

Efficient Generation of Functional Hepatocytes From Human Embryonic Stem Cells and Induced Pluripotent Stem Cells by HNF4 α Transduction

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Hepatocyte-like cells from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are expected to be a useful source of cells drug discovery. Although we recently reported that hepatic commitment is promoted by transduction of SOX17 and HEX into human ESC- and iPSC-derived cells, these hepatocyte-like cells were not sufficiently mature for drug screening. To promote hepatic maturation, we utilized transduction of the hepatocyte nuclear factor 4 α (HNF4 α) gene, which is known as a master regulator of liver-specific gene expression. Adenovirus vector-mediated overexpression of HNF4 α in hepatoblasts induced by SOX17 and HEX transduction led to upregulation of epithelial and mature hepatic markers such as cytochrome P450 (CYP) enzymes, and promoted hepatic maturation by activating the mesenchymal-to-epithelial transition (MET). Thus HNF4 α might play an important role in the hepatic differentiation from human ESC-derived hepatoblasts by activating the MET. Furthermore, the hepatocyte like-cells could catalyze the toxication of several compounds. Our method 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 predicting drug toxicity.

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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 of the body's cell types.^{1,2} They could provide an unlimited source of cells for various applications. Hepatocyte-like cells, which are differentiated from human ESCs and iPSCs,

would be useful for basic research, regenerative medicine, and drug discovery.³ In particular, it is expected that hepatocyte-like cells will be utilized as a tool for cytotoxicity screening in the early phase of pharmaceutical development. To catalyze the toxication of several compounds, hepatocyte-like cells need to be mature enough to exhibit hepatic functions, including high activity levels of the cytochrome P450 (CYP) enzymes. Because the present technology for the generation of hepatocyte-like cells from human ESCs and iPSCs, which is expected to be utilized for drug discovery, is not refined enough for this application, it is necessary to improve the efficiency of hepatic differentiation. Although conventional methods such as growth factor-mediated hepatic differentiation are useful to recapitulate liver development, they lead to only a heterogeneous hepatocyte population.⁴⁻⁶ Recently, we showed that transcription factors are transiently transduced to promote hepatic differentiation in addition to the conventional differentiation method which uses only growth factors.⁷ Ectopic expression of Sry-related HMG box 17 (SOX17) or hematopoietically expressed homeobox (HEX) by adenovirus (Ad) vectors in human ESC-derived mesendoderm or definitive endoderm (DE) cells markedly enhances the endoderm differentiation or hepatic commitment, respectively.^{7,8} However, further hepatic maturation is required for drug screening.

The transcription factor hepatocyte nuclear factor 4 α (HNF4 α) is initially expressed in the developing hepatic diverticulum on E8.75,^{9,10} and its expression is elevated as the liver develops. A previous loss-of-function study showed that HNF4 α plays a critical role in liver development; conditional deletion of HNF4 α in fetal hepatocytes results in the faint expression of many mature hepatic enzymes and the impairment of normal liver morphology.¹¹ The genome-scale chromatin immunoprecipitation assay showed that HNF4 α binds to the promoters of nearly half of the genes expressed in the mouse liver,¹² including cell adhesion and junctional proteins,¹³ which are important in

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the hepatocyte epithelial structure.¹⁴ In addition, HNF4 α plays a critical role in hepatic differentiation and in a wide variety of liver functions, including lipid and glucose metabolism.^{15,16} Although HNF4 α could promote transdifferentiation into hepatic lineage from hematopoietic cells,¹⁷ the function of HNF4 α in hepatic differentiation from human ESCs and iPSCs remains unknown. A previous study showed that hepatic differentiation from mouse hepatic progenitor cells is promoted by HNF4 α , although many of the hepatic markers that they examined were target genes of HNF4 α .¹⁸ They transplanted the HNF4 α -overexpressed mouse hepatic progenitor cells to promote hepatic differentiation, but they did not examine the markers that relate to hepatic maturation such as CYP enzymes, conjugating enzymes, and hepatic transporters.

In this study, we examined the role of HNF4 α in hepatic differentiation from human ESCs and iPSCs. The human ESC- and iPSC-derived hepatoblasts, which were efficiently generated by sequential transduction of SOX17 and HEX, were transduced with HNF4 α -expressing Ad vector (Ad-HNF4 α), and then the expression of hepatic markers of the hepatocyte-like cells were assessed. In addition, we examined whether or not the hepatocyte-like cells, which were generated by sequential transduction of SOX17, HEX, and HNF4 α , were able to predict the toxicity of several compounds.

RESULTS

Stage-specific HNF4 α transduction in hepatoblasts selectively promotes hepatic differentiation

The transcription factor HNF4 α plays an important role in both liver generation¹¹ and hepatic differentiation from human ESCs and iPSCs (Supplementary Figure S1). We expected that hepatic differentiation could be accelerated by HNF4 α transduction. To examine the effect of forced expression of HNF4 α in the hepatic differentiation from human ESC- and iPSC-derived cells, we used a fiber-modified Ad vector.¹⁹ Initially, we optimized the time period for Ad-HNF4 α transduction. Human ESC (H9)-derived DE cells (day 6) (Supplementary Figures S2 and S3a), hepatoblasts (day 9) (Supplementary Figures S2 and S3b), or a heterogeneous population consisting of hepatoblasts, hepatocytes, and cholangiocytes (day 12) (Supplementary Figures S2 and S3c) were transduced with Ad-HNF4 α and then the Ad-HNF4 α -transduced cells were cultured until day 20 of differentiation (Figure 1). We ascertained the expression of exogenous HNF4 α in human ESC-derived hepatoblasts (day 9) transduced with Ad-HNF4 α (Supplementary Figure S4). The transduction of Ad-HNF4 α into human ESC-derived hepatoblasts (day 9) led to the highest expression levels of the hepatocyte markers *albumin* (*ALB*)²⁰ and *α -1-antitrypsin* (Figure 1a). In contrast, the expression levels of the cholangiocyte markers *cytokeratin 7* (*CK7*)²¹ and *SOX9*²² were

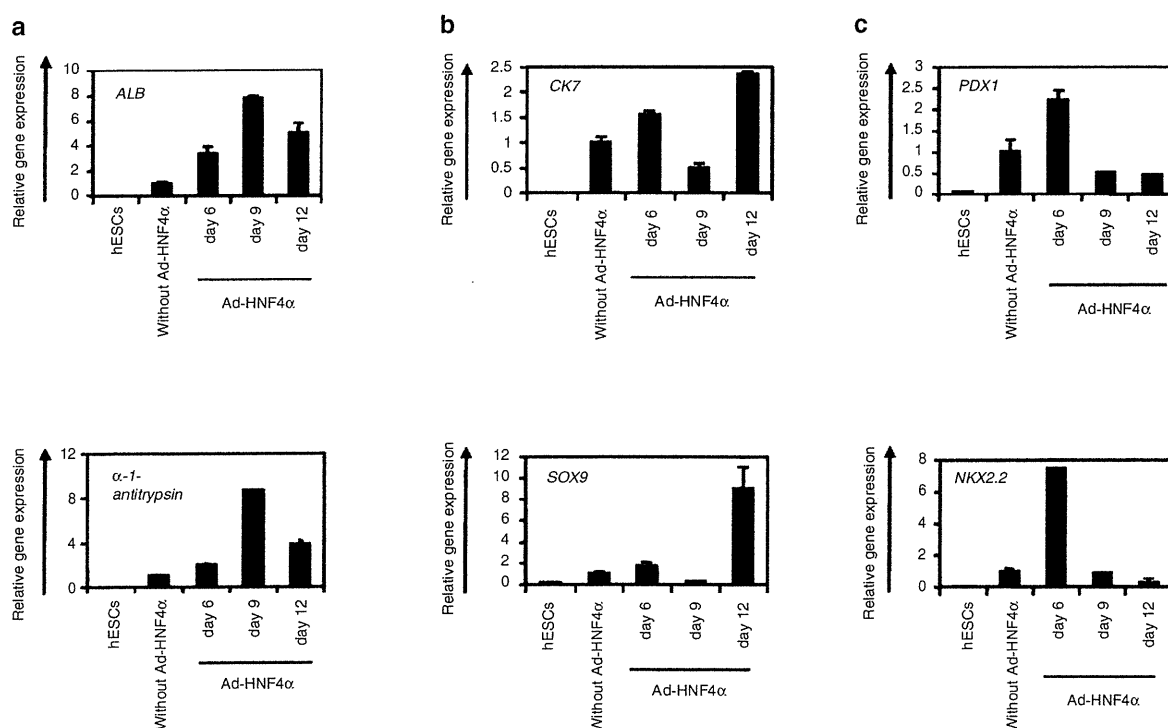


Figure 1 Transduction of HNF4 α into hepatoblasts promotes hepatic differentiation. (a–c) The human ESC (H9)-derived cells, which were cultured for 6, 9, or 12 days according to the protocol described in Figure 2a, were transduced with 3,000 vector particles (VP)/cell of Ad-HNF4 α for 1.5 hours and cultured until day 20. The gene expression levels of (a) hepatocyte markers (*ALB* and *α -1-antitrypsin*), (b) cholangiocyte markers (*CK7* and *SOX9*), and (c) pancreas markers (*PDX1* and *NKX2.2*) were examined by real-time RT-PCR on day 0 (human ESCs (hESCs)) or day 20 of differentiation. The horizontal axis represents the days when the cells were transduced with Ad-HNF4 α . On the y-axis, the level of the cells without Ad-HNF4 α transduction on day 20 was taken as 1.0. All data are represented as means \pm SD ($n = 3$). ESC, embryonic stem cell; HNF4 α , hepatocyte nuclear factor 4 α ; RT-PCR, reverse transcription-PCR.

downregulated in the cells transduced on day 9 as compared with nontransduced cells (Figure 1b). This might be because hepatic differentiation was selectively promoted and biliary differentiation was repressed by the transduction of HNF4 α in hepatoblasts. The expression levels of the pancreas markers *PDX1*²³ and *NKX2.2*²⁴ did not make any change in the cells transduced on day 9 as compared with nontransduced cells (Figure 1c). Interestingly, the expression levels of the pancreas markers were upregulated, when Ad-HNF4 α transduction was performed into DE cells (day 6) (Figure 1c). These results suggest that HNF4 α might promote not only hepatic differentiation but also pancreatic differentiation, although the optimal stage of HNF4 transduction for the differentiation of each cell is different. We have confirmed that there was no difference between nontransduced cells and Ad-LacZ-transduced cells in the gene expression levels of all the markers investigated in Figure 1a–c (data not shown). We also confirmed that Ad vector-mediated gene expression in the human ESC-derived hepatoblasts (day 9) continued until day 14 and almost disappeared on day 18 (Supplementary Figure S5). These results indicated that the stage-specific HNF4 α overexpression in human ESC-derived hepatoblasts (day 9) was essential for promoting efficient hepatic differentiation.

Transduction of HNF4 α into human ESC- and iPSC-derived hepatoblasts efficiently promotes hepatic maturation

From the results of Figure 1, we decided to transduce hepatoblasts (day 9) with Ad-HNF4 α . To determine whether hepatic maturation is promoted by Ad-HNF4 α transduction, Ad-HNF4 α -transduced cells were cultured until day 20 of differentiation according to the schematic protocol described in Figure 2a. After the hepatic maturation, the morphology of human ESCs was gradually changed into that of hepatocytes: polygonal with distinct round nuclei (day 20) (Figure 2b). Interestingly, a portion of the hepatocyte-like cells, which were ALB²⁰-, CK18²¹-, CYP2D6⁻, and CYP3A4²⁵-positive cells, had double nuclei, which was also observed in primary human hepatocytes (Figure 2b,c, and Supplementary Figure S6). We also examined the hepatic gene expression levels on day 20 of differentiation (Figure 3a,b). The gene expression analysis of *CYP1A2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4*, and *CYP7A1*²⁵ showed higher expression levels in all of Ad-SOX17-, Ad-HEX-, and Ad-HNF4 α -transduced cells (three factors-transduced cells) as compared with those in both Ad-SOX17- and Ad-HEX-transduced cells (two factors-transduced cells) on day 20 (Figure 3a). The gene expression level of NADPH-CYP reductase

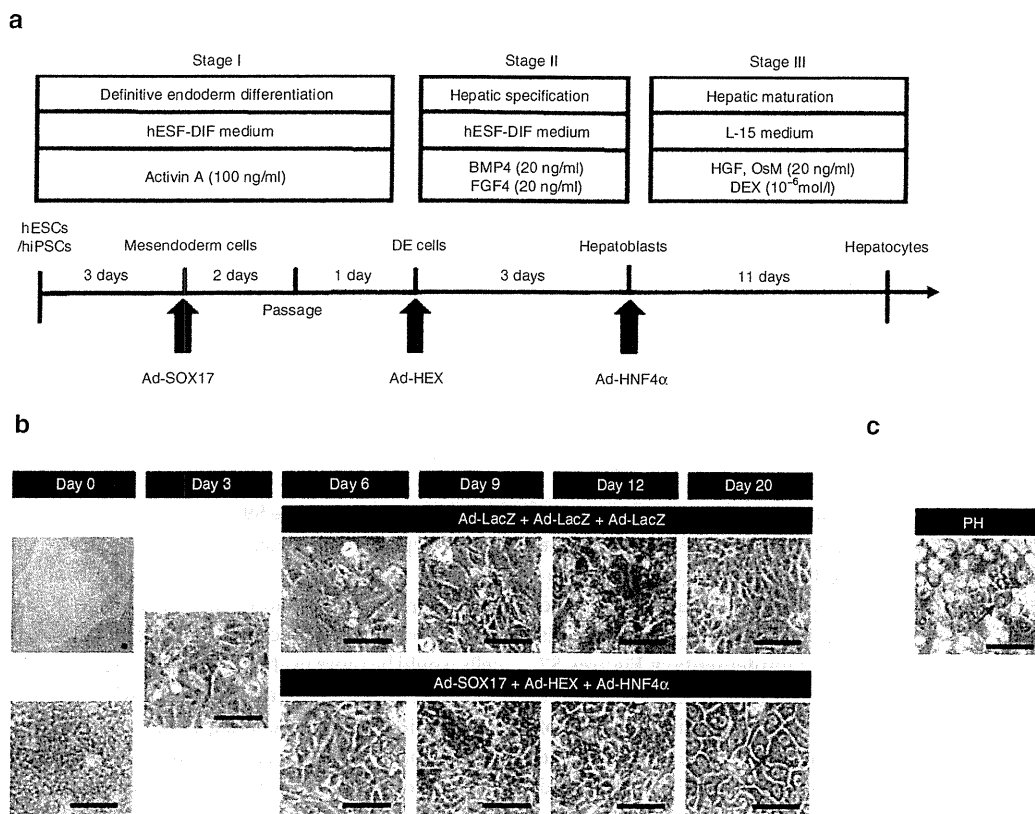


Figure 2 Hepatic differentiation of human ESCs and iPSCs transduced with three factors. (a) The procedure for differentiation of human ESCs and iPSCs into hepatocytes via DE cells and hepatoblasts is presented schematically. The hESF-DIF medium was supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, and 0.5 mg/ml fatty-acid-free BSA. The L15 medium was supplemented with 8.3% tryptose phosphate broth, 8.3% FBS, 10 μ mol/l hydrocortisone 21-hemisuccinate, 1 μ mol/l insulin, and 25 mmol/l NaHCO₃. (b) Sequential morphological changes (day 0–20) of human ESCs (H9) differentiated into hepatocytes via DE cells and hepatoblasts are shown. Red arrow shows the cells that have double nuclei. (c) The morphology of primary human hepatocytes is shown. Bar represents 50 μ m. BSA, bovine serum albumin; DE, definitive endoderm; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell.

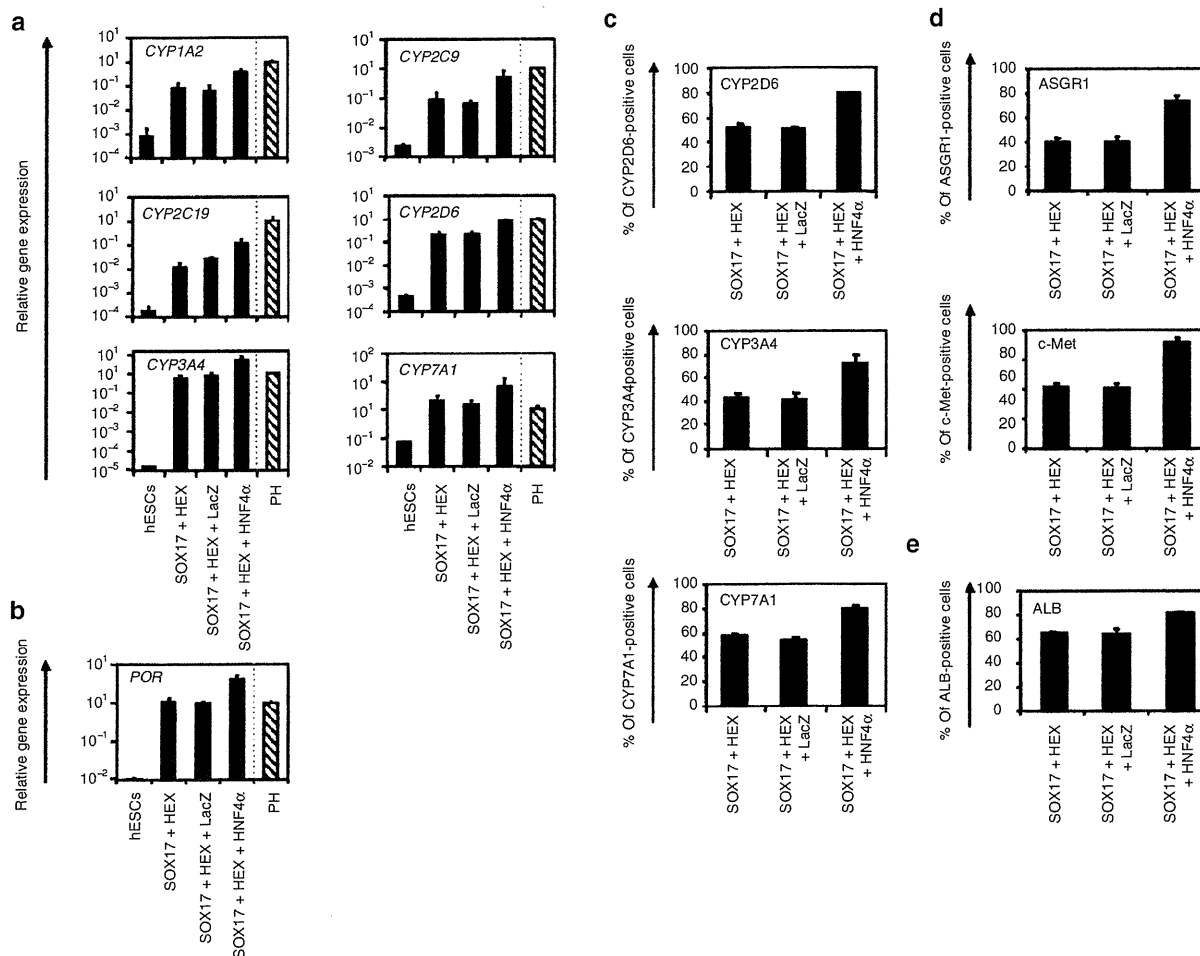


Figure 3 Transduction of HNF4 α promotes hepatic maturation from human ESCs and iPSCs. **(a,b)** The human ESCs were differentiated into hepatocytes according to the protocol described in Figure 2a. On day 20 of differentiation, the gene expression levels of **(a)** CYP enzymes (*CYP1A2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4*, and *CYP7A1*) and **(b)** *POR* were examined by real-time RT-PCR in undifferentiated human ESCs (hESCs), the hepatocyte-like cells, and primary human hepatocytes (PH, hatched bar). On the y-axis, the expression level of primary human hepatocytes, which were cultured for 48 hours after the cells were plated, was taken as 1.0. **(c–e)** The hepatocyte-like cells (day 20) were subjected to immunostaining with **(c)** anti-drug-metabolizing enzymes (*CYP2D6*, *CYP3A4*, and *CYP7A1*), **(d)** anti-hepatic surface protein (*ASGR1* and *c-Met*), and **(e)** anti-ALB antibodies, and then the percentage of antigen-positive cells was examined by flow cytometry on day 20 of differentiation. All data are represented as means \pm SD ($n = 3$). ESC, embryonic stem cell; HNF4 α , hepatocyte nuclear factor 4 α ; iPSC, induced pluripotent stem cell.

(*POR*)²⁶, which is required for the normal function of CYPs, was also higher in the three factors-transduced cells (Figure 3b). The gene expression analysis of ALB, α -1-antitrypsin (α -1-AT), transthyretin, hepatic conjugating enzymes, hepatic transporters, and hepatic transcription factors also showed higher expression levels in the three factors-transduced cells (Supplementary Figures S7 and S8). Moreover, the gene expression levels of these hepatic markers of three factor-transduced cells were similar to those of primary human hepatocytes, although the levels depended on the type of gene (Figure 3a,b, and Supplementary Figures S7 and S8). To confirm that similar results could be obtained with human iPSCs, we used three human iPSC cell lines (201B7, Dotcom, and Tic). The gene expression of hepatic markers in human ESC- and iPSC-derived hepatocytes were analyzed by real-time reverse transcription-PCR on day 20 of differentiation. Three human iPSC cell lines as well as human ESCs also effectively differentiated into hepatocytes in response to transduction of the three factors

(Supplementary Figure S9). Interestingly, we observed differences in the hepatic maturation efficiency among the three human iPSC cell lines. That is, two of the human iPSC cell lines (Tic and Dotcom) were more committed to the hepatic lineage than another human iPSC cell line (201B7). Because almost homogeneous hepatocyte-like cells would be more useful in basic research, regenerative medicine, and drug discovery, we also examined whether our novel methods for hepatic maturation could generate a homogeneous hepatocyte population by flow cytometry analysis (Figure 3c–e). The percentages of CYP2D6-, CYP3A4-, and CYP7A1-positive cells were ~80% in the three factors-transduced cells, while they were ~50% in the two factors-transduced cells (Figure 3c). The percentages of hepatic surface antigen (asialoglycoprotein receptor 1 [*ASGR1*] and met proto-oncogene (*c-Met*))-positive cells (Figure 3d) and ALB-positive cells (Figure 3e) were also ~80% in the three factors-transduced cells. These results indicated that a nearly homogeneous population was obtained by our differentiation protocol

using the transduction of three functional genes (SOX17, HEX, and HNF4 α).

The three factors-transduced cells have characteristics of functional hepatocytes

The hepatic functions of the hepatocyte-like cells, such as the uptake of low-density lipoprotein (LDL) and CYP enzymes activity, of the hepatocyte-like cells were examined on day 20 of differentiation. Approximately 87% of the three factors-transduced cells uptook LDL in the medium, whereas only 44% of the two factors-transduced cells did so (Figure 4a). The activities of CYP enzymes of the hepatocyte-like cells were measured according to the metabolism of the CYP3A4, CYP2C9, or CYP1A2 substrates (Figure 4b). The metabolites were detected in the three factors-transduced cells and their activities were higher than those of the two factors-transduced cells (dimethyl sulfoxide (DMSO) column). We further tested the induction of CYP3A4, CYP2C9, and CYP1A2 by chemical stimulation, since CYP3A4, CYP2C9, and CYP1A2 are the important prevalent CYP isozymes in the liver and are involved in the metabolism of a significant proportion of the currently available commercial drugs (rifampicin or omeprazole column). It is well known that CYP3A4 and CYP2C9 can be induced by rifampicin, whereas CYP1A2 can be induced by omeprazole. The hepatocyte-like cells were treated with either of these. Although undifferentiated human ESCs responded to neither rifampicin nor omeprazole (data not shown), the hepatocyte-like cells produced more metabolites in response to chemical stimulation as well as primary hepatocytes (Figure 4b). The activity levels of the hepatocyte-like cells as compared with those of primary human hepatocytes depended on the types of CYP; the CYP3A4 activity of the hepatocyte-like cells was similar to that of primary human hepatocytes, whereas the CYP2C9 and CYP1A2 activities of the hepatocyte-like cells were slightly lower than those of primary human hepatocytes (Figure 3a). These results indicated that high levels of functional CYP enzymes were detectable in the hepatocyte-like cells.

The metabolism of diverse compounds involving uptake, conjugation, and the subsequent release of the compounds is an important function of hepatocytes. Uptake and release of Indocyanine green (ICG) can often be used to identify hepatocytes in ESC differentiation models.²⁷ To investigate this function in our hepatocyte-like cells, we compared this ability of the three factors-transduced cells with that of the two factors-transduced cells on day 20 of differentiation (Figure 4c). The three factors-transduced cells had more ability to uptake ICG and to excrete ICG by culturing without ICG for 6 hours. We also examined whether the hepatocyte-like cells could store glycogen, a characteristic of functional hepatocytes (Figure 4d). On day 20 of differentiation, the three factors-transduced cells and the two factors-transduced cells were stained for cytoplasmic glycogen using the Periodic Acid-Schiff staining procedure. The three factors-transduced cells exhibited more abundant storage of glycogen than the two-factors-transduced cells. These results showed that abundant hepatic functions, such as uptake and excretion of ICG and storage of glycogen, were obtained by the transduction of three factors.

Many adverse drug reactions are caused by the CYP-dependent activation of drugs into reactive metabolites.²⁸ In order to examine

metabolism-mediated toxicity and to improve the safety of drug candidates, primary human hepatocytes are widely used.²⁸ Because primary human hepatocytes have quite different characteristics among distinct lots and because it is difficult to purchase large amounts of primary human hepatocytes that have the same characteristics, hepatocyte-like cells are expected to be used for this purpose. To examine whether our hepatocyte-like cells could be used to predict metabolism-mediated toxicity, the hepatocyte-like cells were incubated with four substrates (troglitazone, acetaminophen, cyclophosphamide, and carbamazepine), which are known to generate toxic metabolites by CYP enzymes, and then the cell viability was measured (Figure 4e). The cell viability of the two factors plus Ad-LacZ-transduced cells were higher than that of the three factors-transduced cells at each different concentration of four test compounds. These results indicated that the three factors-transduced cells could more efficiently metabolize the test compounds and thereby induce higher toxicity than either the two factors-transduced cells or undifferentiated human ESCs. The cell viability of the three factors-transduced cells was slightly higher than that of primary human hepatocytes.

HNF4 α promotes hepatic maturation by activating mesenchymal-to-epithelial transition

HNF4 α is known as a dominant regulator of the epithelial phenotype because its ectopic expression in fibroblasts (such as NIH 3T3 cells) induces mesenchymal-to-epithelial transition (MET)¹¹, although it is not known whether HNF4 α can promote MET in hepatic differentiation. Therefore, we examined whether HNF4 α transduction promotes hepatic maturation from hepatoblasts by activating MET. To clarify whether MET is activated by HNF4 α transduction, the human ESC-derived hepatoblasts (day 9) were transduced with Ad-LacZ or Ad-HNF4 α , and the resulting phenotype was analyzed on day 12 of differentiation (Figure 5). This time, we confirmed that HNF4 α transduction decreased the population of N-cadherin (hepatoblast marker)-positive cells,²⁹ whereas it increased that of ALB (hepatocyte marker)-positive cells (Figure 5a). The number of CK7 (cholangiocyte marker)-positive population did not change (Figure 5a). To investigate whether these results were attributable to MET, the alteration of the expression of several mesenchymal and epithelial markers was examined (Figure 5b). The human ESC-derived hepatoblasts (day 9) were almost homogeneously N-cadherin³⁰ (mesenchymal marker)-positive and E-cadherin¹¹ (epithelial marker)-negative, demonstrating that human ESC-derived hepatoblasts have mesenchymal characteristics (Figure 5a,b). After HNF4 α transduction, the number of E-cadherin-positive cells was increased and reached ~90% on day 20, whereas that of N-cadherin-positive cells was decreased and was less than 5% on day 20 (Supplementary Figure S10). These results indicated that MET was promoted by HNF4 α transduction in hepatic differentiation from hepatoblasts. Interestingly, the number of growing cells was decreased by HNF4 α transduction (Figure 5c), and the cell growth was delayed by HNF4 α transduction (Supplementary Figure S11). This decrease in the number of growing cells might have been because the differentiation was promoted by HNF4 α transduction. We also confirmed that MET was promoted by HNF4 α transduction in the gene expression levels (Figure 5d).

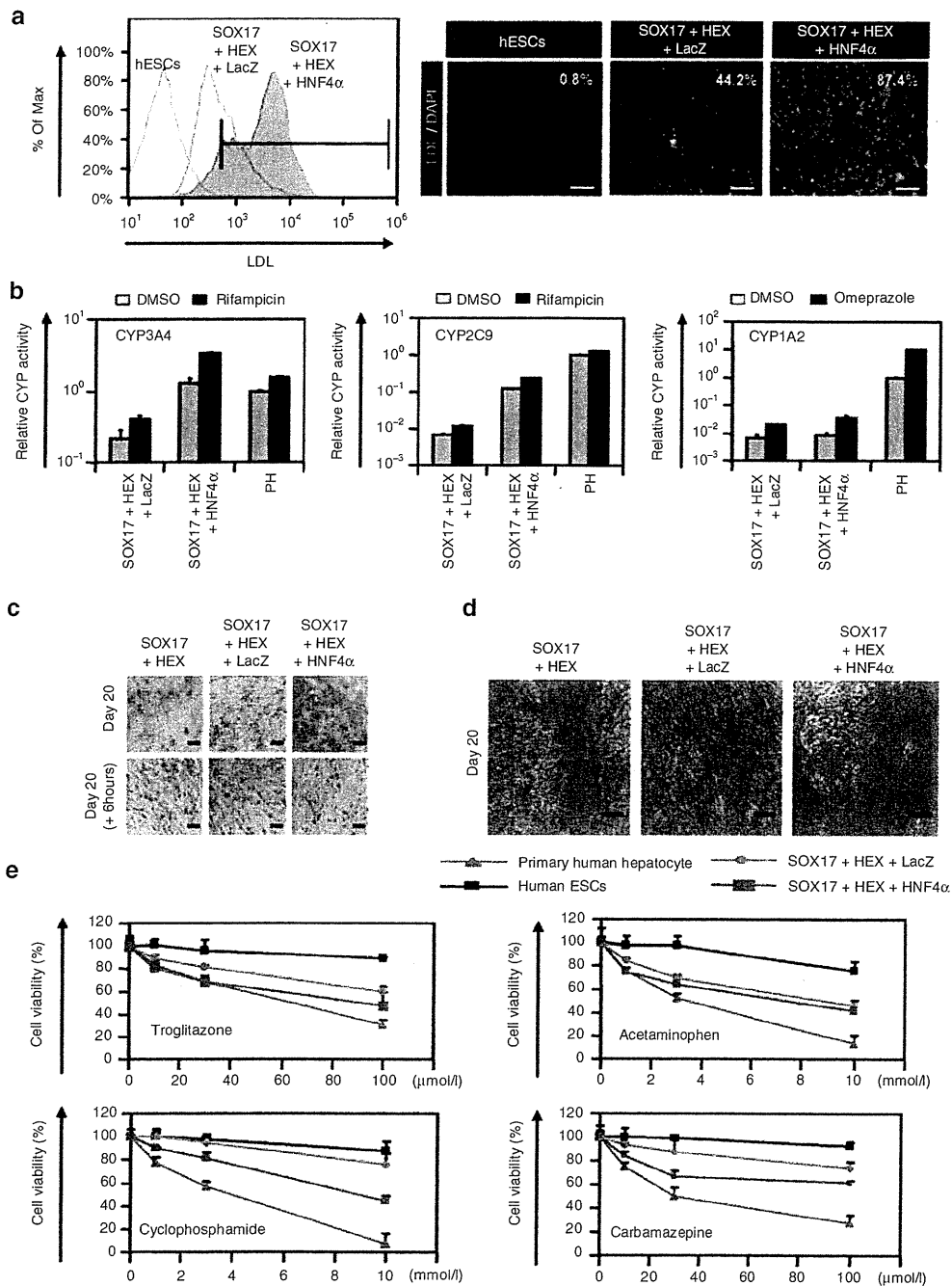


Figure 4 Transduction of the three factors enhances hepatic functions. The human ESCs were differentiated into hepatoblasts and transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4 α for 1.5 hours and cultured until day 20 of differentiation according to the protocol described in Figure 2a. The hepatic functions of the two factors plus Ad-LacZ-transduced cells (SOX17+HEX+LacZ) and the three factors-transduced cells (SOX17+HEX+HNF4 α) were compared. (a) Undifferentiated human ESCs (hESCs) and the hepatocyte-like cells (day 20) were cultured with medium containing Alexa-Fluor 488-labeled LDL (green) for 1 hour, and immunohistochemistry and flow cytometry analysis were performed. The percentage of LDL-positive cells was measured by flow cytometry. Nuclei were counterstained with DAPI (blue). The bar represents 100 μ m. (b) Induction of CYP3A4 (left), CYP2C9 (middle), or CYP1A2 (right) by DMSO (gray bar), rifampicin (black bar), or omeprazole (black bar) in the hepatocyte-like cells (day 20) and primary human hepatocytes (PH), which were cultured for 48 hours after the cells were plated. On the y-axis, the activity of primary human hepatocytes that have been cultured with medium containing DMSO was taken as 1.0. (c) The hepatocyte-like cells (day 20) (upper column) were examined for their ability to take up Indocyanin Green (ICG) and release it 6 hours thereafter (lower column). (d) Glycogen storage of the hepatocyte-like cells (day 20) was assessed by Periodic Acid-Schiff (PAS) staining. PAS staining was performed on day 20 of differentiation. Glycogen storage is indicated by pink or dark red-purple cytoplasm. The bar represents 100 μ m. (e) The cell viability of undifferentiated human ESCs (black), two factors plus Ad-LacZ-transduced cells (green), the three factors-transduced cells (blue), and primary human hepatocytes (red) was assessed by Alamar Blue assay after 48 hours exposure to different concentrations of four test compounds (troglitazone, acetaminophen, cyclophosphamide, and carbamazepine). The cell viability is expressed as a percentage of cells treated with solvent only treat: 0.1% DMSO except for carbamazepine: 0.5% DMSO. All data are represented as means \pm SD ($n = 3$). ESC, embryonic stem cell; DMSO, dimethyl sulfoxide; LDL, low-density lipoprotein.

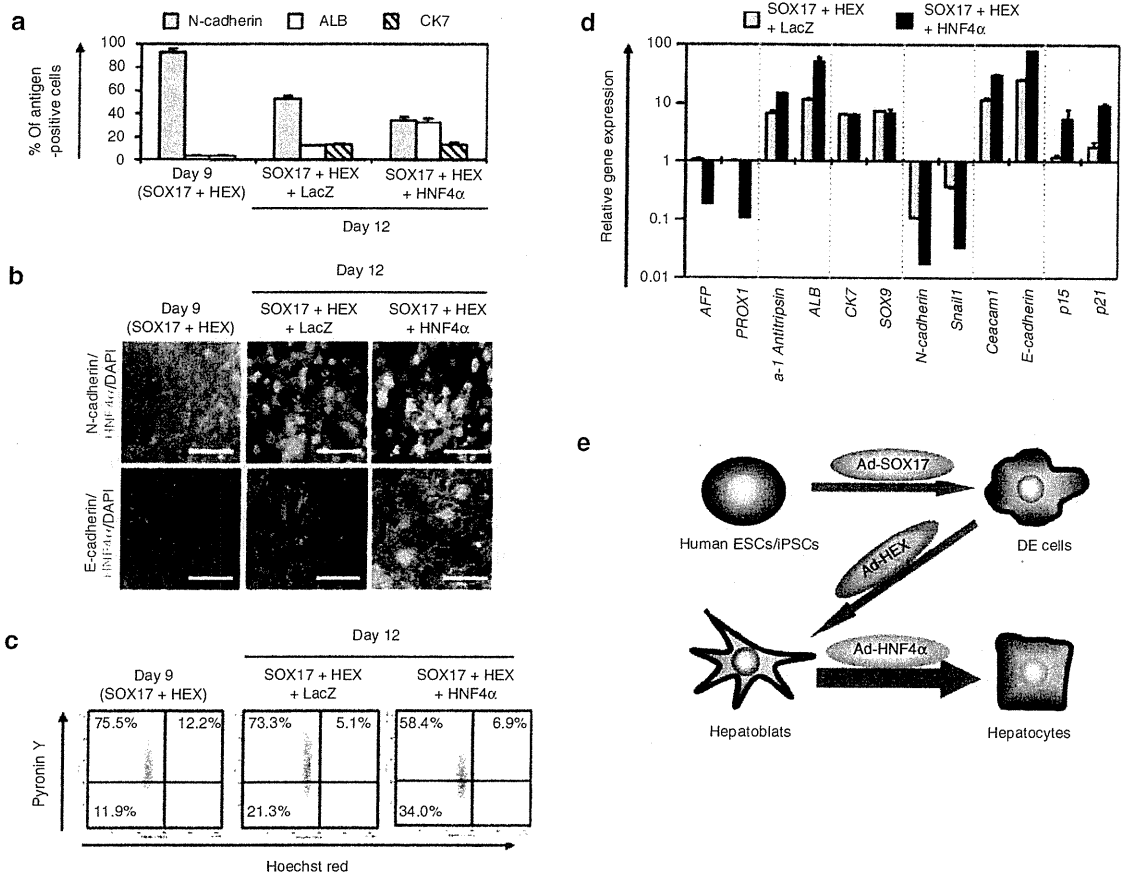


Figure 5 HNF4 α promotes hepatic differentiation by activating MET. Human ESCs were differentiated into hepatoblasts according to the protocol described in Figure 2a, and then transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4 α for 1.5 hours, and finally cultured until day 12 of differentiation. (a) The hepatoblasts, two factors plus Ad-LacZ-transduced cells (SOX17+HEX+LacZ) (day 12), and the three factors-transduced cells (SOX17+HEX+HNF4 α) (day 12) were subjected to immunostaining with anti-N-cadherin, ALB, or CK7 antibodies. The percentage of antigen-positive cells was measured by flow cytometry. (b) The cells were subjected to immunostaining with anti-N-cadherin (green), E-cadherin (green), or HNF4 α (red) antibodies on day 9 or day 12 of differentiation. Nuclei were counterstained with DAPI (blue). The bar represents 50 μ m. Similar results were obtained in two independent experiments. (c) The cell cycle was examined on day 9 or day 12 of differentiation. The cells were stained with Pylonin Y (y-axis) and Hoechst 33342 (x-axis) and then analyzed by flow cytometry. The growth fraction of cells is the population of actively dividing cells (G1/S/G2/M). (d) The expression levels of *AFP*, *PROX1*, α -1-antitrypsin, *ALB*, *CK7*, *SOX9*, *N-cadherin*, *Snail1*, *Ceacam1*, *E-cadherin*, *p15*, and *p21* were examined by real-time RT-PCR on day 9 or day 12 of differentiation. The expression level of hepatoblasts (day 9) was taken as 1.0. All data are represented as means \pm SD ($n = 3$). (e) The model of efficient hepatic differentiation from human ESCs and iPSCs in this study is summarized. The human ESCs and iPSCs differentiate into hepatocytes via definitive endoderm and hepatoblasts. At each stage, the differentiation is promoted by stage-specific transduction of appropriate functional genes. In the last stage of hepatic differentiation, HNF4 α transduction provokes hepatic maturation by activating MET. ESC, embryonic stem cell; HNF4 α , hepatocyte nuclear factor 4 α ; iPSC, induced pluripotent stem cell; MET, mesenchymal-to-epithelial transition; RT-PCR, reverse transcription-PCR; VP, vector particle.

The gene expression levels of hepatocyte markers (α -1-antitrypsin and *ALB*)²⁰ and epithelial markers (*Ceacam1* and *E-cadherin*) were upregulated by HNF4 α transduction. On the other hand, the gene expression levels of hepatoblast markers (*AFP* and *PROX1*)³¹, mesenchymal markers (*N-cadherin* and *Snail*)³², and cyclin dependent kinase inhibitor (*p15* and *p21*)³³ were downregulated by HNF4 α transduction. HNF4 α transduction did not change the expression levels of cholangiocyte markers (*CK7* and *SOX9*). We conclude that HNF4 α promotes hepatic maturation by activating MET.

DISCUSSION

This study has two main purposes: the generation of functional hepatocytes from human ESCs and iPSCs for application to drug toxicity screening in the early phase of pharmaceutical development

and; elucidation of the HNF4 α function in hepatic maturation from human ESCs. We initially confirmed the importance of transcription factor HNF4 α in hepatic differentiation from human ESCs by using a published data set of gene array analysis (Supplementary Figure S1).³⁴ We speculated that HNF4 α transduction could enhance hepatic differentiation from human ESCs and iPSCs.

To generate functional hepatocytes from human ESCs and iPSCs and to elucidate the function of HNF4 α in hepatic differentiation from human ESCs, we examined the stage-specific roles of HNF4 α . We found that hepatoblast (day 9) stage-specific HNF4 α transduction promoted hepatic differentiation (Figure 1). Because endogenous HNF4 α is initially expressed in the hepatoblast,^{9,10} our system might adequately reflect early embryogenesis. However, HNF4 α transduction at an inappropriate stage (day 6 or day 12) promoted

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 iPSC cell lines: two of the human iPSC cell lines (Dotcom and Tic) were more committed to the hepatic lineage than another human iPSC cell line (201B7) (Supplementary Figure S7). Therefore, it would be necessary to select a human iPSC 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 iPSC 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 5 ng/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'-ggcctctagatggaggcagggagaatg-3' and Rev 5'-ccccggcgcagcggcttctagataac-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 $^{\circ}$ 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 VIL0 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 $^{\circ}$ 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-GloTM 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^{47–59} (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 CellDirect (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 (CellDirect) 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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Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection

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ABSTRACT

Host tropism of hepatitis C virus (HCV) is limited to human and chimpanzee. HCV infection has never been fully understood because there are few conventional models for HCV infection. Human induced pluripotent stem cell-derived hepatocyte-like (iPS-Hep) cells have been expected to use for drug discovery to predict therapeutic activities and side effects of compounds during the drug discovery process. However, the suitability of iPS-Hep cells as an experimental model for HCV research is not known. Here, we investigated the entry and genomic replication of HCV in iPS-Hep cells by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively. We showed that iPS-Hep cells, but not iPS cells, were susceptible to infection with HCVpv. The iPS-Hep cells expressed HCV receptors, including CD81, scavenger receptor class B type I (SR-BI), claudin-1, and occludin; in contrast, the iPS cells showed no expression of SR-BI or claudin-1. HCV RNA genome replication occurred in the iPS-Hep cells. Anti-CD81 antibody, an inhibitor of HCV entry, and interferon, an inhibitor of HCV genomic replication, dose-dependently attenuated HCVpv entry and HCV subgenomic replication in iPS-Hep cells, respectively. These findings suggest that iPS-Hep cells are an appropriate model for HCV infection.

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1. Introduction

Hepatitis C virus (HCV), a hepatotropic member of the *Flaviviridae* family, is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. Approximately 130–200 million people are

estimated to be infected with HCV worldwide. Each year, 3–4 million people are newly infected with HCV [1]. Thus, overcoming HCV is a critical issue for the World Health Organization.

HCV contains a positive strand ~9.6 kb RNA encoding a single polyprotein (~3000 aa), which is cleaved by host and viral proteases to form structural proteins (core, E1, E2, and p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [1]. These virus proteins might be potent targets for anti-HCV drugs. However, combination therapy with interferon and ribavirin, which often causes severe side-effects leading to treatment termination, has been the only therapeutic choice [2]. Very recently, new direct antiviral agents have been approved or are under clinical trials; these agents include NS3 protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors [2–4]. However, the emergence of drug resistance is a serious problem associated with the use of direct antiviral agents [5].

Host targets are alternative targets for the development of anti-HCV drugs. A liver-specific microRNA (miRNA), miR-122, facilitates the replication of the HCV RNA genome in cultured liver cells [6]. Administration of a chemically modified oligonucleotide complementary to miR-122 results in long-lasting suppression of HCV with no appearance of resistant HCV in chimpanzees [7]. Epidermal

Abbreviations: HCV, hepatitis C virus; iPS-Hep cells, human induced pluripotent stem cells-derived hepatocyte-like cells; HCVpv, HCV pseudotype virus; SR-BI, scavenger receptor class B type I; miRNA, microRNA; EGF-R, epidermal growth factor receptor; EphA2, ephrin factor A2; iPS cells, human induced pluripotent stem cells; FCS, fetal calf serum; Ad, adenovirus; HNF-4 α , hepatocyte nuclear factor-4 α ; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VSV, vesicular stomatitis virus; VSVpv, VSV pseudotype virus; tet, tetracycline; pol, polymerase; MOI, multiplicity of infection; Dox, doxycycline; IFN, interferon- α ; ES cells, embryonic stem cells.

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growth factor receptor (EGF-R) and ephrin factor A2 (EphA2) are host cofactors for HCV entry [8]. Inhibitors of EGF-R and EphA2 attenuated HCV entry, and prevented the appearance of viral escape variants [8]. These findings strongly indicate that identification of host factors associated with infection of human liver by HCV is a potent strategy for anti-HCV drug development. Because the host tropism of HCV is limited to human and chimpanzee [9], there is no convenient model for the evaluation of HCV infections. This has led to a delay in the development of anti-HCV agents targeting host factors.

Takahashi and Yamanaka developed human induced pluripotent stem (iPS) cells from human somatic cells [10]. The stem cells can be redifferentiated *in vitro*, leading to new models for drug discovery, including iPS-based models for drug discovery, toxicity assessment, and disease modeling [11,12].

Recently, several groups reported that iPS cells can be successfully differentiated into hepatocyte-like (iPS-Hep) cells that show many functions associated with mature hepatocytes [13–19]. However, whether iPS-Hep cells are suitable as a model for HCV infection has not been fully determined. Here, we investigated HCV entry and genomic replication in iPS-Hep cells by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively.

2. Materials and methods

2.1. Cell culture

Huh7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). An iPS cell-line (Dot-com) generated from the human embryonic lung fibroblast cell-line MCR5 was obtained from the Japanese Collection of Research Bioresources Cell Bank [20,21]. The iPS cells were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore, Billerica, MA) in iPSellon culture medium (Cardio, Hyogo, Japan) supplemented with 10 ng/ml fibroblast growth factor-2.

2.2. *In vitro* differentiation

Before the initiation of cellular differentiation, the medium of the iPS cells was replaced with a defined serum-free medium, hESF9, and the cells were cultured as previously reported [22]. The iPS cells were differentiated into iPS-Hep cells by using adenovirus (Ad) vectors expressing SOX17, the homeotic gene HEX or hepatocyte nuclear factor 4 α (HNF-4 α) in addition to the appropriate growth factors, cytokines, and supplements, as described previously [19].

2.3. Reverse transcription (RT)-polymerase chain reaction (PCR) analysis of HCV receptors

Total RNA samples were reverse-transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), and the resultant cDNAs were PCR amplified by using Ex Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) and specific paired-primers for CD81 (5'-cgccaaggatgtgaagcagttc-3' and 5'-tcccggagaagaggtcatgat-3'), scavenger receptor class B type I (SR-BI; 5'-attccgatcatgcaacatga-3' and 5'-cagttttgcttctgcagcacag-3'), claudin-1 (5'-tcagcactgcctgccccagt-3' and 5'-tggtgttggttaagaggtgt-3'), occludin (5'-tca gggaatccacctatcacttcag-3' and 5'-catcagcagcagccatgactcttcac-3'), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5'-tcttcaccacatggagaag-3' and 5'-accactggtgctcagtga-3'). The expected sizes of the PCR products were 245 bp for CD81, 788 bp for SR-BI, 521 bp for claudin-1, 189 bp for occludin, and 544 bp for GAPDH. The PCR products were separated on 2% agarose gels and visualized by staining with ethidium bromide.

2.4. HCVpv infection

Pseudotype vesicular stomatitis virus (VSV) bearing HCV envelope glycoproteins (HCVpv) and VSV envelope glycoproteins (VSVpv) were prepared as described previously [23]. iPS, iPS-Hep and Huh7 cells were treated with HCVpv or mixtures of HCVpv or VSVpv and anti-CD81 monoclonal antibody (JS-81; BD Biosciences, Franklin Lakes, NJ) or control mouse IgG for 2 h. After an additional 24 h of culture, the luciferase activities were measured by using a commercially available kit (PicaGene, Toyo Ink, Tokyo, Japan).

2.5. Preparation of Ad vector expressing the HCV replicon

Ad vectors expressing a tetracycline (tet)-controllable and RNA polymerase (pol) I promoter-driven HCV subgenomic replicon containing renilla luciferase (AdP₂₃₅-HCV), a replication-incompetent HCV subgenomic replicon containing renilla luciferase (AdP₂₃₅- Δ GDD), tet-responsive trans-activator (Ad-tTA) or a tet-controllable RNA pol-I driven firefly luciferase (AdP₂₃₅-fluc) were prepared by using an *in vitro* ligation method as described previously [24–26]. The biological activity (infectious unit) of the Ad vectors was measured by using an Adeno-X rapid titer kit (Clontech, Mountain View, CA).

2.6. HCV replication assay

iPS, iPS-Hep and Huh7 cells were infected with AdP₂₃₅-HCV or AdP₂₃₅- Δ GDD at multiplicity of infection (MOI; infectious unit per cell) of 3, and Ad-tTA at MOI of 15. After 24 h, the cells were treated with 10 μ g/ml of doxycycline (Dox) for 48 h. Renilla luciferase activities in the lysates were then measured with the use of the Renilla Luciferase Assay System (Promega, Madison, WI). To normalize for the infectivity of Ad vector, iPS, iPS-Hep and Huh7 cells were co-infected with AdP₂₃₅-fluc (3 MOI) and Ad-tTA (15 MOI). After a 72-h incubation, the firefly luciferase activities in the lysates were measured, and the renilla luciferase activities were normalized by dividing by the corresponding firefly luciferase activities.

2.7. Quantitative analysis of plus- and minus-strand HCV RNA

iPS, iPS-Hep and Huh7 cells were co-infected with AdP₂₃₅-HCV or AdP₂₃₅- Δ GDD (3 MOI), and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10 μ g/ml of Dox for 48 h. Total RNA was reverse-transcribed into cDNA by using the Thermoscript reverse transcriptase kit (Invitrogen) as described previously [27,28]. Real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa Bio Inc.) by using Applied Biosystems StepOne Plus (Applied Biosystems, Foster City, CA). The transcription products of the HCV plus-strand RNA, minus-strand RNA, and GAPDH gene, were amplified by using specific primers for HCV plus-strand RNA (RC1 primer, 5'-gtctagc-catggcgtagta-3'; and RC21 primer, 5'-ctccggggcactcgcaagc-3'), HCV minus-strand RNA (tag primer, 5'-ggcgcgtcatggtggcgaataa-3'; and RC21 primer), and GAPDH (5'-ggtggtctcctctgacttcaaca-3' and 5'-gtggtcgttgaggccaatg-3'), respectively. The copy numbers of the transcription products of the HCV plus- and minus-strand RNA were normalized with those of the GAPDH gene and infectivity of Ad vector as described in the Section 2.6.

2.8. Inhibition of HCV replication by interferon- α 8

iPS-Hep and Huh7 cells were infected with AdP₂₃₅-HCV (3 MOI) and Ad-tTA (15 MOI). After 24 h of infection, the cells were treated with 10 μ g/ml of Dox and recombinant human interferon- α 8 (IFN) at the indicated concentration. After an additional 48-h incubation, renilla luciferase activity in the lysates was measured with the use of the Renilla Luciferase Assay System. Cell

viability was measured with the use of a WST-8 kit (Nacalai Tesque, Kyoto, Japan).

3. Results

3.1. Infection of iPS-Hep cells with HCVpv

HCV entry requires sequential interaction between the envelope proteins and multiple cellular factors, including CD81, SR-BI, claudin-1, and occludin [29]. To investigate expression of these receptors in iPS-Hep cells, we performed RT-PCR analysis. iPS cells expressed CD81 and occludin, but not SR-BI and claudin-1. In contrast, iPS-Hep and Huh7 cells expressed all four receptors (Fig. 1A). HCVpv have been widely used in studies of the mechanism of HCV entry and in screens for inhibitors of HCV infection [30]. We therefore investigated HCVpv infection in iPS-Hep cells. iPS cells showed no susceptibility to HCVpv infection. In contrast, HCVpv dose-dependently infected iPS-Hep cells as well as Huh7 cells, a popular model cell line for HCV research (Fig. 1B). Treatment of the cells with IgG did not affect susceptibility of iPS-Hep or Huh7 cells to HCVpv infection, even at IgG concentrations of 1 $\mu\text{g}/\text{ml}$. In contrast, anti-CD81 antibody dose-dependently inhibited HCVpv infection of iPS-Hep and Huh7 cells, and the antibody treatment did not affect infection of VSVpv with iPS-Hep (Fig. 1C). These findings suggest that iPS-Hep cells are a useful model for HCV infection.

3.2. Replication of subgenomic HCV RNA in iPS-Hep cells

We previously developed Ad vectors containing tet-controllable and RNA pol I-driven HCV RNA subgenomic replicons (AdP₂₃₅-HCV [replication competent], and AdP₂₃₅- Δ GDD [replication incompetent]). The replicons encoded luciferase, and monitoring of luciferase activity in infected cells was a simple and convenient method to evaluate HCV replication [24]. Here, we found cells transduced with the replication-competent HCV replicon expressed luciferase in iPS-Hep cells, but not in iPS cells (Fig. 2A). In contrast, cells transduced with the replication-incompetent HCV replicon did not express luciferase (Fig. 2A). Taken together, these results suggest that replication of the HCV RNA genome occurred in the iPS-Hep cells. To confirm replication of the HCV genome, we investigated production of minus-strand HCV RNA from the positive-strand HCV RNA genome by performing real time-PCR analysis. The results of this analysis showed that minus-strand HCV RNA was produced in iPS-Hep cells and Huh7 cells, but not in iPS cells (Fig. 2B). To investigate whether the iPS-Hep cells could be used to screen for drugs that suppress HCV replication, we treated the cells with a suppressor of HCV replication, IFN. Treatment with IFN resulted in dose-dependent attenuated replication of the HCV genome with no cytotoxicity (Fig. 3A and B). These findings suggest that the iPS-Hep cells are a suitable system to use for monitoring the replication of the HCV RNA genome.

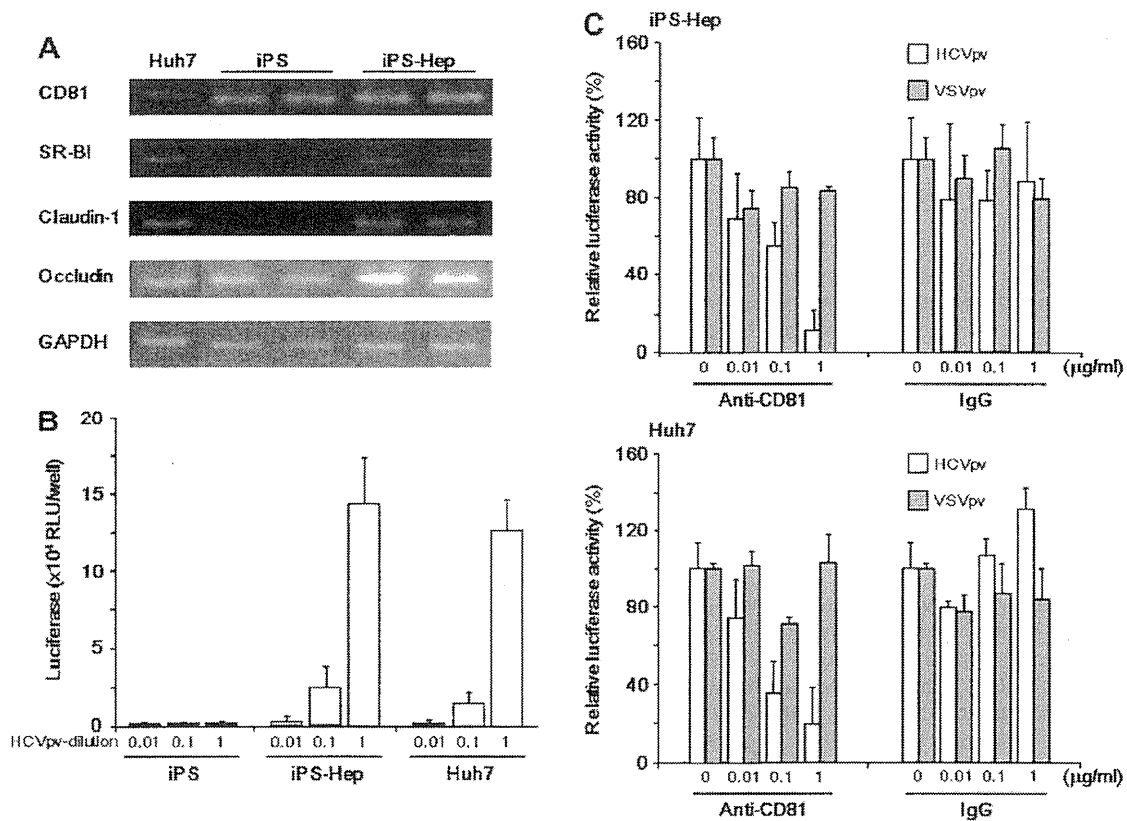


Fig. 1. HCV infection assay in iPS-Hep cells. (A) Expression of HCV receptors in iPS-Hep cells. Total RNA samples from Huh7, iPS, and iPS-Hep cells were subjected to RT-PCR analysis as described in the Section 2. The PCR products were separated on 2% agarose gels, followed by staining with ethidium bromide. (B) Infection of iPS-Hep cells with HCVpv. iPS, iPS-Hep and Huh7 cells were infected with HCVpv at the indicated dilution. After 2 h of infection, the cells were cultured with fresh medium for 24 h. Then, luciferase activities were measured. Data are presented as means \pm SD ($n = 3$). (C) Effect of anti-CD81 antibody on infection of iPS-Hep cells with HCVpv. iPS-Hep (upper panel) and Huh7 (lower panel) cells were treated with mixtures of HCVpv (open column) or VSVpv (gray column) and anti-CD81 antibody or control mouse IgG at the indicated concentrations. After a 2-h incubation, the cells were cultured with fresh medium for 24 h. Then, the luciferase activities were measured. Data represent the percentage of vehicle-treated cells. Data are presented as means \pm SD ($n = 3$).

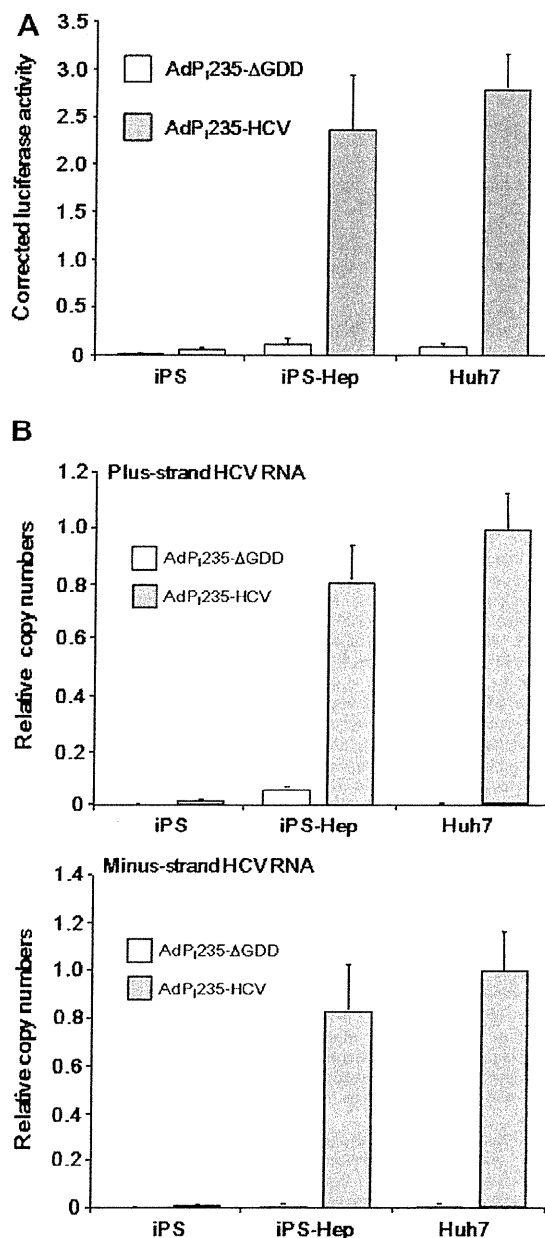


Fig. 2. HCV replication assay in iPS-Hep cells. (A) Comparison of replication of HCV subgenomic replicons, AdP₂₃₅-HCV (gray column) and AdP₂₃₅-ΔGDD (open column), in iPS, iPS-Hep and Huh7 cells. The cells were infected with replicons, treated with Dox, and renilla luciferase activity was measured, as described in the Section 2. To normalize for infectivity of Ad vector, cells were co-infected with AdP₂₃₅-fluc and Ad-tTA. After 72 h, firefly luciferase activity was measured. Corrected luciferase activity was calculated as the ratio of renilla luciferase activity to firefly luciferase activity. (B) Real-time PCR analysis of HCV plus- and minus-strand RNA in iPS-Hep cells. iPS-Hep cells were infected with replicons, and total RNA was subjected to real-time PCR analysis, as described in the Section 2. The copy numbers were shown as ratio of those of Huh7. Data are presented as means \pm SD ($n = 3$).

4. Discussion

Tropism of HCV is limited to human and chimpanzee. Our understanding of HCV infection has been delayed by the lack of appropriate model systems. In the present study, we demonstrated that iPS-Hep cells are suitable *in vitro* models of hepatocytes for use in the study of HCV infection.

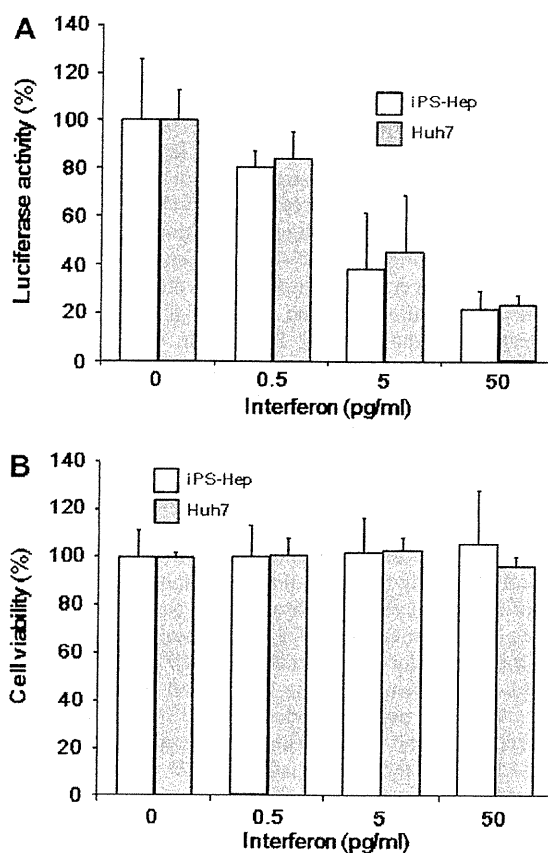


Fig. 3. Effect of interferon on HCV replication in iPS-Hep cells. iPS-Hep (open column) and Huh7 (gray column) cells were infected with AdP₂₃₅-HCV and Ad-tTA. After 24 h, the cells were treated with Dox and the indicated concentration of interferon for 48 h. Luciferase activities (A) and cell viabilities (B) were measured as described in the Section 2. Data represent the percentage of the value for vehicle-treated cells, and are presented as means \pm SD ($n = 3$).

Other *in vitro* model systems of hepatocytes may not accurately reflect the biology of hepatocytes *in vivo*. For instance, expression profiles of mRNAs in embryonic stem (ES) cell-derived hepatocyte-like cells are different from those of primary human hepatocytes [31]. The development of efficient methods to differentiate stem cells into hepatocytes has been a critical issue in the application of stem cell technology to drug discovery. Recently, Mizuguchi and colleagues established efficient differentiation protocols for iPS cells by using adenoviral transfer of SOX17 [17], HEX [18], and HNF-4 α [19] in addition to growth factors. Approximately 80% of the differentiated cells showed expression of hepatic-specific proteins, including cytochrome P-450s (CYP2D6, CYP3A4, and CYP7A1) [19]. The iPS-Hep cells were also used as a simple system to evaluate the hepatotoxicity of drugs that are metabolized into toxic substances by cytochromes [19]. Here, we showed that the essential host factors for HCV infection (occludin, claudin-1, SR-BI, and CD81) are expressed in the iPS-Hep cells. HCV RNA genome replication occurred in the cells, and HCVpv infected the cells. An inhibitor of HCV entry (anti-CD81 antibody), and an anti-HCV agent (IFN), attenuated the entry of HCVpv and the replication of the HCV genome in the cells, respectively. These findings suggest that the iPS-Hep cells are useful for understanding HCV infection and for screening anti-HCV drugs.

We found that iPS cells express CD81 and occludin, and are not susceptible to HCV entry, whereas iPS-Hep cells express all four HCV receptors and are susceptible to HCV entry. These findings are consistent with previous studies showing that CD81, occludin,

SR-BI, and claudin-1 are key receptors for HCV [29]. HNF-4 α , which promotes the differentiation of iPS cells to iPS-Hep cells, is essential for the expression of a multitude of genes encoding cell junction and adhesion proteins during embryonic development of the mouse liver [32]. For instance, claudin-1 expression is not detected in the liver of HNF-4 α -deficient mice [32]. HNF-4 α enhances peroxisome proliferator-activated receptor-mediated SR-BI transcription [33]. Thus, the susceptibility to HCV entry observed in iPS-Hep cells may be the result of the additional expression of claudin-1 and SR-BI following HNF-4 α treatment.

miR-122 is a liver specific miRNA that constitutes 70% of the total miRNA population [34] and is essential for replication of the HCV genome in the liver [6]. ES cells do not express miR-122, whereas expression of miRNA is observed during differentiation into hepatocyte-like cells [35]. Replication of HCV subgenomic replicons was observed in iPS-Hep cells, but not iPS cells (Fig. 2A). Expression of miR-122 might be a key factor controlling the replication of the HCV RNA genome in iPS-Hep cells.

The reasons that 15–20% of people infected with HCV can clear the virus without pharmaceutical intervention, and patients vary in their sensitivity to pharmaceutical treatments, are still unclear [36]. Understanding the basis of these variable responses to infection and treatment would facilitate the discovery of potent targets for drug development for HCV. iPS-derived hepatocytes are a promising system for drug discovery for HCV infection. In the present study, we showed that the iPS-derived hepatocyte-like cells can be used with popular models of HCV infection: HCV subgenomic replicons and HCVpv. Our findings will contribute to our understanding of the mechanisms of HCV infection and to the identification of novel targets for HCV therapy by means of iPS technology.

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Efficient and Directive Generation of Two Distinct Endoderm Lineages from Human ESCs and iPSCs by Differentiation Stage-Specific SOX17 Transduction

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Abstract

The establishment of methods for directive differentiation from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) is important for regenerative medicine. Although Sry-related HMG box 17 (SOX17) overexpression in ESCs leads to differentiation of either extraembryonic or definitive endoderm cells, respectively, the mechanism of these distinct results remains unknown. Therefore, we utilized a transient adenovirus vector-mediated overexpression system to mimic the SOX17 expression pattern of embryogenesis. The number of alpha-fetoprotein-positive extraembryonic endoderm (ExEn) cells was increased by transient SOX17 transduction in human ESC- and iPSC-derived primitive endoderm cells. In contrast, the number of hematopoietically expressed homeobox (HEX)-positive definitive endoderm (DE) cells, which correspond to the anterior DE *in vivo*, was increased by transient adenovirus vector-mediated SOX17 expression in human ESC- and iPSC-derived mesendoderm cells. Moreover, hepatocyte-like cells were efficiently generated by sequential transduction of SOX17 and HEX. Our findings show that a stage-specific transduction of SOX17 in the primitive endoderm or mesendoderm promotes directive ExEn or DE differentiation by SOX17 transduction, respectively.

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Introduction

There are two distinct endoderm lineages in early embryogenesis, the extraembryonic endoderm (ExEn) and the definitive endoderm (DE). The first of these lineages, the ExEn plays crucial roles in mammalian development, although it does not contribute to the formation of body cells. In early embryogenesis, a part of the inner cell mass of the blastocyst differentiates into the primitive endoderm (PrE). The PrE differentiates into the ExEn that composes the parietal endoderm, which contributes to the primary yolk sac, and the visceral endoderm, which overlies the epiblast [1,2]. In contrast, the second of the endoderm lineages, the DE arises from the primitive streak (PS), which is called the mesendoderm [3]. The DE has the ability to differentiate into the hepatic and pancreatic tissue [4].

The establishment of human embryonic stem cells (ESCs) [5] and human induced pluripotent stem cells (iPSCs) [6,7] has opened up new opportunities for basic research and regenerative medicine. To exploit the potential of human ESCs and iPSCs, it is

necessary to understand the mechanisms of their differentiation. Although growth factor-mediated ExEn or DE differentiation is widely performed, it leads to a heterogeneous population [8,9,10,11]. Several studies have utilized not only growth factors but also modulation of transcription factors to control downstream signaling cascades [10,12,13]. Sox17, an Sry-related HMG box transcription factor, is required for development of both the ExEn and DE. In mice, during ExEn and DE development, Sox17 expression is first observed in the PrE and in the anterior PS, respectively [14]. Previous study showed that stable Sox17 overexpression promotes ExEn differentiation from mouse ESCs [12]. On the other hand, another previous study has demonstrated that DE progenitors can be established from human ESCs by stable expression of SOX17 [10]. The mechanism of these discrepancies which occurs in SOX17 transduction still remains unknown. Also, the role of SOX17 in human ExEn differentiation still remains unknown. Therefore, it is quite difficult to promote directive differentiation into either ExEn or DE cells by SOX17 transduction.

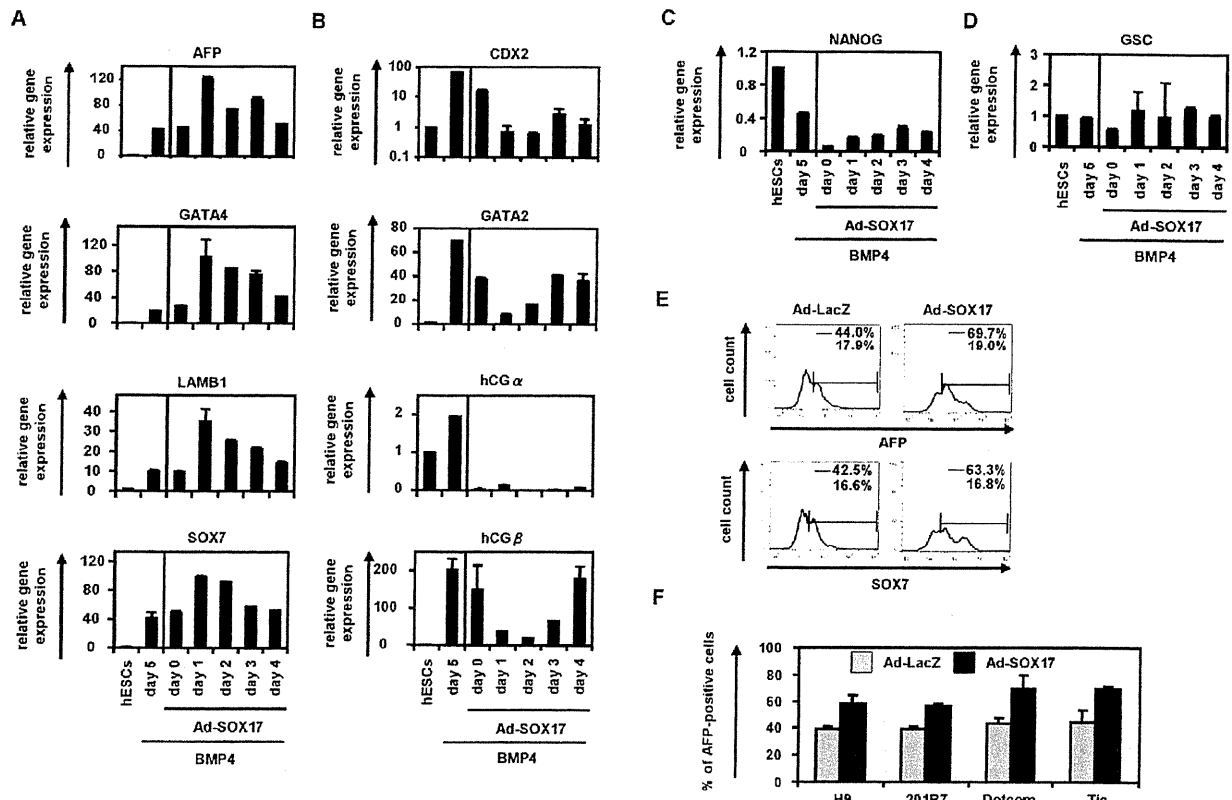


Figure 1. Efficient ExEn differentiation from human ESC- and iPSC-derived PrE cells by SOX17 transduction. (A–D) Undifferentiated human ESCs (H9) and BMP4-induced human ESC-derived cells, which were cultured with the medium containing BMP4 (20 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 VP/cell of Ad-SOX17 for 1.5 h. Ad-SOX17-transduced cells were cultured with 20 ng/ml of BMP4, and then the gene expression levels of (A) the ExEn markers (AFP, GATA4, LAMB1, and SOX7), (B) the trophoctoderm markers (CDX2, GATA2, hCG α , and hCG β), (C) the pluripotent marker (NANOG), and (D) the DE marker (GSC) were examined by real-time RT-PCR on day 5 of differentiation. The horizontal axis represents the day on which the cells were transduced with Ad-SOX17. The expression levels of undifferentiated human ESCs on day 0 were defined 1.0. (E) On day 1, human ESC-derived PrE cells, which were cultured with the medium containing BMP4 for 1 day, were transduced with Ad-LacZ or Ad-SOX17 and cultured until day 5. The ExEn cells were subjected to immunostaining with anti-AFP or anti-SOX7 antibodies, and then analyzed by flow cytometry. (F) After Ad-LacZ or Ad-SOX17 transduction, the efficacies of ExEn differentiation from the human ES cell line (H9) and the three human iPSC cell lines (201B7, Dotcom, and Tic) were compared on day 5 of differentiation. All data are represented as the means \pm SD ($n=3$). doi:10.1371/journal.pone.0021780.g001

In this study, we utilized SOX17 as a stage-specific regulator of ExEn and DE differentiation from human ESCs and iPSCs. The human ESC- and iPSC-derived cells were transduced with SOX17-expressing adenovirus vector (Ad-SOX17), and the resulting phenotypes were assessed for their ability to differentiate into ExEn and DE cells *in vitro*. In addition, we examined whether SOX17-transduced cells have the ability to differentiate into the hepatic lineage. The results showed that stage-specific overexpression of the SOX17 transcription factor promotes directive differentiation into either ExEn or DE cells.

Results

The induction of human ESC-derived PrE cells and human ESC-derived mesendoderm cells

To determine the appropriate stage for SOX17 transduction, ExEn or DE cells were differentiated from human ESCs by a conventional method using BMP4 (20 ng/ml) or Activin A (100 ng/ml), respectively (Figures S1 and S2). Experiments for bidirectional differentiation using BMP4 and Activin A indicated that PrE cells were obtained on day 1 (Figure S1) and mesendoderm

cells were obtained on day 3 (Figure S2). We expected that stage-specific SOX17 transduction into PrE cells or mesendoderm cells could promote ExEn or DE differentiation, because the time period of initiation of SOX17 expression was correlated with the time period of formation of PrE cells (day 1) (Figure S1C) and mesendoderm cells (day 3) (Figure S2C), respectively.

PrE stage-specific SOX17 overexpression promotes directive ExEn differentiation from human ESCs

To examine the effect of forced and transient expression of SOX17 on the differentiation of human ESC- and iPSC-derived cells, we used a fiber-modified adenovirus (Ad) vector containing the EF-1 α promoter and a stretch of lysine residues (KKKKKKK, K7) peptides in the C-terminal region of the fiber knob. The K7 peptide targets heparan sulfates on the cellular surface, and the fiber-modified Ad vector containing the K7 peptides has been shown to be efficient for transduction into many kinds of cells [15,16].

Because the time period of initiation of SOX17 expression was correlated with the time period of formation of PrE cells (day 1) (Figure S1), we expected that stage-specific SOX17 transduction

into PrE cells would promote ExEn differentiation. Therefore, we examined the stage-specific role of SOX17 in ExEn differentiation. Ad-SOX17 transduction was performed in human ESCs treated with BMP4 for 0, 1, 2, 3, or 4 days, and the Ad-SOX17-transduced cells were cultured with medium containing BMP4 until day 5 (Figures 1A–1D). We confirmed the expression of exogenous SOX17 in the human ESC-derived mesendoderm cells transduced with Ad-SOX17 (Figure S3). Since BMP4 is known for its capability to induce both ExEn and trophoderm [8,9], we analyzed not only the expression levels of ExEn markers but also those of trophoderm markers by real-time RT-PCR after 5 days of differentiation (Figures 1A and 1B). The transduction of Ad-SOX17 on day 1 led to the highest expression levels of ExEn markers, alpha-fetoprotein (AFP), GATA4, laminin B1 (LAMB1), and SOX7 [17,18,19]. In contrast, the expression levels of the trophoderm markers CDX2, GATA2, hCG α (human chorionic gonadotropin), and hCG β [20] were down-regulated in Ad-SOX17-transduced cells as compared with non-transduced cells (Figure 1B). The expression levels of the pluripotent marker NANOG and DE marker GSC were not increased by SOX17 transduction (Figures 1C and 1D). We confirmed that there were no differences between non-transduced cells and Ad-LacZ-transduced cells in gene expression levels of all the markers investigated in Figures 1A–1D (data not shown). Therefore, we concluded that ExEn cells were efficiently induced from Ad-SOX17-transduced PrE cells.

The effects of SOX17 transduction on the ExEn differentiation from human ESC-derived PrE cells were also assessed by quantifying AFP- or SOX7-positive ExEn cells. The percentage of AFP- or SOX7-positive cells was significantly increased in Ad-SOX17-transduced cells (69.7% and 63.3%, respectively) (Figure 1E). Similar results were observed in the human iPS cell lines (201B7, Dotcom, and Tic) (Figure 1F). These findings indicated that stage-specific SOX17 overexpression in human ESC-derived PrE cells enhances ExEn differentiation.

Mesendoderm stage-specific SOX17 overexpression promotes directive DE differentiation from human ESCs

To examine the effects of transient SOX17 overexpression on DE differentiation from human ESCs, we optimized the timing of the Ad-SOX17 transduction. Ad-SOX17 transduction was performed in human ESCs treated with Activin A (100 ng/ml) for 0, 1, 2, 3, or 4 days, and the Ad-SOX17-transduced cells were cultured with medium containing Activin A (100 ng/ml) until day 5 (Figures 2A–2C). Using a fiber-modified Ad vector, both undifferentiated human ESCs and Activin A-induced human ESC-derived cells were efficiently transduced (Figure S4). The transduction of SOX17 on day 3 led to the highest expression levels of the DE markers FOXA2 [21], GSC [22], GATA4 [17], and HEX [23] (Figure 2A). In contrast to the DE markers, the expression levels of the pluripotent marker NANOG [24] were down-regulated in Ad-SOX17-transduced cells as compared with non-transduced cells (Figure 2B). The expression levels of the ExEn marker SOX7 [14] were up-regulated, when Ad-SOX17 transduction was performed into human ESCs treated with Activin A (100 ng/ml) for 0, 1, or 2 days (Figure 2C). On the other hand, the expression levels of the ExEn marker SOX7 were significantly down-regulated, when Ad-SOX17 transduction was performed into human ESCs treated with Activin A (100 ng/ml) for 3 or 4 days, indicating that SOX17 overexpression prior to mesendoderm formation (day 0, 1, and 2) promoted not only DE differentiation but also ExEn differentiation. Similar results were obtained with the human iPS cell line (Tic) (Figure S5). Although the expression

levels of the mesoderm marker FLK1 [25] did not exhibit any change when Ad-SOX17 transduction was performed into human ESCs treated with Activin A (100 ng/ml) for 0, 1, or 2 days (Figure 2D), their expression levels were significantly down-regulated when Ad-SOX17 transduction was performed into human ESCs treated with Activin A (100 ng/ml) for 3 or 4 days. These results suggest that SOX17 overexpression promotes directive differentiation from mesendoderm cells into the DE cells, but not into mesoderm cells. We also confirmed that Ad-vector mediated gene expression in the human ESC-derived mesendoderm cells (day 3) continued until day 6 and disappeared on day 10 (Figure S6). SOX17 transduction in the human ESC-derived cells on day 3 and 4 had no effect on cell viability, while that in the cells on day 0, 1, and 2 resulted in severely impaired cell viability (Figure S7), probably because SOX17 transduction directed the cells on day 0, 1, and 2 to differentiate into ExEn cells but the medium containing Activin A (100 ng/ml) was inappropriate for ExEn cells. We confirmed that there were no differences between non-transduced cells and Ad-LacZ-transduced cells in gene expression levels of all the markers investigated in Figures 2A–2D (data not shown). These results indicated that stage-specific SOX17 overexpression in human ESC-derived mesendoderm cells is essential for promoting efficient DE differentiation.

It has been previously reported that human ESC-derived mesendoderm cells and DE cells became CXCR4-positive (>80%) by culturing human ESCs with Activin A (100 ng/ml) [26]. However, Activin A is not sufficient for homogenous differentiation of c-Kit/CXCR4-double-positive DE cells [10,11] or HEX-positive anterior DE cells [23]. Seguin et al. and Morrison et al. reported that the differentiation efficiency of c-Kit/CXCR4-double-positive DE cells was approximately 30% in the absence of stable Sox17 expression and that of HEX-positive anterior DE cells was only about 10% [10,23]. Therefore, we next examined whether Ad-SOX17 transduction improves the differentiation efficiency of c-Kit/CXCR4-double-positive DE cells and HEX-positive anterior DE cells. Human ESC-derived mesendoderm cells were transduced with Ad-SOX17, and the number of CXCR4/c-Kit-double-positive cells was analyzed by using a flow cytometer. The percentage of CXCR4/c-Kit-double-positive cells was significantly increased in Ad-SOX17-transduced cells (67.7%), while that in Ad-LacZ-transduced cells was only 22% (Figure 2E). The percentage of HEX-positive cells was also significantly increased in Ad-SOX17-transduced cells (53.7%), while that in Ad-LacZ-transduced cells was approximately 11% (Figure 2F). Similar results were also observed in the three human iPS cell lines (201B7, Dotcom, and Tic) (Figure 2G). These findings indicated that stage-specific SOX17 overexpression in human ESC-derived mesendoderm cells promotes efficient differentiation of DE cells.

Ad-SOX17-transduced cells tend to differentiate into the hepatic lineage

To investigate whether Ad-SOX17-transduced cells have the ability to differentiate into hepatoblasts and hepatocyte-like cells, Ad-SOX17-transduced cells were differentiated according to our previously described method [13]. Our previous report demonstrated that transient HEX transduction efficiently generates hepatoblasts from human ESC- and iPSC-derived DE cells. The hepatic differentiation protocol used in this study is illustrated in Figure 3A. After the hepatic differentiation, the morphology of human ESCs transduced with Ad-SOX17 followed by Ad-HEX was gradually changed into a hepatocyte morphology: polygonal in shape with distinct round nuclei by day 18 (Figure 3B). We also