

Colony formation assay

Dissociated single hPS cells were seeded at 10,000–250,000 cells/well on a 6-well plate coated with FN ($2 \mu\text{g}/\text{cm}^2$) in hESF9 medium supplemented with and without $1 \mu\text{M}$ GFX. After 5-days-culture, the colonies were fixed in 4.5 mM citric acid, 2.25 mM sodium citrate, 3.0 mM sodium chloride, 65% methanol, and 3% formaldehyde for 5 min, and stained with ALP fast-red substrate (Sigma) for 15 min at room temperature.

Immunocytochemistry

Immunocytochemistry was performed as described previously [20,60]. The image analysis was performed with In Cell analyzer 2000 and Developer tool box software (GE Healthcare, Little Chalfont, Buckinghamshire, UK), or a confocal microscope (Carl Zeiss). The primary and secondary antibodies used were listed in Table S2.

Western blotting

Western blots were performed as described previously [8,20,60]. Protein ($2 \mu\text{g}/\text{lane}$) was separated by 12.5% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were reacted with primary antibodies, peroxidase-conjugated secondary antibodies, and ECL Plus reagent (GE Healthcare). Protein bands were visualized using LAS-4000 imager (Fujifilm, Tokyo, Japan). The primary antibodies used were listed in Table S2.

AlphaScreen assay

AlphaScreen[®] SureFire[®] Cell-based Assay (Perkin-Elmer, Waltham, MA, USA) was performed to measure phosphorylation of AKT-1/2/3, ERK-1/2, and GSK- β in the cells according to the manufacturer's instructions. Materials used were listed in Table S2. The fluorescence signal was measured using an EnSpire[™] plate reader (PerkinElmer).

Gene expression analysis

Total RNA extracted from cultured cells using RNeasy Mini kit (Qiagen, Valencia, CA, USA) were treated with DNase I to remove any genomic contamination, and reverse-transcribed using Superscript VILO cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. For RT-PCR, PCR products were amplified with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), following manufacturer's instruction. The DNA was separated by gel electrophoresis and visualized under ultraviolet light for photography. For quantitative real-time RT-PCR, PCR was performed based on the TaqMan or the SYBR Green gene expression technology in a 7300 Real Time PCR System (Applied Biosystems), following manufacturer's instruction. Threshold cycles were normalized to the housekeeping gene GAPDH and translated to relative values. Specific primers used are listed in Tables S3 and S4. For PCR-array, TaqMan low-density human stem cell pluripotency card PCR array (Applied Biosystems, Foster City, CA) was performed as previously described [61]. Expression levels were all normalized against the housekeeping gene β -actin. The relative expression levels of each gene in embryoid bodies were compared to the levels in H9 hES cells or 201B7 hiPS cells grown on feeders in KSR-based medium.

Transfections with siRNA

Transfections with siRNA were performed using Dharmafect1 (Dharmacon, Chicago, USA) as previously described [62]. Prior to transfection, the hPS cells were incubated with ROCK inhibitor Y-27632 ($10 \mu\text{M}$) for 1 hour and dissociated with TrypLE

(Invitrogen) and pelleted by centrifugation. To prepare siRNA/lipid solutions, 50 pmol of siRNAs were diluted in $100 \mu\text{l}$ of hESF9 medium. In a separate tube, $6 \mu\text{l}$ of Dharmafect1 was diluted in $100 \mu\text{l}$ of hESF9 medium. The solution of the two tubes were mixed and incubated at room temperature for 20 mins. The resulting $200 \mu\text{l}$ of siRNA/lipid solution in hESF9 medium was used to resuspend the cell pelleted containing from 1×10^4 to 1×10^5 cells, and suspension incubated at room temperature for 10 min. After incubation, 1.5 ml of prewarmed hESF9 medium containing ROCK inhibitor ($10 \mu\text{M}$) was added and the suspension transferred into a FN-coated well of 24-well or 6-well plate, followed by culture for 24 hour. After recovery in fresh hESF9 medium, cells were transfected again at 24 hours. Total RNAs or proteins were extracted for analysis 72 hours after the fast transfection. siRNAs were listed as Table S4.

Live cell imaging analysis

After seeded on a 6-well plate coated with FN, the cells were incubated in a live cell imaging system, BioStation CT (Nikon Instruments Inc., Tokyo, Japan) at 37°C $10\% \text{ CO}_2$. The images were captured every 12 hours and analyzed by a soft ware CL-Quant (Nikon Instruments Inc.).

Cell Growth

The cells were inoculated on a 6-well plate coated with FN at the cell density of 250,000 cells/well in hESF9 medium including $10 \text{ ng}/\text{ml}$ FGF-2, supplemented with 0.1% DMSO, GFX in H_2O , or G66976 in DMSO. After 5 days culture, the cell numbers were counted by Coulter Counter (Beckman Coulter, Inc.).

Flow cytometry

Flow cytometry was performed as described previously [61] with a FACS Canto flow cytometer (BD Biosciences). The primary antibodies used were listed in Table S2.

In vitro cell differentiation

In vitro differentiation was induced by the formation of embryoid bodies as described previously [61]. Floating embryoid bodies were maintained in DMEM with $10\% \text{ FCS}$ for more 14 days.

Teratoma formation

The cells were harvested by dispase treatment, collected into tubes, and centrifuged, and the pellets were suspended in DMEM supplemented ROCK inhibitor. The cells from a confluent one-well in 6-well plate were injected to the rear leg muscle or thigh muscle of a SCID (C.B-17/lcr-scid/scid) mouse (CLEA Japan, Tokyo, Japan). Nine weeks after injection, tumors were dissected, weighted, and fixed with 10% formaldehyde Neutral Buffer Solution (Nacalai tesque, Kyoto, Japan). Paraffin-embedded tissue was sliced and stained with hematoxylin and eosin. All animal experiments were conducted in accordance with the guidelines for animal experiments of the National Institute of Biomedical Innovation, Osaka, Japan.

Karyotype analysis

Log phase hPS cells (day 3–4 after subculture) were treated with metaphase arresting solution (Genial Genetic Solutions Ltd., Cheshire, UK) for 5 hr. The treated hPS cells were collected with 0.1% EDTA and processed according to the quality control protocol in the JCRB Cell Bank (<http://cellbank.nibio.go.jp/cellbank.html>). Chromosome numbers were counted in 20

metaphases, and G-banding karyotype analysis was performed on 20 metaphase cells per sample.

Supporting Information

Figure S1 The phosphorylation of AKT, GSK-3 β , and ERK-1/2 was confirmed by western blot analysis using an antibody to AKT, GSK-3 β , and ERK-1/2 and their phosphorylated forms. Each gel image is a representative of independent three to five experiments. (A) Time course of phosphorylation level of AKT, GSK-3 β , and ERK-1/2. H9 hES cells were stimulated with FGF-2 (100 ng/ml) with or without GFX (5 μ M) for 180 minutes after overnight starvation of FGF-2 and insulin. (B) Effect of inhibitors on phosphorylation level of AKT, GSK-3 β , and ERK-1/2. After starvation of FGF-2 and insulin overnight, 201B7 hiPS cells were stimulated with FGF-2 (100 ng/ml) for 15 min with LY294002, GFX, U0126, or BIO or without GFX (5 μ M). (C) Effect of BMP-4 or activin A on phosphorylation level of AKT, GSK-3 β , and ERK-1/2. After starvation of FGF-2 and insulin overnight, 201B7 hiPS cells were stimulated with FGF-2 (100 ng/ml), BMP-4 (10 ng/ml) or activin A (100 ng/ml). (D) Effect of addition of activin A with and without inhibitors on phosphorylation level of AKT, GSK-3 β , and ERK-1/2. After starvation of FGF-2 and insulin overnight, H9 hES cells were stimulated with FGF-2 (10 ng/ml) and activin A (10 or 100 ng/ml) together with U0126 (5 μ M) and GFX (5 μ M) or G66976 (5 μ M) for 15 minutes. (E) Effect of GFX concentration on phosphorylation level of AKT, GSK-3 β , and ERK-1/2. After starvation of FGF-2 and insulin overnight, H9 hES cells were stimulated with FGF-2 (100 ng/ml) with GFX at 1–10 μ M. The phosphorylation levels in the cells were measured by AlphaScreen[®] SureFire[®] assay kit. The values of the y-axis are the ratio of each phosphorylation to each total signal protein. The data are represented as means \pm SD (n = 3). *P < 0.05. (TIF)

Figure S2 Summary of the result of the effect of PI3K, MEK-1/2, or PKCs inhibitor on FGF-2-induced phosphorylation of AKT, GSK-3 β , and ERK-1/2. (TIF)

Figure S3 Knockdown efficacy and effect of siRNA targeting PKC δ , ϵ , and ζ . (A) Total RNAs were extracted for analysis 72 hours after the fast transfected to 201B7 iPS cells. The efficacy of siRNA was evaluated by quantitative RT-PCR. siRNAs and primers were listed as Table S4. (B) Summary of the result of the PKC δ -, PKC ϵ -, PKC ζ -knockdown effect on phosphorylation of GSK-3 β and AKT in FGF-2 signaling. (TIF)

Figure S4 Effect of inhibitory peptides for PKCs on phosphorylation level of ERK-1/2. After starvation of FGF-2 and insulin, the H9 hES cells (right panel) or the 201B7 iPS cells (left panel) were stimulated with FGF-2 (100 ng/ml) for 15 mins with indicated combination of membrane-permeable specific inhibitory peptides for PKC isoforms; PKC α , β , and γ inhibitory peptide (50 μ M), PKC δ inhibitory peptide (50 μ M), PKC ϵ inhibitory peptide (50 μ M), or PKC ζ inhibitory peptide (20 μ M). The phosphorylation levels in the cells were measured by AlphaScreen[®] SureFire[®] assay kit. The values of the y-axis are the ratio of each phosphorylation to each total signal protein. The data are represented as means \pm SD (n = 3). *P < 0.05. (TIF)

Figure S5 Culture of hiPS cells in the hESF9 + activin A + 2i or the hESF9 + activin A + GFX conditions. (A) Phase-contrast image of H9 hES cells serially cultured in hESF9 + activin

A + 2i (hESF9_{a2i}) or hESF9 + activin A + GFX mediums at three passages, as described in Figure 5A and 5B. Scale bars, 200 μ m. (B) Immunocytochemical staining for OCT3/4 expression of H9 cells cultured as described (A). The H9 hES cells stained with anti-OCT3/4 antibody were visualized with Alexa Fluor 488 (green). Nuclei were stained with Hoechst 33342 (blue). Scale bars, 50 μ m. (C) Anti-OCT3/4 staining intensity profiles in the cell population grown in the hESF9 + activin A + 2i or the hESF9 + activin A + GFX conditions were analyzed by IN Cell image analyzer (lower panels). Antigen histogram (red); control histogram (green); Y axis is cell numbers and X axis is fluorescence intensity for anti-OCT3/4 antibody. (TIF)

Figure S6 Immunocytochemical staining of H9, KhES-4, 201B7, and Tic hPS cells for TRA-1-60. The cells grown on FN in hESF9_{a2i} as described in Figure 5C were stained with TRA-1-60 antibody and Alexa Fluor 647-conjugated secondary antibody. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 200 μ m. (TIF)

Figure S7 Long-term culture of hiPS cells in the hESF9_{a2i} medium. Human iPS 201B7 cells were cultured on FN in hESF9_{a2i} medium serially for more than 30 passages. The cells were split at a ratio of 1:3–1:5 every five days. (A) Phase-contrast image of 201B7 hiPS cells cultured on FN in hESF9_{a2i} medium. (B) A comparison of the growth of 201B7 cells in hESF9_{a2i} medium or KSR-based media. The cells were seeded on feeders in KSR-based medium (closed circles) or on FN in hESF9_{a2i} medium (open circles; mean + s.d. of three experiments). Cell numbers were counted every 2 days. (C) Immunocytochemical staining for SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81 (red) expression of 201B7 cells (passage 10) cultured on FN in hESF9_{a2i}. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 200 μ m. (D) FACS profiles for SSEA-1, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-54, A2B5, CD90, and HLA-Class1 expression of hiPS 201B7 cells (passage 22) cultured on FN in hESF9_{a2i} medium. Antigen histogram (red); control histogram (green); the horizontal bar indicates the gating used to score the percentage of antigen-positive cells. (TIF)

Figure S8 Long-term culture of hES cells in the hESF9_{a2i} medium. Human ES H9 cells were cultured on FN in hESF9_{a2i} medium serially for more than 30 passages. The cells were split at a ratio of 1:3–1:5 every five days. (A) Phase-contrast image of H9 hES cells cultured on FN in hESF9_{a2i} medium. (B) A comparison of the growth of H9 hES cells (passage 13, 16, and 17) in hESF9_{a2i} (open circles) or KSR-based media (closed circles). Mean + s.d. of three experiments. (C) Immunocytochemical staining for SSEA-1, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-54, A2B5, CD90, and HLA-Class1 expression (red) in H9 hES cells (passage 13). Nuclei were stained with Hoechst 33342 (blue). (D) FACS profiles of H9 hES cells (passage 14). Antigen histogram (red); control histogram (green). Scale bars = 200 μ m. (TIF)

Figure S9 Karyotype analysis and differentiation potential of H9 hES cells and 201B7 hiPS cells maintained in hESF9_{a2i} conditions. (A) Karyotype analysis of H9 hES cells at passage 15 and 201B7 hiPS cells at passage 21, showing a normal diploid 46, xx karyotype. (B) Heat-map of gene expression in H9 hES cells (at passage 10–13) and 201B7 hiPS cells (at passage 10–20) those during in vitro differentiation in triplicate experiments (Sample No. 3–5). TaqMan low density PCR arrays

(Applied BioSystems) were performed as previously described [61]. Expression levels were all normalized against β -ACTIN. The relative level of each gene expression were generated from the undifferentiated H9 hES cell or 201B7 hiPS cells cultured on mitomycin-inactivated mouse embryonic fibroblasts (MEF) in KSR-based medium (Sample No. 1–2). Heat-map colors (red for up-regulation, blue for down-regulation) depict gene expression. (C) Teratomas derived from H9 hES cells at passage 44 or 201B7 iPS cells at passage 26 maintained in hESF9a₂₁ conditions. (TIF)

Table S1 The composition of media used for serum-free culture. * The composition of the basal medium, ESF for culturing mouse ES cells, is described in Furue et al., 2005 [22]. ** hESF9 medium is described in Furue et al., 2008 [8]. *** hESF9a medium is described in Hayashi and Furue et al., 2010 [23]. (DOC)

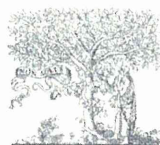
Table S2 A list of the used antibodies. (DOC)

Table S3 A list of the used primers for RT-PCR. (DOC)

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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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ECM (extracellular matrix)

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 HNF1 α . 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 (HNF1 α) (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 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 (Kyoto University). 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 μ g/ml), and penicillin (200 μ g/ml).

2.3. Ad vectors

The human eukaryotic translation elongation factor 1 alpha 1 (EF-1 α) promoter-driven HNF1 α - and FOXA2-expressing Ad vectors (Ad-HNF1 α 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 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ M 2-mercaptoethanol, 10 μ M ethanolamine, 10 μ M sodium selenite, and 0.5 mg/ml bovine serum albumin (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-HNF1 α for 1.5 h on day 6 and cultured for 3 days on Matrigel in hepatocyte culture medium (HCM) (Lonza) supplemented with 30 ng/ml bone morphogenetic protein 4 (BMP4) 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-HNF1 α for 1.5 h on day 9 and cultured for 3 days on Matrigel in HCM supplemented with 10 ng/ml hepatocyte growth factor (HGF), 10 ng/ml FGF1, 10 ng/ml FGF4, and 10 ng/ml FGF10 (all from R&D Systems). 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 μ M hydrocortisone 21-hemisuccinate (Sigma), 1 μ M 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 vertical section

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 an 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 plate 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 \times 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 $^{\circ}\text{C}$. Next, the hESC-derived cells were cultured in a type I collagen gel solution for 3h, and then the medium was changed 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 \times g at 4 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$ 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 *HNF1 α* 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

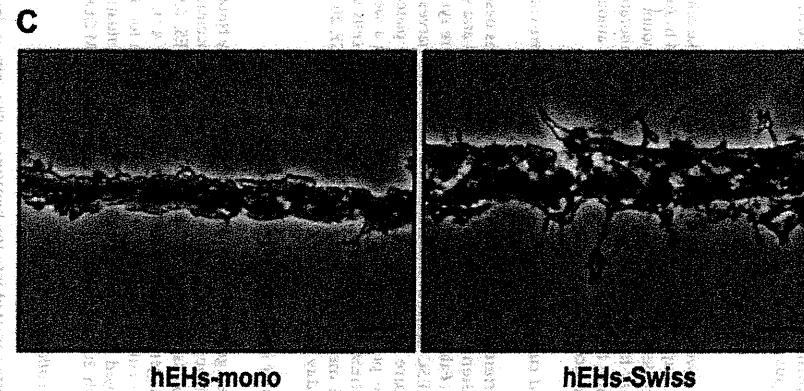
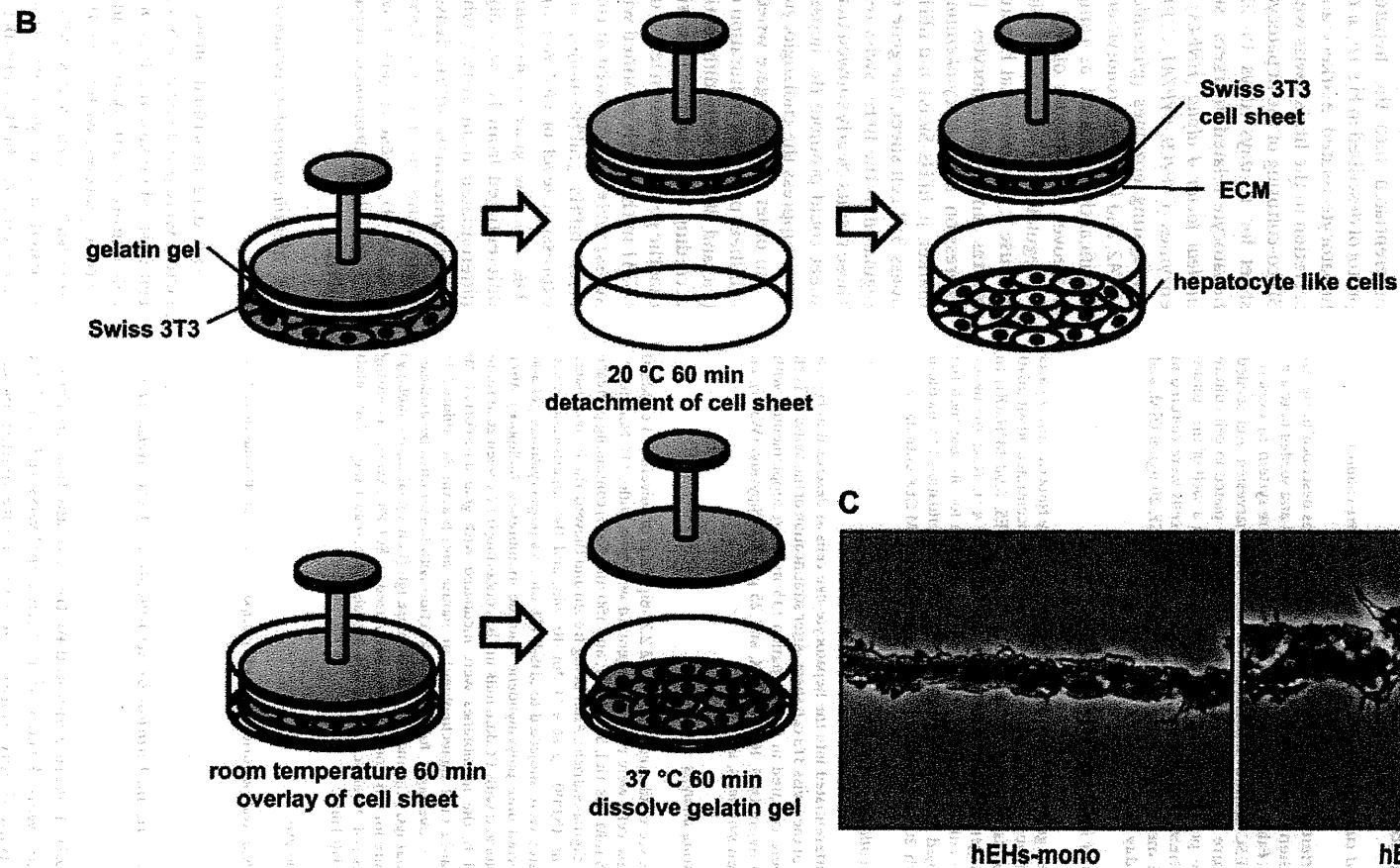
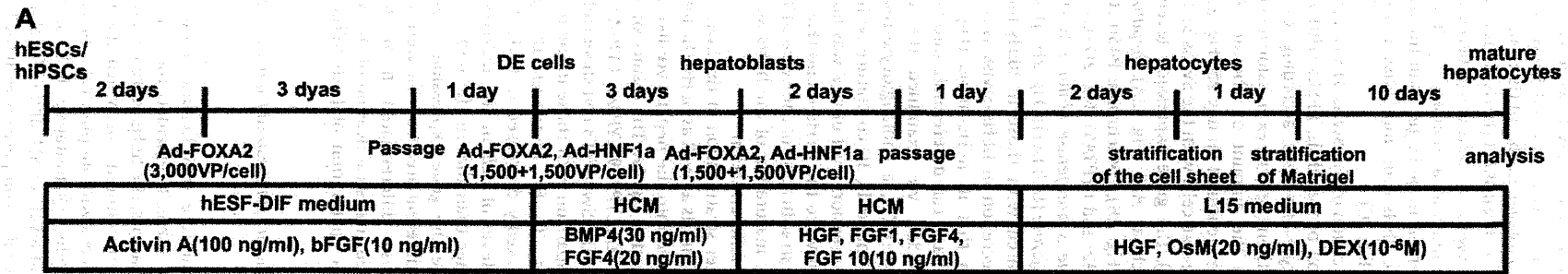


Fig. 1. Experimental protocol and schematic illustration of the procedure to stratify Swiss 3T3 cells on hepatocyte-like cells. (A) The procedure for hepatic differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) using stratification of the Swiss 3T3 cell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

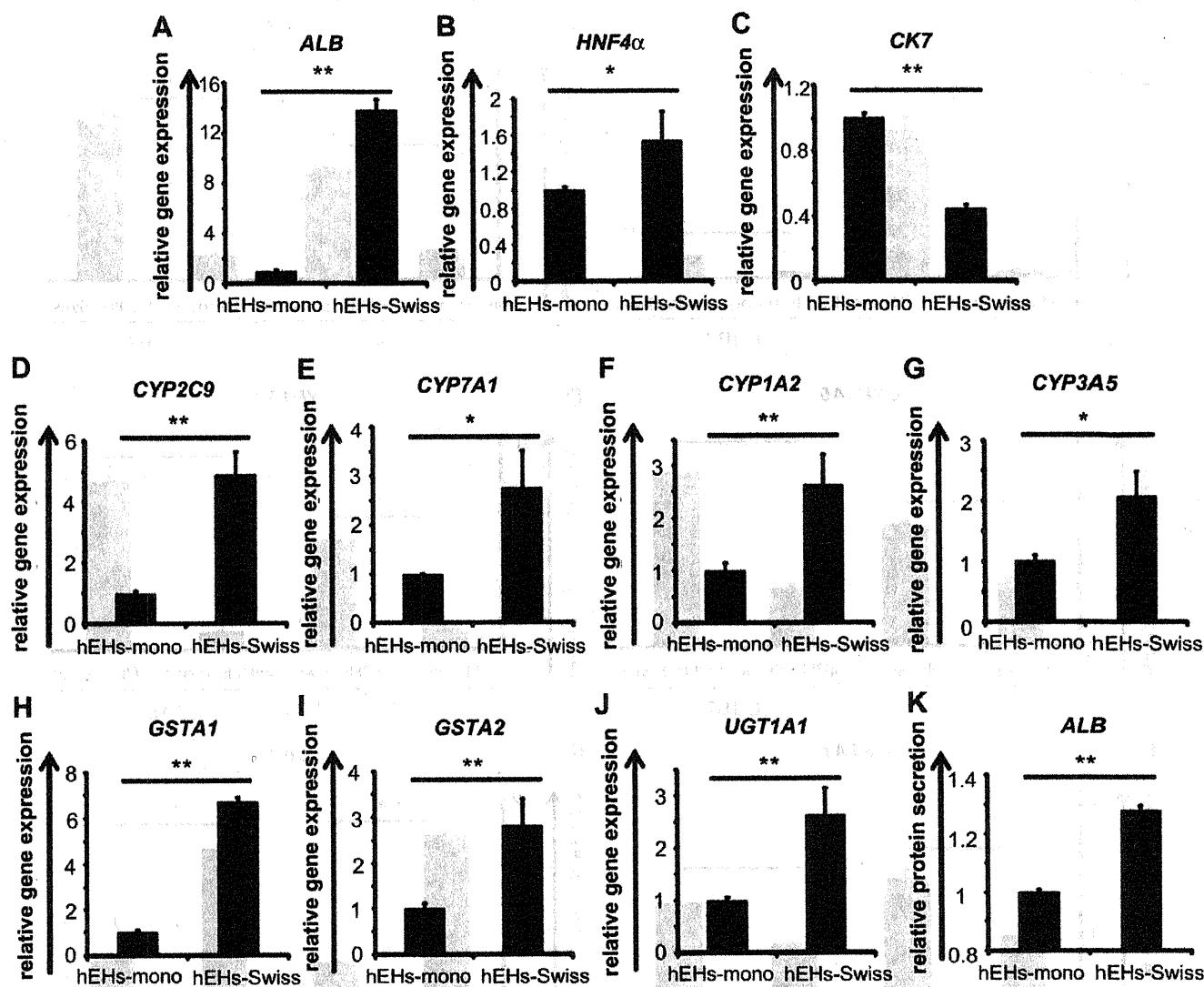


Fig. 2. Stratification of Swiss 3T3 cell sheet on hEHs promotes hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–K): On day 25, the gene expression levels of *ALB* (A), *HNF4 α* (B), *CK7* (C), *CYP2C9* (D), *CYP7A1* (E), *CYP1A2* (F), *CYP3A5* (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 \pm 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 synthesis. 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

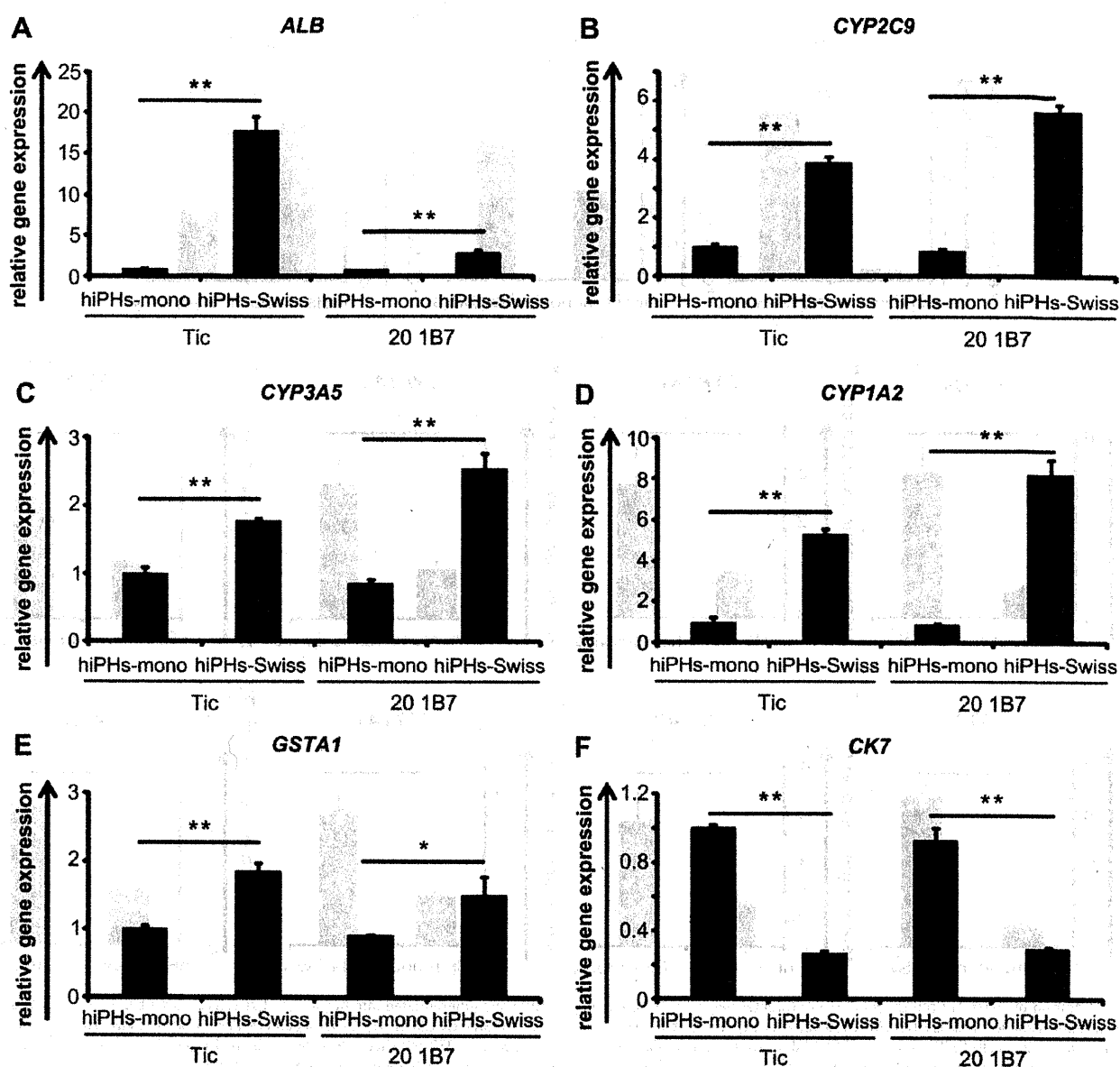


Fig. 3. Stratification of Swiss 3T3 cell sheet on hiPHs promotes hepatic maturation. Human induced pluripotent stem cells (hiPSCs) (Tic and 201B7) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–F): On day 25, the gene expression levels of *ALB* (A), *CYP2C9* (B), *CYP3A5*(C), *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 \pm 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 mature 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

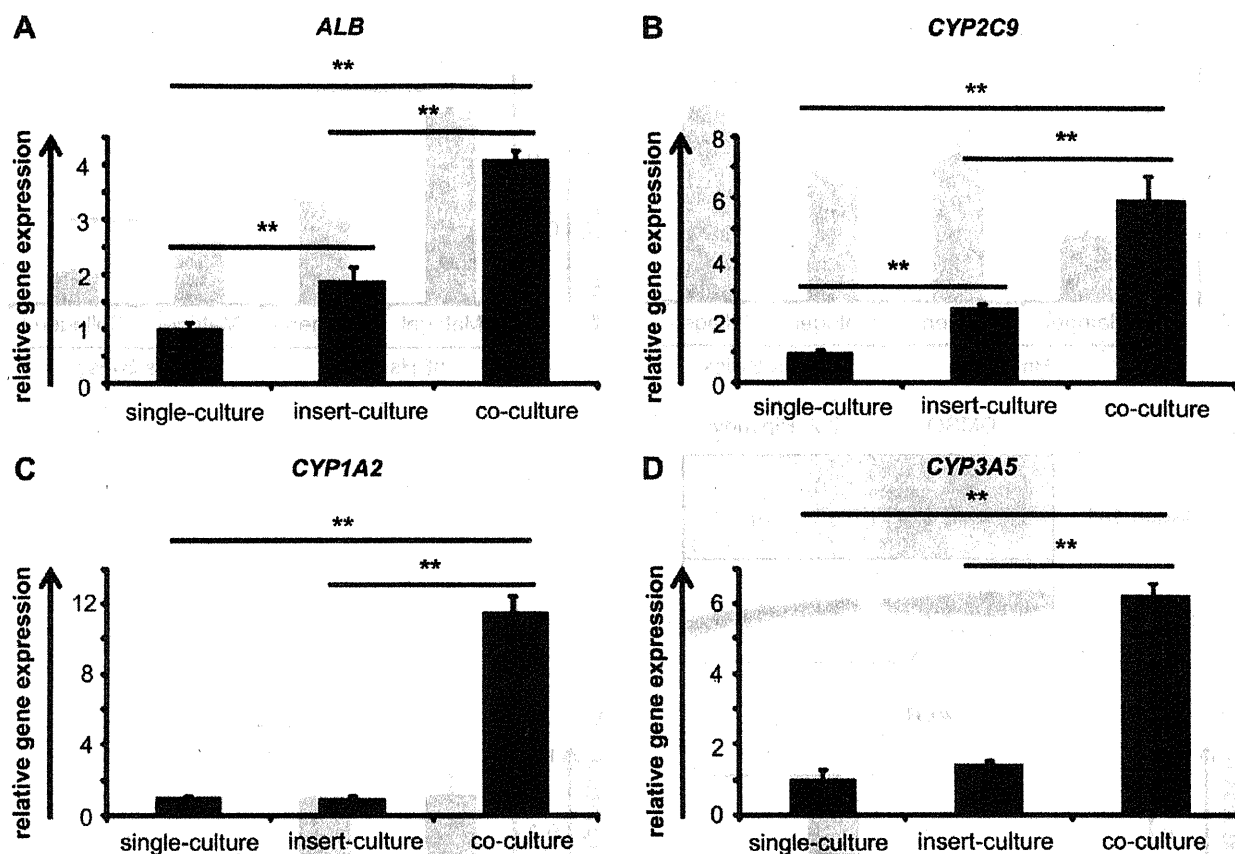


Fig. 4. Physical contacts between hESC-derived hepatocyte-like cells and Swiss 3T3 cells promote hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then the cells were differentiated into hepatocyte-like cells by single-culture, insert-culture, 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 \pm 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

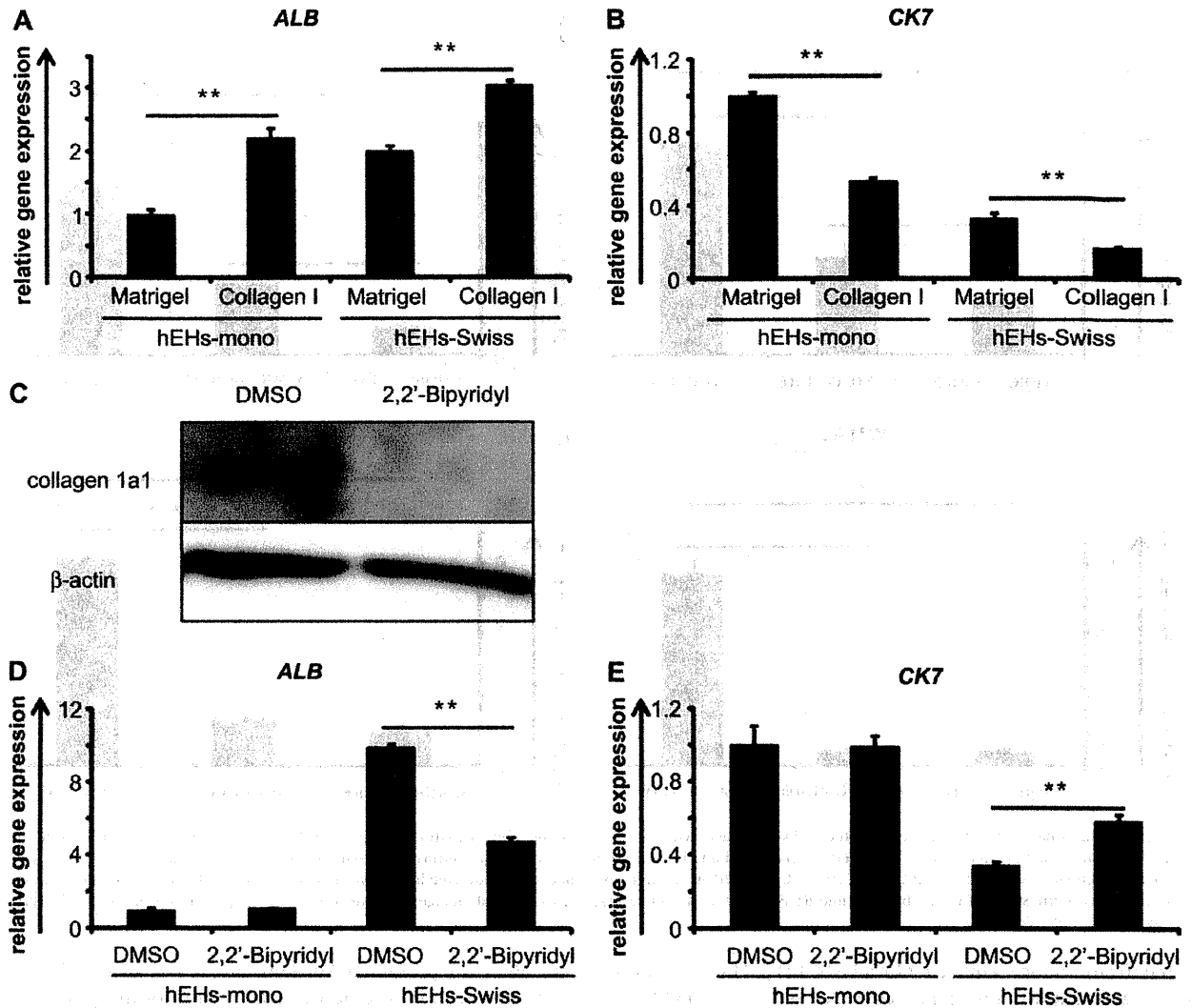


Fig. 5. Stratification of type I collagen gel promotes hepatic maturation. (A and B) hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then type I collagen gel (collagen I) or Matrigel are stratified on monolayer hESC-derived hepatocyte-like cells (hEHs-mono) 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, *coll1a1*, 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 \pm 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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