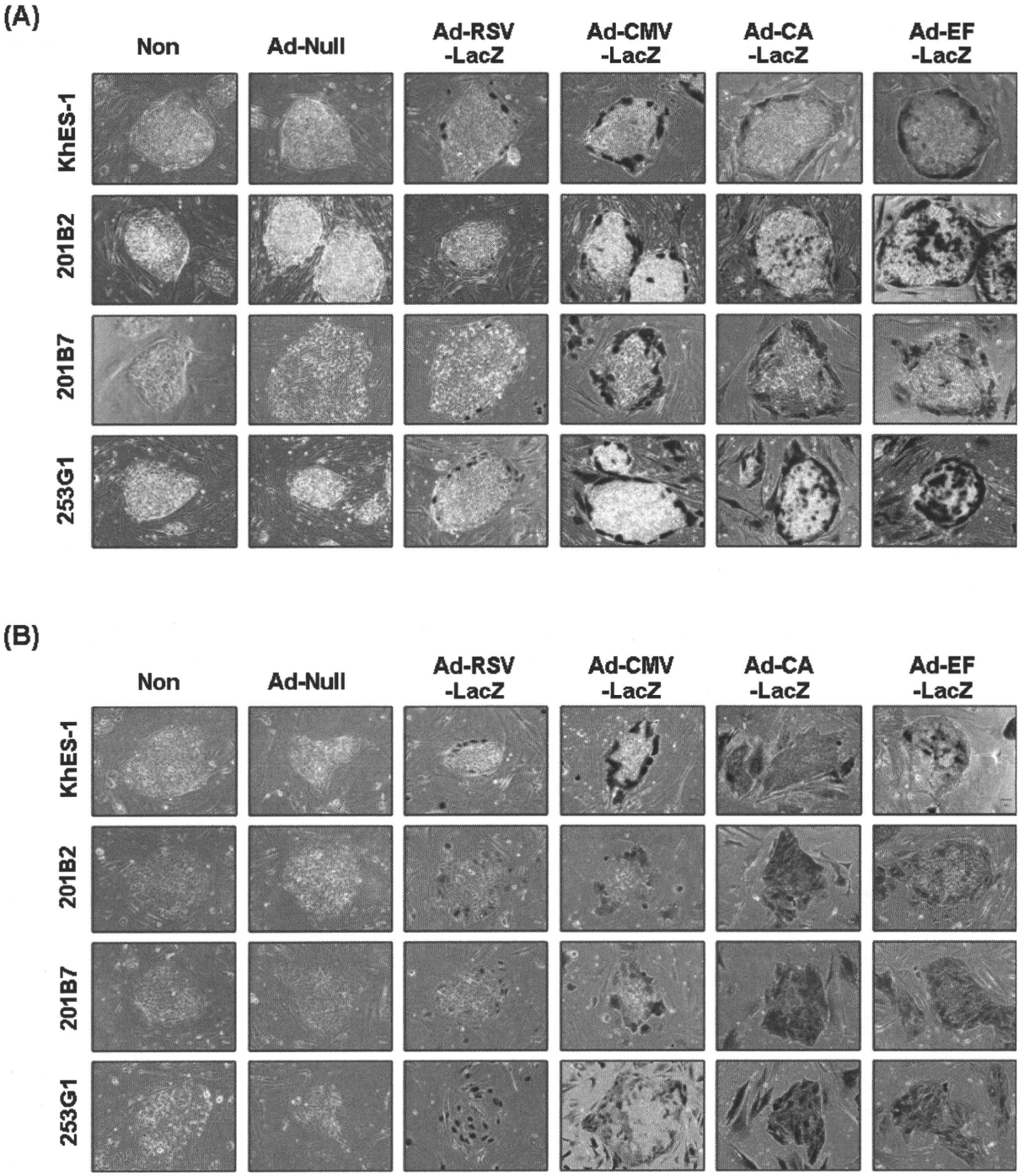

Figure 10 LacZ expression in human ES and iPS cells transduced with Ad vectors containing various types of promoters in the absence or presence of ROCK inhibitor, Y-27632.

Human ES cells (KhES-1) and iPS cells (201B2, 201B7, and 253G1) were passaged into culture plates in the absence (A) or presence (B) of ROCK inhibitor, Y-27632. In the case of the absence of Y-27632, the cells were passaged as small clumps to prevent cell death. On the following day, they were transduced with LacZ-expressing Ad vectors containing various types of promoters at 3,000 VP/cell for 1.5 hr. Forty-eight hours later, X-gal staining was performed. Abbreviations: Ad, adenovirus; RSV, rous sarcoma virus; CMV, cytomegalovirus; CA, CMV enhancer/β-actin promoter with β-actin intron; EF, elongation factor.

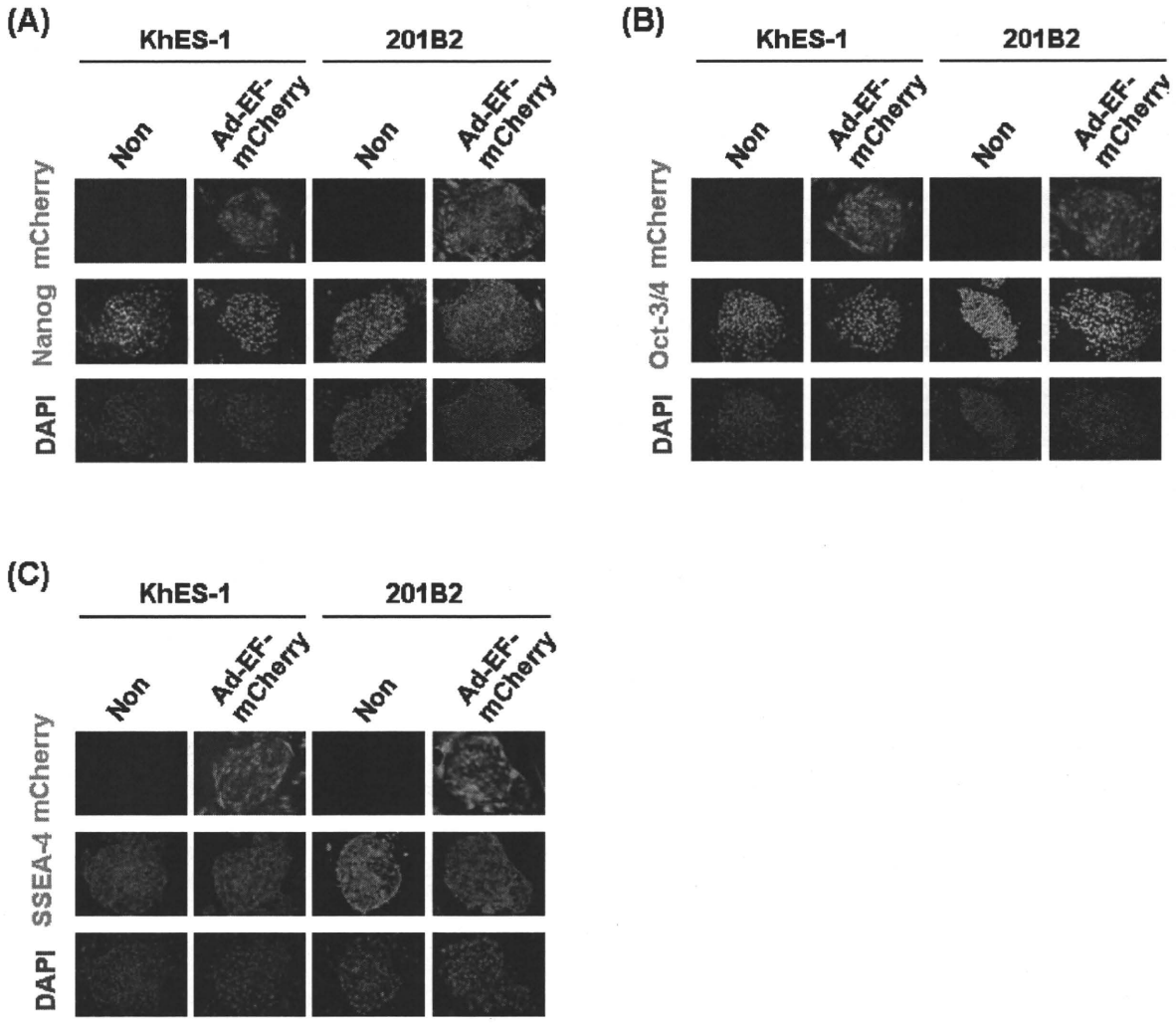

Figure 11 The expression of undifferentiated markers in human ES and iPS cells after the transduction with Ad-EF-mCherry.

Human ES cells (KhES-1) and iPS cells (201B2) were plated into culture plates using Y-27632. On the following day, they were transduced with Ad-EF-mCherry at 3,000 VP/cell for 1.5 hr. Two days later, the expression of Nanog (A), Oct-3/4 (B), and SSEA-4 (C) was detected by immunostaining. Similar results were obtained in the other human iPS cell lines. Abbreviations: SSEA, stage specific embryonic antigen; DAPI, 4',6-diamino-2-phenylindole.

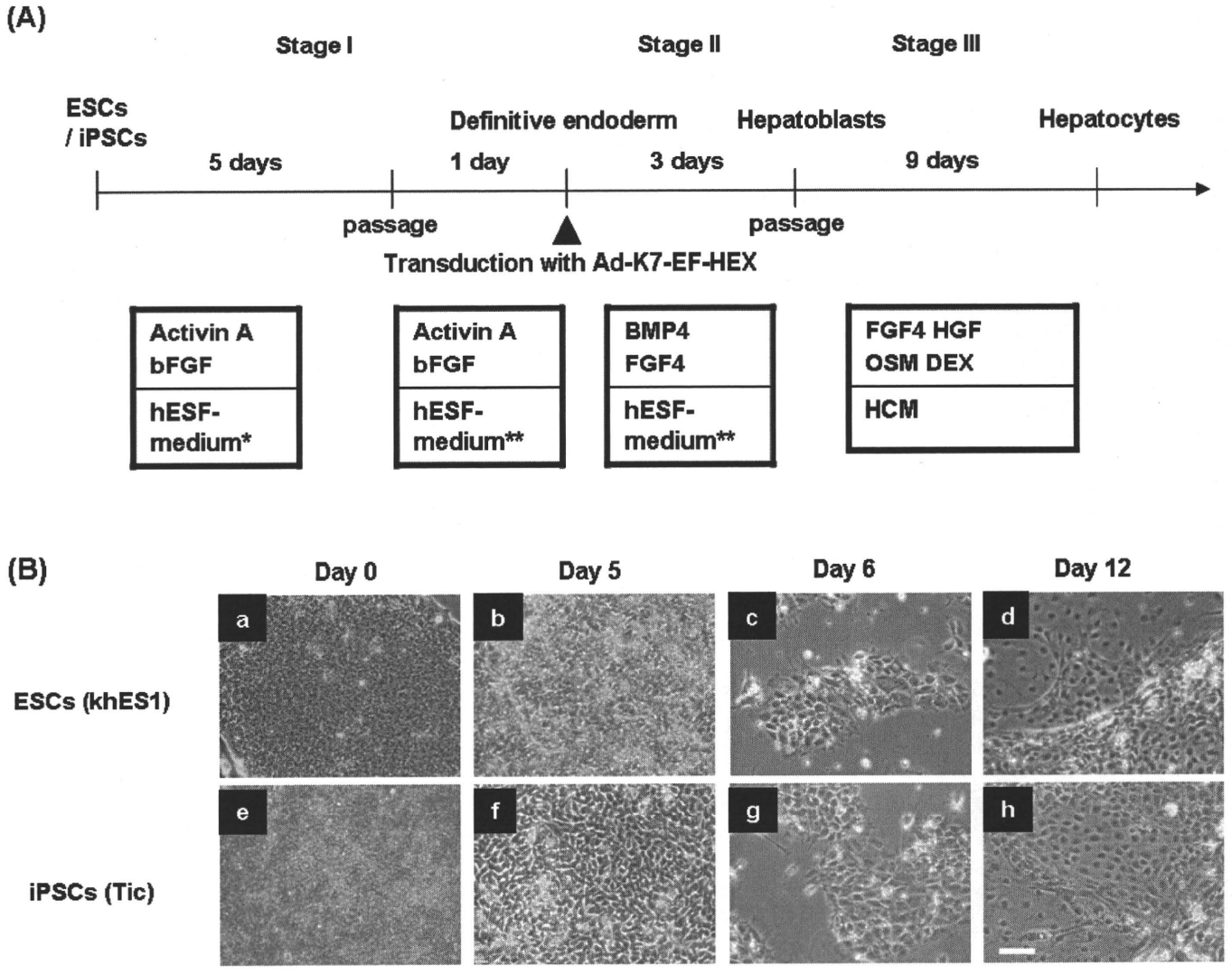

Figure 12 A strategy of differentiation of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) into hepatoblasts and hepatocytes.

(A) Schematic representation illustrating the procedure for differentiation of human ESCs (khES1) and iPSCs (Tic) into hepatocytes. (B) Sequential morphological changes (day 0-12) from human ESCs (khES1) (a-d) and iPSCs (Tic) (e-h) to hepatoblasts via the definitive endoderm. Scale bar represents 50 μ m. Abbreviations: bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein 4; FGF4, fibroblast growth factor 4; HGF, hepatocyte growth factor; OSM, Oncostatin M; DEX. dexamethasone; *, hESF-GRO medium that was supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ M 2-mercaptoethanol, 10 μ M ethanolamine, 10 μ M sodium selenite, 0.5 mg/ml fatty acid free BSA; **, hESF-DIF medium that was supplemented with 10 μ g/ml insulin, 5 μ g/ml apotransferrin, 10 μ M 2-mercaptoethanol, 10 μ M ethanolamine, 10 μ M sodium selenite, 0.5 mg/ml BSA; HCM, hepatocytes culture medium

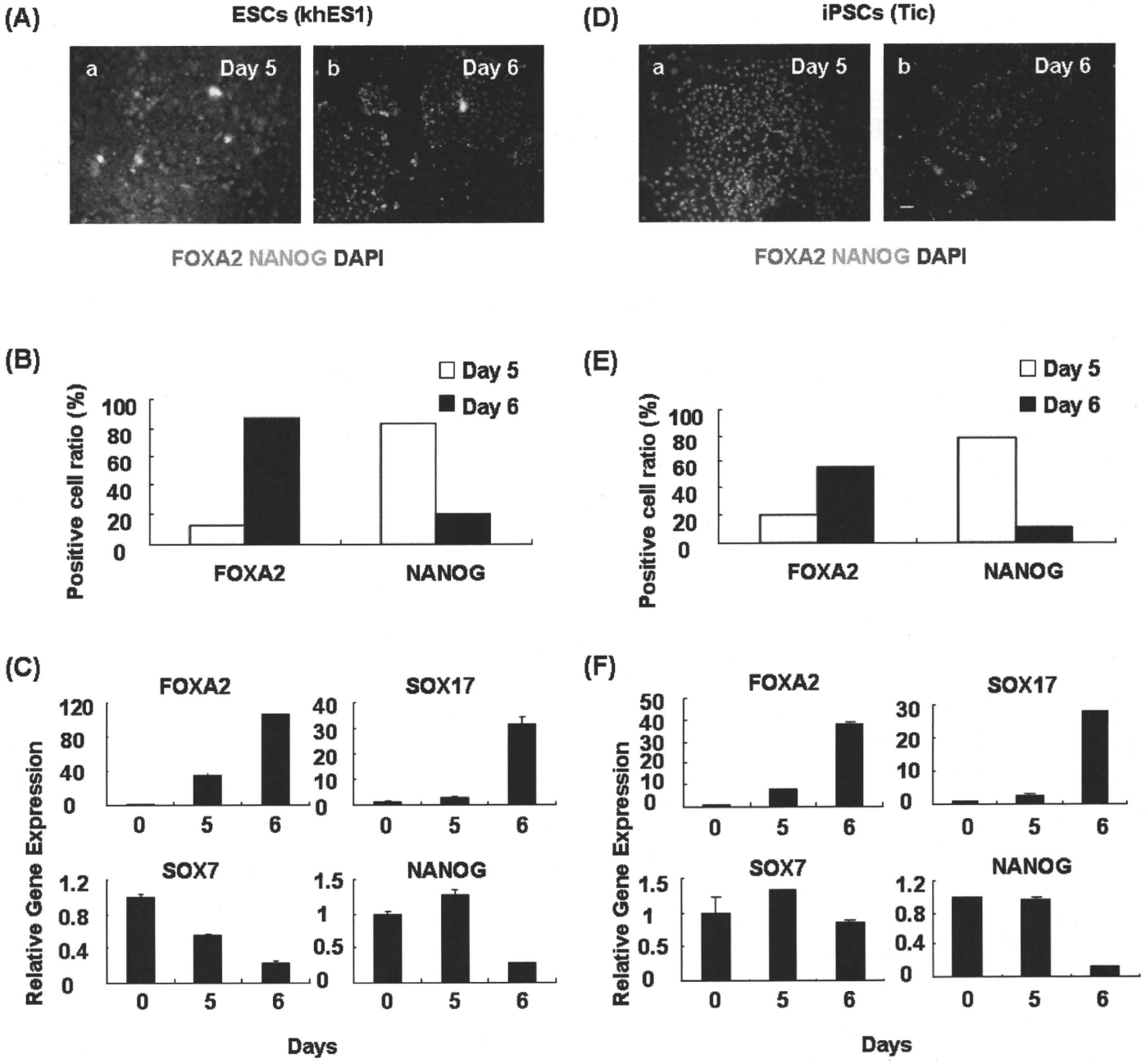

Figure 13 Characterization of the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms.

(A, D) The immunofluorescent staining of the human ESC (khES1)- and iPSC (Tic)-derived differentiated cells before (a; day 5) and after passaging (b; day 6). The cells were immunostained with antibodies against FOXA2 and NANOG. Nuclei were stained with DAPI. (B, E) Semi-quantitative analysis of the immunofluorescent staining in (A, D). Data are presented as the mean of immunopositive cells counted in 8 independent fields. (C, F) Real-time RT-PCR analysis of the level of definitive endoderm (FOXA2 and SOX17), pluripotent (NANOG), and extraembryonic endoderm (SOX7) gene expression at day 5 and 6. At day 5, the cells were passaged. Therefore, the data at day 5 and 6 show the levels of gene expression before (at day 5) or after the passage (at day 6). Data are presented as the mean ± SD from triplicate experiments. The graphs represent the relative gene expression level when the level of undifferentiated cells at day 0 was taken as 1. The scale bar represents 50 μm.

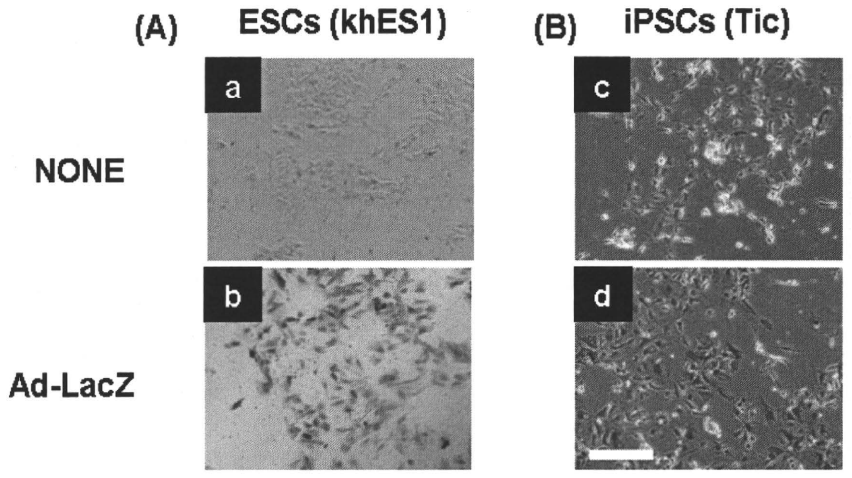

Figure 14 Efficient transgene expression in the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms by using a fiber-modified Ad vector containing the EF-1 α promoter.

Human ESC (khES1)-derived (A (a and b)) and iPSC (Tic)-derived (B (c and d)) definitive endoderms were transduced with 3,000 VP/cell Ad-LacZ for 1.5 h. The next day after transduction, X-gal staining was performed as described in the Materials and Methods. Similar results were obtained in two independent experiments. The scale bar represents 50 μm. Abbreviations: NONE, non-transduced cells; LacZ, Ad-K7-EF-LacZ-transduced cells.

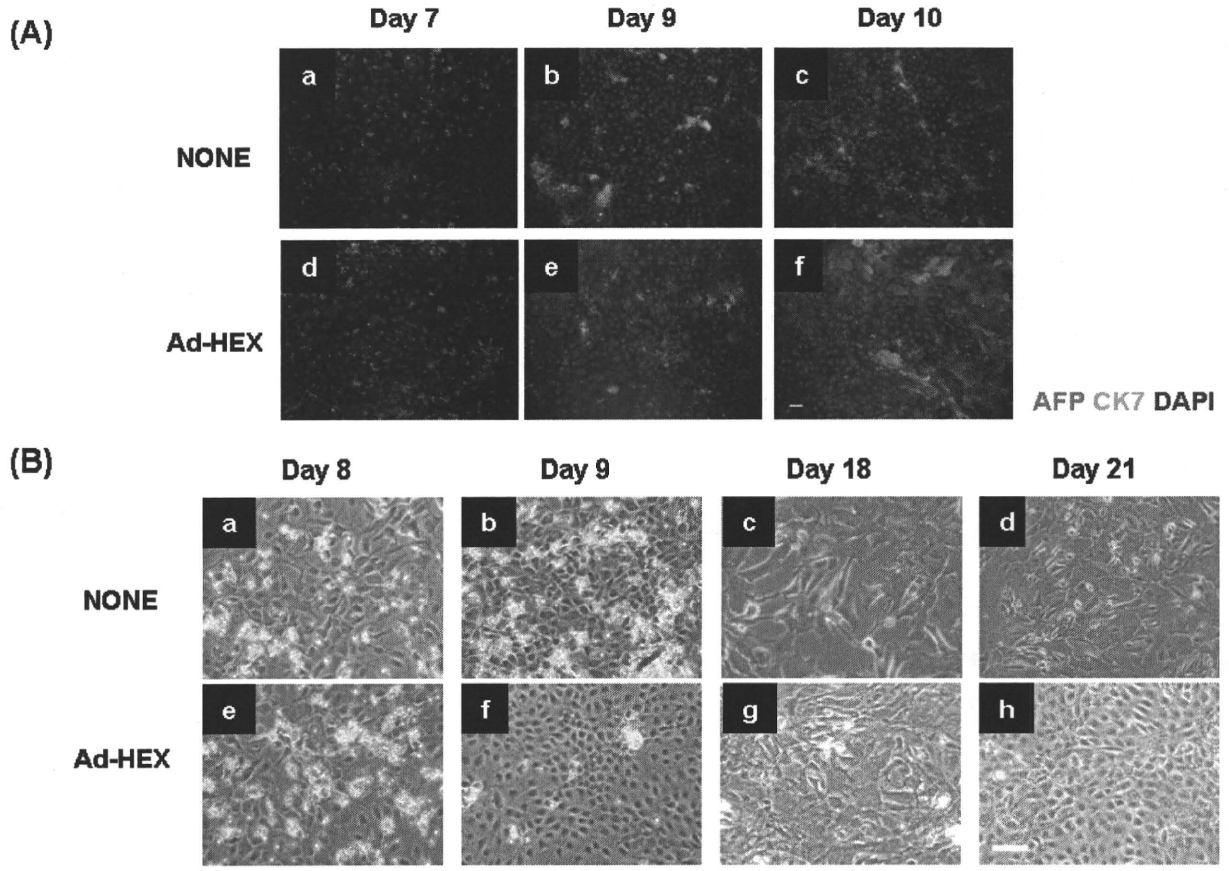

Figure 15 Progression of differentiation of the definitive endoderm to hepatoblasts by transduction of the HEX gene.

(A) Immunocytochemistry of AFP and CK7 expression from day 7 to day 10 in non-transduced cells (a-c) and Ad-HEX-transduced cells (d-f), all of which were induced from the human iPSC (Tic)-derived definitive endoderm. Nuclei were stained with DAPI. (B) Sequential morphological changes from day 8 to day 21 in non-transduced cells (a-d) and Ad-K7-EF-HEX-transduced cells (e-h), all of which were induced from the human iPSC (Tic)-derived definitive endoderm. The Scale bar represents 50 μm. Abbreviations: AFP, α-fetoprotein; CK7, cytokeratin 7; NONE, non-transduced cells; HEX, Ad-K7-EF-HEX-transduced cells.

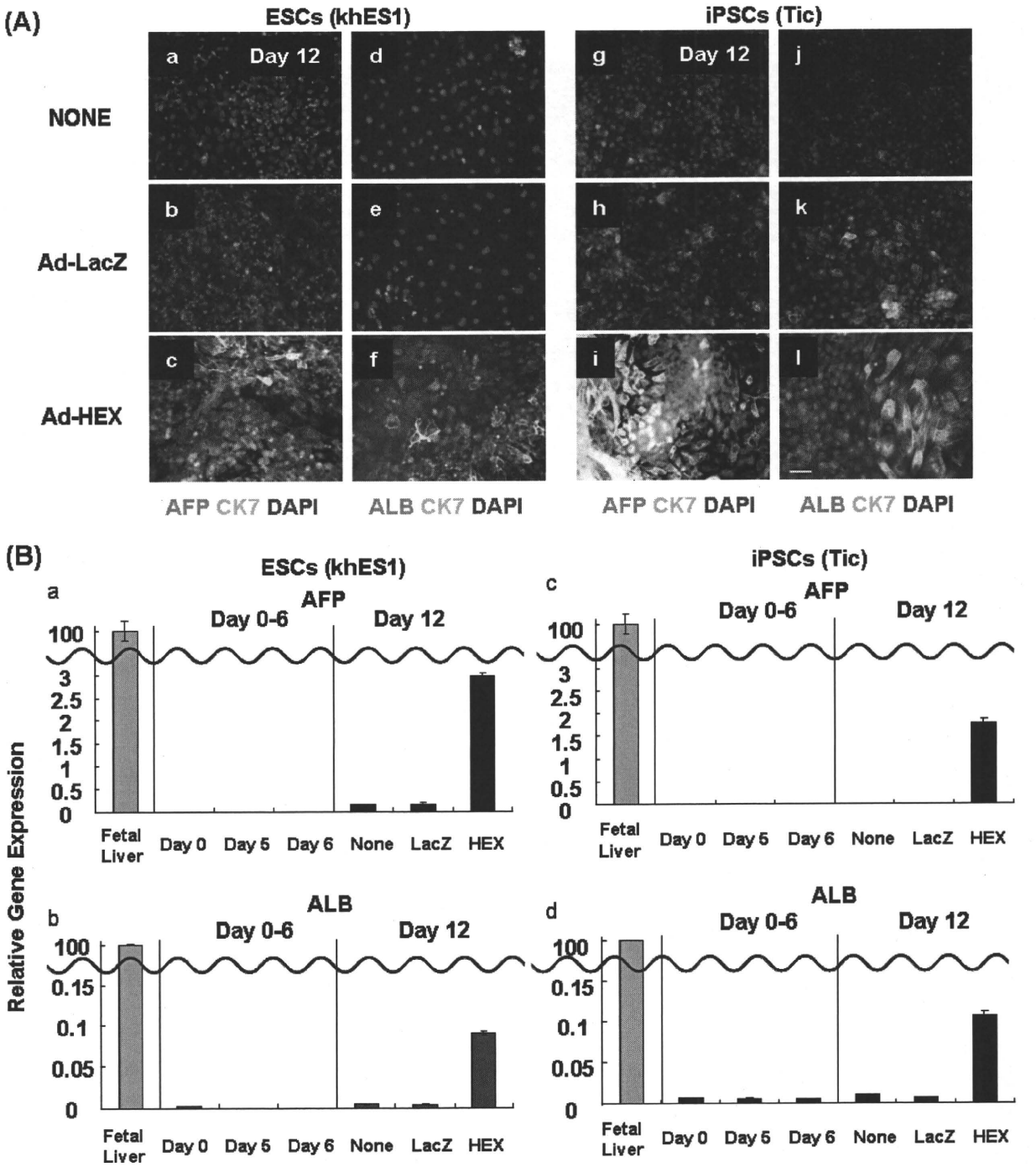

Figure 16 Efficient hepatoblast differentiation from the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms by transduction of the HEX gene.

(A) Immunocytochemistry of AFP, ALB and CK7 expression in non-transduced cells (a, d, g and j), Ad-K7-EF-LacZ-transduced cells (b, e, h and k), and Ad-K7-EF-HEX-transduced cells (c, f, i and l) at day 12, all of which were induced from the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms. Nuclei were stained with DAPI. Scale bar represents 50 μm. (B) Real-time RT-PCR analysis of the level of AFP (a and c) and ALB (b and d) expression in non-transduced cells, Ad-K7-EF-LacZ-transduced cells, and Ad-K7-EF-HEX-transduced cells, all of which were induced from the human ESC (khES1)- and iPSC (Tic)-derived definitive endoderms (day 0, 5, 6 and 12). The cells were transduced with Ad-K7-EF-LacZ or Ad-K7-EF-HEX at day 6 as described in Figure 3A. The data at day 6 was obtained before the transduction with Ad-K7-EF-HEX. The graphs represent the relative gene expression levels when the level in the fetal liver was taken as 100. Abbreviations: NONE, non-transduced cells; LacZ, Ad-K7-EF-LacZ-transduced cells; and HEX, Ad-K7-EF-HEX-transduced cells; AFP, α-fetoprotein; ALB, albumin; CK7, cytokeratin 7.

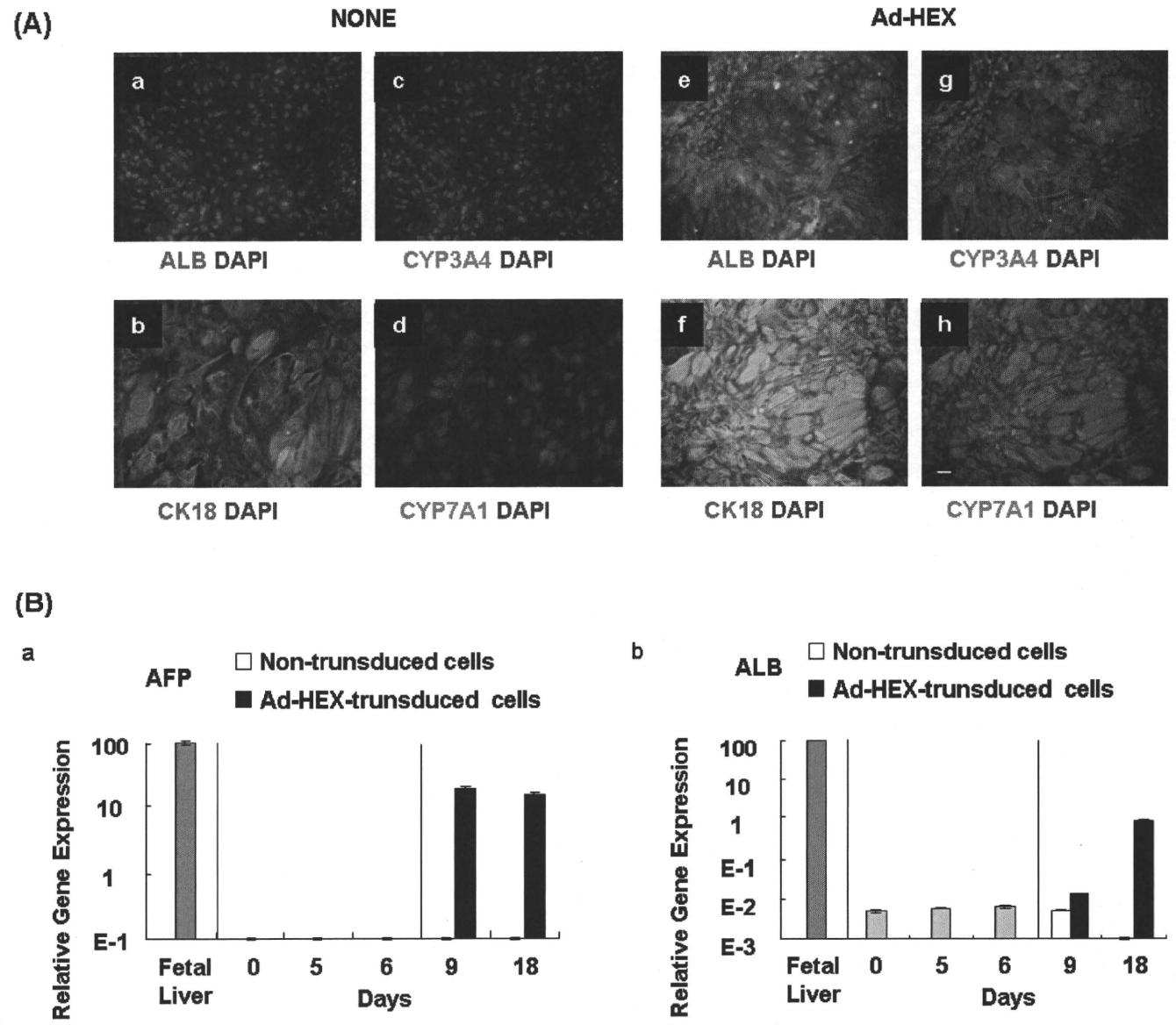

Figure 17 Efficient differentiation of Ad-HEX-transduced hepatoblasts into hepatocytes.

(A) Immunocytochemistry of ALB, CYP3A4, CYP7A1 and CK18 expression in non-transduced cells (a-d) and Ad-K7-EF-HEX-transduced cells (e-h), all of which were induced from the human iPSC (Tic)-derived definitive endoderm. Nuclei were stained with DAPI. The scale bar represents 50 μm. (B) Real-time RT-PCR analysis of AFP (a) and ALB (b) expression in non-transduced cells and Ad-K7-EF-HEX-transduced cells, both of which were induced from the human iPSC (Tic)-derived definitive endoderm (day 0, 5, 6 and 12). The cells were transduced with Ad-K7-EF-HEX at day 6 as described in Figure 3A. The data at day 6 were obtained before the transduction with Ad-K7-EF-HEX. The graphs represent the relative gene expression level when the level in the fetal liver was taken as 100. Abbreviations: NONE, non-transduced cells; HEX, Ad-K7-EF-HEX-transduced cells; AFP, α-fetoprotein; ALB, albumin, CK18, cytokeratin 18.

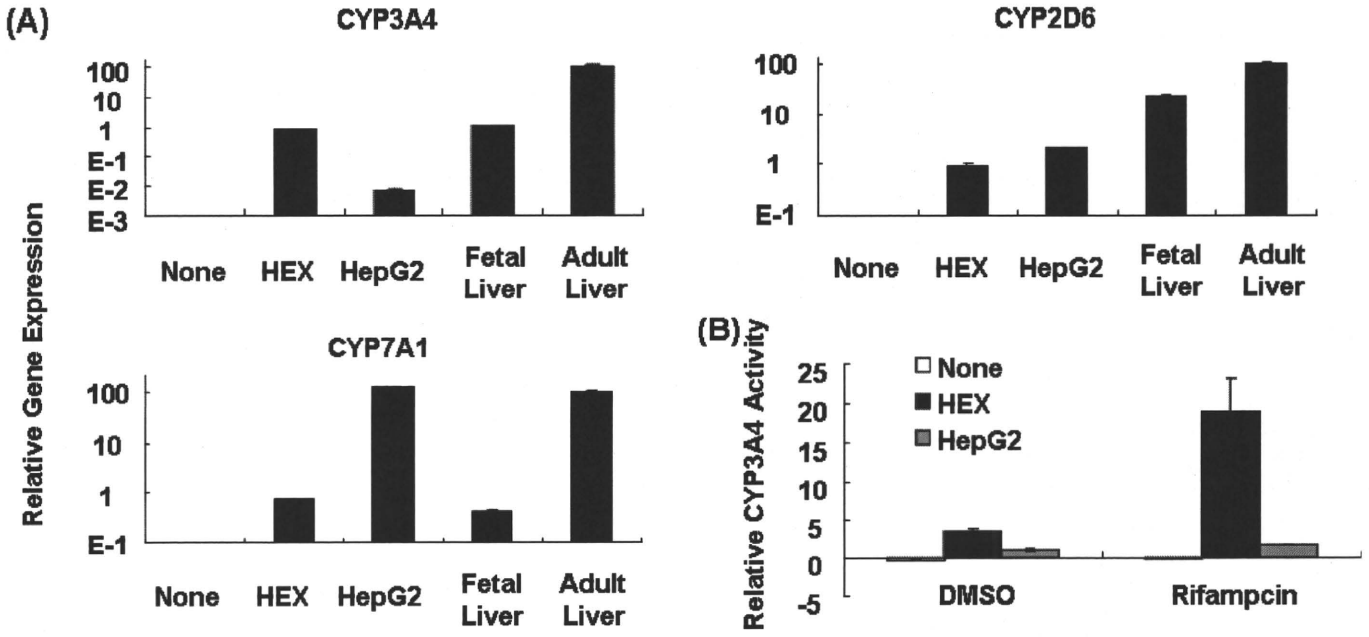

Figure 18 Cytochrome P450 isozymes in human iPSC (Tic)-derived hepatocytes.

(A) Real-time RT-PCR analysis of CYP3A4, CYP7A1 and CYP2D6 expression in iPSC (Tic)-derived non-transduced cells, Ad-HEX-transduced cells, and fetal and adult liver tissues. (B) Induction of CYP3A4 by rifampicin in human iPSC (Tic)-derived non-transduced cells, Ad-K7-EF-HEX-transduced cells, and the HepG2 cell line. Data are presented as the mean ± SD from triplicate experiments. The graphs represent the relative gene expression level when the level in the adult liver was taken as 100. Abbreviations: AFP, α-fetoprotein; ALB, albumin; NONE, non-transduced cells; HEX, Ad-K7-EF-HEX-transduced cells; DMSO, dimethyl sulfoxide.

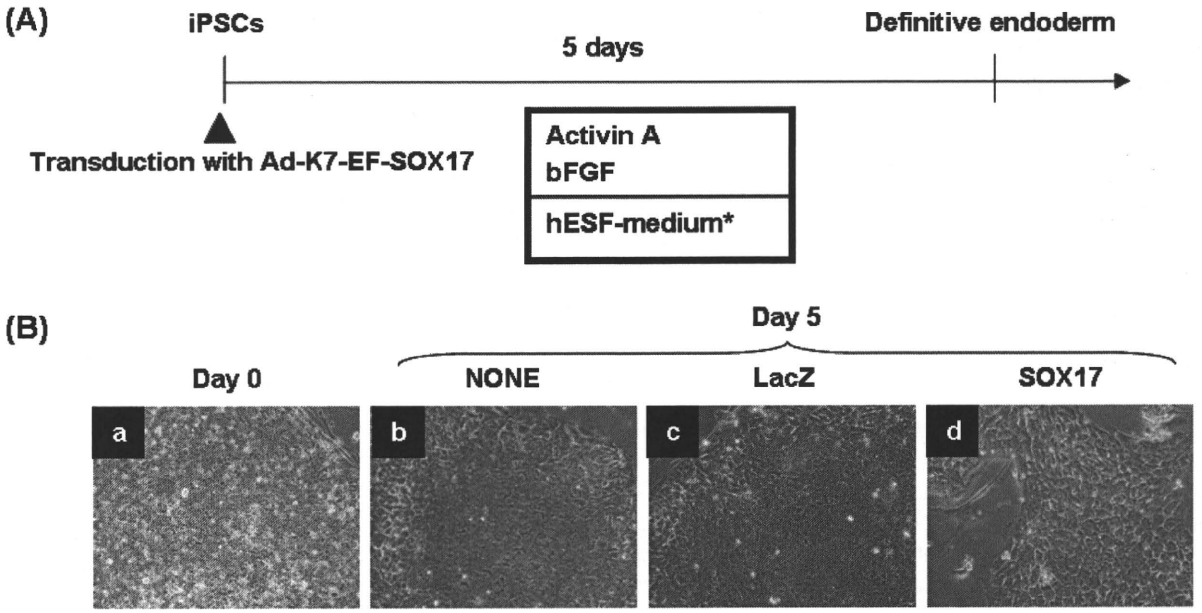

Figure 19 A strategy of differentiation of human induced pluripotent stem cells (iPSCs) to definitive endoderms.

(A) Schematic representation illustrating the procedure for differentiation of human iPSCs (201B7) to definitive endoderms. (B) Morphological changes (day 0-5) from human iPSCs (201B7) (a) to definitive endoderms (b-d). Abbreviations: bFGF, basic fibroblast growth factor; *, hESF-GRO medium that was supplemented with 10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, 0.5 mg/ml fatty acid free BSA. Abbreviations: NONE, non-transduced cells; LacZ, Ad-K7-EF-LacZ-transduced cells; and HEX, Ad-K7-EF-SOX17-transduced cells.

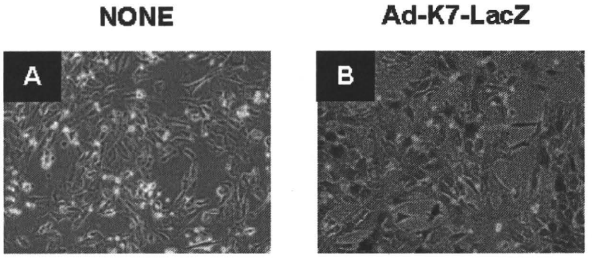

Figure 20 Efficient transgene expression in the human iPSCs (201B7) by using a fiber-modified Ad vector containing the EF-1 α promoter.

Human iPSCs (201B7) were transduced with 3,000 VP/cell Ad-LacZ for 1.5 h. The next day after transduction, X-gal staining was performed as described in the Materials and Methods. Abbreviations: NONE, non-transduced cells; LacZ, Ad-K7-EF-LacZ-transduced cells.

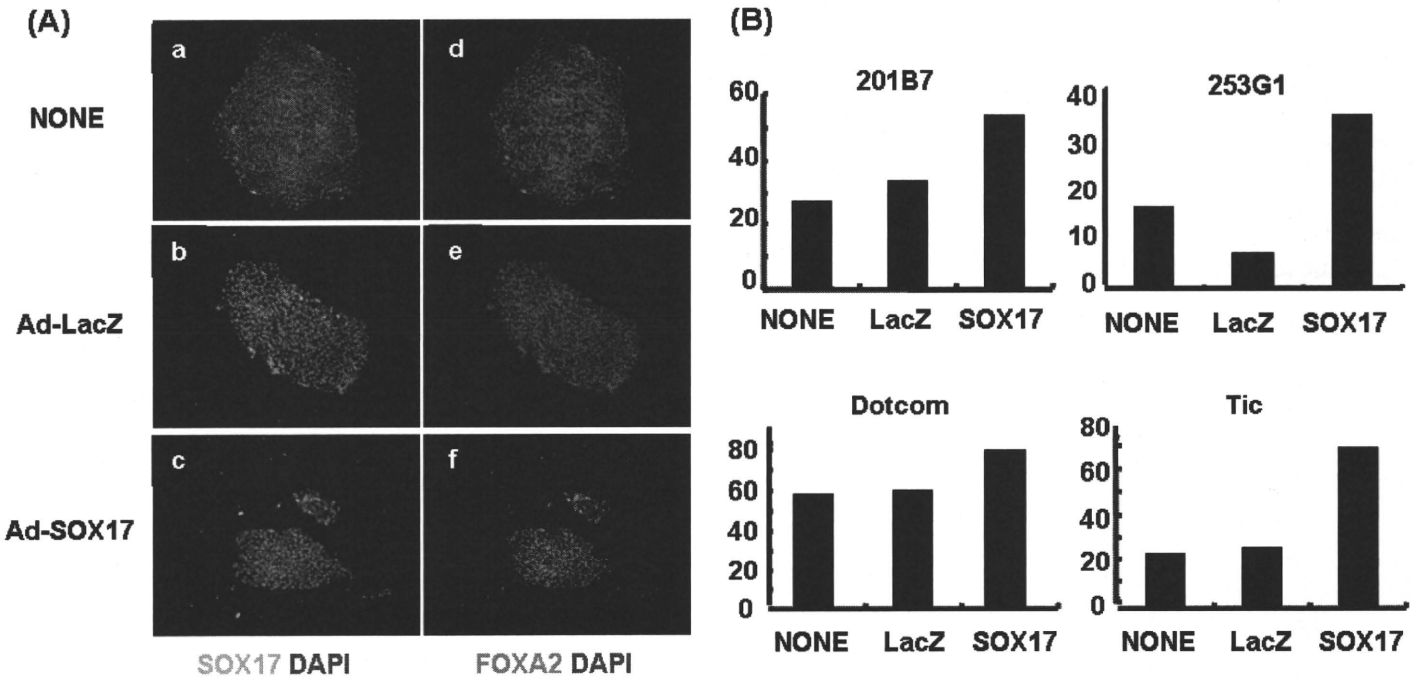

Figure 21 Efficient generation of definitive endoderms from human iPSC lines by transient overexpression of SOX17.

(A) The immunofluorescent staining of the human iPSC (201B7) -derived differentiated cells at day 5. The cells were immunostained with antibodies against SOX17 and FOXA2. Nuclei were stained with DAPI. (B) Semi-quantitative analysis of the immunofluorescent staining in iPSC (201B7, 253G1, Dotcom and Tic) -derived differentiated cells at day 5. Data are presented as the mean of immunopositive cells counted in 8 independent fields. Abbreviations: NONE, non-transduced cells; LacZ, Ad-K7-EF-LacZ-transduced cells; and SOX17, Ad-K7-EF-SOX17-transduced cells.

Table 2 List of Taqman gene expression assays and primers

Genes	Assay ID
NANOG	Hs02387400_g1
FOXA2	Hs00232764_m1
SOX17	Hs00751752_s1
SOX7	Hs00846731_s1
HEX	Hs00242160_m1
AFP	Hs01040607_m1
ALB	Hs00910225_m1
CYP3A4	Hs00430021_m1
CYP7A1	Hs00167982_m1
CYP2D6	Hs02576168_g1

Table 3 List of antibodies used

Primary antibodies

Antigen	Type	Company	fixing condition	blocking condition
OCT3/4	MOUSE	Santa Cruz	4% PFA	2% BSA / PBS
NANOG	RABBIT	ReproCELL	4% PFA	2% BSA / PBS
SSEA-4	MOUSE	Upstat	4% PFA	2% BSA / PBS
FOXA2	GOAT	R and D Systems	4% PFA	2% BSA / PBS
SOX17	MOUSE	R and D Systems	4% PFA	10% FCS/ 2% BSA / PBS
AFP	RABBIT	DAKO	4% PFA	2% BSA / PBS
ALB	RABBIT	SIGMA	Methanol	2% BSA / PBS
CK7	MOUSE	Invitrogen	4% PFA / Methanol	2% BSA / PBS
CK18	MOUSE	Invitrogen	Methanol	2% BSA / PBS
CYP3A4	GOAT	Santa Cruz	Methanol	2% BSA / PBS
CYP7A1	GOAT	Santa Cruz	Methanol	2% BSA / PBS

Secondary antibodies

Type	Company	Dilution
Alexa Fluor 488 anti-mouse	Molecular Probes	1/1000
Alexa Fluor 488 anti-rabbit	Molecular Probes	1/1000
Alexa Fluor 594 anti-rabbit	Molecular Probes	1/1000
Alexa Fluor 594 anti-goat	Molecular Probes	1/1000

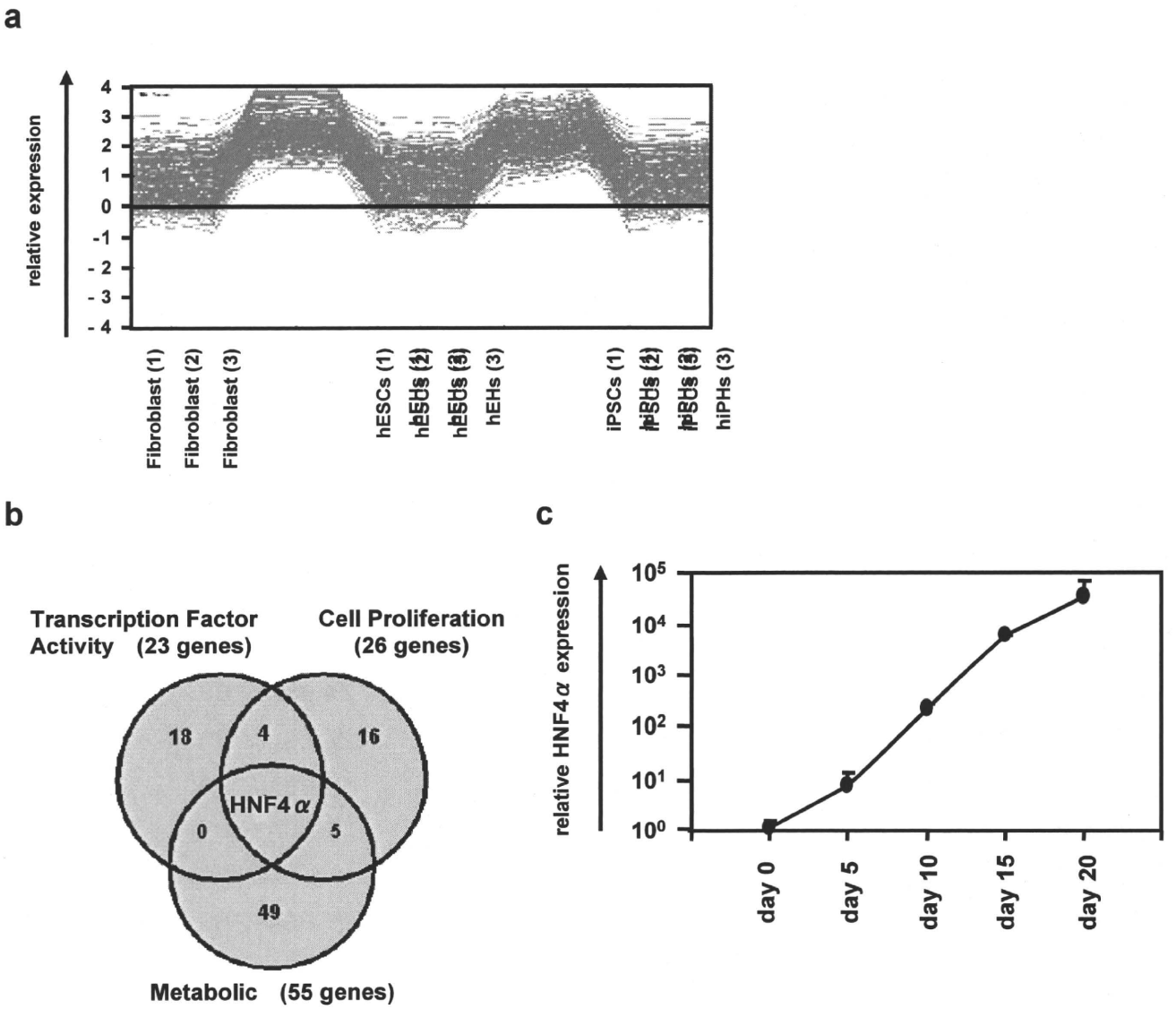

Fig. 22 Genome-wide screening of transcription factors involved in hepatic differentiation emphasizes the importance of the transcription factor *HNF4 α*.

Si-Tayeb et al. had provided a data set of gene array analysis, which compared undifferentiated human ESCs or iPSCs and human ESC- or iPSC-derived hepatocytes, in the NCBI Gene Expression Omnibus (GEO) repository (GEO accession: GSE14897). (**a**) According to the method of Significant Analysis of Microarray (SAM) method, 302 genes that were differentially upregulated in hepatic differentiation from human ESCs and iPSCs were picked up. (**b**) As we hypothesized that the hepatic transcriptional network, metabolic function, and regulating proliferation of cells are important for efficient generation of functional hepatocytes, the transcriptional factors and the genes related to cell proliferation and metabolism were extracted from these 302 genes according to the description in the Gene Ontology (GO). This selection emphasized that transcriptional factor *HNF4 α* is an important functional gene that fulfilled all these conditions. (**c**) Human ESCs (H9) were differentiated into hepatocytes according to the protocol described in **Fig. 6a** (without HNF4 α transduction). On day 0, 5, 10, 15, and 20 of differentiation, the gene expression level of *HNF4 α* was examined by real-time RT-PCR. All data are represented as means ± SD (n=3).

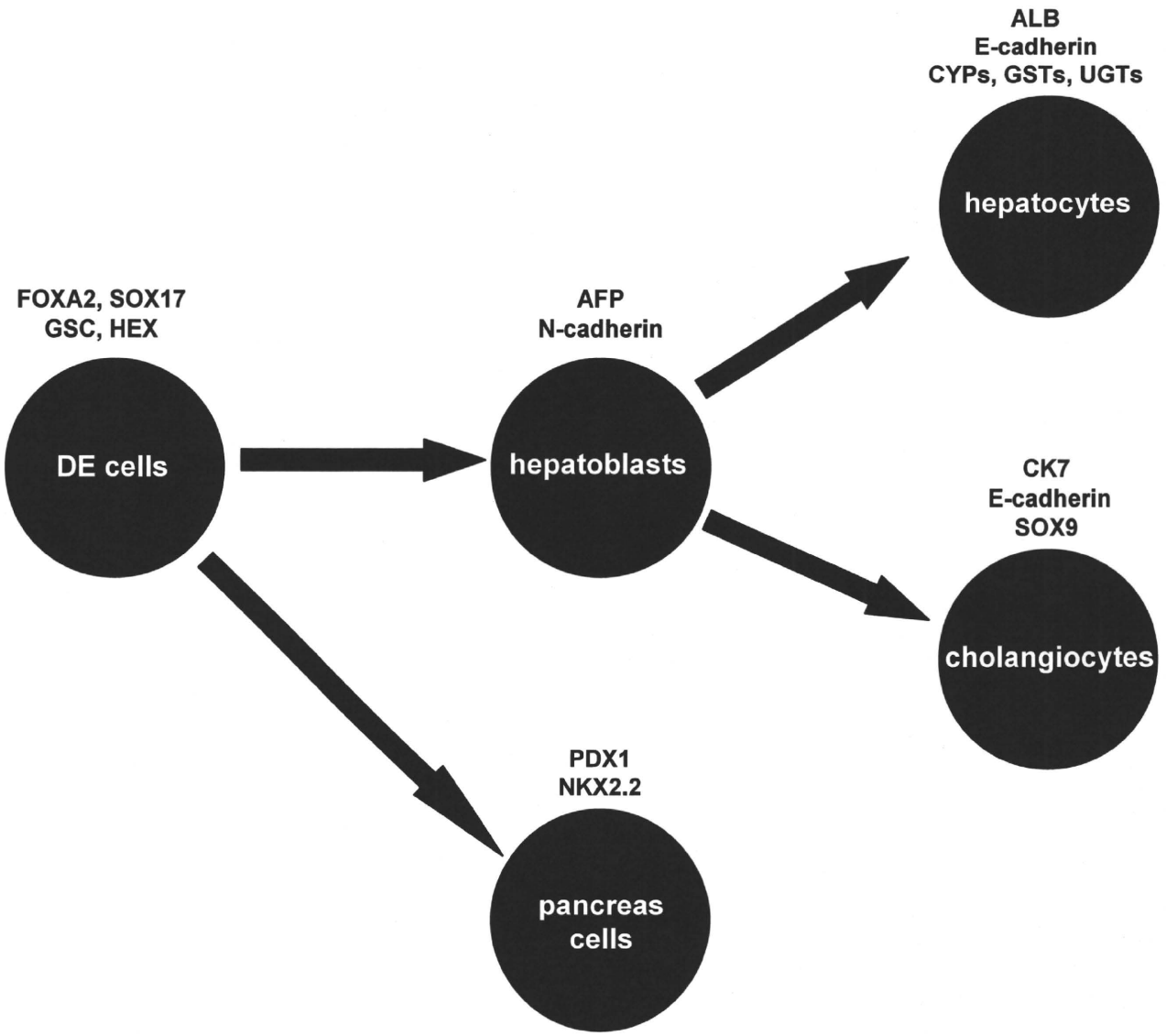

Fig. 23 Summary of specific markers for DE cells, hepatoblasts, hepatocytes, cholangiocytes, and pancreas cells.

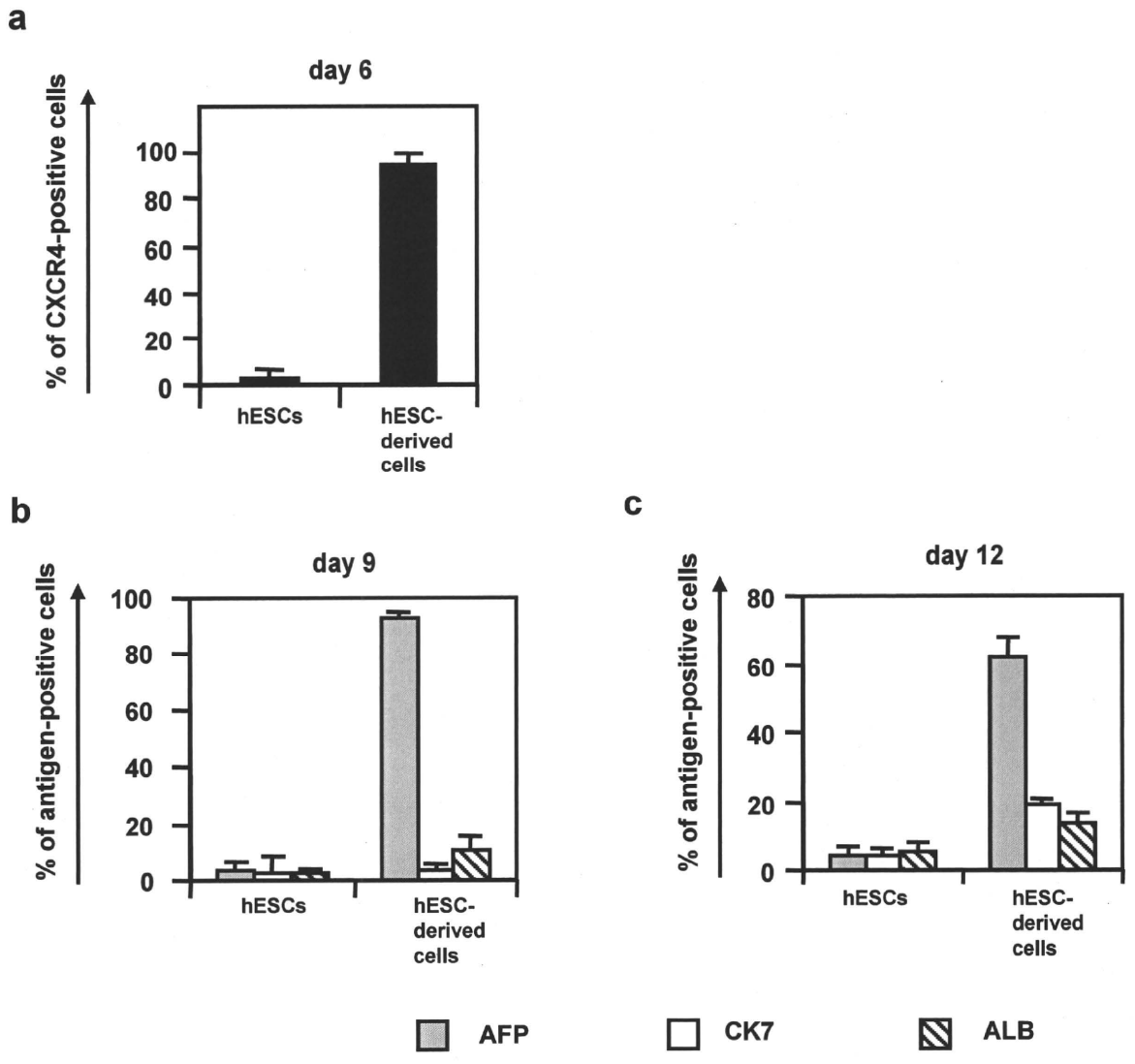

Fig. 24 The formation of DE cells, hepatoblasts, hepatocytes, and cholangiocytes from human ESCs. (**a**) Human ESCs (H9) were differentiated into DE cells according to the protocol described in **Fig. 6a** and subjected to immunostaining with anti-CXCR4 antibodies on day 6 of differentiation. On (**b**) day 9 or (**c**) day 12, human ESC-derived cells were subjected to immunostaining with anti-AFP, CK7, and ALB antibodies. The percentage of antigen-positive cells was measured by flow cytometry. The gray, white, or hatched bars represent the percentage of AFP-, CK7-, or ALB-positive cells, respectively. All data are represented as means ± SD (*n*=3).

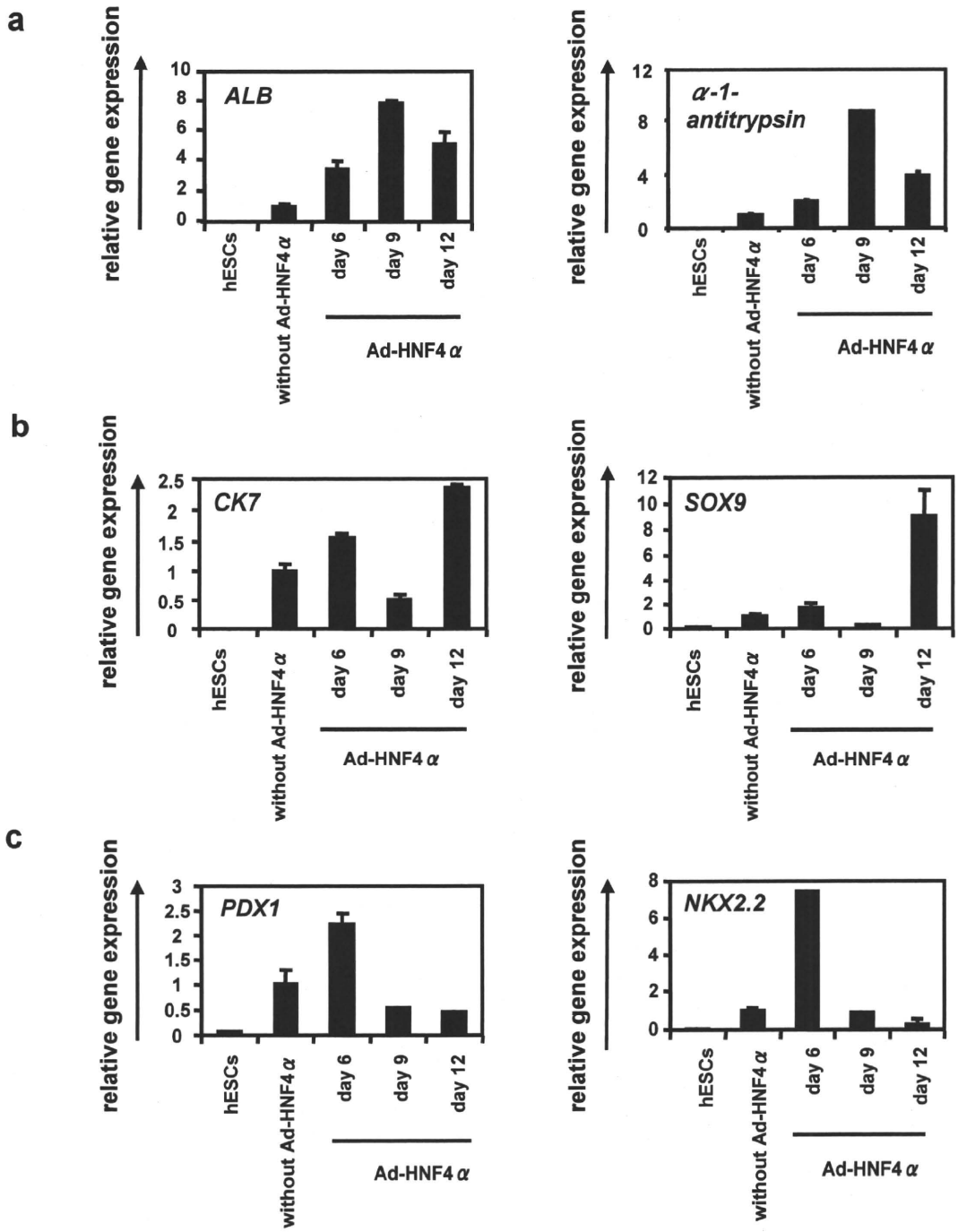

Fig. 25 Transduction of HNF4α into hepatoblasts promotes hepatic differentiation.
(**a-c**) The human ESC (H9)-derived cells, which were cultured for 6, 9, or 12 days according to the protocol described in Fig. 27a, were transduced with 3,000 vector particles (VP)/cell of Ad-HNF4α for 1.5 hr and cultured 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-HNF4α. On the y axis, the level of the cells without Ad-HNF4α transduction on day 20 was taken as 1.0. All data are represented as means ± SD (*n*=3).

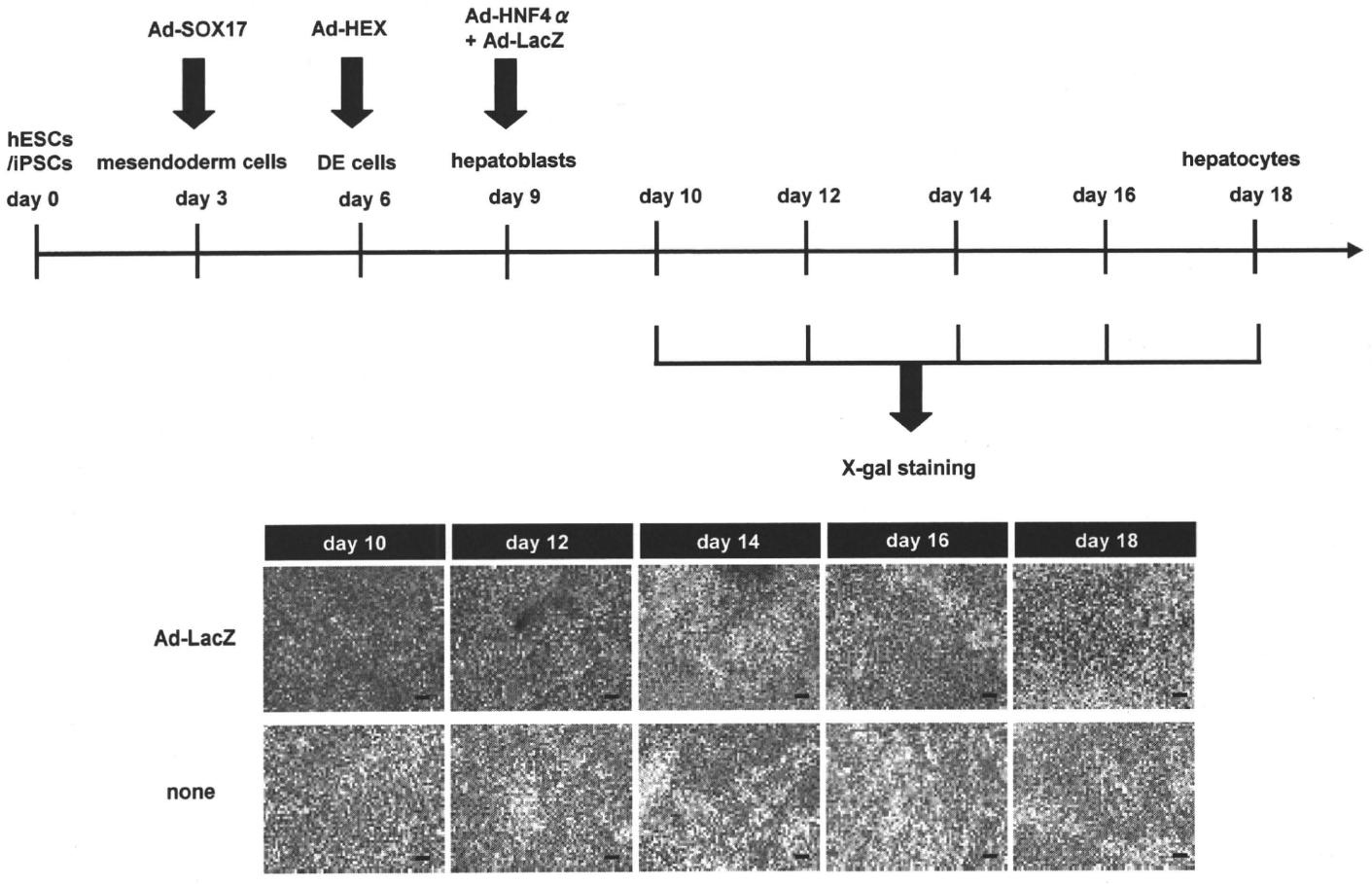

Fig. 26 Time course of LacZ expression in hepatoblasts transduced with Ad-LacZ.
Human ESCs were differentiated into hepatoblasts according to the protocol described in **Fig. 6a**, and then transduced with 3,000 VP/cell of Ad-LacZ for 1.5 hr. On days 10, 12, 14, 16, and 18, X-gal staining was performed. The scale bar represents 100 μm. Similar results were obtained in two independent experiments.

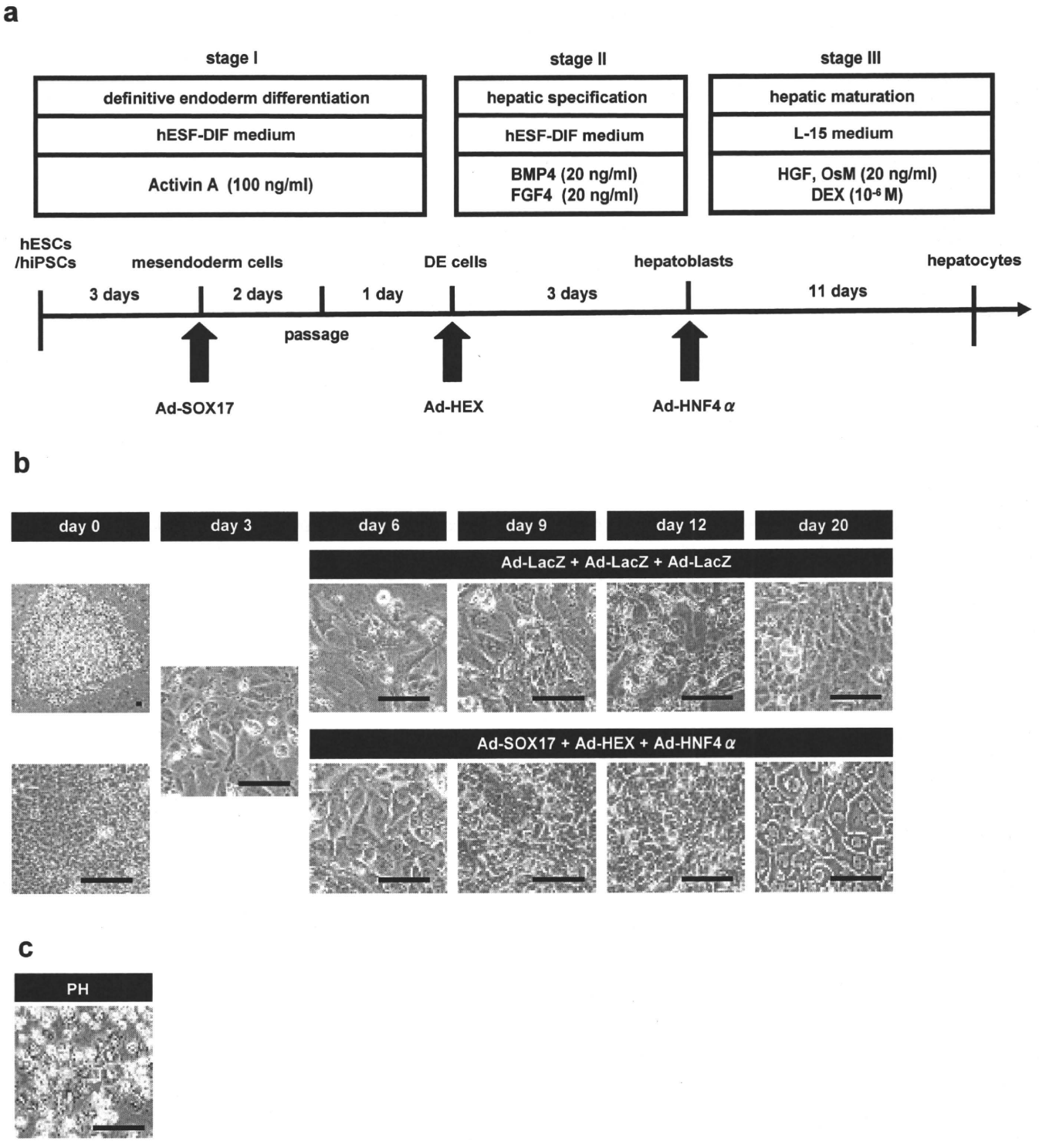

Fig. 27 Hepatic differentiation of human ESCs and iPSCs transduced with 3 factors.
(**a**) The procedure for differentiation of human ESCs and iPSCs into hepatocytes via DE cells and hepatoblasts is presented schematically. (**b**) Sequential morphological changes (day 0-20) of human ESCs (H9) differentiated 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. Scale bar represents 50 μm.

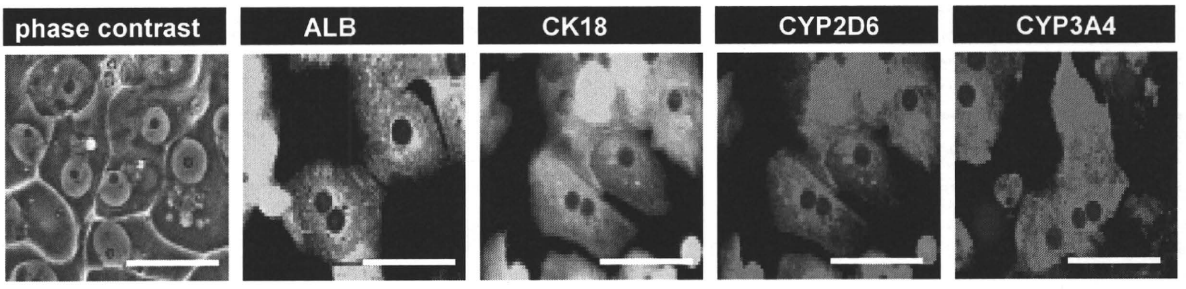

Fig. 28 The morphology of induced hepatocytes that have two nuclei.
Human ESCs were differentiated into hepatocytes according to the protocol described in **Fig. 6a**. The induced hepatocytes (day 20) were subjected to immunostaining with anti-ALB (green), CK18 (green), CYP2D6 (red), or CYP3A4 (red) antibodies. Nuclei were counterstained with DAPI (blue). The scale bar represents 25 μm. Similar results were obtained in two independent experiments.

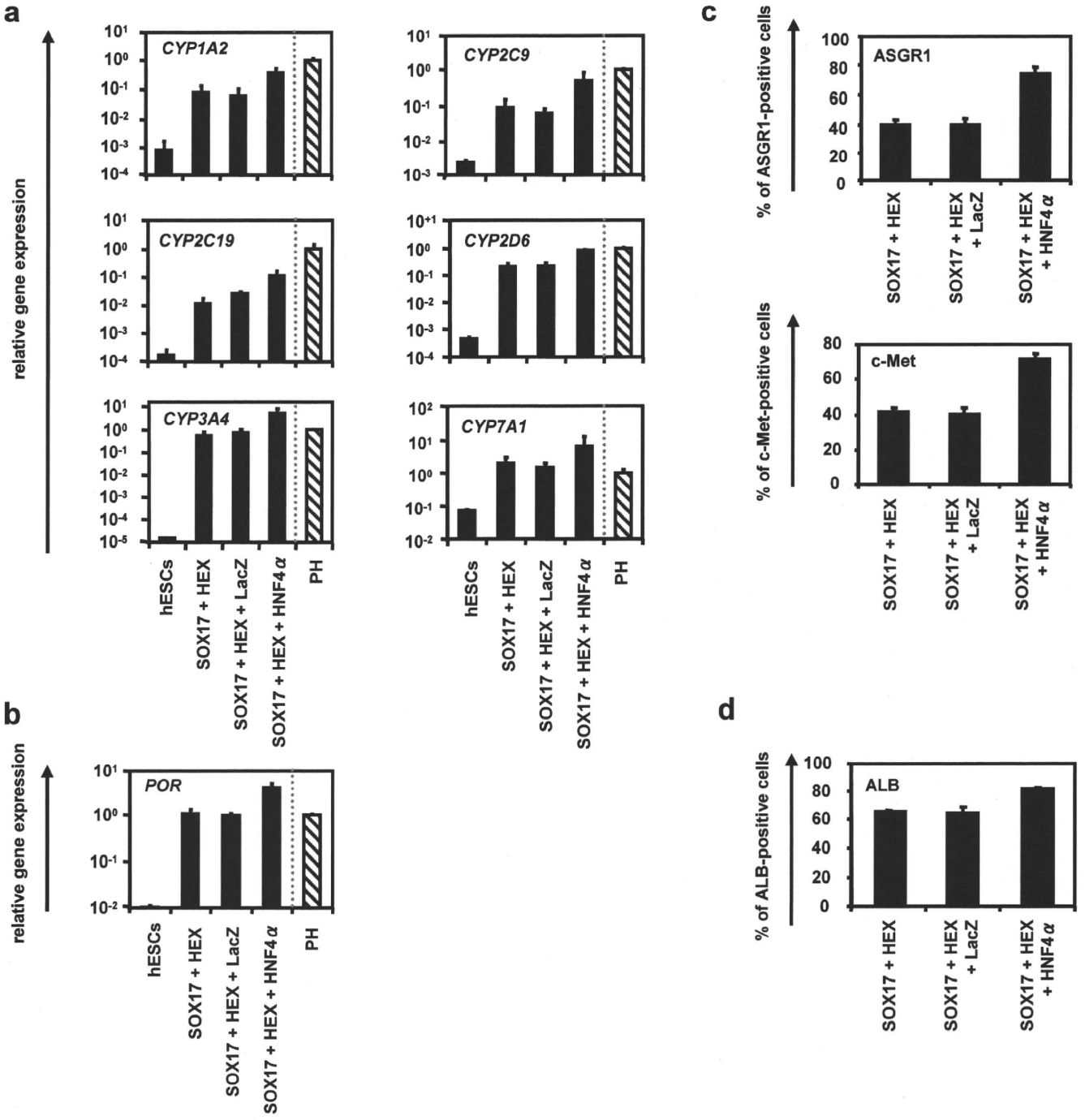

Fig. 29 Transduction of HNF4 α promotes hepatic maturation from human ESCs and iPSCs.
(**a, b**) The human ESCs were differentiated into hepatocytes according to the protocol described in **Fig. 6a**. On day 20 of differentiation, the gene expression levels of (**a**) CYP enzymes (*CYP1A2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4*, and *CYP7A1*) and (**b**) *POR* were examined by real-time RT-PCR in undifferentiated human ESCs (hESCs), induced hepatocytes, and primary human hepatocytes (PH, hatched bar). On the y axis, the expression level of primary human hepatocytes, which were cultured for 48 h after the cells were plated was taken as 1.0. (**c** and **d**) The induced hepatocytes (day 20) were subjected to immunostaining with (**c**) anti-hepatic surface protein (ASGR1 and c-Met) and (**d**) 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 ± SD (n=3).

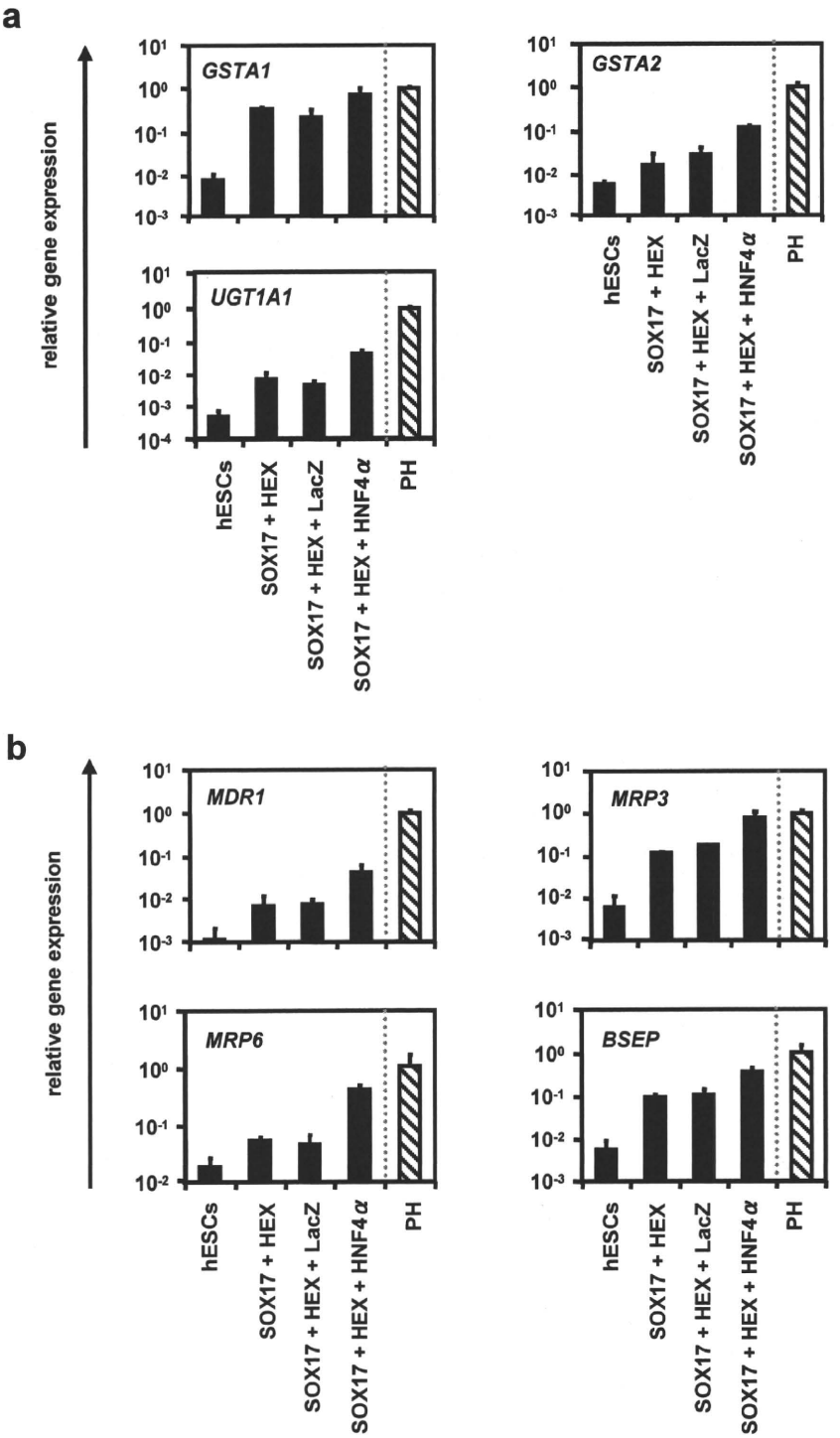

Fig. 30 Upregulation of the expression levels of conjugating enzymes and hepatic transporters by HNF4 α transduction.

(**a, b**) The human ESCs were differentiated into hepatocytes according to the protocol described in **Fig. 6a**. On day 20 of differentiation, the gene expression analysis of (**a**) hepatic conjugating enzymes (*glutathione s-transferase A1 [GSTA1]*, *GSTA2*, and *uridine diphosphate glucuronosyltransferase 1A1* [*UGT1A1*]) and (**b**) hepatic transporters (*multi-drug resistant gene 1 [MDR1], multi-drug resistance protein 3 [MRP3]*, *MRP6*, and *bile salt export pump [BSEP]*) showed higher expression levels in the 3 factors-*transduced cells* (SOX17 + HEX + HNF4 α) as compared with control cells on day 20. On the y axis, the expression level of primary human hepatocytes (PH, hatched bar), which were cultured for 48 h after the cells were plated was taken as 1.0. All data are represented as means ± SD (*n*=3).

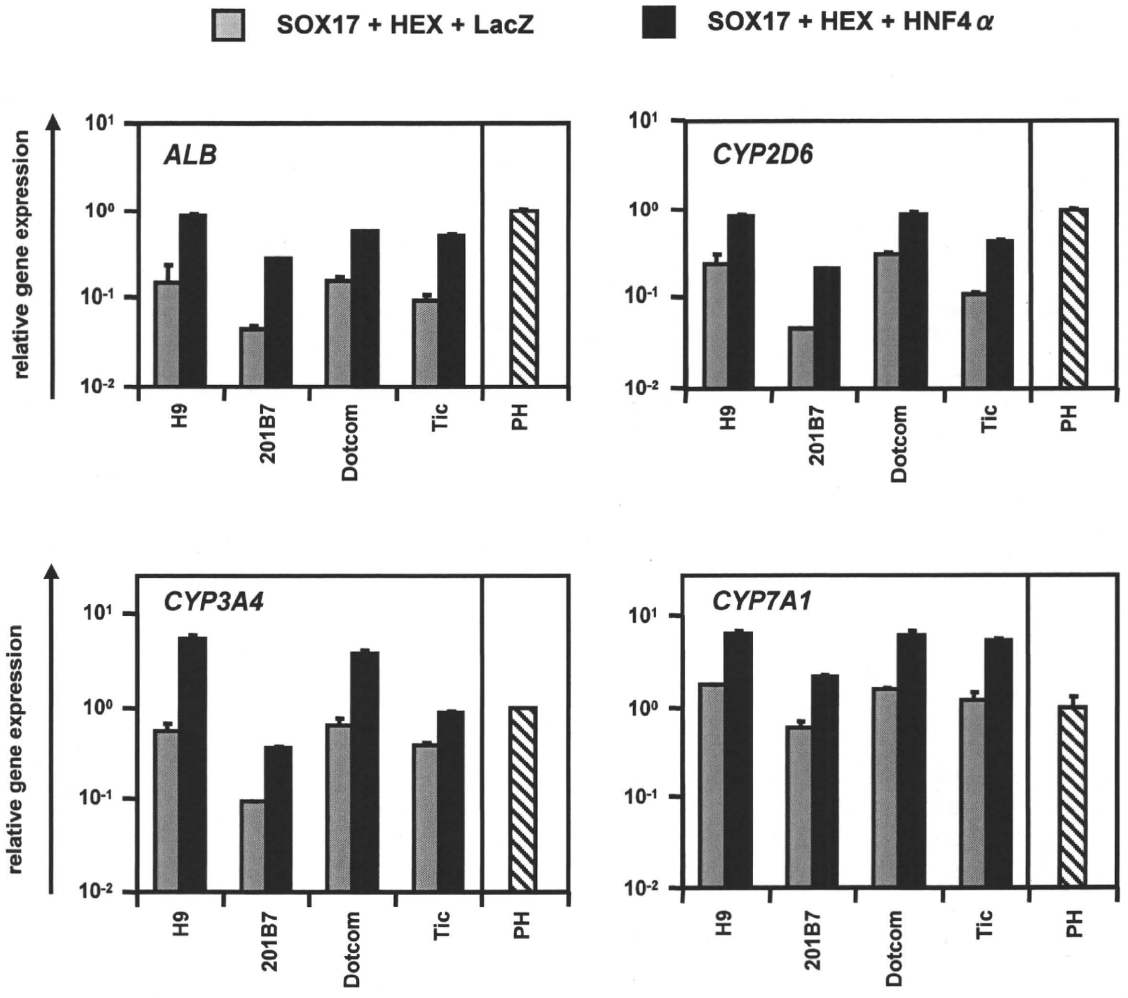

Fig. 31 Generation of hepatocytes from various human ES or iPS cell lines.
Hepatoblasts were transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4 α for 1.5 h and cultured until day 20 of differentiation according to the protocol described in **Fig. 6a**. The expression levels of the hepatocyte markers (*ALB*, *CYP2D6*, *CYP3A4*, and *CYP7A1*) were examined by real-time RT-PCR in human ESC (H9)-derived hepatocytes and human iPSC (201B7, Dotcom, or Tic)-derived hepatocytes. The gene expression profiles of cells transduced with 3 factors (black bar) were compared with those of cells transduced with 2 factors plus Ad-LacZ (gray bar). The level of primary human hepatocytes (PH, hatched bar), which were cultured 48 hr after plating the cells were plated, was taken as 1.0. All data are represented as means ± SD (n = 3).