(**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.

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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

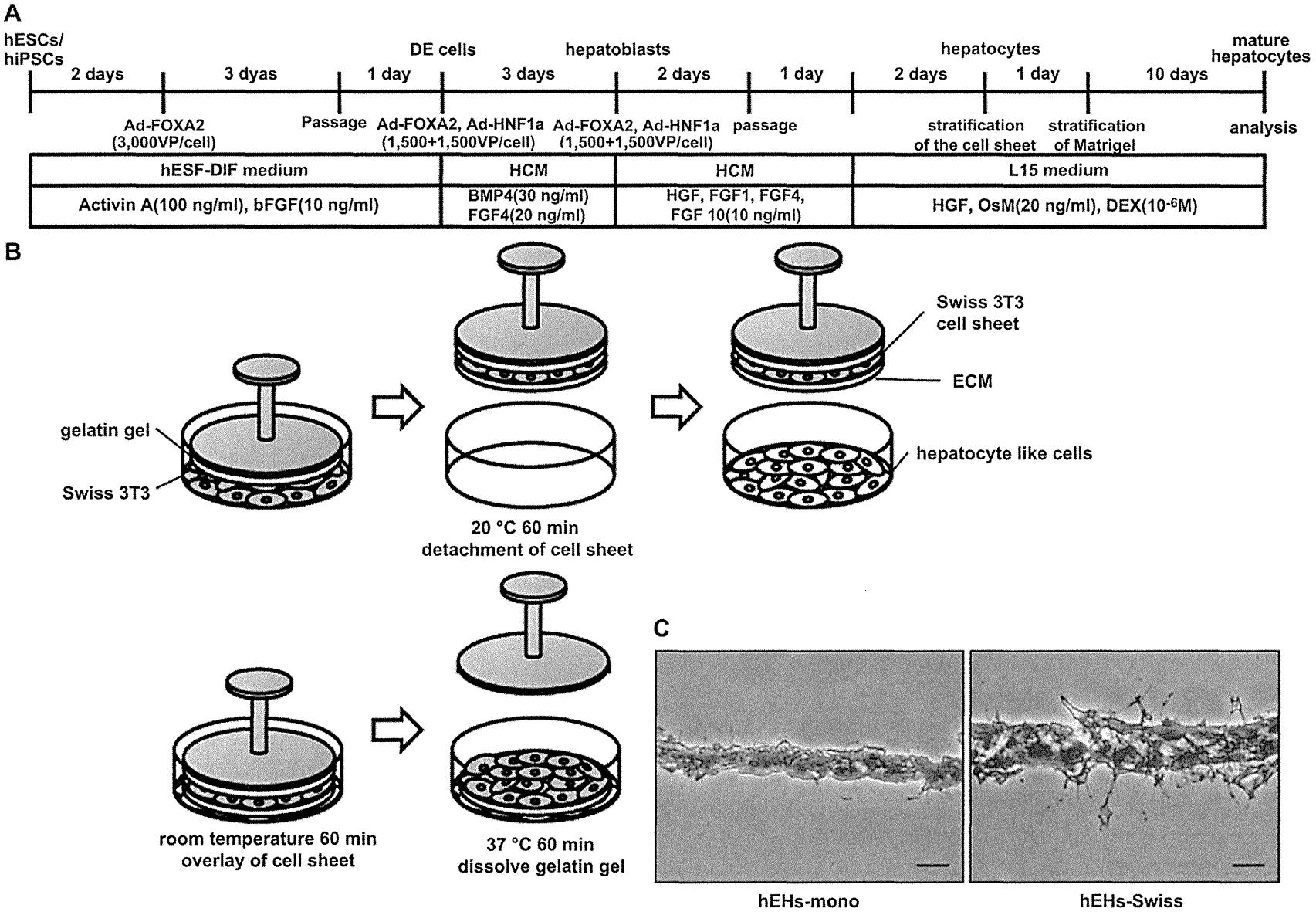

Fig. 1. Experimental protocol and schematic illustration of the procedure to stratify Swiss 3T3 cells on hepatocyte-like cells. (A) The procedure for hepatic differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) using stratification of the Swiss 3T3 cell 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.

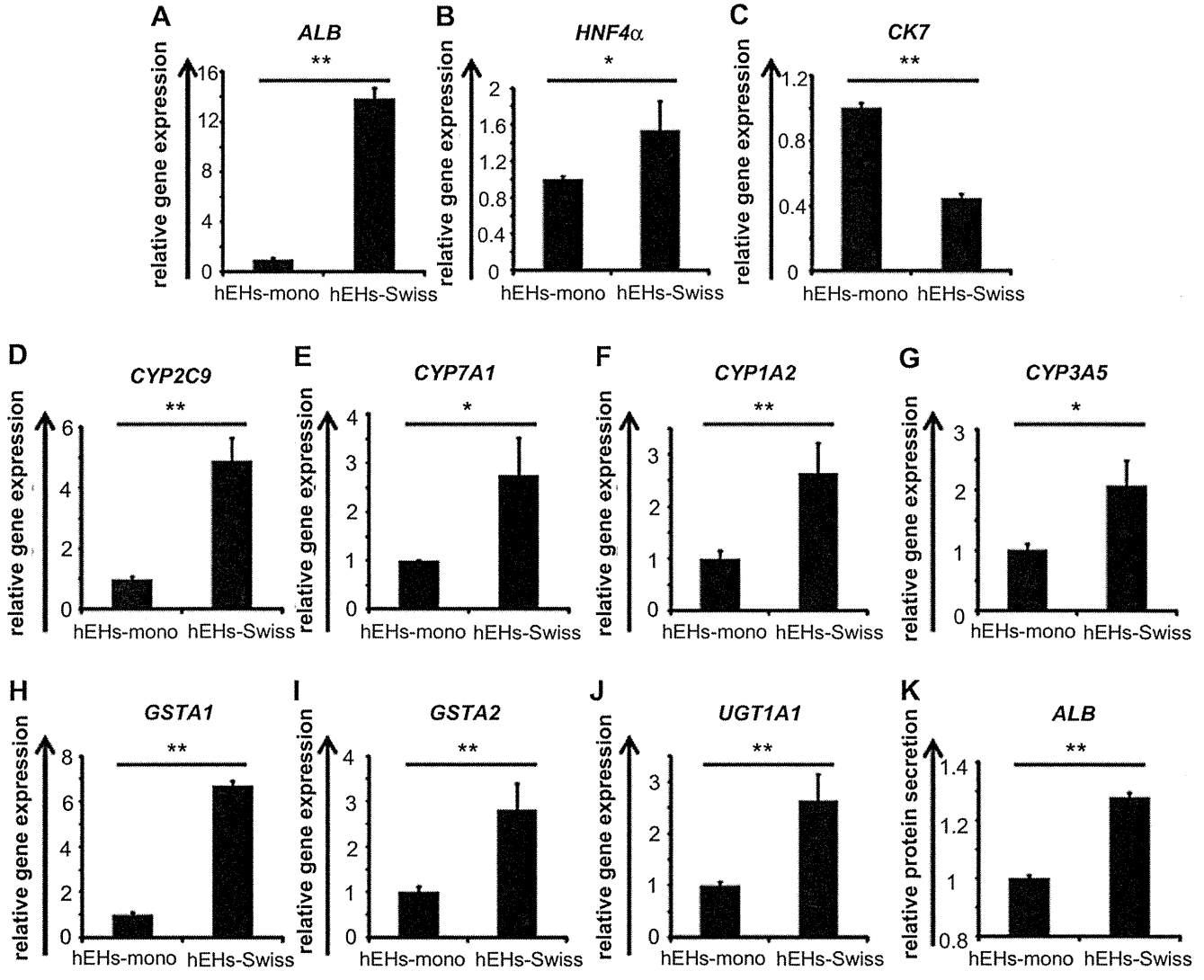

Fig. 2. Stratification of Swiss 3T3 cell sheet on hEHs promotes hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–K): On day 25, the gene expression levels of *ALB* (A), *HNF4A* (B), *CK7*(C), *CYP2C9* (D), *CYP7A1* (E), *CYP1A2* (F), *CYP3A5* (G), *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

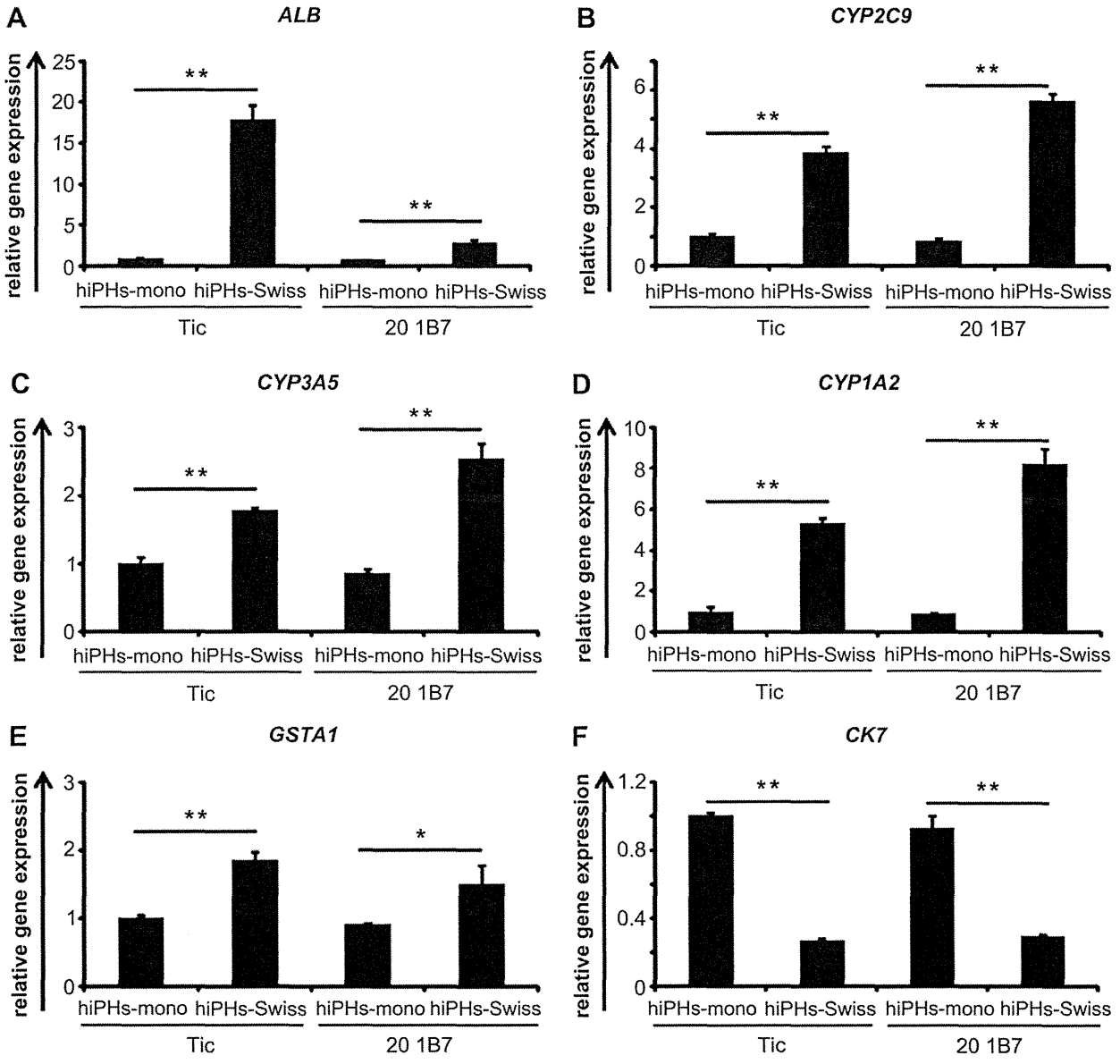

Fig. 3. Stratification of Swiss 3T3 cell sheet on hiPHs promotes hepatic maturation. Human induced pluripotent stem cells (hiPSCs) (Tic and 201B7) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–F): On day 25, the gene expression levels of *ALB* (A), *CYP2C9* (B), CYP3A5(C), *CYP1A2* (D), *GSTA1* (E), and *CK7* (F) were examined in monolayer hiPSC-derived hepatocyte-like cells (hiPHs-mono) and hiPSC-derived hepatocyte-like cells stratified with Swiss 3T3 cell sheet (hiPHs-Swiss) by real-time RT-PCR. The values were graphed as the fold-changes relative to hiPHs-mono differentiated from Tic. All data are represented as means ± SD ($n = 3$). $^{*}P < 0.05$ $^{**}P < 0.01$.

attempted to employ a cell sheet engineering technology to further induce maturation of the hEHs and hiPHs.

We observed a significant increase in the expression of hepatocyte-related genes in the hEHs- and hiPHs-Swiss as compared with those in the hEHs- and hiPHs-mono, respectively (Figs. 2 and 3), indicating that 3D co-culture with the Swiss 3T3 cell sheet was effective to promote hepatic maturation of the hEHs and hiPHs. On the other hand, Han et al. have recently shown that hESC-derived DE cells cannot be promoted to differentiate into hepatoblasts by co-culture of mouse fibroblast 3T3 cells [38]. Considering that primary rat hepatocytes are also able to grow and retain their functions for a long period of time in the presence of Swiss 3T3 cells [19,20], Swiss 3T3 cells would probably have the capacity to support the functions of freshly isolated mature hepatocytes and hESC- or hiPSC-derived hepatocyte-like cells, but not DE cells. Besides Swiss 3T3 cells, we attempted to maturate the hEHs using 3D co-culture with the bovine carotid artery endothelial cell sheet, because Kim et al. recently succeeded in creating a functional hepatocyte culture system by stacking bovine carotid artery endothelial cell sheets on primary rat hepatocytes [25]. However, our preliminary data showed that Swiss 3T3 cell sheets were superior to the bovine carotid artery endothelial cell sheets in terms of hepatic maturation of hEHs (data not shown). Thus, we conducted the present experiments to facilitate hepatic differentiation of human pluripotent stem cells using Swiss 3T3 cell sheets.

Interestingly, we found a difference in hepatic differentiation efficiency among hiPSC lines (Fig. 3). This might have been due to epigenetic memory of the hiPSC line, because several studies showed that the epigenetic memory of iPSCs affected the differentiation capacity [39,40]. Kleger et al. showed that iPSCs generated from mouse liver progenitor cells, could be more effectively differentiated into hepatocyte-like cells in comparison with iPSCs

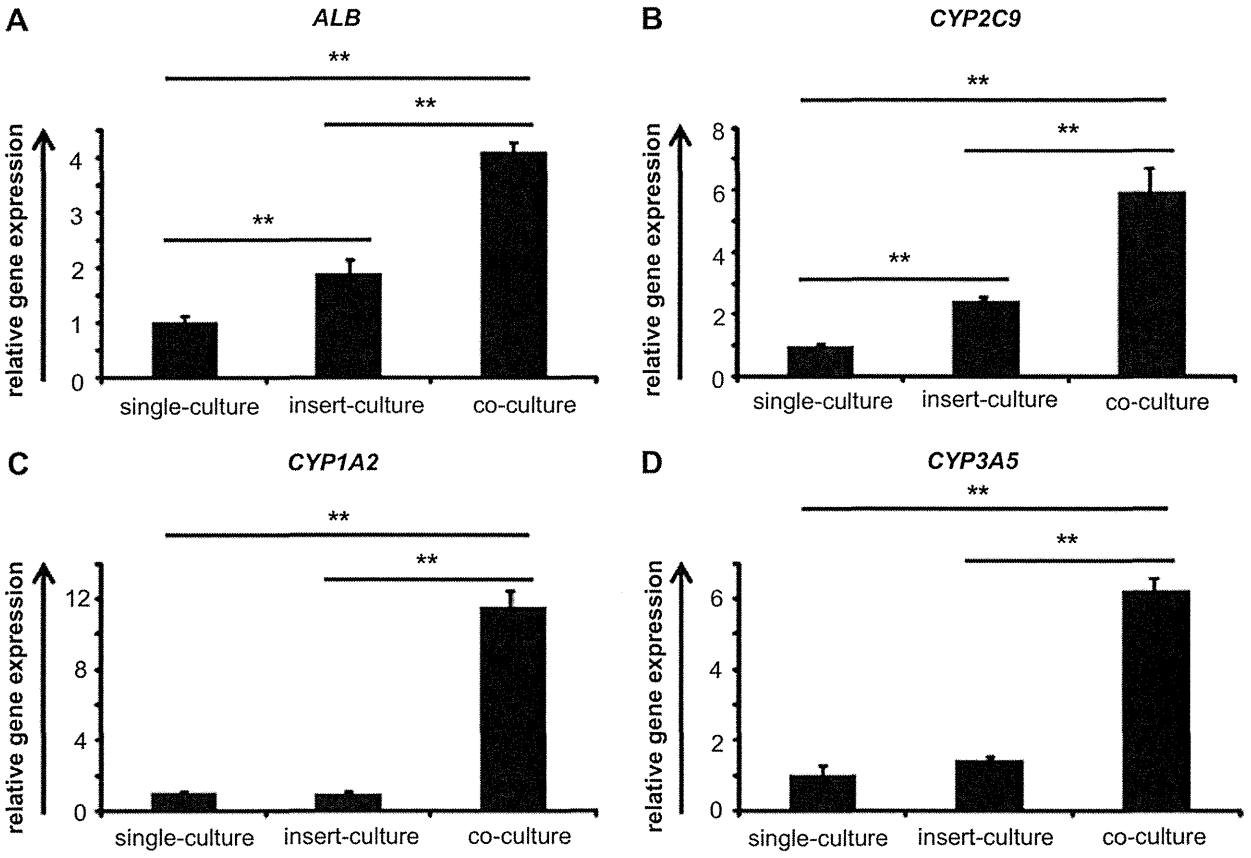

Fig. 4. Physical contacts between hESC-derived hepatocyte-like cells and Swiss 3T3 cells promote hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then the cells were differentiated into hepatocyte-like cells by single-culture, insert-culture, or co-culture with Swiss 3T3 cells. (A–D): On day 25, the gene expression levels of *ALB* (A), *CYP2C9* (B), *CYP1A2* (C) and *CYP3A5* (D) were examined in hESC-derived hepatocyte-like cells (hEHs) differentiated by single-culture, insert-culture, or co-culture with Swiss 3T3 cells by real-time RT-PCR. The values were graphed as the fold-changes relative to hEHs by single-culture. All data are represented as means ± SD ($n = 3$). $^{**}P < 0.01$.

generated from mouse embryo fibroblasts [41]. Thus, to more efficiently differentiate into hepatocyte-like cells from hiPSCs, it might be valuable to employ hiPSCs generated from freshly isolated human hepatocytes. Moreover, by using our 3D co-culture system, such hiPSCs would be differentiated into more mature hepatocyte-like cells.

We investigated the Swiss 3T3 cell-derived hepatic maturation factors by using cell culture inserts, and found that the physical contacts between Swiss 3T3 cells and the hEHs were the major factors contributing to the hepatic maturation of hEHs (Fig. 4). Because Swiss 3T3 cell-derived soluble factors partially induce maturation of hEHs (Fig. 4A and B), it would also be interesting to search for hepatic maturation factors secreted from Swiss 3T3 cells.

To further investigate the maturation factors, we examined whether type I collagen, which is abundantly synthesized by Swiss 3T3 cells, could promote hepatic maturation. Stratification of type I collagen gel could lead to a promotion of hepatic maturation of hEHs-mono as well as hEHs-Swiss (Fig. 5A). We also found that hepatic maturation by 3D co-culture with the Swiss 3T3 cell sheet was suppressed by inhibition of collagen synthesis (Fig. 5D). Taken together, these results show that type I collagen is one of the key molecules in promotion of hepatic maturation by stratification of Swiss 3T3 cells. It is known that the space of Disse, which faces hepatocytes directly, contains various kinds of ECM proteins, including type I collagen [42]. Because the conditions in 3D co-culture, which contains type I collagen synthesized from Swiss 3T3 cells, can mimic the *in vivo* liver microstructure, including the space of Disse, the hepatic maturation from hEHs and hiPHs might be efficiently promoted. Furthermore, it was also reported that, by the stratification of type I collagen gel in primary rat hepatocyte culture, the cytoskeletal organizations, such as actin localization, in primary rat hepatocytes were changed and stress fibers were obliterated just as in the *in vivo* state [43]. They also showed that the stratification of type I collagen gel in primary rat hepatocyte culture maintained ALB secretion in primary rat hepatocyte. Thus, the alteration of the cytoskeletal organization might also be changed in the hEHs and hiPHs by 3D co-culture with the Swiss 3T3 cell sheet. For these reasons, it could be speculated that stratification of Swiss 3T3 cell sheets positively affects the maturation process of hEHs and hiPHs mediated by cell-to-cell and cell-type I collagen–cell interactions. The expression level of the *CK7* gene in the hEHs was down-regulated by stratification of the Swiss 3T3 cell sheet or type I collagen gel (Figs. 2C and 5B). Although Matrigel, which contains large amount of type IV collagen, is widely used to differentiate hESCs and hiPSCs into hepatocyte-like cells, it is reported that type IV collagen promotes cholangiocyte differentiation [44]. Therefore, it would be important to note that stratification of Swiss 3T3 cell sheet inhibits the cholangiocyte differentiation and thereby allows the cells to drive the way to hepatic differentiation. Although we showed that a Swiss 3T3 cell-derived type I collagen plays an important role in hepatic maturation, it was likely that the other soluble factors would also be involved in the promotion of hepatic maturation.

We employed Swiss 3T3 cells for 3D co-culture with the hEHs and hiPHs. However, it would be an attractive study to employ other kinds of cells such as liver sinusoidal endothelial cells, stellate

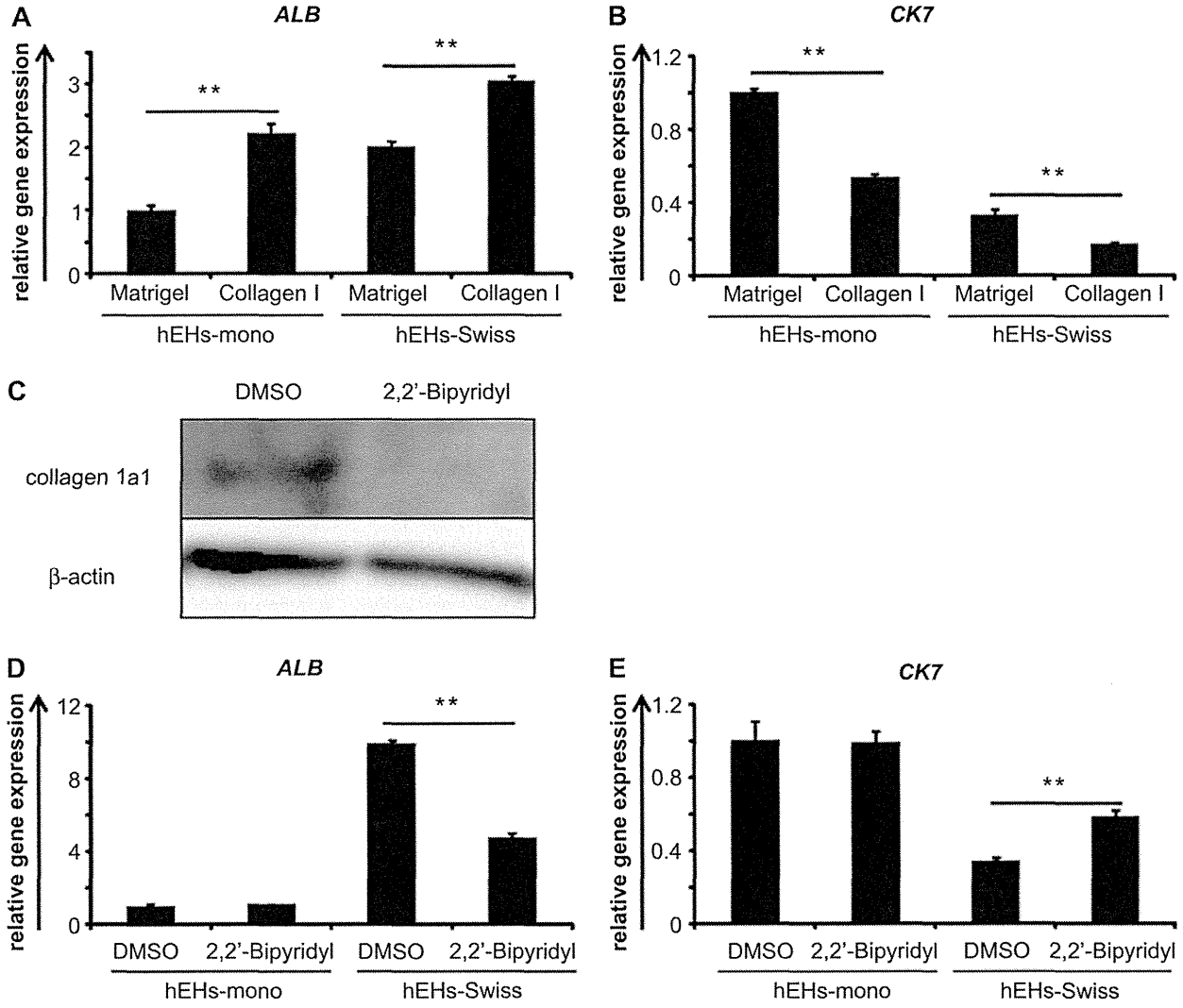

Fig. 5. Stratification of type I collagen gel promotes hepatic maturation. (A and B) hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then type I collagen gel (collagen I) or Matrigel are stratified on monolayer hESC-derived hepatocyte-like cells (hEHs-mono) and hESC-derived hepatocyte-like cells stratified with Swiss 3T3 cell sheet (hEHs-Swiss). On day 25, the gene expression levels of *ALB* (A) and *CK7* (B) were examined in hEHs-mono and hEHs-Swiss cultured with Matrigel or type I collagen gel by real-time RT-PCR. (C) Swiss 3T3 cells were cultured with 2,2′-Bipyridyl or solvent (0.1% DMSO) for 3 days, and then the expression of type I collagen precursor, col1a1, in these cells were detected by Western blot analysis. (D and E) hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A. After stratification of Swiss 3T3 cells on day 14, these cells were treated with 2,2′-Bipyridyl or solvent (0.1% DMSO). On day 25, the gene expression levels of *ALB* (D) and *CK7* (E) were examined in hEHs-mono and hEHs-Swiss treated with 2,2′-Bipyridyl or solvent (0.1% DMSO) by real-time RT-PCR. The values were graphed as the fold-changes relative to hEHs-mono cultured with Matrigel. All data are represented as means ± SD ($n = 3$). $^{**}P < 0.01$.

cells, and Kupffer cells, to mimic the *in vivo* liver microstructure. By mimicking the *in vivo* liver microstructure, basic molecular mechanisms, including cell–cell interactions, in liver development would be clarified. Moreover, because our cell sheet technology allows us to stratify the multiple cell sheets and create layered 3D tissue constructs, combinations with multiple layers consisting of various types of cells might be able to develop an efficient method for hepatic maturation of the hEHs and hiPHs. In addition, by using new biomaterials with cell patterning techniques, more mature hepatocyte-like cells would be probably generated from human pluripotent stem cells, and thereby accelerate the research into tissue generation.

5. Conclusions

We succeeded in promoting the hepatic maturation of both the hEHs and hiPHs by stratification of the Swiss 3T3 cell sheet using a cell sheet engineering technology. We also determined that type I collagen, which is synthesized in Swiss 3T3 cells, plays an important role in hepatic maturation. Since our cell sheet engineering technology enables us to stratify multiple cell sheets, this technology would have the potential to mimic the *in vivo* liver microstructure and to generate hepatocyte-like cells, which have functions similar to primary hepatocytes. Our methods would be powerful tools for *in vitro* applications, such as drug toxicity screening in the early phase of pharmaceutical development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.03.011.

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総　説

ヒト iPS 細胞から肝細胞への分化誘導の現状と創薬応用

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ヒトiPS細胞から肝細胞への分化誘導の現状と創薬応用

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Current Status of Hepatic Differentiation from Human iPS cells and Application for Drug Development

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1. はじめに

創薬のプロセスは，一般的に開発費に1000億円超，1つの医薬品が製品化されるまでに10～15年を要する．その過程で数万～100万件の候補化合物の中から薬効，毒性などの評価を経て，1つが医薬品として承認を受ける．この過程を迅速化させ，開発成功率を向上させるための新しい技術のひとつとして，iPS細胞（induced pluripotent stem cells）技術に注目が集まっている．

ヒトiPS細胞から分化させた細胞（特に，肝臓，心筋，神経細胞等）は，医薬品開発研究の最上流の疾患のメカニズム解明や創薬ターゲット分子の検索研究だけでなく，化合物スクリーニングや薬効評価試験・安全性薬理試験・毒性試験・薬物動態試験等の前臨床試験においても活用が期待されている．細胞を用いた*in vitro*アッセイ系は，薬理作用（有効性）の評価や毒性評価のためにこれまでも活用されてきたが，多くは株化細胞や（ヒト）初代培養細胞を用いたものである．株化細胞はスループット性に優れているが，生体の状態（病態）を必ずしも反映しておらず，一方で，ヒト初代培養細胞は入手が限られ，ロット差も大きいこと，単一ロットの細胞を大量に得ることが困難であるという課題がある．また，動物由来の初代培養細胞や動物実験では，『種差の壁』のために，ヒト固有の薬理・毒性作用を見落とす可能性がある．ヒトiPS細胞由来分化誘導細胞は，これらの問題点の克服が期待できることから，大きな注目を集めている．

本稿では，産業界からのニーズが特に高い肝細胞に焦点をあて，ヒトiPS細胞から肝細胞への分化誘導の現状と創薬応用（特に毒性評価）への可能性について，著者らの最新の知見を中心に概説する．

2. ヒトiPS細胞由来肝細胞を用いた創薬研究

肝臓（肝細胞）は生体内外の物質の代謝，解毒，排出等に関与する主要な臓器（細胞）であり，医薬品は主に肝細胞で薬物代謝酵素により代謝され，抱合系酵素により解毒を受け，トランスポーターにより排出される．肝毒性は医薬品候補化合物の開発中止原因の主要なものであり，正常肝細胞を用いて将来起こりえる高い潜在的毒

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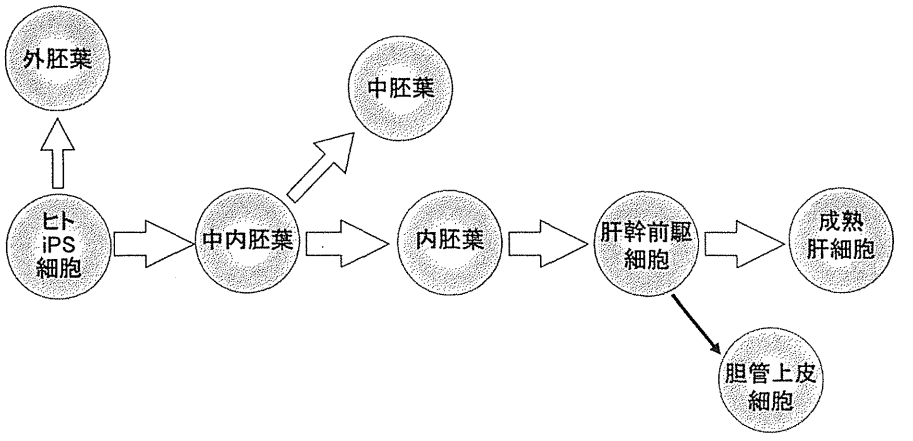

Fig. 1　ヒトiPS細胞から肝細胞への分化誘導
ヒトiPS細胞は中内胚葉，内胚葉，肝幹前駆細胞を経由して成熟した肝細胞へと分化する．

性発現を研究開発の初期段階に予測できれば，より安全性の高い医薬品を効率良く開発することにつながると考えられる．現在は，主にヒト初代培養（凍結）肝細胞（本稿では，ヒト凍結肝細胞も含めてヒト初代培養肝細胞と記載する）や肝ミクロソームを用いて，薬剤あるいは薬剤の代謝過程で生成する反応性代謝物による細胞傷害性等を試験する毒性試験や，薬物代謝酵素の誘導や阻害等の薬物動態評価試験が施行されている．しかしながら，ヒト初代培養肝細胞は高価であり，高機能なヒト肝細胞ロットの安定供給が難しいといった問題等から，ヒトiPS細胞由来分化誘導肝細胞を用いた毒性・薬物動態評価系の開発が期待されている．

また，薬物代謝酵素の活性は個人差が大きいことが知られているが（薬物代謝酵素の種類によるが，数十倍～千倍程度），将来的には，様々な個人由来のヒトiPS細胞由来分化誘導肝細胞を用いることで，個人差を反映した評価系が開発できる可能性もある．

3.　ヒトiPS細胞から肝細胞への分化誘導

3.1　ヒトiPS細胞から肝細胞への分化誘導の現状

ヒトiPS細胞から肝細胞への分化誘導は，先行して進められてきたヒトES細胞（embryonic stem cells）から肝細胞への分化誘導を応用して進められてきており，両者は共通の方法を用いて分化誘導できる．そこで本稿では，両者を区別することなく，紹介する．

ヒトiPS細胞は中内胚葉，内胚葉，肝幹前駆細胞を経由して成熟した肝細胞へと分化する（Fig. 1）．一般に，外胚葉由来の神経細胞や，中胚葉由来の心筋細胞への分化誘導に比べ，内胚葉に属する肝細胞や膵臓細胞への分化誘導は研究が遅れていた（Fig. 2）．しかしながら，

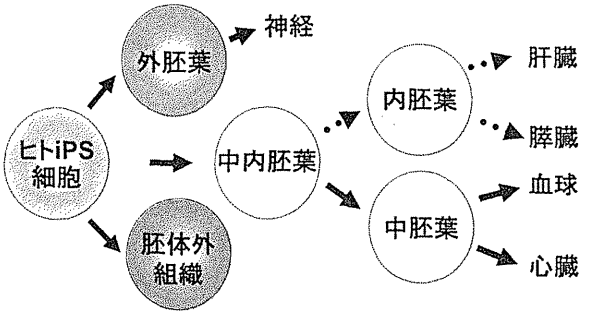

Fig. 2　ヒトiPS細胞から各胚葉への分化
神経細胞は外胚葉を，心筋細胞は中胚葉を，肝細胞や膵臓細胞は内胚葉を経由して分化する．

2005年にD'Amourらによって，アクチビンAが内胚葉を分化誘導できることが発見されて以来[1]，急速に研究が進展している．これまでに，ヒトiPS細胞から肝細胞への様々な分化誘導法が開発されているが（前述のように，ヒトES細胞から肝細胞への分化誘導法も含める），未分化ヒトiPS細胞から肝細胞への分化過程を，以下の3ステップあるいは4ステップに分けて分化誘導する方法が一般的である．即ち，(1) 未分化iPS細胞から内胚葉への分化ステップ（内胚葉分化），(2) 内胚葉から肝幹前駆細胞への分化ステップ（肝特異化），(3) 肝幹前駆細胞から肝細胞への分化ステップ（肝成熟化）〔あるいは肝幹前駆細胞から肝細胞への分化ステップを，(3) 肝（幹前駆）細胞の増幅と (4) 肝細胞の成熟化のステップに分ける〕に分け，個々の分化ステージで，発生段階を模倣したように，分化に必要な増殖因子やサイトカイン等を付加して分化させることが試みられている（詳細は代表的な総説[2, 3]を参照）．

(1)の内胚葉への分化ステップでは，アクチビンAの