocyte characteristics of PHs. Therefore, we employed both 3D

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spheroid culture and Matrigel overlay culture systems to promote hepatocyte maturation of the hepatocyte-like cells.

The hepatocyte-like cells generated from hESCs/hiPSCs are expected to be used in drug development. To the best of our knowledge, however, few studies have tried to predict widespread drug-induced cytotoxicity *in vitro* using the hepatocyte-like cells. To precisely determine the applicability of the hepatocyte-like cells to drug screening, it is necessary to investigate the responses of these hepatocyte-like cells to many kinds of hepatotoxic drugs.

In this study, 3D spheroid and Matrigel overlay cultures of the hepatocyte-like cells were performed to promote hepatocyte maturation. The gene expression analysis of cytochrome P450 (CYP) enzymes, conjugating enzymes, hepatic transporters, and hepatic nuclear receptors in the 3D spheroid-cultured hESC- or hiPSC-derived hepatocyte-like cells (3D ES-hepa or 3D iPSC-hepa), were analyzed. In addition, CYP induction potency and drug metabolism capacity were estimated in the 3D ES/iPSC-hepa. To determine the suitability of these cells for drug screening, we examined whether the drug-induced cytotoxicity is induced by treatment of various kinds of hepatotoxic drugs in 3D ES/iPSC-hepa.

2. Materials and methods

2.1. hESCs and hiPSCs culture

A hESC line, H1 and H9 (WiCell Research Institute), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore) with Repro Stem medium (Repro CELL) supplemented with 5 ng/ml fibroblast growth factor 2 (FGF2) (Sigma). Both H1 and H9 were used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan and furthermore, and the study was approved by Independent Ethics Committee.

Three human iPSC lines were provided from the JCRB Cell Bank (Tic, JCRB Number: JCRB1331; Dotcom, JCRB Number: JCRB1327; Toe, JCRB Number: JCRB1338) [14,15]. These human iPSC lines were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with iPSELLON (Cardio) supplemented with 10 ng/ml FGF2. Other three human iPSC lines, 201B6, 201B7 and 253G1 were kindly provided by Dr. S. Yamanaka (Kyoto University) [2]. These human iPSC lines were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with Repro Stem supplemented with 5 ng/ml FGF2.

2.2. *In vitro* differentiation

Before the initiation of cellular differentiation, the medium of hESCs was exchanged into a defined serum-free medium, hESF9, and cultured as previously reported [16]. The differentiation protocol for the induction of definitive endoderm cells, hepatoblasts, and hepatocytes was based on our previous reports with some modifications [3–5,17]. Briefly, in mesendoderm differentiation, hESCs 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 was purchased from Cell Science & Technology Institute; differentiation hESF-DIF medium was supplemented with 10 µg/ml human recombinant insulin, 5 µg/ml human apotransferrin, 10 µM 2-mercaptoethanol, 10 µM ethanolamine, 10 µM sodium selenite, and 0.5 mg/ml bovine fatty acid free serum albumin [all from sigma]). To generate definitive endoderm cells, the mesendoderm cells were transduced with 3000 vector particle (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-HNF1α 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-HNF1α 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). To perform hepatocyte maturation on Nanopillar Plate (a prototype multi-well culturing plate for spheroid culture developed and prepared by Hitachi High-Technologies Corporation) shown in Fig. 1B, the cells were seeded at 2.5×10^5 cells/cm² (Fig. S1) in hepatocyte culture medium (Fig. S2) supplemented with 10 ng/ml HGF, 10 ng/ml FGF1, 10 ng/ml FGF4, and 10 ng/ml FGF10 on day 11. In the first stage of hepatocyte maturation (from day 12 to day 25), the cells were cultured for 13 days on Matrigel in HCM supplemented with 20 ng/ml HGF,

20 ng/ml oncostatin M (OsM), 10 ng/ml FGF4, and 10^{-6} M dexamethasone (DEX). In the second stage of hepatocyte maturation (from day 25 to day 35), Matrigel was overlaid on the hepatocyte-like cells. Matrigel were diluted to a final concentration of 0.25 mg/ml with William's E medium (Invitrogen) containing 4 mM L-glutamine, 50 µg/ml gentamycin sulfate, $1 \times$ ITS (BD Biosciences), 20 ng/ml OsM, and 10^{-6} M DEX. The culture medium was aspirated, and then the Matrigel solution (described above) was overlaid on the hepatocyte-like cells. The cells were incubated overnight, and the medium was replaced with HCM supplemented with 20 ng/ml OsM and 10^{-6} M DEX.

2.3. Adenovirus (Ad) vectors

Ad vectors were constructed by an improved *in vitro* ligation method [18,19]. The human EF-1α promoter-driven LacZ-, FOXA2-, or HNF1α-expressing Ad vectors (Ad-LacZ, Ad-FOXA2, or Ad-HNF1α, respectively) were constructed previously [3,4,20]. All of Ad vectors contain a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob for more efficient transduction of hESCs, hiPSCs, and DE cells, in which transfection efficiency was almost 100%, and purified as described previously [3–5]. The vector particle (VP) titer was determined by using a spectrophotometric method [21].

2.4. Flow cytometry

Single-cell suspensions of hESC/hiPSC-derived cells were fixed with 2% paraformaldehyde (PFA) at 4°C for 20 min, and then incubated with the primary antibody (described in Table S1), followed by the secondary antibody (described in Table S1). Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences).

2.5. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from hESCs or hiPSCs and their derivatives using ISO-GENE (Nippon Gene). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Real-time RT-PCR was performed with Taqman gene expression assays (Applied Biosystems) or SYBR Premix Ex Taq (TaKaRa) using an ABI PRISM 7000 Sequence Detector (Applied Biosystems). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used in this study are described in Table S2.

2.6. Immunohistochemistry

The cells were fixed with 4% PFA. After incubation with 1% Triton X-100, blocking with Blocking One (Nakalai tesque), the cells were incubated with primary antibody (described in Table S1) at 4°C for overnight, followed by incubation with a secondary antibody (described in Table S1) at room temperature for 1 h.

2.7. ELISA

The hESCs or hiPSCs were differentiated into hepatocytes as described in Fig. 1A. The culture supernatants, which were incubated for 24 h after fresh medium was added, were collected and analyzed for the amount of ALB secretion by ELISA. ELISA kits for ALB were purchased from Bethyl. ELISA was performed according to the manufacturer's instructions. The amount of ALB secretion was calculated according to each standard followed by normalization to the protein content per well.

2.8. Urea secretion

The hESCs or hiPSCs were differentiated into hepatocytes as described in Fig. 1A. The culture supernatants, which were incubated for 24 h after fresh medium was added, were collected and analyzed for the amount of urea secretion. Urea measurement kits were purchased from BioAssay Systems. The experiment was performed according to the manufacturer's instructions. The amount of urea secretion was calculated according to each standard followed by normalization to the protein content per well.

2.9. Canalicular secretory assay

At cellular differentiation, the hepatocyte-like cell spheroids were treated with 5 mM choly-lysyl-fluorescein (CLF) (BD Biosciences) for 30 min. The cells were washed with culture medium, and then observed by fluorescence microscope. To inhibit the function of BSEP, the cells were pretreated with Cyclosporin A 24 h before of the CLF treatment.

2.10. Assay for CYP activity and CYP induction

To measure the cytochrome P450 2C9 and 3A4 activity of the cells, we performed lytic assays by using a P450-Glo™ CYP2C9 (catalog number; V8791) and

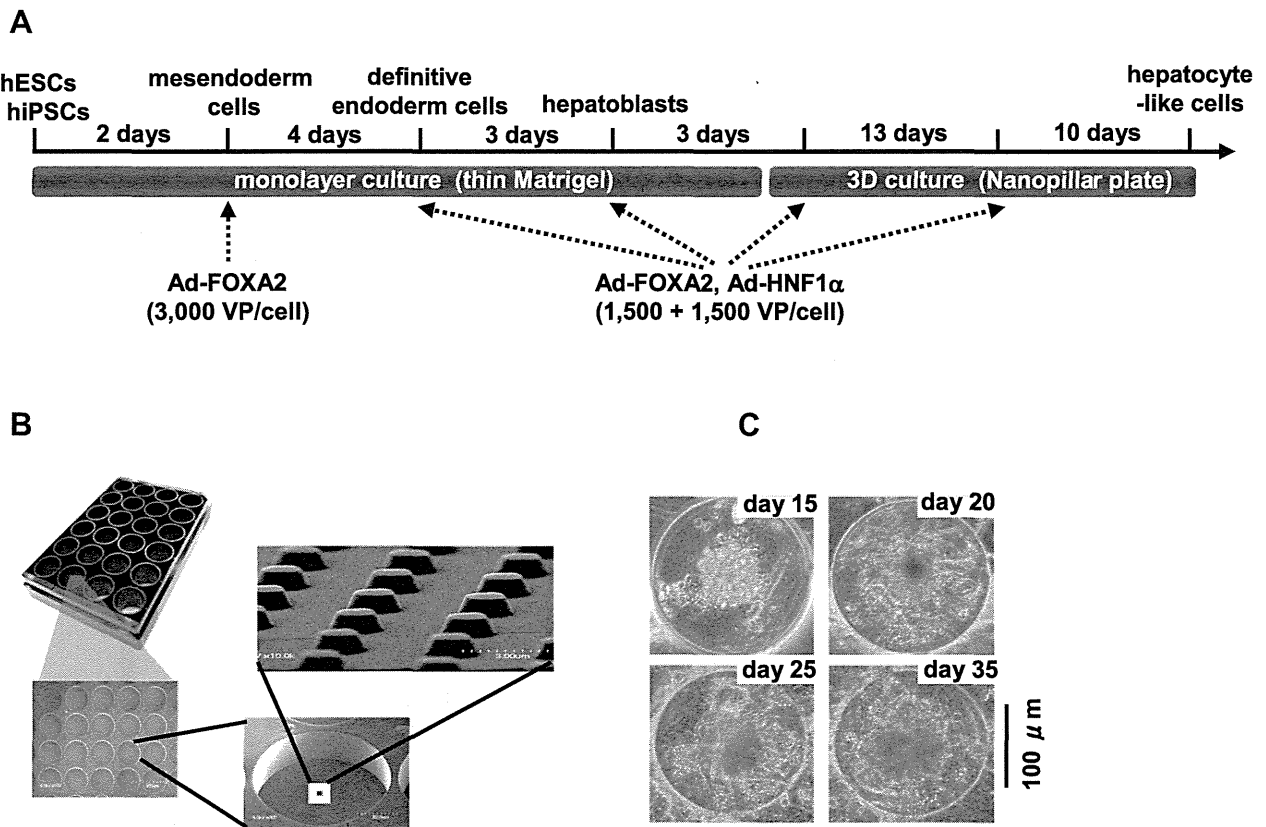


Fig. 1. Hepatocyte-like cells were differentiated from hESCs/hiPSCs by using Nanopillar Plate. (A) The procedure for differentiation of hESCs into 3D ES/iPS-hepa via mesendoderm cells, definitive endoderm cells, and hepatoblasts is presented schematically. In the differentiation, not only the addition of growth factors but also stage-specific transient transduction of both FOXA2- and HNF1 α -expressing Ad vector (Ad-FOXA2 and Ad-HNF1 α , respectively) was performed. The cellular differentiation procedure is described in detail in the materials and methods section. (B) Photograph display of a 24-well format Nanopillar Plate and its microstructural appearances of the hole and pillar structure. (C) Phase-contrast micrographs of the hESC-hepa spheroids on the Nanopillar Plate are shown. Scale bar represents 100 μ m.

3A4 (catalog number: V9001) Assay Kit (Promega), respectively. We measured the fluorescence activity with a luminometer (Lumat LB 9507; Berthold) according to the manufacturer's instructions. The CYP activity was normalized with the protein content per well.

To measure CYP2C9 and 3A4 induction potency, the CYP activity was measured by using a P450-GloTM CYP2C9 and 3A4 Assay Kit, respectively. The cells were treated with rifampicin, which is known to induce both CYP2C9 and 3A4, at a final concentration of 10 μ M for 48 h. The cells were also treated with Ketoconazole (Sigma) or Sulfaphenazole (Sigma), which are inhibitors of CYP3A4 or 2C9, at a final concentration of 1 μ M or 2 μ M, respectively, for 48 h. Controls were treated with DMSO (final concentration 0.1%). Inducer compounds were replaced daily.

2.11. Cell viability tests

Cell viability was assessed by the WST-8 assay kit (Dojindo) in Fig. 2D. After treatment with test compounds, such as Acetaminophen (Wako), Allopurinol (Wako), Amiodaron (Sigma), Benzbromarone (Sigma), Clozapine (Wako), Cyclizine (MP bio), Dantrone (Wako), Desipramine (Wako), Disulfiram (Wako), Erythromycin (Wako), Felbamate (Sigma), Flutamide (Wako), Isoniazid (Sigma), Labetalol (Sigma), Lefunomide (Sigma), Maprotiline (Sigma), Nefazodone (Sigma), Nitrofurantoin (Sigma), Sulindac (Wako), Tacrine (Sigma), Tebinafine (Wako), Tolcapone (TRC), Troglitazone (Wako), and Zafirlukast (Cayman) for 24 h, the cell viability was measured. The cell viability of the 3D iPSC-hepa were assessed by WST-8 assay after 24 h exposure to different concentrations of Aflatoxin B1 (Sigma) and Benzbromarone in the presence or absence of the CYP3A4 or 2C9 inhibitor, Ketoconazole (1 μ M) or Sulfaphenazole (10 μ M), respectively. The control refers to incubations in the absence of test compounds and was considered as 100% viability value. Controls were treated with DMSO (final concentration 0.1%). ATP assay (BioAssay Systems), Alamar Blue assay (Invitrogen), and Crystal Violet (Wako) staining assay were performed according to the manufacturer's instructions.

2.12. Primary human hepatocytes

Three lots of cryopreserved human hepatocytes (lot Hu8072 [CellzDirect], HC2-14, and HC10-101 [Xenotech]) were used. These three lots of cryopreserved human hepatocytes were cultured according to our previous report [5].

2.13. Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student's *t*-test. All data are represented as means \pm SD ($n = 3$).

3. Results

The 3D ES/iPS-hepa were generated from hESCs/hiPSCs as shown in Fig. 1A. Hepatocyte differentiation of hESCs/hiPSCs was efficiently promoted by stage-specific transient transduction of FOXA2 and HNF1 α in addition to the treatment with appropriate soluble factors (growth factors and cytokines) [6]. On day 11, the hESC-derived cells were seeded at 2.5×10^5 cells/cm² (Fig. S1) on Nanopillar Plate (Fig. 1B), in hepatocyte culture medium (Fig. S2) to promote hepatocyte maturation. In addition, Matrigel was overlaid on the 3D ES-hepa to promote further hepatocyte maturation. The 3D ES-hepa with compact morphology that were adhesive to the substratum and had an optimal size (approximately 100 μ m in diameter) were formed by using the Nanopillar Plate (Fig. 1C). The spheroids seem to be stable because they could be cultured for more than 20 days. We have confirmed that more than 90% of the cells that constitute the spheroids were alive, indicating that the necrotic centers are absent.

To investigate whether or not a 3D spheroid culture could promote hepatocyte maturation of the hepatocyte-like cells, various hepatocyte characteristics of the 3D ES/iPS-hepa were compared with those of the monolayer-cultured hESC- or hiPSC-derived hepatocyte-like cells (mono ES-hepa or mono iPS-hepa). The gene expression level of *ALB* peaked on day 20 in the mono ES-hepa, and then it was dramatically decreased after day 25 (Fig. 2A). In contrast, the gene expression level of *ALB* was

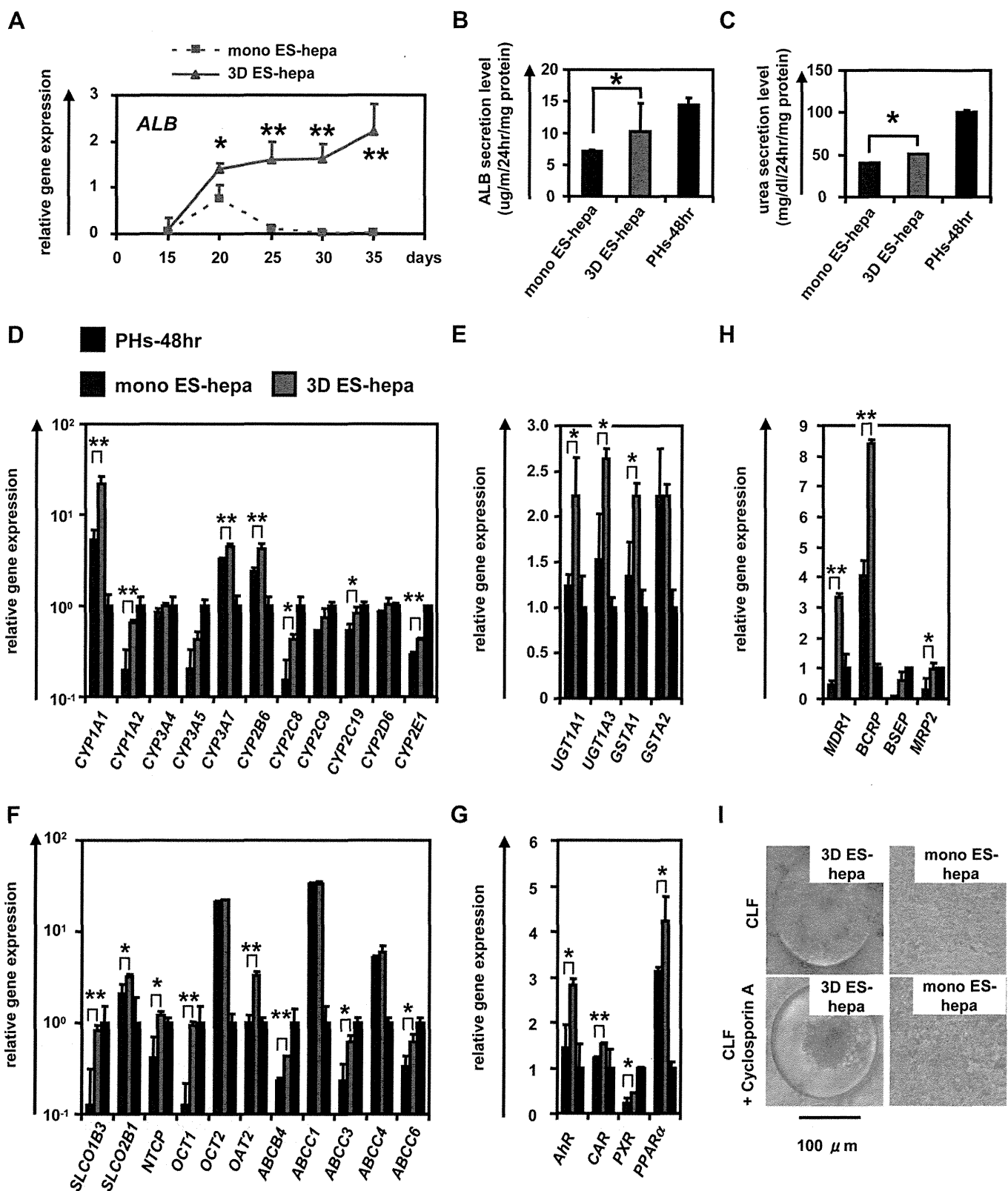


Fig. 2. Hepatocyte functions in hESC-derived hepatocyte-like cells were enhanced by using Nanopillar Plate. (A) The gene expression levels of *ALB* were measured by real-time RT-PCR on day 15, 20, 25, 30, and 35. On the y axis, the gene expression levels in PHs (three lots of PHs were used in all studies), which were cultured for 48 h after plating (PHs-48hr), were taken as 1.0. (B, C) The amount of ALB (B) and urea (C) secretion were examined in the mono ES-hepa (day 20), the 3D ES-hepa (day 35), and PHs-48hr. (D–H) The gene expression levels of CYP enzymes (D), conjugating enzymes (E), hepatic transporters (F), hepatic nuclear receptors (G), and bile canalicular transporters (H) were examined by real-time RT-PCR in the mono ES-hepa, the 3D ES-hepa, and PHs-48hr. On the y axis, the expression levels in PHs-48hr were taken as 1.0. (I) The ability of bile acid uptake and efflux was examined in the mono ES-hepa and 3D ES-hepa. Choly-lysyl-fluorescein (CLF) (5 μ M) was used for the observation of bile canalicular uptake and efflux. To inhibit transportation by BSEP, the cells were pretreated with 1 μ M Cyclosporin A. **P* < 0.05; ***P* < 0.01.

moderately increased in the 3D ES-hepa until day 35 (Fig. 2A). These results suggest that the hepatocyte functions of the 3D ES-hepa are sustained for more than 2 weeks on the Nanopillar Plate, although those of the mono ES-hepa are rapidly devitalized (Fig. 2A and Fig. S4). Other hepatocyte characteristics, such as ability of ALB and urea secretion and gene expression levels of hepatocyte-related markers in the 3D ES-hepa were compared with those of the mono ES-hepa (Fig. 2B–H). Because the gene expression level of *ALB* in the 3D ES-hepa was the highest on day 35 and that in mono ES-hepa was the highest on day 20, various hepatocyte characteristics were compared on day 35 or day 20, respectively. The amount of ALB (Fig. 2B) and urea (Fig. 2C) secretion in the 3D ES-hepa was higher than those of the mono ES-hepa. The gene expression levels of CYP enzymes (Fig. 2D), conjugating enzymes (Fig. 2E), hepatic transporters (Fig. 2F), hepatic nuclear receptors (Fig. 2G), and hepatic transcription factors (Fig. S5) in the 3D ES-hepa were higher than those in the mono ES-hepa. The expression levels of most of the genes in the 3D ES-hepa were higher than those in the mono ES-hepa. Because the previous study [11] showed that hepatocyte spheroids expressed hepatocyte transporters similar to those of the bile canaliculi in native liver tissue, the gene expression levels of bile canaliculi transporters (Fig. 2H), as well as the ability of bile acid uptake and efflux, (Fig. 2I) were examined in the 3D ES-hepa. The gene expression levels of bile canaliculi transporters were increased in the 3D ES-hepa compared with those of mono ES-hepa and PHs (Fig. 2H). The bile canaliculi formation was visualized by BSEP fluorescent substrate: Cholyl-L-lysyl-fluorescein (CLF), which is inhibited by BSEP

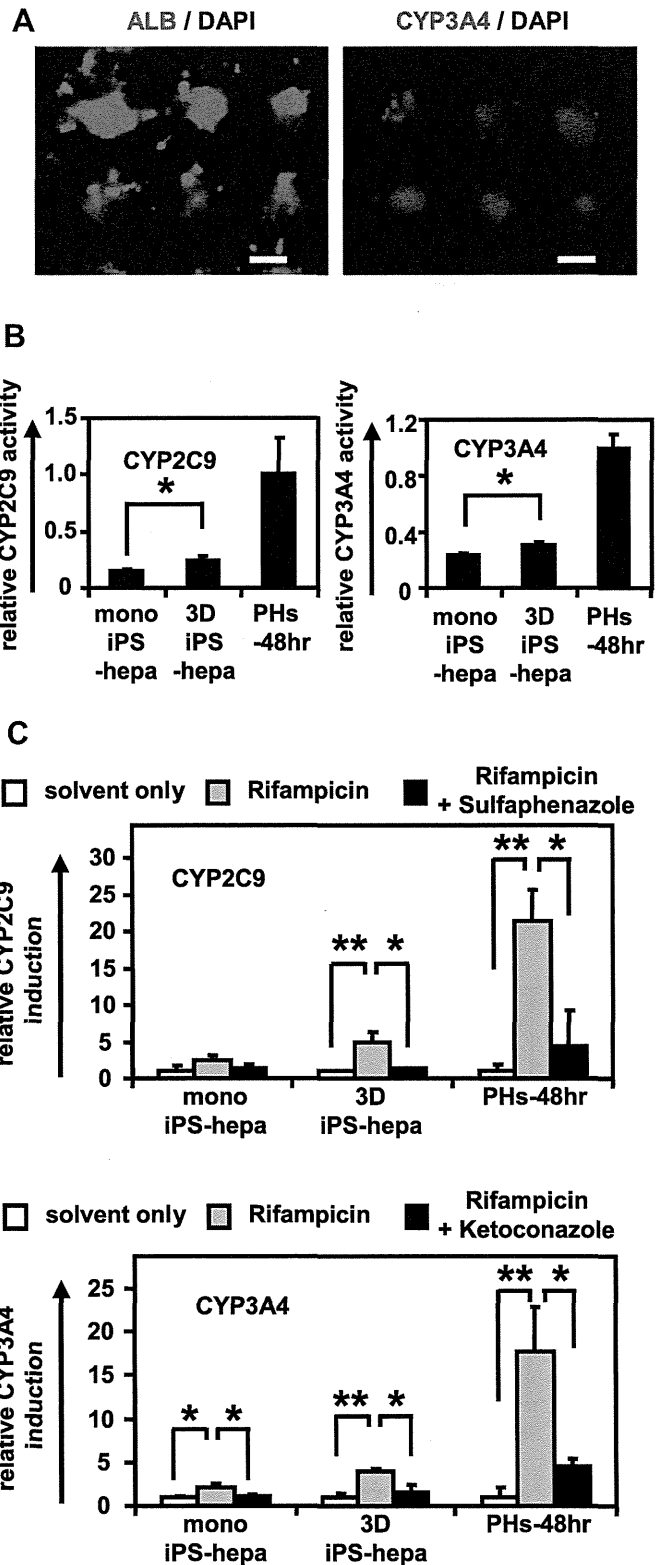


Fig. 4. Drug metabolism capacity and CYP induction potency were examined in the 3D iPS-hepa. (A) The 3D iPS-hepa (day 35) were subjected to immunostaining with anti-ALB (green) or CYP3A4 (red) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar represents 100 μ m. (B) The CYP activity was measured in the mono iPS-hepa (day 20), the 3D iPS-hepa (day 35), and PHs-48hr. On the y axis, the CYP activity in PHs-48hr was taken as 1.0. (C) Induction of CYP2C9 (left) or CYP3A4 (right) by DMSO (solvent only; white bar), Rifampicin (gray bar), or rifampicin and CYP inhibitor (Sulfaphenazole or Ketoconazole, black bar) in the mono iPS-hepa, the 3D iPS-hepa, and PHs-48hr. On the y axis, the CYP activity of the cells that have been cultured in DMSO-containing medium was taken as 1.0. * $P < 0.05$; ** $P < 0.01$.

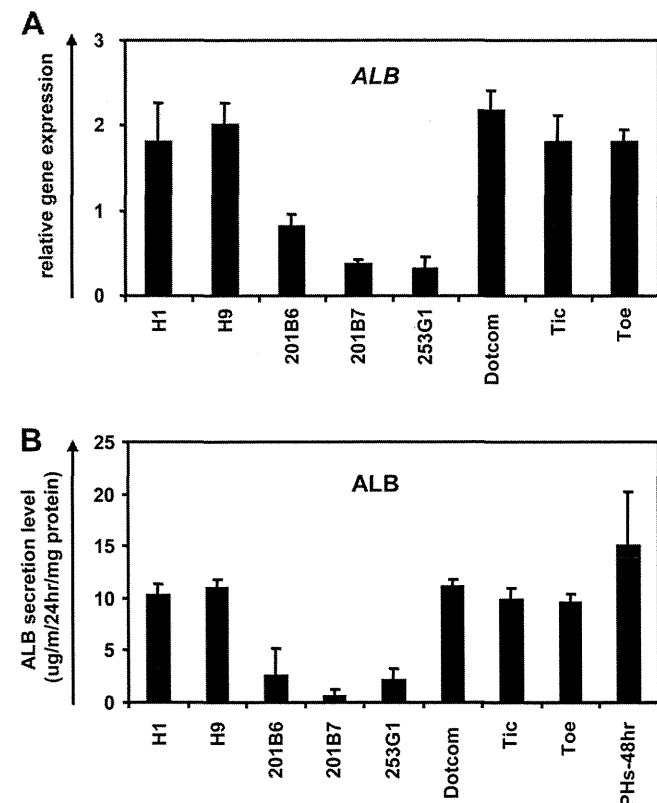
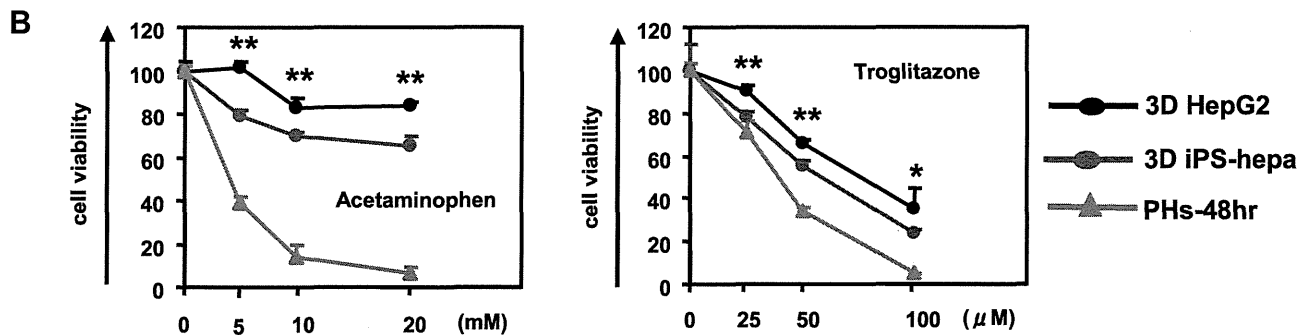
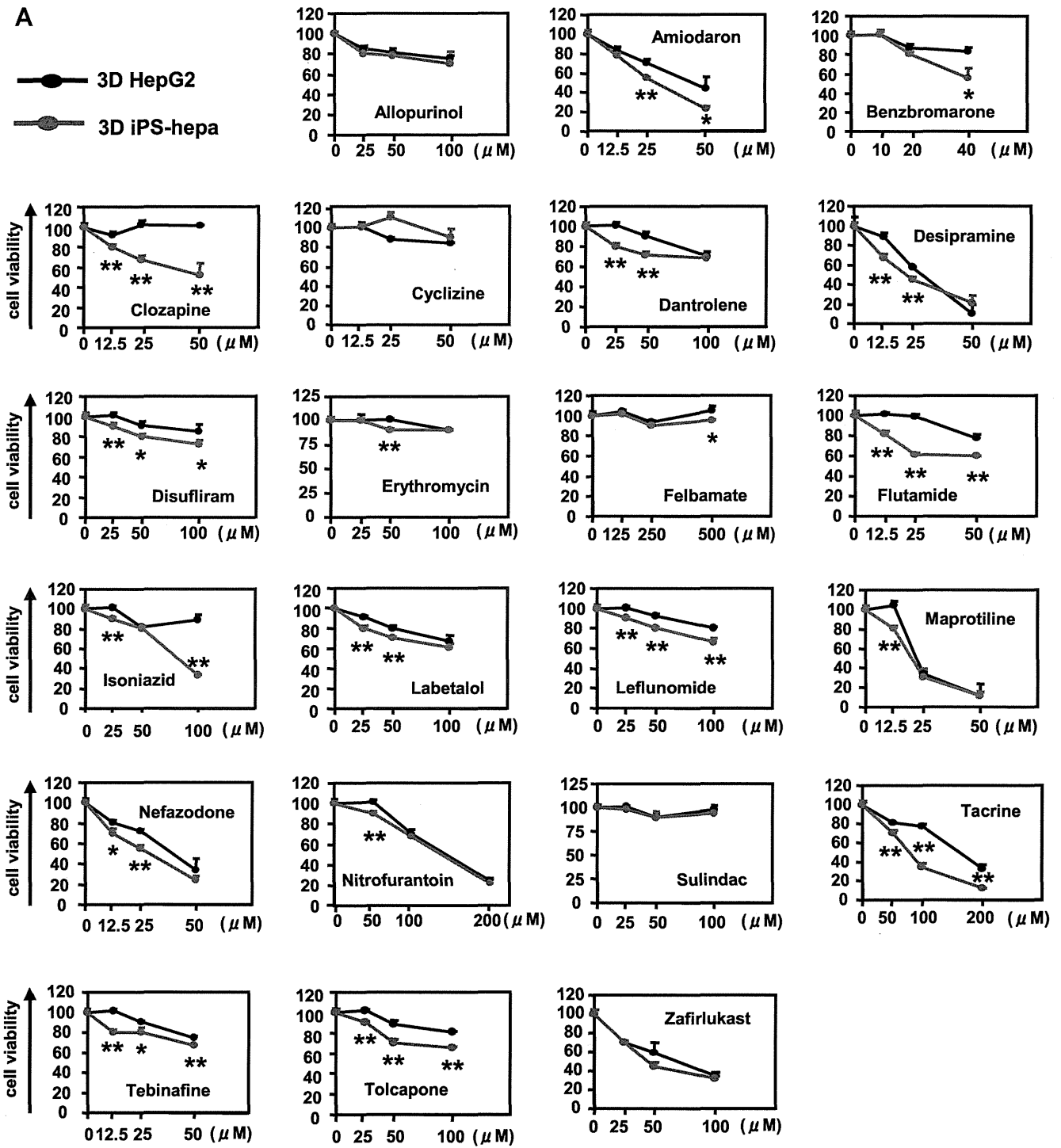


Fig. 3. Comparison of the hepatic differentiation capacities of various hESC and hiPSC lines hESCs (H1 and H9) and hiPSCs (201B6, 201B7, 253G1, Dotcom, Tic, and Toe) were differentiated into the 3D ES/iPS-hepa as described in Fig. 1A. (A) On day 20, the gene expression level of *ALB* was examined by real-time RT-PCR. On the y axis, the gene expression level of *ALB* in PHs-48hr was taken as 1.0. (B) On day 20, the amount of ALB secretion was examined by ELISA. The amount of ALB secretion was calculated according to each standard followed by normalization to the protein content per well.



inhibitor Cyclosporin A [22,23]. More CLF was accumulated in the 3D ES-hepa than in the mono ES-hepa (Fig. 2I upper panel). Moreover, CLF accumulation was inhibited by Cyclosporin A treatment only in the 3D ES-hepa (Fig. 2I lower panel), demonstrating that the functionality of BSEP transporter in 3D ES-hepa was greater than that in mono ES-hepa. These results suggested that hepatocyte maturation was promoted by the culture on the Nanopillar Plate. It is likely that, compared to the monolayer culture condition, the 3D spheroid-culture condition is more similar to the *in vivo* condition.

It is important to select an hESC/hiPSC line that has a strong ability to differentiate into hepatocyte-like cells in the case of medical applications such as drug screening. In this study, two hESC lines and six hiPSC lines were differentiated into the hepatocyte-like cells, and then their gene expression levels of *ALB* (Fig. 3A) and *ALB* secretion levels (Fig. 3B) were compared. These results suggest that the iPSC line, Dotcom, was the suitable cell line for hepatocyte maturation. Therefore, the iPSC line, Dotcom, was used to examine the possibility of the 3D iPS-hepa for drug screening. The drug metabolism capacity and the CYP induction potency of the 3D iPS-hepa were compared with those of the mono iPS-hepa. We confirmed the expression of *ALB* and *CYP3A4* protein in the 3D ES-hepa (Fig. 4A). The activity levels of CYP enzymes in the 3D iPS-hepa were measured according to the metabolism of the *CYP2C9* or *CYP3A4* substrates (Fig. 4B); the levels were higher than those of the mono iPS-hepa (Fig. 4B). We further tested the induction of *CYP2C9* and *CYP3A4* by chemical stimulation (rifampicin was used as a *CYP2C9* or *CYP3A4* inducer). Compared with mono iPS-hepa, the 3D iPS-hepa produced more metabolites in response to chemical stimulation (Fig. 4C). In addition, the CYP induction was inhibited by using *CYP2C9* or *CYP3A4* inhibitor (Sulfaphenazole or Ketoconazole, respectively). These results indicated that drug metabolism capacity and CYP induction potency in 3D iPS-hepa were higher than those in mono iPS-hepa.

Many researchers have tried to predict the drug-induced cytotoxicity *in vitro* using hepatocarcinoma-derived cells such as HepG2 cells [24,25]. HepG2 cells are less expensive than PHs and the reproducible experiments are easier to perform than they are with PHs, although 30% of the compounds were incorrectly classified as nontoxic [24,25]. To overcome these problems, hESC/hiPSC-derived hepatocyte-like cells are expected to be used to predict drug-induced cytotoxicity. To examine its applicability to drug screening, the 3D iPS-hepa were treated with various drugs, that cause hepatotoxicity. WST-8 assay was performed to evaluate cell viability (Fig. S6). The susceptibility of the 3D iPS-hepa to most of the hepatotoxic drugs was higher than that of the mono iPS-hepa (Fig. S7). Compared to the mono iPS-hepa, the 3D iPS-hepa were more suitable tools for drug screening. Next, the susceptibility of the 3D iPS-hepa to the hepatotoxic drugs was compared with that of the 3D spheroid cultured HepG2 cells (3D HepG2; the hepatocyte functions of 3D HepG2 cells are higher than those of monolayer cultured HepG2 cells [Fig. S8]). With most of the drugs, the cell viability of the 3D iPS-hepa was lower than that of the 3D HepG2 (Fig. 5A). These results indicated that the 3D iPS-hepa are more valuable tools for drug screening than the 3D HepG2. However, the susceptibility of the 3D iPS-hepa to Acetaminophen and Troglitazone was lower than that of the PHs which were cultured for 48 h after the cells were plated (Fig. 5B). These results might be due to the lower activity levels of CYPs in 3D iPS-hepa as compared as those in PHs. Taken together, 3D iPS-hepa are more valuable tools for drug screening than the 3D HepG2, although further maturation

of 3D iPS-hepa is still required for 3D iPS-hepa to be an alternative cell source of PHs in the drug screening.

To examine whether drug-induced cytotoxicity is caused by CYP metabolites in 3D iPS-hepa, Aflatoxin B1 (mainly metabolized by *CYP3A4* [26]) and Benzbromarone (mainly metabolized by *CYP2C9* [27]) were treated in the presence or absence of a *CYP3A4* and a *2C9* inhibitor, Ketoconazole and Sulfaphenazole, respectively (Fig. 6). The cell viability of 3D iPS-hepa was partially rescued by treatment with the CYP inhibitor. These results indicated that drug-induced cytotoxicity was caused by CYP metabolites of Aflatoxin B1 and Benzbromarone.

4. Discussion

Recently, it has been expected that human pluripotent stem cells and their derivatives, including hepatocyte-like cells, will be utilized in applications for the safety assessment of drugs. We have previously reported that combinational overexpression of *SOX17*, *HEX*, and *HNF4 α* , or combinational overexpression of *FOXA2* and *HNF1 α* could promote hepatocyte differentiation [5,6]. However, the drug metabolism capacity of the hepatocyte-like cells generated by our previous protocol was still lower than that of primary human hepatocytes [6]. To generate more matured hepatocyte-like cells as compared with our previous protocol, we established a hepatocyte differentiation method employing not only stage-specific transient overexpression of hepatocyte-related transcription factors but also a 3D culture systems using a Nanopillar Plate, was established. Although the use of hepatocyte-like cells generated from hESCs/hiPSCs in application for drug toxicity testing has begun to be focused, to the best of our knowledge, there have been few studies that have investigated whether hepatocyte-like cells could predict many kinds of drug-induced toxicity.

3D culture spheroids were generated from hESCs/hiPSCs by using a Nanopillar Plate. The diameter of the spheroids was approximately 100 μm on day 35 of differentiation (Fig. 1C). Because it is known that the no-oxygen limitation would take place in spheroids up to 100 μm in diameter [28], the size of the spheroid might be important to generate spheroids with high viability. A Nanopillar Plate has a potential to regulate the spheroid diameter simply by culturing under optimized seeding condition, on its suitably designed pillar and hole structure [11]. Therefore, a Nanopillar Plate would be a suitable environment for the generation of 3D ES/iPS-hepa that show high viability and possess high level of hepatocellular functions.

The levels of many hepatocyte functions, such as *ALB* secretion ability (Fig. 2B), urea secretion ability (Fig. 2C), hepatocyte-related gene expressions (Fig. 2D–H), drug metabolism capacity (Fig. 4B), and CYP induction potency (Fig. 4C), of 3D ES/iPS-hepa were higher than those of mono ES/iPS-hepa. This might have been because the structural and functional polarity, which can be seen in the naïve environment of hepatocytes, of the hepatocyte-like cells was configured by a 3D culturing condition. Previous studies have shown that a 3D culture condition is suitable to maintain the hepatic characteristics of the isolated hepatocytes because this condition mimic *in vivo* environment [29,30]. These facts indicated that the 3D culture condition is a more suitable condition for the hepatocyte-like cells than the monolayer culture condition.

Two hES cell lines and six hiPS cell lines were differentiated into the hepatocyte-like cells in this study. The hiPS cell line, Dotcom, seemed to be a suitable cell line for hepatic differentiation (Fig. 3). Because the hepatic differentiation propensity differs among the

Fig. 5. The possibility of applying 3D iPS-hepa to drug testing was examined. (A) The cell viability of the 3D HepG2 (black) and 3D iPS-hepa (red) were assessed by WST-8 assay after 24 h exposure to different concentrations of 22 test compounds. (B) The cell viability of the 3D HepG2 (black), 3D iPS-hepa (red), and PHs-48hr (green) were assessed by WST-8 assay after 24 h exposure to different concentrations of Acetaminophen and Troglitazone. Cell viability is expressed as a percentage of cells treated with solvent only. * $P < 0.05$; ** $P < 0.01$.

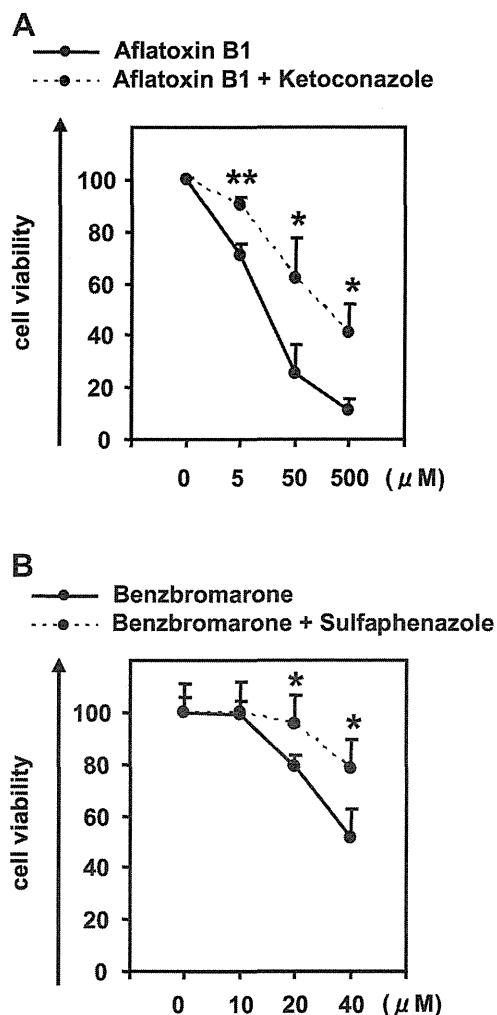


Fig. 6. Drug-induced cytotoxicity in the 3D iPSC-hepa is mediated by cytochrome P450. (A, B) The cell viability of the 3D iPSC-hepa was assessed by WST-8 assay after 24 h exposure to different concentrations of (A) Aflatoxin B1 and (B) Benzbromarone in the presence or absence of the CYP3A4 or 2C9 inhibitor, Ketoconazole or Sulfaphenazole, respectively. Cell viability was expressed as the percentage of cells treated with solvent only. * $P < 0.05$; ** $P < 0.01$.

hES/hiPS cell lines, it would be important to select an appropriate cell line for medical applications such as drug screening. However, the dominant reason for this hepatic differentiation propensity is not been well known. It would be interesting study to elucidate the mechanism of this propensity.

Although the drug metabolism capacity and CYP induction potency of 3D iPSC-hepa were higher than those of mono iPSC-hepa (Fig. 4B and C), they were still lower than those of primary human hepatocytes. The hepatic nuclear factors are known to be key molecules in the CYP induction of hepatocytes [30]. Therefore, overexpression of hepatic nuclear factors, which are not abundantly expressed in the hepatocyte-like cells (such as *PXR*), might upregulate the CYP induction potency of the hepatocyte-like cells.

3D iPSC-hepa were more sensitive for detection of the drug-induced cytotoxicity than HepG2 cells that are widely used to predict hepatotoxicity [31,32] (Fig. 5). In addition, the decrease of cell viability, which was caused by hepatotoxic drugs, of 3D iPSC-hepa was partially rescued by treatment with a CYP inhibitor (Fig. 6). These data suggest that the hepatocyte-like cells could detect the toxicity of the reactive metabolites that were generated by drug metabolizing enzymes such as CYP enzymes. Because in many cases, drug-induced hepatotoxicity is caused by the reactive

metabolites produced by drug metabolizing enzymes [33], our finding that the hepatocyte-like cells could detect the toxicity of reactive metabolites should be of great potential for toxicological screening. Moreover, it might be possible to predict idiosyncratic liver toxicity by using hepatocyte-like cells generated from hiPSCs that were established from a patient with a rare CYP polymorphism. However, some compounds did not show any cytotoxicity (such as Cyclizine, Felbamate, and Sulindac) (Fig. 5). To apply the hepatocyte-like cells for wide-spread drug screening, generation of the hepatocyte-like cells are required to detect hepatotoxicity in more sensitive manner. Previous studies showed that the depletion of conjugating enzymes [32] or knockdown of *Nrf2* [34] expression are useful to upregulate the sensitivity to hepatotoxic drugs. Therefore, these approaches would be useful to generate more sensitive hepatocytes to toxic drugs.

5. Conclusions

In this study, we established the efficient hepatocyte differentiation method which employs not only stage-specific transient overexpression of hepatocyte-related transcription factors but also 3D spheroid culture systems by using Nanopillar Plate. To the best of our knowledge, this is the first study in which the hepatocyte-like cells, having enough hepatocyte functions, mediate drug-induced cytotoxicity against many compounds. Our hepatocyte-like cells differentiated from hESCs or hiPSCs have potential to be applied in drug toxicity testing.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2012.11.029>.

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CCAAT/enhancer binding protein-mediated regulation of TGF β receptor 2 expression determines the hepatoblast fate decision

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ABSTRACT

Human embryonic stem cells (hESCs) and their derivatives are expected to be used in drug discovery, regenerative medicine and the study of human embryogenesis. Because hepatocyte differentiation from hESCs has the potential to recapitulate human liver development *in vivo*, we employed this differentiation method to investigate the molecular mechanisms underlying human hepatocyte differentiation. A previous study has shown that a gradient of transforming growth factor beta (TGF β) signaling is required to segregate hepatocyte and cholangiocyte lineages from hepatoblasts. Although CCAAT/enhancer binding proteins (c/EBPs) are known to be important transcription factors in liver development, the relationship between TGF β signaling and c/EBP-mediated transcriptional regulation in the hepatoblast fate decision is not well known. To clarify this relationship, we examined whether c/EBPs could determine the hepatoblast fate decision via regulation of TGF β receptor 2 (TGFBR2) expression in the hepatoblast-like cells differentiated from hESCs. We found that *TGFBR2* promoter activity was negatively regulated by c/EBP α and positively regulated by c/EBP β . Moreover, c/EBP α overexpression could promote hepatocyte differentiation by suppressing TGFBR2 expression, whereas c/EBP β overexpression could promote cholangiocyte differentiation by enhancing TGFBR2 expression. Our findings demonstrated that c/EBP α and c/EBP β determine the lineage commitment of hepatoblasts by negatively and positively regulating the expression of a common target gene, *TGFBR2*, respectively.

KEY WORDS: Hepatoblasts, c/EBP, CEBP, Human ESCs

INTRODUCTION

Many animal models, such as chick, *Xenopus*, zebrafish and mouse, have been used to investigate the molecular mechanisms of liver development. Because many functions of the key molecules in liver

development are conserved in these species, studies on liver development in these animals can be highly informative with respect that in humans. However, some functions of important molecules in liver development might differ between human and other species. Although analysis using genetically modified mice has been successfully performed, it is not of course possible to perform genetic experiments to elucidate molecular mechanisms of liver development in human. Pluripotent stem cells, such as human embryonic stem cells (hESCs), are expected to overcome some of these problems in the study of human embryogenesis, including liver development, because the gene expression profiles of this model are similar to those in normal liver development (Agarwal et al., 2008; DeLaForest et al., 2011).

During liver development, hepatoblasts differentiate into hepatocytes and cholangiocytes. A previous study has shown that a high concentration of transforming growth factor beta (TGF β) could give rise to cholangiocyte differentiation from hepatoblasts (Clotman et al., 2005). To transmit the TGF β signaling, TGF β receptor 2 (TGFBR2) has to be stimulated by TGF β 1, TGF β 2 or TGF β 3 (Kitisin et al., 2007). TGF β binding to the extracellular domain of TGFBR2 induces a conformational change, resulting in the phosphorylation and activation of TGFBR1. TGFBR1 phosphorylates SMAD2 or SMAD3, which binds to SMAD4, and then the SMAD complexes move into the nucleus and function as transcription factors to express various kinds of differentiation-related genes (Kitisin et al., 2007). Although the function of TGFBR2 in regeneration of the adult liver has been thoroughly examined (Oe et al., 2004), the function of TGFBR2 in the hepatoblast fate decision has not been elucidated.

CCAAT/enhancer binding protein (c/EBP) transcription factors play decisive roles in the differentiation of various cell types, including hepatocytes (Tomizawa et al., 1998; Yamasaki et al., 2006). The analysis of c/EBP α (*Cebpa*) knockout mice has shown that many abnormal pseudoglandular structures, which co-express antigens specific for both hepatocytes and cholangiocytes, are present in the liver parenchyma (Tomizawa et al., 1998). These data demonstrated that c/EBP α plays an important role in hepatocyte differentiation. It is also known that the suppression of c/EBP α expression in periportal hepatoblasts stimulates cholangiocyte differentiation (Yamasaki et al., 2006). Although the function of c/EBP α in liver development is well known, the relationship between TGF β signaling and c/EBP α -mediated transcriptional regulation in the hepatoblast fate decision is poorly understood. c/EBP β is also known to be an important factor for liver function (Chen et al., 2000), although the function of c/EBP β in the cell fate decision of hepatoblasts is not well known. c/EBP α and c/EBP β bind to the same DNA binding site. However, the promoter activity of hepatocyte-specific genes, such as those encoding hepatocyte nuclear factor 6 (HNF6, also known as ONECUT1) and UGT2B1,

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is positively regulated by *c/EBP* α but not *c/EBP* β (Hansen et al., 1998; Plumb-Rudewicz et al., 2004), suggesting that the functions of *c/EBP* α and *c/EBP* β in the hepatoblast fate decision might be different.

In the present study, we first examined the function of TGFBR2 in the hepatoblast fate decision using hESC-derived hepatoblast-like cells, which have the ability to self-replicate, differentiate into both hepatocyte and cholangiocyte lineages, and repopulate the liver of carbon tetrachloride (CCl₄)-treated immunodeficient mice. *In vitro* gain- and loss-of-function analyses and *in vivo* transplantation analysis were performed. Next, we investigated how TGFBR2 expression is regulated in the hepatoblast fate decision. Finally, we examined whether our findings could be reproduced in delta-like 1 homolog (*Dlk1*)-positive hepatoblasts obtained from the liver of E13.5 mice. To the best of our knowledge, this study provides the first evidence of *c/EBP*-mediated regulation of TGFBR2 expression in the human hepatoblast fate decision.

RESULTS

Hepatoblast-like cells are generated from hESCs

First, we investigated whether the hepatoblast-like cells (HBCs), which were differentiated from hESCs as described in supplementary material Fig. S1A, have similar characteristics to human hepatoblasts. We recently found that hESC-derived HBCs could be purified and maintained on human laminin 111 (LN111)-coated dishes (Takayama et al., 2013). The long-term cultured HBC population (HBCs passaged more than three times were used in this study) were nearly homogeneous and expressed human hepatoblast markers such as alpha-fetoprotein (AFP), albumin (ALB), cytokeratin 19 (CK19, also known as KRT19) and EPCAM (Schmelzer et al., 2007) (supplementary material Fig. S1B). In addition, most of the colonies observed on human LN111-coated plates were ALB and CK19 double positive, although a few colonies were ALB single positive, CK19 single positive, or ALB and CK19 double negative (supplementary material Fig. S1C). To examine the hepatocyte differentiation capacity of the HBCs *in vivo*, these cells were transplanted into CCl₄-treated immunodeficient mice. The hepatocyte functionality of the transplanted cells was assessed by measuring secreted human ALB levels in the recipient mice (supplementary material Fig. S1D). Human ALB serum was detected in the mice that were transplanted with the HBCs, but not in the control mice. These results demonstrated that the HBCs generated from hESCs have similar characteristics to human hepatoblasts and would therefore provide a valuable tool to investigate the mechanisms of human liver development. In the present study, HBCs generated from hESCs were used to elucidate the mechanisms of the hepatoblast fate decision.

TGFBR2 expression is decreased in hepatocyte differentiation but increased in cholangiocyte differentiation

The HBCs used in this study have the ability to differentiate into both hepatocyte-like cells [cytochrome P450 3A4 (CYP3A4) positive; Fig. 1B] and cholangiocyte-like cells (CK19 positive; Fig. 1C) (the protocols are described in Fig. 1A). Because the expression pattern of TGFBR2 during differentiation from hepatoblasts is not well known, we examined it in hepatocyte and cholangiocyte differentiation from HBCs. *TGFBR2* was downregulated during hepatocyte differentiation from HBCs (Fig. 1D), but upregulated in cholangiocyte differentiation from HBCs (Fig. 1E). After the HBCs were cultured on Matrigel, the cells were fractionated into three populations according to the level of TGFBR2 expression (TGFBR2-negative, -lo or -hi; Fig. 1F). The

HBC-derived TGFBR2-lo cells strongly expressed *α AT* and *CYP3A4* (hepatocyte markers), whereas the HBC-derived TGFBR2-hi cells strongly expressed *SOX9* and integrin β 4 (*ITGB4*) (cholangiocyte markers). These data suggest that the TGFBR2 expression level is decreased in hepatic differentiation, but increased in biliary differentiation of the HBCs.

The cell fate decision of HBCs is regulated by TGF β signals

To examine the function of TGF β 1, β 2 and β 3 (all of which are ligands of TGFBR2) in the hepatoblast fate decision, HBCs were cultured in medium containing TGF β 1, β 2 or β 3 (Fig. 2A,B). The expression levels of cholangiocyte marker genes were upregulated by addition of TGF β 1 or TGF β 2, but not TGF β 3 (Fig. 2A), whereas those of hepatocyte markers were downregulated by addition of TGF β 1 or TGF β 2 (Fig. 2B). To ascertain that TGFBR2 is also important in the hepatoblast fate decision, HBCs were cultured in medium containing SB-431542, which inhibits TGF β signaling (Fig. 2C,D). Hepatocyte marker genes were upregulated by inhibition of TGF β signaling (Fig. 2C), whereas cholangiocyte markers were downregulated (Fig. 2D). To confirm the function of TGF β 1, β 2 and β 3 in the hepatoblast fate decision, colony assays of the HBCs were performed in the presence or absence of TGF β 1, β 2 or β 3 (Fig. 2E). The number of CK19 single-positive colonies was significantly increased in TGF β 1- or β 2-treated HBCs. By contrast, the number of ALB and CK19 double-positive colonies was reduced in TGF β 1-, β 2- or β 3-treated HBCs. These data indicated that TGF β 1 and β 2 positively regulate the biliary differentiation of HBCs. Taken together, the findings suggested that TGFBR2 might be a key molecule in the regulation of hepato-biliary lineage segregation.

TGFBR2 plays an important role in the cell fate decision of HBCs

To examine whether TGFBR2 plays an important role in the hepatoblast fate decision, *in vitro* gain- and loss-of-function analysis of TGFBR2 was performed in the HBCs. We used siRNA in knockdown experiments (supplementary material Fig. S2) during HBC differentiation on Matrigel. Whereas TGFBR2-suppressing siRNA (si-TGFBR2) transfection upregulated the expression of hepatocyte markers, it downregulated cholangiocyte markers (Fig. 3A). si-TGFBR2 transfection increased the percentage of asialoglycoprotein receptor 1 (ASGR1)-positive hepatocyte-like cells (Fig. 3B). By contrast, it decreased the percentage of aquaporin 1 (AQP1)-positive cholangiocyte-like cells. These results suggest that TGFBR2 knockdown promotes hepatocyte differentiation, whereas it inhibits cholangiocyte differentiation. Next, we used Ad vector to perform efficient transduction into the HBCs (supplementary material Fig. S3) and ascertained *TGFBR2* gene expression in TGFBR2-expressing Ad vector (Ad-TGFBR2)-transduced cells (supplementary material Fig. S4). Ad-TGFBR2 transduction downregulated the expression of hepatocyte markers, whereas it upregulated cholangiocyte markers (Fig. 3C). Ad-TGFBR2 transduction decreased the percentage of ASGR1-positive hepatocyte-like cells but increased the percentage of AQP1-positive cholangiocyte-like cells (Fig. 3D). These results suggest that TGFBR2 overexpression inhibits hepatocyte differentiation, whereas it promotes cholangiocyte differentiation. Taken together, these results suggest that TGFBR2 plays an important role in deciding the differentiation lineage of HBCs.

To investigate whether hepatoblasts would undergo differentiation in a TGFBR2-associated manner *in vivo*, HBCs transfected/transduced with si-control, si-TGFBR2, Ad-LacZ or Ad-

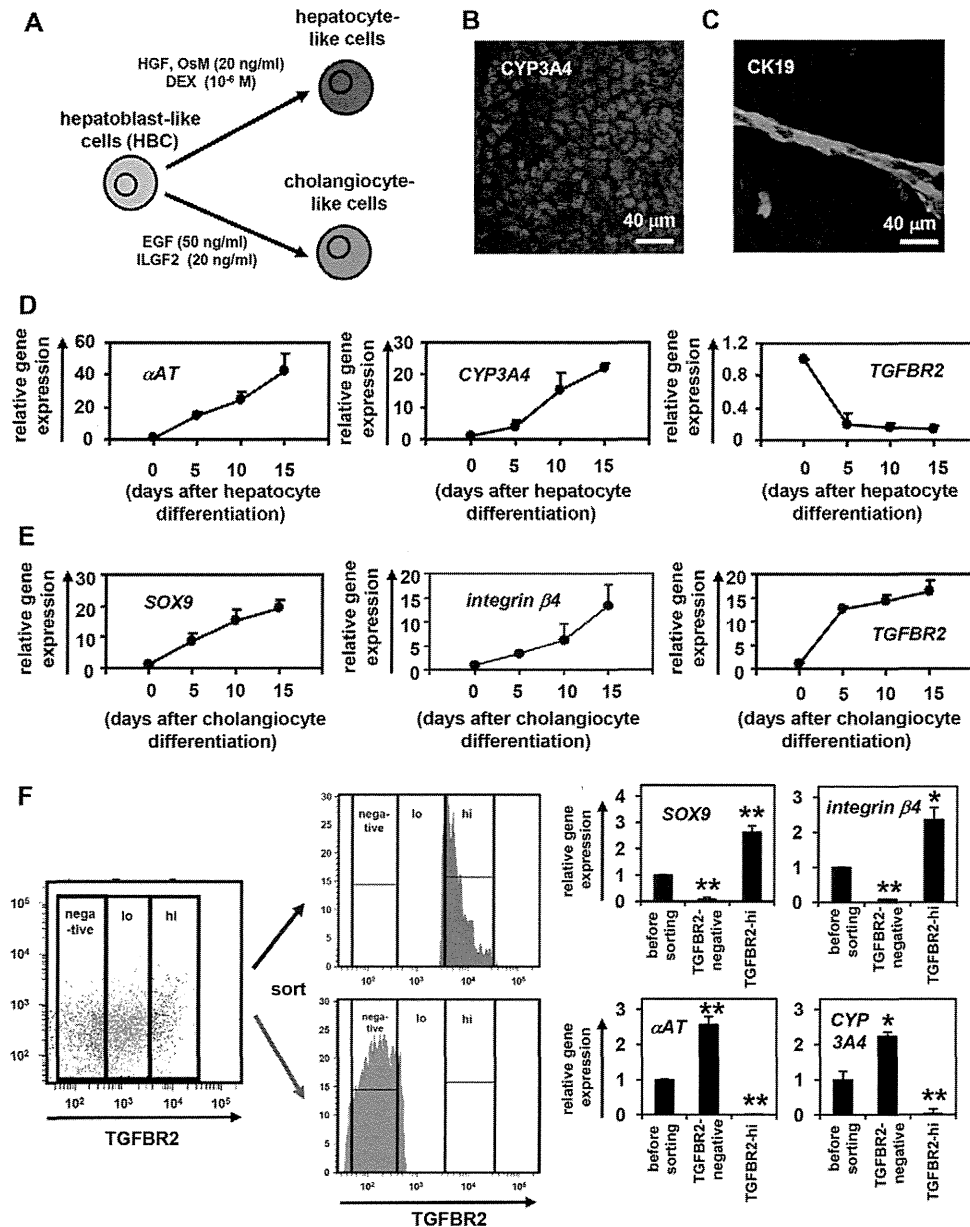


Fig. 1. HBCs can differentiate into both hepatocyte and cholangiocyte lineages.

(A) The strategy for hepatocyte and cholangiocyte differentiation from HBCs. (B, C) The HBC-derived hepatocyte-like cells or cholangiocyte-like cells were subjected to immunostaining with anti-CYP3A4 (red, B) or anti-CK19 (green, C) antibodies, respectively. (D, E) Temporal gene expression levels of hepatocyte markers (α AT and CYP3A4) (D) or cholangiocyte markers (SOX9 and integrin β 4) (E) during hepatocyte or cholangiocyte differentiation as measured by real-time RT-PCR. The gene expression of *TGFBR2* was also examined. The gene expression levels in HBCs were taken as 1.0. (F) HBCs were cultured on Matrigel for 5 days, and then the expression level of *TGFBR2* was examined by FACS analysis. *TGFBR2*-negative, -lo and -hi populations were collected and real-time RT-PCR analysis was performed to measure the expression levels of hepatocyte markers (α AT and CYP3A4) and cholangiocyte markers (SOX9 and integrin β 4). * P <0.05, ** P <0.01 (compared with 'before sorting'). Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test (n =3).

TGFBR2 were transplanted into CCl₄-treated immunodeficient mice (Fig. 3E, F). Although some of the si-control-transfected or Ad-LacZ-transduced HBCs remained as HBCs (HNF4 α and CK19 double positive), most of them showed *in vitro* differentiation toward hepatocyte-like cells (HNF4 α single positive) (Fig. 3E, top row). By contrast, Ad-*TGFBR2*-transduced HBCs were predominantly committed to cholangiocyte-like cells (CK19 single positive) and si-*TGFBR2*-transfected HBCs were predominantly committed to hepatocyte-like cells (HNF4 α single positive) (Fig. 3E, bottom row). Ad-*TGFBR2* transduction decreased the percentage of HNF4 α -positive hepatocyte-like cells, whereas it increased the percentage of CK19-positive cholangiocyte-like cells (supplementary material Fig. S5). The hepatocyte functionality of the *in vivo* differentiated HBCs was assessed by measuring secreted human ALB levels in the recipient mice (Fig. 3F). Mice that were transplanted with Ad-*TGFBR2*-transduced HBCs showed lower human ALB serum levels than those transplanted with Ad-LacZ-transduced HBCs, and the mice that were transplanted with si-*TGFBR2*-transfected HBCs showed higher human ALB serum

levels than those transplanted with si-control-transfected HBCs. These data suggest that cholangiocyte or hepatocyte differentiation was promoted by *TGFBR2* overexpression or knockdown, respectively. Thus, based on these data from *in vitro* and *in vivo* experiments, *TGFBR2* plays an important role in deciding the differentiation lineage of HBCs.

***TGFBR2* promoter activity and expression are negatively regulated by c/EBP α and positively regulated by c/EBP β**

A previous study has shown that *TGFBR2* expression is upregulated in *Hnf6* knockout mice (Clotman et al., 2005), although we confirmed by ChIP assay that HNF6 does not bind to the *TGFBR2* promoter region (data not shown). Because c/EBP α is important in the hepatoblast fate decision (Suzuki et al., 2003), we expected that c/EBPs might directly regulate *TGFBR2* expression. The *TGFBR2* promoter region was analyzed to examine whether *TGFBR2* expression is regulated by c/EBPs. Some c/EBP binding sites (supplementary material Fig. S6) were predicted by rVista 2.0 (<http://rvista.dcode.org/>) (Fig. 4A). By performing a ChIP assay, one

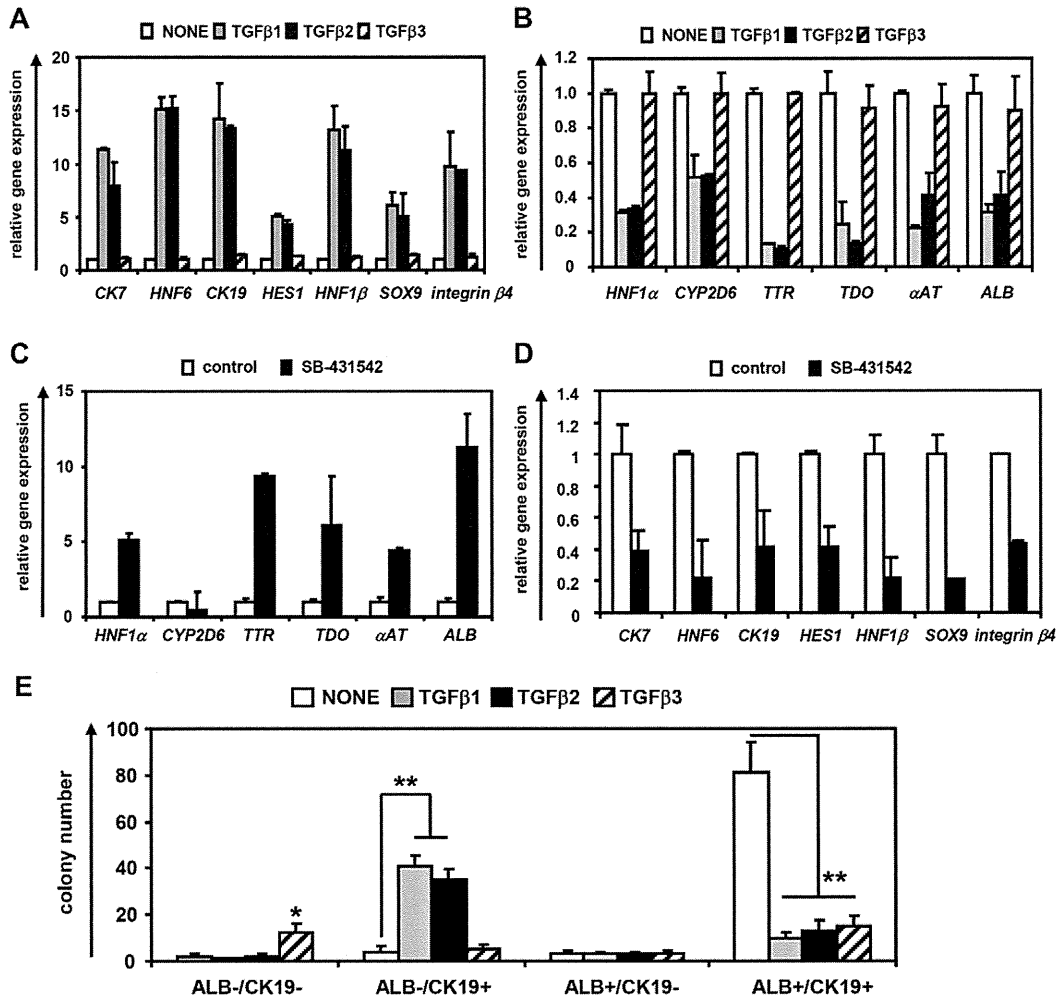


Fig. 2. Hepatocyte and cholangiocyte differentiation from HBCs is regulated by TGF β signaling. (A,B) HBCs were cultured in differentiation hESF-DIF medium containing 10 ng/ml TGF β 1, TGF β 2 or TGF β 3 for 10 days. The expression levels of cholangiocyte (A) and hepatocyte (B) marker genes were measured by real-time RT-PCR. On the y-axis, the gene expression level of cholangiocyte markers in untreated cells (NONE) was taken as 1.0. (C,D) HBCs were cultured in differentiation hESF-DIF medium containing SB-431542 (10 μ M) for 10 days. Control cells were treated with solvent only (0.1% DMSO). Expression levels of hepatocyte (C) and cholangiocyte (D) marker genes were measured by real-time RT-PCR. On the y-axis, the gene expression level of hepatocyte markers in untreated cells (control) was taken as 1.0. (E) HBC colony formation assay in the presence or absence of 10 ng/ml TGF β 1, TGF β 2 or TGF β 3. HBCs were plated at 200 cells/cm² on human LN111-coated dishes. The colonies were separated into four groups based on the expression of ALB and CK19: double-negative, ALB negative and CK19 positive, ALB positive and CK19 negative, and double positive. The numbers represent wells in which the colony was observed in three 96-well plates (total 288 wells). Five days after plating, the cells were fixed with 4% PFA and used for double immunostaining. * P <0.05, ** P <0.01 (compared with NONE). Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's t -test (n =3).

c/EBP binding site was found in the *TGFBR2* promoter region (Fig. 4B). A reporter assay of the *TGFBR2* promoter region showed that *c/EBP* β activates *TGFBR2* promoter activity, whereas *c/EBP* α inhibits it (Fig. 4C). In addition, *TGFBR2* expression was downregulated by Ad-*c/EBP* α transduction, whereas *TGFBR2* was upregulated by Ad-*c/EBP* β transduction in HepG2 cells (*TGFBR2* positive) (Fig. 4D). We ascertained the expression of *c/EBP* α or *c/EBP* β (*CEBPA* or *CEBPB* – Human Gene Nomenclature Committee) in the Ad-*c/EBP* α - or Ad-*c/EBP* β -transduced cells, respectively (supplementary material Fig. S4). These results demonstrated that the promoter activity and expression of *TGFBR2* were directly regulated by both *c/EBP* α and *c/EBP* β .

***c/EBPs* determine the cell fate decision of HBCs via regulation of *TGFBR2* expression**

To elucidate the relationship between *TGFBR2* and *c/EBPs* (*c/EBP* α and *c/EBP* β) in the hepatoblast fate decision, we first examined the

temporal gene expression patterns of *TGFBR2*, *c/EBP* α and *c/EBP* β in hepatocyte and cholangiocyte differentiation. During hepatocyte differentiation, *TGFBR2* expression was downregulated, whereas *c/EBP* α was upregulated (supplementary material Fig. S7A, top). During cholangiocyte differentiation, *c/EBP* α was downregulated, whereas *TGFBR2* and *c/EBP* β were upregulated (supplementary material Fig. S7A, bottom). In addition, the ratio of *c/EBP* α to *c/EBP* β was significantly increased in hepatocyte differentiation, but significantly reduced in cholangiocyte differentiation (supplementary material Fig. S7B). High-level expression of *c/EBP* α was detected in *TGFBR2*-negative cells, but not in *TGFBR2*-hi cells (supplementary material Fig. S7C). By contrast, high-level expression of *c/EBP* β was detected in *TGFBR2*-hi cells, but not in *TGFBR2*-negative cells. These results suggest that *TGFBR2* is negatively regulated by *c/EBP* α and positively regulated by *c/EBP* β in the differentiation model from HBCs as well as in the HepG2 cell line (Fig. 4).

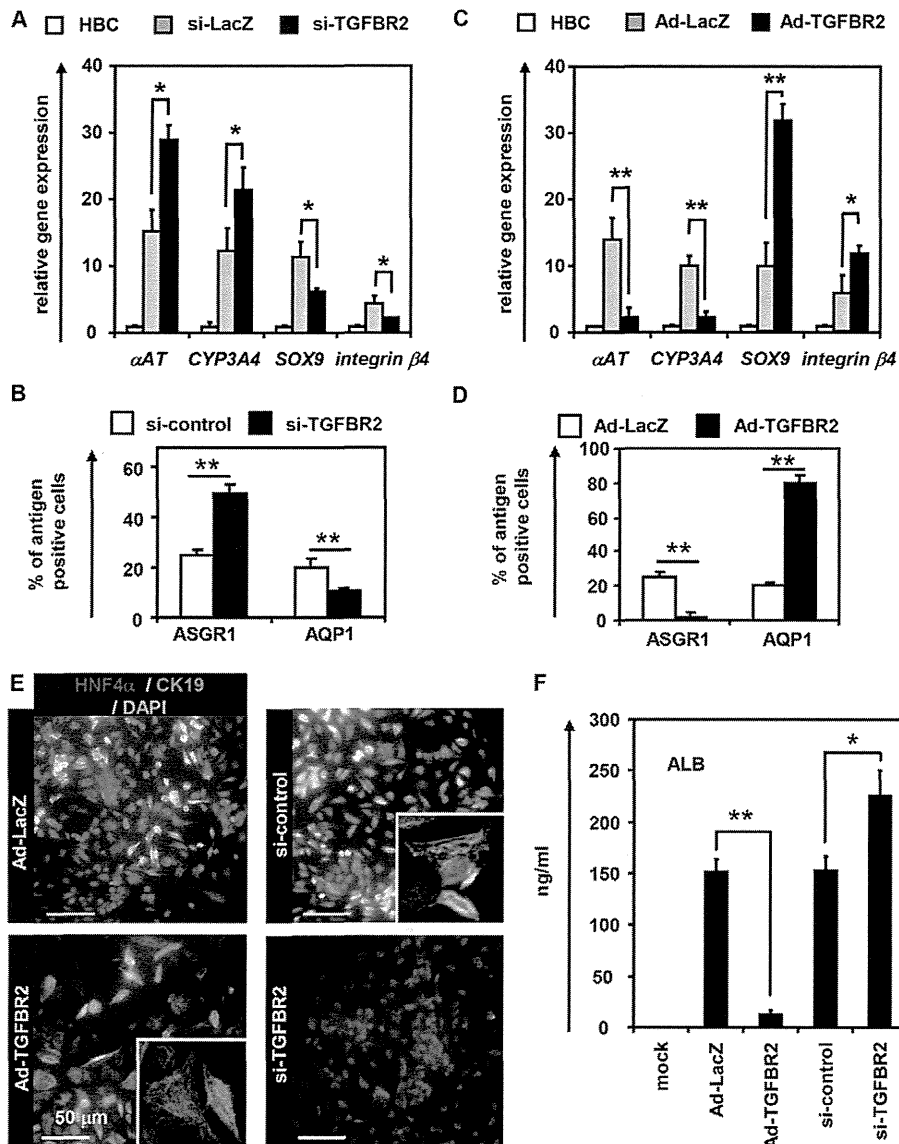


Fig. 3. TGFBR2 regulates bi-directional differentiation of HBCs. (A) HBCs were transfected with 50 nM control siRNA (si-control) or TGFBR2-suppressing siRNA (si-TGFBR2) and cultured in differentiation hESF-DIF medium for 10 days. The expression levels of hepatocyte (α AT and CYP3A4) or cholangiocyte (SOX9 and integrin β 4) markers were measured by real-time RT-PCR. On the y-axis, the gene expression level in HBCs was taken as 1.0. (B) On day 10 after siRNA transfection, the efficiency of hepatocyte or cholangiocyte differentiation was measured by estimating the percentage of ASGR1-positive or AQP1-positive cells, respectively, by FACS analysis. (C) HBCs were transfected with 3000 VPs/cell of Ad-LacZ or Ad-TGFBR2 for 1.5 hours and cultured in differentiation hESF-DIF medium for 10 days. Expression levels of hepatocyte or cholangiocyte marker genes were measured by real-time RT-PCR. On the y-axis, gene expression levels in the HBCs was taken as 1.0. (D) On day 10 after Ad vector transduction, the efficiency of hepatocyte or cholangiocyte differentiation was measured by estimating the percentage of ASGR1-positive or AQP1-positive cells, respectively, by FACS analysis. (E,F) The si-control, si-TGFBR2, Ad-LacZ- or Ad-TGFBR2-transfected/transduced HBCs (1.0×10^6 cells) were transplanted into CCl₄-treated (2 mg/kg) *Rag2/Il2rg* double-knockout mice by intrasplenic injection. (E) Expression of human HNF4 α (red) and CK19 (green) was examined by double immunohistochemistry 2 weeks after transplantation. Nuclei were counterstained with DAPI (blue). (F) Levels of human ALB in recipient mouse serum were measured 2 weeks after transplantation. * $P < 0.05$, ** $P < 0.01$ (compared with Ad-LacZ-transduced or si-control-transfected cells). Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test ($n=3$).

ChIP experiments showed that *c/EBP α* or *c/EBP β* is recruited to the *TGFBR2* promoter region containing the *c/EBP* binding site in hepatocyte-like cells or cholangiocyte-like cells, respectively (Fig. 5A), suggesting that *c/EBP α* and *c/EBP β* oppositely regulate *TGFBR2* promoter activity in the differentiation from HBCs. We confirmed that *c/EBP α* or *c/EBP β* was mainly recruited to the *TGFBR2* promoter region containing the *c/EBP* binding site in TGFBR-negative or TGFBR2-positive cells, respectively (supplementary material Fig. S7D). Taken together, we concluded that *c/EBP α* and *c/EBP β* are able to regulate the cell fate decision of HBCs via regulation of *TGFBR2* expression. During differentiation from HBCs, *TGFBR2* expression was negatively regulated by *c/EBP α* and positively regulated by *c/EBP β* (Fig. 5B). To examine whether *c/EBP α* or *c/EBP β* could regulate the differentiation from HBCs, *in vitro* gain- and loss-of-function analyses were performed. si-*c/EBP α* transfection downregulated hepatocyte marker gene expression, whereas it upregulated cholangiocyte marker genes (Fig. 5C). By contrast, si-*c/EBP β* transfection upregulated hepatocyte marker and downregulated cholangiocyte marker gene expression (Fig. 5C). In accordance, Ad-*c/EBP α* transduction upregulated hepatocyte marker genes and downregulated cholangiocyte markers (Fig. 5D), whereas Ad-

c/EBP β transduction downregulated hepatocyte markers and upregulated cholangiocyte marker genes. Promotion of hepatocyte differentiation by Ad-*c/EBP α* transduction was inhibited by Ad-TGFBR2 transduction, whereas inhibition of cholangiocyte differentiation by Ad-*c/EBP α* transduction was rescued by Ad-TGFBR2 transduction (Fig. 5E). In addition, promotion of hepatocyte differentiation by si-*c/EBP β* transfection was inhibited by Ad-TGFBR2 transduction, whereas inhibition of cholangiocyte differentiation by si-*c/EBP β* transfection was rescued by Ad-TGFBR2 transduction (Fig. 5F). We further confirmed that inhibition of hepatocyte differentiation by si-*c/EBP α* -transfection was rescued by si-TGFBR2 transfection (supplementary material Fig. S8). Taken together, these results led us to conclude that *c/EBP α* and *c/EBP β* could determine the cell fate of HBCs by negatively and positively regulating *TGFBR2* expression, respectively (supplementary material Fig. S9).

***c/EBPs* organize the differentiation of fetal mouse hepatoblasts through regulation of *TGFBR2* expression**

We have demonstrated that *c/EBPs* may determine the HBC fate decision via regulation of the expression level of *TGFBR2*. To examine whether our findings could be replicated in native liver

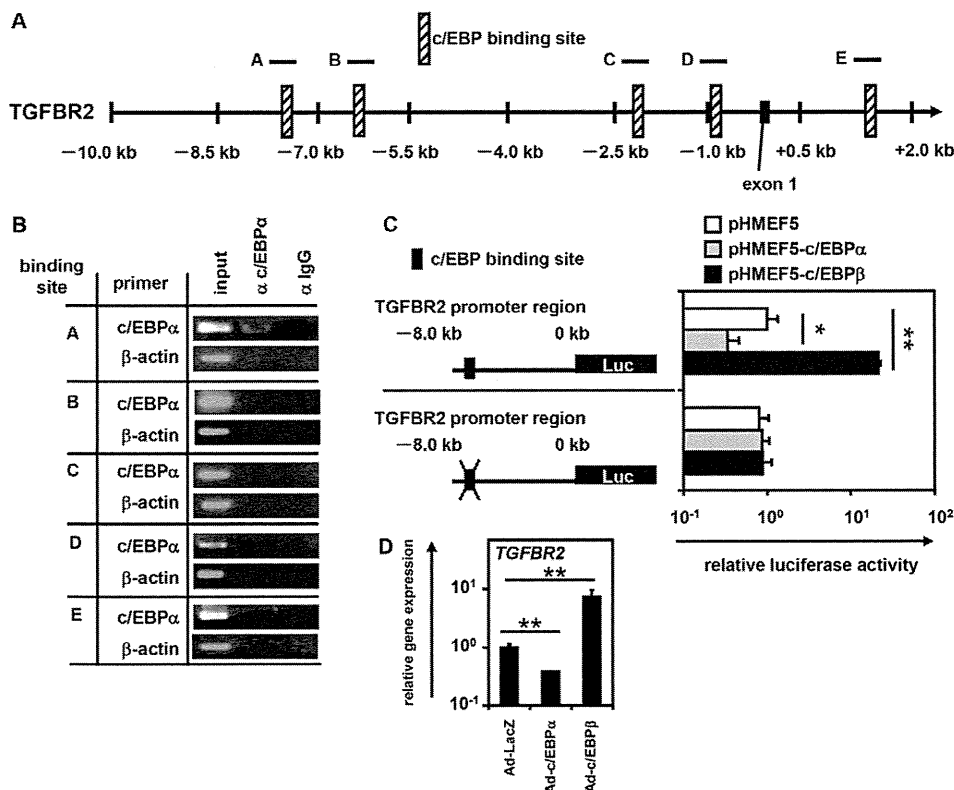


Fig. 4. *TGFBR2* promoter activity and expression are negatively regulated by *c/EBPα* and positively regulated by *c/EBPβ*. (A) Candidate *c/EBP* binding sites (hatched boxes) in the *TGFBR2* promoter region as predicted using *rVista* 2.0 (see supplementary material Fig. S7). (B) hESCs (H9 cells) were differentiated into hepatoblasts and then a ChIP assay performed. The antibodies and primers employed are summarized in supplementary material Tables S1 and S4. (C) HEK293 cells were transfected with firefly luciferase (*Luc*) expression plasmids containing the promoter region of *TGFBR2*. In addition, empty plasmid (pHMEF5), *c/EBPα* expression plasmid (pHMEF5-*c/EBPα*) or *c/EBPβ* expression plasmid (pHMEF5-*c/EBPβ*) was transfected. After 36 hours, a dual luciferase assay was performed. Base pair positions are relative to the translation start site (+1). (D) HepG2 cells (*TGFBR2*-positive cells) were transfected with 3000 VPs/cell of Ad-LacZ, Ad-*c/EBPα* or Ad-*c/EBPβ* for 1.5 hours and cultured for 48 hours. The expression level of *TGFBR2* in HepG2 cells was measured by real-time RT-PCR. On the y-axis, the gene expression level in Ad-LacZ-transduced cells was taken as 1.0. **P* < 0.05, ***P* < 0.01. Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test (*n* = 3).

development, fetal hepatoblasts were purified from E13.5 mice. The gene expression level of *TGFBR2* in fetal mouse hepatoblasts was negatively or positively regulated by *c/EBPα* or *c/EBPβ*, respectively (Fig. 6A,B). The promotion of hepatocyte differentiation by Ad-*c/EBPα* transduction was inhibited by Ad-*TGFBR2* transduction, whereas the inhibition of cholangiocyte differentiation by Ad-*c/EBPα* transduction was rescued by Ad-*TGFBR2* transduction (Fig. 6C). In addition, the promotion of hepatocyte differentiation by si-*c/EBPβ* transfection was inhibited by Ad-*TGFBR2* transduction, whereas the inhibition of cholangiocyte differentiation by si-*c/EBPβ* transfection was rescued by Ad-*TGFBR2* transduction (Fig. 6D). Taken together, these results led us to conclude that *c/EBPα* and *c/EBPβ* could determine the cell fate of fetal mouse hepatoblasts by negatively and positively regulating *TGFBR2* expression, respectively. Our *in vitro* differentiation system could also prove useful in elucidating the molecular mechanisms of human liver development.

DISCUSSION

The purpose of this study was to better understand the molecular mechanisms of the hepatoblast fate decision in humans. To elucidate the molecular mechanisms of liver development, both conditional knockout mouse models and cell culture systems are useful. For example, DeLaForest et al. demonstrated the role of HNF4α in hepatocyte differentiation using hESC culture systems (DeLaForest et al., 2011). The technology for inducing hepatocyte differentiation from hESCs has recently been dramatically advanced (Takayama et al., 2012a). Because it is possible to generate functional HBCs from hESCs, which can self-replicate and differentiate into both hepatocyte and cholangiocyte lineages (supplementary material Fig. S1 and Fig. 1), the differentiation model of HBCs generated from hESCs should provide a powerful tool for analyzing the molecular mechanisms of human liver development.

In this study, the molecular mechanisms of the hepatoblast fate decision were elucidated using hESC culture systems. HBCs cultured on human LN111 expressed hepatoblast markers (supplementary material Fig. S1) and had the ability to differentiate into both hepatocyte-like cells and cholangiocyte-like cells (Fig. 1). Because a previous study showed that low and high concentrations of TGFβ were required for hepatocyte and cholangiocyte differentiation, respectively (Clotman et al., 2005), we expected that *TGFBR2* might contribute to the hepatoblast fate decision. Although TGFβ1, β2 and β3 are all ligands of *TGFBR2*, TGFβ3 did not promote cholangiocyte differentiation (Fig. 2). This might have been because only TGFβ3 is unable to upregulate the expression of *SOX9*, which is the key factor in bile duct development *in vivo* and cholangiocyte differentiation *in vitro* (Antoniou et al., 2009). We examined the function of *TGFBR2* in the hepatoblast fate decision, and found that its overexpression promoted cholangiocyte differentiation, whereas *TGFBR2* knockdown promoted hepatocyte differentiation (Fig. 3). Although an exogenous TGFβ ligand was not added to the differentiation medium, the endogenous TGFβ ligand present in Matrigel, which was used in our differentiation protocol, might have bound to *TGFBR2*. It might also be that the cells committed to the biliary lineage express TGFβ, as a previous study showed that bile duct epithelial cells express TGFβ (Lewindon et al., 2002).

To examine the molecular mechanism regulating *TGFBR2* expression, the *TGFBR2* promoter region was analyzed (Fig. 4). *TGFBR2* promoter activity was negatively regulated by *c/EBPα* and positively regulated by *c/EBPβ*. *c/EBPα* overexpression downregulated *TGFBR2* promoter activity in spite of the fact that *c/EBPα* protein has no repression domain (Yoshida et al., 2006). CTBP1 and CTBP2 (Vernochet et al., 2009) are known to be co-repressors of *c/EBPα*, and as such constitute candidate co-repressors recruited to the *c/EBP* binding site in the *TGFBR2* promoter region.

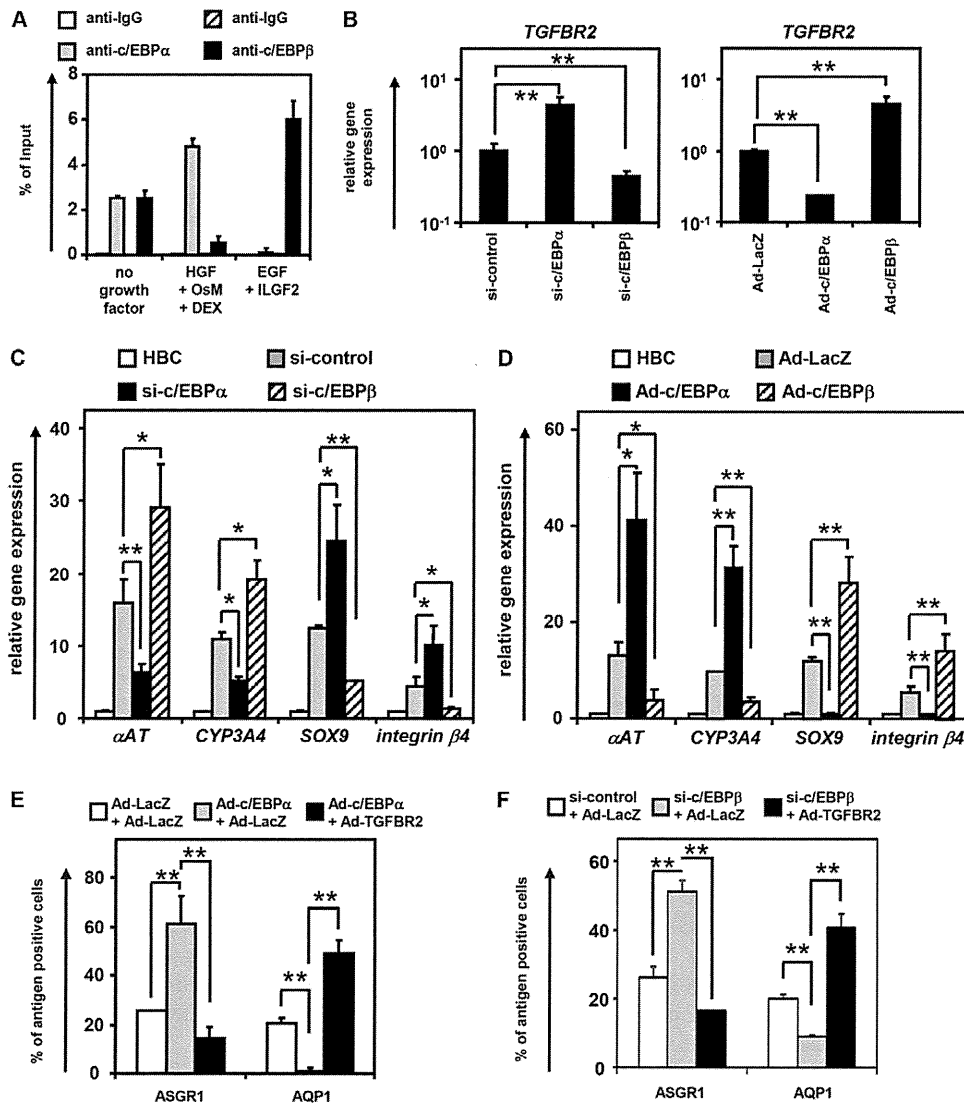


Fig. 5. *c/EBPα* and *c/EBPβ* promote hepatocyte and cholangiocyte differentiation by regulating *TGFBR2* expression, respectively. (A) HBCs were differentiated into hepatocyte-like cells or cholangiocyte-like cells according to the scheme outlined in Fig. 1A. On day 10 after hepatocyte or cholangiocyte differentiation, recruitment of *c/EBPα* or *c/EBPβ* to the *TGFBR2* promoter region was examined by ChIP assay. (B-D) HBCs were transfected with 50 nM si-control, si-*c/EBPα* or si-*c/EBPβ* and cultured in differentiation hESF-DIF medium for 10 days (B left, C). The expression levels of *TGFBR2* and hepatocyte and cholangiocyte markers were then measured by real-time RT-PCR. (B right, D) HBCs were transduced with 3000 VPs/cell of Ad-LacZ, Ad-*c/EBPα* or Ad-*c/EBPβ* for 1.5 hours and cultured in differentiation hESF-DIF medium for 10 days. The expression levels of *TGFBR2* and hepatocyte and cholangiocyte markers were then measured by real-time RT-PCR. On the y-axis, the gene expression level in the si-control-transfected or Ad-LacZ-transduced cells was taken as 1.0 in B, and levels in HBCs were taken as 1.0 in C and D. (E) HBCs were transduced with 3000 VPs/cell each of Ad-LacZ + Ad-LacZ, Ad-*c/EBPα* + Ad-LacZ, or Ad-*c/EBPα* + Ad-TGFBR2 for 1.5 hours and cultured in differentiation hESF-DIF medium for 10 days. The efficiency of hepatocyte or cholangiocyte differentiation was measured by estimating the percentage of ASGR1-positive or AQP1-positive cells, respectively, by FACS analysis. (F) HBCs were transduced with 3000 VPs/cell of Ad-LacZ or Ad-TGFBR2 and then transfected with 50 nM si-control or si-*c/EBPβ* and cultured in hESF-DIF medium for 10 days. The efficiency of hepatocyte or cholangiocyte differentiation was measured by estimating the percentage of ASGR1-positive or AQP1-positive cells, respectively, by FACS analysis. * $P < 0.05$, ** $P < 0.01$. Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test ($n = 3$).

Proteome analysis of *c/EBPα* would provide an opportunity to identify the co-repressor of *c/EBPα*. Because large numbers of nearly homogeneous hepatoblasts can be differentiated from hESCs, as compared with the isolation of fetal liver hepatoblasts, hepatocyte differentiation technology from hESCs might prove useful in proteome analysis.

We found that Ad-*c/EBPα* transduction could promote hepatocyte differentiation by suppressing *TGFBR2* expression (Fig. 5). Our findings might thus provide a detailed explanation of the phenotype of *c/EBPα* knockout mice; that is, hepatocyte differentiation is

inhibited and cholangiocyte differentiation is promoted in these mice (Yamasaki et al., 2006). We also found that Ad-*c/EBPβ* transduction could promote cholangiocyte differentiation by enhancing *TGFBR2* expression. Because both *c/EBPα* and *c/EBPβ* can bind to the same binding site, reciprocal competition for binding is likely to be influenced by regulating *c/EBPα* or *c/EBPβ* expression. Therefore, the expression ratio between *c/EBPα* and *c/EBPβ* might determine the cell fate of hepatoblasts by regulating the expression level of *TGFBR2*. We confirmed that our findings could be reproduced in fetal mouse hepatoblasts (Fig. 6). Because a previous study had

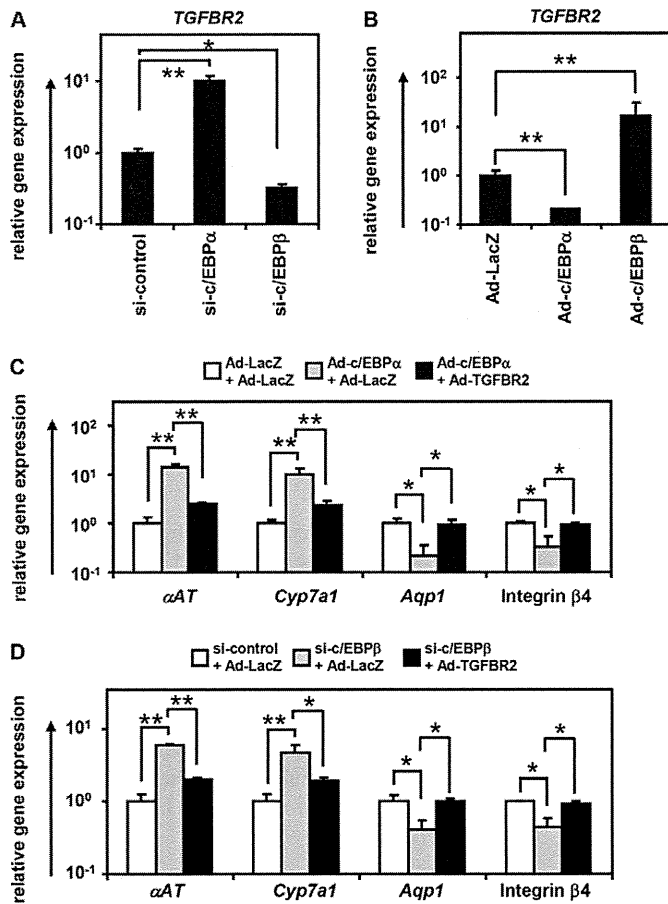


Fig. 6. c/EBPs control the differentiation of fetal mouse hepatoblasts through regulation of TGFBR2 expression. Fetal mouse hepatoblasts (Dlk1-positive cells; the purity was over 98%) were sorted from E13.5 mouse liver. (A) Fetal mouse hepatoblasts were transfected with 50 nM si-control, si-c/EBP α or si-c/EBP β and cultured for 5 days. The expression of *TGFBR2* was measured by real-time RT-PCR. (B) Fetal mouse hepatoblasts were transfected with 3000 VPs/cell of Ad-LacZ, Ad-c/EBP α or Ad-c/EBP β for 1.5 hours and cultured for 5 days. The expression of *TGFBR2* was measured by real-time RT-PCR. On the y-axis, the gene expression level in the si-control-transfected cells or Ad-LacZ-transduced cells was taken as 1.0. (C) Fetal mouse hepatoblasts were transfected with 3000 VPs/cell each of Ad-LacZ + Ad-LacZ, Ad-c/EBP α + Ad-LacZ, or Ad-c/EBP α + Ad-TGFBR2 for 1.5 hours and cultured for 5 days. On day 5, the expression levels of hepatocyte (*α AT* and *Cyp7a1*) and cholangiocyte (*Aqp1* and integrin β 4) markers were measured by real-time RT-PCR. (D) Fetal mouse hepatoblasts were transfected with 3000 VPs/cell of Ad-LacZ or Ad-TGFBR2 and then transfected with 50 nM si-control or si-c/EBP β and cultured for 5 days. On day 5, the gene levels of hepatocyte (*α AT* and *Cyp7a1*) and cholangiocyte (*Aqp1* and integrin β 4) markers were measured by real-time RT-PCR. On the y-axis, the gene expression level in the si-control-transfected or Ad-LacZ-transduced cells was taken as 1.0. * P <0.05, ** P <0.01. Error bars indicate s.d. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test ($n=3$).

shown that the addition of hepatocyte growth factor (HGF) to hepatoblasts upregulated the expression of *c/EBP α* and downregulated the expression of *c/EBP β* (Suzuki et al., 2003), the ratio between *c/EBP α* and *c/EBP β* might be determined by HGF during hepatocyte differentiation.

In this study, we have identified for the first time that *TGFBR2* is a target of *c/EBPs* in the hepatoblast fate decision (supplementary material Fig. S9). *c/EBP α* promotes hepatocyte differentiation by downregulating the expression of *TGFBR2*, whereas *c/EBP β*

promotes cholangiocyte differentiation by upregulating *TGFBR2* expression. This study might have revealed a molecular mechanism underlying the lineage commitment of human hepatoblasts controlled by a gradient of TGF β signaling. We believe that similar procedures that adopt the model of human pluripotent stem cell (including human iPS cell) differentiation will be used not only for the elucidation of molecular mechanisms underlying human hepatocyte and biliary differentiation but also for investigating the causes of congenital anomalies of the human liver and biliary tract.

MATERIALS AND METHODS

Ad vectors

Ad vectors were constructed by an improved *in vitro* ligation method (Mizuguchi and Kay, 1998; Mizuguchi and Kay, 1999). The human *c/EBP α* and *c/EBP β* genes (accession numbers NM_004364 and NM_005194, respectively) were amplified by PCR using the following primers: *c/EBP α* , Fwd 5'-GCTCTAGATGCCGGGAGAACTCTAACTC-3' and Rev 5'-GCGGTACCAAACCCTCCCTGGGTCC-3'; *c/EBP β* , Fwd 5'-GCATCTAGATTTCATGCAACGCCTGGTG-3' and Rev 5'-ATAGGTACCTAAAATTACCGACGGGCTCC-3'. The human *TGFBR2* gene was purchased from Addgene (plasmid 16622). The human *c/EBP α* , *c/EBP β* or *TGFBR2* gene was inserted into pBSKII (Invitrogen), resulting in pBSKII-*c/EBP α* , -*c/EBP β* or -*TGFBR2*. Then, human *c/EBP α* , *c/EBP β* or *TGFBR2* was inserted into pHMEF5 (Kawabata et al., 2005), which contains the human elongation factor 1 α (*EF1 α* , also known as *EEF1A1*) promoter, resulting in pHMEF5-*c/EBP α* , -*c/EBP β* or -*TGFBR2*. pHMEF5-*c/EBP α* , -*c/EBP β* or -*TGFBR2* was digested with *I-CeuI/PI-SceI* and ligated into *I-CeuI/PI-SceI*-digested pAdHM41-K7 (Koizumi et al., 2003), resulting in pAd-*c/EBP α* , -*c/EBP β* or -*TGFBR2*. The human *EF1 α* promoter-driven *lacZ*- or *FOXA2*-expressing Ad vectors (Ad-*LacZ* or Ad-*FOXA2*, respectively) were constructed previously (Takayama et al., 2012b; Tashiro et al., 2008). All Ad vectors contain a stretch of lysine residues (K7) in the C-terminal region of the fiber knob for more efficient transduction of hESCs, definitive endoderm cells and HBCs, in which transfection efficiency was almost 100%, and the Ad vectors were purified as described previously (Takayama et al., 2012a; Takayama et al., 2011). The vector particle (VP) titer was determined by a spectrophotometric method (Maizel et al., 1968).

hESC culture

The H9 hESC line (WiCell Research Institute) was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Merck Millipore) in ReproStem medium (ReproCELL) supplemented with 5 ng/ml FGF2 (Katayama Kagaku Kogyo). H9 was used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan and the study was approved by the Independent Ethics Committee.

Generation and maintenance of hESC-derived HBCs

Before the initiation of cellular differentiation, the hESC medium was exchanged for a defined serum-free medium, hESF9, and cultured as previously reported (Furue et al., 2008). The differentiation protocol for the induction of definitive endoderm cells and HBCs was based on our previous reports with some modifications (Takayama et al., 2012a; Takayama et al., 2012b; Takayama et al., 2011). Briefly, in mesendoderm differentiation, hESCs were cultured for 2 days on Matrigel Matrix (BD Biosciences) in differentiation hESF-DIF medium, which contains 100 ng/ml activin A (R&D Systems); hESF-DIF medium was purchased from 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, 0.5 mg/ml bovine fatty acid-free serum albumin (all from Sigma) and 1 \times B27 Supplement (without vitamin A; Invitrogen). To generate definitive endoderm cells, the mesendoderm cells were transfected with 3000 VPs/cell of *FOXA2*-expressing Ad vector (Ad-*FOXA2*) for 1.5 hours on day 2 and cultured until day 6 on Matrigel in differentiation hESF-DIF medium supplemented with 100 ng/ml activin A. For induction of the HBCs, the

definitive endoderm cells were cultured for 3 days on Matrigel in differentiation hESF-DIF medium supplemented with 20 ng/ml BMP4 (R&D Systems) and 20 ng/ml FGF4 (R&D Systems). Transient overexpression of FOXA2 in the mesendoderm cells is not necessary for establishing HBCs, but it is helpful for efficient generation of the HBCs. The HBCs were first purified from the hESC-derived cells (day 9) by selecting attached cells on a human recombinant LN111 (BioLamina)-coated dish 15 minutes after plating (Takayama et al., 2013). The HBCs were cultured on a human LN111-coated dish (2.0×10^4 cells/cm²) in maintenance DMEM/F12 medium [DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1× insulin/transferrin/selenium, 10 mM nicotinamide, 0.1 μM dexamethasone (DEX) (Sigma), 20 mM HEPES, 25 mM NaHCO₃, 2 mM L-glutamine, and penicillin/streptomycin] which contained 40 ng/ml HGF (R&D Systems) and 20 ng/ml epidermal growth factors (EGF) (R&D Systems). The medium was refreshed every day. The HBCs were dissociated with Accutase (Millipore) into single cells, and subcultured every 6 or 7 days. The HBCs used in this study were passaged more than three times.

In vitro hepatocyte and cholangiocyte differentiation

To induce hepatocyte differentiation, the HBCs were cultured on a Matrigel-coated dish (7.5×10^4 cells/cm²) in Hepatocyte Culture Medium (HCM without EGF; Lonza) supplemented with 20 ng/ml HGF, 20 ng/ml Oncostatin M (OsM) (R&D Systems) and 1 μM DEX. To induce cholangiocyte differentiation, the HBCs were cultured in collagen gel. To establish collagen gel plates, 500 μl collagen gel solution [400 μl type I-A collagen (Nitta gelatin), 50 μl 10× DMEM and 50 μl 200 mM HEPES buffer containing 2.2% NaHCO₃ and 0.05 M NaOH] was added to each well, and then the plates were incubated at 37°C for 30 minutes. The HBCs (5×10^4 cells) were resuspended in 500 μl differentiation DMEM/F12 medium [DMEM/F12 medium supplemented with 20 mM HEPES, 2 mM L-glutamine, 100 ng/ml EGF and 40 ng/ml ILGF2 (IGF2)], and then mixed with 500 μl of the collagen gel solution and plated onto the basal layer of collagen. After 30 minutes, 2 ml differentiation DMEM/F12 medium was added to the well.

Inhibition of TGFβ signaling

SB-431542 (Santa Cruz Biotechnology), which is a small molecule that acts as a selective inhibitor of activin receptor-like kinase (ALK) receptors [ALK4, ALK5 and ALK7 (also known as ACVR1B, TGFBR1 and ACVR1C)], was used to inhibit TGFβ signaling in HBCs.

Flow cytometry

Single-cell suspensions of hESC-derived cells were fixed with 2% paraformaldehyde (PFA) at 4°C for 20 minutes, and then incubated with primary antibody (supplementary material Table S1) followed by secondary antibody (supplementary material Table S2). Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences). Cell sorting was performed using a FACS Aria (BD Biosciences).

RNA isolation and reverse transcription (RT)-PCR

Total RNA was isolated from hESCs and their derivatives using ISOGENE (Nippon Gene). cDNA was synthesized using 500 ng total RNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using an Applied Biosystems StemOnePlus real-time PCR system. Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene *GAPDH*. Primers are described in supplementary material Table S3.

Immunohistochemistry

Cells were fixed with 4% PFA. After incubation with 0.1% Triton X-100 (Wako), blocking with Blocking One (Nakalai Tesque) or PBS containing 2% FBS, 2% BSA and 0.1% Triton X-100, the cells were incubated with primary antibody (supplementary material Table S1) at 4°C overnight, followed by secondary antibody (supplementary material Table S2) at room

temperature for 1 hour. Immunopositive cells were counted in at least eight randomly chosen fields.

HBC colony formation assay

For the colony formation assay, HBCs were cultured at a low density (200 cells/cm²) on a human LN111-coated dish in maintenance DMEM/F12 medium supplemented with 25 μM LY-27632 (ROCK inhibitor; Millipore).

Transplantation of clonally derived HBCs

Clonally derived HBCs were dissociated using Accutase and then suspended in maintenance DMEM/F12 medium without serum. The HBCs (1×10^6 cells) were transplanted 24 hours after administration of CCl₄ (2 mg/kg) by intrasplenic injection into 8- to 10-week-old *Rag2/Il2rg* double-knockout mice. Recipient mouse livers and blood were harvested 2 weeks after transplantation. Grafts were fixed with 4% PFA and processed for immunohistochemistry. Serum was extracted and subjected to ELISA. All animal experiments were conducted in accordance with institutional guidelines.

ELISA

Levels of human ALB in mouse serum were examined by ELISA using kits from Bethyl Laboratories according to the manufacturer's instructions.

Culture of mouse Dlk1⁺ cells

Dlk1⁺ hepatoblasts were isolated from E13.5 mouse livers using anti-mouse Dlk1 monoclonal antibody (MBL International Corporation, D187-4) as described previously (Tanimizu et al., 2003). Dlk1⁺ cells were resuspended in DMEM/F12 (Sigma) containing 10% FBS, 1× insulin/transferrin/selenium (ITS), 10 mM nicotinamide (Wako), 0.1 μM DEX and 5 mM L-glutamine. Cells were plated on laminin-coated dishes and cultured in medium containing 20 ng/ml HGF, EGF and 25 μM LY-27632 (ROCK inhibitor).

lacZ assay

Hepatoblasts were transduced with Ad-LacZ at 3000 VPs/cell for 1.5 hours. The day after transduction (day 10), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining was performed as described previously (Kawabata et al., 2005).

Reporter assays

The effects of *c/EBPα* or *c/EBPβ* overexpression on *TGFBR2* promoter activity were examined using a reporter assay. An 8 kb fragment of the 5' flanking region of the *TGFBR2* gene was amplified by PCR using the following primers: Fwd, 5'-CCGAGCTCATGTTTGTATGAAGTGTCTAG-CTTCCAAGG-3'; Rev, 5'-GGCTCGAGCCTCGACGTCCAGCCCCT-3'. The fragment was inserted into the *SacI/XhoI* sites of pGL3-basic (Promega), resulting in a pGL3-*TGFBR2* promoter region (pGL3-TGFBR2-PR). To generate a *TGFBR2* promoter region containing mutations in the *c/EBP* binding site, the following primers were used in PCR (mutations are indicated by lowercase letters): Fwd, 5'-CACTAGTATTCaGTG-AtCcgAAAATATGG-3'; Rev, 5'-CACTAGTATTCaGTGAtCcgAAAATATGG-3'; this resulted in pGL3-mTGFBR2-PR. HEK293 cells were maintained in DMEM (Wako) supplemented with 10% FBS, penicillin and streptomycin, and 2 mM L-glutamine. In reporter assays, 60 ng pGL3-TGFBR2-PR or pGL3-mTGFBR2-PR was transfected together with 720 ng each expression plasmid (pHMEF5, pHMEF5-*c/EBPα* and pHMEF5-*c/EBPβ*) and 60 ng internal control plasmid (pCMV-Renilla luciferase) using Lipofectamine 2000 reagent (Invitrogen). Transfected cells were cultured for 36 hours, and a Dual Luciferase Assay (Promega) was performed according to the manufacturer's instructions.

siRNA-mediated knockdown

Pre-designed siRNAs targeting *c/EBPα*, *c/EBPβ* and *TGFBR2* mRNAs were purchased from Thermo Scientific Dharmacon. Cells were transfected with 50 nM siRNA using RNAiMAX (Invitrogen) transfection reagent according to the manufacturer's instructions. As a negative control, we used scrambled siRNA (Qiagen) of a sequence showing no significant similarity to any mammalian gene.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay kit was purchased from Upstate. Cells were crosslinked using formaldehyde at a final concentration of 1% at 37°C for 10 minutes, and then genomic DNA was fragmented by sonicator. The resulting DNA-protein complexes were immunoprecipitated using the antibodies described in supplementary material Table S1 or control IgG as described in supplementary material Table S2. The precipitated DNA fragments were analyzed by real-time RT-PCR using the primers shown in supplementary material Table S4 to amplify the *TGFBR2* promoter region including the c/EBP binding sites or β -actin locus as a control. The results of quantitative ChIP analysis (Fig. 5A) were expressed as the amount of amplified *TGFBR2* promoter region relative to input DNA taken as 100%.

Statistical analysis

Statistical analysis was performed using an unpaired two-tailed Student's *t*-test. All data are represented as mean \pm s.d. ($n=3$).

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Competing interests

The authors declare no competing financial interests.

Author contributions

K. Takayama, K.K. and H.M. developed the concepts or approach; K. Takayama, Y.N., K.O., H.O. and T.Y. performed experiments; K. Takayama, K.K., M.I., K. Tashiro, F.S., T.H., T.O., M.F.K. and H.M. performed data analysis; K. Takayama, K.K. and H.M. prepared or edited the manuscript prior to submission.

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Supplementary material

Supplementary material available online at
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