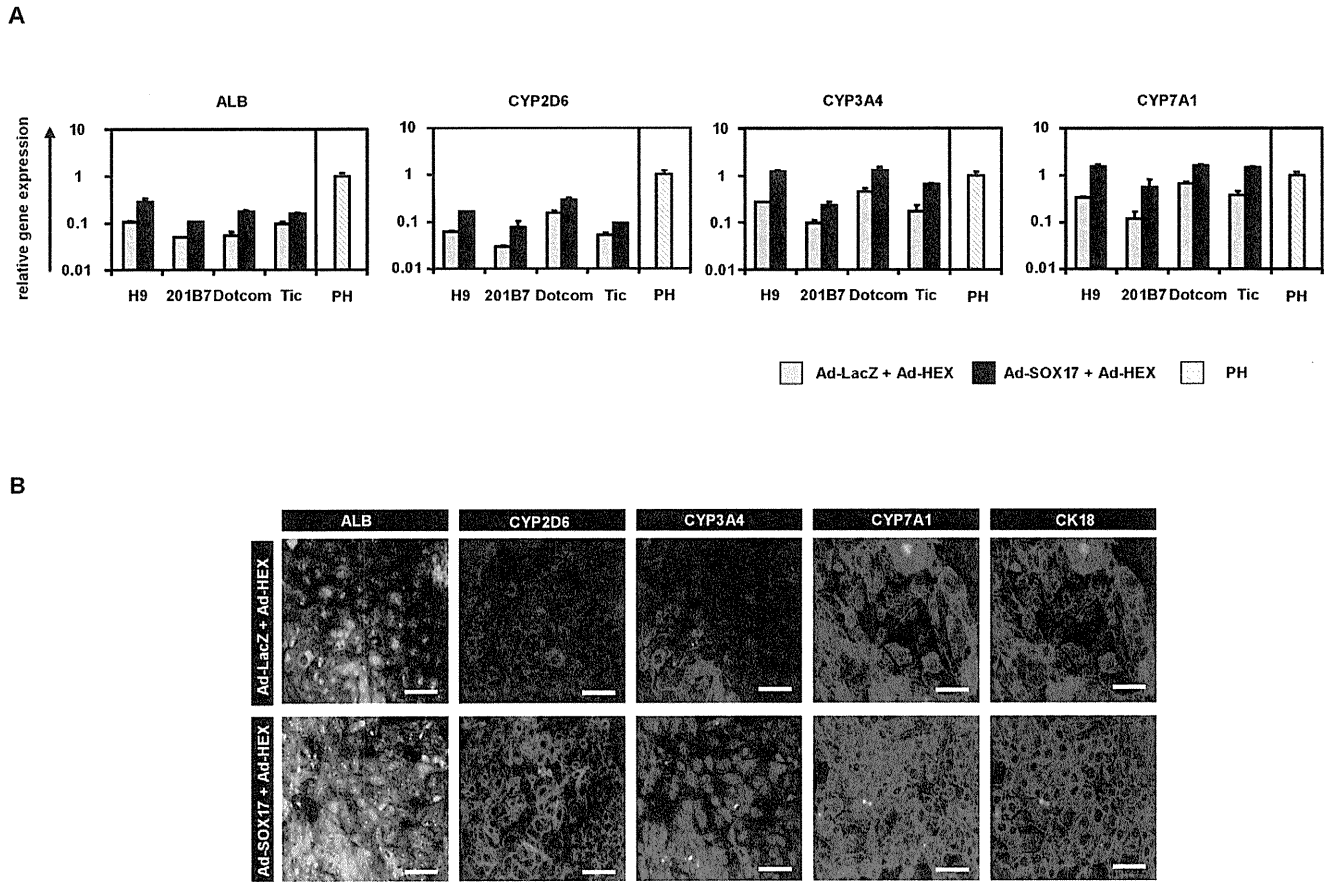

Figure 4. Characterization of hepatocyte-like cells from human ESC- and iPSC-derived DE cells. (A) The Ad-LacZ-transduced cells and Ad-SOX17-transduced cells were transduced with 3,000 VP/cell of Ad-HEX for 1.5 h on day 6. On day 18 of differentiation, the levels of expression of the hepatocyte markers (ALB, CYP2D6, CYP3A4, and CYP7A1) were examined by real-time RT-PCR in human ESC (H9)-derived hepatocyte-like cells and human iPSC (201B7, Dotcom, or Tic)-derived hepatocyte-like cells. The gene expression profiles of cells transduced with both Ad-SOX17 and Ad-HEX (black bar) were compared with those of cells transduced with both Ad-LacZ and Ad-HEX (gray bar). The expression level of primary human hepatocytes (PH, hatched bar), which were cultured 48 h after plating the cells, were defined as 1.0. All data are represented as the means±SD ($n = 3$). (B) The expression of the hepatocyte markers ALB (green), CYP2D6 (red), CYP3A4 (red), CYP7A1 (red), and CK18 (green) was also examined by immunohistochemistry on day 18 of differentiation. Nuclei were counterstained with DAPI (blue). The scale bar represents 50 μm.
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differentiation but also ExEn differentiation even in the presence of Activin A (Figures 2A and 2C), demonstrating that transduction at an inappropriate stage of differentiation prevents directed differentiation. These results suggest that stage-specific SOX17 transduction mimicking the gene expression pattern in embryogenesis could selectively promote DE differentiation.

Another important finding about DE differentiation is that the protocol in the present study was sufficient for nearly homogeneous DE and anterior DE differentiation by mesendoderm stage-specific SOX17 overexpression; the differentiation efficacies of c-Kit/CXCR4-double-positive DE cells and HEX-positive anterior DE cells were approximately 70% and 54%, respectively (Figures 2E and 2F). The conventional differentiation protocols without gene transfer were not sufficient for homogenous DE and anterior DE differentiation; the differentiation efficacies of DE and anterior DE were approximately 30% and 10%, respectively [10,11,23]. One of the reasons for the efficient DE differentiation by SOX17 transduction might be the activation of the FOXA2 gene which could regulate many endoderm-associated genes [35]. Moreover, SOX17-transduced cells were more committed to the hepatic lineage (Figure 4). This might be because the number of HEX-positive anterior DE cell populations was increased by SOX17 transduction. Recent studies have shown that the conditional expression of Sox17 in the pancreas at E12.5, when it is not normally expressed, is sufficient to promote biliary differentiation at the expense of endocrine cells [36]. Therefore, we reconfirmed that our protocol in which SOX17 was transiently transduced at the appropriate stage of differentiation was useful for DE and hepatic differentiation from human ESCs and iPSCs.

Using human iPSCs as well as human ESCs, we confirmed that stage-specific overexpression of SOX17 could promote directive differentiation of either ExEn or DE cells (Figures 1F, 2G, and 4A). Interestingly, a difference of DE and hepatic differentiation efficacy among human iPS cell lines was observed (Figures 1F and 2G). Therefore, it would be necessary to select a human iPS cell line that is suitable for hepatic differentiation in the case of medical applications, such as liver transplantation.

To control cellular 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 [37]. Recently, we have also demonstrated that ectopic HEX expression by Ad vectors in human ESC-derived DE cells markedly enhances the hepatic differentiation [13]. Thus, Ad vector-mediated transient gene

transfer should be a powerful tool for regulating cellular differentiation.

In summary, the findings presented here demonstrate a stage-specific role of SOX17 in the ExEn and DE differentiation from human ESCs and iPSCs (Figure S8). Although previous reports showed that SOX17 overexpression in ESCs leads to differentiation of either ExEn or DE cells, we established a novel method to promote directive differentiation by SOX17 transduction. Because we utilized a stage-specific overexpression system, our findings provide further evidence that the lineage commitment in this method seems to reflects what is observed in embryonic development. In the present study, both human ESCs and iPSCs (3 lines) were used and all cell lines showed efficient ExEn or DE differentiation, indicating that our novel protocol is a powerful tool for efficient and cell line-independent endoderm differentiation. Moreover, the establishing methods for efficient hepatic differentiation by sequential SOX17 and HEX transduction would be useful for *in vitro* applications such as screening of pharmacological compounds as well as for regenerative therapy.

Materials and Methods

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 [38] and cultured as we previously reported. hESF9 consists of hESF-GRO medium (Cell Science & Technology Institute) supplemented with 5 factors (10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, and 10 μM sodium selenite), oleic acid conjugated with fatty acid free bovine albumin, 10 ng/ml FGF2, and 100 ng/ml heparin (all from Sigma).

To induce, ExEn cells, human ESCs and iPSCs were cultured for 5 days on a gelatin-coated plate in mouse embryonic conditioned-medium supplemented with 20 ng/ml BMP4 (R&D system) and 1% FCS (GIBCO-BRL).

The differentiation protocol for induction of DE cells, hepatoblasts, and hepatocyte-like cells was based on our previous report with some modifications [13]. Briefly, in DE differentiation, human ESCs and iPSCs were cultured for 5 days on a Matrigel (BD)-coated plate in hESF-DIF medium (Cell Science & Technology Institute) supplemented with the above-described 5 factors, 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 h and cultured in hESF-DIF (Cell Science & Technology Institute) medium supplemented with the above-described 5 factors, 0.5 mg/ml BSA, 10 ng/ml bone morphology protein 4 (BMP4) (R&D Systems), and 10 ng/ml FGF4 (R&D systems). In hepatic differentiation, the cells were cultured in hepatocyte culture medium (HCM) supplemented with SingleQuots (Lonza), 10 ng/ml hepatocyte growth factor (HGF) (R&D Systems), 10 ng/ml Oncostatin M (OsM) (R&D Systems), and 10^{-7} M dexamethasone (DEX) (Sigma).

Human ESC and iPSC Culture

A human ES cell line, H9 (WiCell Research Institute), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore) with Repro Stem (Repro CELL), supplemented with 5 ng/ml fibroblast growth factor 2 (FGF2) (Sigma). Human ESCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics) into small clumps, and subcultured every 4 or 5 days. 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), supplemented with 10 ng /ml FGF2. Another human iPS cell line, 201B7, generated from human dermal fibroblasts (HDF) was kindly provided by Dr. S. Yamanaka (Kyoto University) [6]. 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 subcultured every 5 or 6 days.

Adenovirus (Ad) Vectors

Ad vectors were constructed by an improved *in vitro* ligation method [41,42]. The human SOX17 gene (accession number NM_022454) was amplified by PCR using primers designed to incorporate the 5′ BamHI and 3′ XbaI restriction enzyme sites: Fwd 5′-gcagggatccagcgccatgagcagcccgg-3′ and Rev 5′-cttctagagatcagggacctgtcacacgtc-3′. The human SOX17 gene was inserted into pcDNA3 (Invitrogen), resulting in pcDNA-SOX17, and then the human SOX17 gene was inserted into pHMEF5 [15], which contains the human EF-1α promoter, resulting in pHMEF-SOX17. The pHMEF-SOX17 was digested with I-CeuI/PI-SceI and ligated into I-CeuI/PI-SceI-digested pAdHM41-K7 [16], resulting in pAd-SOX17. The human elongation factor-1α (EF-1α) promoter-driven LacZ- or HEX-expressing Ad vectors, Ad-LacZ or Ad-HEX, were constructed previously. [13,43]. Ad-SOX17, Ad-HEX, and Ad-LacZ, which contain 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 [13,15,43]. The vector particle (VP) titer was determined by using a spectrophotometric method [44].

Flow Cytometry

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

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from human ESCs, iPSCs, and their derivatives using ISOGENE (Nippon Gene) according to the manufacture's instructions. Primary human hepatocytes were purchased from CellzDirect. 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 (Applied Biosystems) 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 (GAPDH). The primer sequences used in this study are described in Table S1.

Immunohistochemistry

The cells were fixed with methanol or 4% PFA. After blocking with PBS containing 2% BSA and 0.2% Triton X-100 (Sigma), the cells were incubated with primary antibody at 4°C for 16 h, followed by incubation with a secondary antibody that was labeled

with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) at room temperature for 1 h. All the antibodies are listed in Table S2.

Crystal Violet Staining

The human ESC-derived cells that had adhered to the wells were stained with 200 µl of 0.3% crystal violet solution at room temperature for 15 min. Excess crystal violet was then removed and the wells were washed three times. Fixed crystal violet was solubilized in 200 µl of 100% ethanol at room temperature for 15 min. Cell viability was estimated by measuring the absorbance at 595 nm of each well using a microtiter plate reader (Sunrise, Tecan).

LacZ Assay

The human ESC- and iPSC-derived cells were transduced with Ad-LacZ at 3,000 VP/cell for 1.5 h. 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 [15].

Supporting Information

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

Table S2 List of antibodies used in this study.
(DOC)

Figure S1 PrE cells formation from human ESCs on day 1 of differentiation. (A) The procedure for differentiation of human ESCs and iPSCs to ExEn cells by treatment with BMP4 (20 ng/ml) is presented schematically. (B) Human ESCs (H9) were morphologically changed during ExEn differentiation; when human ESCs were cultured with the medium containing BMP4 (20 ng/ml) for 5 days, the cells began to show flattened epithelial morphology. The scale bar represents 50 µm. (C–E) The tTemporal protein expression analysis during ExEn differentiation was performed by immunohistochemistry. The PrE markers COUP-TF1 [21] (red), SOX17 [14] (red), and SOX7 [14] (red) were detected on day 1. In contrast to the PS markers, the expression of the DE marker GSC [22] (red) was not detected and the level of the pluripotent marker NANOG (green) declined between day 0 and day 1. Nuclei were counterstained with DAPI (blue). The scale bar represents 50 µm.
(PDF)

Figure S2 Mesendoderm cells formation from human ESCs on day 3 of differentiation. (A) The procedure for differentiation of human ESCs and iPSCs to DE cells by treatment with Activin A (100 ng/ml) is presented schematically. hESF-GRO medium was supplemented with 5 factors and 0.5 mg/ml fatty acid free BSA, as described in the Materials and Methods. (B) Human ESCs (H9) were morphologically changed during DE differentiation; when human ESCs were cultured with the medium containing Activin A (100 ng/ml) for 5 days, the morphology of the cells began to show visible cell-cell boundaries. The scale bar represents 50 µm. (C–E) The tTemporal protein expression analysis during DE differentiation was performed by immunohistochemistry. The anterior PS markers FOXA2 [21] (red), GSC [22] (red), and SOX17 [14] (red) were adequately detected on day 3. The PS marker T [45] (red) was detected until day 3. In contrast to the PS markers, the expression of the pluripotent marker NANOG [24] (green) declined between day 2 and day 3. Nuclei were counterstained with DAPI (blue). The scale bar represents 50 µm.
(PDF)

Figure S3 Overexpression of SOX17 mRNA in human ESC (H9)-derived PS cells by Ad-SOX17 transduction. Human ESC-derived PS cells (day 1) were transduced with 3,000VP/cell of Ad-SOX17 for 1.5 h. On day 3 of differentiation, real-time RT-PCR analysis of the SOX17 expression was performed in Ad-LacZ-transduced cells and Ad-SOX17-transduced cells. On the y axis, the expression levels of undifferentiated human ESCs on day 0 was were taken defined as 1.0. All data are represented as the means±SD ($n = 3$).
(PDF)

Figure S4 Efficient transduction in Activin A-induced human ESC (H9)-derived cells by using a fiber-modified Ad vector containing the EF-1α promoter. Undifferentiated human ESCs and Activin A-induced human ESC-derived cells, which were cultured with the medium containing Activin A (100 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 vector particles (VP)/cell of Ad-LacZ for 1.5 h. The day after transduction, X-gal staining was performed. The scale bar represents 100 µm. Similar results were obtained in two independent experiments.
(PDF)

Figure S5 Optimization of the time period for Ad-SOX17 transduction to promote DE differentiation from human iPSCs (Tic). Undifferentiated human iPSCs and Activin A-induced human iPSC-derived cells, which were cultured with the medium containing Activin A (100 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 VP/cell of Ad-SOX17 for 1.5 h. Ad-SOX17-transduced cells were cultured with Activin A (100 ng/ml) until day 5, and then real-time RT-PCR analysis was performed. The horizontal axis represents the day on which the cells were transduced with Ad-SOX17. On the y axis, the expression levels of undifferentiated cells on day 0 was were taken defined as 1.0. All data are represented as the means±SD ($n = 3$).
(PDF)

Figure S6 Time course of LacZ expression in human ESC (H9)-derived mesendoderm cells transduced with Ad-LacZ. The hHuman ESC-derived mesendoderm cells (day 3) were transduced with 3,000 VP/cell of Ad-LacZ for 1.5 h. On days 4, 5, 6, 8, and 10, X-gal staining was performed. Note that human ESC-derived cells were passaged on day 5. The scale bar represents 100 µm. Similar results were obtained in two independent experiments.
(PDF)

Figure S7 Optimization of the time period for Ad-SOX17 transduction into Activin A-induced human ESC (H9)-derived cells. Undifferentiated human ESCs and Activin A-induced hESC-derived cells, which were cultured with the medium containing Activin A (100 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 VP/cell of Ad-LacZ or Ad-SOX17 for 1.5 h. Ad-SOX17-transduced cells were cultured with Activin A (100 ng/ml) until day 5, then the cell viability was evaluated with crystal violet staining. The horizontal axis represents the day on which the cells were transduced with Ad-SOX17. On the y axis, the level of non-transduced cells was taken defined as 1.0. All data are represented as the means±SD (n = 3).
(PDF)

Figure S8 Model of differentiation of human ESCs and iPSCs into ExEn and DE cells by stage-specific SOX17 transduction. The ExEn and DE differentiation process is divided into at least two stages. In the first stage, human ESCs differentiate into either PrE cells by treatment with BMP4 (20 ng/ml) or mesendoderm cells by treatment with Activin A (100 ng/

ml). In the second stage, SOX17 promotes the further differentiation of each precursor cell into ExEn and DE cells, respectively. We have demonstrated that the efficient differentiation of these two distinct endoderm lineages is accomplished by stage-specific SOX17 transduction.
(PDF)

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Author Contributions

Conceived and designed the experiments: K. Takayama MI K. Kawabata MFK HM. Performed the experiments: K. Takayama MI K. Tashiro. Analyzed the data: K. Takayama MI K. Kawabata K. Tashiro K. Katayama FS HM. Contributed reagents/materials/analysis tools: K. Kawabata K. Katayama FS TH MFK HM. Wrote the paper: K. Takayama K. Kawabata HM.

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Efficient Generation of Hepatoblasts From Human ES Cells and iPS Cells by Transient Overexpression of Homeobox Gene *HEX*

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Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the potential to differentiate into all cell lineages, including hepatocytes, *in vitro*. Induced hepatocytes have a wide range of potential application in biomedical research, drug discovery, and the treatment of liver disease. However, the existing protocols for hepatic differentiation of PSCs are not very efficient. In this study, we developed an efficient method to induce hepatoblasts, which are progenitors of hepatocytes, from human ESCs and iPSCs by overexpression of the *HEX* gene, which is a homeotic gene and also essential for hepatic differentiation, using a HEX-expressing adenovirus (Ad) vector under serum/feeder cell-free chemically defined conditions. Ad-HEX-transduced cells expressed α-fetoprotein (AFP) at day 9 and then expressed albumin (ALB) at day 12. Furthermore, the Ad-HEX-transduced cells derived from human iPSCs also produced several cytochrome P450 (CYP) isozymes, and these P450 isozymes were capable of converting the substrates to metabolites and responding to the chemical stimulation. Our differentiation protocol using Ad vector-mediated transient HEX transduction under chemically defined conditions efficiently generates hepatoblasts from human ESCs and iPSCs. Thus, our methods would be useful for not only drug screening but also therapeutic applications.

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INTRODUCTION

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most cell types of the body,[1–4] and thereby have the potential to provide an unlimited source of cells for a variety of applications.[5] Hepatocytes are useful cells for biomedical research, regenerative medicine, and drug discovery. They are particularly applicable to drug screenings, such as for the determination of metabolic and toxicological properties of drug compounds in *in vitro* models, because the liver is the main detoxification organ in the body.[6] For these applications, it is necessary to prepare a large number of functional hepatocytes from human ESCs and iPSCs. Many of the existing methods for cell differentiation of human ESCs and iPSCs into hepatocytes employ undefined, serum-containing medium and feeder cells.[7–9] Preparation of human ESC- and iPSC-derived hepatocytes for therapeutic applications and drug toxicity testing in humans should be done in nonxenogenic culture systems to avoid potential contamination with pathogens. Furthermore, the efficiency of the differentiation of the human ESCs and iPSCs into hepatocytes is not particularly high using these methods.[9–14]

In vertebrate development, the liver is derived from the primitive gut tube, which is formed by a flat sheet of cells called the definitive endoderm.[5,15] Shortly afterwards, the definitive endoderm is separated into endoderm derivatives containing the liver bud, the cells of which are referred to as hepatoblasts. The hepatoblasts have the potential to proliferate and differentiate into both hepatocytes and cholangiocytes. In the process of hepatic differentiation, the maturation is characterized by the expression of liver- and stage-specific genes. For example, α-fetoprotein (AFP) is an early hepatic marker, which is expressed in hepatoblasts in the liver bud until birth, and its expression is dramatically reduced after birth.[16] In contrast, albumin (ALB), which is the most abundant protein synthesized by hepatocytes, is initially expressed at lower levels in early fetal hepatocytes, but its expression level is increased as the hepatocytes mature, reaching a maximum in adult hepatocytes.[17] Furthermore, isoforms of cytochrome P450 (CYP) proteins also exhibit differential expression levels according to the developmental stages

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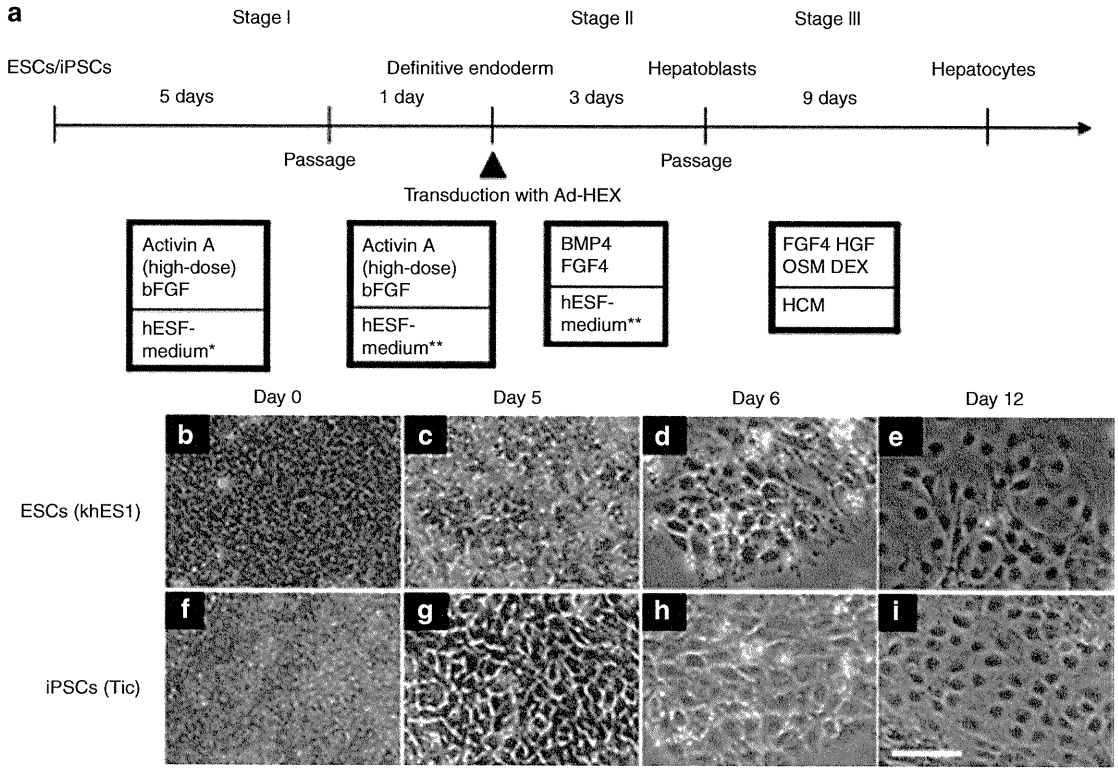

Figure 1 A strategy of differentiation of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to hepatoblasts and hepatocytes. (**a**) Schematic representation illustrating the procedure for differentiation of human ESCs (khES1) and iPSCs (Tic) to hepatocytes. (**b–i**) Phase contrast microscopy showing sequential morphological changes (day 0–12) from (**b–e**) human ESCs (khES1) and (**f–i**) iPSCs (Tic) to hepatoblasts via the definitive endoderm. Bar = 50 µm. bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein 4; DEX, dexamethasone; FGF4, fibroblast growth factor 4; HGF, hepatocyte growth factor; OSM, Oncostatin M; HCM, hepatocytes culture medium; *, hESF-GRO medium that was supplemented with 10 µg/ml human recombinant insulin, 5 µg/ml human apotransferrin, 10 µmol/l 2-mercaptoethanol, 10 µmol/l ethanolamine, 10 µmol/l sodium selenite, 0.5 mg/ml fatty acid free BSA; **, hESF-DIF medium that was supplemented with 10 µg/ml insulin, 5 µg/ml apotransferrin, 10 µmol/l 2-mercaptoethanol, 10 µmol/l ethanolamine, 10 µmol/l sodium selenite, 0.5 mg/ml BSA.

of the liver. Although most CYPs (including CYP3A4, CYP7A1, and CYP2D6) are only slightly expressed or not detected in the fetal liver tissue, the expression levels are dramatically increased after birth.[18]

For the development of hepatoblasts, numerous transcription factors are required, such as hematopoietically expressed homeobox (*HEX*), GATA-binding protein 6, prospero homeobox 1, and hepatocyte nuclear factor 4A.[15,19] Among them, *HEX* is suggested to function at the earliest stage of hepatic linage.[20] HEX is first expressed in the definitive endoderm and becomes restricted to the future hepatoblasts. Targeted deletion of the *HEX* gene in the mouse results in embryonic lethality and a dramatic loss of the fetal liver parenchyma.[19,21,22] The hepatic genes, including *ALB*, prospero homeobox1, and hepatocyte nuclear factor 4A, are transiently expressed in the definitive endoderm of *HEX*-null embryos, and further morphogenesis of the hepatoblasts does not occur.[23] In general, then, *HEX* is essential for the definitive endoderm to adopt a hepatic cell fate.

Adenovirus (Ad) vectors are one of the most efficient gene delivery vehicles and have been widely used in both experimental studies and clinical trials.[24] Ad vectors are attractive vehicles for gene transfer because they are easily constructed, can be prepared in high titers, and provide high transduction efficiency in both dividing and nondividing cells. We have developed efficient methods for Ad vector-mediated transient transduction into mouse ESCs and iPSCs.[25,26] We have also showed that the differentiations of mouse ESCs and iPSCs into adipocytes and osteoblasts were dramatically promoted by Ad vector-mediated peroxisome proliferator activated receptor γ and runt related transcription factor 2 transduction, respectively.[25,26]

In this study, we hypothesized that transient *HEX* transduction could efficiently induce hepatoblasts from human ESCs and iPSCs. A previous study demonstrated that *HEX* regulates the differentiation of hemangioblasts and endothelial cells from mouse ESCs,[27] whereas the role of *HEX* in the differentiation of hepatoblasts from human ESCs and iPSCs remains unknown. We found that differentiation of hepatoblasts from the human ESC- and iPSC-derived definitive endoderms, but not from undifferentiated human ESCs and iPSCs, could be facilitated by Ad vector-mediated transient transduction of a *HEX* gene. Furthermore, the Ad-HEX-transduced cells that were derived from human iPSCs were able to differentiate into functional hepatocytes *in vitro*. All the processes for cellular differentiation were performed under serum/feeder cell- free chemically defined conditions. Our culture systems and differentiation method based on Ad vector-mediated transient transduction under chemically defined conditions would provide a platform for drug screening as well as safe therapies.

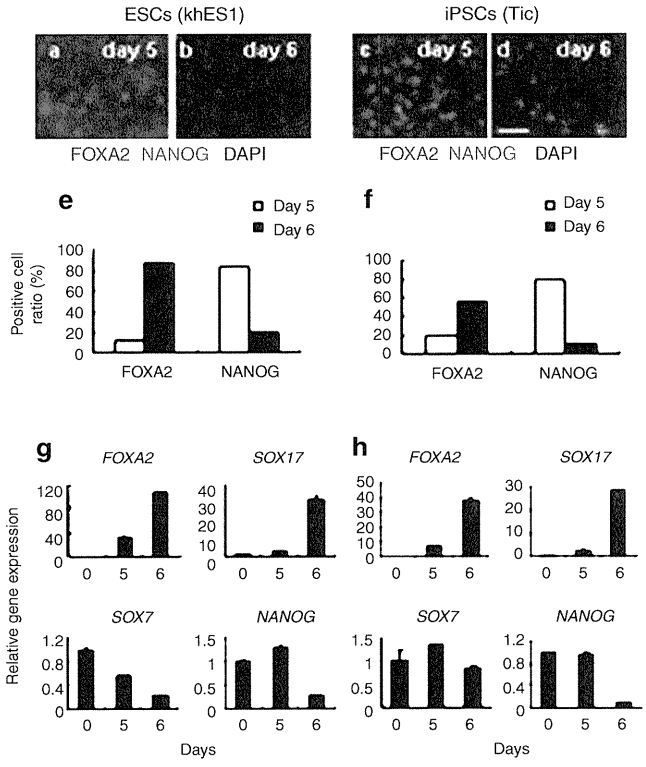

Figure 2 Characterization of the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. (**a–d**) The immunofluorescent staining of the human ESC (khES1)- and iPSC (Tic) derived differentiated cells before (**a** and **c**; day 5) and after passaging (**b** and **d**; day 6). The cells were immunostained with antibodies against FOXA2 and NANOG. Nuclei were stained with DAPI. (**e**,**f**) Semiquantitative analysis of the immunofluorescent staining in **a–d**. Data are presented as the mean of immunopositive cells counted in eight independent fields. (**g**,**h**) Real-time RT-PCR analysis of the level of definitive endoderm (*FOXA2* and *SOX17*), pluripotent (*NANOG*), and extra-embryonic endoderm (*SOX7*) gene expression at day 5 and 6. At day 5, the cells were passaged. Therefore, the data at day 5 and 6 show the levels of gene expression before (at day 5) or after the passage (at day 6). Data are presented as the mean ± SD from triplicate experiments. The graphs represent the relative gene expression level when the level of undifferentiated cells at day 0 was taken as 1. Bar = 50 μm. ESC, embryonic stem cells; iPSC, induced pluripotent stem cells.

RESULTS

Differentiation of human ESC- and iPSC-derived definitive endoderms

Our three-step differentiation protocol is illustrated in Figure 1a. After treatment with 50 ng/ml of Activin A (high-dose) and basic fibroblast growth factor (bFGF) for 5 days on a laminin-coated plate, morphologically, the human ESCs and iPSCs were gradually transformed from typical, defined, tight human ESC, and iPSC colonies (day 0) into less dense, flatter cells containing prominent nuclei (day 5), even though the majority of the cells had a morphology resembling that of undifferentiated cells (**Figure 1b,c,f,g**). FACS analysis showed that ~46% of human iPSC-derived differentiated cells expressed CXCR4 (expressed in the definitive endoderm but not the primitive endoderm) (**Supplementary Figure S1a**). Human ESC- and iPSC-derived differentiated cells were immunostained with the definitive endoderm marker, FOXA2 (**Figure 2a,c**). However, the majority of the cells expressed the pluripotent marker NANOG, indicating that undifferentiated cells remain in the induced cultures at day 5. After the cells were passaged with trypsin-EDTA and seeded on a laminin-coated plate a second time, the resultant cells were found to be more homogeneous and flatter at day 6 (**Figure 1d,h**). Semiquantitative analysis by counting immunopositive cells revealed that the number of FOXA2-positive cells was increased and, in turn, the number of NANOG-positive cells was decreased at day 6 after passaging (**Figure 2e,f**). Real-time reverse transcriptase (RT)-PCR analysis showed that the definitive endoderm markers *FOXA2* and *SOX17* mRNA were upregulated, whereas the pluripotent marker *NANOG* mRNA was downregulated at day 6 (**Figure 2g,h**). These results were consistent with the immunofluorescence results (**Figure 2a–d**). The expression levels of the mesoderm marker *FLK1* mRNA and ectoderm marker *PAX6* mRNA were downregulated or unchanged at day 6 (**Supplementary Figure S1b–e**). Importantly, the expression of *SOX7* mRNA (expressed in the extra-embryonic endoderm but not the definitive endoderm) was downregulated (**Figure 2g,h**). These results indicate that the definitive endoderm is induced or selected from human ESCs and iPSCs after passaging. We obtained the same results using another human iPSC line (**Supplementary Figure S2a–d**).

HEX induces hepatoblasts from the human ESC- and iPSC-derived definitive endoderms

To investigate whether forced expression of transcription factors could promote hepatic differentiation, the human ESC- and iPSC-derived definitive endoderms were transduced with Ad vectors. We used a fiber-modified Ad vector containing the elongation factor-1α promoter and a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob to examine the transduction efficiency in the human ESC- and iPSC-derived definitive endoderms. The elongation factor-1α promoter was found to be highly active in human ESCs.[28] The K7 peptide targets heparan sulfates on the cellular surface, and the fiber-modified Ad vector containing K7 peptides was shown to be efficient for transduction into many kinds of cells.[29,30] The human ESC- and iPSC-derived definitive endoderms were transduced with a LacZ-expressing Ad vector (Ad-LacZ) at 3,000 vector particle/cell. X-Gal staining showed that the Ad-LacZ-transduced human ESC- and iPSC-derived definitive endoderms successfully expressed LacZ (**Figure 3**). Nearly 100% of the cells transduced with Ad-LacZ were strongly X-gal positive. The transduction efficiency in the human ESC- and iPSC-derived definitive endoderms transduced with the conventional Ad vector containing the wild-type capsid at 3,000 vector particle/cell was ~80% and X-gal staining was much weaker than that in the cells transduced with fiber-modified Ad vectors (**Supplementary Figure S6**).

Next, the human ESC- and iPSC-derived definitive endoderms were transduced with a HEX-expressing fiber-modified Ad vector (Ad-HEX). Although HEX is known to be a transcription factor that is essential for liver development, it remains unclear what the effect of transient *HEX* overexpression is on differentiation from human ESCs and iPSCs or their derivatives *in vitro*. We confirmed the overexpression of *HEX* in the human ESC- and iPSC-derived definitive endoderms transduced with Ad-HEX (**Supplementary Figure S3a–f**). Gene expression analysis revealed the upregulation of *AFP* mRNA, which was expressed by hepatoblasts or early hepatocytes, in Ad-HEX-transduced cells as

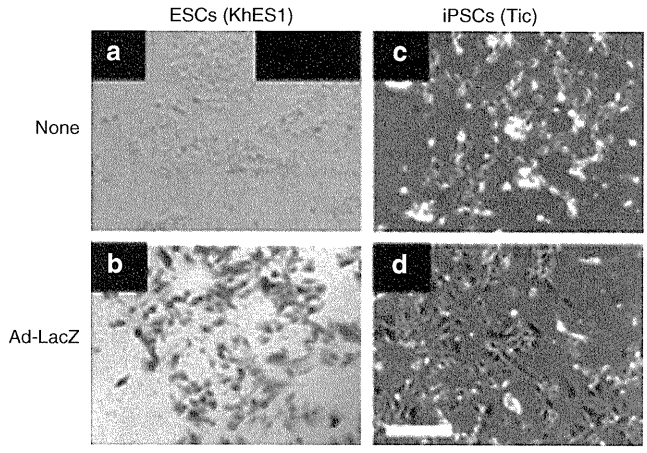

Figure 3 Efficient transgene expression in the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by using a fiber-modified Ad vector containing the EF-1α promoter. (**a,b**) Human ESC (khES1)-derived and (**c,d**) iPSC (Tic) derived definitive endoderms were transduced with 3,000 VP/cell of Ad-LacZ for 1.5 hours. The next day after transduction, X-gal staining was performed as described in the Materials and Methods section. Similar results were obtained in two independent experiments. Scale = 50 μm. Ad, adenovirus; EF-1α, elongation factor-1α; ESC, embryonic stem cells; iPSC, induced pluripotent stem cells; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

compared with nontransduced cells or Ad-LacZ-transduced cells (**Figure 4a,c**). Expression of *ALB* mRNA, which is the most abundant protein in liver, was also observed in Ad-HEX-transduced cells (**Figure 4b,d**).

During liver development, both hepatocytes and cholangiocytes were differentiated from the hepatoblasts. We examined the protein expression of AFP, ALB, and the cholangiocyte marker cytokeratin 7 (CK7) in Ad-HEX-transduced cells by immunostaining (**Figure 4e–p**). The AFP-positive populations were detected in Ad-HEX-transduced cells (**Figure 4g,m**). ALB-positive cells were also detected, although the detection efficiency was very low (**Figure 4j,p**). CK7-positive cells were observed among the Ad-HEX-transduced cells, and all CK7-positive cells were found near the AFP- and ALB-positive cells, suggesting that hepatoblasts are generated by the transient overexpression of a *HEX* gene. Semiquantitative RT-PCR analysis showed that the expression levels of the liver-enriched transcription factors hepatocyte nuclear factor 1A, hepatocyte nuclear factor 1B, hepatocyte nuclear factor 4A, and hepatocyte nuclear factor 6 mRNA were upregulated in Ad-HEX-transduced cells (**Supplementary Figure S4a,b**). The expressions of CCAAT/enhancer binding protein α and prospero homeobox 1 mRNA, two transcription factors known to play a pivotal role in the establishment of the hepatoblasts, were also induced in Ad-HEX-transduced cells (**Supplementary Figure S4a, b**). Taken together, these findings indicate that *HEX* enhances the specification of hepatoblasts from the human ESC- and iPSC-derived definitive endoderms. Similar results were obtained with another human iPSC line (**Supplementary Figure S2e–g**).

Time course of differentiation of the definitive endoderm to hepatoblasts

Next, we examined the time course of AFP and CK7 expression during differentiation of human iPSCs to hepatoblasts in Ad-HEX-

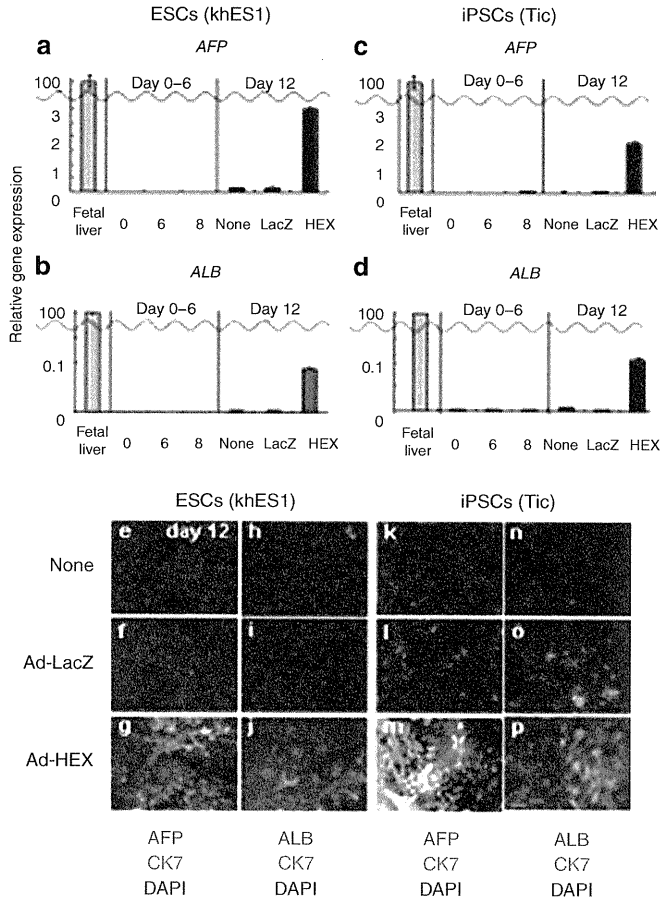

Figure 4 Efficient hepatoblast differentiation from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by transduction of the *HEX* gene. (**a–d**) Real-time RT-PCR analysis of the level of (**a,c**) *AFP* and (**b,d**) *ALB* expression in nontransduced cells, Ad-LacZ-transduced cells, and Ad-HEX-transduced cells, all of which were induced from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms (day 0, 5, 6, and 12). The cells were transduced with Ad-LacZ or Ad-HEX at day 6 as described in **Figure 1a**. The data at day 6 was obtained before the transduction with Ad-HEX. The graphs represent the relative gene expression levels when the level in the fetal liver was taken as 100. (**e–p**) Immunocytochemistry of AFP, ALB, and CK7 expression in nontransduced cells (**e,h,k,** and **n**), Ad-LacZ-transduced cells (**f,i,l**, and **o**), and Ad-HEX-transduced cells (**g,j,m,** and **p**) at day 12, all of which were induced from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. Nuclei were stained with DAPI. Bar = 50 μm. Ad, adenovirus; AFP, α-fetoprotein; ALB, albumin; CK7, cytokeratin 7; HEX, Ad-HEX-transduced cells; ESC, embryonic stem cells; iPSC, induced pluripotent stem cell; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

transduced cells and nontransduced cells. At day 7 (the day after transduction), the expression of AFP was not detectable in Ad-HEX-transduced or nontransduced cells (**Supplementary Figure S5a,d**). At day 8–9, morphological changes to hepatocyte-like cells were observed in Ad-HEX-transduced cells (**Supplementary Figure S5h,i**). We also observed homogeneous AFP-positive cells at day 9 (**Supplementary Figure S5e**). At day 10, CK7-positive cells appeared, indicating that hepatoblasts started to differentiate into hepatocytes and cholangiocytes at day 9–10 (**Supplementary Figure S5f**). At day 12, ALB-positive cells appeared, indicating that hepatocytes were differentiated from Ad-HEX-transduced cells (**Figure 4p**). These results showed that *HEX* induces the hepatoblasts from the

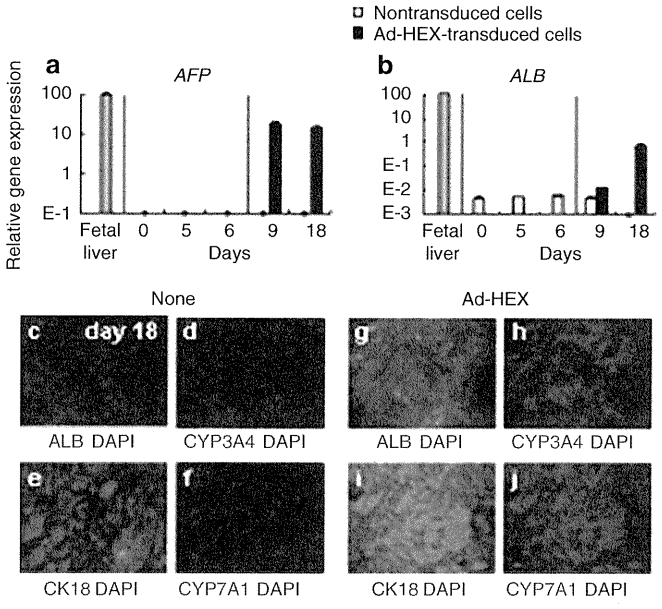

Figure 5 Efficient differentiation of Ad-HEX-transduced hepatoblasts into hepatocytes. (**a**,**b**) Real-time RT-PCR analysis of (**a**) *AFP* and (**b**) *ALB* expression in nontransduced cells and Ad-HEX-transduced cells, both of which were induced from the human iPSC (Tic) derived definitive endoderm (day 0, 5, 6, and 12). The cells were transduced with Ad-HEX at day 6 as described in **Figure 1a**. The data at day 6 were obtained before the transduction with Ad-HEX. The graphs represent the relative gene expression level when the level in the fetal liver was taken as 100. (**c–j**) Immunocytochemistry of ALB, CYP3A4, CYP7A1, and CK18 expression in (**c–f**) nontransduced cells and (**g–j**) Ad-HEX-transduced cells, all of which were induced from the human iPSC (Tic) derived definitive endoderm at day 18. Nuclei were stained with DAPI. Bar = 50 μm. Ad, adenovirus; AFP, α-fetoprotein; ALB, albumin; CK18, cytokeratin 18; ESC, embryonic stem cells; HEX, Ad-HEX-transduced cells; iPSC, induced pluripotent stem cell; None, nontransduced cells; RT-PCR, reverse transcriptase-PCR.

definitive endoderm, and the Ad-HEX-transduced cells could differentiate into both hepatocytes and cholangiocytes.

Directed hepatic differentiation from hepatoblasts

With the protocol described above, heterogeneous populations containing CK7-positive cholangiocytes were observed at day 12 (**Figure 4p**). To promote the differentiation of hepatoblasts to hepatocytes, the human iPSC-derived differentiated cells at day 9 (**Supplementary Figure S5e**) were dislodged with trypsin-EDTA and plated on collagen I-coated dishes as previously reported.[11] After 8–11 days in culture with medium containing FGF4, HGF, OSM, and DEX, the Ad-HEX-transduced cells became more flattened (**Supplementary Figure S5m**), whereas the nontransduced cells became fibroblast-like cells (**Supplementary Figure S5i**). Gene expression analysis showed the upregulation of *ALB* mRNA in Ad-HEX-transduced cells under this culture condition, whereas the expression of *ALB* mRNA was reduced in the nontransduced cells at day 18 (**Figure 5b**). Immunostaining showed that only a small percentage of Ad-HEX-transduced cells expressed ALB at day 12 (**Figure 4p**), whereas most of the Ad-HEX-transduced cells were ALB-positive at day 18 (**Figure 5g**). Most of the Ad-HEX-transduced cells also expressed CYP3A4 at day 18 (**Figure 5h**). More importantly, in the Ad-HEX-transduced cells, CYP7A1 and cytokeratin 18 were detected and these proteins are known

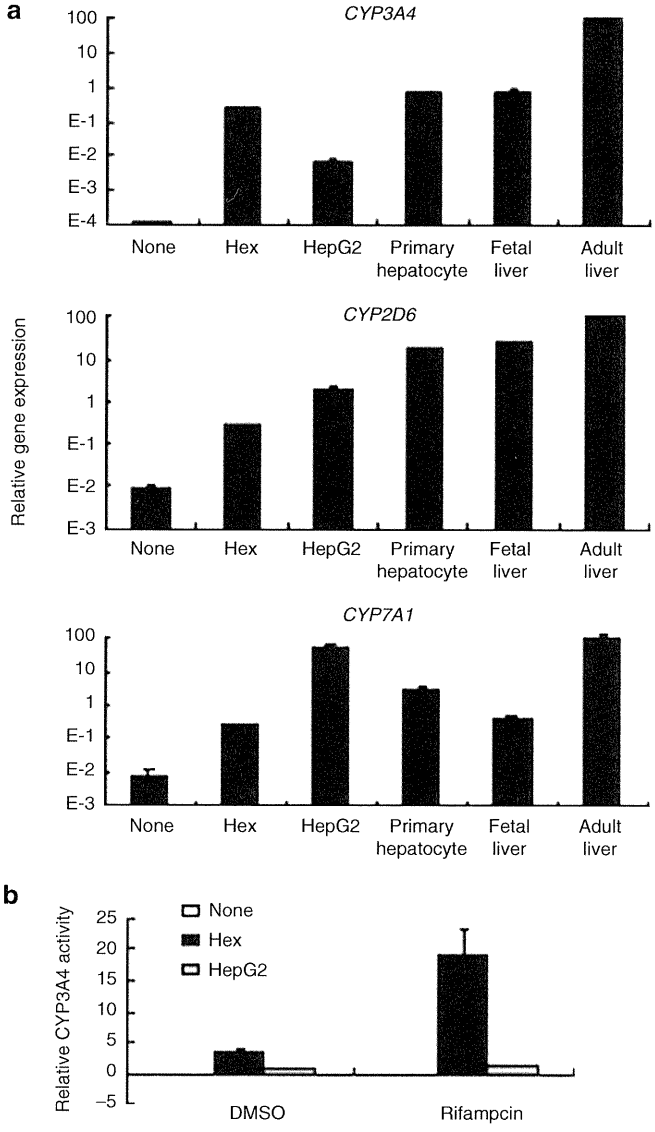

Figure 6 Cytochrome P450 isozymes in human iPSC (Tic) derived hepatocytes. (**a**) Real-time RT-PCR analysis of *CYP3A4*, *CYP7A1*, and *CYP2D6* expression in iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, and fetal and adult liver tissues. (**b**) Induction of CYP3A4 by rifampicin in human iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, the HepG2 cell line and primary human hepatocytes, which were cultured 48 hours after plating the cells. Data are presented as the mean ± SD from triplicate experiments. The graphs represent the relative gene expression level when the level in the adult liver was taken as 100. AFP, α-fetoprotein; ALB, albumin; DMSO, dimethyl sulfoxide; ESC, embryonic stem cells; HEX, Ad-HEX-transduced cells; iPSC, induced pluripotent stem cell; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

to be detected in hepatocytes but not in extra-embryonic cells[31,32] (**Figure 5i,j**). Quantitative analysis showed that ~84, 80, 88, and 92% of Ad-HEX-transduced cells expressed ALB, CYP3A4, CYP7A1, and cytokeratin 18, respectively. These results indicate that Ad-HEX-transduced cells could differentiate to hepatic cells. However, the expression level of *ALB* mRNA in Ad-HEX-transduced cells was lower than that in fetal liver tissue and in turn, the expression of *AFP* mRNA was maintained (**Figure 5a**). Therefore, Ad-HEX-transduced cells are committed to the hepatic linage, but are not yet mature hepatocytes.

Ad-HEX-transduced cells exhibit hepatic functions

To test the hepatic function in the Ad-HEX-transduced cells, we investigated the liver metabolism, because P450 cytochrome enzymes play a critical role in this function. We examined the expression level of several members of this multigene family, *i.e.*, *CYP3A4*, *CYP7A1*, mRNA and *CYP2D6* in Ad-HEX-transduced cells by real-time RT-PCR. The real-time RT-PCR analysis showed that the mRNAs for *CYP3A4*, *CYP7A1*, and *CYP2D6* were expressed in Ad-HEX-transduced cells, whereas none of these mRNAs were expressed in the nontransduced cells (**Figure 6a**). The expression levels of *CYP3A4* in Ad-HEX-transduced cells were similar to those observed in primary human hepatocytes, which were cultured 48 hours after plating the cells, or fetal liver tissues but lower than those in adult liver. The *CYP2D6* and *CYP7A1* mRNA expressions in Ad-HEX-transduced cells were lower than those in primary hepatocytes or adult tissues. Next, we investigated the metabolism of the P450 3A4 substrates by measuring the activity of P450 isozymes. The metabolites were detected in Ad-HEX-transduced cells, and their activity was 3.4-fold higher than that in the most commonly used human hepatocyte cell line, HepG2 (**Figure 6b**; DMSO column). This result was consistent with the real-time RT-PCR data (**Figure 6a**). We further tested the induction of CYP3A4 upon chemical stimulation, because CYP3A4 is the most prevalent P450 isozyme in the liver and is involved in the metabolism of a significant proportion of the currently available commercial drugs. Because CYP3A4 can be induced with rifampicin, both Ad-HEX-transduced cells and HepG2 cells were treated with rifampicin, followed by treatment with CYP3A4 substrate. Ad-HEX-transduced cells produced 5.4-fold higher levels of metabolites in response to rifampicin treatment (**Figure 6b**; rifampicin column). This result indicates that P450 isozymes are active in Ad-HEX-transduced cells.

DISCUSSION

The object of this study was to develop an efficient method for generating hepatoblasts and hepatocytes from human ESCs and iPSCs for application to drug toxicity screening tests as well as therapeutics such as regenerative medicine. We found that transient HEX transduction in the definitive endoderm together with a culture under chemically defined conditions was useful for this purpose.

It has been reported that a high concentration of Activin A induces differentiation of human ESCs into the definitive endoderm.[8,33,34] On the other hand, undifferentiated human ESCs are maintained by a low concentration of Activin A.[35] Several studies have shown that bFGF promotes the differentiation of ESCs into the definitive endoderm and inhibits the differentiation of ESCs into the extra-embryonic endoderm.[35–38] bFGF has been reported to inhibit the BMP signaling, which can promote the extra-embryonic lineage differentiation.[39] The extra-embryonic endoderm expresses most of the hepatocyte markers, such as AFP.[40] Contamination of the extra-embryonic endoderm makes it difficult to estimate the hepatic differentiation from human ESCs and iPSCs.[11,14,40] In this study, we showed that both Activin A and bFGF induce definitive endoderm populations, while they repress the extra-embryonic endoderm differentiation (**Figure 2g,h**). Interestingly, after the differentiated cells that were cultured on laminin-coated plates with Activin A and bFGF were passaged at day 5, FOXA2-positive cells (definitive endoderm) were enriched in the resultant cells at day 6 (**Figure 2a–f**). This may have been because FOXA2-positive cells efficiently adhered to the laminin-coated plate and/or because trypsinized, single undifferentiated ESCs/iPSCs cannot survive. The passaging of differentiated cells might be attributed to the reduction in the number of not only the extra-embryonic endoderm cells but also the undifferentiated cells. However, the efficiency of the definitive endoderm differentiation in this study was not as efficient as that reported by other groups.[8,33,34] Other cell lineages, such as the mesoderm and extra-embryonic endoderm, might remain at day 6 (**Figure 2g,h** and **Supplementary Figure S1**). Further improvement of the culture conditions will thus be needed in order to enhance the definitive endoderm differentiation.

Hepatoblasts and hepatocytes were differentiated from the human ESC- and iPSC-derived definitive endoderms by transient overexpression of the homeobox gene *HEX*. A fiber-modified Ad vector containing K7 peptides mediated much higher gene expression than conventional Ad vectors in the human ESC- and iPSC-derived definitive endoderms (**Supplementary Figure S6**). This new hepatic differentiation protocol shows that *HEX* induces AFP-positive hepatoblasts at day 9 and ALB-positive hepatocytes at day 12 from human ESCs and iPSCs, whereas the previous protocols require a few weeks or months to induce AFP- and ALB-positive hepatocytes from PSCs.[9–11] Previous studies suggested that *HEX* could regulate liver-enriched transcription factors such as hepatocyte nuclear factor 4A and hepatocyte nuclear factor 6.[19,23] Overexpression of the *HEX* gene under the conditions employed in the present study could activate several transcription factors that are required for hepatic differentiation (**Supplementary Figure S4a,b**). However, the Ad-HEX-transduced cells showed a low level of expression of *ALB* and some CYP450 species, as well as a high level of *AFP* expression, indicating that the cells were still immature. To promote further hepatic differentiation or maturation, it may be effective to culture the hepatic cells in a 3D environment or on feeder cells such as cardiomyocyte- or endothelium-derived cells.[41,42] In addition, the function of our hepatic cells was still limited. Further analysis of the other functions of our hepatic cells, such as glycogen storage, uptake of indocyanine green and organic anion low-density lipoprotein, and transplantation of Ad-HEX-transduced cells into the liver of immunodeficient mice, is clearly needed for the appreciation to drug screening and therapeutic treatment modalities.

During the preparation of this article, Kubo *et al.* have reported that *HEX* could promote hepatoblast differentiation from mouse ESCs.[43] Their report is consistent with our data, suggesting that *HEX* plays a pivotal regulatory role in not only mouse but also human hepatic differentiation. They also showed that the overexpression of *HEX* at the definitive endoderm stage is critical for hepatic specification of the mouse ESCs. We also confirmed that forced expression of *HEX* in the undifferentiated human ESCs and iPSCs did not elevate the expression of *ALB* and *CK7* (**Supplementary Figure S7**), indicating that *HEX* enhances the hepatic differentiation not from the undifferentiated cells but from the definitive endoderm. However, Kubo *et al.* used recombinant mouse ESCs (tet-HEX ESCs), in which the tetracycline-regulated *HEX* expression cassette

is integrated into the host cell genome to induce *HEX* in a stage-specific manner. Their system would not be appropriate for clinical use because the transgene is randomly integrated into the host cell genome and this leads to a risk of mutagenesis.[44] On the other hand, we generated human hepatoblasts by Ad vector-mediated transient *HEX* transduction, method which avoids the integration of exogenous DNA into the host chromosome.

Touboul *et al.* reported that human ESCs and iPSCs can differentiate into functional hepatocytes under chemically defined conditions.[34] In the present study, hepatoblasts were generated in a chemically defined serum-free medium, which minimized exposure to animal cells and proteins, and on a defined extracellular matrix, such as laminin or collagen, which do not contain undefined growth factors. To generate hepatocytes, hepatocyte culture medium, which is serum-free but not defined, was used in the stage III. When defined hESF-medium was used in the stage III, the expression levels of *ALB* and *CYP3A4* mRNA were half the levels seen in the cells cultured with hepatocyte culture medium in the preliminary experiment (data not shown). Human ESCs and iPSCs were also grown for maintaining the undifferentiated state on a feeder layer, which contains xenoantigen such as bovine apolipoprotein B-100. Bovine apolipoprotein B-100 is known to be a dominant xenoantigen for cell-based therapies.[45] Human ESC- and iPSC-derived hepatocytes should be generated and cultured under chemically defined conditions not only to avoid potential contamination with pathogens for the safer therapeutic application, but also to obtain reproducible results using the differentiation protocols.[34,46] Development of differentiation protocols using other genes of transcription factors as well as *HEX* genes based on a chemically defined medium is under way. Overall, our strategy should provide a novel protocol for hepatic differentiation from human ESCs and iPSCs, which could be useful for regenerative medicine and drug screening.

MATERIALS AND METHODS

Ad vectors. Ad vectors were constructed by an improved *in vitro* ligation method.[47,48] The human *HEX* complementary DNA derived from pDNR-LIB-*HEX* (Invitrogen, Carlsbad, CA) was inserted into pHMEF5,[29] which contains the human elongation factor-1α promoter, resulting in pHMEF-HEX. The pHMEF-HEX was digested with I-CeuI/PI-SceI and ligated into I-CeuI/PI-SceI-digested pAdHM41-K7,[30] resulting in pAd-HEX. Ad-HEX and Ad-LacZ, both of which contain the elongation factor-1α promoter and a stretch of lysine residues (K7) peptides in the C-terminal region of the fiber knob, were generated and purified as described previously.[26,29] The vector particle titer was determined by using a spectrophotometric method.[49]

Human ESCs and iPSCs culture. A human ESC line, khES1, was obtained from Kyoto University (Kyoto, Japan).[50] khES1 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 after approval by the review board at Kyoto University. Human ESCs were maintained on a feeder layer of mitomycin-inactivated mouse embryonic fibroblasts (ICR; ReproCELL Incorporated, Tokyo, Japan) with Dulbecco's modified Eagle's medium/F-12 (Sigma, St Louis, MO) supplemented with 0.1 mmol/l 2-mercaptoethanol, 0.1 mmol/l nonessential amino acids, 2 mmol/l L-glutamine, 20% GIBCO knockout serum replacement (Invitrogen), and 5 ng/ml bFGF (Sigma) in a humidified atmosphere of 3% CO_2 and 97% air at 37 °C. Human ESCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics, Burgess Hill, UK) into small clumps, and subcultured every 5 or 6 days.

Two human iPS clones derived from the embryonic human lung fibroblast cell line MCR5 were provided from JCRB Cell Bank (Tic, JCRB Number: JCRB1331; and Dotcom, JCRB Number: JCRB1327).[3,4] In the present study, we mainly used the Tic cell line, but similar results were obtained using the Dotcom cell line, and these are shown in the supplementary figures. Human iPSCs were maintained on a feeder layer of mitomycin-inactivated mouse embryonic fibroblasts (Hygro Resistant Strain C57/BL6; Hygro, Millipore, MA) on a gelatin-coated flask in human iPS medium. Human iPS medium consists of knockout Dulbecco's modified Eagle's medium/F12 (Invitrogen), supplemented with 0.1 mmol/l 2-mercaptoethanol, 0.1 mmol/l nonessential amino acids, 2 mmol/l L-glutamine, 20% knockout serum replacement, and 10 ng/ml bFGF in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Human iPSCs were dissociated with 0.1 mg/ml dispase (Roche) into small clumps and subcultured every 7 or 8 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 in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C.[46] hESF9 consists of hESF-GRO medium (Cell Science & Technology Institute, Sendai, Japan) supplemented with five factors (10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μmol/l 2-mercaptoethanol, 10 μmol/l ethanolamine, 10 μmol/l sodium selenite), oleic acid conjugated with fatty acid free bovine ALB, 10 ng/ml bFGF, and 100 ng/ml heparin (all from Sigma). For induction of definitive endoderm, human ESCs and iPSCs were dissociated into single cells with Accutase (Invitrogen) and cultured for 5 days on a mouse laminin-coated tissue 12-well plate (6.0×10^4 cells/cm^2) in hESF-GRO medium (Cell Science & Technology Institute) supplemented with the five factors, 0.5 mg/ml fatty acid free bovine ALB (BSA) (Sigma), 10 ng/ml bFGF, and 50 ng/ml Activin A (R&D Systems, Minneapolis, MN) in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C. The medium was refreshed every day.

For induction of hepatoblasts, the human ESC- and iPSC-derived definitive endoderms (day 5) were dissociated with 0.0125% trypsin-0.01325 mmol/l EDTA, and then the trypsin was inactivated with 0.1% soybean trypsin inhibitor (Sigma). The cells were seeded at 1.2×10^5 cells/cm^2 on a laminin-coated 12-well plate with hESF-DIF (Cell Science & Technology Institute) medium supplemented with the five factors, 0.5 mg/ml BSA, 10 ng/ml bFGF, and 50 ng/ml Activin A in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C. The next day, the cells were transduced with 3,000 vector particle/cell of Ad vectors (Ad-HEX and Ad-LacZ) for 1.5 hours in hESF-DIF medium supplemented with the five factors, BSA, 10 ng/ml FGF4 (R&D Systems) and 10 ng/ml BMP4 (R&D Systems).[10] The medium was refreshed every day.

For induction of hepatocytes, human iPSC-derived hepatoblasts in one well (day 9) were passaged onto two wells with 0.0125% trypsin-0.01325mmol/l EDTA and 0.1% trypsin inhibitor, on type I collagen-coated tissue 12-well plate (15 μg/cm^2) (Nitta Gelatin, Osaka, Japan). The cells were cultured in hepatocyte culture medium supplemented with SingleQuots (Lonza, Walkersville, MD), 10 ng/ml FGF4, 10 ng/ml HGF (R&D Systems), 10 ng/ml Oncostatin M (R&D Systems), and 0.392 ng/ml dexamethasone (Sigma).[11] The medium was refreshed every 2 days.

RNA isolation, RT-PCR, immunostaining, flow cytometry, lacz assay, and assay for cytochrome P4503A4 activity. For details of these procedures, See **Supplementary Materials and Methods, Supplementary Tables S1 and S2.**

SUPPLEMENTARY MATERIAL

Figure S1. Characterization of the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms.

Figure S2. Efficient differentiation of another human iPSC line (Dotcom) into hepatoblasts by overexpression of the *HEX* gene.

Figure S3. Overexpression of *HEX* in the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms.

Figure S4. Characterization of Ad-HEX-transduced hepatoblasts.
Figure S5. Progression of differentiation of the definitive endoderm to hepatoblasts.
Figure S6. X-gal staining of human iPSC (Tic)-derived definitive endoderms transduced with a conventional or a fiber-modified Ad vector containing the EF-1α promoter.
Figure S7. *HEX* promotes the differentiation into the hepatic lineage, not from undifferentiated iPSCs (Tic), but from iPSC (Tic)-derived definitive endoderm.
Table S1. List of Taqman gene expression assays and primers.
Table S2. List of antibodies used.
Materials and Methods.

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