

Fig. (4). (A) Total serum cholesterol levels. ADMPC transplantation in WHHL rabbits was followed for 12 weeks. Total serum cholesterol was measured in five rabbits that each received 3×10^7 ADMPC, three rabbits that each received 3×10^7 ADSC, and in six rabbits that received saline (control). Data are mean \pm SEM. # $P < 0.05$; control vs. the ADMPC-transplanted WHHL rabbit; & $P < 0.05$; the ADSC-transplanted WHHL rabbit vs. the ADMPC-transplanted WHHL rabbit. (B) Lipoprotein profiles in a representative WHHL rabbit with ADMP transplantation after gel filtration. Serum samples from the WHHL rabbit before and 4 weeks after transplantation were fractionated. Note the marked reduction in low-density lipoprotein (LDL) peak and appearance of a high-density lipoprotein (HDL) peak. (C) Rate of clearance of LDL from the serum of rabbits with and without transplantation of ADMPC. Animals were injected with ^{125}I -labeled human LDL, and the time course of clearance was monitored following trichloroacetic acid precipitation of serum at 5 min, 1 h, 2 h, 4 h, 6 h, and 28 h. Residual ^{125}I -LDL was expressed as a percentage of the signal at 5 min. The panel is the representative of two independent experiments. (D) DiO-LDL uptake into ADMPC-derived hepatocytes in the WHHL rabbit liver. Thin slices of recipient liver were incubated with DiO-labeled LDL in the serum-free medium for 24 h. After washing and fixation, the incubated slices were viewed by fluorescence microscopy. DiO-LDL-uptake cells (green) and no-uptake parenchymal cells are observed in the section. Bar = 100 μm .

3.5. In Situ Stem Cell Therapy by ADMPC

To determine the effects of ADMPC transplantation on the recipient rabbit lipid profile, serum cholesterol levels were monitored over 12 weeks (Fig. 4, cited from reference 12 with modification). Significant reductions in total serum cholesterol were observed within 4 weeks of the transplantation, and the reductions were maintained for the entire period (Fig. 4A). Furthermore, ADMPC-recipient animals showed significantly greater reductions than those in the control group. To determine the effects of ADMPC transplantation on the fractions of high-density lipoprotein and LDL in re-

ipient animals, fractionation by fast protein liquid chromatography was performed (Fig. 4B). Transplantation of ADMPC resulted in marked reduction of the peak LDL cholesterol and increment of high-density lipoprotein cholesterol fraction (right panel). Next, clearance experiments were performed with human LDL to confirm that the transplanted ADMPC contributed to the fall in serum cholesterol through uptake of LDL via LDL receptors. The rate of LDL clearance was significantly higher in the WHHL rabbits with transplanted ADMPC than WHHL rabbits without transplanted ADMPC (Fig. 4C). Rabbits with ADMPC transplants showed ~2.4-fold (high-dose; 3×10^7 cells/rabbit) and

1.4-fold (low-dose; 5×10^6 cells/rabbit) increases in the rate of LDL cholesterol clearance over non-transplanted rabbits. To evaluate the uptake of DiO-LDL by transplants *ex vivo*, thin liver slices of WHHL rabbit were incubated with DiO-labeled LDL for 24 h and the uptake was examined as for the clearance experiments (Fig. 4D). DiO-LDL was taken up by some but not all of the cells in the WHHL rabbit liver transplanted with ADMPC, with the positive cells observed dispersed, contacting, and integrating among the non DiO-LDL-positive parenchymal cells. This finding suggests that ADMPC differentiated into hepatocytes *in vivo*, thus lowering the serum cholesterol directly via LDL uptake.

3.6. ADMPC as a Promising Tool for Regenerative Medicine

For any successful regenerative medicine program, three issues must be considered: 1) what kind of tissues should be nominated as cell sources, 2) how should the cells be obtained from tissues, and 3) how will the cells behave after transplantation/administration. The following section discusses these considerations with respect to this study.

The source of stem cells for regenerative medicine should be easily and safely accessible as well as free of any ethical issues, thus allowing allogenic as well as autologous cellular therapy applications, and finally, the cell source should be available in large amounts. Adipose tissue is therefore a suitable cell source under these criteria. Liposuction surgery provides a safe method for collection of adipose tissue, harvesting from 100 ml to >3 L of lipoaspirate from material that is routinely discarded, thus avoiding any ethical concerns [16].

As to the second issue of cell processing, methods to isolate cells from adipose tissue were first reported in the 1960s [17-19]. Such methods involved mincing rat fat pads and incubating the resultant tissue fragments with collagenase. The digested material was then centrifuged to separate the floating population of mature adipocytes from the pelleted stromal vascular fraction (SVF). The SVF, which are sometimes referred to adipose tissue-derived regenerative cells (ADRC), consists of a heterogeneous cell population including fibroblasts, endothelial cells, pre-adipocytes, and mesenchymal stem cells [17-19]. However, as mentioned, this non-cultured cell population is too heterogeneous to apply therapeutically, necessitating the isolation of pre-adipocyte and/or mesenchymal stem cell-rich adherent cells from the SVF. The original procedure for this separation step was subsequently modified for the isolation of cells from human adipose tissue SVFs [20-23], and Zuk *et al.* [7] reported that the processed lipoaspirates exhibited mesenchymal stem cell-like features and could differentiate into adipocytes, osteocytes, and chondrocytes. Such cells are currently labeled as adipose tissue-derived stem cells (ADSC). The procedure used for obtaining these stem cells from lipoaspirates was described by Bjornorp *et al.* [24] to obtain pre-adipocytes.

We have subsequently developed a novel isolation method for stem-like cells according to their adhesion properties [8-12]. As shown in Fig. (1Aa), fibroblast- and endothelial-like cells completely adhered onto the culture dish after 24 h in culture following the first-plating of the SVF. Most of the SVF cells were difficult to detach by conven-

tional pipetting after this length of culture (24 h). However, a population of round self-aggregating cells that could only be detached by treatment with EDTA solution showed multi-lineage differentiation potency. In the EDTA-treating method, only cells of their properties with self-aggregation and EDTA-sensitiveness could be selected as ADMPC from plated SVF. In the conventional pre-plating methods to obtain ADSC [7, 24], the cells with EDTA-resistance could not be excluded. ADMPC exhibited high differentiation capacities for osteocytic, adipocytic, and chondrocytic lineages compared with ADSC, and could differentiate into hepatocyte-like cells, insulin-producing cells, and cardiomyoblast-like cells *in vitro* as non-mesenchymal lineages. We therefore named these cells ADMPC.

ADMPC differ from ADSC with regard to gene expression profiling. ADMPC express islet-1, a marker of undifferentiated cells and of cardiac, hepatic, and pancreatic progenitor cells [9-11]. Based on our findings, we propose that the islet-1-expressing ADMPC could be differentiated or reprogrammed into hepatocytes in the recipient liver *in vivo* after transplantation. In other words, the appropriate cells can show *in situ reprogramming* when transplanted and recruited into an appropriate environment.

Finally, we considered how the ADMPC would behave after transplantation/administration *in situ*. Traditionally, stem/progenitor cells are differentiated into terminally differentiated cells prior to transplantation/administration. For example, iPS cells are differentiated into neuronal cells and then applied for neuronal disease therapies. In these processes, researchers could mimic the relevant microenvironment and then differentiate the transplanted cells into the desired cell lineages. In contrast, we hypothesized that *in vitro* or *ex vivo* reprogramming could not sufficiently recapitulate the desired transplant microenvironment. Our concept is that the microenvironment *in situ* might supply cytokines to exert paracrine effects and form appropriate extracellular matrices, thus prompting the progenitor cells toward the desired terminal differentiation. ADMPC express islet-1, indicating that they might be appropriate progenitor cells on their own for *in situ* reprogramming as hepatocytes in the liver microenvironment. If these reprogrammed cells could correct given disease defects, clinical applications for *in situ* stem cell therapy become feasible.

In this review, we propose *in situ* stem cell therapy as a new tool for regenerative medicine and *in situ* reprogramming as a mechanism for the correction of disease. Yamana *et al.* [25] presented terminally differentiated cells that could be reprogrammed into the pluripotent state using only four factors. Followers confirmed the fact and named the concept "reprogramming". Melton *et al.* [26] subsequently showed that gene-modified cells alone could direct differentiation along a terminal path, and renamed the mechanism "direct reprogramming", and some cases of *ex vivo* gene therapy might be included in this concept. Here, we presented stem-like cells that could differentiate terminally *in situ* in the appropriate microenvironment.

4. CONCLUSIONS

In this review we describe ADMPC, novel adipose tissue-derived cells with stem cell-like properties and higher

differentiation potential than previously reported adipose tissue-derived cells. Not only could ADMPC differentiate into hepatocyte-like cells *in vitro*, but ADMPC *per se* also showed the same capacity in the hepatic environment and the *in situ* reprogrammed cells could correct the metabolic defect of diseased animals. The mechanisms described for *in situ* reprogramming hold great promise for applications in regenerative medicine as “*in situ* stem cell therapy”.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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DISCLOSURE

It should be noted that the authors have previously published much of the material covered in this review article in “Tissue Eng Part C Methods”, Volume 17, 2011, Pages 145-154.

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

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

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

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

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

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Introduction

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

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

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

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

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

Materials and methods

In vitro differentiation

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

Results

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

generated by the optimized protocol for pharmaceutical use in detail.

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

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

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

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

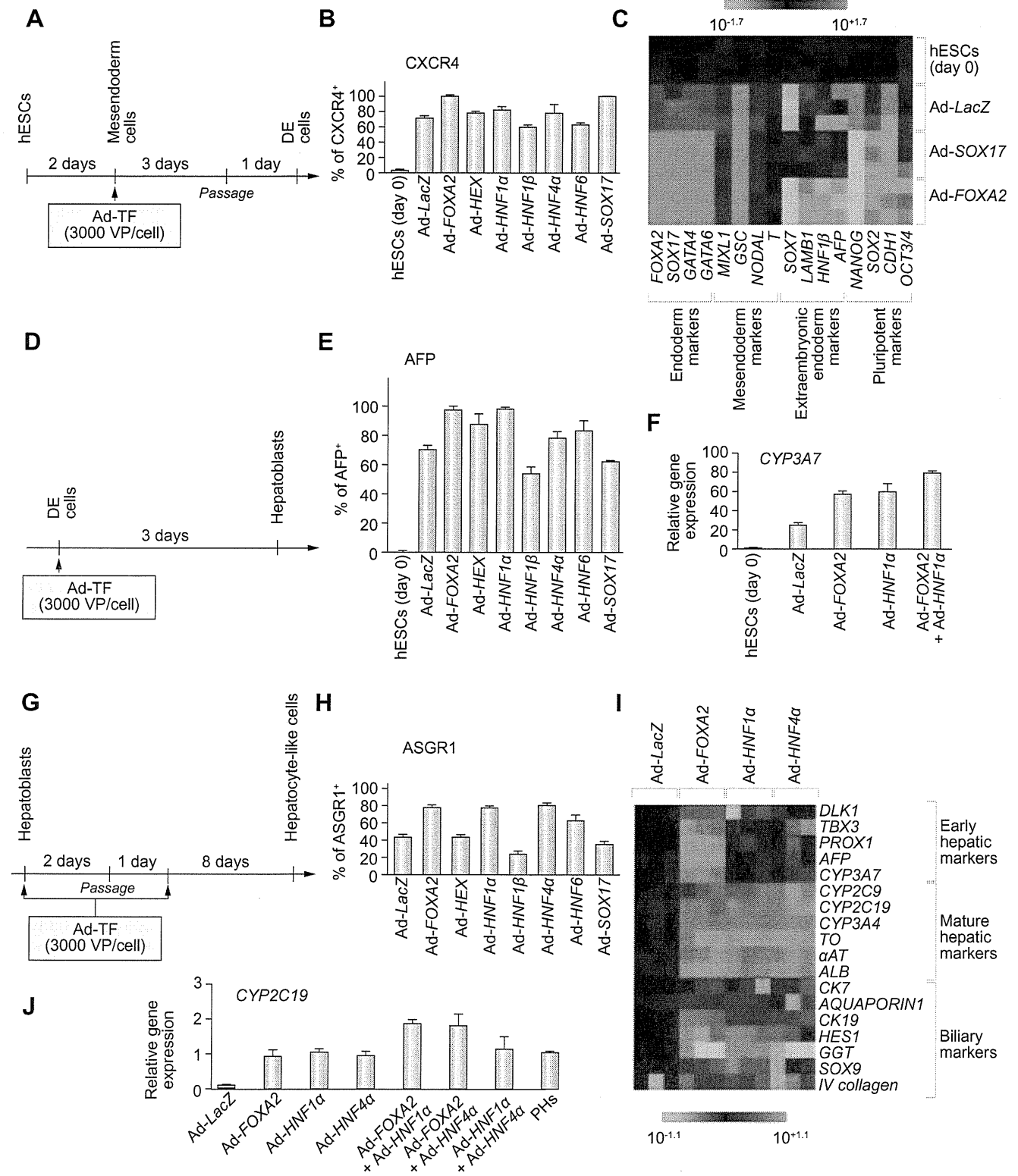


Fig. 1. Efficient hepatic differentiation from hESCs by FOXA2 and HNF1 α transduction. (A) The schematic protocol describes the strategy for DE differentiation from hESCs (H9). Mesendoderm cells (day 2) were transduced with 3000 VP/cell of transcription factor (TF)-expressing Ad vector (Ad-TF) for 1.5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

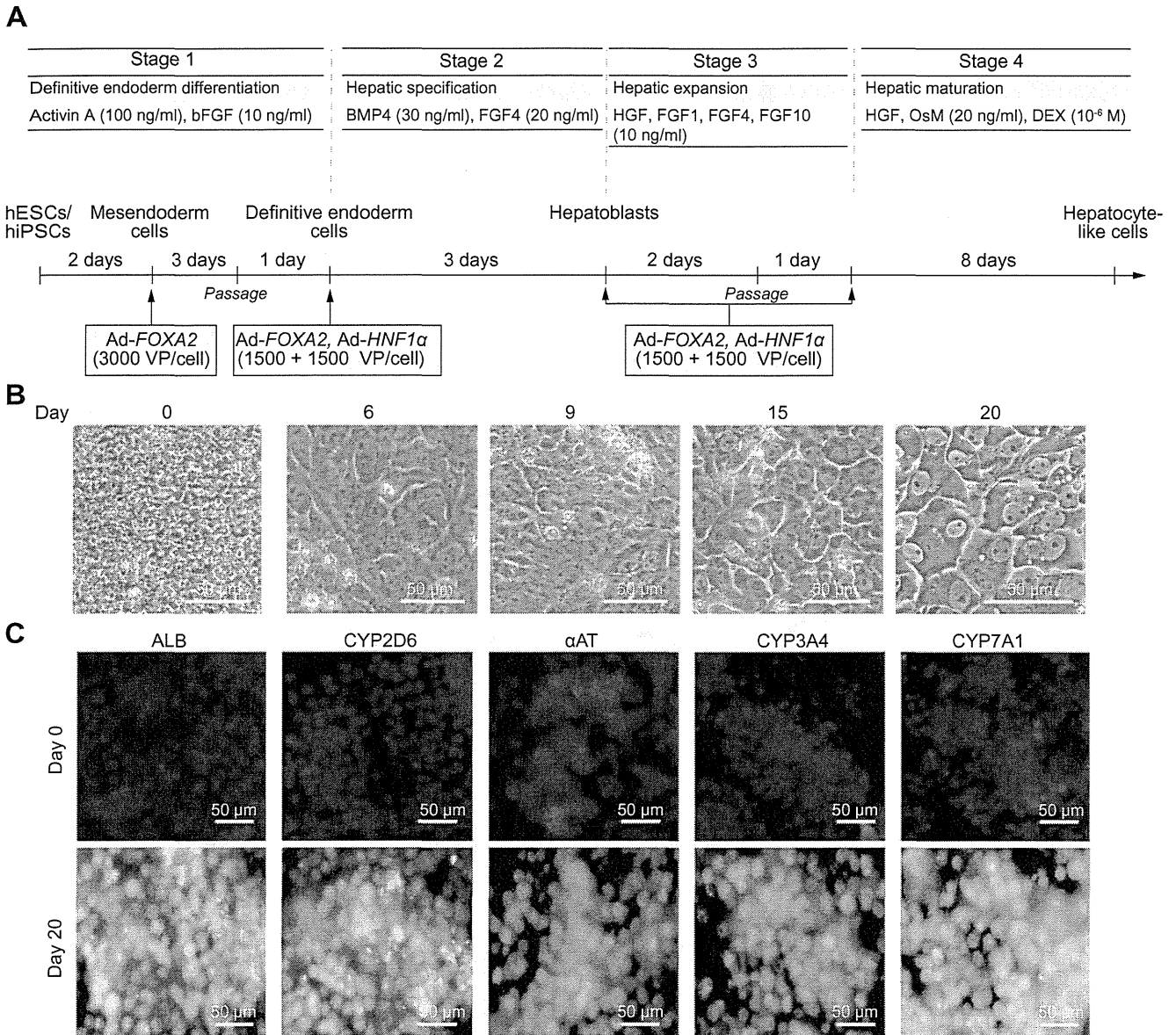


Fig. 2. Hepatic differentiation of hESCs and hiPSCs by FOXA2 and HNF1 α transduction. (A) The differentiation procedure of hESCs and hiPSCs into hepatocytes via DE cells and hepatoblasts is schematically shown. Details of the hepatic differentiation procedure are described in Materials and methods. (B)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

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

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

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

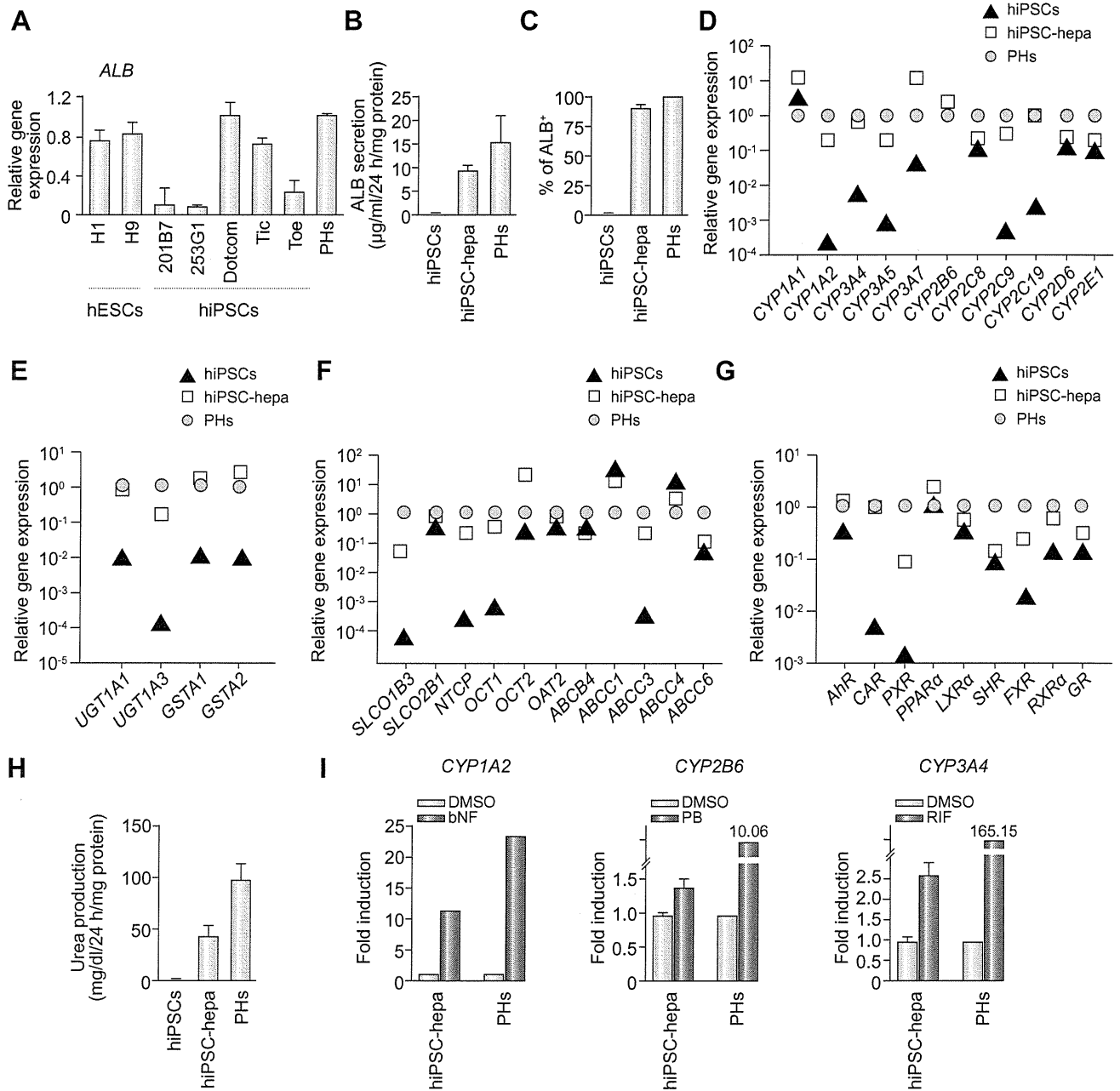


Fig. 3. The hepatic characterization of hiPSC-hepa. hESCs (H1 and H9) and hiPSCs (201B7, 253G1, Dotcom, Tic, and Toe) were differentiated into hepatocyte-like cells as described in Fig. 2A. (A) On day 20, the gene expression level of *ALB* was examined by real-time RT-PCR. On the y axis, the gene expression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

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

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

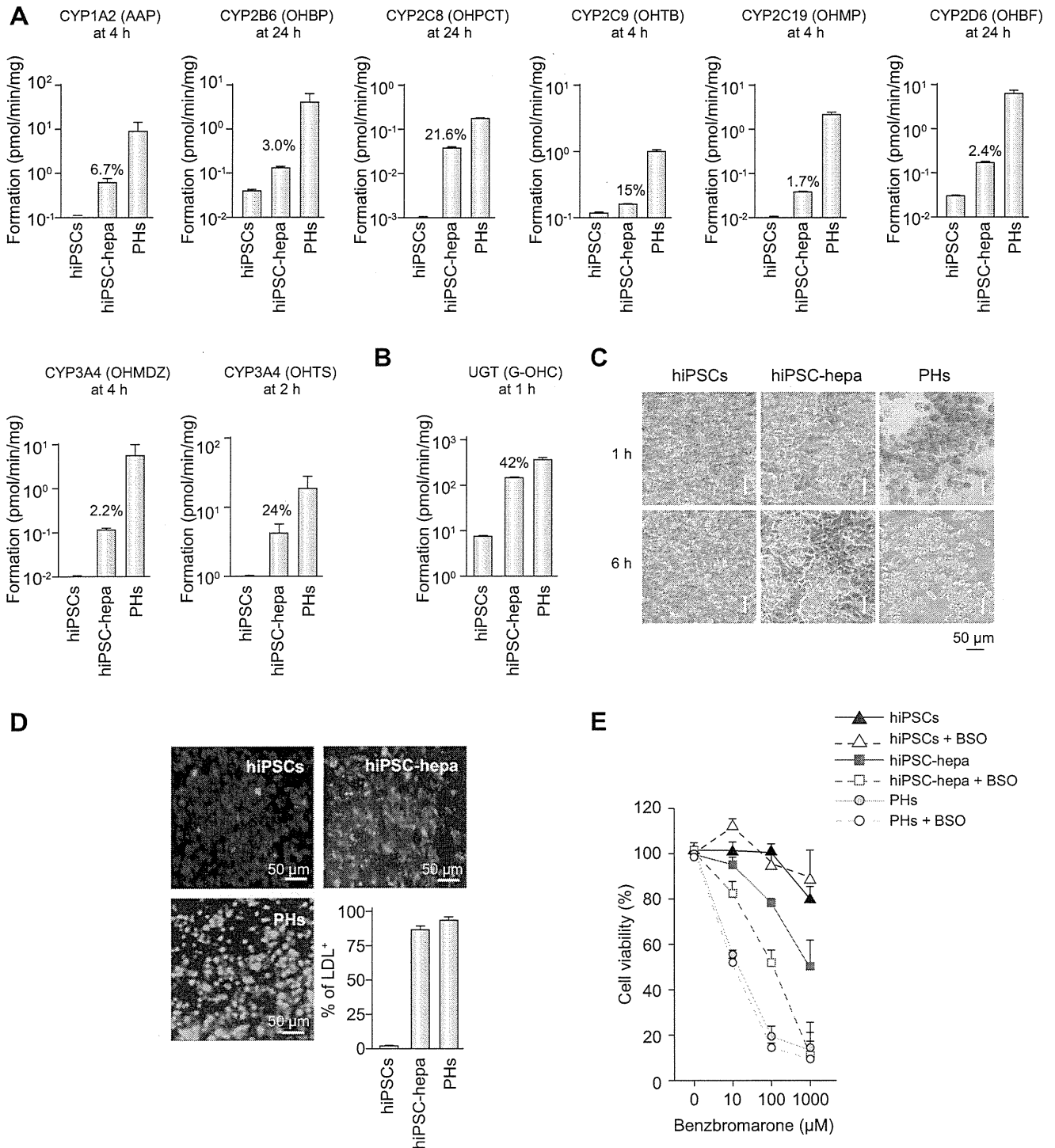


Fig. 4. Evaluation of the drug metabolism capacity and hepatic transporter activity of hiPSC-hepa. hiPSCs (Dotcom) were differentiated into hepatocytes as described in Fig. 2A. (A and B) Quantitation of metabolites in hiPSCs, hiPSC-hepa, and PHs, which were cultured for 48 h after the cells were plated,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

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

Characterization of the hESC-hepa/hiPSC-hepa

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

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

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

Drug metabolism capacity and hepatic transporter activity of hiPSC-hepa

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

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

Discussion

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

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

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

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

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

Conflict of interest

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

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Supplementary data

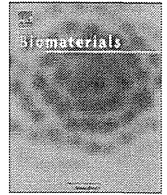
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2012.04.038>.

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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 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 mesoderm 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 mesoderm 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 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 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 HCl (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 °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 °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 *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

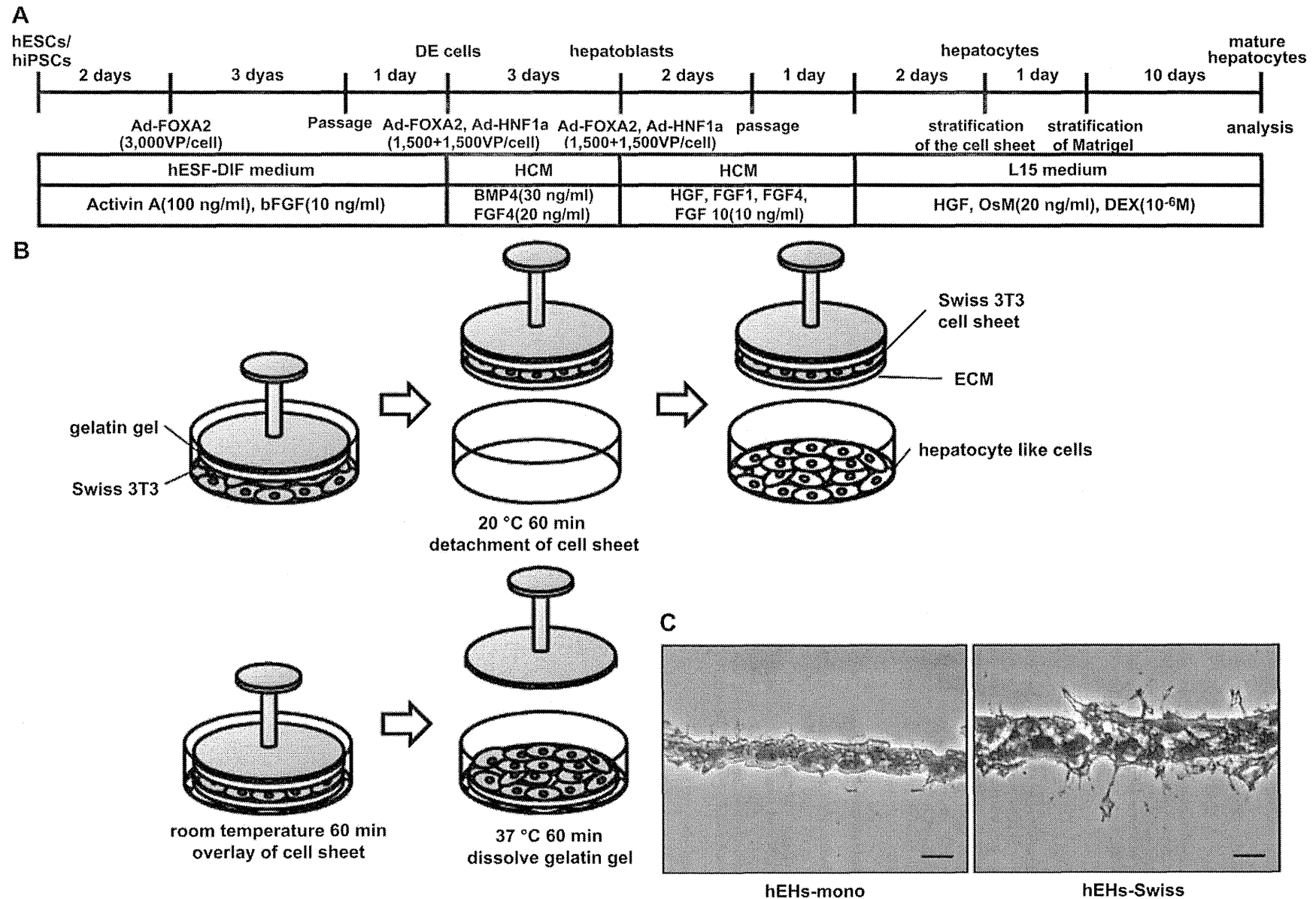


Fig. 1. Experimental protocol and schematic illustration of the procedure to stratify Swiss 3T3 cells on hepatocyte-like cells. (A) The procedure for hepatic differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) using stratification of the Swiss 3T3 cell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

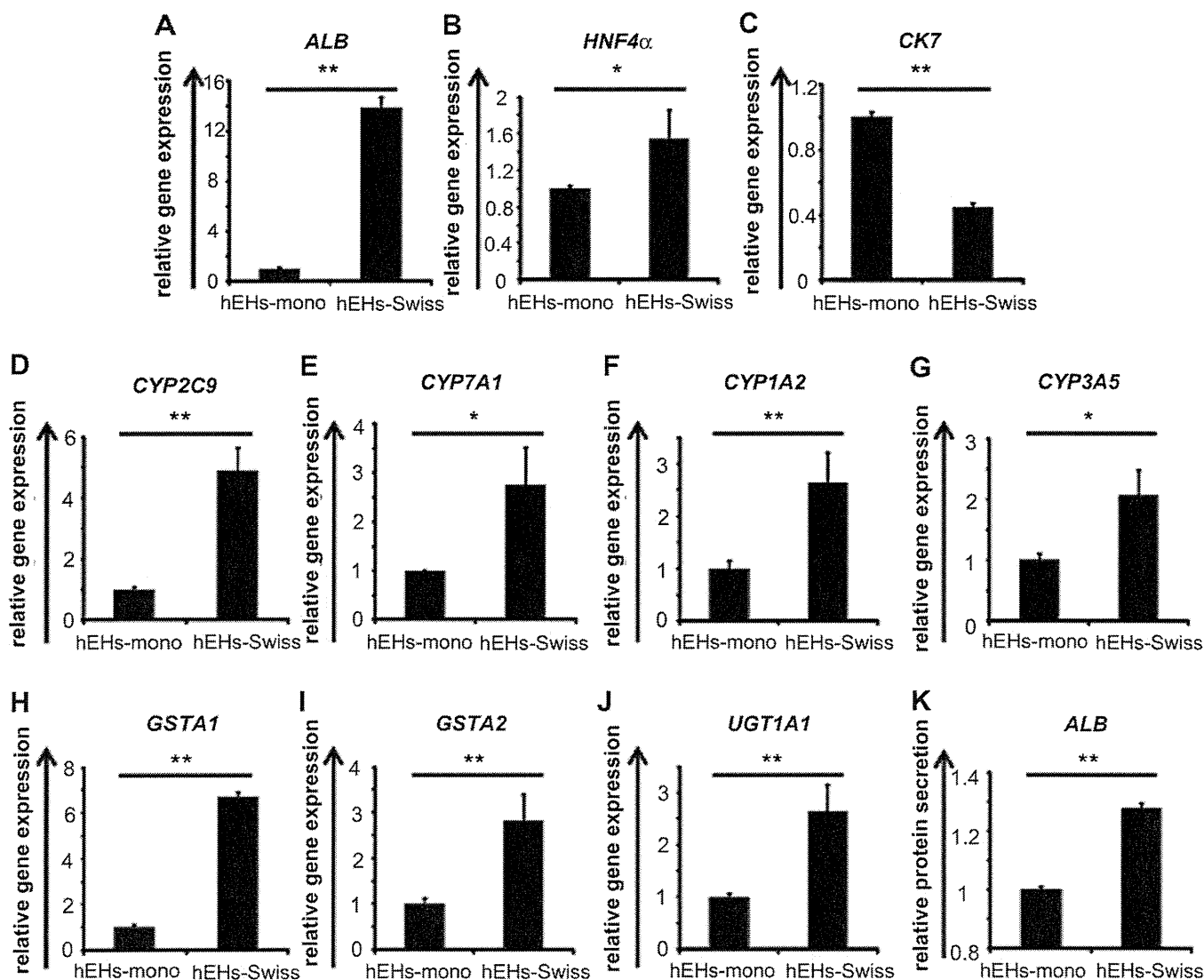


Fig. 2. Stratification of Swiss 3T3 cell sheet on hEHs promotes hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–K): On day 25, the gene expression levels of *ALB* (A), *HNF4A* (B), *CK7* (C), *CYP2C9* (D), *CYP7A1* (E), *CYP1A2* (F), *CYP3A5*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

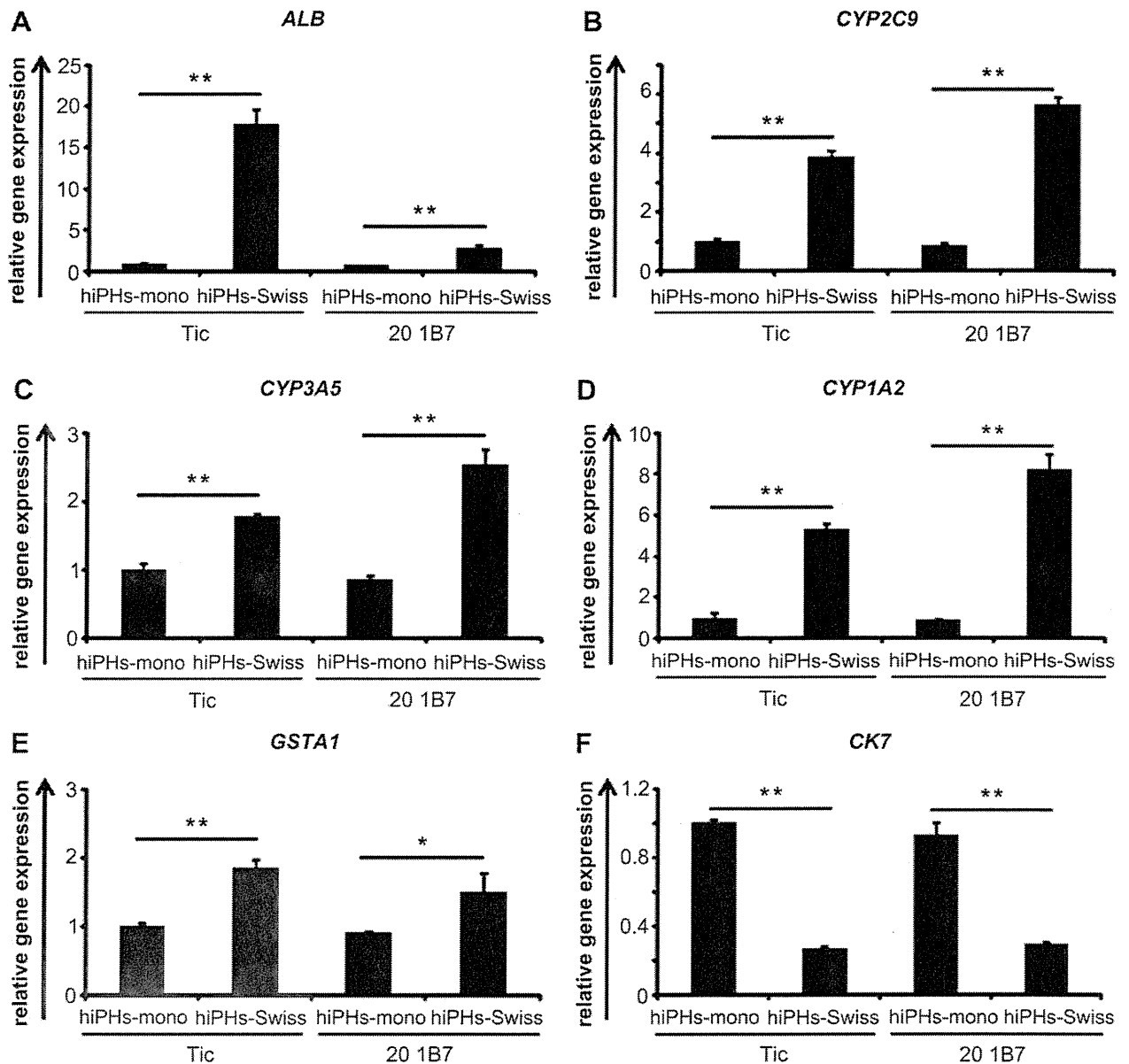


Fig. 3. Stratification of Swiss 3T3 cell sheet on hiPHs promotes hepatic maturation. Human induced pluripotent stem cells (hiPSCs) (Tic and 201B7) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–F): On day 25, the gene expression levels of *ALB* (A), *CYP2C9* (B), *CYP3A5*(C),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

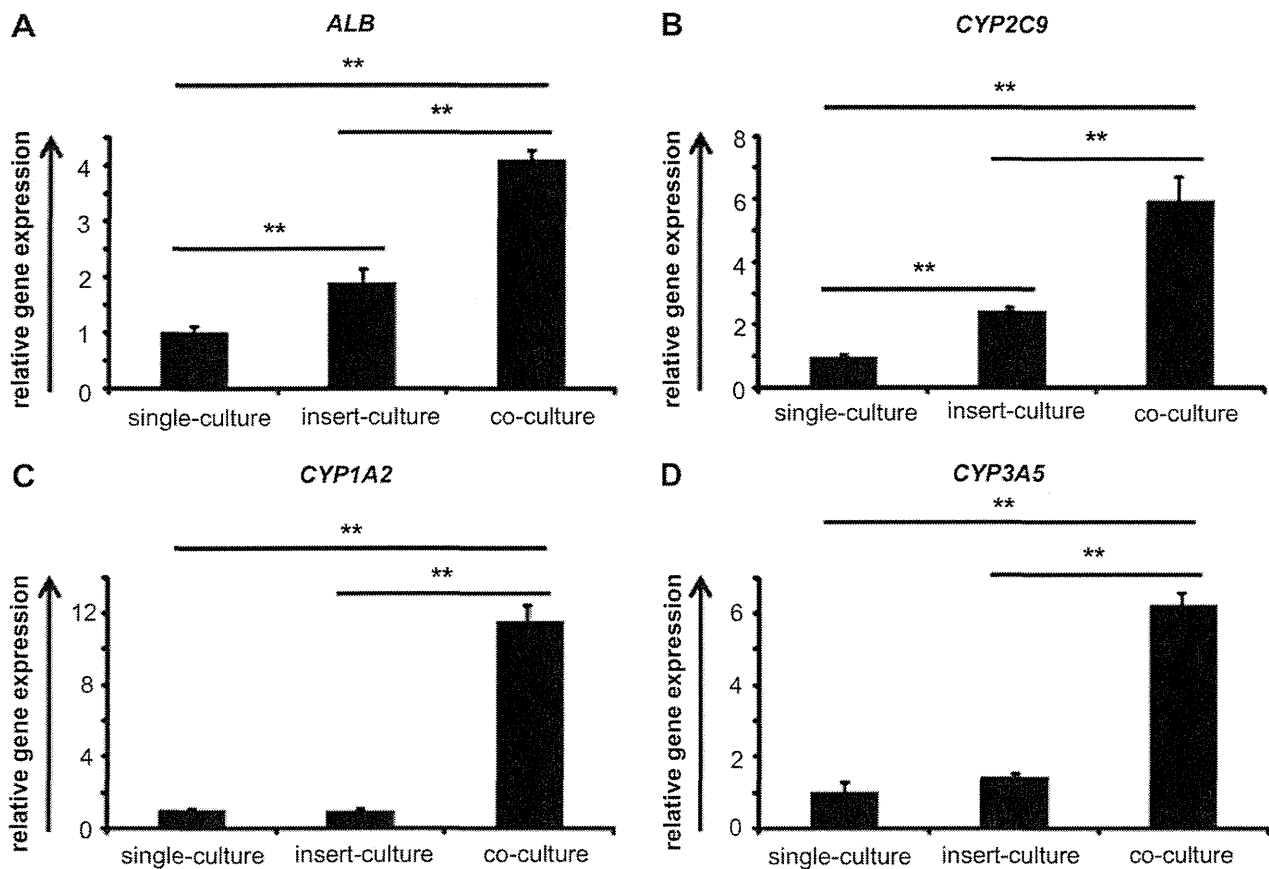


Fig. 4. Physical contacts between hESC-derived hepatocyte-like cells and Swiss 3T3 cells promote hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then the cells were differentiated into hepatocyte-like cells by single-culture, insert-culture,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

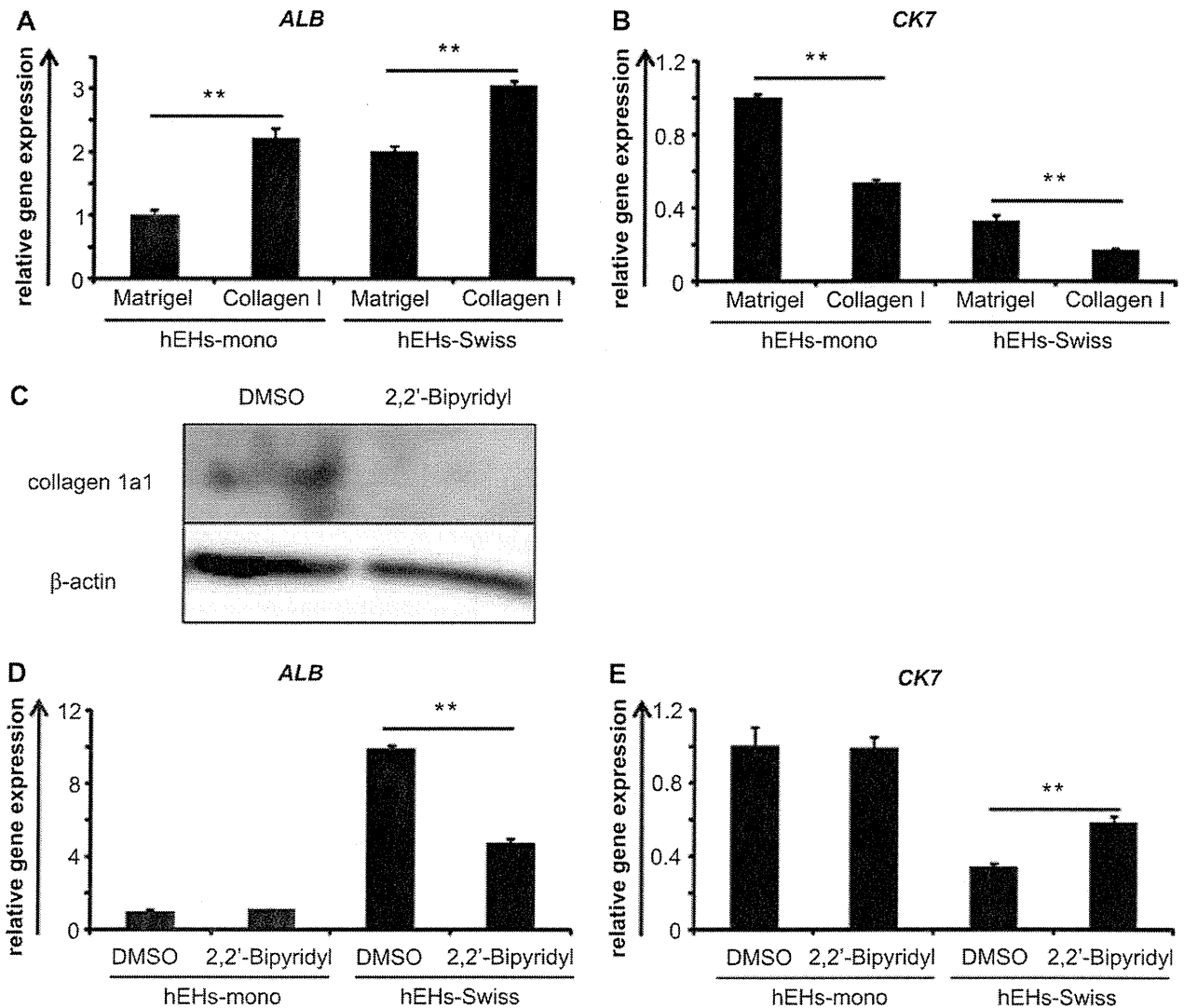


Fig. 5. Stratification of type I collagen gel promotes hepatic maturation. (A and B) hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then type I collagen gel (collagen I) or Matrigel are stratified on monolayer hESC-derived hepatocyte-like cells (hEHs-mono)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