Supplemental Materials and Methods

hESCs and hiPSCs culture

Two hESC lines, H1 and H9 (WiCell Research Institute), were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore) with Repro Stem (ReproCELL) supplemented with 5 ng/ml fibroblast growth factor 2 (FGF2) (Sigma). 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.

Three human iPSC lines generated from the human embryonic lung fibroblast cell line MCR5 were provided from the JCRB Cell Bank (Tic, JCRB Number: JCRB1331; Dotcom, JCRB Number: JCRB1327; Toe, JCRB Number: JCRB1338) [1, 2]. 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 human iPSC lines, 201B7 and 253G1, were generated from human dermal fibroblasts (HDF) was kindly provided by Dr. S. Yamanaka (Kyoto University) [3]. The human iPSC lines, 201B7 and 253G1, were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with Repro Stem (Repro CELL) supplemented with 5 ng/ml FGF2 (Sigma).

Adenovirus (Ad) vectors

Ad vectors were constructed by an improved *in vitro* ligation method [4, 5]. The human HNF1α, FOXA2, and HNF6 gene (accession number NM_000545, NM_021784, and NM_004498, respectively) were amplified by PCR using primers: HNF1α Fwd 5'-GATCTCTAGACTGTGGCAGCCGAGAG-3' and HNF1α Rev 5'-CTAAGGAATTCCCTGCTATCTTGAGGTCCTGGTC -3'; FOXA2 Fwd 5'-AAAGAATTCACTGCCATGCACTCGGCTTCCAG-3' and FOXA2 Rev 5'-CCTGCAACAACAGCAATGGAGGAGAAC -3'; HNF6 Fwd 5'-CATCCTCGAGGTGTCCGCCGCCTGCTC-3' and HNF6 Rev 5'-CTAAGGAATTCCCTGCTATCTTGAGGTCCTGGTC -3'. The human HNF1β gene (accession number BC_017714) was purchased from Excellgen. The human HNF1α, FOXA2, or HNF6 gene was inserted into pBSKII (Invitrogen), resulting in pBSKII-HNF1α, -FOXA2, or -HNF6, respectively. Then, the human HNF1α, HNF1β, FOXA2, or HNF6 gene was inserted into pHMEF5 [6], which contains the human

elongation factor-1α (EF-1α) promoter, resulting in pHMEF-HNF1α, -HNF1β, -FOXA2, or -HNF6, respectively. The pHMEF-HNF1α, -HNF1β, -FOXA2, or -HNF6 was digested with I-CeuI/PI-SceI and ligated into I-CeuI/PI-SceI-digested pAdHM41-K7 [7], resulting in pAd-HNF1α, -HNF1β, -FOXA2, or -HNF6, respectively. The human EF-1α promoter-driven LacZ-, SOX17-, HEX-, HNF4α-expressing Ad vectors (Ad-LacZ, Ad-SOX17, Ad-HEX, or Ad-HNF4α, respectively) were constructed previously [8-10]. 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 [8, 10, 11]. The vector particle (VP) titer was determined by using a spectrophotometric method [12].

Flow cytometry

Single-cell suspensions of hESCs, hiPSCs, and their derivatives were fixed with methanol at 4°C for 20 min and then incubated with the primary antibody described in **Table S2**, followed by the secondary antibody. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD biosciences).

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

Total RNA was isolated from hESCs, hiPSCs, and their derivatives using ISOGENE (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 S3**.

Immunohistochemistry

The cells were fixed with methanol or 4% paraformaldehyde (PFA) (Wako). After blocking with PBS containing 2% BSA (Sigma) and 0.2% Triton X-100 (Sigma), the cells were incubated with primary antibody at 4°C for 16 hr, followed by incubation

with a secondary antibody that was labeled with Alexa Fluor 488 (Invitrogen) or Alexa Fluor 594 (Invitrogen) at room temperature for 1 hr. All the antibodies are listed in **Table S2**.

ELISA

The hiPSCs were differentiated into hepatocytes as described in **Figure 2A**. The culture supernatants, which were incubated for 24 hr 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.

Urea secretion

The hiPSCs were differentiated into hepatocytes as described in **Figure 2A**. The culture supernatants, which were incubated for 24 hr 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.

CYP induction

To measure CYP1A2, 2B6, and 3A4 induction potency, real-time RT-PCR was performed. The undifferentiated hiPSCs, hiPSC-hepa, and PHs were treated with b-naphthoflavone [bNF], phenobarbital [PB], or rifampicin [RIF], which is an inducer for CYP1A2, 2B6, or 3A4, at a final concentration of 10 μM, 750 μM, or 10 μM, respectively, for 48 hr. The CYP inducers used in this study are summarized in **Table S4**. Controls were treated with DMSO (final concentration 0.1%). Inducer compounds were replaced daily.

Drug metabolism capacity of hiPSC-hepa

All hiPSCs, hiPSC-hepa, and PHs were treated with Phenacetin [PHE], Bupropion [BP], Paclitaxel [PCT], Tolbutamide [TB], *S*-mephenytoin [MP], Bufuralol [BF], Midazolam

[MDZ], Testosterone [TS], and Hydroxyl coumarin [OHC] at a working concentration of 10 μM for PHE, 150 μM for BP, 20 μM for PCT, 500 μM for TB, 200 μM for MP, 50 μM for BF, 10 μM for MDZ, 100 μM for TS, and 10 μM for OHC in the medium. The substrates (PHE, BP, PCT, TB, MP, BF, MDZ, RS, and OHC) and that metabolites (Acetaminophen [AAP], Hydroxybupropion [OHBP], 6α-hydroxypaclitaxel [OHPCT], Hydroxytolbutamide [OHTB], 4'-hydroxymephenytoin [OHMP], 1'-hydroxybufuralol [OHBF], 1'-hydroxymidazolam [OHMDZ], 6β-hydroxytestosterone [OHTS], 7-Hydroxycoumarin glucuronide [G-OHC]), which are used in this study, are summarized in **Table S5**. The cocktail assays were adopted for group 1; PHE and MDZ and group 2; PCT, TB, MP and BF. The other substrates were incubated alone. The supernatant was collected at 1, 2, 4, and 24 hr respectively after treatment, and immediately mixed with two volumes of acetonitrile and methanol (1:1, v/v) containing 50 μM of dextrophan and 50 μM of propranolol as internal standards. Samples were filtrated with MutiScreen (Nihon Millipore K.K.) for 5 min at 1750 *g*, and an aliquot (5 μl) of the supernatant was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) to determine the concentration of metabolite quantitatively according to each standard curve. The LC-MS/MS system consisting of a Prominence HPLC system (Shimadzu Corporation) and an API 5000 triple quadrupole mass spectrometer (AB SCIEX) with an L-Column ODS (150 mm x 2.1 mm i.d., 5 μm; Chemicals Evaluation and Research Institute) was used. The mass spectrometer was set to the multiple-reaction monitoring mode and was operated with the electrospray ionization source in positive ion mode. The mobile phase was delivered at a flow rate of 0.5 ml/min using a gradient elution profile consisting of solvent A (0.02% formic acid/distilled water) and solvent B (0.02% formic acid/ acetonitrile). The details of the LC gradient conditions and mass spectrometer conditions are described in **Tables S6** and **S7**, respectively. The concentrations of each metabolite were calculated according to each standard followed by normalization to the protein content per well.

Primary human hepatocytes

In **Figures 3C, 4C, 4D, 4E** and **S8**, one lot of cryopreserved human hepatocytes (lot Hu8072 [CellzDirect]) was used. In **Figure 1, 3A, 3B, 3D-3H, S6,** and **S7**), three lots of cryopreserved human hepatocytes (lot Hu8072 [CellzDirect], HC2-14, and HC10-101 [Xenotech]) were used. These three lots of crypreserved human hepatocytes (lot Hu8072 [CellzDirect], HC2-14, and HC10-101 [Xenotech]) were cultured according to our previous report [11]. In **Figures 3I, 4A, 4C** and **S9**, three

lots of cryopreserved human hepatocytes (lot HH309, HEP187087 [Tissue Transformation Technologies], and Hu1126 [CellzDirect]) were used. The vials of hepatocytes were rapidly thawed in a shaking water bath at 37°C; the contents of the vial were emptied into prewarmed Williams' medium E (Sigma) and the suspension was centrifuged at 100*g* for 5 min at room temperature. The hepatocyte pellet was resuspended in 2.0 ml of prewarmed Williams' medium E by gentle inversion, and the cell number and viability were assessed using the trypan blue exclusion test. The hepatocytes were seeded at $2.5x10^5$ cells/well in Williams' medium E onto a collagen-coated 24-well plate. The medium was replaced with Hepato-STIM (BD Biosciences) after 24 hr after seeding and then changed daily. The hepatocytes were subjected to inducer treatment at 48 hr after cell seeding.

Cell Viability Tests

Cell viability was assessed by using an Alamar Blue assay kit (Biosource). The culture medium was replaced with culture medium containing 0.5 mg/ml solution of Alamar Blue and the cells were incubated for 3 hr at 37°C. The supernatants of the cells were measured at a wavelength of 570 nm with background subtraction at 600 nm in a plate reader. Control refers to incubations in the absence of test compounds and was considered as 100% viability value. In the case of Buthionine-SR-sulfoximine (BSO) addition, the cells were pre-treated with BSO for 24 hr.

Assay for Cytochrome P450 Activity

To measure the cytochrome P450 3A4 and 2C9 activity of the cells, we performed Lytic assays by using a P450-GloTM CYP3A4 and 2C9 Assay Kit (Promega) in **Supplemental figures 6C** and **7B**. We measured the fluorescence activity with a luminometer (Lumat LB 9507; Berthold) according to the manufacturer's instructions.

Cellular Uptake and Excretion of Indocyanine Green

Indocyanine Green (ICG) (Sigma) was dissolved in DMSO at 100 mg/ml, then added to a culture medium of the hepatocyte-like cells to a final concentration of 1 mg/ml on day 20. After incubation at 37°C for 60 min, the medium with ICG was discarded and the cells were washed with PBS. The cellular uptake of ICG was then examined by microscopy. PBS was then replaced by the culture medium and the cells

were incubated at 37°C for 6 hr. The excretion of ICG was examined by microscopy.

Uptake of LDL

The cells were cultured with medium containing Alexa-488-labeled LDL (Invitrogen) for 1 hr, and then the cells that could uptake LDL were assessed by immunohistochemistry and FACS analysis.

Periodic Acid-Schiff (PAS) Assay for Glycogen

The cells were fixed with 4% PFA and stained using a PAS staining system (Sigma) on day 20 of differentiation according to the manufacturer's instructions.

Supplemental figure legends

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 ± SD (n=3).

Fig. S2 Overexpression of FOXA2, HNF1α, HNF4α, SOX17, HEX, HNF1β, or HNF6 mRNA in mesendoderm cells by Ad- FOXA2, Ad-HNF1α, Ad-HNF4α, Ad-SOX17, Ad-HEX, Ad-HNF1β, 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-HNF1α, Ad-HNF4α, Ad-SOX17, Ad-HEX, Ad-HNF1β, or Ad-HNF6 for 1.5 hr. On day 4, real-time RT-PCR analysis of *FOXA2*, *HNF1α*, *HNF4α*, *SOX17*, *HEX*, *HNF1β*, or *HNF6* expression was performed in Ad-FOXA2-, Ad-HNF1α-, Ad-HNF4α-, Ad-SOX17-, Ad-HEX-, Ad-HNF1β-, or Ad-HNF6-transduced cells, respectively. On the y axis, the gene expression levels of *FOXA2*, *HNF1α*, *HNF4α*, *SOX17*, *HEX*, *HNF1β*, or *HNF6* in Ad-LacZ-transduced cells on day 4 were taken as 1.0. All data are represented as means ± SD (n=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 ± SD (n=3).

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 ± SD (n=3). The results showed that addition of HGF, FGF1, FGF4, and FGF10 increased the number of the hepatoblasts.

Fig. S5 Arrest of hepatoblast proliferation by FOXA2 and HNF1α 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-HNF1α 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 ± SD (n = 3).

Fig. S6 FOXA2 and HNF1α transduction promote more efficient hepatic differentiation as compared with SOX17, HEX, and HNF4α 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 *αAT* were examined by real-time RT-PCR in Ad-LacZ-transduced cells (Ad-LacZ), Ad-SOX17-, Ad-HEX-, and Ad-HNF4α-transduced cells (Ad-SOX17 + Ad-HEX + Ad-HNF4α), Ad-FOXA2- and Ad-HNF1α-transduced cells (Ad-FOXA2 + Ad-HNF1α), 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 *αAT* 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-HNF4α, Ad-FOXA2 + Ad-HNF1α, PHs-48hr, and PH-0hr. (**C**) The CYP2C9 activity level was examined in Ad-LacZ, Ad-SOX17 + Ad-HEX + Ad-HNF4α, Ad-FOXA2 + Ad-HNF1α, 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 ± SD (n=3).

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 ± SD (n=3).

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 μm.

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], Paclitazel [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], 6α-hydroxypaclitaxel [OHPCT], Hydroxytolbutamide [OHTB], 4'-hydroxymephenytoin [OHMP], 1'-hydroxybufuralol [OHBF], 1'-hydroxymidazolam [OHMDZ], 6β-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 ± SD (n=3).

Supplemental tables

Table S1 The detail results of FACS analysis in Figure 1

	% of CXCR4-positive cells	% of AFP-positive cells	% of ASGR1-positive cells
hESCs (day 0)	1.2 ±1.1	0.2 ± 0.2	
Ad-LacZ	71.6 ± 0.1	69.3 ± 2.5	41.4 ± 4.3
Ad-FOXA2	99.2 ± 0.2	95.5 ± 2.3	76.7 ± 3.3
Ad-HEX	77.4 ± 1.2	86.1 ± 7.2	41.4 ± 3.6
Ad-HNF1α	80.5 ±5.8	96.1 ± 1.1	75.7 ± 1.7
Ad-HNF1β	58.3 ± 4.6	53.3 ± 4.1	22.5 ± 3.6
Ad-HNF4α	76.5 ± 12.5	77.7 ± 3.3	78.6 ± 3.1
Ad-HNF6	62.7 ±1.6	81.1 ± 6.3	61.3 ± 6.6
Ad-SOX17	99.7 ± 0.0	61.1 ± 1.3	34.5 ± 3.7

Table S2 List of antibodies used in this study

Antigen	Type	Company
Alpha-1-Fetoprotein	rabbit	DAKO
ASGR1	goat	Santa Cruz Biotechnoogy
ALB	goat	Bethyl
T	goat	R&D Systems
CXCR4	mouse	R&D Systems
CYP2D6	goat	Santa Cruz Biotechnology
CYP3A4	goat	Santa Cruz Biotechnology

CYP7A1	goat	Santa Cruz Biotechnology
αAT	rabbit	DAKO

Table S3 List of Taqman probes and primers used in this study

Gene Symbol	Primers (forward/reverse; 5' to 3')
FOXA2	GCGACCCCAAGACCTACAG/GGTTCTGCCGGTAGAAGGG
SOX17	GTGGACCGCACGGAATTTG/GAGGCCCATCTCAGGCTTG
GATA4	CATCAAGACGGAGCCTGGCC/TGACTGTCGGCCAAGACCAG
GATA6	CCATGACTCCAACTTCCACC/ACGGAGGACGTGACTTCGGC
MIXL1	CCGAGTCCAGGATCCAGGTA/CTCTGACGCCGAGACTTGG
GSC	TCTCAACCAGCTGCACTGTC/CGTTCTCCGACTCCTCTGAT
NODAL	CCGAGGGCAGACATCATCC/CCATCCACTGCCACATCTTCT
T	TGCTTCCCTGAGACCCAGTT/GATCACTTCTTTCCTTTGCATCAAG
SOX7	ACGCCGAGCTCAGCAAGAT/TCCACGTACGGCCTCTTCTG
LAMB1	AGGAACCCGAGTTCAGCTACG/CACGTCGAGGTCACCGAAA
HNF1β	TCACAGATACCAGCAGCATCAGT/GGGCATCACCAGGCTTGTA
AFP	TGGGACCCGAACTTTCCA/GGCCACATCCAGGACTAGTTTC
NANOG	AGAAGGCCTCAGCACCTAC/GGCCTGATTGTTCCAGGATT
SOX2	GGCAGCTACAGCATGATGATGCAGGAGC/CTGGTCATGGAGTTGTACTGCAGG
E-cadherin	CGAGAGCTACACGTTCACGG/GTGTCGAGGGAAAAATAGGCTG
OCT3/4	CTTGAATCCCGAATGGAAAGGG/GTGTATATCCCAGGGTGATCCTC
DLK1	GGGCACAGGAGCATTCATAG/GACGGGGAGCTCTGTGATAG
TBX3	GCCATGTACGTGTAGGGGTA/CTTCCACCTCCAGCAGCA
PROX1	TTGACATTGGAGTGAAAAGGACG/TGCTCAGAACCTTGGGGATTC
CYP3A7	AAGGTCGCCTCAAAGAGACA/TGCACTTTCTGCTGGACATC
CYP2C9	GGACAGAGACGACAAGCACA/CATCTGTGTAGGGCATGTGG
CYP2C19	ACTTGGAGCTGGGACAGAGA/CATCTGTGTAGGGCATGTGG
CYP3A4	AAGTCGCCTCGAAGATACACA/AAGGAGAGAACACTGCTCGTG
TO	GGCAGCGAAGAAGACAAATC/TCGAACAGAATCCAACTCCC
αAT	ACTGTCAACTTCGGGGACAC/CATGCCTAAACGCTTCATCA
ALB	GCACAGAATCCTTGGTGAACAG/ATGGAAGGTGAATGTTTTCAGCA
CK7	AGACGGAGTTGACAGAGCTG/GGATGGCCCGGTTCATCTC
Aquaporin1	GGTGGGGAACAACCAGACG/TACATGAGGGCACGGAAGATG
CK19	CTCCCGCGACTACAGCCACT/TCAGCTCATCCAGCACCCTG
HES1	ATGGAGAAAAATTCCTCGTCCC/TTCAGAGCATCCAAAATCAGTGT

GGT	GGAGAGCACCTCTTCCTCAG/GCCTGGATTCTCCCAGAGAT
SOX9	TTTCCAAGACACAAACATGA/AAAGTCCAGTTTCTCGTTGA
IV collagen	CCTTTCTCTCCTGAAAGCCC/TGTGTTCCTGAAAAGGGGTC
CYP1A1	GAGGCCAGAAGAAACTCCGT/CCCAGCTCAGCTCAGTACCT
CYP1A2	CAATCAGGTGGTGGTGTCAG/GCTCCTGGACTGTTTTCTGC
CYP3A5	CGGCATCATAGGTAGGTGGT/TATGAACTGGCCACTCACCC
CYP2B6	GTCCCAGGTGTACCGTGAAG /CCCTTTTGGGAAACCTTCTG
CYP2C8	CAGTGCCAACCAAGTTTTCA/CTCGGGACTTTATGGATTGC
CYP2D6	CTTTCGCCCCAACGGTCTC/TTTTGGAAGCGTAGGACCTTG
CYP2E1	ACCCGAGACACCATTTTCAG/TCCAGCACACACTCGTTTTC
UGT1A1	TAAGTGGCTACCCCAAAACG/GCTTTGCATTGTCCATCTGA
UGT1A3	TCAGATGGACAATGCAAAGCGC/GGCGCATGATGTTCTCCTTGTA
GSTA1	CCGTGCATTGAAGTAGTGGA/AATTCAGTTGTCGAGCCAGG
GSTA2	TGCAACAATTAAGTGCTTTACCTAAGTG/TTAACTAAGTGGGTGAATAGGAGTTGTATT
SLCO1B1	TAAAGCTGAGTGACAGAGCTGC/AAACAGCAGAGGCACAACCT
SLCO2B1	AGGGCTCTGCTTAGAGGGAG/GGAAATGCCCAAGGAAAAAC
NTCP	AGAAGGTGGAGCAGGTGGT/ATCTTGGTCTGTGGCTGCTC
ABCB4	AATTTATCCTGCCAATCGGA/GCATCAGCAGCAAACAAAAA
ABCC1	TGGGCAGGGATTCTCTTTTA/TCATGCTCACTTTCTGGCTG
ABCC3	GTCCGCAGAATGGACTTGAT/TCACCACTTGGGGATCATTT
ABCC4	TCTCCGTTTATGGCCAATTT/CCGTGTACCAGGAGGTGAAG
ABCC6	TGTCGCTCTTTGGAAAATCC/AGGAACACTGCGAAGCTCAT
OCT1/SLC22A1	TAATGGACCACATCGCTCAA/AGCCCCTGATAGAGCACAGA
OCT2/SLC22A2	ATACAGTTGGGCTCCTGGTG/GAGGCGGGTAGAGATTTTCC
AhR	AGTTATCCTGGCCTCCGTTT/TCAGTTCTTAGGCTCAGCGTC
CAR	AGTTGCACAGGTGTTTGCTG/GTGCTTAGATGCTGGCATGA
PXR	TCCGGAAAGATCTGTGCTCT/AGGGAGATCTGGTCCTCGAT
LXRα	TGGGGTTGATGAATTCCACT/ GGTACAACCCTGGGAGTGAG
SHR	ACTTCACACAGCACCCAGTG/AGGGACCATCCTCTTCAACC
FXR	CACAGCGTTTTTGGTAATGC/TTGTTTGTGGAGACAGAGCCT
RXRα	TGTCAATCAGGCAGTCCTTG/GAGTGTACAGCTGCGAGGG
GR	TGTTGTTGCTGTTGAGGAGC/TTCCCTGGTCGAACAGTTTT
PPARA	AGAGTGGGCTTTCCGTGTC/GCCGCCTTCAGGTACAGTAG

Table S4 List of CYP inducers used in this study

CYP	Inducer	Conc. (μM)

CYP1A2	β-naphthoflavone	10
CYP2B6	Phenobarbital	750
CYP3A4	Rifampicin	10

Table S5 List of CYP substrates and that metabolites used in this study

CYP	Substrate	Sub Abbr.	Conc. (μM)	Reaction
CYP1A2	Phenacetin	PHE	10	O-de-ethylation
CYP2B6	Bupropion	BP	150	Hydroxylation
CYP2C8	Paclitaxel	PCT	20	6α-Hydroxylaion
CYP2C9	Tolbtamide	TB	500	Hydroxylation
CYP2C19	S-mephenytoin	MP	200	4'-hydroxylation
CYP2D6	Bufuralol	BF	50	1'-hydroxylation
CYP3A4	Midazolam	MDZ	10	1'-hydroxylation
CYP3A4	Testosterone	TS	100	6β-hydroxylation
UGT	Hydroxy coumarin	OHC	10	Glucuronidation

CYP	Metabolites	Metabolites Abbr.	Detection limit of metabolite (μM)
CYP1A2	Acetaminophen	AAP	0.0031
CYP2B6	Hydroxybupropion	OHBP	0.006
CYP2C8	6α-hydroxypaclitaxel	OHPCT	0.0031
CYP2C9	Hydroxytolbutamide	OHTB	0.001
CYP2C19	4'-hydroxymephenytoin	OHMP	0.003
CYP2D6	1'-hydroxybufuralol	OHBF	0.003
CYP3A4	1'-hydroxymidazolam	OHMDZ	0.003
CYP3A4	6β-hydroxytestosterone	OHTS	0.049
UGT	7-Hydroxycoumarin glucuronide	G-OHC	0.015

Table S6 The LC conditions of AAP, OHMDZ, G-OHC, OHTS, OHPCT, OHBP, OHTB, OHMP, OHBF The mobile phase was delivered at a flow rate of 0.5 ml/min using a gradient elution profile consisting of solvent A (0.02% formic acid/distilled water) and solvent B (0.02% formic acid/ acetonitrile). The details of the LC gradient conditions are described in this table.

Gradient:

Time (min)	B Conc. (%)
0.00 – 1.00	20
1.00 - 3.00	20 → 70
3.00 - 5.00	70
5.00 - 5.01	70 → 20
5.01 – 7.01	20

LC conditions for OHTS

Gradient:

Time (min)	B Conc. (%)
0.00 – 1.00	30
1.00 - 3.00	30 → 70
3.00 - 5.00	70
5.00 - 5.01	70 → 30
5.01 – 7.01	30

LC conditions for OHPCT, OHBP, OHTB, OHMP and OHBF

Flow rate:

Time (min)	Flow rate (mL/min)
0.00 – 4.72	0.5
4.72 – 4.73	0.5 → 0.7
4.73 – 8.00	0.7
8.00 – 8.01	0.7 → 0.5
8.01 – 8.50	0.5

Gradient:

Time (min)	B Conc. (%)
0.00 – 1.00	15
1.00 - 3.00	15 → 50
3.00 – 4.70	67
4.70 – 4.71	67 → 100
4.71 – 6.00	100
6.00 – 6.01	100 → 15
6.01 – 8.50	15

Table S7 The Mass spectrometer conditions for multiple reaction monitoring metabolite quantification

The details of the mass spectrometer conditions are described in this table.

Metabolite	Parent (m/z)	Daughter (m/z)	Declustering potential (V)	Collision energy (V)
AAP	152.10	110.10	20	25
OHMDZ	341.90	203.30	60	40
G-OHC	339.30	163.00	80	30
OHTS	305.10	269.10	80	20
OHPCT	870.50	286.20	60	25
OHBP	256.10	139.00	60	40
OHTB	287.00	171.10	50	25
OHMP	235.30	150.10	70	30
OHBF	278.10	159.10	60	35
Dextrophan (IS)	257.90	156.90	60	50
Propranolol (IS)	259.90	115.70	70	25

IS; internal standard

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The promotion of hepatic maturation of human pluripotent stem cells in 3D co-culture using type I collagen and Swiss 3T3 cell sheets

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ABSTRACT

Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) are known to be a useful cell source for drug screening. We recently developed an efficient hepatic differentiation method from hESCs and hiPSCs by sequential transduction of FOXA2 and HNF1α. It is known that the combination of three-dimensional (3D) culture and co-culture, namely 3D co-culture, can maintain the functions of primary hepatocytes. However, hepatic maturation of hESC- or hiPSC-derived hepatocyte-like cells (hEHs or hiPHs, respectively) by 3D co-culture systems has not been examined. Therefore, we utilized a cell sheet engineering technology to promote hepatic maturation. The gene expression levels of hepatocyte-related markers (such as cytochrome P450 enzymes and conjugating enzymes) and the amount of albumin secretion in the hEHs or hiPHs, which were 3D co-cultured with the Swiss 3T3 cell sheet, were significantly up-regulated in comparison with those in the hEHs or hiPHs cultured in a monolayer. Furthermore, we found that type I collagen synthesized in Swiss 3T3 cells plays an important role in hepatic maturation. The hEHs or hiPHs that were 3D co-cultured with the Swiss 3T3 cell sheet would be powerful tools for medical applications, such as drug screening.

1. Introduction

Several studies have recently shown the ability of human embryonic stem cells (hESCs) [1] and human induced pluripotent stem cells (hiPSCs) [2] to differentiate into hepatocyte-like cells [3–6]. Although primary human hepatocytes are generally employed for drug toxicity screening in the early phase of pharmaceutical development, these cells have some drawbacks, such as their limited range of sources, difference in variability and functions from batch to batch, and de-differentiation. Because hESC- or hiPSC-derived hepatocyte-like cells (hEHs or hiPHs, respectively) have potential to resolve these problems, they are expected to be applied to drug screening. The hepatic differentiation processes from hESCs and hiPSCs are divided into three-stages, differentiation into definitive endoderm (DE) cells, hepatoblasts, and mature hepatocytes. Hepatic differentiation methods based on the treatment of growth factors have been widely used to generate hepatocyte-like cells from hESCs or hiPSCs [5–9]. However, the hepatic differentiation efficiency is not high enough for medical applications such as drug screening [10]. To promote the efficiency of hepatic differentiation and hepatic maturation, we have developed hepatic differentiation methods that combine the transduction of transcription factor genes involved in liver development

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with stimulation by growth factors [11–13]. The hepatocyte-like cells generated by our protocols have levels of expression of hepatocyte-related genes similar to the levels in (cryopreserved) primary human hepatocytes cultured for 48 h after plating [12]. Moreover, we have recently established more efficient and simple methods for hepatic differentiation from hESCs and hiPSCs by sequential transduction of forkhead box A2 (FOXA2) and hepatocyte nuclear factor 1 homeobox A (HNF1α) (in submitted). In that recent study, we showed that the hEHs or hiPHs expressed the genes of hepatocyte-related markers at levels similar to those in primary human hepatocytes and could metabolize various types of drugs.

It is known that cell–cell interactions between hepatocytes and their surrounding cells are essential for liver development and maintenance of liver functions [14–17]. Although primary human hepatocytes rapidly lose their functions under a monolayer culture condition, they could retain their functions, such as albumin secretion and urea synthesis, in three-dimensional (3D) culture and co-culture [18–21]. Moreover, it has been reported that the primary hepatocytes maintain their functions for a long time by the combination of 3D culture and co-culture, namely 3D co-culture [22–24]. In particular, the functions of primary rat hepatocytes cultured in a 3D co-culture, were shown to be more efficiently preserved than the functions of primary rat hepatocytes cultured in monolayer a co-culture [24]. Recently, Kim et al. reported that primary rat hepatocytes are able to maintain their functions in 3D co-culture with an endothelial cell sheet [25]. To perform 3D co-culture with a cell sheet, they employed cell sheet engineering technology using temperature-responsive culture dishes grafted with a temperature-responsive polymer, poly(*N*-isopropylacrylamide). This cell sheet engineering technology make it possible to manipulate a monolayer cell sheet with the extracellular matrices (ECMs) synthesized from the cells [26]. Although 3D culture or co-culture methods have been individually applied to promote hepatic differentiation from ESCs or iPSCs [27–29], few studies have investigated the hepatic differentiation from hESCS or hiPSCs using a 3D co-culture method.

In this study, we examined whether 3D co-culture, which uses the cell sheet engineering technology, could promote hepatic differentiation, and particularly the differentiation into mature hepatocyte-like cells, from hESCs and hiPSCs. Because Swiss 3T3 cells are widely used for co-culture with primary hepatocytes [18–20], we employed Swiss 3T3 cells for 3D co-culture with the hEHs or hiPHs. After hEHs and hiPHs were 3D co-cultured with a Swiss 3T3 cell sheet, we examined the expression levels of hepatocyte-related genes. Moreover, we investigated a Swiss 3T3 cell-derived factor that can promote hepatic maturation from hESCs and hiPSCs.

2. Materials and methods

2.1. hESC and hiPSC culture

A hESC line, H9 (WiCell Research Institute), was maintained on a feeder layer of mitomycin C (MMC)-treated mouse embryonic fibroblasts (MEF, Millipore) with ReproStem (ReproCELL) supplemented with 5 ng/ml fibroblast growth factor 2 (FGF2) (Sigma). hESCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics) into small clumps and were then subcultured every 4 or 5 days. H9 cells were 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. One hiPSC line generated from the human embryonic lung fibroblast cell line MCR5 was provided from the JCRB Cell Bank (Tic, JCRB Number: JCRB1331). Another hiPSC line, 201B7, generated from human dermal fibroblasts was kindly provided by Dr. S. Yamanaka (KyotoUniversity). These hiPSC lines were maintained on a feeder layer of MMC-treated MEF with iPSellon (for Tic, Cardio) or ReproStem (for 201B7, ReproCELL) supplemented with 10 ng/ml (for Tic) or 5 ng/ml (for 201B7) FGF2.hiPSCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics) into small clumps and were then subcultured every 5 or 6 days.

2.2. Swiss 3T3 cell culture

A mouse fibroblast line, Swiss 3T3, was maintained with RPMI-1640 medium (Sigma) supplemented with fetal bovine serum (10%) (FBS), streptomycin (120 μg/ml), and penicillin (200 μg/ml).

2.3. Ad vectors

The human eukaryotic translation elongation factor 1 alpha 1 (EF-1α) promoter-driven HNF1α- and FOXA2-expressing Ad vectors (Ad-HNF1α and Ad-FOXA2, respectively) were constructed previously (in submitted). 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 transduction efficiency was almost 100%, and purified as described previously [11,12,30]. The vector particle (VP) titer was determined by using a spectrophotometric method [31].

2.4. 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 hESCs and hiPSCs were cultured as previously reported [32]. The differentiation protocol for the induction of DE cells, hepatoblasts, and hepatocytes was based on our previous report with some modifications (in submitted). 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 hESF-DIF medium (Cell Science & Technology Institute) 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 serum albumin (BSA) (all from Sigma) (differentiation hESF-DIF medium) containing 100 ng/ml Activin A (R&D Systems) and 10 ng/ml FGF2. To generate DE cells, hESC- or hiPSC-derived 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 FGF2. For induction of the hepatoblasts, the hESC- or hiPSC-derived 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) and 20 ng/ml FGF4 (all from R&D Systems). To expand the hepatoblasts, 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 induce hepatic maturation, the cells were cultured for 2 days on Matrigel in L15 medium (Invitrogen) supplemented with 8.3% tryptose phosphate broth (BD Biosciences), 10% FBS (Vita), 10 μM hydrocortisone 21-hemisuccinate (Sigma), 1 μM insulin, and 25 mM NaHCO3 (Wako) (differentiation L15 medium) containing 20 ng/ml hepatocyte growth factor (HGF), 20 ng/ml Oncostatin M (OsM) (R&D Systems), and 10^{-6} M Dexamethasone (DEX) (Sigma). As described below, the Swiss 3T3 cell sheet was stratified onto hepatocyte-like cells on day 14 and cultured in differentiation L15 medium supplemented with 20 ng/ml HGF, 20 ng/ml OsM, and 10^{-6} M DEX until day 15. On day 15, Matrigel was stratified onto the cells and cultured in differentiation L15 medium supplemented with 20 ng/ml HGF, 20 ng/ml OsM, and 10^{-6} M DEX until day 25.

2.5. Cell sheet harvesting and stratifying procedure utilizing a gelatin-coated manipulator

The stratifying protocol was performed as previously described with some modifications [25,33]. Briefly, Swiss 3T3 cells were seeded on a 24-well temperature-responsive culture plate (TRCP) (Cell Seed Inc, Tokyo) on day 12. Two days after seeding (day 14), Swiss 3T3 cells were grown to confluence. On the same day (day 14), a gelatin-coated cell sheet manipulator was placed on the Swiss 3T3 cells, and the culture temperature was reduced to 20 °C for 60 min. By removing the manipulator, cultured Swiss 3T3 cells were harvested as a contiguous cell sheet that attached on the gelatin. The Swiss 3T3 cell sheet was then stratified on the hEHs or hiPHs. The culture plate with the manipulator was incubated at room temperature for 60 min to induce adherence between the hEHs or hiPHs and Swiss 3T3 cell sheet. To dissolve the gelatin, the culture plate was incubated at 37 °C for 60 min, and this was followed by several washing steps.

2.6. RNA isolation and reverse transcription-PCR

Total RNA was isolated from the hESC- or hiPSC-derived cells using ISOGENE (Nippon Gene) according to the manufacturer's instructions. 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 or Fast SYBR Green Master Mix using an ABI Step One Plus (all from 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 Supplementary Tables 1 and 2.

2.7. Preparation of verticalsection

On day 15, the hEHs cultured with or without the Swiss 3T3 cell sheet were frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek), then vertically sectioned and fixed with 4% paraformaldehyde. These sections were monitored by a phase contrast microscope (Olympus).

2.8. ELISA

hESCs or hiPSCs were differentiated into the hepatocyte-like cells as described in Fig. 1A. The culture supernatants, which were incubated for 24 h after fresh medium was added, were collected and analyzed to determine the amount of ALB secretion by ELISA. ELISA kits for ALB were purchased from Bethyl Laboratories. ELISA was performed according to the manufacturer's instructions. The amount of ALB secretion was calculated according to each standard.

2.9. Co-culture and culture in a cell culture insert system (insert-culture)

hESCs were differentiated into the hepatocyte-like cells as described in Fig. 1A until day 14, and then the hESC-derived cells were harvested and seeded onto a 6-well culture plate (Falcon) with Swiss 3T3 (1:1) in a co-culture system. In a insert-culture system, hESC-derived hepatocyte-like cells were harvested and seeded onto a 6-well culture plate alone, and Swiss 3T3 cells were plated in cell culture inserts (membrane pore size 1.0 μm; Falcon), and placed in a well of the culture palate containing hESC-derived hepatocyte-like cells. These cells were cultured in differentiation L15 medium supplemented with 20 ng/ml HGF, 20 ng/ml OsM, and 10^{-6} M DEX until day 25.

2.10. Stratification of type I collagen gel

A type I collagen gel solution was prepared as suggested by Nitta Gelatin: 7 parts of solubilized collagen in HC1 (pH 3.0) 2 parts of 5× concentrated RPMI-1640 medium, and 2 parts of reconstitution buffer (0.2 M HEPES, 0.08 M NaOH) to neutralize the collagen gel, were mixed gently but rapidly at 4 °C. Next, the hESC-derived cells were cultured in a type I collagen gel solution for 3h, and then the medium waschanged and the cells were cultured in differentiation L15 medium supplemented with 20 ng/ml HGF, 20 ng/ml OsM, and 10^{-6} M DEX until day 25.

2.11. Inhibition of collagen synthesis

hESCs were differentiated into the hepatocyte-like cells as described in Fig. 1A until stratification of the Swiss 3T3 cell sheet. After stratification of the Swiss 3T3 cell sheet, the cells were cultured in differentiation L15 medium supplemented with 20 ng/ml HGF, 20 ng/ml OsM, 10^{-6} M DEX, and 25 μM 2,2′-Bipyridyl (Wako), an inhibitor of collagen synthesis, until day 25.

2.12. Western blotting analysis

Swiss 3T3 cells were cultured with 25 μM 2,2′-Bipyridyl or solvent (0.1% DMSO) for 3 days, and these cells were then homogenized with lysis buffer (1% Nonidet P-40, 1 mM EDTA, 25 mM Tris-HCl, 5 mM NaF, and 150 mM NaCl) containing protease inhibitor mixture (Sigma-Aldrich). After being frozen and thawed, the homogenates were centrifuged at 15,000× g at 4 °C for 10 min, and the supernatants were collected. The lysates were subjected to SDS-PAGE on 7.5% polyacrylamide gel and were then transferred onto polyvinylidene fluoride membranes (Millipore). After the reaction was blocked with 1% skim milk in TBS containing 0.1% Tween 20 at room temperature for 1 h, the membranes were incubated with goat anti-col1a1 Ab (diluted 1/200; Santa Cruz Biotechnology) or mouse anti-β-actin Ab (diluted 1/5000; Sigma) at 4 °C overnight, followed by reaction with horseradish peroxidase-conjugated anti-goat IgG (Chemicon) or anti-mouse IgG (Cell Signaling Technology) at room temperature for 1 h. The band was visualized by ECL Plus Western blotting detection reagents (GE Healthcare) and the signals were read using a LAS-3000 imaging system (FUJI Film).

2.13. Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student's *t*-test.

3. Results

3.1. Efficient hepatic maturation by stratification of the Swiss 3T3 cell sheet

The hEHs, which were generated by the transduction of *HNF1α* and *FOXA2* genes, were 3D co-cultured with the Swiss 3T3 cell sheet to promote hepatic differentiation and to generate mature hepatocytes from hESCs and hiPSCs. Our differentiation strategy using the stratification of the Swiss 3T3 cell sheet is illustrated in Fig. 1A. The stratifying procedure was performed on day 14 as described in Fig. 1B. The day after stratifying the Swiss 3T3 cell sheet on the hEHs, vertical sections of the monolayer hEHs (hEHs-mono) and the hEHs stratified with the Swiss 3T3 cell sheet (hEHs-Swiss) were prepared (Fig. 1C). We found that Swiss 3T3 cells were successfully harvested and overlaid onto the hEHs as a monolayer cell sheet (Fig. 1C). Moreover, the hEHs seemed to be larger than the Swiss 3T3 cells. The space between the hEHs cells and Swiss 3T3 cells suggests the formation of ECMs (Fig. 1C).

To investigate whether stratification of the Swiss 3T3 cell sheet could promote hepatic maturation of the hEHs, hESCs (H9) were differentiated into the hepatocyte-like cells according to the protocol described in Fig. 1A, and then the gene expression levels of hepatocyte-related markers and the amount of albumin (ALB) secretion in the hEHs-Swiss were measured on day 25 (Fig. 2). By 3D co-culturing of the hepatocyte-like cells with the Swiss 3T3 cell sheet for 10 days (days 15–25), the gene expression levels of hepatocyte-related markers, such as *ALB* (Fig. 2A), *hepatocyte nuclear factor 4 alpha* (*HNF4A*) (Fig. 2B), cytochrome P450 (CYP) enzymes (*CYP2C9*, *CYP7A1*, *CYP1A2*, and *CYP3A5*) (Fig. 2D–G), and conjugating enzymes (*glutathione S-transferase alpha 1* [*GSTA1*], *GSTA2*, and *UDP glucuronosyltransferase* [*UGT1A1*]) (Fig. 2H–J) were significantly increased as compared with those in hEHs-mono. Moreover, the amount of ALB secretion in hEHs-Swiss was also up-regulated as compared with that in hEHs-mono (Fig. 2K). Because it is known that hepatoblasts can differentiate into hepatocytes and cholangiocytes [34,35], we examined the gene expression level of *cytokeratin 7* (*CK7*), a cholangiocyte-related marker, in hEHs-Swiss and hEHs-mono. In 3D co-culture with the Swiss 3T3 cell sheet, the gene expression level of *CK7* was down-regulated in the hEHs-Swiss relative to the hEHs-mono (Fig. 2C). These results clearly showed that stratification of the Swiss 3T3 cell sheet could promote the hepatic maturation of the hEHs and, in turn, suppress the cholangiocyte differentiation.

In order to investigate whether stratification of the Swiss 3T3 cell sheet promotes maturation of hiPHs as well as hEHs, the hiPSCs (Tic and 201B7) were differentiated into the hepatocyte-like cells according to the protocol described in Fig. 1A. The results showed that the gene expression levels of *ALB*, *CYP2C9*, *CYP3A5*, *CYP1A2*, and *GSTA1* in the hiPHs stratified with the Swiss 3T3 cell sheet (hiPHs-Swiss) were up-regulated in comparison with those in the monolayer hiPHs (hiPHs-mono) (Fig. 3A–E). Moreover, the gene expression level of *CK7* was markedly decreased in hiPHs-Swiss (Fig. 3F). The gene expression level of *ALB* in the hiPHs-Swiss differentiated from Tic was higher than that in the hiPHs-Swiss differentiated from 201B7, while the gene expression levels of CYP enzymes in the hiPHs-Swiss differentiated from Tic were lower than those in the hiPHs-Swiss differentiated from 201B7 (Fig. 3A–D). These results showed that stratification of the Swiss 3T3 cell sheet promoted hepatic maturation of both hEHs and hiPHs.

3.2. Identification of maturation factors synthesized from Swiss 3T3 cells

The data described above indicate that hepatic maturation factors were produced in Swiss 3T3 cells. To elucidate the Swiss 3T3 cell-derived hepatic maturation factors, the hEHs were cultured in cell culture-insert systems (insert-cultured), in which the hEHs were co-cultured with Swiss 3T3 cells without physical contacts, or co-cultured with Swiss 3T3 cells. Quantitative PCR analysis revealed that the gene expression levels of *ALB* and *CYP2C9* in the insert-cultured hEHs were increased in comparison with the hEHs-mono, while the expression levels of these genes were lower than

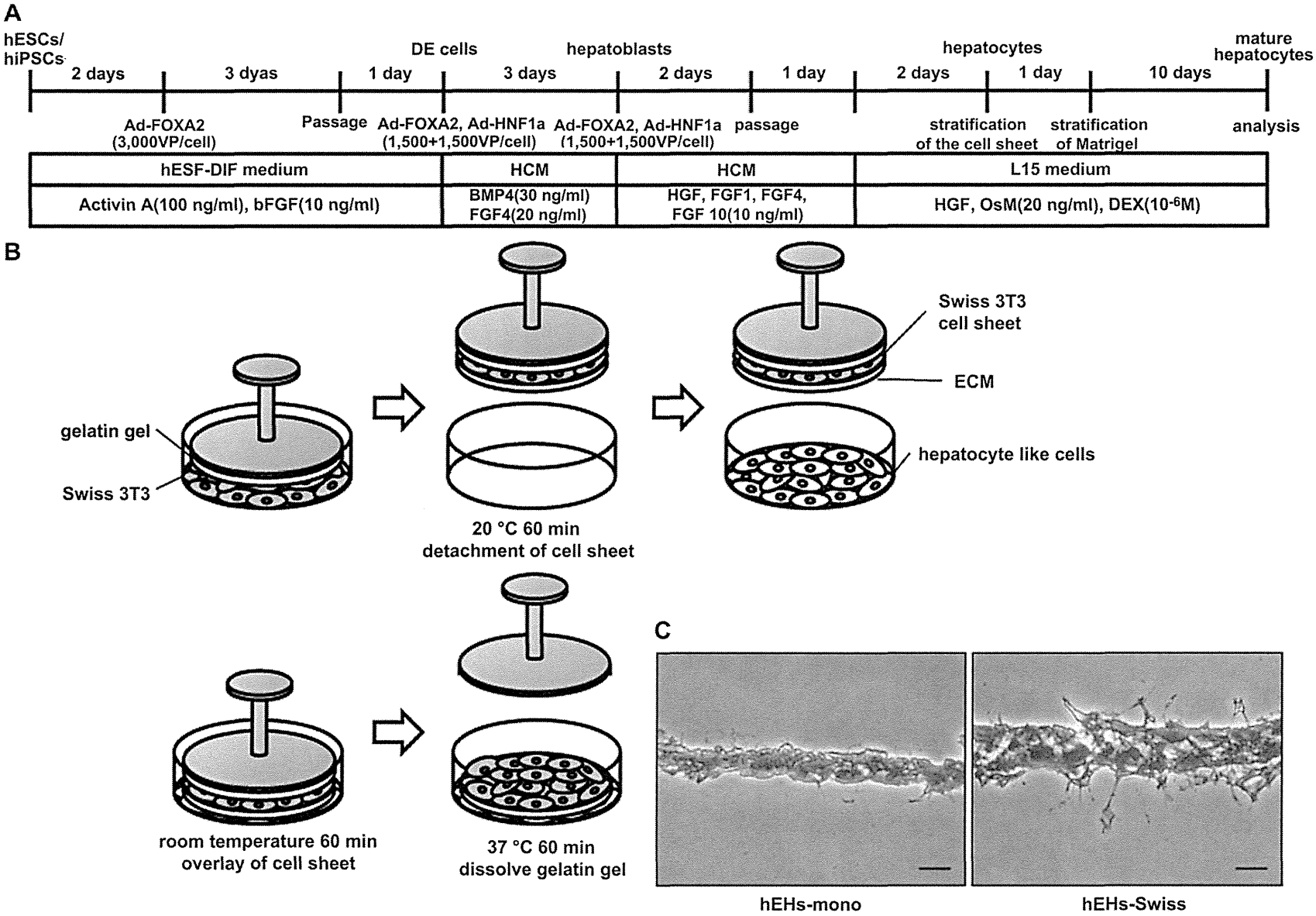

Fig. 1. Experimental protocol and schematic illustration of the procedure to stratify Swiss 3T3 cells on hepatocyte-like cells. (A) The procedure for hepatic differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) using stratification of the Swiss 3T3 cell sheet. Details of the hepatic differentiation procedure are described in the Materials and methods section. (B) The stratifying protocol was performed by using gelatin-coated manipulator. Details of the stratifying procedure are described in the Materials and methods section. (C) Phase-contrast micrographs of the vertical sections with monolayer hESC (H9)-derived hepatocyte-like cells (hEHs-mono) or hepatocyte-like cells stratified with Swiss 3T3 cell sheet (hEHs-Swiss) on day 15. Scale bars represent 25 μM.

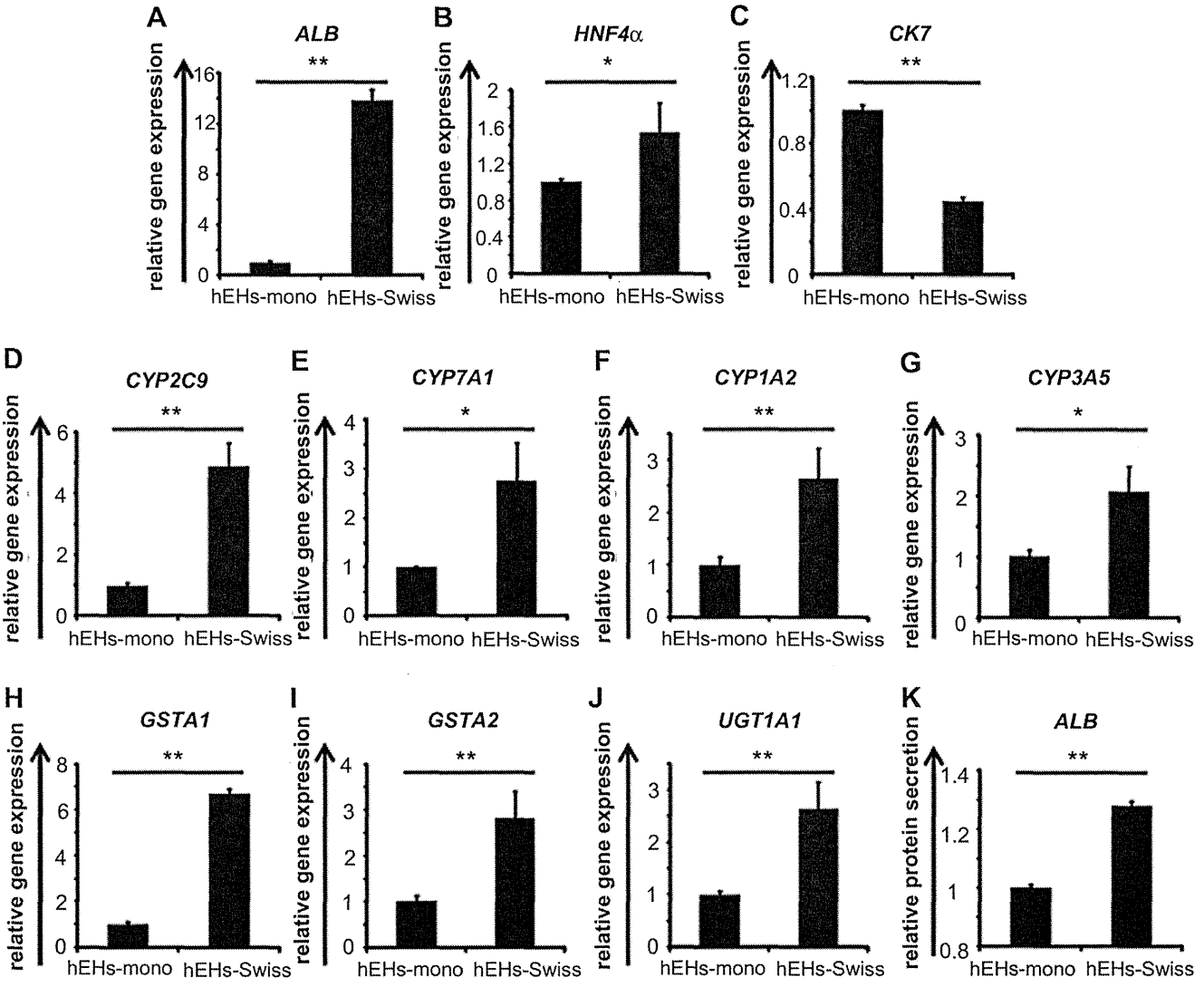

Fig. 2. Stratification of Swiss 3T3 cell sheet on hEHs promotes hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–K): On day 25, the gene expression levels of *ALB* (A), *HNF4A* (B), *CK7*(C), *CYP2C9* (D), *CYP7A1* (E), *CYP1A2* (F), *CYP3A5* (G), *GSTA1* (H), *GSTA2* (I), and *UGT1A1* (J) were examined in monolayer hESC-derived hepatocyte-like cells (hEHs-mono) and hESC-derived hepatocyte-like cells stratified with Swiss 3T3 cell sheet (hEHs-Swiss) by real-time RT-PCR. The values were graphed as the fold-changes relative to hEHs-mono. (K) On day 25, the amounts of ALB secretion were examined in hEHs-mono or hEHs-Swiss by ELISA. The values were graphed as the fold-changes relative to hEHs-mono. All data are represented as means ± Standard Deviation (SD) ($n = 3$). *$P < 0.05$ **$P < 0.01$.

those in the co-cultured hEHs (Fig. 4A and B). Furthermore, a significant elevation of *CYP1A2* and *CYP3A5* gene expression was observed only in the co-cultured hEHs (Fig. 4C and D). Therefore, these data indicate that physical contacts between hEHs and Swiss 3T3 cells play an important role in hepatic maturation of the hEHs, although Swiss 3T3 cell-derived soluble factors also played a small role in the hepatic maturation.

Because ECMs are important factors in hepatic differentiation [36], we examined the effect of Swiss 3T3 cell-derived ECMs on hepatic maturation of the hEHs. Swiss 3T3 cells abundantly synthesize collagen and almost all of the synthesized collagen is type I collagen [37]. To mimic 3D co-culture with Swiss 3T3 cell sheet, type I collagen gel was stratified onto the hEHs. As a control, Matrigel, which contains abundant type IV collagen but not type I collagen, was stratified onto the hEHs. As with the case of the Swiss 3T3 cell sheet stratification, the hEHs-mono stratified with type I collagen gel showed an elevation of hepatocyte-related marker, but a reduction of cholangiocyte marker (Fig. 5A and B, hEHs-mono). In addition, stratification of type I collagen augmented the hepatic maturation of the Swiss 3T3 cell sheet-stratified hEHs (Fig. 5A and B, hEHs-Swiss). We further examined the role of Swiss 3T3 cell-derived type I collagen on hepatic maturation using 2,2′-Bipyridyl, an inhibitor of collagen synthesize. The collagen synthesis in Swiss 3T3 cells could be efficiently inhibited by treatment with 2,2′-Bipyridyl, as determined by Western blotting analysis (Fig. 5C). Quantitative RT-PCR analysis revealed that the gene expression level of *ALB* was significantly down-regulated, but that of CK7 was up-regulated in the hEHs-Swiss cultured in the presence of 2,2′-Bipyridyl (Fig. 5D and E). Taken together, our findings indicated that type I collagen, which was synthesized from Swiss 3T3 cells, was indispensable for the maturation of the hEHs by Swiss 3T3 cell sheet.

4. Discussion

Our main purpose in the current study was to develop a more efficient method for hepatic maturation of the hEHs and hiPHs, because such a method will be needed to generate more mature hepatocyte-like cells, which have potent activity to metabolize drugs, for wide-spread use of drug screening. Therefore, we