

**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, Paclitazell, 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 $\alpha$ -hydroxypaclitaxel [OHPCT], Hydroxytolbutamide [OHTB], 4'-hydroxymephenytoin [OHMP], 1'-hydroxybufuralol [OHBF], 1'-hydroxymidazolam [OHMDZ], 6 $\beta$ -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 [ $\alpha$ 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 $\alpha$ -transduced cells were significantly higher than those of Ad-SOX17-, Ad-HEX-, and Ad-HNF4 $\alpha$ -transduced cells. These results indicated that FOXA2 and HNF1 $\alpha$  transduction promotes more efficiently hepatic differentiation than SOX17, HEX, and HNF4 $\alpha$  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 $\alpha$ . Taken together, endogenous hepatocyte-related genes in hiPSC-hepa should have been upregulated by FOXA2 and HNF1 $\alpha$  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  $\beta$ -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, Paclitazell, 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 $\alpha$  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 $\alpha$  transduction strongly promoted hepatic commitment and maturation (Fig. 1F and J), although in the stage of hepatic expansion and maturation, HNF4 $\alpha$  transduction was as efficient as that of HNF1 $\alpha$  (Fig. 1J). Since HNF1 $\alpha$  is one of the target genes of HNF4 $\alpha$  [13], the signaling through HNF4 $\alpha$  to HNF1 $\alpha$  would be important for efficient hepatic expansion and maturation. Considering these results together, we ascertained a pair of two transcription factors, FOXA2 and HNF1 $\alpha$ , that could promote efficient hepatic differentiation from hESCs. In embryogenesis, the expression of FOXA2 and HNF1 $\alpha$  is initially detected in DE or hepatoblasts, respectively and the expression levels of both FOXA2 and HNF1 $\alpha$  are elevated as the liver develops [14,15]. Therefore, our hepatic differentiation technology, which employs FOXA2 and HNF1 $\alpha$  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 $\alpha$  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 HNF1 $\alpha$  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.

### Acknowledgements

We thank Misae Nishijima, Nobue Hirata, Miki Yoshioka, and Hiroko Matsumura for their excellent technical support. We thank Ms. Ong Tyng Tyng for critical reading of the manuscript. HM, MKF, and TH were supported by grants from the Ministry of Health, Labor, and Welfare of Japan. HM was also supported by Japan Research foundation For Clinical Pharmacology, The Nakatomi Foundation, and The Uehara Memorial Foundation. K. Kawabata was supported by Grants from the Ministry of Education, Sports, Science and Technology of Japan (20200076) and the Ministry of Health, Labor, and Welfare of Japan. K. Katayama and FS were supported by Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

### Supplementary data

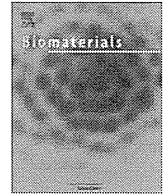
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2012.04.038>.

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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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### ARTICLE INFO

#### Article history:

Received 16 February 2012

Accepted 3 March 2012

Available online 23 March 2012

#### Keywords:

Hepatocyte

Co-culture

Collagen

Fibroblast

Liver

ECM (extracellular matrix)

### ABSTRACT

Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) are known to be a useful cell source for drug screening. We recently developed an efficient hepatic differentiation method from hESCs and hiPSCs by sequential transduction of FOXA2 and HNF1 $\alpha$ . 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 $\alpha$ ) (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  $\mu$ g/ml), and penicillin (200  $\mu$ g/ml).

### 2.3. Ad vectors

The human eukaryotic translation elongation factor 1 alpha 1 (EF-1 $\alpha$ ) promoter-driven HNF1 $\alpha$ - and FOXA2-expressing Ad vectors (Ad-HNF1 $\alpha$  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  $\mu$ g/ml human recombinant insulin, 5  $\mu$ g/ml human apotransferrin, 10  $\mu$ M 2-mercaptoethanol, 10  $\mu$ M ethanolamine, 10  $\mu$ M sodium selenite, and 0.5 mg/ml bovine serum albumin (BSA) (all from Sigma) (differentiation hESF-DIF medium) containing 100 ng/ml Activin A (R&D Systems) and 10 ng/ml FGF2. To generate DE cells, hESC- or hiPSC-derived mesendoderm cells were transduced with 3000 VP/cell of Ad-FOXA2 for 1.5 h on day 2 and cultured until day 6 on Matrigel in differentiation hESF-DIF medium supplemented with 100 ng/ml Activin A and 10 ng/ml FGF2. For induction of the hepatoblasts, the hESC- or hiPSC-derived DE cells were transduced with each 1500 VP/cell of Ad-FOXA2 and Ad-HNF1 $\alpha$  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 $\alpha$  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  $\mu$ M hydrocortisone 21-hemisuccinate (Sigma), 1  $\mu$ M insulin, and 25 mM NaHCO<sub>3</sub> (Wako) (differentiation L15 medium) containing 20 ng/ml hepatocyte growth factor (HGF), 20 ng/ml Oncostatin M (OsM) (R&D Systems), and 10<sup>-6</sup> 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<sup>-6</sup> 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<sup>-6</sup> 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  $\mu\text{m}$ ; 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  $\mu\text{M}$  2,2'-Bipyridyl (Wako), an inhibitor of collagen synthesis, until day 25.

### 2.12. Western blotting analysis

Swiss 3T3 cells were cultured with 25  $\mu\text{M}$  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-coll1a1 Ab (diluted 1/200; Santa Cruz Biotechnology) or mouse anti- $\beta$ -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 $\alpha$*  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

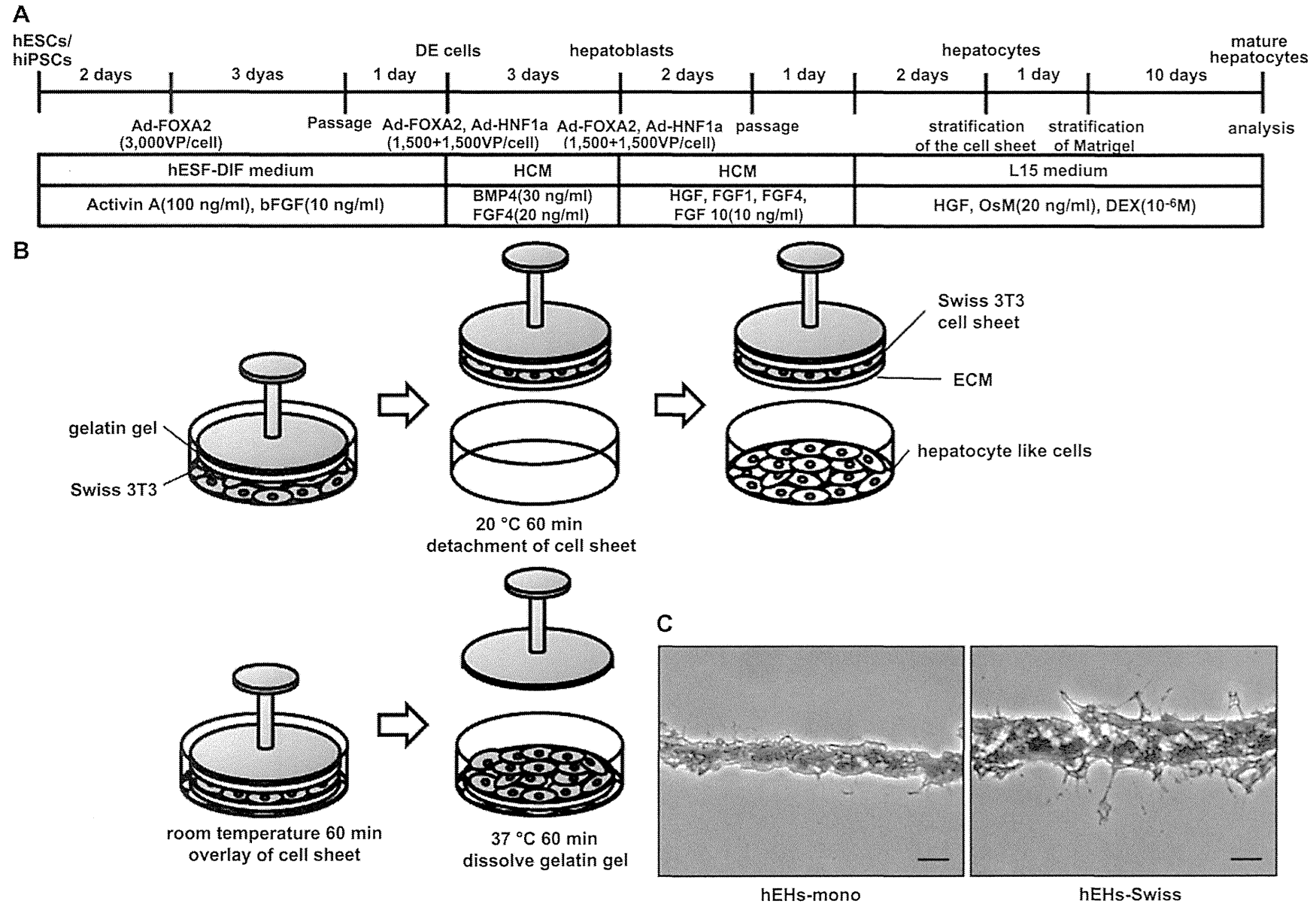
the stratification of the Swiss 3T3 cell sheet is illustrated in Fig. 1A. The stratifying procedure was performed on day 14 as described in Fig. 1B. The day after stratifying the Swiss 3T3 cell sheet on the hEHs, vertical sections of the monolayer hEHs (hEHs-mono) and the hEHs stratified with the Swiss 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.

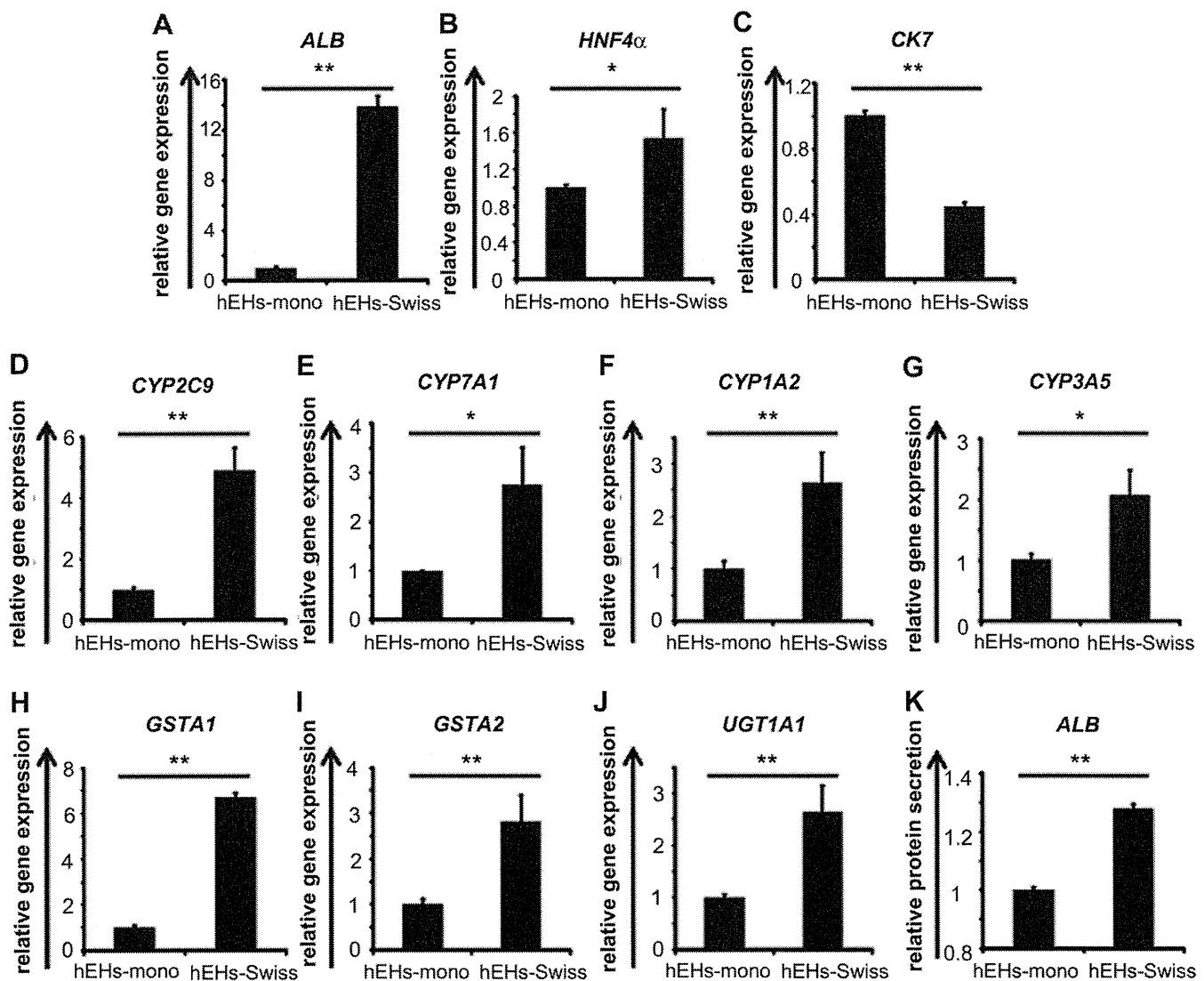
### 3.2. Identification of maturation factors synthesized from Swiss 3T3 cells

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 procedure 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  $\mu$ 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) ( $n = 3$ ). \* $P < 0.05$  \*\* $P < 0.01$ .

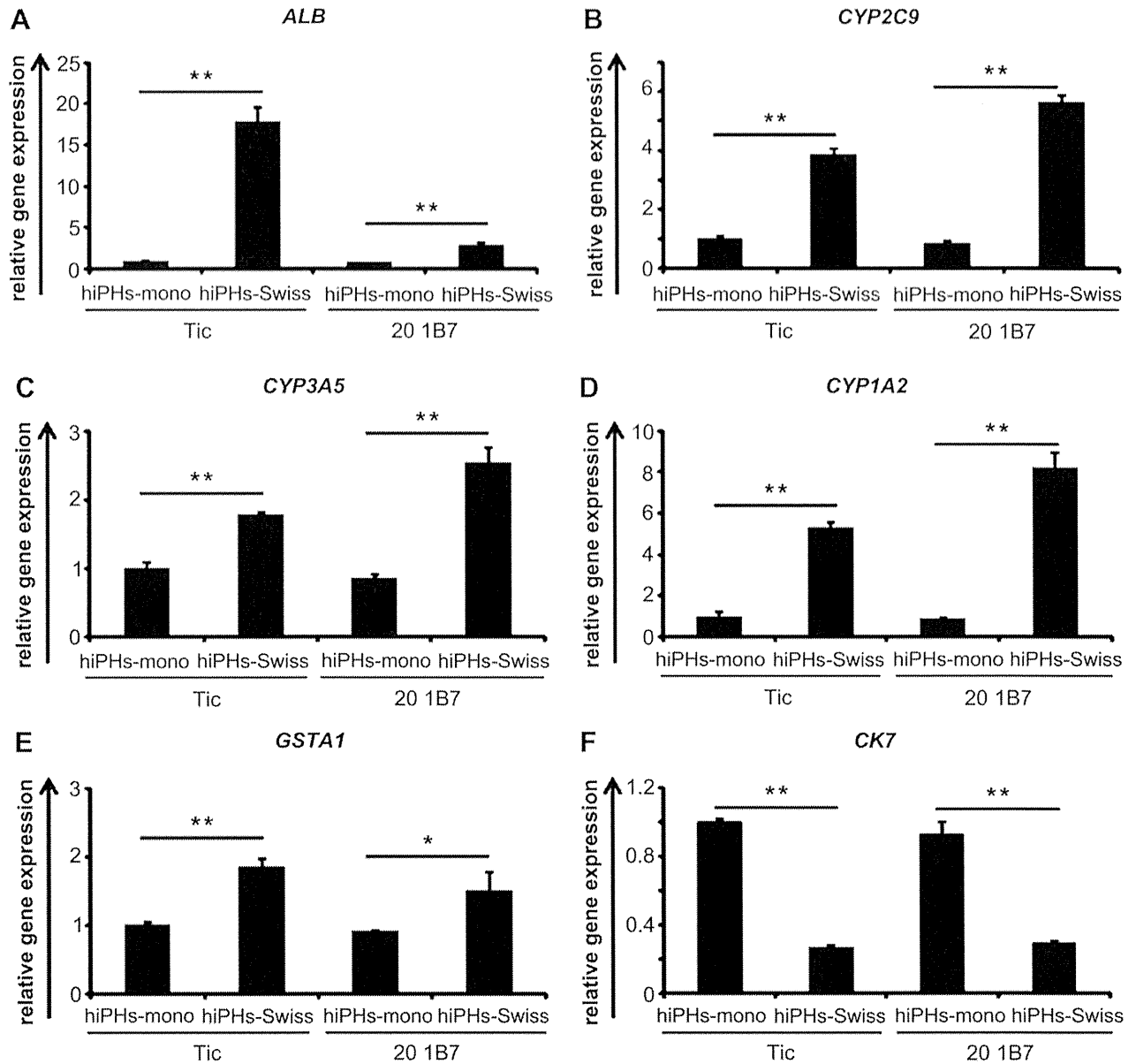
those in the co-cultured hEHs (Fig. 4A and B). Furthermore, a significant elevation of *CYP1A2* and *CYP3A5* gene expression was observed only in the co-cultured hEHs (Fig. 4C and D). Therefore, these data indicate that physical contacts between hEHs and Swiss 3T3 cells play an important role in hepatic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



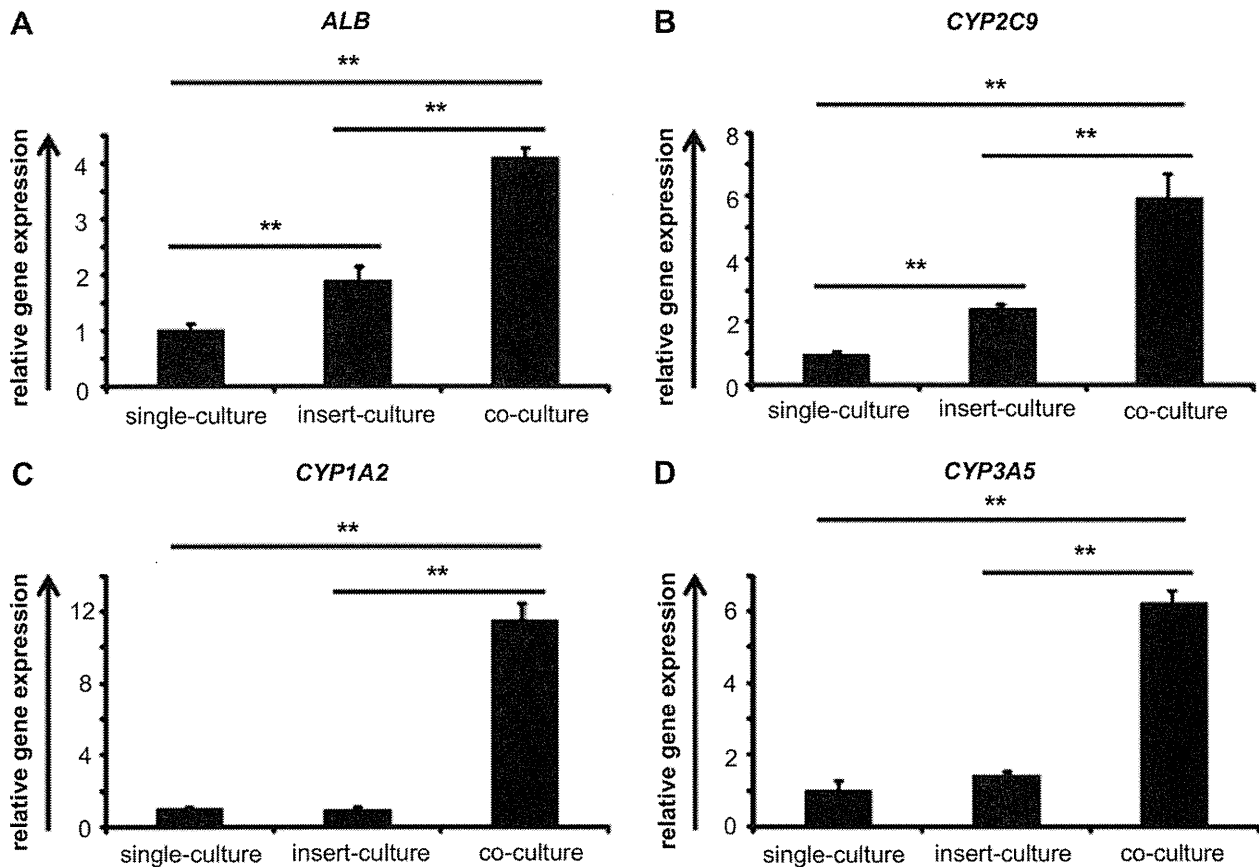
**Fig. 3.** Stratification of Swiss 3T3 cell sheet on hiPHs promotes hepatic maturation. Human induced pluripotent stem cells (hiPSCs) (Tic and 201B7) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–F): On day 25, the gene expression levels of *ALB* (A), *CYP2C9* (B), *CYP3A5* (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 ( $n = 3$ ). \*\* $P < 0.01$ .

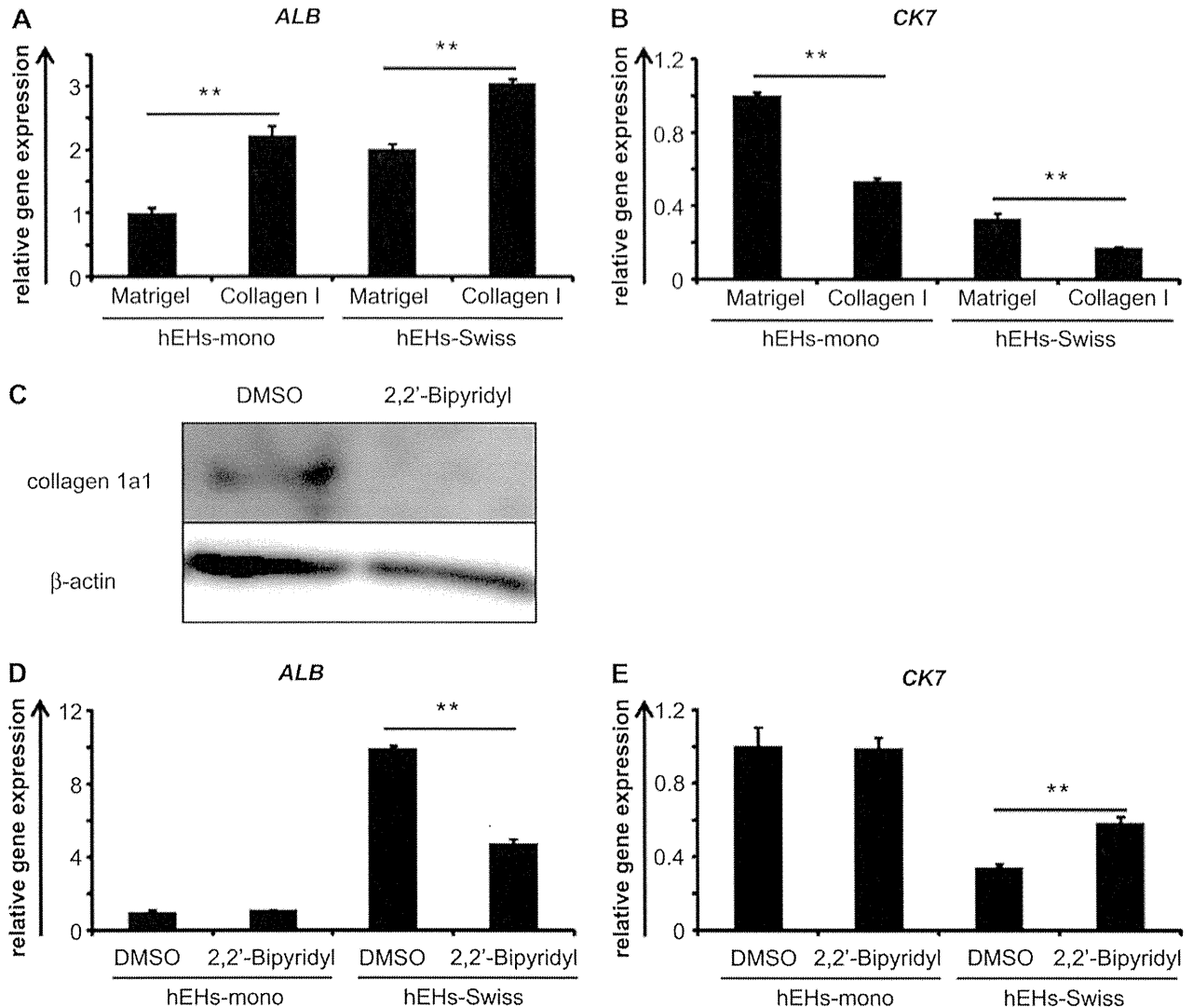
generated from mouse embryo fibroblasts [41]. Thus, to more efficiently differentiate into hepatocyte-like cells from hiPSCs, it might be valuable to employ hiPSCs generated from freshly isolated human hepatocytes. Moreover, by using our 3D co-culture system, such hiPSCs would be differentiated into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

## Acknowledgements

We thank Misae Nishijima, Miki Yoshioka, Nobue Hirata, and Hiroko Matsumura for their excellent technical support. We also thank Tetsutaro Kikuchi (Cell Seed Inc) for providing a cell sheet stamp manipulator system. HM, KK, MKF, and TH were supported by grants from the Ministry of Health, Labor, and Welfare of Japan

(MEXT). HM was also supported by Japan Research foundation For Clinical Pharmacology and The Uehara Memorial Foundation. KO was supported by Special Coordination Funds for Promoting Science and Technology from MEXT. FS was supported by Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

## 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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SHORT REPORT

# Promotion of hematopoietic differentiation from mouse induced pluripotent stem cells by transient HoxB4 transduction

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Received 25 April 2011; received in revised form 1 September 2011; accepted 5 September 2011

Available online 16 September 2011

**Abstract** Ectopic expression of HoxB4 in embryonic stem (ES) cells leads to an efficient production of hematopoietic cells, including hematopoietic stem/progenitor cells. Previous studies have utilized a constitutive HoxB4 expression system or tetracycline-regulated HoxB4 expression system to induce hematopoietic cells from ES cells. However, these methods cannot be applied therapeutically due to the risk of transgenes being integrated into the host genome. Here, we report the promotion of hematopoietic differentiation from mouse ES cells and induced pluripotent stem (iPS) cells by transient HoxB4 expression using an adenovirus (Ad) vector. Ad vector could mediate efficient HoxB4 expression in ES cell-derived embryoid bodies (ES-EBs) and iPS-EBs, and its expression was decreased during cultivation, showing that Ad vector transduction was transient. A colony-forming assay revealed that the number of hematopoietic progenitor cells with colony-forming potential in HoxB4-transduced cells was significantly increased in comparison with that in non-transduced cells or LacZ-transduced cells. HoxB4-transduced cells also showed more efficient generation of CD41-, CD45-, or Sca-1-positive cells than control cells. These results indicate that transient, but not constitutive, HoxB4 expression is sufficient to augment the hematopoietic differentiation of ES and iPS cells, and that our method would be useful for clinical applications, such as cell transplantation therapy.

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## Introduction

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, each of which is derived from the inner cell mass of blastocysts and somatic cells by transducing three or four

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transcription factors, respectively, can differentiate into various types of cells *in vitro*. They are thus considered as a valuable model to understand the processes involved in the differentiation of lineage-committed cells as well as an unlimited source of cells for therapeutic applications such as hematopoietic stem/progenitor cell (HSPC) transplantation (Evans and Kaufman, 1981; Thomson et al., 1998; Keller, 2005; Takahashi and Yamanaka, 2006; Takahashi et al., 2007).

Differentiation of ES and iPS cells into mature hematopoietic cells, including erythrocytes, myeloid cells, and lymphoid cells, has been performed by embryoid body (EB) formation or coculture with stromal cells (Nakano et al., 1994; Chadwick et al., 2003; Schmitt et al., 2004; Vodyanik et al., 2005). However, the development of an efficient differentiation method for immature hematopoietic cells, including HSPCs, from ES and iPS cells has been challenging. Previously, Daley and his colleagues have shown that enforced expression of HoxB4 in mouse ES cells by a retrovirus vector robustly enhanced the differentiation of ES cells into HSPCs *in vitro*, and these ES cell-derived HSPCs had a long-term reconstitution potential *in vivo* (Kyba et al., 2002; Wang et al., 2005). In addition, constitutive expression of HoxB4 was shown to induce the hematopoietic differentiation from human ES cells (Bowles et al., 2006). These findings indicated that manipulation of HoxB4 expression would be effective for production of HSPCs from ES and iPS cells. However, it is known that long-term constitutive HoxB4 expression in HSPCs has an inhibitory effect on the differentiation of certain hematopoietic lineages, such as lymphoid cells and erythroid cells (Kyba et al., 2002; Pilat et al., 2005), and can lead to a significant risk of leukemogenesis in large animals (Zhang et al., 2008). Although a tetracycline-inducible HoxB4 expression system has been utilized to overcome these unwanted effects, this gene expression system is complex, and cannot be directly applied to therapeutic use. Foreign genes can be integrated into the host chromosome in a stable gene expression system that includes a tetracycline-regulated system, and this could cause an increased risk of cellular transformation (Li et al., 2002; Hacein-Bey-Abina et al., 2003; Williams and Baum, 2004). Therefore, to apply ES cell- and iPS cell-derived HSPCs to clinical medicine, development of a simple and transient HoxB4 transduction method in ES and iPS cells is required.

We have utilized an adenovirus (Ad) vector as a tool for transduction of functional genes into stem cells, because Ad vectors are relatively easy to construct, can be produced at high titers, and mediate efficient and transient gene expression in both dividing and nondividing cells. We have demonstrated that Ad vectors could efficiently transduce a foreign gene in stem cells, including ES and iPS cells (Kawabata et al., 2005; Tashiro et al., 2009, 2010). We also succeeded in promoting the differentiation of osteoblasts, adipocytes, or hepatoblasts from ES and iPS cells by Ad vector-mediated transient transduction of Runx2, PPAR $\gamma$ , or Hex, respectively (Tashiro et al., 2009, 2008; Inamura et al., 2011).

Our data led us to examine whether HSPCs could also be efficiently differentiated from ES and iPS cells by Ad vector-mediated transduction of a HoxB4. In the present study, we investigated whether or not differentiation of HSPCs from mouse ES and iPS cells could be promoted by

transient HoxB4 expression. Our results showed that Ad vector-mediated transient HoxB4 expression in mouse ES and iPS cells are sufficient to augment the differentiation of hematopoietic cells, including HSPCs, from mouse ES and iPS cells. This result indicates that an Ad vector-mediated transient gene expression system would be a powerful and safe tool to induce hematopoietic differentiation from mouse ES and iPS cells.

## Results

### Transduction with Ad vectors in ES-EBs or iPS-EBs

A previous study using a tetracycline-inducible HoxB4 expression system showed that hematopoietic stem/progenitor cells (HSPCs) were generated by induction of HoxB4 expression in ES cell-derived embryoid bodies (ES-EBs) from day 4 to day 6 of differentiation (Kyba et al., 2002), suggesting that HoxB4 expression within this time range would be effective for induction of hematopoietic cells. In addition, CD41<sup>+</sup>c-kit<sup>+</sup> cells in EBs are reported to be early hematopoietic progenitor cells (Mitjavila-Garcia et al., 2002; Mikkola et al., 2003). Thus, we planned to transduce HoxB4 in total cells derived from ES- or iPS-EBs on day 5 of differentiation or in CD41<sup>+</sup>c-kit<sup>+</sup> cells derived from ES- or iPS-EBs on day 6. We initially investigated the expression of coxsackievirus and adenovirus receptor (CAR) in ES-EB- or iPS-EB-derived cells, because CAR was indispensable for transduction of an exogenous gene using Ad vector (Bergelson et al., 1997; Tomko et al., 1997). Flow cytometric analysis showed the expression of CAR in ES-EB- and iPS-EB-derived total cells and CD41<sup>+</sup>c-kit<sup>+</sup> cells, although the expression levels of CAR in CD41<sup>+</sup>c-kit<sup>+</sup> cells were decreased in comparison with that in total cells (Figs. 1a and b). These results indicate that ES-EB- and iPS-EB-derived total cells and CD41<sup>+</sup>c-kit<sup>+</sup> cells could be transduced with Ad vectors. We also observed the expression of green fluorescent protein (GFP) in iPS-EB-derived total cells. Because the mouse iPS cells used in this study express GFP under the control of Nanog promoter (Okita et al., 2007), the existence of GFP-positive cells showed that undifferentiated iPS cells would still be present in iPS-EB-derived total cells.

We next examined the transduction efficiency in EB-derived total cells or EB-derived CD41<sup>+</sup>c-kit<sup>+</sup> cells using DsRed- or GFP-expressing Ad vectors, respectively. After transduction with Ad-DsRed or Ad-GFP at 3000 vector particles (VPs)/cell, the cells were cultured with the hematopoietic cytokines for 2 days. The results showed that, at 3000 VPs/cell, approximately 60% or 40% of the EB-derived total cells or EB-derived CD41<sup>+</sup>c-kit<sup>+</sup> cells, respectively, expressed transgenes (Figs. 1c and d). Although the number of transgene-expressing cells was increased in the case of transduction with Ad vectors at 10,000 VPs/cell, the number of viable cells was markedly reduced (data not shown). Therefore, we decided to use Ad vectors at 3000 VPs/cell for transducing human HoxB4 (hHoxB4) into ES-EBs and iPS-EBs. RT-PCR analysis on day 3 after transduction with Ad-hHoxB4 into EB-derived total cells showed an elevation of hHoxB4 mRNA expression in hHoxB4-transduced cells, while neither non-transduced cells nor LacZ-transduced cells showed hHoxB4 expression (Fig. 1e). Importantly, the expression level of hHoxB4 in the cells was markedly decreased on day 6 after Ad

transduction. This result showed that the ES-EB- or iPS-EB-derived cells could express transgenes by Ad vectors, and that Ad vector mediated the transient transgene expression in these cells.

### Transient HoxB4 expression augments the generation of hematopoietic cells from mouse ES and iPS cells

To induce and expand the hematopoietic cells from the iPS cell line 38C2, EB-derived total cells were plated and cultured on OP9 stromal cells with the hematopoietic cytokines. On day 10 after plating on OP9 cells, the number of 38C2-derived hematopoietic cells in LacZ-transduced cells was similar to that in non-transduced cells. On the other hand, transient transduction of HoxB4 with Ad-hHoxB4 resulted in a significant increase in the number of hematopoietic cells compared with non-transduced cells or LacZ-transduced cells (Fig. 2a, middle). Likewise, an increase in the hematopoietic cell number by Ad vector-mediated hHoxB4 transduction was also observed in ES cell derived-hematopoietic cells or the other iPS line 20D17-derived hematopoietic cells (Fig. 2a, left and right). Additionally, ES-EB- or iPS-EB-derived CD41<sup>+</sup>c-kit<sup>+</sup> cells, which were transiently transduced with hHoxB4, could proliferate on OP9 stromal cells for over 20 days (Fig. 2b). This result is mostly in agreement with the previous report that ES cell-derived hematopoietic cells stably expressing HoxB4 had a growth advantage in the presence of hematopoietic cytokines (Pilat et al., 2005). Transient, but not stable, HoxB4 expression in ES-EB- or iPS-EB-derived cells would be sufficient to augment the generation of hematopoietic cells from ES and iPS cells.

We next investigated the surface antigen expression in non-transduced cells, LacZ-transduced cells, or hHoxB4-transduced cells after expansion on OP9 stromal cells. Flow cytometric analysis revealed an increase of CD45 and CD41 expressions in HoxB4-transduced cells, compared with non-transduced cells and LacZ-transduced cells (Figs. 3a and b). CD45 is known as a marker of hematopoietic cells. In both *in vitro* ES cell differentiation and a developing mouse embryo, the expression of CD45 was developmentally controlled, and CD45 expression was observed on hematopoietic cells after expression of CD41 (Mitjavila-Garcia et al., 2002; Mikkola et al., 2003). Thus, a higher percentage of CD45<sup>+</sup> cells in HoxB4-transduced cells would be due, at least in part, to an increase of CD41 expression in HoxB4-transduced cells relative to non-transduced cells and LacZ-transduced cells. We also

found a significant elevation of Sca-1 in hHoxB4-transduced cells (Figs. 3a and b). Sca-1 is expressed in fetal and adult HSPCs (Arai et al., 2004; McKinney-Freeman et al., 2009), although Sca-1 expression was observed in other types of cells. Therefore, our data suggest that immature hematopoietic cells would be generated in hHoxB4-transduced cells more efficiently than in non-transduced cells or LacZ-transduced cells.

In parallel with the flow cytometric analysis, we also analyzed the expression levels of hematopoietic marker genes in iPS cell-derived hematopoietic cells by RT-PCR (Fig. 3c). The expression levels of marker genes in LacZ-transduced cells were mostly equal to those in non-transduced cells. In contrast, among the genes we assayed, the expression levels of *Gata-1*, *c-myb*, and *Cxcr4* mRNA were slightly but significantly up-regulated in hHoxB4-transduced cells. GATA-1 reflects early hematopoietic development, whereas c-Myb is a marker of definitive hematopoiesis (Godin and Cumano, 2002). Increased expression of these genes in HoxB4-transduced cells suggests that transient hHoxB4 expression promotes the production of both primitive and definitive hematopoietic progenitor cells from mouse ES and iPS cells. We could not detect the hHoxB4 mRNA expression in Ad-hHoxB4-transduced cells, confirming the transient hHoxB4 expression by Ad vectors (Fig. 3c).

### HoxB4 expression enhances development of hematopoietic progenitor cells from mouse ES and iPS cells

To examine whether hematopoietic immature cells with hematopoietic colony-forming potential could be generated from ES and iPS cells, ES cell-derived hematopoietic cells and iPS cell-derived hematopoietic cells, both of which were cultured on OP9 stromal cells for 10 days, were plated and cultured in methylcellulose-containing media with hematopoietic cytokines. Without Ad transduction, the number of total hematopoietic colonies in the iPS cell line 38C2 was five times as high as that in ES cells, whereas another iPS cell line, 20D17, had nearly the same hematopoietic differentiation potential as ES cells (Fig. 4a). These results indicate that there is a difference in hematopoietic differentiation potential among iPS cell lines.

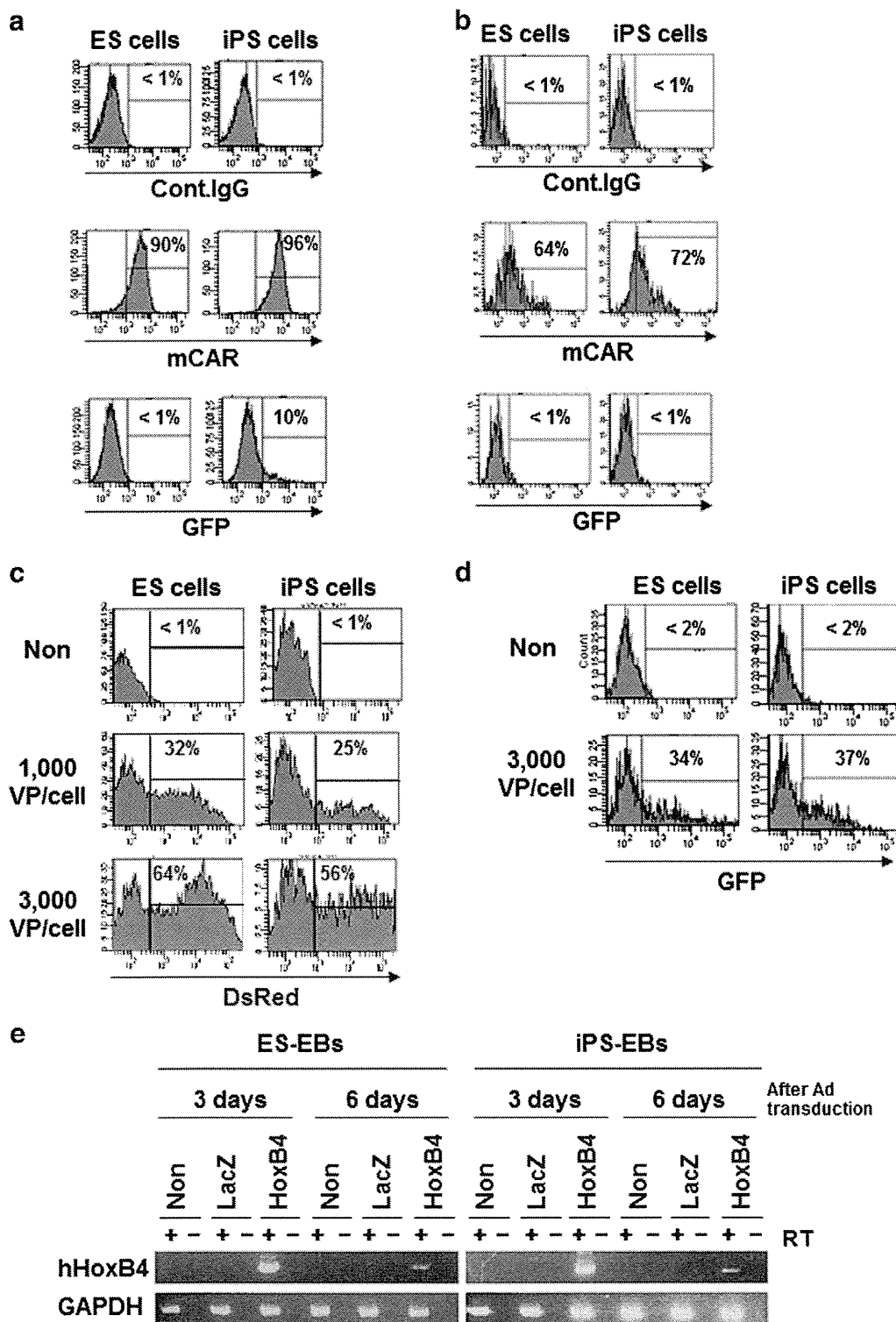
We next examined the hematopoietic colony potential in LacZ-transduced cells or HoxB4-transduced cells. The colony assay revealed a significant increase in the number of total hematopoietic colonies in hHoxB4-transduced cells compared with control cells, whereas there was no significant difference in the number of hematopoietic colonies between

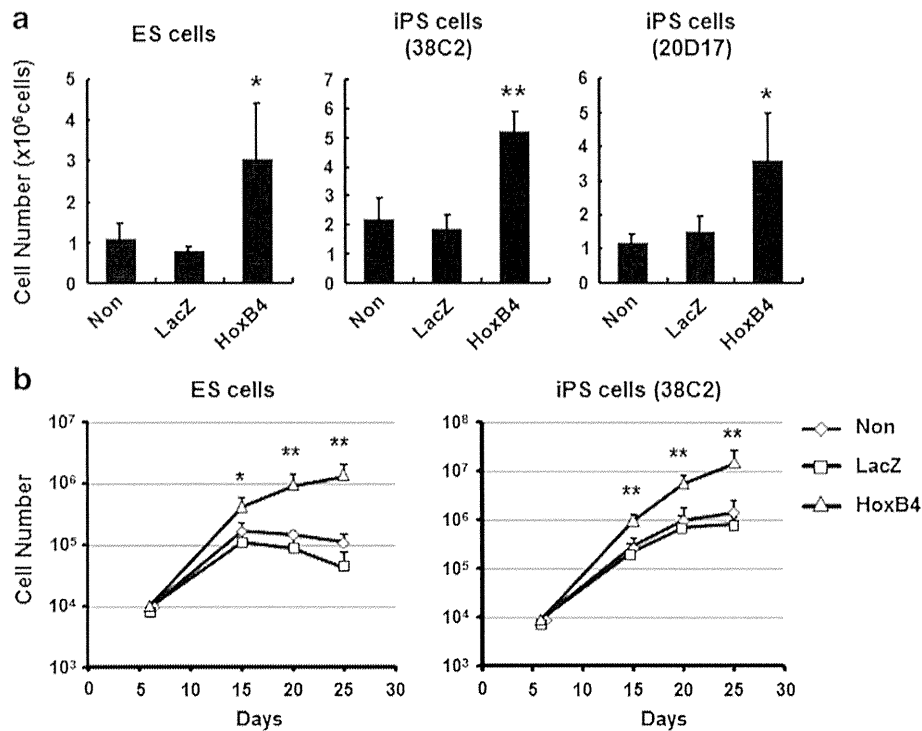
**Figure 1** Transduction with Ad vectors in ES-EB- or iPS-EB-derived cells. (a, b) The expression levels of CAR, a primary receptor for Ad, in ES-EB- or iPS-EB-derived total cells (a) or CD41<sup>+</sup>c-kit<sup>+</sup> cells (b) were detected with anti-mouse CAR monoclonal antibody by flow cytometric analysis. As a negative control, the cells were incubated with an irrelevant antibody. Data shown are from one representative experiment of three performed. (c, d) EB-derived total cells (c) or CD41<sup>+</sup>c-kit<sup>+</sup> cells (d), purified by FACS (Supplemental Fig. 1), were transduced with Ad-DsRed or Ad-GFP for 1.5 h, and transgene-expressing cells were then analyzed by flow cytometry. Because CD41<sup>+</sup>c-kit<sup>+</sup> cells do not express GFP (Fig. 1b), Ad-GFP was used for transduction into CD41<sup>+</sup>c-kit<sup>+</sup> cells. Similar results were obtained in three independent experiments. (e) The expression level of human HoxB4 mRNA in the cells was examined by conventional RT-PCR on days 3 and 6 after transduction with Ad-hHoxB4 at 3000 VPs/cell into EB-derived total cells. Abbreviations: ES, embryonic stem; iPS, induced pluripotent stem; mCAR, mouse coxsackievirus and adenovirus receptor; GFP, green fluorescent protein; Cont., control.; VP, vector particle; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Transduction with Ad vectors



non-transduced cells and LacZ-transduced cells (Fig. 4a). Note that the number of the most immature multipotent progenitor cells, CFU-GEMM/CFU-Mix, in hHoxB4-transduced cells was approximately seven times as great as that in non-transduced cells or LacZ-transduced cells, and that large CFU-Mix colonies were more frequently observed in hHoxB4-trans-

duced cells than control cells (Fig. 4b and data not shown). A colony assay after culturing on OP9 stromal cells for 20 days also revealed that much number of myeloid (CFU-G, M, and GM) colonies and CFU-Mix colonies were observed by transient hHoxB4 transduction (Figs. 4c and d). Thus, our data clearly showed that Ad vector-mediated transient hHoxB4 expression





**Figure 2** The number of ES cell- or iPS cell-derived hematopoietic cells was significantly increased in Ad-hHoxB4-transduced cells. (a, b) ES-EB- or iPS-EB-derived total cells (a) or CD41<sup>+</sup>c-kit<sup>+</sup> cells (b) were transduced with Ad-LacZ or Ad-hHoxB4 at 3000 VPs/cell for 1.5 h, and the cells were then plated on OP9 feeder cells. As a control, non-transduced cells were also plated on OP9 cells. After culturing on OP9 feeders with the hematopoietic cytokines for 10 days (a) or 20 days (b), the number of hematopoietic cells per 2 wells of a 6-well plate was counted. (a) Left, ES cells; middle, iPS cell line 38C2; right, iPS cell line 20D17. Results shown were the mean of four independent experiments with indicated standard deviations. \*  $p < 0.05$ , \*\*  $p < 0.01$  as compared with non-transduced or Ad-LacZ-transduced cells.

enhances the differentiation of hematopoietic immature cells, including HSPCs, from mouse ES and iPS cells.

## Discussion

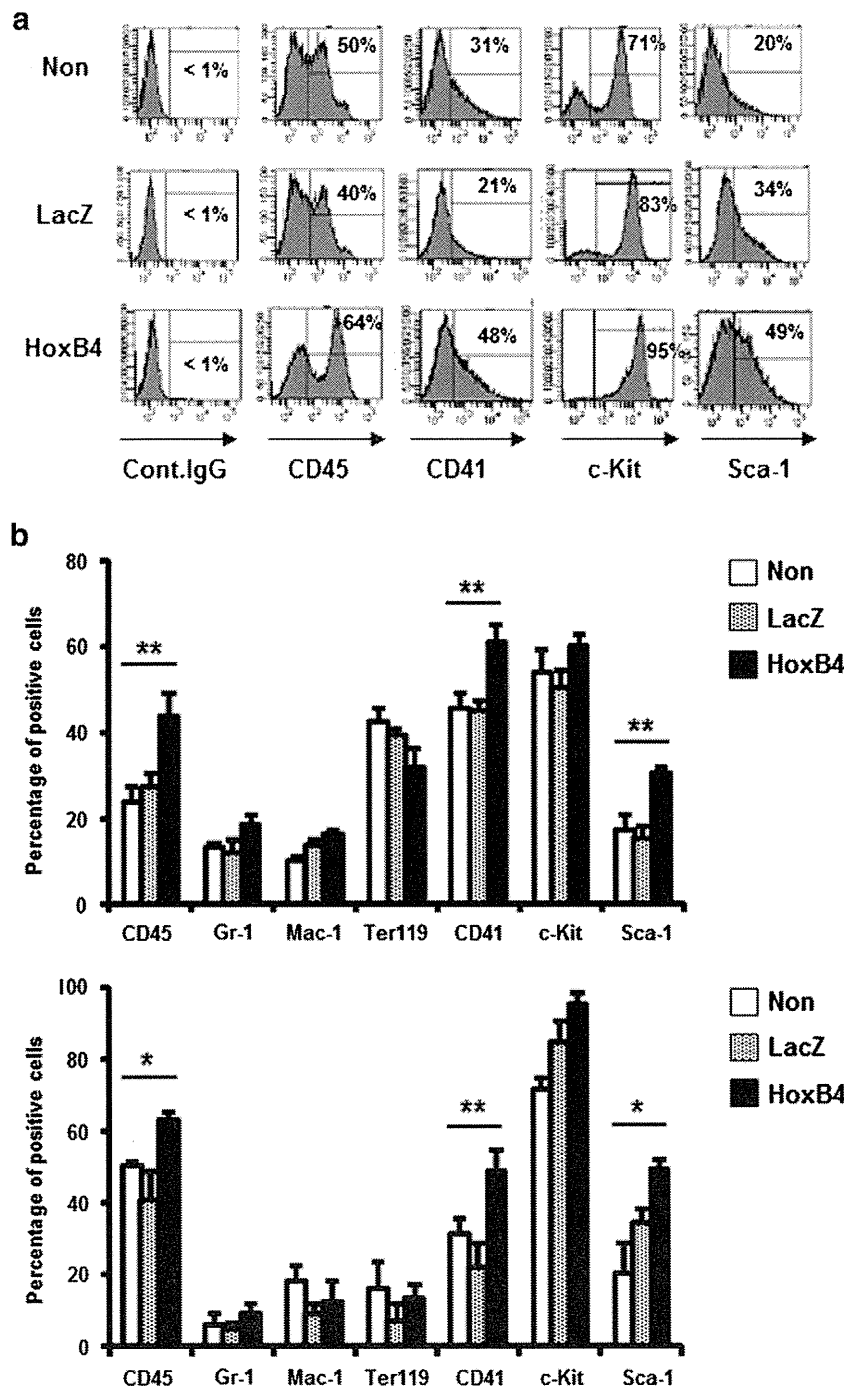
Previous studies have shown that enforced expression of HoxB4 is an effective strategy for hematopoietic differentiation from both mouse and human ES cells (Kyba et al., 2002; Bowles et al., 2006; Pilat et al., 2005; Schiedlmeier et al., 2007). These studies usually used recombinant ES cells, such as ES cells constitutively expressing HoxB4 (Pilat et al., 2005) or ES cells containing a tetracycline (Tet)-inducible HoxB4 expression system (Kyba et al., 2002), to induce hematopoietic cells. However, this expression system might raise clinical concerns, including the risk of oncogenesis due to integration of transgenes into host genomes. In the present study, we showed that Ad vector-mediated transient hHoxB4 expression in mouse ES-EB- or iPS-EB-derived cells could result in an efficient production of hematopoietic cells, including HSPCs with a hematopoietic colony-forming ability, from mouse ES and iPS cells (Figs. 2, 3, and 4). Our data obtained in this report are largely consistent with previous reports (Kyba et al., 2002) in which HSPCs were generated by using ES cells containing the Tet-regulated HoxB4 expression cassette. Therefore, a transient HoxB4 expression system using an Ad vector, instead of a Tet-inducible HoxB4 expression

system, would contribute to safer clinical applications of ES or iPS cell-derived hematopoietic cells.

Conventional Ad vector is known to infect the cells through an entry receptor, CAR, on the cellular surface (Bergelson et al., 1997; Tomko et al., 1997). Previously, we showed that undifferentiated ES and iPS cells expressed CAR, and conventional Ad vector could easily transduce a foreign gene in more than 90% of the undifferentiated ES and iPS cells at 3000 VPs/cell (Kawabata et al., 2005; Tashiro et al., 2009). Like undifferentiated ES and iPS cells, we could detect the CAR expression in more than 90% or 70% of EB-derived total cells or EB-derived CD41<sup>+</sup>c-kit<sup>+</sup> cells, respectively (Figs. 1a and b). However, the transduction efficiency in EB-derived total cells or CD41<sup>+</sup>c-kit<sup>+</sup> cells was only 60% or 40%, respectively, of the cells at most (Figs. 1c and d). Although we are not certain why transgene expression was not observed in all of CAR<sup>+</sup> EB-derived cells, it is possible that the promoter might not have worked in all of the cells because the EB-derived total cells and CD41<sup>+</sup>c-kit<sup>+</sup> cells were heterogeneous, unlike in the case of undifferentiated ES and iPS cells. It is also possible that the Ad binding site of CAR might be disrupted by trypsin treatment during the preparation of the EB-derived cells (Carson, 2000). Because the development of efficient transduction methods in EB-derived cells is considered to be a powerful tool to promote the hematopoietic differentiation from ES and iPS cells, further improvement of the transduction conditions will be needed.

We found a difference in the hematopoietic differentiation potential among mouse iPS cell lines (Fig. 4). Consistent with our data, Kulkeaw et al. showed a difference in the

hematopoietic differentiation capacity among six iPS cell lines (Kulkeaw et al., 2010). In addition, recent studies have reported that iPS cells leave an epigenetic memory of



**Figure 3** Expression of surface antigen and hematopoietic marker genes in mouse ES cell- or iPS cell-derived cells. (a, b) ES cell- or iPS cell line 38C2-derived cells were reacted with each antibody, and were then subjected to flow cytometric analysis. (a) Representative data from iPS cell line 38C2 are shown. (b) Percentage of each antigen positive cells in ES cell-derived cells (upper) or iPS cell-derived cells (lower) is shown. The data expressed the mean of three independent experiments with indicated standard deviations. \*  $p < 0.05$ , \*\*  $p < 0.01$  as compared with non-transduced or Ad-LacZ-transduced cells. (c) Total RNA was extracted from undifferentiated iPS cells (Day 0), iPS-EB (Day 5), iPS cells-derived hematopoietic cells (day 15), OP9 stromal cells, and MEF feeder, and semi-quantitative PCR (left) or quantitative real-time PCR (right) was then carried out as described in the Materials and methods. The data expressed the mean of three independent experiments with indicated standard deviations. \*  $p < 0.05$ , \*\*  $p < 0.01$  as compared with non-transduced or Ad-LacZ-transduced cells. Abbreviation: EBs, embryoid bodies; MEF, mouse embryonic fibroblast; GATA, GATA-binding protein.

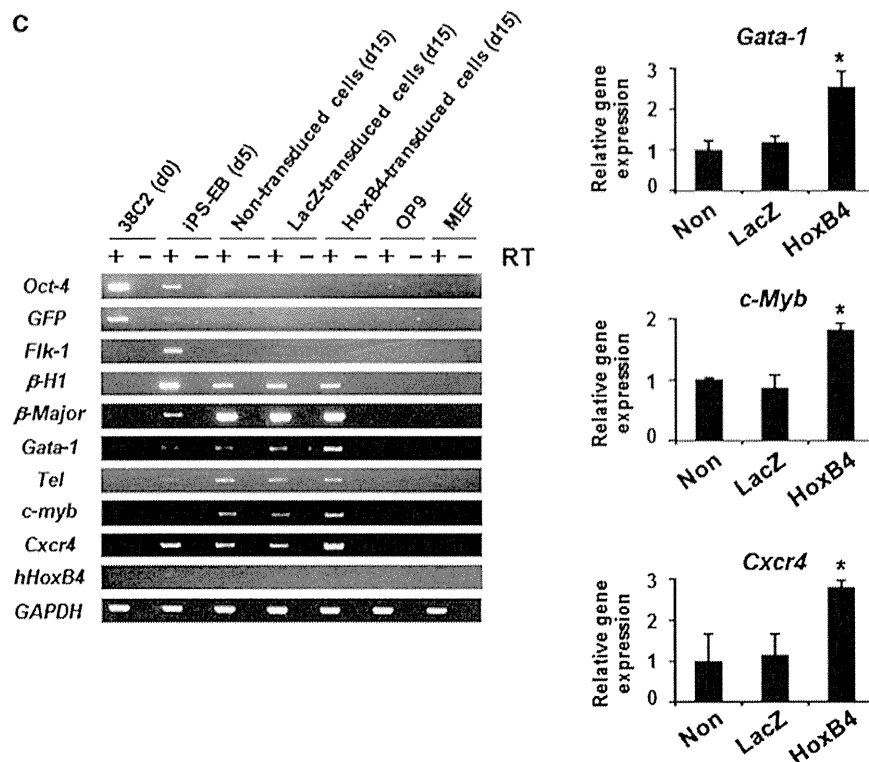


Figure 3 (continued).

their cellular origin, and this memory influences their functional properties, including *in vitro* differentiation (Kim et al., 2010; Polo et al., 2010). Thus, these reports indicate that, in order to obtain a large number of HSPCs from iPS cells, it is necessary to choose an appropriate iPS cell line, such as HSPC-derived iPS cells (Okabe et al., 2009). Importantly, using mouse embryonic fibroblast-derived iPS cells (38C2 and 20D17), we showed that the use of transient hHoxB4 transduction in iPS-EB-derived cells achieved more effective differentiation into HSPCs than the use of non-transduced cells (Fig. 4). Our method therefore should be efficient for the production of HSPCs from any iPS cell line.

An important but unsolved question in this study is whether ES cell-derived hematopoietic cells and iPS cell-derived hematopoietic cells transduced with Ad-hHoxB4 have long-term hematopoietic reconstitution potential *in vivo*. Recent studies have demonstrated that some surface antigen expressions were different between bone marrow-derived HSPCs and ES cell-derived HSPCs, and that CD41<sup>+</sup> cells had long-term repopulation ability in ES cell-derived HSPCs (McKinney-Freeman et al., 2009; Matsumoto et al., 2009). Our flow cytometric analysis revealed an increase of CD41<sup>+</sup> cells in hHoxB4-transduced cells compared with non-transduced cells and LacZ-transduced cells (Fig. 3b). We also showed that Ad-hHoxB4-transduced cells could proliferate on OP9 stromal cells more efficiently than control cells (Fig. 2). Thus, these results suggest that immature hematopoietic cells were generated by transient hHoxB4 transduction, and that hHoxB4-transduced cells might have reconstitution potential *in vivo*. This *in vivo* transplantation analysis is now on-going in our laboratory.

In the present study, we succeeded in the promotion of hematopoietic differentiation from mouse ES and iPS cells by Ad vector-mediated hHoxB4 transduction. Ad vector transduction can avoid the integration of transgene into host genomes, and multiple genes can be transduced by Ad vectors in an appropriate differentiation period. Thus, an even more efficient protocol for hematopoietic differentiation from ES and iPS cells could likely be established by co-transduction of HoxB4 and other genes involved in the hematopoiesis, such as Cdx4 (Wang et al., 2005) and Scl/Tal1 (Kurita et al., 2006), using Ad vectors. Taken together, our results show that Ad vector-mediated transient gene expression is valuable tool to induce hematopoietic cell from ES and iPS cells, and this strategy would be applicable to safe therapeutic applications, such as HSPC transplantation.

## Materials and methods

### Antibodies

The following primary monoclonal antibodies (Abs), conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or PE-Cy7, were used for flow cytometric analysis: anti-CD45 (30-F11, eBioscience, San Diego, CA), anti-CD11b (M1/70, eBioscience), anti-Sca-1 (D7, eBioscience), anti-Ter-119 (Ter-119, eBioscience), anti-Gr-1 (RB6-8C5, eBioscience), anti-c-Kit (ACK2 or 2B8, eBioscience), anti-CD41 (MWRReg30, BD Bioscience San Jose, CA). Purified rat anti-coxsackievirus and adenovirus receptor (CAR) was kindly provided from Dr. T. Imai (KAN Research Institute, Hyogo, Japan). For detection of CAR, the PE-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West