

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

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

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

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

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

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Introduction

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

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

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

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

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

Materials and methods

In vitro differentiation

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

Results

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

generated by the optimized protocol for pharmaceutical use in detail.

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

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

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

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

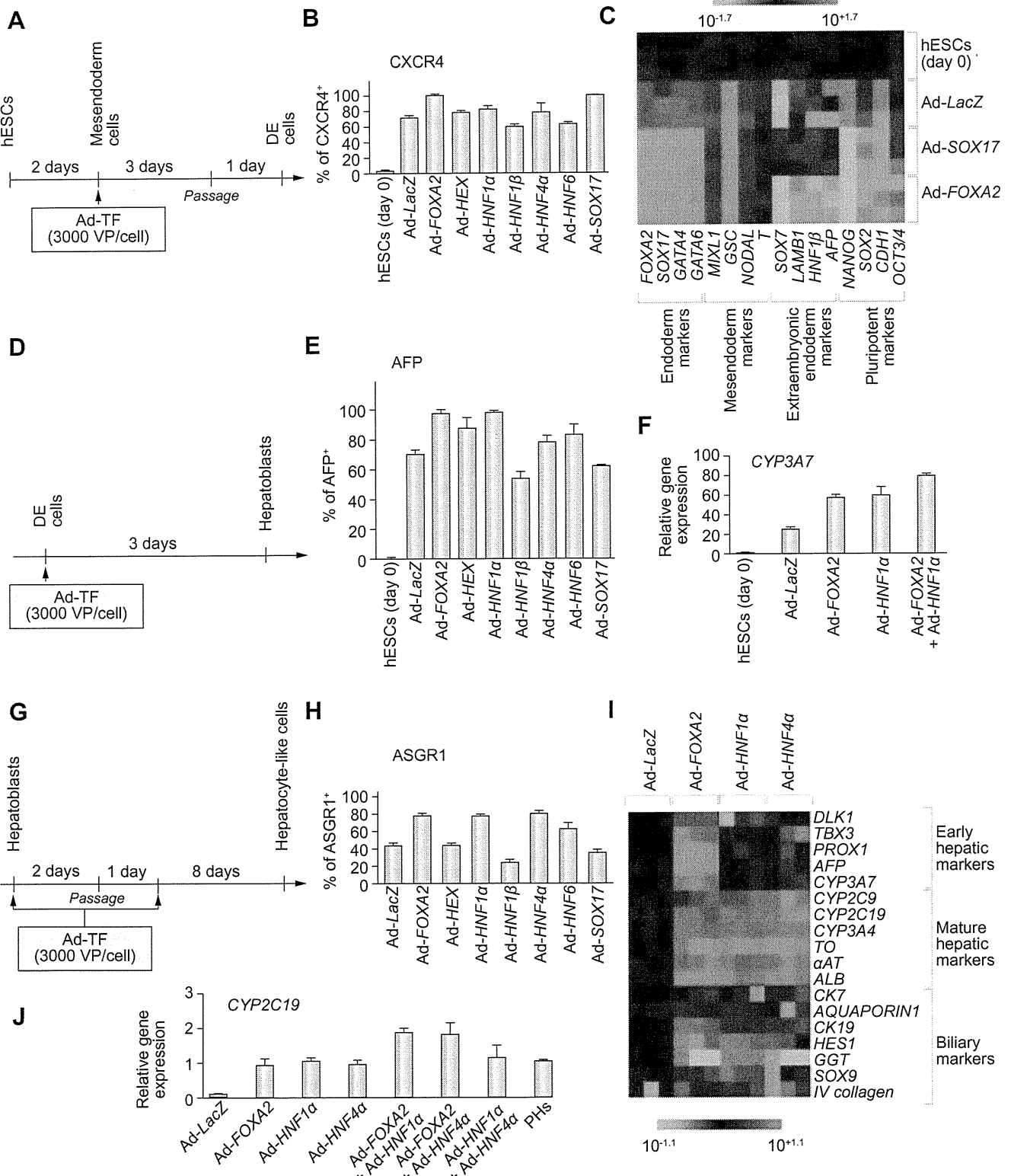


Fig. 1. Efficient hepatic differentiation from hESCs by FOXA2 and HNF1α transduction. (A) The schematic protocol describes the strategy for DE differentiation from hESCs (H9). Mesoderm cells (day 2) were transduced with 3000 VP/cell of transcription factor (TF)-expressing Ad vector (Ad-TF) for 1.5 h and cultured as described in Fig. 2A. (B) On day 5, the efficiency of DE differentiation was measured by estimating the percentage of CXCR4-positive cells using FACS analysis. (C) The gene expression profiles were examined on day 5. (D) Schematic protocol describing the strategy for hepatoblast differentiation from DE. DE cells (day 6) were transduced with 3000 VP/cell of Ad-TF for 1.5 h and cultured as described in Fig. 2A. (E) On day 9, the efficiency of hepatoblast differentiation was measured by estimating the percentage of AFP-positive

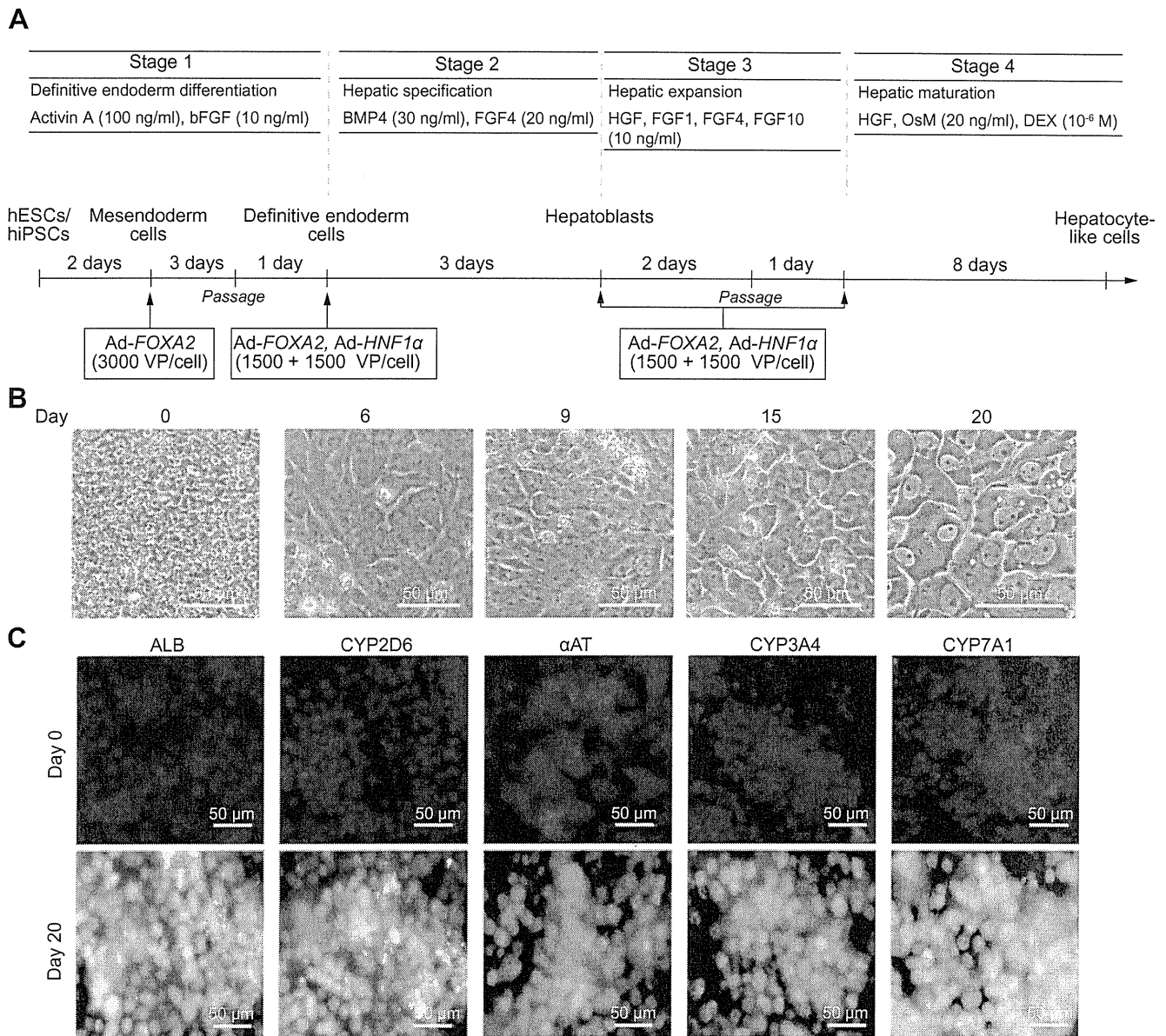


Fig. 2. Hepatic differentiation of hESCs and hiPSCs by FOXA2 and HNF1 α transduction. (A) The differentiation procedure of hESCs and hiPSCs into hepatocytes via DE cells and hepatoblasts is schematically shown. Details of the hepatic differentiation procedure are described in Materials and methods. (B)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

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

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

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

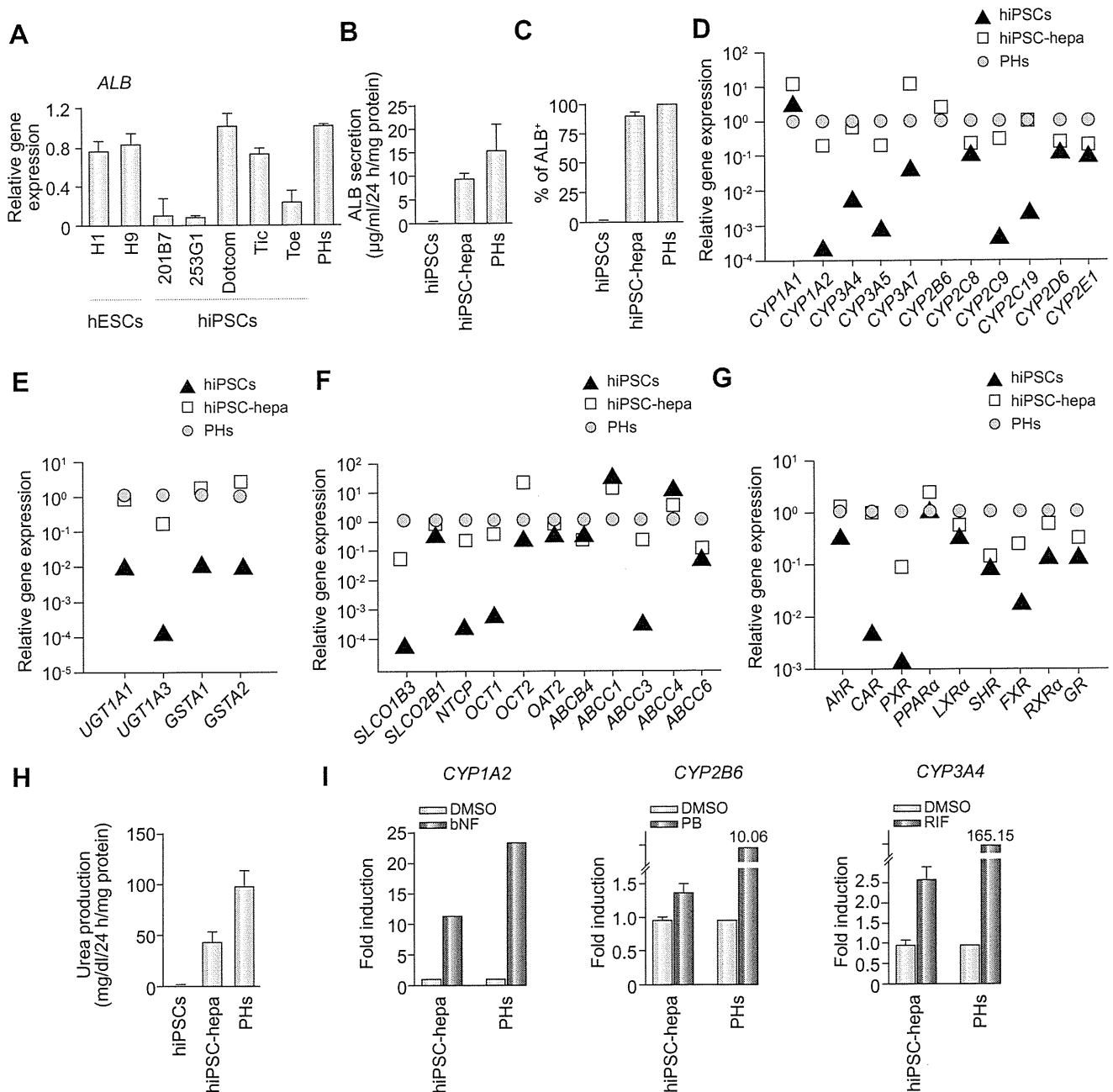


Fig. 3. The hepatic characterization of hiPSC-hepa. hESCs (H1 and H9) and hiPSCs (201B7, 253G1, Dotcom, Tic, and Toe) were differentiated into hepatocyte-like cells as described in Fig. 2A. (A) On day 20, the gene expression level of *ALB* was examined by real-time RT-PCR. On the y axis, the gene expression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

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

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

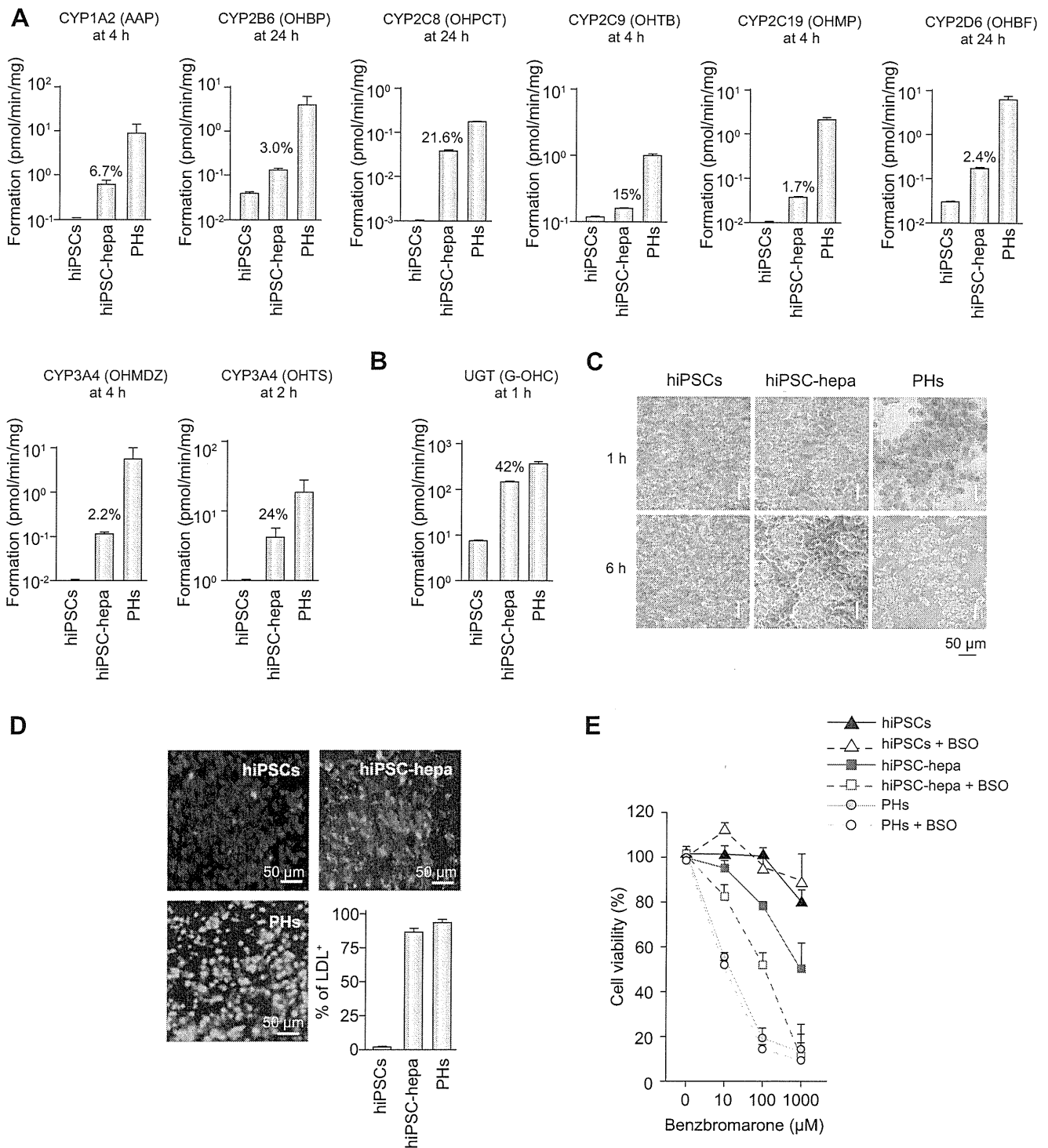


Fig. 4. Evaluation of the drug metabolism capacity and hepatic transporter activity of hiPSC-hepa. hiPSCs (Dotcom) were differentiated into hepatocytes as described in Fig. 2A. (A and B) Quantitation of metabolites in hiPSCs, hiPSC-hepa, and PHs, which were cultured for 48 h after the cells were plated,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

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

Characterization of the hESC-hepa/hiPSC-hepa

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

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

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

Drug metabolism capacity and hepatic transporter activity of hiPSC-hepa

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

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

Discussion

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

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

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

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

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

Conflict of interest

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

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Supplementary data

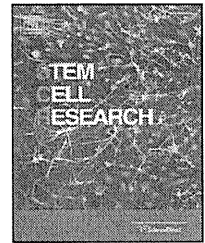
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2012.04.038>.

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SHORT REPORT

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

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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; Hacey-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 γ , 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⁺c-kit⁺ 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⁺c-kit⁺ 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⁺c-kit⁺ cells, although the expression levels of CAR in CD41⁺c-kit⁺ 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⁺c-kit⁺ 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⁺c-kit⁺ 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⁺c-kit⁺ 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⁺c-kit⁺ 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⁺ 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

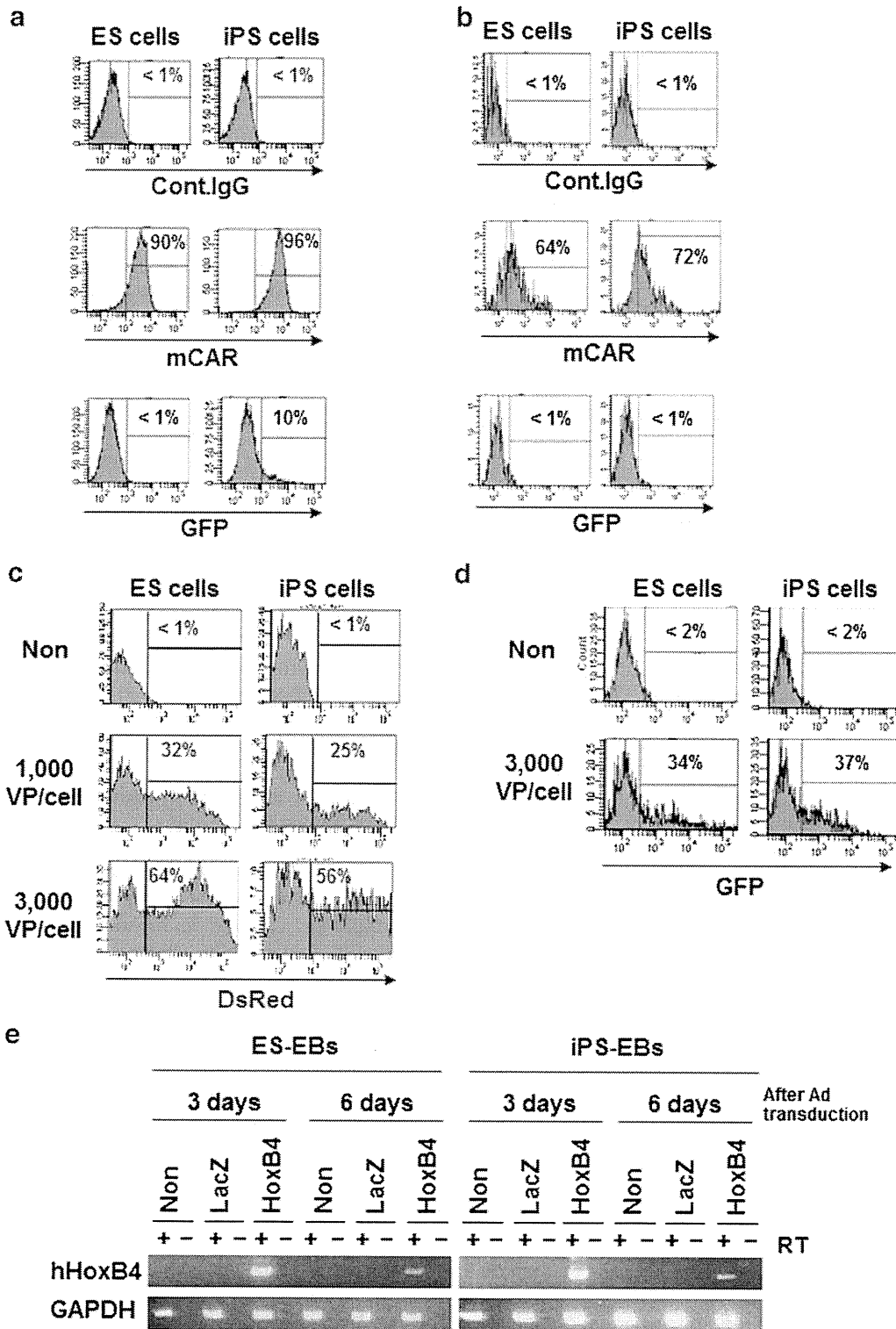
To examine whether hematopoietic immature cells with hematopoietic colony-forming potential could be generated from ES and iPS cells, ES cell-derived hematopoietic cells and iPS cell-derived hematopoietic cells, both of which were cultured on OP9 stromal cells for 10 days, were plated and cultured in 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⁺c-kit⁺ 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⁺c-kit⁺ 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⁺c-kit⁺ cells do not express GFP (Fig. 1b), Ad-GFP was used for transduction into CD41⁺c-kit⁺ 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



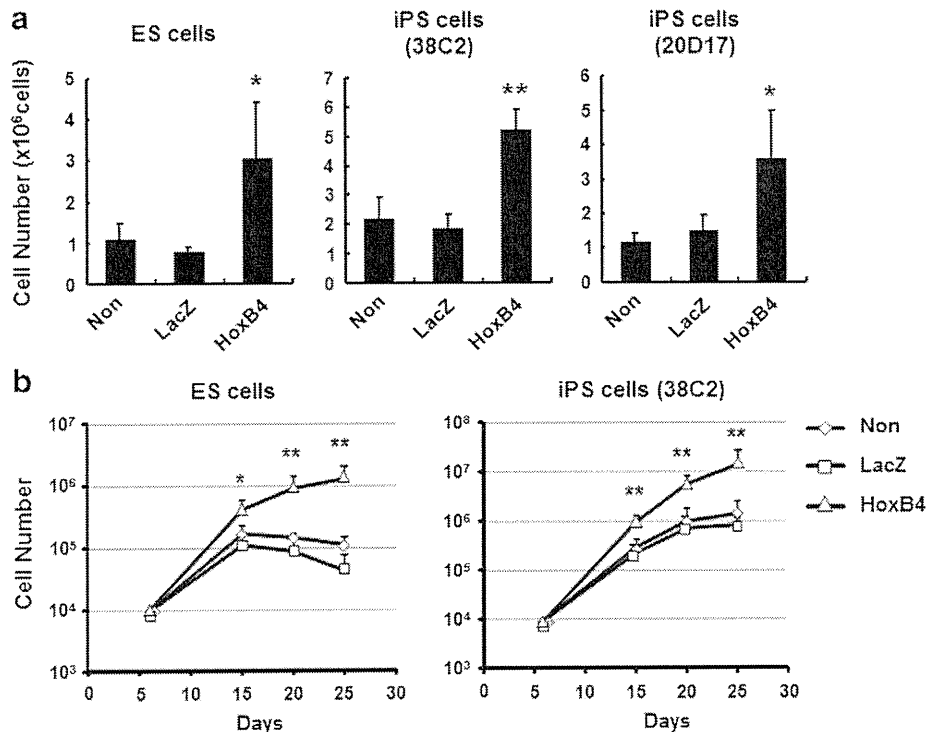


Figure 2 The number of ES cell- or iPS cell-derived hematopoietic cells was significantly increased in Ad-hHoxB4-transduced cells. (a, b) ES-EB- or iPS-EB-derived total cells (a) or CD41⁺c-kit⁺ cells (b) were transduced with Ad-LacZ or Ad-hHoxB4 at 3000 VPs/cell for 1.5 h, and the cells were then plated 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⁺c-kit⁺ cells, respectively (Figs. 1a and b). However, the transduction efficiency in EB-derived total cells or CD41⁺c-kit⁺ 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⁺ 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⁺c-kit⁺ 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

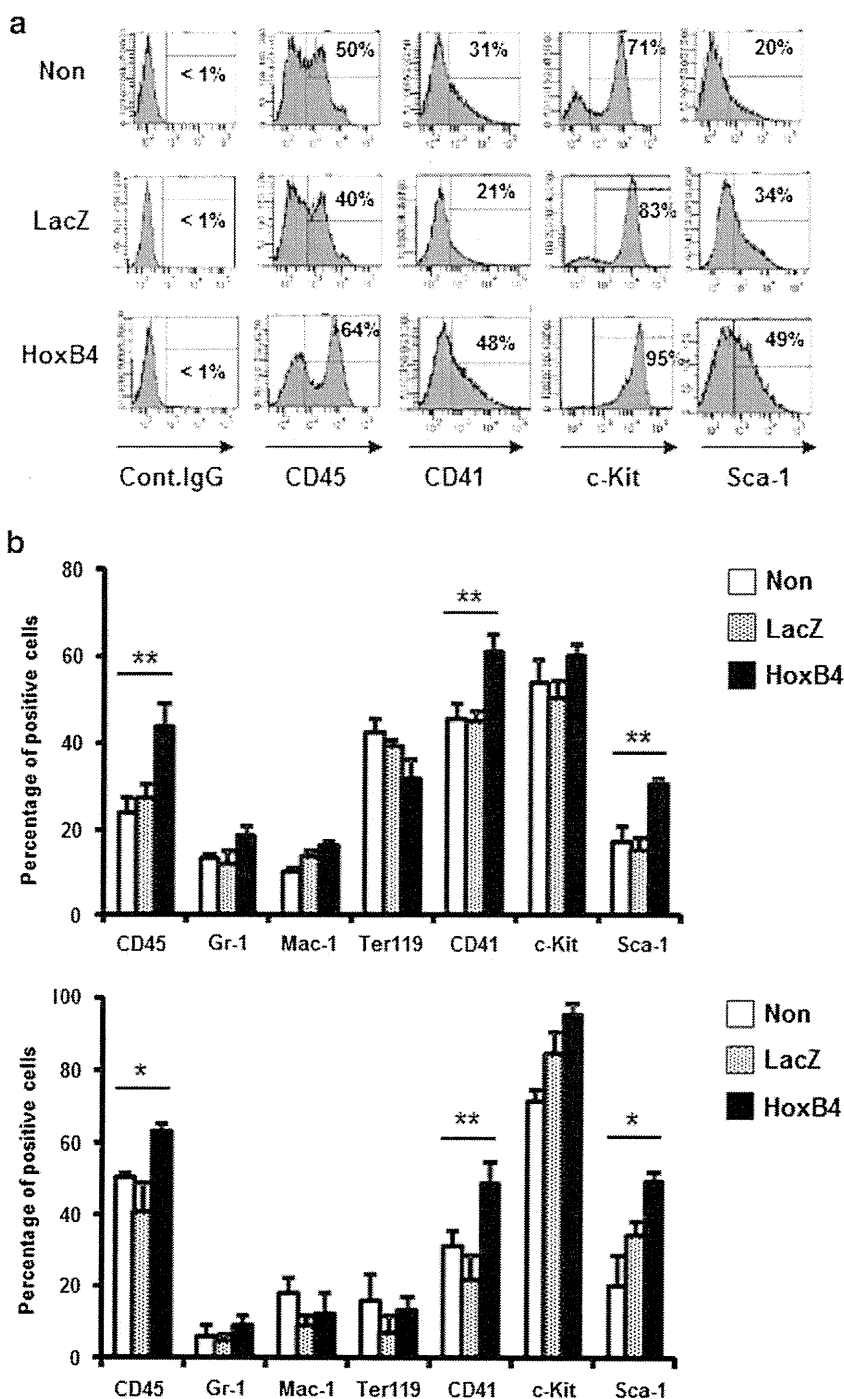


Figure 3 Expression of surface antigen and hematopoietic marker genes in mouse ES cell- or iPS cell-derived cells. (a, b) ES cell- or iPS cell line 38C2-derived cells were reacted with each antibody, and were then subjected to flow cytometric analysis. (a) Representative data from iPS cell line 38C2 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.

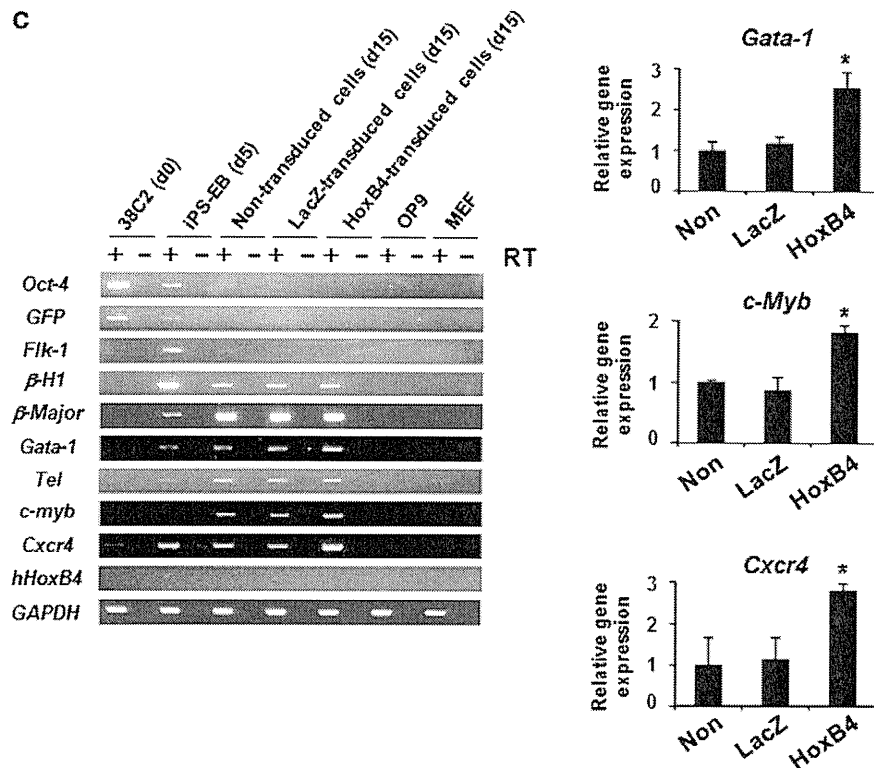


Figure 3 (continued).

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⁺ 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⁺ 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

Grove, PA) or DyLight649-conjugated goat anti-rat IgG (BioLegend, San Diego, CA) was used as secondary Abs.

Cell cultures

The mouse ES cell line E14 and two mouse iPS cell lines, 38C2 and 20D17, both of which were generated by Yamanaka and his colleagues (Okita et al., 2007), were used in this study. 38C2 was kindly provided by Dr. S. Yamanaka (Kyoto University, Kyoto, Japan), and 20D17 was purchased from Riken Biore-source Center (Tsukuba, Japan). In the present study, we mainly used 38C2 iPS cells except where otherwise indicated. Mouse ES and iPS cells were cultured in leukemia inhibitory factor-

containing medium on a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts (MEF) as described previously (Tashiro et al., 2009). OP9 stromal cells were cultured in α -minimum essential medium (α MEM: Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and non-essential amino acid (Invitrogen).

Ad vectors

Ad vectors were constructed by an improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999). The shuttle

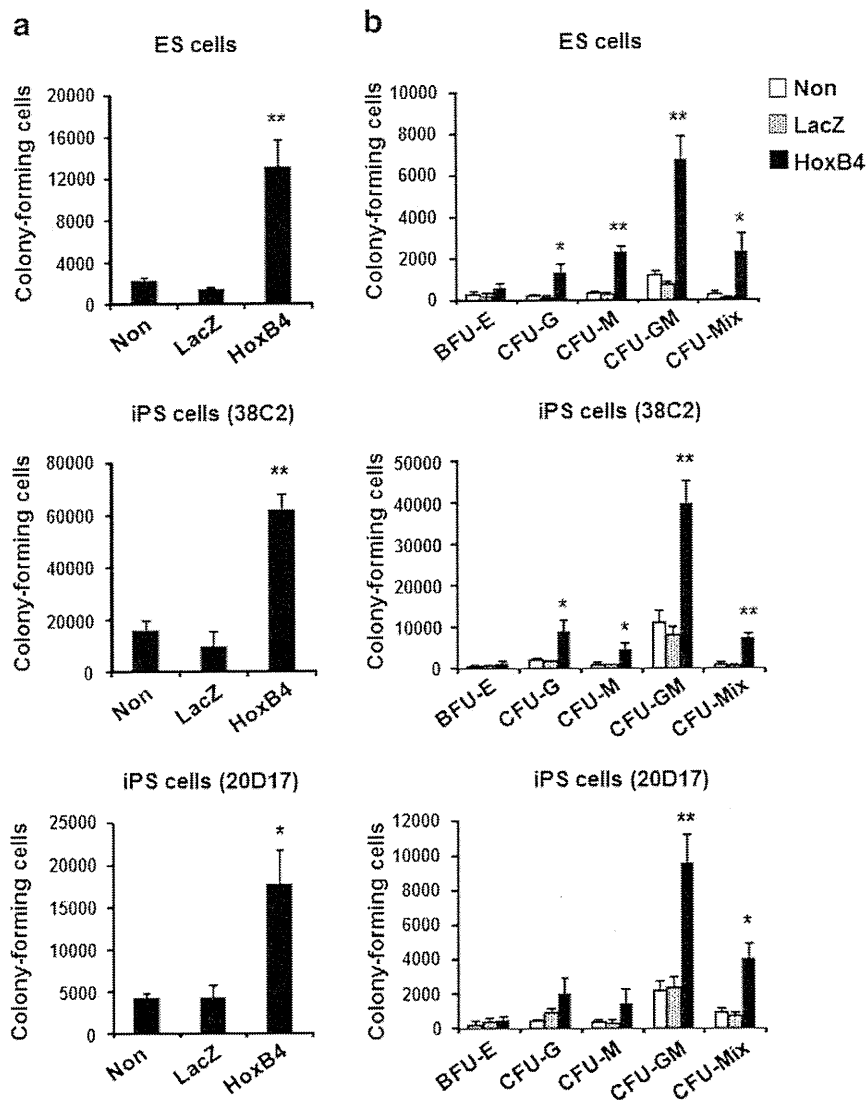


Figure 4 Significant increase of hematopoietic colony-forming cells in Ad-HoxB4-transduced hematopoietic cells. After ES-EB- or iPS-EB-derived cells were transduced with Ad-LacZ or Ad-hHoxB4, hematopoietic cells were generated by co-culturing with OP9 cells in the presence of hematopoietic cytokines for 10 days (a, b) or 20 days (c, d). A colony-forming assay was performed using methylcellulose medium, and the number of hematopoietic colonies was then counted under light microscopy. The number of total colonies (a, c) or subdivided colonies by morphological subtype (BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-Mix) (b, d) generated from ES cells (E14) or iPS cells (38C2 and 20D17) was shown. Colony number was normalized to total number of the cells. Results shown were the mean of three (c, d) or four (a, b) independent experiments with indicated standard deviations. * $p < 0.05$, ** $p < 0.01$ as compared with non-transduced or Ad-LacZ-transduced cells. Abbreviation: BFU-E, burst-forming unit; CFU-G, colony-forming unit-granulocyte; CFU-M, CFU-monocyte; CFU-GM, CFU-granulocyte, monocyte; CFU-GEMM/CFU-Mix, CFU-granulocyte, erythrocyte, monocyte, megakaryocyte.

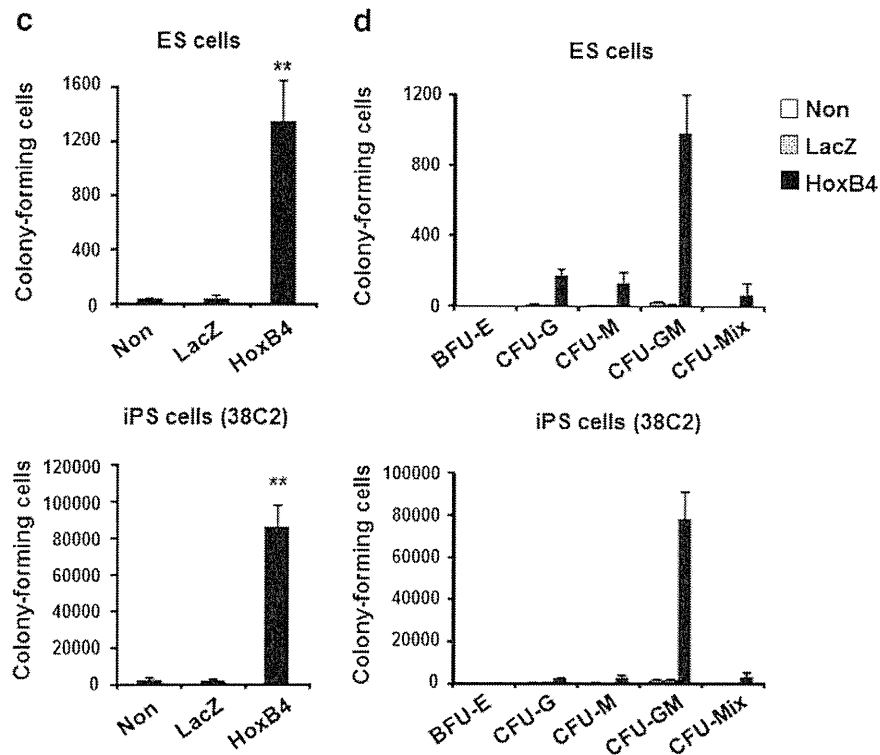


Figure 4 (continued).

plasmid pHMCA5, which contains the CMV enhancer/ β -actin promoter with β -actin intron (CA) promoter (a kind gift from Dr. J. Miyazaki, Osaka University, Osaka, Japan) (Niwa et al., 1991), was previously constructed (Kawabata et al., 2005). The human HoxB4 (hHoxB4)-expressing plasmid, pHMCA-hHoxB4, and DsRed-expressing plasmid, pHMCA-DsRed, were generated by inserting a hHoxB4 cDNA (a kindly gift from Dr. S. Karlsson, Lund University Hospital, Lund, Sweden) and a DsRed cDNA (Clontech, Mountain View, CA), respectively, into pHMCA5. pHMCA-hHoxB4 or pHMCA-DsRed were digested with *I-CeuI*/*PI-SceI* and ligated into *I-CeuI*/*PI-SceI*-digested pAdHM4 (Mizuguchi and Kay, 1998), resulting in pAd-hHoxB4 or pAd-DsRed, respectively. Ad-hHoxB4 and Ad-DsRed were generated and purified as described previously (Tashiro et al., 2008). The CA promoter-driven β -galactosidase (LacZ)-expressing Ad vector, Ad-LacZ, and the CA promoter-driven GFP-expressing Ad vector, Ad-CA-GFP, were generated previously (Tashiro et al., 2008). The vector particle (VP) titer was determined by using a spectrophotometrical method (Maizel et al., 1968).

In vitro differentiation

Prior to embryoid body (EB) formation, mouse ES or iPS cells were suspended in differentiation medium (Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 15% FBS, 0.1 mM 2-mercaptoethanol (Nacalai tesque, Kyoto, Japan), 1 \times non-essential amino acid (Specialty Media, Inc.), 1 \times nucleosides (Specialty Media, Inc.), 2 mM L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen)) and cultured on a culture dish at 37 $^{\circ}$ C for 45 min to remove MEF layers. Mouse ES cell- or iPS cell-derived EBs (ES-EBs or iPS-

EBs, respectively) were generated by culturing ES or iPS cells on a round-bottom low cell binding 96-well plate (Lipidure-coat plate; Nunc) at 1×10^3 cells per well. ES-EBs or iPS-EBs were collected on day 5, and a single cell suspension was prepared by trypsin/EDTA treatment (Invitrogen) at 37 $^{\circ}$ C for 2 min. ES-EB- or iPS-EB-derived CD41⁺c-kit⁺ cells were sorted by FACSaria (BD Bioscience). The purity of the CD41⁺c-kit⁺ cells was greater than 90% based on flow cytometric analysis (Supplemental Fig. 1). Cells were then transduced with an Ad vector at 3000 vector particles (VPs)/cell for 1.5 h in a 15 ml tube. After transduction, total cells (2×10^5) or CD41⁺c-kit⁺ cells (1×10^4) were cultured on OP9 feeder cells in a well of a 6-well plate in α MEM supplemented with 20% FBS, 2 mM L-glutamine, non-essential amino acid, 0.05 mM 2-mercaptoethanol, and hematopoietic cytokines (50 ng/ml mouse stem cell factor (SCF), 50 ng/ml human Flt-3 ligand (Flt-3L), 20 ng/ml thrombopoietin (TPO), 5 ng/ml mouse interleukin (IL)-3, and 5 ng/ml human IL-6 (all from Peprotec, Rocky Hill, NJ)). After culturing with OP9 stromal cells, both non-adherent hematopoietic cells and adherent hematopoietic cells were collected as follows. The non-adherent hematopoietic cells were collected by pipetting and were transferred to 15 ml tubes. The adherent hematopoietic cells were harvested with the use of trypsin/EDTA, and then incubated in a tissue culture dish for 30 min to eliminate the OP9 cells. Floating cells were collected as hematopoietic cells and transferred to the same 15 ml tubes. These hematopoietic cells were kept on ice for further analysis.

Flow cytometry

Cells (1×10^5 to 5×10^5) were incubated with monoclonal Abs at 4 $^{\circ}$ C for 30 min and washed twice with staining buffer

Table 1 List of primers used for RT-PCR.

Gene name	Species	(5') Sense primers (3')	(5')Antisense primers (3')
GAPDH	Ms	ACCACAGTCCATGCCATCAC	TCCACCACCTGTTGCTGTA
HoxB4	Hs	AGAGGCGAGAGAGCAGCTT	TTCTTCTCCAGCTCCAAGA
Oct-3/4	Ms	GTTTGCCAAGCTGCTGAAGC	TCTAGCCCAAGCTGATTGGC
GFP	–	CACATGAAGCAGCAGCAGCTT	TGCTCAGGTAGTGGTTGTCG
Flk-1	Ms	TCTGTGGTTCTGCGTGGAGA	GTATCATTTCACACCACCC
Gata1	Ms	TTGTGAGGCCAGAGAGTGTG	TTCTCGTCTGGATTCCATC
Gata1 (real-time PCR)	Ms	GTCAGAACCGCCCTCTCATC	GTGGTCGTTTGACAGTTAGTGCAT
Tel	Ms	CTGAAGCAGAGGAAATCTCGAATG	GGCAGGCAGTATTATTCTCGA
c-myb	Ms	CCTCACCTCCATCTCAGCTC	GCTGGTGAGGCACCTTTCTTC
β -H1	Ms	AGTCCCATGGAGTCAAAGA	CTCAAGGAGACCTTTGCTCA
β -Major	Ms	CTGACAGATGCTCTTTGGG	CACAACCCAGAAACAGACA
CXCR4	Ms	GTCTATGTGGCGTCTGGAT	GGCAGAGCTTTTGAACTTGG

(PBS/2%FBS). Dead cells were excluded from the analysis by 7-amino actinomycin D (7-AAD, eBioscience). Analysis was performed on an LSRFortessa flow cytometer by using FACS-*Diva* software (BD Bioscience). For detection of transgene expression by Ad vectors, EB-derived total cells or CD41⁺c-kit⁺ cells were transduced with Ad-DsRed or Ad-CA-GFP, respectively, for 1.5 h. At 48 h of incubation with the hematopoietic cytokines as described above, transgene expression in the cells was analyzed by flow cytometry.

Colony assay

A colony-forming assay was performed by plating ES cell-derived hematopoietic cells or iPS cell-derived hematopoietic cells into methylcellulose medium M3434 (Stem Cell Technologies, Vancouver, BC, Canada). After incubation at 37 °C and 5% CO₂ for 10 to 14 days in a humidified atmosphere, colony numbers were counted. The morphology of colonies was observed using an inverted light microscope.

RT-PCR

Total RNA was isolated with the use of ISOGENE (Nippon Gene, Tokyo, Japan). cDNA was synthesized by using Super-Script II reverse transcriptase (Invitrogen) and the oligo(dT) primer. Semi-quantitative PCR was performed with the use of TaKaRa ExTaq HS DNA polymerase (Takara, Shiga, Japan). The PCR conditions were 94 °C for 2 min, followed by the appropriate number of cycles of 94 °C for 15 s, 55 °C for 30 s with 72 °C for 30 s and a final extension of 72 °C for 1 min, except for the addition of 5% dimethyl sulfoxide in the case of hHoxB4 cDNA amplification. The product was assessed by 2% agarose gel electrophoresis followed by ethidium bromide staining. Quantitative real-time PCR was performed using StepOnePlus real-time PCR system with FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The sequences of the primers used for in this study are listed in Table 1.

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2011.09.001.

Conflict of interest

The authors have no financial conflict of interest.

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