tion of SOX17, HEX, and HNF4α 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 HNF1α transduction strongly promoted hepatic commitment and maturation (Fig. 1F and J), although in the stage of hepatic expansion and maturation, HNF4α transduction was as efficient as that of HNF1α (Fig. 1J). Since HNF1α is one of the target genes of HNF4α [13], the signaling through HNF4α to HNF1α would be important for efficient hepatic expansion and maturation. Considering these results together, we ascertained a pair of two transcription factors, FOXA2 and HNF1α, that could promote efficient hepatic differentiation from hESCs. In embryogenesis, the expression of FOXA2 and HNF1α is initially detected in DE or hepatoblasts, respectively and the expression levels of both FOXA2 and HNF1α are elevated as the liver develops [14,15]. Therefore, our hepatic differentiation technology, which employs FOXA2 and HNF1α 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 HNF1α 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; infact, 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 HNF1α 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 HNF1α. It is known that the combination of three-dimensional (3D) culture and co-culture, namely 3D co-culture, can maintain the functions of primary hepatocytes. However, hepatic maturation of hESC- or hiPSC-derived hepatocyte-like cells (hEHs or hiPHs, respectively) by 3D co-culture systems has not been examined. Therefore, we utilized a cell sheet engineering technology to promote hepatic maturation. The gene expression levels of hepatocyte-related markers (such as cytochrome P450 enzymes and conjugating enzymes) and the amount of albumin secretion in the hEHs or hiPHs, which were 3D co-cultured with the Swiss 3T3 cell sheet, were significantly up-regulated in comparison with those in the hEHs or hiPHs cultured in a monolayer. Furthermore, we found that type I collagen synthesized in Swiss 3T3 cells plays an important role in hepatic maturation. The hEHs or hiPHs that were 3D co-cultured with the Swiss 3T3 cell sheet would be powerful tools for medical applications, such as drug screening.

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1. Introduction

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

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

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

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

2. Materials and methods

2.1. hESC and hiPSC culture

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

2.2. Swiss 3T3 cell culture

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

2.3. Ad vectors

The human eukaryotic translation elongation factor 1 alpha 1 (EF-1α) promoter-driven HNF1α- and FOXA2-expressing Ad vectors (Ad-HNF1α and Ad-FOXA2, respectively) were constructed previously (in submitted). All of Ad vectors contain a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob for more efficient transduction of hESCs, hiPSCs, and DE cells, in which transduction efficiency was almost 100%, and purified as described previously [11,12,30]. The vector particle (VP) titer was determined by using a spectrophotometric method [31].

2.4. In vitro differentiation

Before the initiation of cellular differentiation, the medium of hESCs and hiPSCs was exchanged for a defined serum-free medium, hESF9, and hESCs and hiPSCs were cultured as previously reported [32]. The differentiation protocol for the induction of DE cells, hepatoblasts, and hepatocytes was based on our previous report with some modifications (in submitted). Briefly, in mesendoderm differentiation, hESCs and hiPSCs were dissociated into single cells by using Accutase (Millipore) and cultured for 2 days on Matrigel (BD Biosciences) in hESF-DIF medium (Cell Science & Technology Institute) supplemented with 10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, and 0.5 mg/ml bovine serum albumin (BSA) (all from Sigma) (differentiation hESF-DIF medium) containing 100 ng/ml Activin A (R&D Systems) and 10 ng/ml FGF2. To generate DE cells, hESC- or hiPSC-derived mesendoderm cells were transduced with 3000 VP/cell of Ad-FOXA2 for 1.5 h on day 2 and cultured until day 6 on Matrigel in differentiation hESF-DIF medium supplemented with 100 ng/ml Activin A and 10 ng/ml FGF2. For induction of the hepatoblasts, the hESC- or hiPSC-derived DE cells were transduced with each 1500 VP/cell of Ad-FOXA2 and Ad-HNF1α for 1.5 h on day 6 and cultured for 3 days on Matrigel in hepatocyte culture medium (HCM) (Lonza) supplemented with 30 ng/ml bone morphogenetic protein 4 (BMP4) and 20 ng/ml FGF4 (all from R&D Systems). To expand the hepatoblasts, the hepatoblasts were transduced with each 1500 VP/cell of Ad-FOXA2 and Ad-HNF1α for 1.5 h on day 9 and cultured for 3 days on Matrigel in HCM supplemented with 10 ng/ml hepatocyte growth factor (HGF), 10 ng/ml FGF1, 10 ng/ml FGF4, and 10 ng/ml FGF10 (all from R&D Systems).To induce hepatic maturation, the cells were cultured for 2 days on Matrigel in L15 medium (Invitrogen) supplemented with 8.3% tryptose phosphate broth (BD Biosciences), 10% FBS (Vita), 10 μM hydrocortisone 21-hemisuccinate (Sigma), 1 μM insulin, and 25 mM NaHCO3 (Wako) (differentiation L15 medium) containing 20 ng/ml hepatocyte growth factor (HGF), 20 ng/ml Oncostatin M (OsM) (R&D Systems), and 10^{-6} M Dexamethasone (DEX) (Sigma). As described below, the Swiss 3T3 cell sheet was stratified onto hepatocyte-like cells on day 14 and cultured in differentiation L15 medium supplemented with 20 ng/ml HGF, 20 ng/ml OsM, and 10^{-6} M DEX until day 15. On day 15, Matrigel was stratified onto the cells and cultured in differentiation L15 medium supplemented with 20 ng/ml HGF, 20 ng/ml OsM, and 10^{-6} M DEX until day 25.

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

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

2.6. RNA isolation and reverse transcription-PCR

Total RNA was isolated from the hESC- or hiPSC-derived cells using ISOGENE (Nippon Gene) according to the manufacturer's instructions. cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Real-time RT-PCR was performed with Taqman gene expression assays or Fast SYBR Green Master Mix using an ABI Step One Plus (all from Applied Biosystems). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). The primer sequences used in this study are described in Supplementary Tables 1 and 2.

2.7. Preparation of verticalsection

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

2.8. ELISA

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

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

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

2.10. Stratification of type I collagen gel

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

2.11. Inhibition of collagen synthesis

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

2.12. Western blotting analysis

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

2.13. Statistical analysis

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

3. Results

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

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

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

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

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

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

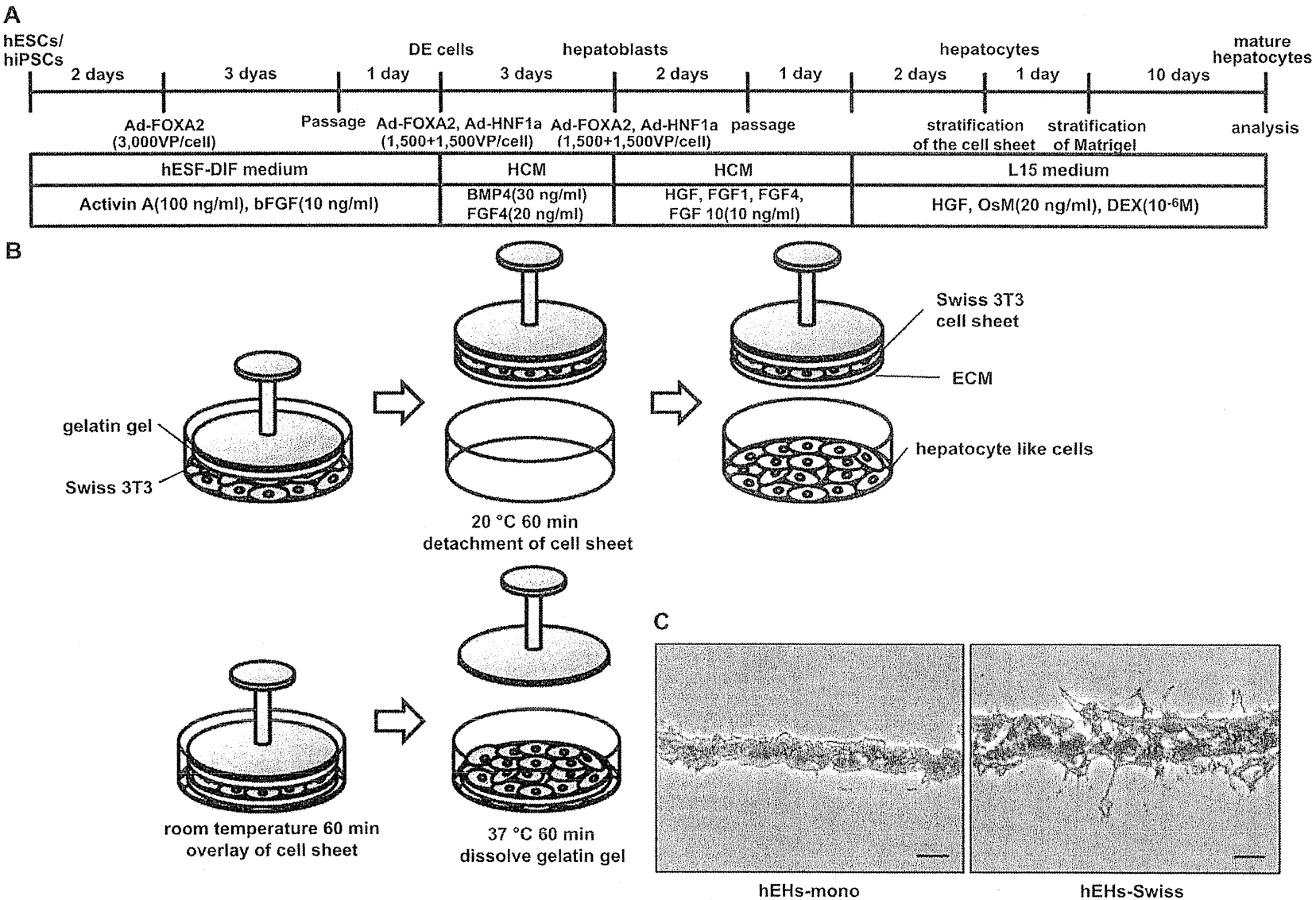

Fig. 1. Experimental protocol and schematic illustration of the procedure to stratify Swiss 3T3 cells on hepatocyte-like cells. (A) The procedure for hepatic differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) using stratification of the Swiss 3T3 cell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

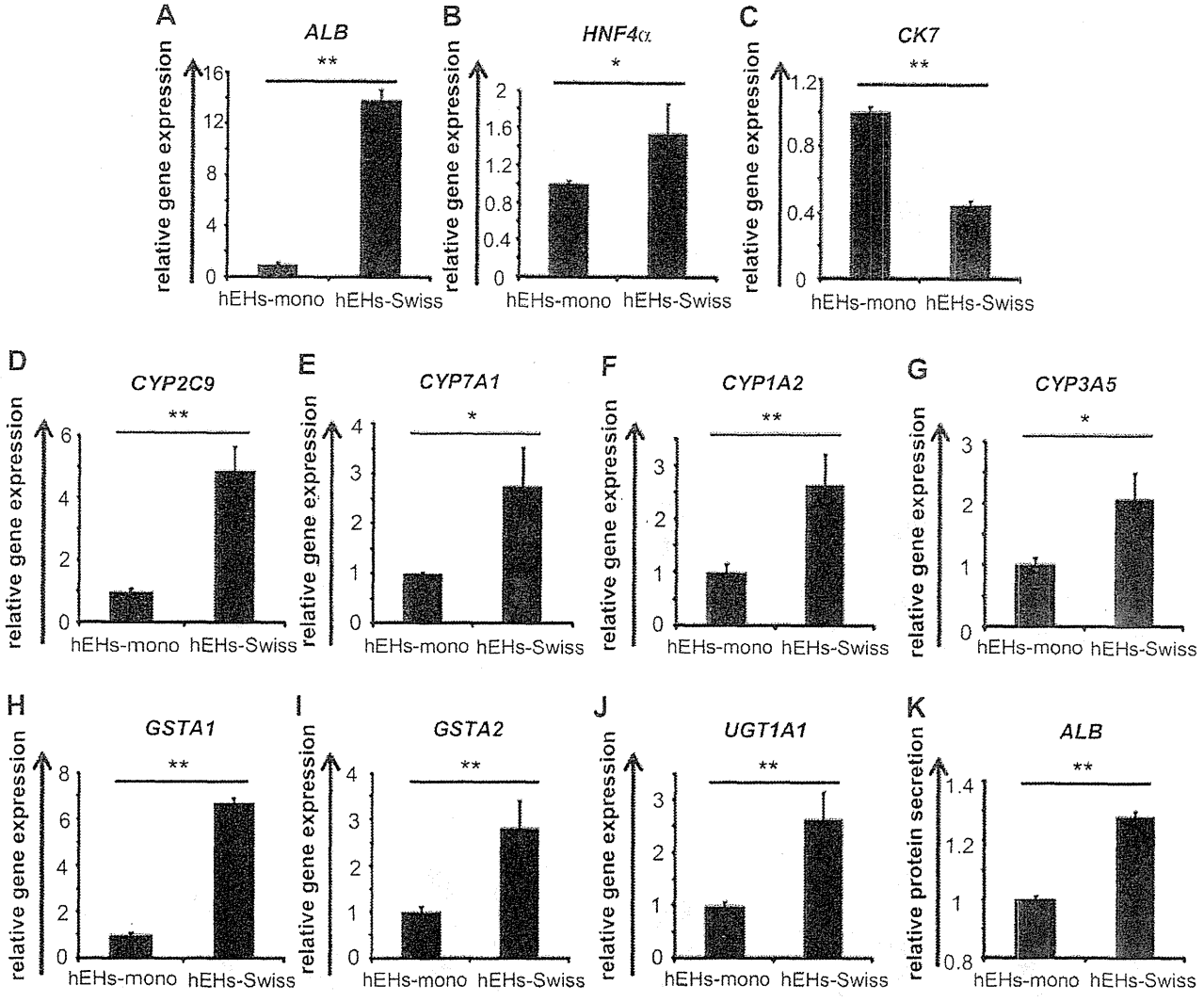

Fig. 2. Stratification of Swiss 3T3 cell sheet on hEHs promotes hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–K): On day 25, the gene expression levels of *ALB* (A), *HNF4A* (B), *CK7*(C), *CYP2C9* (D), *CYP7A1* (E), *CYP1A2* (F), *CYP3A5* (G),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

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

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

4. Discussion

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

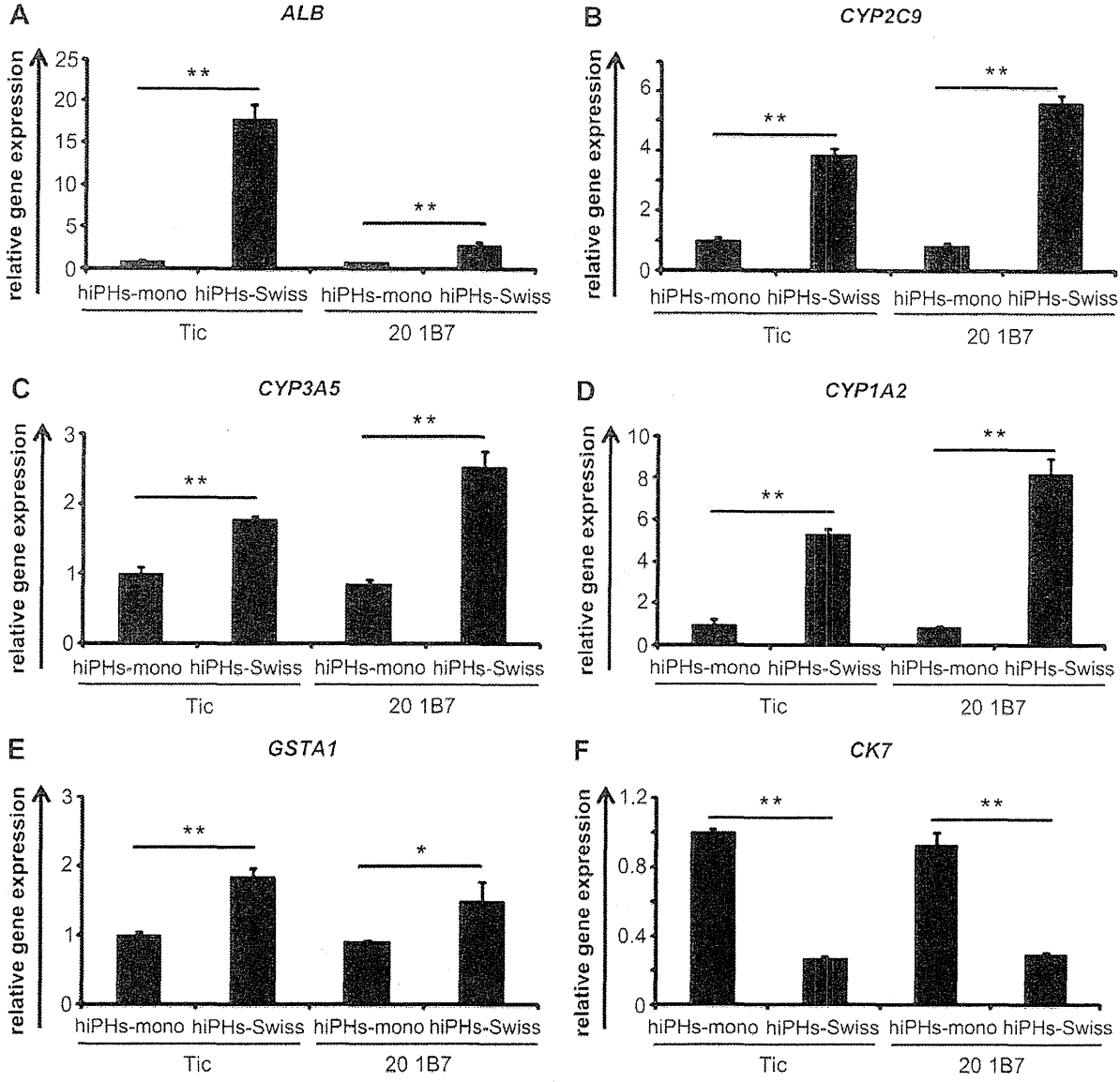

Fig. 3. Stratification of Swiss 3T3 cell sheet on hiPHs promotes hepatic maturation. Human induced pluripotent stem cells (hiPSCs) (Tic and 201B7) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–F): On day 25, the gene expression levels of *ALB* (A), *CYP2C9* (B), CYP3A5(C),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

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

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

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

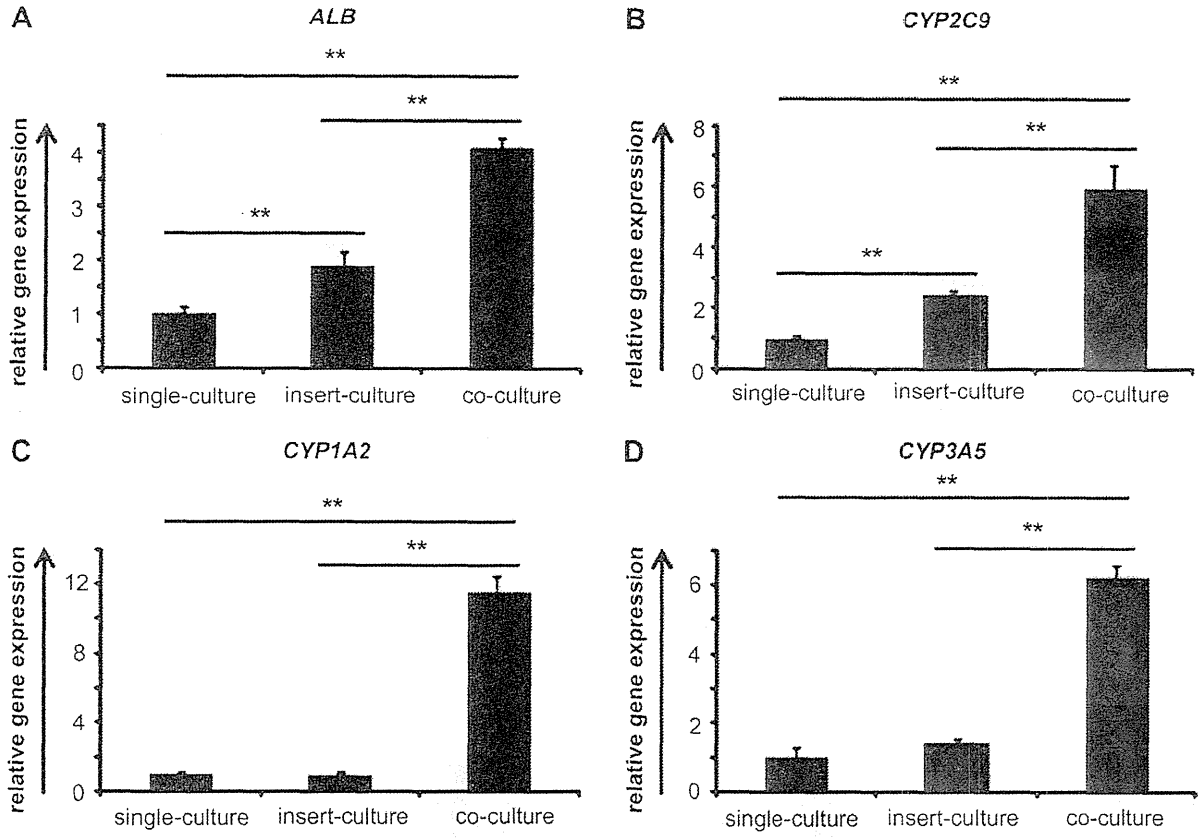

Fig. 4. Physical contacts between hESC-derived hepatocyte-like cells and Swiss 3T3 cells promote hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then the cells were differentiated into hepatocyte-like cells by single-culture, insert-culture,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

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

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

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

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

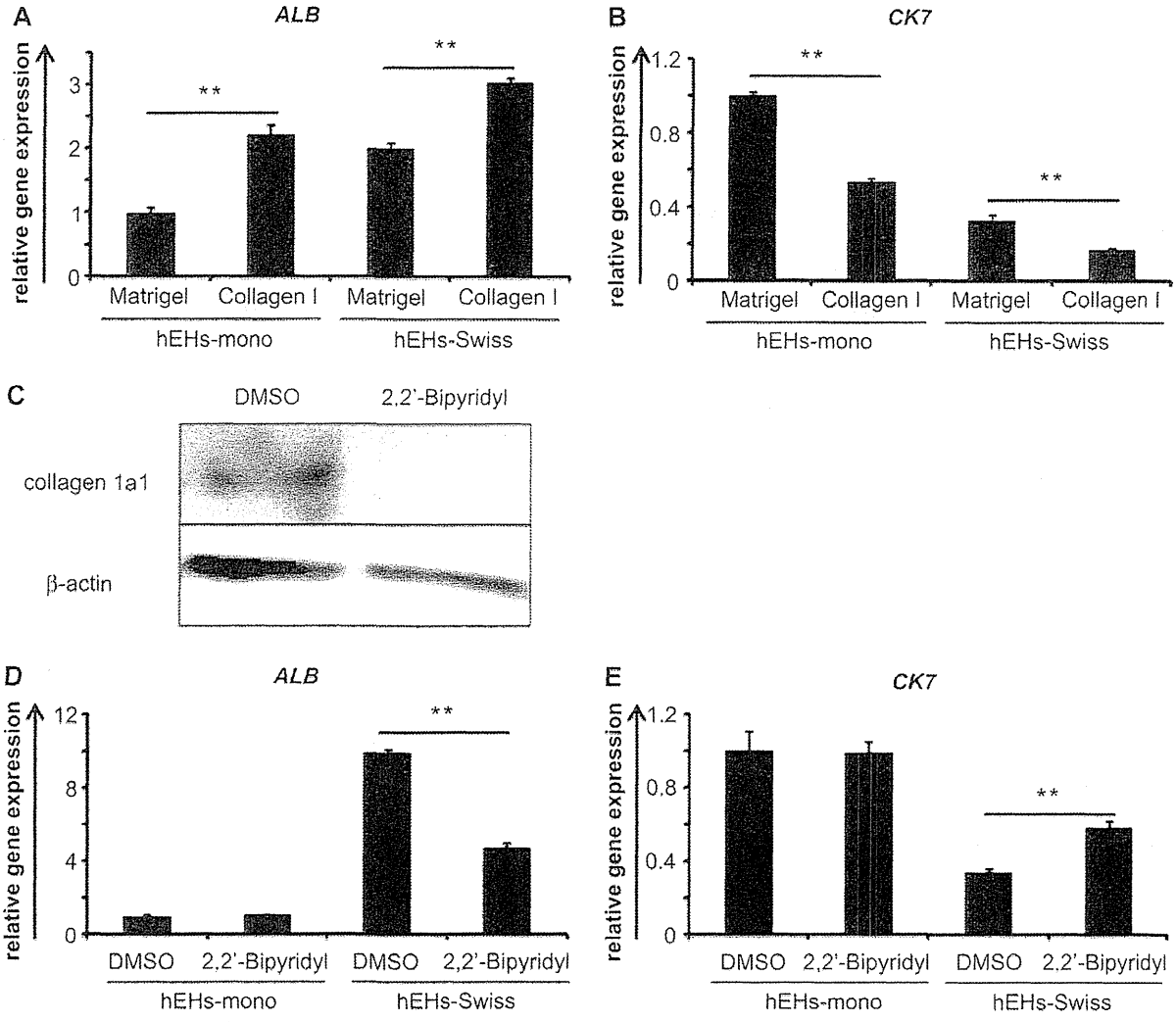

Fig. 5. Stratification of type I collagen gel promotes hepatic maturation. (A and B) hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then type I collagen gel (collagen I) or Matrigel are stratified on monolayer hESC-derived hepatocyte-like cells (hEHs-mono)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

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

5. Conclusions

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

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Appendix A. Supplementary data

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

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総　説

ヒト iPS 細胞から肝細胞への分化誘導の現状と創薬応用

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

Hiroyuki MIZUGUCHI[*1,2,3], Kazuo TAKAYAMA[*1], Yasuhito NAGAMOTO[*1], Kenji KAWABATA[*3]

1. はじめに

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

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

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

2. ヒト iPS 細胞由来肝細胞を用いた創薬研究

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

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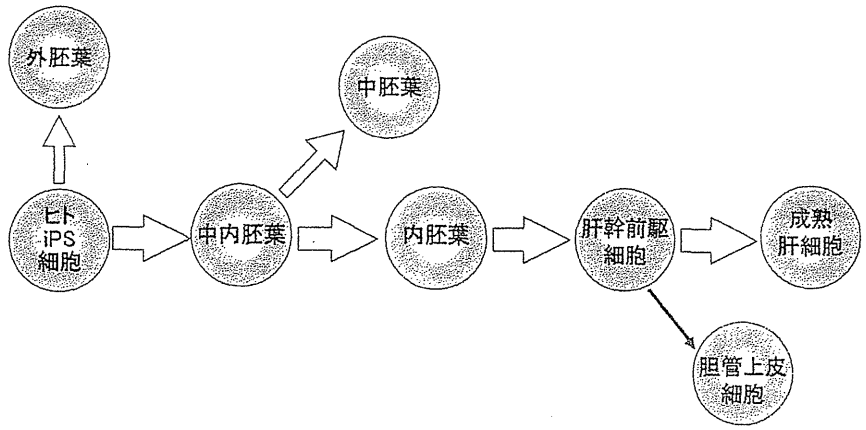

Fig. 1　ヒトiPS細胞から肝細胞への分化誘導
ヒトiPS細胞は中内胚葉，内胚葉，肝幹前駆細胞を経由して成熟した肝細胞へと分化する．

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

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

3.　ヒトiPS細胞から肝細胞への分化誘導

3.1　ヒトiPS細胞から肝細胞への分化誘導の現状

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

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

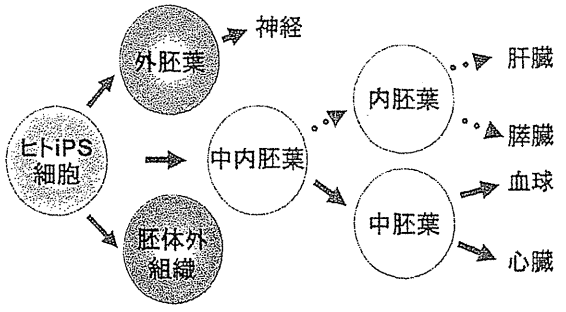

Fig. 2　ヒトiPS細胞から各胚葉への分化
神経細胞は外胚葉を，心筋細胞は中胚葉を，肝細胞や膵臓細胞は内胚葉を経由して分化する．

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

(1) の内胚葉への分化ステップでは，アクチビンAの

付加がほぼ全てのプロトコールで用いられており，アクチビンAに加えFGF2（fibroblast growth factor 2）やWnt3aを付加して分化誘導する方法も知られている．

(2)の内胚葉から肝幹前駆細胞への分化ステップには，BMP（bone morphogenetic protein）シグナルとFGF（fibroblast growth factor）シグナルが必要なことが判明しており，BMP4やFGF4などを付加する方法が汎用されている．また，肝細胞への方向付けにおいてはDMSO（dimethyl sulfoxide）によるヒストンのアセチル化が有効であることも知られており，DMSOを用いた方法も報告されている[4]．

(3)の肝幹前駆細胞から肝細胞への分化には，HGF（hepatocyte growth factor）やオンコスタチンM（OsM），デキサメタゾン（DEX）などを用いて分化誘導する方法が一般的である．更に各分化ステップで，培地や細胞外マトリックス（I型コラーゲンやマトリゲルが汎用される）の種類，血清やフィーダー細胞の有無等が各プロトコールで工夫されている．ヒトiPS細胞由来分化誘導肝細胞を再生医療に利用する場合には，血清やフィーダー細胞等の異種動物由来成分を排除し，かつ組成の明らかな培地（chemically defined mediumと呼ばれる）で分化誘導する必要があるが，同細胞を創薬研究に応用する場合にはそのような制限は必要ない．むしろ，創薬応用には可能な限り成熟度が高い肝細胞を分化誘導する必要があり，特に血清の付加は現時点では有用である（ただし，血清のロットチェックは必須である）．

以前は，胚様体（embryoid body: EB）形成法を用いて肝細胞への分化が試みられてきたが，最近では，EB形成を介さず，上述のように直接分化させる方法が一般的である．しかしながら，これらの増殖因子やサイトカインの添加だけからなる分化誘導法は，肝細胞への分化効率もまだまだ不十分なのが現状であり，更なる分化効率の向上が必要となっている．

3.2 分化ステージに応じた最適な転写因子の過剰発現を組み合わせたヒトiPS細胞から肝細胞への高効率分化誘導

著者らは，付加する増殖因子やサイトカインを単に最適化しただけの分化誘導法の改良では，劇的なヒトiPS細胞から肝細胞への分化効率の向上が期待できないのではないかと考え，個々の分化ステップの細胞に（肝細胞への分化に）適した転写因子を一過性に過剰発現させることで，効率よく肝細胞への分化を誘導する方法を開発した（Fig. 3）．すなわち，増殖因子やサイトカインを付加した従来の方法で細胞の外部環境を分化に適した状態にした上に，細胞内部から強制的に分化を生じさせるように適切な転写因子を発現させることで，分化効率を飛躍的に向上させる方法を考案した．

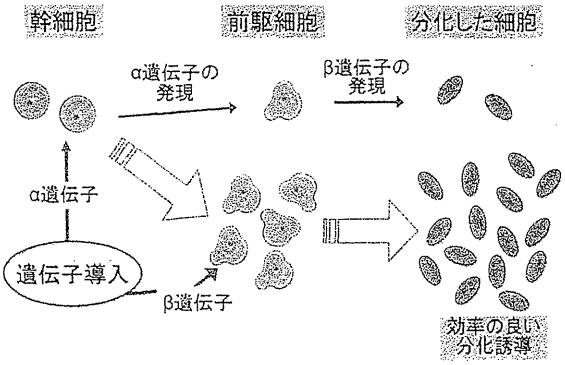

Fig. 3 機能遺伝子の導入による分化誘導効率の向上
適切な分化状態の細胞に効率よくかつ一過性に機能遺伝子を発現させることにより，目的の機能細胞を効率よく分化誘導することが期待できる．

当初は，未分化iPS細胞からアクチビンA処理で分化させた中内胚葉にSOX17（Sry-related HMG box 17）遺伝子を，内胚葉から肝幹前駆細胞への分化ステップではHEX（hematopoietically expressed homeobox）遺伝子を，肝幹前駆細胞から肝細胞への分化ステップではHNF4α（hepatocyte nuclear factor 4α）遺伝子を導入することで，高いアルブミン産生能や薬物代謝機能を有した肝細胞を効率よく分化誘導することに成功した[5-7]．更に最近では，ヒトES/iPS細胞から肝細胞への各分化ステップにおいて7種類の肝関連転写因子（FOXA2, SOX17, HEX, HNF1α, HNF1β, HNF4α, HNF6）を導入し，最も効率良く肝分化を促進できる転写因子を探索した結果，FOXA2（forkhead box protein A2）及びHNF1α（hepatocyte nuclear factor 1α）遺伝子を組み合わせて発現させることにより，更に効率良く成熟肝細胞を分化誘導することに成功した（Fig. 4）[8]．

このようにして作製した肝細胞は，80～90%以上の細胞がアルブミン，アシアロ糖タンパク質受容体，LDL（low density lipoprotein）取り込み能，インドシアニングリーン取り込み能，薬物代謝酵素（シトクロムP450 3A4, CYP7A1, CYP2D6等）陽性であり，ヒト初代培養肝細胞に匹敵する薬物代謝酵素の遺伝子発現レベルを示した．また，シトクロムP450酵素などで代謝される9種類の薬物の代謝プロファイルを調べたところ，ヒトiPS細胞由来分化誘導肝細胞の薬物代謝能はヒト初代培養肝細胞より低いものの（シトクロムP450酵素の種類により異なるが，ヒトiPS細胞由来分化誘導肝細胞はヒト初代培養肝細胞の1～40%程度の活性），いずれの薬物に対しても代謝能を有していることが確認された．各

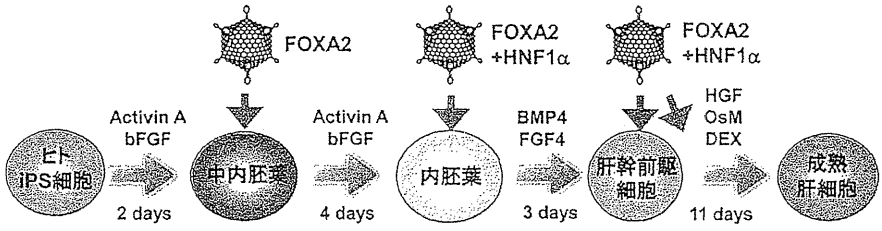

Fig.4　液性因子と転写因子の導入を組み合わせることによるヒトiPS細胞から肝細胞への高効率分化誘導

ヒトiPS細胞をアクチビンAで培養することによって得られた培養3日目の中内胚葉に対して，FOXA2発現アデノウイルスベクターを作用させた．更に，アクチビンAで4日間培養した後，培養6日目の内胚葉に対してFOXA2及びHNF1α発現アデノウイルスベクターを作用させた．BMP4とFGF4を用いて3日間培養した後，培養9日目の肝幹前駆細胞に対してFOXA2及びHNF1α発現アデノウイルスベクターを作用させた．その後，肝幹前駆細胞をHGF，オンコスタチンM（OsM），デキサメタゾン（DEX）を用いて11日間培養することによって（培養12日目にFOXA2及びHNF1α発現アデノウイルスベクターを更に作用），高い薬剤代謝機能やアルブミン産生能等を有した肝細胞へ分化させることができる．

シトクロムP450酵素の遺伝子発現と代謝能との間に，ヒトiPS細胞由来分化誘導肝細胞とヒト初代培養肝細胞で乖離が認められたが，この原因としては，そもそもシトクロムP450酵素の活性は個人差が大きいことが知られており（数十倍～千倍程度の個人差），用いたヒトiPS細胞が低いシトクロムP450酵素活性の個人から樹立されていた可能性や，シトクロムP450酵素の活性発現に必要な補酵素群の発現が未だ分化誘導肝細胞では十分でないこと等が考えられた．今後，異なった個人から樹立したヒトiPS細胞由来分化誘導肝細胞を用いて，同様の検討する必要があるであろう．

一方，作製したヒトiPS細胞由来分化誘導肝細胞を用いて，薬剤に対する毒性評価についても検討した（論文投稿中），肝毒性を生じることが知られている多種類の薬剤について，本分化誘導肝細胞を用いて細胞毒性評価試験を行ったところ，株化細胞であるHepG2細胞を用いた場合に比べ，より感度良く毒性（細胞傷害性）を示し，かつその毒性はシトクロムP450酵素の阻害剤を加えると部分的に消失した．したがって，シトクロムP450酵素で代謝された代謝物（反応性代謝物）によって生じた細胞傷害性を，分化誘導肝細胞が検出できることが明らかとなった．反応性代謝物は薬物性肝障害の主な原因と考えられており，ヒトiPS細胞由来分化誘導肝細胞で反応性代謝物による細胞傷害性を検出できたことは，極めて大きな意義をもつと考えられる．以上のことから，FOXA2及びHNF1α遺伝子を導入することにより，ヒトiPS細胞から薬物代謝能を有する肝細胞を効率良く分化誘導できるだけでなく，同細胞が薬物の毒性スクリーニングに使用可能であることが示唆された．

なお，細胞分化の各ステップでの転写因子（遺伝子）の導入には，機能性に優れ，独自開発した改良型アデノウイルスベクターを用いた．iPS細胞から肝細胞への分化のように，分化の各ステップが階層的に起こる場合には，各分化ステップでだけ導入遺伝子が機能するように（後の細胞分化に影響を与えないように），遺伝子発現期間は一過性であること，そして効率よく細胞集団を分化させるためには，100%の遺伝子発現効率で遺伝子発現させることが必須となるが，改良型アデノウイルスベクターはこのような目的に唯一叶うベクターである．本研究で用いた改良型アデノウイルスベクターは，細胞への感染に関与するウイルス表面タンパク質のファイバータンパク質のC末端領域にポリリジン配列（KKKKKKK：リジン（K）が7つ続くのでK7と略称）を遺伝子工学的に付与しており，細胞表面のヘパラン硫酸を認識して多くの細胞種に効率よく遺伝子導入が可能となる（Fig. 5）．本K7型アデノウイルスベクターは，未分化ヒトiPS細胞や，ヒトiPS細胞から分化した細胞に対しても，100%の効率で遺伝子導入が可能であった[9]．

著者らは，機能面で優れた様々なアデノウイルスベクターを開発しており，詳細は文献[9]を参照されたい．

3.3　3次元培養によるヒトiPS細胞由来分化誘導肝細胞の成熟化

ヒト初代培養肝細胞は，培養すると急速に肝細胞特異的な性質が失われていくことが知られている．例えば，アルブミンやシトクロムP450酵素の遺伝子発現は，最適化された培養条件で培養しても，48時間も培養すると，解凍（凍結肝細胞の場合）直後の遺伝子発現と比較すると10～100分の1程度にまで低下する．一方で，スフェロイド培養等の3次元培養や，繊維芽細胞や血管

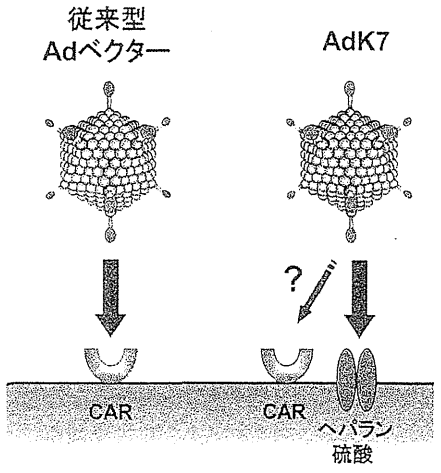

Fig.5 改良型アデノウイルスベクターの遺伝子導入特性
従来のアデノウイルス(Ad)ベクターはCAR(coxsackievirus adenovirus receptor)を認識して感染する．ポリリジン配列をファイバータンパク質のC末端領域に遺伝子工学的に付与したアデノウイルスベクター（AdK7）は，多くの細胞で発現しているヘパラン硫酸を認識して感染できるため，CAR陰性の細胞を含む多くの細胞への高効率な遺伝子導入が可能となる．

内皮細胞との共培養系でヒト初代培養肝細胞を培養すると，アルブミンやシトクロムP450酵素等の肝特異的な機能の減弱は，ある程度抑制されることが知られている．

そこで，細胞シート工学技術を用いることで，シート状に回収したSwiss3T3細胞とヒトiPS細胞から分化誘導した肝細胞とを積層3次元共培養し，肝機能の向上が可能か検討した（東京女子医大・先端生命医科学研究所大橋一夫先生，岡野光夫先生との共同研究）[10]．その結果，単層のヒトiPS細胞由来分化誘導肝細胞と比較し，肝細胞特異的な遺伝子発現量やアルブミン分泌量が有意に増加することが明らかとなった．また，ヒトiPS細胞由来分化誘導肝細胞の成熟化には，肝細胞とSwiss3T3細胞との物理的な接触が重要であることを見出した．更に，ヒトiPS細胞由来分化誘導肝細胞へ，1型コラーゲンゲルを重層することにより肝細胞成熟化が促進される一方で，コラーゲン合成阻害剤存在下においてはSwiss3T3細胞との積層3次元共培養時の成熟化が抑制されたことから，Swiss3T3細胞が産生する1型コラーゲンが肝細胞成熟化を担う主要な因子の1つであることが明らかとなった．最近では，簡便に3次元培養が可能な基材が各社から販売されており，培養法の改良によってもヒトiPS細胞由来分化誘導肝細胞の成熟化亢進が期待できる．

3.4 Direct-reprogrammingによる肝細胞への直接分化

近年，繊維芽細胞等の分化した細胞から，iPS細胞を介さずに，直接他の細胞に分化を誘導するDirect-reprogrammingに関する研究がトピックスとなっている．

古くは，膵臓細胞を肝細胞に分化誘導した研究（2000年）や，B細胞をマクロファージに分化誘導した研究（2004年）があるが，2008年以降，膵β細胞や神経細胞，心筋細胞，肝細胞等を，通常複数の転写因子を発現する遺伝子を導入して，繊維芽細胞から直接分化誘導した研究が相次いでいる．肝細胞についても，マウスの系であるが，繊維芽細胞からのDirect-reprogrammingの報告がある[11, 12]（ヒト細胞を用いた肝細胞へのDirect-reprogrammingについてはまだ報告例はない）．iPS細胞から分化誘導した細胞同様に，Direct-reprogrammingによって得られた細胞（肝細胞を含む）も，創薬研究に有用なツールとなる可能性はあるが，重要なのは最終的に得られる分化細胞の“分化度”と，分化細胞を大量供給できるか？という観点であり，この2点が満たされれば，iPS細胞から分化させたのか，あるいはDirect-reprogrammingであるのかは問題ではない．

分化細胞の大量供給という観点では，Direct-reprogrammingによって終末分化した細胞に直接分化させた場合には，通常，細胞は増殖能を失うことから大量供給は難しく，その前駆細胞を分化誘導する方が有用かもしれない．その場合，前駆細胞を成熟細胞に分化させる技術が必要になり，iPS細胞から目的細胞への分化誘導研究は，この過程での技術開発にも役立つことが期待される．

4. おわりに

従来のヒトES/iPS細胞から分化誘導させた肝細胞は，機能面において初代培養肝細胞に比べて大きく劣っており，創薬研究への応用は困難であった．しかしながら，著者らが開発した分化誘導法により，創薬応用に向けて，ようやく最低限の解析が可能なレベルにまで分化した肝細胞を得ることが可能になった．

本稿では触れなかったが，著者らが分化誘導した肝細胞は，C型肝炎ウイルスに対する感染能も有しており[13]，肝炎研究のための有力な培養モデル系にもなる（同様な報告が最近，海外のグループからも報告された[14-16]）．一方で，ヒトiPS細胞由来分化誘導肝細胞を幅広く創薬研究に応用するためには，実験毎に3週間に及ぶ分化誘導を行うことは細胞供給の観点から効率が悪い．そこで現

在著者らは，分化途中の肝幹前駆細胞の段階で，分化細胞を大量に増幅できないかという課題にも取り組んでいる．今度，より一層高機能な（成熟度が高い）ヒトiPS細胞由来分化誘導肝細胞の作製法の開発（改良）を進めるとともに，本分化誘導肝細胞が創薬研究で広く活用されることを期待している．

なお，本稿で紹介した分化誘導法で作製されたヒトiPS細胞由来分化誘導肝細胞は，リプロセル社よりReproHepatoとして市販されている．

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特 集　iPS細胞と神経疾患

ヒトiPS細胞の再生医療および創薬研究への応用の現状と展望

Perspectives Regarding the Potential Use of Human Induced Pluripotent Stem Cells for the Development of and Research on Medicinal Products

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Takao Hayakawa[1)], Hiroyuki Mizuguchi[2,3)]

Abstract

Human induced pluripotent stem cells (hiPSCs) are expected to be used in various life science areas, ranging from basic research to medical applications. This article describes perspectives regarding the potential use of hiPSCs, especially in Japan, for manufacturing products related to regenerative medicine as well as for establishing cell-based assay/screening systems that can be used for effective and efficient assessment of candidates for new drugs. The applications of hiPSCs include the following: hiPSC-derived retinal pigment epithelial cells for treating age-related macular degeneration; potential corneal reconstruction by using a combination of various relevant hiPSC-derived differentiated cells; potential treatment of Parkinson's disease by using dopaminergic neurons generated from hiPSCs; potential treatment of spinal cord injury by using neural stem/progenitor cells generated from hiPSCs; potential treatment of chronic heart failure by using hiPSC-derived functional cardiomyocytes; and development of cell-based drug toxicity screening and drug effect assay systems involving cells such as cardiomyocytes, hepatocytes, and neural cells that are differentiated from hiPSCs and can be used in the early phase of new drug development. The current situation regarding the development of guidelines for ensuring the quality and safety of hiPSC-derived medicinal products has also been described.

Key words : human induced pluripotent stem cells, regenerative medicine, cell-based medicinal products, cell-based drug assay/screening system, regulatory guidelines

はじめに

2007年にヒト人工多能性幹細胞（human induced pluripotent stem cells：hiPScs：以下，ヒトiPS細胞）が山中らにより発明されて以来早5年が経過した。これは，分化したヒト細胞をリプログラミング（初期化）できることを示したものであり，ヒト細胞の分化・脱分化が人為的に自在に操作できる可能性を示唆する金字塔である。その活用により，生命現象解明のための基礎研究，病因や発症機構解明などの医学研究，毒性・薬効評価系確立などを通した創薬研究，さらに再生医療の実用化にも無限の可能性が拓かれた。

ヒトiPS細胞は，胚性幹（embryonic stem：ES）細胞と異なり倫理的な問題が少なく，また自己iPS細胞由来の製品では，ドナーとレシピエントが同一人であり，移植した場合の拒絶反応の回避が期待できるなど，再生医療のための素材として大きな脚光を浴びている。ヒト

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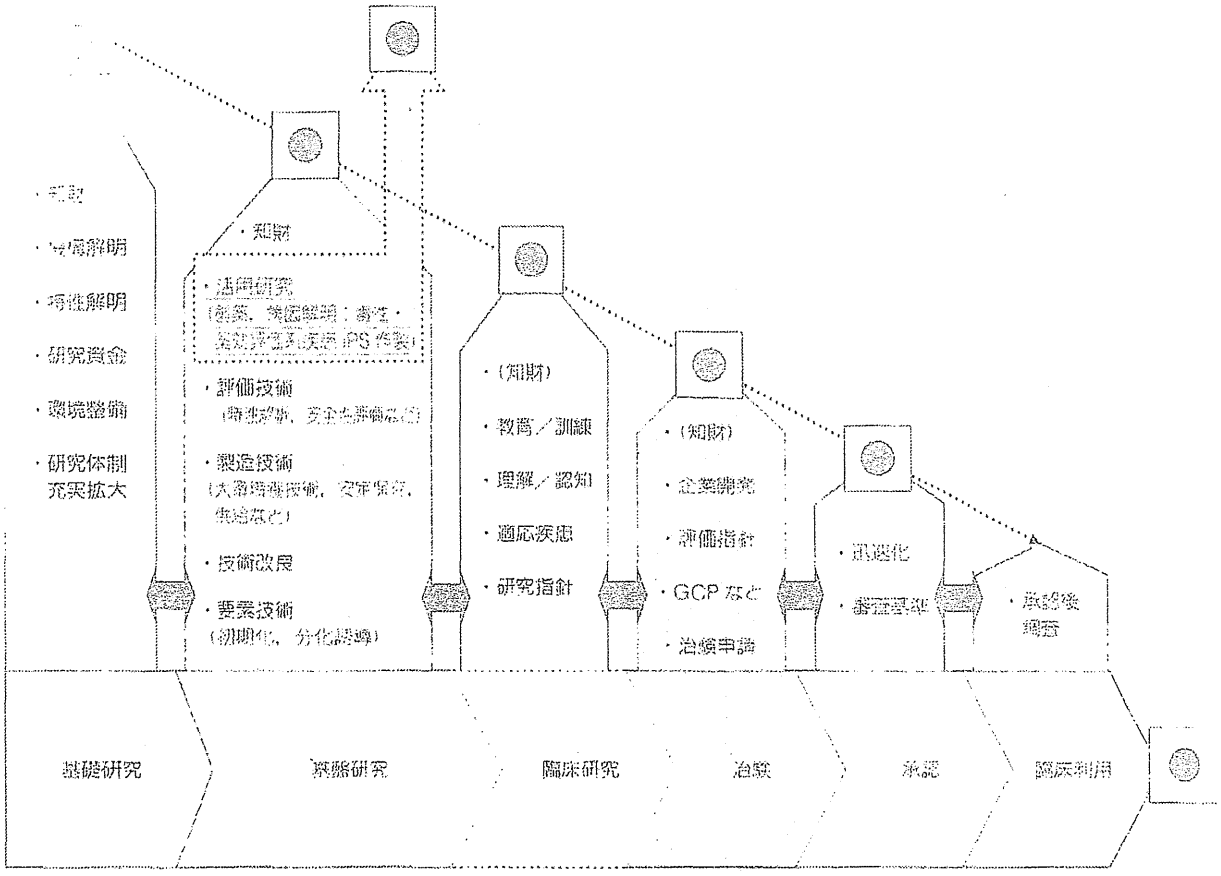

Fig. 1　ヒト iPS 細胞の再生医療，創薬研究への応用に必要な段階と要素

・各ベクトルにおいて世界をリードするためのオールジャパン体制の構築。
・各ベクトルの成果物を相互活用し，効率的，効果的，相乗的に各要素を進展させるためのオールジャパン体制の構築。
・臨床利用に各要素が最も有効に活用されるには，臨床目的や最終製品とそれに至る過程をイメージしながら個々の研究・開発を進めることが肝要。
・行政指針の役割は，最も効率的，効果的に所定の目標に達するための要素と方策の提示。当初は基本的留意事項の提示。各研究の進展や製品化に合わせ充実を図る。

iPS 細胞による再生医療の本格的な実用化には時間を要すると思われるが，一部では着実な進展もみられている。一方，創薬研究のため iPS 細胞に由来する細胞アッセイ系を活用しようとするアプローチは，再生医療用製品におけるような安全性の観点からの課題を多くの場合考慮に入れる必要はなく，より早期の実用化が期待されている。本稿では，主にわが国での再生医療および創薬研究（医薬品開発研究）へのヒト iPS 細胞の応用に関する現状と今後の展望について解説する。

1．ヒト iPS 細胞由来製品を用いた再生医療

ヒト iPS 細胞を用いた再生医療に関連して実用化の重要として研究面でヒト iPS 細胞から神経細胞を含め各種分化細胞を作製したという報告は多数ある。これらがヒトに適用されるまでには，Fig. 1 および Fig. 2 に示したような多くのステップと検討が必要であり，ヒト iPS 細胞由来製品がヒトに適応された例はいまだない。ここでは，実用化に向けてわが国で進捗がみられる事例のいくつかについて現状と展望を以下に紹介する。

1．ヒト iPS 細胞から作製した網膜色素上皮細胞の加齢黄斑変性症への適応

ヒトへの臨床適用が最も早いと予測されるのが，理化学研究所の高橋政代博士のグループによるヒト iPS 細胞から網膜色素上皮（retinal pigment epithelial：PRE）細胞を作製し，加齢黄斑変性症に適応しようとする試みである。加齢黄斑変性は，高齢者の視力低下の主原因であるが，現存の治療法では疾患の沈静化や視力の回復が得られる症例は限定的であり，大多数の症例は線維性瘢

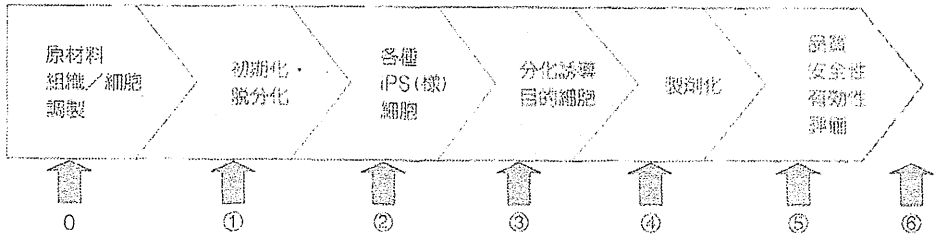

Fig. 2　ヒト iPS（様）細胞加工医薬品等の製造，評価のポイント
①初期化／脱分化（遺伝子導入もしくは今後開発されるかも知れない別の手法X）
①増殖因子などによる培養，細胞大量培養
②細胞株樹立，細胞のバンク化，品質維持・管理，安定供給，細胞大量培養
②初期化／脱分化細胞［iPS（様）細胞］の特性解析（identity，purity，potency），安定性，安全性など
③目的細胞への確実な分化誘導条件（分化誘導剤，培地，培養条件など）
③分化した目的細胞の特性解析（特異的細胞マーカ，細胞の不均一性・純度，造腫瘍性など）
④目的細胞の製剤化，非細胞成分との組み合わせなど
⑤目的製品の安定性を含む品質特性解析，非臨床／臨床試験による安全性・有効性評価（臨床研究v.s. 治験），安全性上の関心事：異所性の組織形成，不適切な分化／造腫瘍性，目的外の表現型発現，免疫拒絶反応
⑥製造販売承認後の品質管理，安全性モニターなど

痕の除去とともに網膜色素上皮の再生さらには視細胞の再生が必要とされている。

本アプローチの根拠となる実験結果（proof of concept）は霊長類のES細胞を用いて疾患モデルの治療効果を世界で初めて確認したことに遡る[1]。細胞移植に治療効果があることは，胎仔細胞のPRE障害モデル動物への移植などから確認されている[2]。しかし，他家移植であるES細胞由来PRE細胞の場合，拒絶反応が起こることも含めて自己iPS細胞由来PREに比べてリスクが高いことから後者を利用する臨床応用が目指すこととなった。

その基盤となる研究として，最近，高橋らは，iPS細胞から，生体内の細胞と同様の機能（貪食機能）を持つRPE細胞を分化誘導することに成功した。また，iPS細胞から得られたRPE細胞から，タイトジャンクションを持った細胞シートを作製することにも成功している[3,4]。そして，ヒト皮膚から誘導したiPS細胞をもとに(1)生体内で機能する成熟した細胞を，(2)移植して機能回復に必要な量を通常の培養で，(3)100%純化した状態で得ることができるに至っているという。このように(1)質（成熟度），(2)量，(3)純度の3点を満たす細胞種は他には存在しないため，PRE細胞が最も臨床応用に近い細胞といえる。

詰めの作業として，(i)宿主細胞のゲノムに組み込まれる恐れのないベクターを用いてiPS細胞を樹立する技術の確認[5]，(ii)適度な強度と生体内と同じ組成の基底膜を有し，移植に適した柔軟性を持ったRPE細胞のシートを製造する技術の確認，(iii)従来の分化誘導法の改良により高純度のPRE細胞の集団を取得する技術の改良・開発，(iv)非臨床安全性試験の実施，(v)生物由来成分の適合性の確認を含む製造工程の確立などを進めつつあり，平成24年（2012年）度中には臨床研究（first-in-human）を開始する予定と聞く。シナリオどおりだと世界初のヒトiPS細胞由来製品の臨床研究となる可能性が高い。

本細胞移植は，移植細胞が1度生着すれば生涯にわたり効果を発揮すると考えられ，また技術が確立すれば段階を経てより早期の患者に適応を広げることにより広範な患者が恩恵を受ける新たな医療となると期待されている。

2．ヒトiPS細胞を用いた角膜全層［…］皮）の再生治療法

現在のところ角膜疾患のため重篤な［…］患者に対しては献眼に依存した［…］る。しかし，ドナー角膜が［…］界における待機患者約1［…］万眼/年）。また，重篤な角［…］膜移植が奏功しない，そこ［…］法が期待されている。

角膜は上皮、［…］が国では，大［…］の大和雅之教［…］口腔粘膜［…］