

bidirectional differentiation; heterogeneous populations, which contain the hepatocytes and pancreas cells or hepatocytes and cholangiocytes, were obtained, respectively (Figure 1), consistent with a previous report that HNF4 α plays an important role not only in the liver but also in the pancreas.¹² Therefore, we concluded that HNF4 α plays a significant stage-specific role in the differentiation of human ESC- and iPSC-derived hepatoblasts to hepatocytes (Figure 5e).

We found that the expression levels of the hepatic functional genes were upregulated by HNF4 α transduction (Figure 3a,b, and **Supplementary Figures S7** and **S8**). Although the *c/EBP α* and *GATA4* expression levels of the three factors-transduced cells were higher than those of primary human hepatocytes, the *FOXA1*, *FOXA2*, *FOXA3*, and *HNF1 α* , which are known to be important for hepatic direct reprogramming and hepatic differentiation,^{35,36} expression levels of three factors-transduced cells were slightly lower than those of primary human hepatocytes (**Supplementary Figure S8**). Therefore, additional transduction of *FOXA1*, *FOXA2*, *FOXA3*, and *HNF1 α* might promote further hepatic maturation. Some previous hepatic differentiation protocols that utilized growth factors without gene transfer led to the appearance only of heterogeneous hepatocyte populations.^{4–6} The HNF4 α transduction led not only to the upregulation of expression levels of several hepatic markers but also to an almost homogeneous hepatocyte population; the differentiation efficacy based on *CYPs*, *ASGR1*, or *ALB* expression was ~80% (Figure 3c–e). The efficient hepatic maturation in this study might be attributable to the activation of many hepatocyte-associated genes by the transduction of HNF4 α , which binds to the promoters of nearly half of the genes expressed in the liver.¹² In the later stage of hepatic maturation, hepatocyte-associated genes would be strongly upregulated by endogenous transcription factors but not exogenous HNF4 α because transgene expression by Ad vectors was almost disappeared on day 18 (**Supplementary Figure S5**). Another reason for the efficient hepatic maturation would be that sequential transduction of *SOX17*, *HEX*, and HNF4 α could mimic hepatic differentiation in early embryogenesis.

Next, we examined whether or not the hepatocyte-like cells had hepatic functions. The activity of many kinds of *CYPs* was upregulated by HNF4 α transduction (Figure 4b). Ad-HNF4 α -transduced cells exhibit many characteristics of hepatocytes: uptake of LDL, uptake and excretion of ICG, and storage of glycogen (Figure 4a,c,d). Many conventional tests of hepatic characteristics have shown that the hepatocyte-like cells have mature hepatocyte functions. Furthermore, the hepatocyte-like cells can catalyze the toxication of several compounds (Figure 4e). Although the activities to catalyze the toxication of test compounds in primary human hepatocytes are slightly higher than those in the hepatocyte-like cells, the handling of primary human hepatocytes is difficult for a number of reasons: since their source is limited, large-scale primary human hepatocytes are difficult to prepare as a homogeneous population. Therefore, the hepatocyte-like cells derived from human ESCs and iPSCs would be a valuable tool for predicting drug toxicity. To utilize the hepatocyte-like cells in a drug toxicity study, further investigation of the drug metabolism capacity and *CYP* induction potency will be needed.

We also investigated the mechanisms underlying efficient hepatic maturation by HNF4 α transduction. Although the

number of cholangiocyte populations did not change by HNF4 α transduction, we found that the number of hepatoblast populations decreased and that of hepatocyte populations increased, indicating that HNF4 α promotes selective hepatic differentiation from hepatoblasts (Figure 5a). As previously reported, HNF4 α regulates the expression of a broad range of genes that code for cell adhesion molecules,¹³ extracellular matrix components, and cytoskeletal proteins, which determine the main morphological characteristics of epithelial cells.^{14,35,37} In this study, we elucidated that *MET* was promoted by HNF4 α transduction (Figure 5b,d). Thus, we conclude that HNF4 α overexpression in hepatoblasts promotes hepatic differentiation by activating *MET* (Figure 5e).

Using human iPSCs as well as human ESCs, we confirmed that the stage-specific overexpression of HNF4 α could promote hepatic maturation (**Supplementary Figure S9**). Interestingly, the differentiation efficacies differed among human iPS cell lines: two of the human iPS cell lines (*Dotcom* and *Tic*) were more committed to the hepatic lineage than another human iPS cell line (*201B7*) (**Supplementary Figure S7**). Therefore, it would be necessary to select a human iPS cell line that is suitable for hepatic maturation in the case of medical applications, such as drug screening and liver transplantation. The difference of hepatic differentiation efficacy among the three iPS cell lines might be due to the difference of epigenetic memory of original cells or the difference of the inserted position of the foreign genes for the reprogramming.

To control hepatic differentiation mimicking embryogenesis, we employed Ad vectors, which are one of the most efficient transient gene delivery vehicles and have been widely used in both experimental studies and clinical trials.³⁸ We used a fiber-modified Ad vector containing the *EF-1 α* promoter and a stretch of lysine residue (KKKKKKK, K7) peptides in the C-terminal region of the fiber knob.¹⁹ The K7 peptide targets heparan sulfates on the cellular surface, and the fiber-modified Ad vector containing the K7 peptides was shown to be efficient for transduction into many kinds of cells including human ESCs and human ESC-derived cells.^{7–8,19} Thus, Ad vector-mediated transient gene transfer should be a powerful tool for regulating cellular differentiation.

In summary, the findings described here demonstrate that transcription factor HNF4 α plays a crucial role in the hepatic differentiation from human ESC-derived hepatoblasts by activating *MET* (Figure 5e). In the present study, both human ESCs and iPSCs (three lines) were used and all cell lines showed efficient hepatic maturation, indicating that our protocol would be a universal tool for cell line-independent differentiation into functional hepatocytes. Moreover, the hepatocyte-like cells can catalyze the toxication of several compounds as primary human hepatocytes. Therefore, our technology, by sequential transduction of *SOX17*, *HEX*, and HNF4 α , would be a valuable tool for the efficient generation of functional hepatocytes derived from human ESCs and iPSCs, and the hepatocyte-like cells could be used for the prediction of drug toxicity.

MATERIALS AND METHODS

Human ESC and iPSC culture. A human ES cell line, H9 (WiCell Research Institute, Madison, HI), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore, Billerica, MA) with Repro Stem (Repro CELL, Tokyo, Japan) supplemented with 5ng/ml fibroblast

growth factor 2 (FGF2) (Sigma, St Louis, MO). Human ESCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics, Indianapolis, IN) into small clumps and then were subcultured every 4 or 5 days. H9 was used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan. Two human iPS cell lines generated from the human embryonic lung fibroblast cell line MCR5 were provided from the JCRB Cell Bank (Tic, JCRB Number: JCRB1331; and Dotcom, JCRB Number: JCRB1327).^{39,40} These human iPS cell lines were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with iPSELLon (Cardio, Kobe, Japan) supplemented with 10 ng/ml FGF2. Another human iPS cell line, 201B7, generated from human dermal fibroblasts was kindly provided by Dr S. Yamanaka (Kyoto University).² The human iPS cell line 201B7 was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with Repro Stem (Repro CELL) supplemented with 5 ng/ml FGF2 (Sigma). Human iPSCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics) into small clumps and were then subcultured every 5 or 6 days.

In vitro differentiation. Before the initiation of cellular differentiation, the medium of human ESCs and iPSCs was exchanged for a defined serum-free medium, hESF9, and cultured as we previously reported.⁴¹ hESF9 consists of hESF-GRO medium (Cell Science & Technology Institute, Sendai, Japan) supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, oleic acid conjugated with fatty-acid-free bovine albumin (BSA), 10 ng/ml FGF2, and 100 ng/ml heparin (all from Sigma).

The differentiation protocol for the induction of DE cells, hepatoblasts, and hepatocytes was based on our previous report with some modifications.⁷ Briefly, in mesendoderm differentiation, human ESCs and iPSCs were dissociated into single cells and cultured for 3 days on Matrigel (Becton, Dickinson and Company, Tokyo, Japan) in hESF-DIF medium (Cell Science & Technology Institute) supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, 0.5 mg/ml BSA, and 100 ng/ml Activin A (R&D Systems, Minneapolis, MN). To generate mesendoderm cells and DE cells, human ESC-derived cells were transduced with 3,000 vector particles (VP)/cell of Ad-SOX17 for 1.5 hours on day 3 and cultured until day 6 on Matrigel (BD) in hESF-DIF medium (Cell Science & Technology Institute) supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, 0.5 mg/ml BSA, and 100 ng/ml Activin A (R&D Systems). For induction of hepatoblasts, the DE cells were transduced with 3,000 VP/cell of Ad-HEX for 1.5 hours on day 6 and cultured for 3 days on a Matrigel (BD) in hESF-DIF (Cell Science & Technology Institute) medium supplemented with the 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, 0.5 mg/ml BSA, 20 ng/ml bone morphogenetic protein 4 (R&D Systems), and 20 ng/ml FGF4 (R&D Systems). In hepatic differentiation, hepatoblasts were transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4 α for 1.5 hr on day 9 and were cultured for 11 days on Matrigel (BD) in L15 medium (Invitrogen, Carlsbad, CA) supplemented with 8.3% tryptose phosphate broth (BD), 8.3% fetal bovine serum (Vita, Chiba, Japan), 10 μ mol/l hydrocortisone 21-hemisuccinate (Sigma), 1 μ mol/l insulin, 25 mmol/l NaHCO₃ (Wako, Osaka, Japan), 20 ng/ml hepatocyte growth factor (R&D Systems), 20 ng/ml Oncostatin M (R&D Systems), and 10⁻⁶ mol/l Dexamethasone (Sigma).

Ad vectors. Ad vectors were constructed by an improved *in vitro* ligation method.^{42,43} The human HNF4 α gene (accession number NM_000457) was amplified by PCR using primers designed to incorporate the 5' Not I and 3' Xba I restriction enzyme sites: Fwd 5'-ggcctctagatggaggcaggagaatg-3' and Rev 5'-ccccgcggccgagcggcttctagataac-3'. The human HNF4 α gene was inserted into pBSKII (Invitrogen), resulting in pBSKII-HNF4 α , and

then the human HNF4 α gene was inserted into pHMEF5,⁴⁴ which contains the human elongation factor-1 α (EF-1 α) promoter, resulting in pHMEF-HNF4 α . The pHMEF-HNF4 α was digested with I-CeuI/PI-SceI and ligated into I-CeuI/PI-SceI-digested pAdHM41-K7,¹⁹ resulting in pAd-HNF4 α . The human EF-1 α promoter-driven LacZ-, SOX17-, or HEX-expressing Ad vectors, Ad-LacZ, Ad-SOX17, or Ad-HEX, were constructed previously.^{7,8,45} Ad-LacZ, Ad-SOX17, Ad-HEX, and Ad-HNF4 α , each of which contains a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob for more efficient transduction of human ESCs, iPSCs, and DE cells, were generated and purified as described previously.⁷ The VP titer was determined by using a spectrophotometric method.⁴⁶

LacZ assay. Human ESC- and iPSC-derived cells were transduced with Ad-LacZ at 3,000 VP/cell for 1.5 hours. After culturing for the indicated number of days, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) staining was performed as described previously.⁴⁴

Flow cytometry. Single-cell suspensions of human ESCs, iPSCs, and their derivatives were fixed with methanol at 4 °C for 20 minutes and then incubated with the primary antibody, followed by the secondary antibody. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD).

RNA isolation and reverse transcription-PCR. Total RNA was isolated from human ESCs, iPSCs, and their derivatives using ISOGENE (Nippon Gene) according to the manufacturer's instructions. Primary human hepatocytes were purchased from CellzDirect, Durham, NC. complementary DNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Real-time reverse transcription-PCR was performed with Taqman gene expression assays (Applied Biosystems, Foster City, CA) or SYBR Premix Ex Taq (TaKaRa) using an ABI PRISM 7000 Sequence Detector (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. The primer sequences used in this study are described in **Supplementary Table S1**.

Immunohistochemistry. The cells were fixed with methanol or 4% paraformaldehyde (Wako). After blocking with phosphate-buffered saline containing 2% BSA (Sigma) and 0.2% Triton X-100 (Sigma), the cells were incubated with primary antibody at 4 °C for 16 hours, followed by incubation with a secondary antibody that was labeled with Alexa Fluor 488 (Invitrogen) or Alexa Fluor 594 (Invitrogen) at room temperature for 1 hour. All the antibodies are listed in **Supplementary Table S2**.

Assay for CYP activity. To measure cytochrome P450 3A4, 2C9, and 1A2 activity, we performed Lytic assays by using a P450-Glo™ CYP3A4 Assay Kit (Promega, Madison, WI). For the CYP3A4 and 2C9 activity assay, undifferentiated human ESCs, the hepatocyte-like cells, and primary human hepatocytes were treated with rifampicin (Sigma), which is the substrate for CYP3A4 and CYP2C9, at a final concentration of 25 μ mol/l or DMSO (0.1%) for 48 hours. For the CYP1A2 activity assay, undifferentiated human ESCs, the hepatocyte-like cells, and primary human hepatocytes were treated with omeprazole (Sigma), which is the substrate for CYP1A2, at a final concentration of 10 μ M or DMSO (0.1%) for 48 hours. We measured the fluorescence activity with a luminometer (Lumat LB 9507; Berthold, Oak Ridge, TN) according to the manufacturer's instructions.

Pyronin Y/Hoechst Staining. Human ESC-derived cells were stained with Hoechst33342 (Sigma) and Pyronin Y (PY) (Sigma) in Dulbecco's modified Eagle medium (Wako) supplemented with 0.2 mmol/l HEPES and 5% FCS (Invitrogen). Samples were then placed on ice for 15 minutes, and 7-AAD was added to a final concentration of 0.5 mg/ml for exclusion of dead cells. Fluorescence-activated cell-sorting analysis of these cells was

performed on a FACS LSR Fortessa flow cytometer (Becton Dickinson) equipped with a UV-laser.

Cellular uptake and excretion of ICG. ICG (Sigma) was dissolved in DMSO at 100 mg/ml, then added to a culture medium of the hepatocyte-like cells to a final concentration of 1 mg/ml on day 20 of differentiation. After incubation at 37°C for 60 minutes, the medium with ICG was discarded and the cells were washed with phosphate-buffered saline. The cellular uptake of ICG was then examined by microscopy. Phosphate-buffered saline was then replaced by the culture medium and the cells were incubated at 37°C for 6 hours. The excretion of ICG was examined by microscopy.

Periodic Acid-Schiff assay for glycogen. The hepatocyte-like cells were fixed with 4% paraformaldehyde and stained using a Periodic Acid-Schiff staining system (Sigma) on day 20 of differentiation according to the manufacturer's instructions.

Cell viability tests. Cell viability was assessed by Alamar Blue assay kit (Invitrogen). After treatment with test compounds^{47–50} (troglitazone, acetaminophen, cyclophosphamide, and carbamazepine) (all from Wako) for 2 days, the culture medium was replaced with 0.5 mg/ml solution of Alamar Blue in culturing medium and cells were incubated for 3 hours at 37°C. The supernatants of the cells were measured at a wavelength of 570 nm with background subtraction at 600 nm in a plate reader. Control refers to incubations in the absence of test compounds and was considered as 100% viability value.

Uptake of LDL. The hepatocyte-like cells were cultured with medium containing Alexa-488-labeled LDL (Invitrogen) for 1 hour, and then the cells that could uptake LDL were assessed by immunohistochemistry and flow cytometry.

Primary human hepatocytes. Cryopreserved human hepatocytes were purchased from CellzDirect (lot Hu8072). The vials of hepatocytes were rapidly thawed in a shaking water bath at 37°C; the contents of the vial were emptied into prewarmed Cryopreserved Hepatocyte Recovery Medium (CellzDirect) and the suspension was centrifuged at 100g for 10 minutes at room temperature. The hepatocytes were seeded at 1.25×10^5 cells/cm² in hepatocyte culture medium (Lonza, Walkersville, MD) containing 10% FCS (GIBCO-BRL) onto type I collagen-coated 12-well plates. The medium was replaced with hepatocyte culture medium containing 10% FCS (GIBCO-BRL) 6 hours after seeding. The hepatocytes, which were cultured 48 hours after plating the cells, were used in the experiments.

SUPPLEMENTARY MATERIAL

Figure S1. Genome-wide screening of transcription factors involved in hepatic differentiation emphasizes the importance of the transcription factor HNF4 α .

Figure S2. Summary of specific markers for DE cells, hepatoblasts, hepatocytes, cholangiocytes, and pancreas cells.

Figure S3. The formation of DE cells, hepatoblasts, hepatocytes, and cholangiocytes from human ESCs.

Figure S4. Overexpression of HNF4 α mRNA in hepatoblasts by Ad-HNF4 α transduction.

Figure S5. Time course of LacZ expression in hepatoblasts transduced with Ad-LacZ.

Figure S6. The morphology of the hepatocyte-like cells.

Figure S7. Upregulation of the expression levels of conjugating enzymes and hepatic transporters by HNF4 α transduction.

Figure S8. Upregulation of the expression levels of hepatic transcription factors by HNF4 α transduction.

Figure S9. Generation of hepatocytes from various human ES or iPSC cell lines.

Figure S10. Promotion of MET by HNF4 α transduction.

Figure S11. Arrest of cell growth by HNF4 α transduction.

Table S1. List of Taqman probes and primers used in this study.

Table S2. List of antibodies used in this study.

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