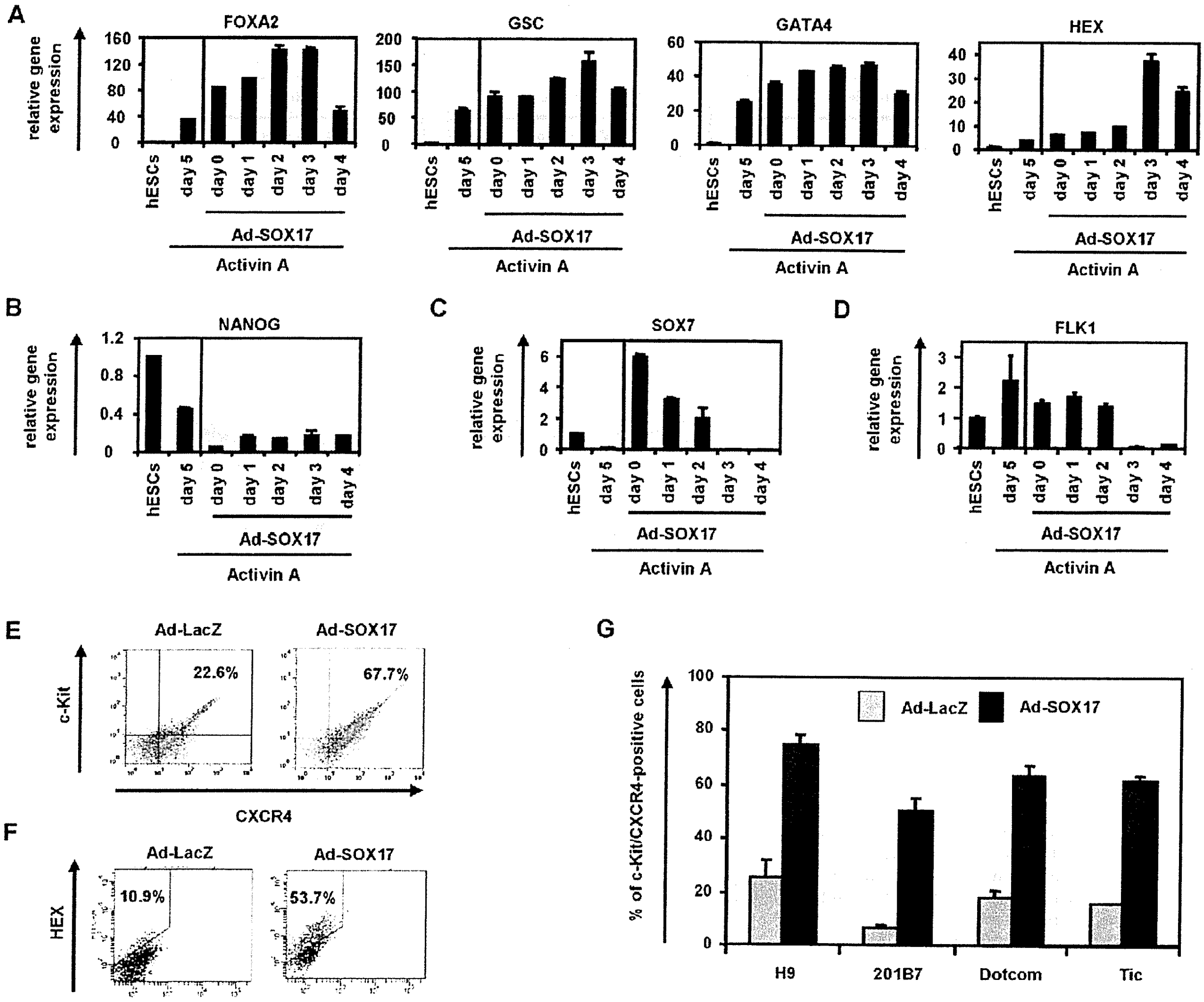

Figure 2. Efficient DE differentiation from human ESC- and iPSC-derived mesendoderm cells by SOX17 transduction. (A–D) Undifferentiated human ESCs (H9) and Activin A-induced human ESC-derived cells, which were cultured with the medium containing Activin A (100 ng/ml) for 0, 1, 2, 3, and 4 days, were transduced with 3,000 VP/cell of Ad-SOX17 for 1.5 h. Ad-SOX17-transduced cells were cultured with 100 ng/ml of Activin A, and the gene expression levels of (A) the DE markers (FOXA2, GSC, and GATA4) and anterior DE marker (HEX), (B) the pluripotent marker (NANOG), (C) the ExEn marker (SOX7), and (D) the mesoderm marker (FLK1) 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 human ESCs on day 0 were defined 1.0. (E, F) After human ESCs were cultured with 100 ng/ml of Activin A for 3 days, human ESC-derived mesendoderm cells were transduced with Ad-LacZ or Ad-SOX17 and cultured until day 5. Ad-LacZ- or Ad-SOX17-transduced DE cells were subjected to immunostaining with anti-c-Kit, anti-CXCR4 (E) and anti-HEX antibodies (F) and then analyzed by flow cytometry. (G) After Ad-LacZ or Ad-SOX17 transduction, the DE differentiation efficacies of the human ES cell line (H9) and three human iPS cell lines (201B7, Dotcom, and Tic) were compared at day 5 of differentiation. All data are represented as the means±SD (*n* = 3).
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examined hepatic gene and protein expression levels on day18 of differentiation. For this purpose, we used a human ES cell line (H9) and three human iPS cell lines (201B7, Dotcom, Tic). On day 18 of differentiation, the gene and protein expression analysis showed up-regulation of the hepatic markers albumin (ALB) [27], cytochrome P450 2D6 (CYP2D6), CYP3A4, and CYP7A1 [28] mRNA and ALB, CYP2D6, CYP3A4, CYP7A1, and cytokeratin (CK)18 proteins in both Ad-SOX17- and Ad-HEX-transduced cells transduced cells as compared with both Ad-LacZ- and Ad-HEX-transduced cells (Figures 4A and 4B). These results indicated that Ad-SOX17-transduced cells were more committed to the hepatic lineage than non-transduced cells.

Discussion

The directed differentiation from human ESCs and iPSCs is a useful model system for studying mammalian development as well as a powerful tool for regenerative medicine [29]. In the present study, we elucidated the bidirectional role of SOX17 on either ExEn or DE differentiation from human ESCs and iPSCs. We initially confirmed that initiation of SOX17 expression was consistent with the time period of PrE or mesendoderm cells formation (Figures S1 and S2). We speculated that stage-specific transient SOX17 transduction in PrE or mesendoderm could enhance ExEn or DE differentiation from human ESCs and iPSCs, respectively.

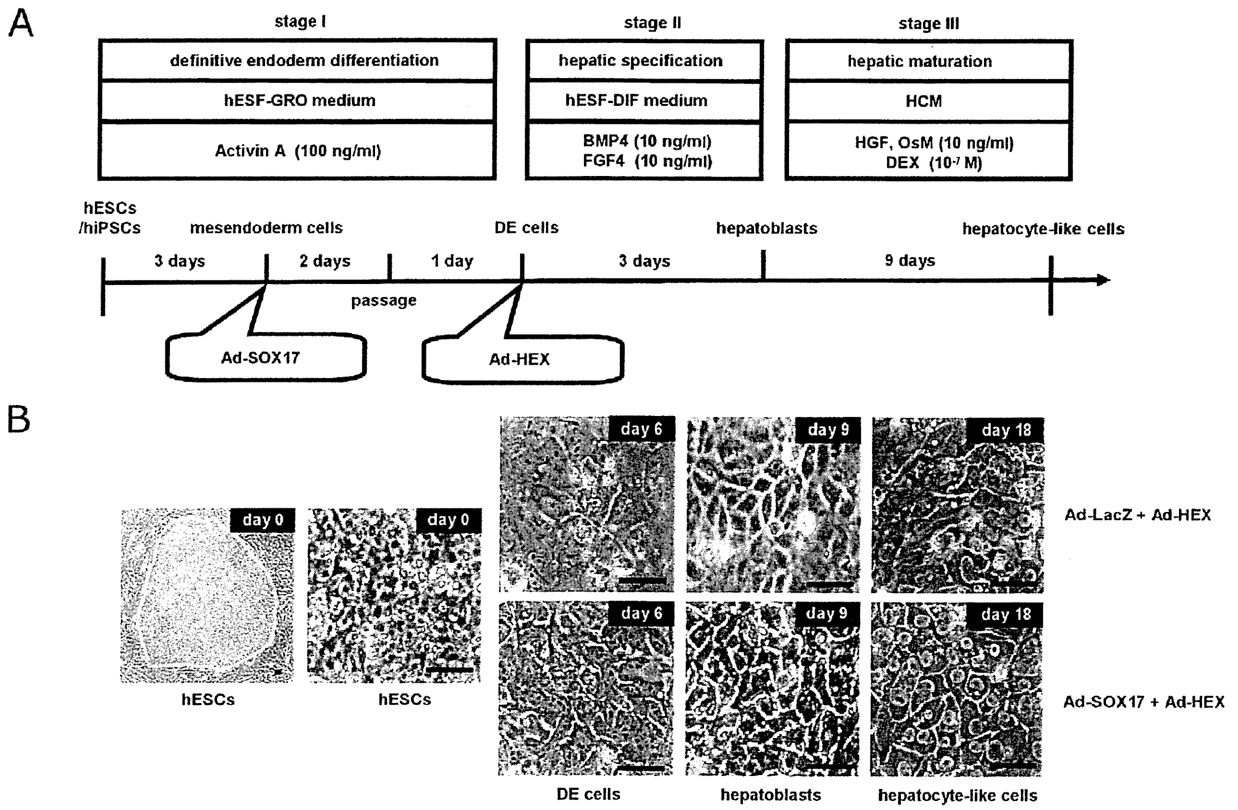

Figure 3. Hepatic Differentiation of Human ESC- and iPSC-Derived DE Cells Transduced with Ad-HEX. (A) The procedure for differentiation of human ESCs and iPSCs into hepatoblasts and hepatocyte-like cells is presented schematically. Both hESF-GRO and hESF-DIF medium were supplemented with 5 factors and 0.5 mg/ml fatty acid-free BSA, as described in the Materials and Methods section. (B) Sequential morphological changes (day 0–18) of human ESCs (H9) differentiated into hepatocyte-like cells via the DE cells and the hepatoblasts are shown. The scale bar represents 50 µm.
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SOX17 transduction at the pluripotent stage promoted random differentiation giving heterogeneous populations containing both ExEn and DE cells were obtained (Figures 2A–2C). Qu et al. reported that SOX17 promotes random differentiation of mouse ESCs into PrE cells and DE cells *in vitro* [30], which is in consistent with the present study. Previously, Niakan et al. and Seguin et al. respectively demonstrated that ESCs could promote either ExEn or DE differentiation by stable SOX17 expression, respectively [10,12]. Although these discrepancies might be attributable to differences in the species used in the experiments (i.e., human versus mice), SOX17 might have distinct functions according to the appropriate differentiation stage. To elucidate these discrepancies, we examined the stage-specific roles of SOX17 in the present study, and found that human ESCs and iPSCs could differentiate into either ExEn or DE cells when SOX17 was overexpressed at the PrE or mesendoderm stage, respectively, but not when it was overexpressed at the pluripotent stage (Figures 1 and 2). This is because endogenous SOX17 is strongly expressed in the PrE and primitive streak tissues but only slightly expressed in the inner cell mass, our system might adequately reflect the early embryogenesis [14,31].

In ExEn differentiation from human ESCs, stage-specific SOX17 overexpression in human ESC-derived PrE cells promoted efficient ExEn differentiation and repressed trophectoderm differentiation (Figures 1A and 1B), although SOX17 transduction at the pluripotent stage did not induce the efficient differentiation of ExEn cells. In our protocol, the stage-specific overexpression of SOX17 could elevate the efficacy of AFP-positive or SOX7-positive ExEn differentiation from human ESCs and iPSCs. The reason for the efficient ExEn differentiation by SOX17 transduction might be due to the fact that SOX17 lies downstream from GATA6 and directly regulates the expression of GATA4 and GATA6 [12]. Although it was previously been reported that Sox17 plays a substantial role in late-stage differentiation of ExEn cells *in vitro* [32], those reports utilized embryoid body formation, in which other types of cells, including endoderm, mesoderm, and ectoderm cells, might have influences on cellular differentiation. The present study showed the role of SOX17 in a homogeneous differentiation system by utilizing a mono-layer culture system.

In DE differentiation from human ESCs, we found that DE cells were efficiently differentiated from the human ESC-derived mesendoderm cells by stage-specific SOX17 overexpression (Figure 2). Therefore, we concluded that SOX17 plays a significant role in the differentiation of mesendoderm cells to DE cells. Although SOX17 overexpression before the formation of mesendoderm cells did not affect mesoderm differentiation, SOX17 transduction at the mesendoderm stage selectively promoted DE differentiation and repressed mesoderm differentiation (Figures 2A and 2D). These results show that SOX17 plays a crucial role in decision of DE differentiation from mesendoderm cells, as previous studies suggested [33,34]. Interestingly, SOX17 transduction at the pluripotent stage promoted not only DE

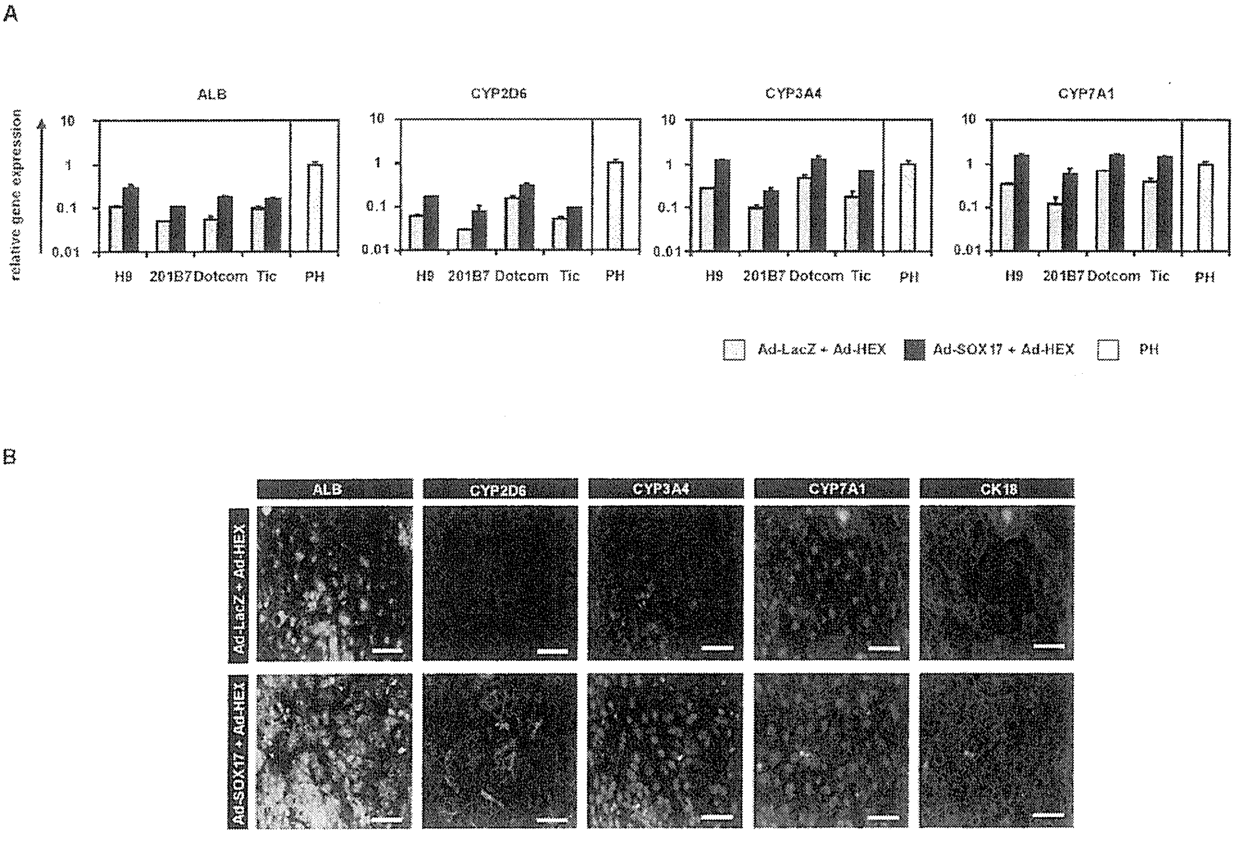

Figure 4. Characterization of hepatocyte-like cells from human ESC- and iPSC-derived DE cells. (A) The Ad-LacZ-transduced cells and Ad-SOX17-transduced cells were transduced with 3,000 VP/cell of Ad-HEX for 1.5 h on day 6. On day 18 of differentiation, the levels of expression of the hepatocyte markers (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-TFI [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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