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Article

Induced hepatic stem cells are suitable for human hepatocyte production

Derivation Process of iTS-L cells

OSKM

HNF1β (+)
HNF4α (+)

HNF1β (-)
HNF4α (-)

Hepatocytes

iTS-L cells
(induced tissue-specific stem cells from liver)

iPSCs

| Hepatocytes | iTS-L(No.1) | iPSCs(201B7) |

100μm · 200μm · 200μm

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Highlights
iTS cells have self-renewal and multipotency

iTS cells express tissue-specific markers

iTS-L cells are less prone to teratoma formation than iPSCs
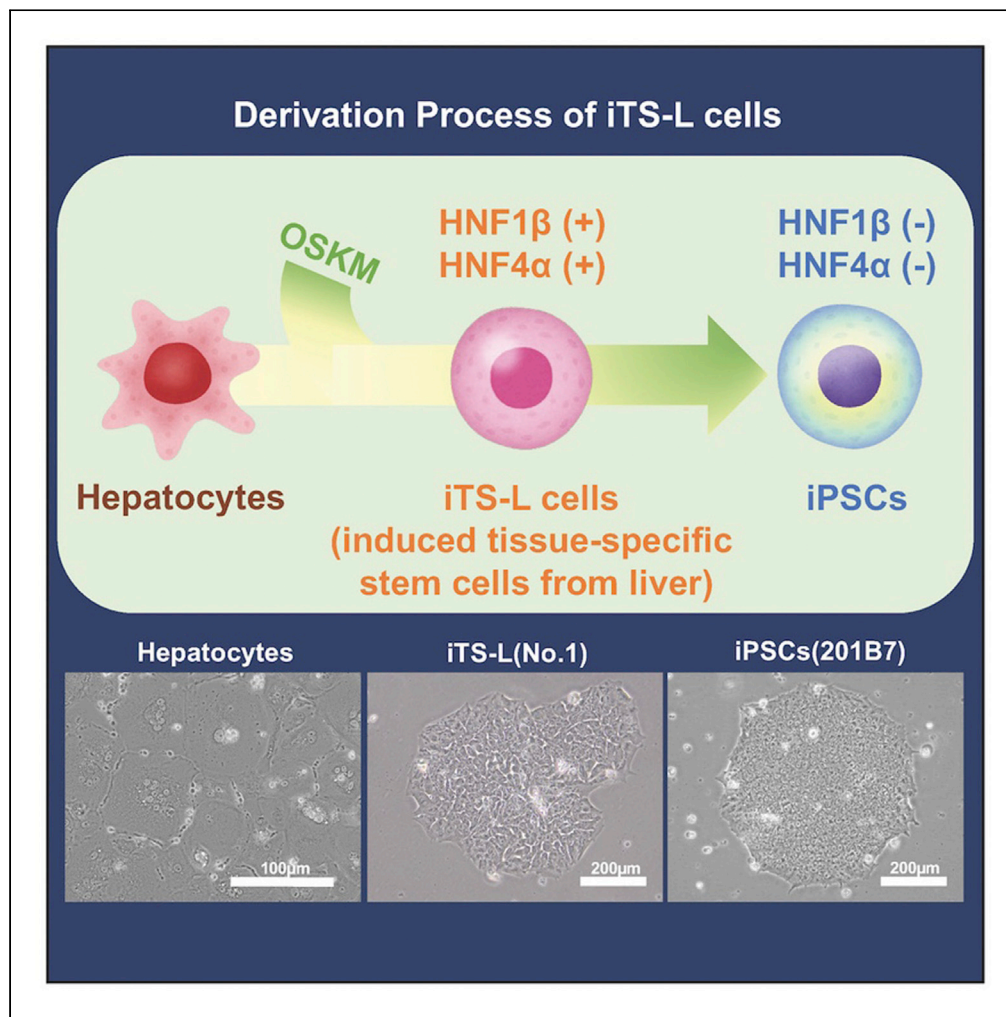
Article

Induced hepatic stem cells are suitable for human hepatocyte production

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SUMMARY

Human hepatocytes were transfected with Sendai virus vectors (SeV) expressing OCT3/4, SOX2, KLF4, and C-MYC to produce hepatocyte-derived induced pluripotent stem cells (iPSCs). The messenger RNA (mRNA) expression of undifferentiated markers (passage 19-21) and hepatocyte-specific markers (HSMs) (passage 0-20) in 48 established hepatocyte-derived iPSC-like colonies was examined. Among the 48 clones, 10 clones continuously expressed HSM mRNA (HNF1β and HNF4α) in passage 0-20. The colonies which expressed HSMs (iTS-L cells: induced tissue-specific stem cells from liver) showed a different tendency in microarray and methylation analyses to fibroblast-derived iPSCs (strain: 201B7). iTS-L cells were less likely to form teratomas in mice than iPSCs (He). The iTS-L cells were differentiated into hepatocyte-like cells more efficiently than iPSCs (He) or iPSCs (201B7). These data suggest that SeV expressing OCT3/4, SOX2, KLF4, and C-MYC induce the generation of iPSCs and iTS-L cells.

INTRODUCTION

Human-induced pluripotent stem cells (hiPSCs), which are similar to embryonic stem (ES) cells in their morphology, gene expression pattern, epigenetic status, and ability to differentiate into cells derived from three embryonic germ layers, are established by gene transfer methods using certain vectors (Takahashi et al., 2007a, 2007b). However, it has been demonstrated that epigenetic memory is inherited from the parental cells after the reprogramming of mouse/human iPSCs. These findings suggest that the phenotype of iPSCs may be influenced by their cells of origin and that their skewed differentiation potential may prove useful in the generation of differentiated cell types that are currently difficult to produce from ES/iPS cells for the treatment of human diseases. Our recent study demonstrated the generation of induced tissue-specific stem/progenitor (iTS/iTP) cells by the transient overexpression of reprogramming factors combined with tissue-specific selection (Miyagi-Shiohira et al., 2018; Noguchi et al., 2015, 2019). iTS/iTP cells are "incompletely reprogrammed" cells that inherit numerous components of epigenetic memory from donor tissue. Hikichi et al. showed that reprogramming is inhibited when the source cell expresses any of the four factors known to promote differentiation into hepatocytes: Foxa2, HNF4α, Foxa3, Hnf1α (Hikichi et al., 2013). Indeed, our group showed that mouse and human pancreatic islets, which express Foxa2 and HNF4α, were partially reprogrammed and iTS/iTP cells from pancreatic tissue were generated (Noguchi et al., 2019).

In this study, we transfected Sendai virus vectors (SeV) expressing OCT3/4, SOX2, KLF4, and C-MYC (OSKM) in human hepatocytes, by which the reprogramming genes were stably expressed in cells (Ban et al., 2011; Fusaki et al., 2009; Nakanishi and Otsu, 2012). Forty-eight iPSC-like clones were established and the difference in the gene expression at passage 1-20 after the establishment was determined. Recently, it has been reported that hiPSCs within 20 passages after establishment show high efficacy in differentiation and basic research has reported that hiPSCs passaged 10-20 times show high efficacy in differentiation, which is influenced by the source cells (Hu et al., 2016). We, therefore, used the clones in passage 1-20. We also compared the ability of iPSCs and iTS-L cells, which expressed hepatocyte-specific markers (HSM) (HNF4α and NHF1β), to induce differentiation into hepatocytes.

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RESULTS

Generation of human hepatocyte-derived human-induced pluripotent stem cell-like clones by Sendai virus vectors

Human primary hepatocytes were reprogrammed using SeV expressing OSKM. Forty-eight iPSC-like clones were generated. Ten clones continuously expressed the mRNA of the HSMs (HNF1β and HNF4α) in passage 0-20 (Figures S1–S22, upper panel). The ten colonies that expressed the HSMs were named iTS-L cells (induced tissue-specific stem cells from liver) (Figure 1A). The other 38 clones in which HSMs were not expressed at passage 20 were named iPSCs (He). The morphology of all colonies was observed using an optical microscope (Figure S1, Lower panel). The reprogramming genes that were transduced into hepatocytes disappeared at the fifth passage (Figure S7). There was no tendency for the increased expression of HSM mRNA when the reprogramming factors had disappeared in the clones (Figure S6, Upper panel). The cell growth rate of iTS-L cells was maintained with an increased number of passages (P10-P20) (Figure 1B). In order to examine the characteristics of iTS-L, the mRNA expression of representative undifferentiated genes was examined. First, the mRNA expression of a typical undifferentiated gene of iPSCs (He) was approximately 1.5 to 3 times higher in comparison to skin fibroblast-derived iPSCs (201B7) (Figure 1C). This result may be considered to be influenced by background differences such as the cell type of origin of the reprogrammed cell, the number of passages after the establishment of iPSCs, and the difference in the vector used to transfer the reprogrammed gene. The mRNA expression of two representative undifferentiated genes, OCT3/4 and SOX2, was significantly lower in iTS-L in comparison to 201B7 (Figure 1C). Changes in the HSM expression of one iPSC (He) clone and two iTS-L clones (No. 33 and 41) were evaluated through passage numbers 0-20. Both iTS-L clones consistently expressed HSM, whereas iPSCs (He) did not (Figure 1D). Although the morphologies of iPSCs (201B7) and iPSCs (He) were similar, the two iTS-L clones were more likely to identify colonies with a relatively large cytoplasmic area in comparison to iPSCs (Figure 1E). We performed additional experiments by obtaining three new types of hepatocytes from different donors. The newly generated iTS-L cells, clone (No. 1), and clone (No. 9) were also re-established using the newly obtained hepatocytes. High-magnification photographs of line 201B7 and iTS-L clones (No. 1) under an optical microscope are shown (Figure S23E). The mRNA expression of albumin, alpha-feto-protein (AFP), cytochrome P450 3A7, and cytochrome P450 3A4, in addition to HNF1β and HNF4α, was used to select iTS-L (Figure S23A, iTS-L (No. 1): left Fig, iTS-L (No. 9): right Fig). In addition, a new higher-magnification image allowing the comparison of iTS-L cells and iPSCs was added (Figure S23E).

The expression of embryonic and tissue-specific markers in human induced pluripotent stem cells (He) and iTS-L cells (No. 33 and 41)

The expression of HSMs (HNF4α, HNF1β, CYP3A4, ALB, AFP, TOD2, TAT, and AAT) in human iTS-L cells (No. 33 and 41) was evaluated by quantitative RT-PCR. Both iTS-L clones expressed HNF4α, HNF1β, CYP3A4, AFP, TOD2, and TAT. In contrast, the expression of ALB and AAT, which were expressed in mature hepatocytes, were lower in both iTS-L clones (Figure 2A). Furthermore, the mRNA expression of albumin, alpha-feto-protein (AFP), cytochrome P450 3A7, and cytochrome P450 3A4, in addition to HNF1β and HNF4α, was used to select iTS-L cells (Figure S23A). The expression of embryonic and tissue-specific markers in human iPSCs (He) and iTS-L cells (No. 33 and 41) was also evaluated using a protein array. Embryonic markers (OCT3/4, NANOG, and SOX2) were expressed in all cell types (Figures 2B and S23). In the protein array, the protein expression of undifferentiated markers (OCT3/4, NANOG, and SOX2) in iPSCs (201B7) was lower than expected (Figure S23). We used whole fractional protein as the source of the protein array. A protein expression analysis of undifferentiated markers may require supplemental evaluation using the nuclear fraction as a source. With the exception of except PDX-1/IPF1 (a pancreatic marker), endodermal markers (E-Cadherin, AFP, GATA-4, HNF-3β/FOXA2, and SOX17) were expressed in iPSCs (He) and iTS-L cells (No. 33 and 41) (Figure 2B). The expression of E-Cadherin, AFP, and SOX17 in iPSCs (He) and iTS-L cells (No. 41) was significantly higher in comparison to iPSCs (201B7). Some mesodermal markers (Gooosecoid (GSC), SNAIL, and VEGFR2/KDR/FLK-1) and ectodermal markers (OTX2, TP63/TP73L, and HCG) were expressed in iPSCs (He) and/or iTS-L cells (No. 33 and 41) (Figure 2B). This may be owing to the contamination of feeder cells and/or naturally differentiated cells from stem cells. To validate the results of these protein arrays, we performed Western blotting on a sample of proteins extracted from line 201B7 and the newly generated iTS-L cells (No. 1) as a control (Figure S23C). The bands were quantified and compared (Figure S23D), and the results were consistent for OCT3/4, Snail, and HCG. However, different results were obtained for the other measurement targets. These results indicate that it is difficult to accurately characterize iTS-L cells at the protein expression.
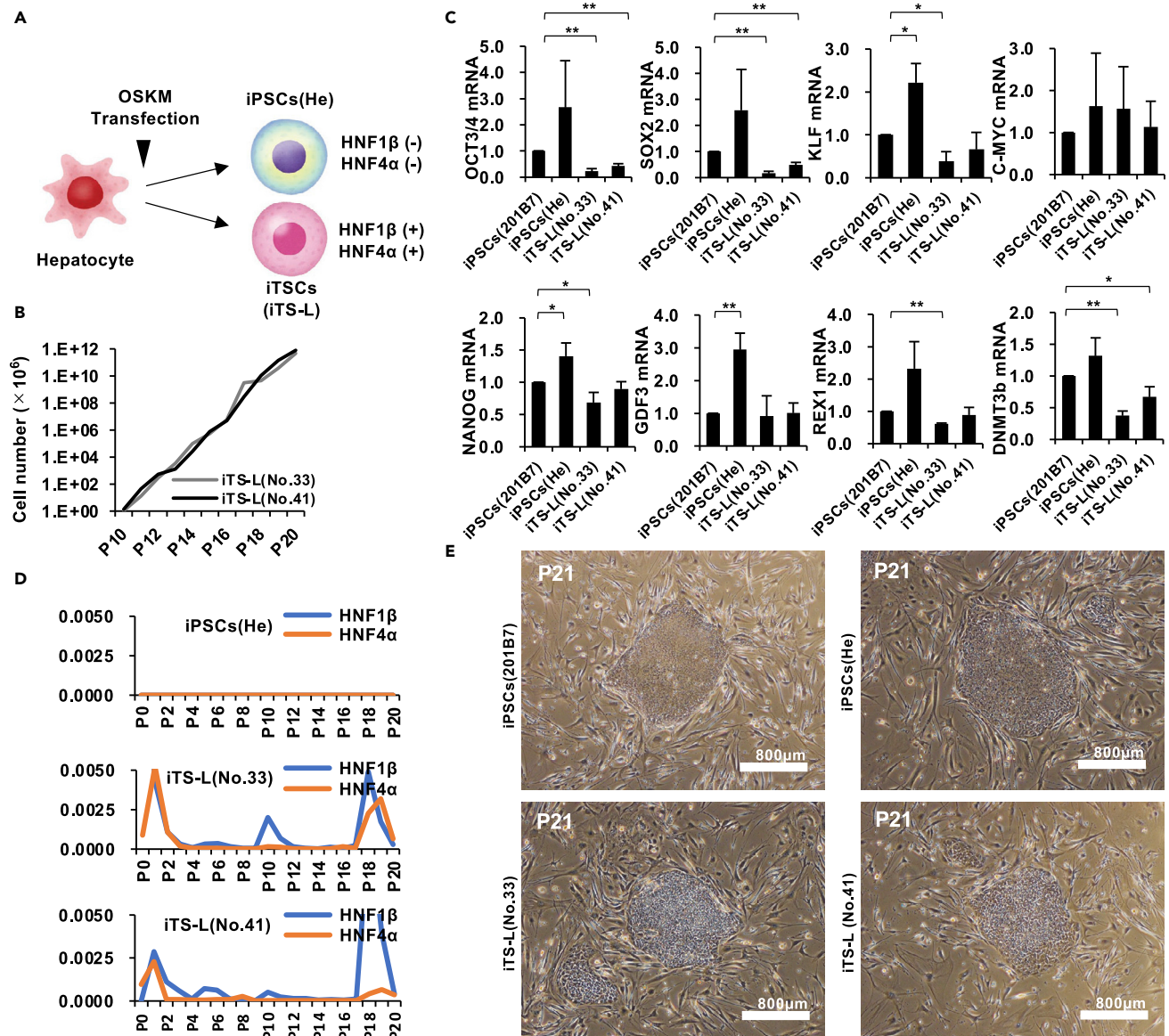
Figure 1. iTS-L and iPSCs (He) were established from human hepatocytes

(A) Illustration of the process of establishing iTS-L and iPSCs (He) by introducing a reprogramming gene into human hepatocytes.

(B) Cell proliferation assay. The cells were detached from the 6-well dish, the cells were mixed with 8 mL of PBS, and the number of cells was counted. At each passage, cells diluted 1/16-fold were seeded. The cells of passage numbers 10-20 were counted. The number of iTS-L cells (No. 33) is shown by the gray line, and the number of iTS-L cells (No. 41) is shown by the black line. (n = 1).

(C) RT-PCR of human embryonic stem cell markers. The Y axis represents the relative position of the mRNA expression of various target genes expressed by the hiPSC reference strain (201B7). The passage number for each sample was 19, 20, and 21 (n = 3). The data represent the mean $\pm$ S.D. *p < 0.05, **p < 0.01.

(D) The time course of the mRNA expression (HNF1β and HNF4α) in colonies at passage numbers 0 to 20 after iPSCs were established. The Y axis represents the relative position of the mRNA expression of the target gene expressed by hepatocytes. (n = 1).

(E) The cell morphology of iPSCs (201B), iPSCs (He), iTS-L cells (No. 33), and iTS-L cells (No. 41) at passage number 21 under MEF feeder culture conditions. Optical microscope images are shown. Scale bar = 800 μm.

Microarray and methylation analyses

We performed a microarray analysis to compare the global gene-expression profiles of human iPSCs (201B7), iTS-L cells (No. 33), and hepatocytes from two different donors. Unsupervised hierarchical clustering of gene expression profiles of iPSCs (201B7), iTS-L cells (No. 33), and hepatocytes showed that iTS-L cells markedly differed from iPSCs and hepatocytes and that iTS-L cells (No. 33) clustered more
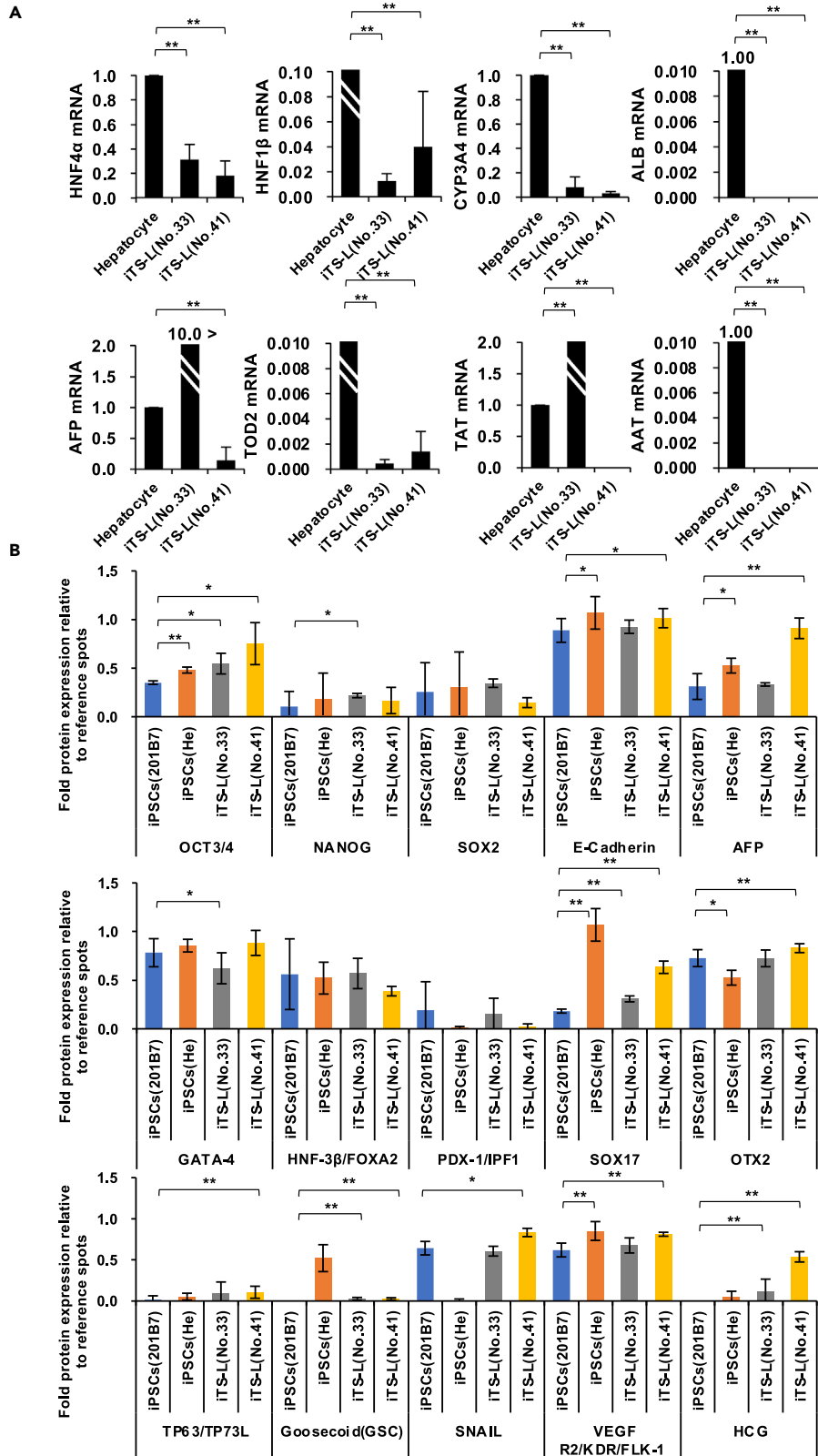
Figure 2. Cellular characteristics of iTS-L cells and iPSCs (He) were examined using hepatocyte and ES cell markers

(A) RT-PCR of human hepatocyte markers. The Y axis represents the relative position of the mRNA expression of various target genes expressed by the human hepatocyte. The passage number for each sample was 19, 20, and 21 (n = 3). The data represent the mean $\pm$ S.D. *p < 0.05, **p < 0.01.

(B) A protein array analysis of human embryonic stem cell markers. The color development of the array was quantified by image analysis using ImageJ. The Y axis shows a relative value when the reference value of the array is 1ste original data of the array are shown in Figure S28 rde passage number of the sample was 20 (n = 2). Protein array measurements were performed in duplicate. The data represent the mean $\pm$ S.D. *p < 0.05, **p < 0.01.

closely with iPSCs (201B7) than hepatocytes from two different donors (Figure 3A). Similar results were displayed in a principal component analysis (Figure 3B).

Next, bisulfite genomic sequencing of the promoter regions of embryonic markers (*OCT3/4* and *NANOG*) and endoderm markers (*HNF1β* and *HNF4α*) were performed. Bisulfite genomic nucleotide sequencing demonstrated that the *OCT3/4* and *NANOG* promoters remained methylated in iTS-L cells (No. 33) but were demethylated in iPSCs (201B7). In contrast, the *HNF1β* and *HNF4α* promoters were demethylated in iTS-L cells (No. 33) (Figure 3C). These results demonstrate that the methylation of these promoters in iTS-L cells differs from that in iPSCs.

Teratoma formation

Teratoma formation was examined by the subcutaneous injection of iPSCs (He) and two iTS-L clones (No. 33 and 41) into immunodeficient mice. The examination of teratoma formation at 10 and 15 weeks after the injection of various iPSCs revealed teratoma formation after a single subcutaneous injection of iPSCs (He). However, there was no teratoma formation when both iTS-L clones (No. 33 and 41) were injected into four subcutaneous sites (Figure 3D). These data suggest that iTS-L cells were less likely to form teratomas than iPSCs (He). Similar results were shown in experiments with immunodeficient nude mice (data not shown).

Differentiation of human induced pluripotent stem cells and iTS-L cells into hepatocytes

The potential of iPSCs and iTS-L cells to differentiate into hepatocytes was examined. iPSCs (201B7), iPSCs (15M66), and two iTS-L clones (No. 1 and 9) were induced to differentiate into hepatocytes. The differentiation process (endoderm induction, hepatic induction, and hepatic maturation) was determined according to a previous protocol (Figure 4A) (Ang et al., 2018). iPSCs (201B7), iPSCs (15M66), and two iTS-L clones (No. 1 and 9) differentiated into hepatocytes (Figure 4B). Large square-polygonal cells were observed in each type of differentiated cells. The cells had 1-2 bright and round nuclei with typical characteristics of cultured hepatocytes. The size of iTSC-L-derived hepatocytes was 5-10 μm in diameter, whereas the size of primary hepatocytes was 50-100 μm in diameter (Figure S29).

At 0, 3, 6, 12, and 18 days after induction, the mRNA expression of hepatocyte markers (Figure 4C) in differentiated iPSCs (201B7), iPSCs (15M66), and iTS-L clones (No. 9) was examined. Most hepatocyte markers (AFP, ALB, AAT, TAT, CYP2Aα, CYP3A7, and TDO2) were increased. Compared to iPSCs (201B7), iTS-L clones (No. 9) showed a significantly increased mRNA expression of AAT on day 12 of differentiation induction and significantly increased mRNA expression of ALB, AAT, and TDO2 on day 16 of differentiation induction. To collect as much data as possible on "differentiation efficiency/maturity/quality," we used iTS-L (No. 1) and iTS-L (No. 9) and two types of iPSCs (201B7 line and 15M66 line), and the data from the mRNA array analyses performed for the corresponding mRNA arrays were added (Figures S30A–S30D).

We also evaluated the expression of HNF4α and epithelial cell adhesion molecule (EpCAM), which is known to be expressed on the cell membrane and in the cytoplasm of hepatocytes. All differentiated iPSCs (201B7), iPSCs (15M66), and two iTS-L clones (No. 1 and 9) expressed HNF4α and EpCAM. (Figure 4D). On day 16 after the induction of differentiation, the culture medium was sampled to measure albumin (Alb) (g/dL) and urea nitrogen (UN) (mg/dL). The Alb concentrations (g/dL) were as follows: iPSCs (201B7): mean, 0.188; standard deviation, 0.034; iPSCs (15M66): mean, 0.200; standard deviation, 0.000; and iTS-L clones (No. 9): mean, 0.200; standard deviation, 0.000. No significant differences were found between the groups. The urea nitrogen (UN) concentrations (mg/dL) were as follows: iPSCs (201B7), mean 0.550 (standard deviation 0.697); iPSCs (15M66), mean 0.650 (standard deviation 0.689); and iTS-L clones (No. 9), mean 0.913 (standard deviation 0.384). No significant differences were found between the groups.
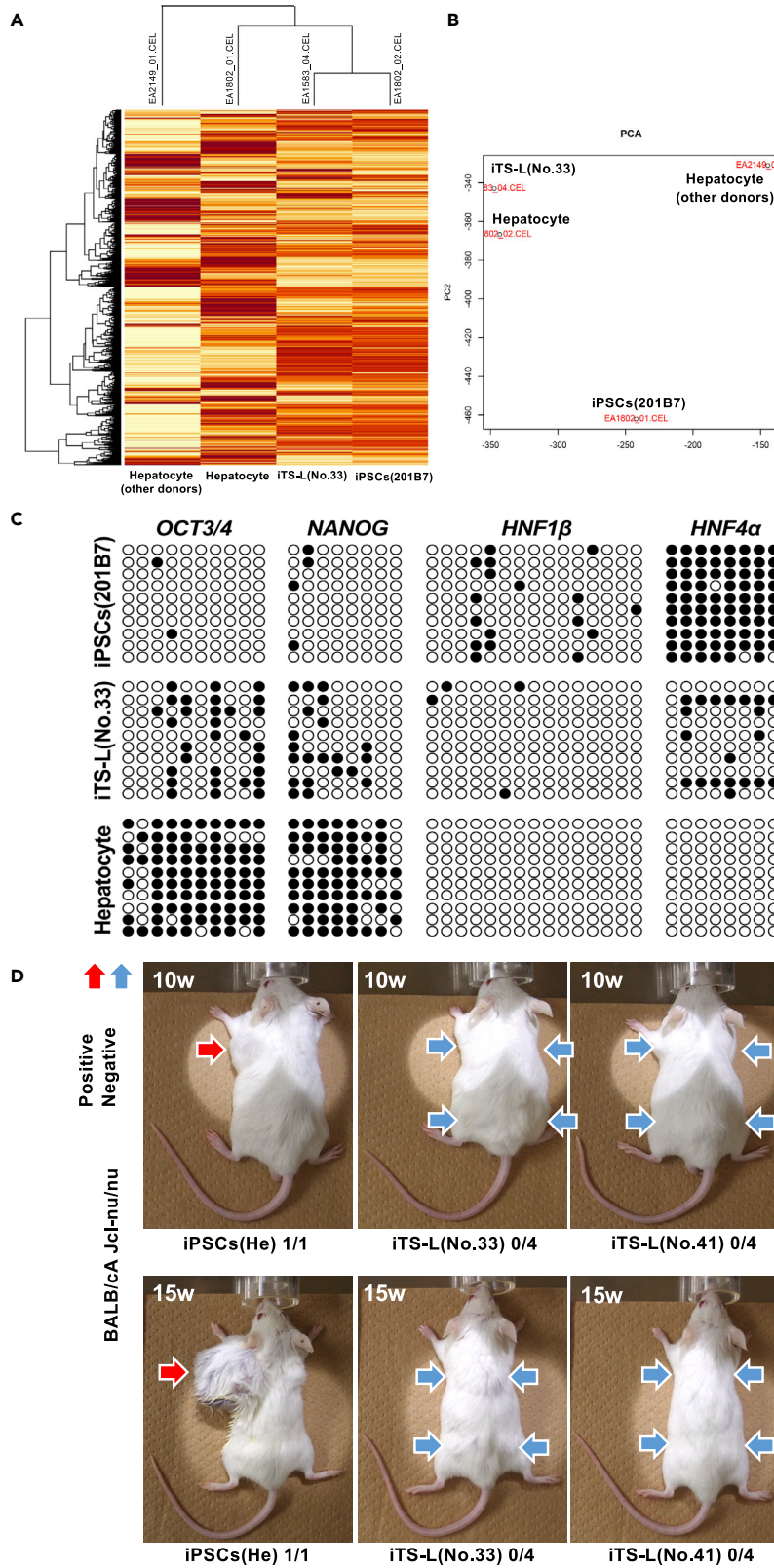
A

EA2149_01.CEL EA1802_01.CEL EA1583_04.CEL EA1802_02.CEL

Hepatocyte (other donors)   Hepatocyte   iTS-L(No.33)   iPSCs(201B7)

B

PCA

iTS-L(No.33)
83o04.CEL

Hepatocyte
802o02.CEL

Hepatocyte (other donors)
EA2149o0

iPSCs(201B7)
EA1802o01.CEL

C

OCT3/4   *NANOG*   *HNF1β*   *HNF4α*

iPSCs(201B7)

iTS-L(No.33)

Hepatocyte

D

Positive Negative

BALB/cA Jcl-nu/nu

10w — iPSCs(He) 1/1   10w — iTS-L(No.33) 0/4   10w — iTS-L(No.41) 0/4

15w — iPSCs(He) 1/1   15w — iTS-L(No.33) 0/4   15w — iTS-L(No.41) 0/4

Figure 3. Cellular properties of iTS-L in which the HNF4α gene expression was activated

(A) A microarray analysis was performed on 38500 human genes using the Human Genome U133 Plus 2.0 Array. The X axis indicates the sample name (Hepatocyte (other donors), Hepatocyte, iTS-L [No. 33], and iPSCs [201B7]). The hierarchical cluster analysis of four types of cells is shown. The Y axis is a heat map of each gene expression. Highly expressed genes are shown in red and lowly expressed genes are shown in white. Each gene was subjected to a hierarchical cluster analysis. A principal component analysis was performed on four types of cells based on the microarray analysis data. Four types of cells were subjected to a hierarchical cluster analysis. EA2149_01.CEL and EA1802_01.CEL were expressed as hepatocytes, EA1802_02.CEL was expressed as iTS-L cells (No. 33), and EA1583_04.CEL was expressed as iPSCs (201B7).

(B) Four types of cells were subjected to a principal component analysis (PCA). EA2149_01.CEL was expressed as hepatocytes (other donors), EA1802_01.CEL was expressed as hepatocytes, EA1802_02.CEL is expressed as iTS-L cells (No. 33), and EA1583_04.CEL was expressed as iPSCs (201B7).

(C) Bisulfite Sequence Analysis of Genomic DNA. A bisulfite sequence analysis of the OCT3/4, NANOG, HNF1β, and HNF4α promoter regions in iPSCs (201B7), iTS-L (No. 33), and hepatocytes. Open circles indicate unmethylated and closed circles indicate methylated CpG dinucleotides.

(D) A teratoma formation test derived from cells injected subcutaneously into mice. The upper panel shows photographs of subcutaneous teratoma formation tests at the base of the forelimbs of the mouse and at the base of the hind limbs 10 weeks after subcutaneous cell transplantation. The lower panel shows photographs of subcutaneous teratoma formation tests at the base of the forelimbs of the mouse and at the base of the hind limbs 15 weeks after subcutaneous cell transplantation. The upper and lower panels show photographs of the same individual mice. Red arrows indicate teratoma formation; blue arrows indicate the absence of teratoma formation.

We also evaluated the potency of the differentiation of these cells into hepatocytes using a different protocol (Figure S24A, left panel) (Nakamura et al., 2012). Hepatocytes differentiated from both iTS-L cells (No. 1 and 9) seemed smaller in comparison to primary hepatocytes (Figure S24B). The mRNA expression of hepatocyte markers was examined. All cells differentiated from iPSCs (201B7) and two iTS-L clones (No. 1 and 9) expressed mRNA of AFP, ALB, AAT, TAT, CYP3Aa, CYP3A7, and TDO2. The *CYP3Aa* mRNA expression was converted to a hepatocyte expression of 1. The *CYP3Aa* mRNA expression level in iTS-L (No. 9) were shown, indicating a transient increase [d0: mean 0.0087, standard deviation 0.104; d3: mean, 0.066; standard deviation, 0.104; d6: mean, 2.353; standard deviation, 2.54; d12: mean, 104.59; standard deviation, 173.65; d16: mean, 150.77; standard deviation, 233.01] (Figure 4C). Data from the hepatocyte differentiation protocol (Ang et al.) (Figure 4C) and the hepatocyte differentiation protocol itself (Nakamura et al.) (Figure S24C) are shown with the same hepatocytes as controls and the corrected values are converted to 1. As a result, the *ALB* mRNA expression of iTS-L (No. 9) on day 16 after the start of the induction of differentiation in the hepatocyte differentiation protocol (Ang et al.) (Figure 4C) was higher than that of the control hepatocytes. In contrast, the *ALB* mRNA expression of iTS-L (No. 9) at 12 days after the start of the induction of differentiation by the hepatocyte differentiation protocol (Nakamura et al.) (Figure S24C) was lower than that of the control hepatocytes. Differentiated cells from iPSCs (201B7) expressed lower levels of CYP3Aa in comparison to iTS-L cells (No. 9). The levels of HNF1β and HNF4α, which are endoderm markers (Mathew et al., 2012), in iPSCs (He) were higher than in other cell types (Figure 1C). Regarding embryonic markers, the expression of KLF4, C-MYC, GDF3, and other embryonic markers (OCT3/4, SOX2, NANOG, REX1, and DNMT3b) in iPSCs (He) were higher in comparison to other cell types (Figure 1C). Other embryonic markers were also positive in all cell types. These data suggest that the cells differentiated by this protocol contained more immature progenitor hepatocytes (hepatoblasts) in comparison to the cells differentiated by the protocol in Figure 4A. More importantly, these data suggest that both iTS-L cells (No. 1 and 9) (Figures 1C and S24C) and iTS-L cells (No. 33 and 41) (data not shown) differentiated into hepatocytes more efficiently in comparison to iPSCs (201B7).

The functional test of differentiated cells from human induced pluripotent stem cells (201B7), human induced pluripotent stem cells (He), and iTS-L cells (No. 33 and 41)

The uptake of Indocyanine Green (ICG) into cells (Figure 5A, left panel) and excretion from cells (Figure 5A, right panel) were examined. Cells differentiated from iPSCs (201B7), iPSCs (He), and iTS-L cells (No. 33 and 41) had the ability to uptake and excrete ICG, similarly to primary hepatocytes (Figure 5A, top panel). A Periodic Acid Schiff (PAS) assay was performed to show glycogen storage in the cells (purple in the light micrograph). Cells differentiated from iPSCs (201B7), iPSCs (He), and iTS-L cells (No. 33 and 41) showed glycogen retention, similarly to primary hepatocytes (Figure 5B, top panel). A hepatocyte toxicity assay was performed. Several factors of the liver function (GOT, GPT, γ-GTP, LAP, and LDH) released from differentiated cells/hepatocytes by stimulation with D-galactosamine (D-GaIN) were biochemically analyzed. The release of GPT by primary hepatocytes was significantly increased after D-GaIN stimulation (Figure 5C, upper left panel). The release of GOT and GPT was significantly increased in iTS-L cells (No. 33) and iTS-L cells (No. 41)
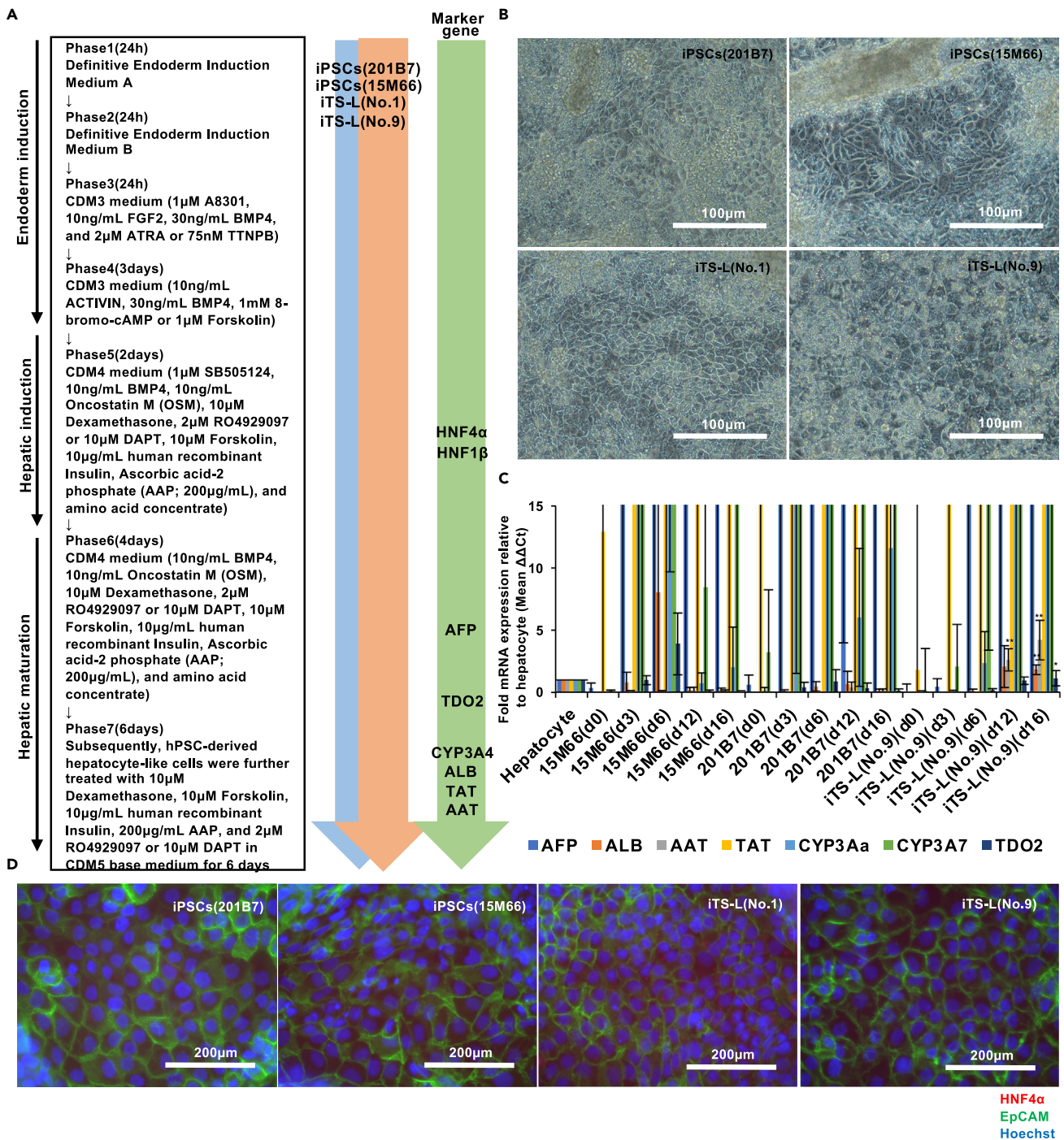
Figure 4. Induction of differentiation into hepatocytes (Protocol 1)

(A) The administration schedule of the differentiation-inducing reagent in each stage of hepatocellular differentiation induction (endoderm induction, hepatic induction, and hepatic maturation) is listed in detail in the left panel. The center arrow indicates the starting point of the differentiation of each cell in the process of inducing hepatocyte differentiation. The arrows on the right indicate the expression of specific marker genes at each stage of hepatocyte differentiation and maturation.

(B) Cell morphology of mature hepatocytes produced from iPSCs (201B7), iPSCs (15M66), iTS-L cells (No. 1), and iTS-L cells (No. 9). Optical microscope images are shown. Scale bar = 100 μm.

Figure 4. Continued

(C) The panel shows changes in the mRNA expression of hepatocyte markers at 0 days, 3 days, 6 days, 12 days, and 16 days after the start of induction of hepatocyte differentiation in iPSCs (201B7), iPSCs (15M66) and iTS-L cells (No. 9). The Y axis shows a relative value in comparison to the expression of hepatocytes as controls and the corrected values converted to 1. The data represent the mean $\pm$ S.D. *p < 0.05, **p < 0.01.

(D) Mature hepatocytes produced by inducing differentiation from each cell were stained by fluorescent immunostaining using HNF4α and EpCAM antibodies, with HNF4α stained in red and EpCAM in green, and then photographed under a microscope. Scale bar = 200 μm.

(Figure 5C, upper right panel, Figure 5C, lower left panel). The release of GOT was significantly increased in iPSCs (201B7) (Figure 5C, lower right panel). The high release of LDH was detected with iTS-L (No. 33), iTS-L cells (No. 41), and iPSCs (201B7) with and without D-GaIN stimulation, while the release of LDH from primary hepatocytes was low.

Hepatocyte differentiation with the omitting protocol

iTS-L cells express endodermal cell markers (HNF1β and HNF4α). We, therefore, suspected that the omission of phases 1-4 of the protocol from Figure 4A does not affect the differentiation of iTS-L cells into hepatocytes. The ability of iTS-L cells (No. 41), iPSCs (201B7), and iPSCs (He) to differentiate into hepatocytes was evaluated using the complete protocol (Phase 1-7) and the omitting protocol (Phase 5-7) of Figure 4A. With the omitting protocol, the levels of ALB, AAT, HNF4α, HNF1β, CYP3Aa, and TDO2 in iTS-L cells (No. 41) were similar to or greater than the levels observed with the complete protocol (Figure 5D, top panel). The expression of ALB, AAT, HNF4α, HNF1β, CYP3Aa, and TDO2 in iPSCs (201B7) and iPSCs (He) was hardly induced when the omitting protocol was used (Figure 5D, iPSCs (201B7); middle panel, iPSCs (He); bottom panel).

DISCUSSION

In this study, we established 48 colonies of hiPSC-like cells using primary human hepatocytes. Most clones expressed HNF1β and HNF4α at passage 0 (colony pick). Then, these colonies were passaged 20 times. Ten clones of 48 iPSC-like colonies continuously expressed HNF1β and HNF4α at 20 passages. We evaluated teratoma formation using 2 of the 10 clones that continuously expressed HNF1β and HNF4α at 20 passages; neither of these clones formed teratomas (Figure 3D). As the definition of human iPSCs includes "teratoma formation," we named these clones as "induced tissue-specific stem cells from liver; iTS-L cells" in this study. We previously reported that mouse iTS-L cells (Noguchi et al., 2015) and the characteristics of human iTS-L cells in this study are similar to those of mouse iTS-L cells. We defined iTS cells by the following properties: 1) self-renewal and multipotency (as the definition of stem cells), 2) the expression of tissue-specific markers, and 3) the absence of teratoma formation. Clones No. 33 and 41 showed no teratoma formation. Therefore, these two clones were iTS cells, not iPSCs. We reconducted the mouse teratoma experiment and found that HNF1β-positive + HNF4α-positive iTS-L (No. 1) and iTS-L (No. 9) cells did not form teratomas (Figures S32F and S32G). Therefore, we can still say at this point that iTS-L cells are characterized as being less prone to teratoma formation than iPSCs. The next question is whether or not HNF1β-positive + HNF4α-positive iTS-L (No. 1) and iTS-L (No. 9) cells exhibit limited differentiation induction. First, we conducted an experiment in which iTS-L (No. 1) cells were induced to differentiate into hepatocytes (Figure S31C). We then followed up with an experiment to induce the differentiation of iTS-L (No. 9) cells into hepatocytes (Figure 4C). The results showed that iTS-L cells tended to be more active in inducing differentiation into hepatocytes than line 201B, as described in this article. We felt that the term "iTS-L(No. 1) cells" complicated our understanding of the experiment. Therefore, we prepared "Human Fetal Liver Poly A + RNA(+) 201B7 cells," which were transfected with mRNA from fetal liver cells, as artificially generated iTS-L cells. First, we examined the protein expression of OCT3/4 by Western blotting using a sample 24 h after introducing fetal hepatocyte mRNA into the 201B7 strain. As a result, fetal hepatocyte mRNA weakened the protein expression of OCT3/4 in strain 201B7 (Figures S32A and S32C). Next, hepatocytes from line 201B7 and Human Fetal Liver Poly A + RNA(+) 201B7 cells were passed three times, and the mRNA was extracted from the cell pellet obtained at each passage. The mRNA expression of hepatocyte markers (HNF1β, HNF4α, albumin, AFP, CYP3A7, and CYP3A4) was then examined. The results showed that the mRNA expression of hepatocyte markers by Human Fetal Liver Poly A + RNA(+) 201B7 cells was significantly increased compared to that by the control line 201B7 (Figure S32B). Furthermore, human fetal liver Poly A + RNA(+) 201B7 cells showed differentiation potential in three germ layers (Figure S33).

Methylation analysis revealed that the promoter region of HNF1β and HNF4α in iTS-L cells was almost demethylated. It has been reported that an epigenetic state called epigenetic memory derived from a source
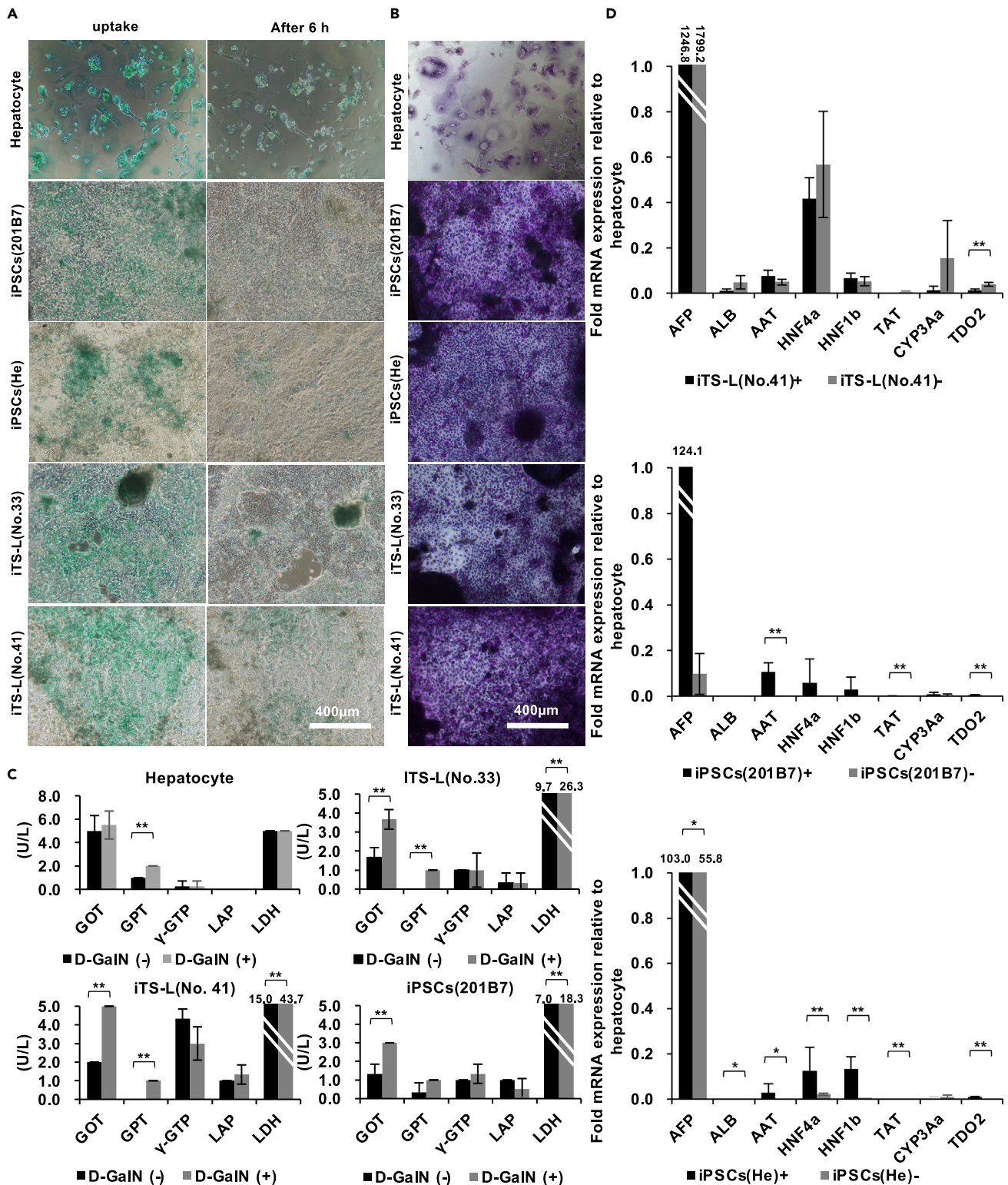
Figure 5. The functional activity of hepatocytes induced from iPSCs (201B7), iPSCs (He), iTS-L cells (No. 33), and iTS-L cells (No. 41)

(A) The indocyanine green (ICG) uptake and release. Hepatocytes induced from four human iPSCs (iPSCs (201B7), iPSCs (He), iTS-L cells (No. 33), and iTS-L cells (No. 41)) (on the 18th day of differentiation culture) and hepatocytes were examined for their ability to take up ICG (left panel) and release it 6 h later (right panel). Scale bar = 400 μm.

Figure 5. *Continued*

(B) Glycogen storage ability, as evaluated by Periodic Acid Schiff (PAS) staining was determined for hepatocytes induced from four human iPSCs (iPSCs (201B7), iPSCs (He), iTS-L cells (No. 33), and iTS-L cells (No. 41)) (on the 18th day of differentiation culture) and hepatocytes. Nuclei were counterstained with hematoxylin. Glycogen storage is indicated by pink or dark red-purple cytoplasm. Scale bar = 400 μm.

(C) D-galactosamine cytotoxic assay. Cells were incubated with 25 mM D-galactosamine (D-GalN) for 24h at 37°C. Liver-specific enzymes (GOT, GPT, γ-GTP, LAP, and isozymes of LDH) released from the cells into the culture medium were measured (n = 3). The data represent the mean $\pm$ S.D. *p < 0.05, **p < 0.01. Hepatocytes induced from four human iPSCs (iPSCs (201B7), iPSCs (He), iTS-L cells (No. 33), and iTS-L cells (No. 41)) (on the 18th day of differentiation culture) and hepatocytes were used in this assay.

(D) The effect of the hepatocyte differentiation induction protocol without endoderm induction. The upper graph shows the expression of the hepatocyte marker mRNA of iTS-L cells (No. 41) induced to differentiate into hepatocytes using the hepatocyte differentiation induction protocol with endoderm induction (+) and the differentiation induction protocol without endoderm induction (−). The relative values are shown with the mRNA expression of hepatocytes as 1ste middle graph shows the expression of the hepatocyte marker mRNA of iPSCs (201B7) induced to differentiate into hepatocytes using the hepatocyte differentiation induction protocol with endoderm induction (+) and the differentiation induction protocol without endoderm induction (−). The relative values are shown with the mRNA expression of hepatocytes as 1ste lower graph shows the expression of the hepatocyte marker mRNA of iPSCs (He) induced to differentiate into hepatocytes using the hepatocyte differentiation induction protocol with endoderm induction (+) and the differentiation induction protocol without endoderm induction (−). The relative values are shown with the mRNA expression of hepatocytes as 1. The data represent the mean $\pm$ S.D. *p < 0.05, **p < 0.01.

cell remains in some iPSCs (Bird, 2002; Stewart-Morgan et al., 2020). In this study, we transfected SeV expressing OSKM in human hepatocytes and 20.8% (10/48) of the clones expressed HNF1β and HNF4α at passage 20. Although it is well-known that reprogramming genes are stably expressed in cells after the transfection of SeV (Ban et al., 2011; Fusaki et al., 2009; Nakanishi and Otsu, 2012), 20% of clones at passage 20 retained epigenetic memory.

The methylation analysis indicated that the promoter region of HNF1β was demethylated in both iTS-L cells and iPSCs. In contrast, the promoter region of HNF4α was demethylated in iTS-L cells, while it was methylated in iPSCs, suggesting that HNF4α could be a more suitable marker than HNF1β for the generation of iTS-L. Indeed, we previously used HNF4α as a "selection marker" to generate mouse iTS-L cells (Noguchi et al., 2015). HNF4α, a member of the nuclear receptor family, was discovered as a transcription factor that binds to the promoter region of genes that are highly expressed in the liver. It is also known as a marker of hepatocyte differentiation. As HNF4α is also expressed in the kidney, pancreas, and intestine, its role as a differentiation marker for these organs has been attracting attention. HNF4α has been shown to inhibit cell reprogramming (Hikichi et al., 2013). The data of Hikichi et al. may suggest that it is easy for "partial" reprogramming to occur in hepatocytes after the transfection of reprogramming factors because of the expression of HNF4α.

In conclusion, we generated human iPSCs and iTS-L cells SeV expressing OSKM factors. iTS-L cells provide advantages over iPSCs, including highly efficient differentiation and the absence of teratoma formation. Moreover, some phases could be omitted during the differentiation protocol, as shown in Figure 5. This is another advantage of iTS-L cells, especially in clinical application, because the cost of the differentiation procedure could be reduced. The generation of iTS cells could have important implications for the clinical application of stem cells.

Limitations of the study

A major bottleneck facing the production of clinical hiPSCs with regard to time and cost is the use of residual Sendai virus vector for hiPSC establishment, which requires five cell passages after initialization when Laminin 511 is used as a coating material. Clinical hiPSC production is shifting from the colony picking method, in which production personnel arbitrarily select colonies after hiPSC establishment, to the bulk method, which does not involve colony picking. The bulk method assumes that initialized cells are homogeneous hiPSCs, and in actual operation, even if the Sendai virus vector that establishes the latest hiPSCs is used, factors such as time lag in cell initialization and depth of cell initialization may overlap. Therefore, when considering the production of cells for clinical use, the established cells must be homogeneous iTS-L cells.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105052.

AUTHOR CONTRIBUTIONS

Author roles: study design, YN, CS, HN; conducted the study, YN, CS, HN; data collection, YN, CS, HN; data analysis, YN, CS, HN; data interpretation, YN, CS, HN; provided materials, IS, MW, MM, MT; drafted the article, YN, HN; revised the content of the article, YN, HN; approval of the final version of the article, YN, CS, IS, MW, MM, MT, HN. YN takes responsibility for the integrity of all of the data analyses.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure diversity in experimental samples through the selection of genomic datasets.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Human Oct-3/4 Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF1759-SP
Anti-Human Nanog Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF1997-SP
Anti-Human SOX2 Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF2018-SP
Anti-Human E-Cadherin Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF648-SP
Anti-Human alpha-Fetoprotein Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF1369-SP
Anti-Human GATA-4 MAb	R&D Systems	Cat# MAB2606-SP
Anti-Human HNF-3 beta/FoxA2 Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF2400-SP
Anti-Human PDX-1/IPF1 Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF2419-SP
Anti-Human SOX17 Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF1924-SP
Anti-Human Otx2 Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF1979-SP
Anti-Human p63/TP73L Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF1916-SP
Anti-Human Goosecoid Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF4086-SP
Anti-Human Snail Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF3639-SP
Anti-Human VEGF R2/KDR Affinity Purified Polyclonal Ab	R&D Systems	Cat# AF357-SP
Anti-Human Chorionic Gonadotropin alpha/beta (HCG) MAb	R&D Systems	Cat# MAB97701-SP
Anti beta-actin (C4)	Santa Cruz Biotechnology, Inc.	Cat# SC-47778
Human EpCAM aa 250	Abcam	Cat# ab71916
goat anti-Rabbit IgG H&L (Alexa Fluor 488)	Abcam	Cat# ab150077
Anti HNF-4α (H-1) (Alexa Fluor 647)	Santa Cruz Biotechnology	Cat# SC-374229 AF647
Cellstain ® - Hoechst 33342 solution	DOJINDO LABORATORIES	Cat# H342
Chemicals, peptides, and recombinant proteins		
iMatrix-511	Matrixome Inc.	Cat# 892 011
2-mercaptoethanol	Sigma-Aldrich	Cat# M3148-25M
trypsin	ThermoFisher Scientific	Cat# 12563011
CultureSure dimethyl sulphoxide (DMSO)	FUJIFILM Wako Pure Chemical Corporation	Cat# 031–24051
collagenase type IV	ThermoFisher Scientific	Cat# 17104–019
Chemically Defined Lipid Concentrate	ThermoFisher Scientific	Cat# 11905031
L-Glutamine, 200 mM Solution	ThermoFisher Scientific	Cat# 25030–081
Recombinant Human/Mouse/Rat Activin A Protein	R&D Systems	Cat# 338-AC-010
Recombinant Human Oncostatin M (OSM) Protein	R&D Systems	Cat# 295-OM-010
Recombinant Human Wnt-3a Protein	R&D Systems	Cat# 5036-WN-010
Recombinant Human Sonic Hedgehog/Shh Protein, High Activity	R&D Systems	Cat# 8908-SH-005
Recombinant Human HGF Protein	R&D Systems	Cat# 294-HG-005
8-Bromo-cAMP, sodium salt	R&D Systems	Cat# 76939-46-3
1 mM CaCl$_2$	FUJIFILM Wako Pure Chemical Corporation	Cat# 036–00485
gelatin	FUJIFILM Wako Pure Chemical Corporation	Cat# 190–15805
gelatin from bovine skin Type B, powder, BioReagent, suitable for cell culture	Sigma-Aldrich	Cat# G9391
CultureSure A-83-01	FUJIFILM Wako Pure Chemical Corporation	Cat# 03924111

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bone Morphogenetic Protein 4 (truncated) (BMP-4), Human, recombinant, Animal-derived-free	FUJIFILM Wako Pure Chemical Corporation	Cat# 020–18851
1% Penicillin-Streptomycin Solution	FUJIFILM Wako Pure Chemical Corporation	Cat# 16823191
Forskolin	FUJIFILM Wako Pure Chemical Corporation	Cat# 067–02191
Dexamethasone	FUJIFILM Wako Pure Chemical Corporation	Cat# 047–18863
DAPT	FUJIFILM Wako Pure Chemical Corporation	Cat# 043–33581
human recombinant Insulin	FUJIFILM Wako Pure Chemical Corporation	Cat# 093–06471
MEM Essential Amino Acids Solution (×50)	FUJIFILM Wako Pure Chemical Corporation	Cat# 132–15641
MEM Non-essential Amino Acids Solution (×100)	FUJIFILM Wako Pure Chemical Corporation	Cat# 139–15651
Transferrin, Human, recombinant	FUJIFILM Wako Pure Chemical Corporation	Cat# 11096-37-0
Albumin, Human, recombinant expressed in plants	FUJIFILM Wako Pure Chemical Corporation	Cat# 018–21541
Y-27632	FUJIFILM Wako Pure Chemical Corporation	Cat# 257–00511
RO4929097	ChemScene LLC	Cat# CS-0480
SB505124	Cayman Chemical	Cat# CAY-11793-1
L-Ascorbic Acid 2-phosphate (magnesium salt)	Cayman Chemical	Cat# 16457
Primate ES Cell Medium	ReproCELL	Cat# RCHEMD001
Freezing Medium for human ES/iPS Cells (DAP213)	ReproCELL	Cat# RCHEFM001
Recombinant human bFGF (FGF2)	ReproCELL	Cat# RCHEOT002
ATRA	Tokyo Chemical Industry Co., Ltd	Cat# R0064
PVA [Poly(vinyl Alcohol)]	Tokyo Chemical Industry Co., Ltd	Cat# P0469
TTNPB	Tocris Bioscience	Cat# 0761/10
D-PBS(−)	Nacalai Tesque	Cat# 11482–15
0.25% trypsin	ThermoFisher Scientific	Cat# 27250018
0.1 mg/mL collagenase IV	ThermoFisher Scientific	Cat# 17104019
Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free	CORNING	Cat# 356230
B-27™ Supplement, minus insulin	Thermo Fisher Scientific K.K	Cat# A1895601
GlutaMAX™ Supplement	Thermo Fisher Scientific K.K	Cat# 35050061
PSC Cardiomyocyte Differentiation Kit	Thermo Fisher Scientific K.K	Cat# A2921201
PSC Neural Induction Medium	Thermo Fisher Scientific K.K	Cat# A1647801
Sufficient 100x Non-Essential Amino Acids (NEAA)	MP Biomedicals, LLC	Cat# IC1681049
Recombinant Human Activin A	BioLegend, Inc.	Cat# BL592006
Recombinant Human/Mouse/Rat Activin A Protein	R&D Systems	Cat# 338-AC-010
Recombinant Human BMP-4	PeproTech	Cat# 02018851
Bone Morphogenetic Protein 4 (truncated) (BMP-4)	FUJIFILM Wako	Cat# 020–18851
Human Fetal Liver Poly A + RNA	Clontech Laboratories, Inc.	Cat# 636108
A TransIT-mRNA Transfection Kit	Mirus Bio LLC	Cat# MIR2225
TOKIWA-Bio SRV iPS-1 Vector	TOKIWA-Bio Inc.	Cat# 383–19131
TOKIWA-Bio SRV iPS-2 Vector	TOKIWA-Bio Inc.	Cat# 380–19141
TOKIWA-Bio SRV iPS-3 Vector	TOKIWA-Bio Inc.	Cat# 385–19711
TOKIWA-Bio SRV iPS-4 Vector	TOKIWA-Bio Inc.	Cat# 385–19691
CytoTune-iPS 2.0	Medical & Biological Laboratories Co., Ltd.	Cat# IDT-DV0304
Freezing Medium for human ES/iPS Cells (DAP213)	ReproCELL	Cat# RCHEFM001
Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free	Corning	Cat# 356230
SB505124	Cayman Chemical	Cat# 11793

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Penicillin-Streptomycin Solution (×100)	FUJIFILM Wako Pure Chemical Corporation	Cat# 16823191
ICG	Sigma-Aldrich	Cat# 155020
D-GaIN	FUJIFILM Wako Pure Chemical Corporation	Cat# 07505013
EzRIPA Lysis kit	ATTO	Cat# WSE-7420
EzApply	ATTO	Cat# 2332330
EzStandard PrestainBlue	ATTO	Cat# 2332347
c-PAGEL 10%	ATTO	Cat# 2331955
EzRun	ATTO	Cat# 2332310
P plus membranes	ATTO	Cat# 2322451
Filter paper	ATTO	Cat# 2392393
EzFastBlot	ATTO	Cat# 2332590
EzBlock Chemi	ATTO	Cat# 2332615
EzTBS	ATTO	Cat# 2332625
EzWestBlue	ATTO	Cat# 2332456
Precision Plus Protein™ All Blue Prestained Protein Standards	Bio-Rad Laboratories, Inc.	Cat# 1610373
Hanks' Balanced Salt Solution (HBSS)	Life Technologies	Cat# 14025092
Critical commercial assays		
RNeasy Mini kit	Qiagen N.V.	Cat# 74106
SuperPREP II Cell Lysis & RT Kit for quantitative PCR	TOYOBO CO., LTD.	Cat# SCQ-401
FastStart Essential DNA Green Master	Roche	Cat# 06402712001
Luna Universal qPCR Master Mix	New England Biolabs Inc.	Cat# M3003E
TaqMan Array 96-Well FAST Plate(Human Stem Cell Pluripotency)	Applied Biosystems	Cat# 4418722
TaqMan™ Fast Advanced Master Mix	Thermo Fisher Scientific	Cat# 4444963
Proteome Profiler Human Pluripotent Stem Cell Array Kit	R&D Systems	Cat# ARY010
PAS staining kit	Muto Pure Chemicals	Cat# 15792
Experimental models: Cell lines		
Cryopreserved Hepatocytes Species:Human, Lot#S1412T, Lot#S1238 and Lot#S1350	KaLy-Cell	Cat# HHCPC-2M
hiPSC lines 201B7	CiRA Foundation	N/A
hiPSC lines 15M66	CiRA Foundation	N/A
MEF cells	ReproCELL Inc.	Cat# RCHEFC003
Oligonucleotides		
oligonucleotides sequences were listed in Table S1	–	–
Software and algorithms		
US National Library of Medicine National Institutes of Health website	National Institutes of Health (NIH)	RRID:SCR_011446 https://www.ncbi.nlm.nih.gov/pubmed/
Primer 3 Plus application	Primer 3 Plus	RRID:SCR_003081 http://www.bioinformatics.nl/cgi-bin/primer3plus/pimer 3 plus.cgi
R software program	R Development Core Team	RRID:SCR_001905 https://www.r-project.org

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
National Institutes of Health (NIH) Image J software program (Version 1.53)	National Institutes of Health (NIH)	RRID:SCR_003070 https://imagej.nih.gov/ij
StatPlus software program(StatPlus:mac LE)	AnalystSoft	RRID:SCR_014635 Build 8.0.1.0/ Core v7.7.11
NCBI Nucleotide	National Center for Biotechnology Information (NCBI)	RRID:SCR_004860 https://www.ncbi.nlm.nih.gov
Other		
LightCycler 96 Real-Time PCR system	Roche	Cat# 05 815 916 001
StepOnePlus system	Life Technologies	Cat# StepOnePlus-01
GeneChip Human Genome Ul33 Plus 2.0 Array	Applied Biosystems™	Cat# 900466
Invitrogen™ EVOS™ FL Auto Imaging System	ThermoFisher Scientific	Cat# AMAFD1000
BZ-X800 fluorescence microscop	KEYENCE CORPORATION	Cat# BZ-X800
myPower II 300	ATTO	Cat# AE-8135
HorizeBLOT 2M-R	ATTO	Cat# WSE-4025
BALB/cAJcl-nu/nu male mice	CLEA Japan	N/A
C.B-17/lcr-scid/scidJcl	CLEA Japan	N/A
StemFit AK03N	AJINOMOTO HEALTHY SUPPLY CO., INC.	N/A
RPMI 1640 with L-Gln, liquid medium	Nacalai Tesque	Cat# 30264–85
Ham's F-12 with L-Gln, liquid	Nacalai Tesque	Cat# 17458–65
IMDM	ThermoFisher Scientific	Cat# 12440053
KLC-TM medium	KaLy-Cell	Cat# KLC-TM
KLC-SM medium	KaLy-Cell	Cat# KLC-SM
Hepatocyte basal medium	Lonza	Cat# CC-3199
Primate ES Cell Medium	ReproCELL Inc.	Cat# RCHEMD001
KNOCKOUT serum replacement (KSR)	ThermoFisher Scientific	Cat# 10828028
Definitive Endoderm Induction Medium A	ThermoFisher Scientific	Cat# A3062601
Definitive Endoderm Induction medium B	ThermoFisher Scientific	Cat# A3062601

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Hirofumi Noguchi (noguchih@med.u-ryukyu.ac.jp).

Materials availability

Unique materials generated in this study are available from the lead contact upon reasonable request following the signing of a Materials Transfer Agreement.

Data and code availability

- The accession number for the microarray analysis data reported in this paper is GSE211436 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE211436).

- Microarray analysis data generated in this study were provided as CEL files (EA1583_04.CEL, EA1802_01.CEL, EA1802_02.CEL, EA2149_01.CEL).

- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Cell lines

Maintenance culture of hepatocytes

Human hepatocytes was obtained commercially (Cryopreserved Hepatocytes Species:Human, HHCPC-2M, Lot#S1412T, Lot#S1238 and Lot#S1350) (KaLy-Cell, Plobsheim, France). The hepatocytes were seeded from vials into culture dishes using the media and scaffold materials described in the designated culture protocol, cultured, and passaged. Briefly, the frozen hepatocytes were dissolved using KLC-TM medium (KaLy-Cell, Plobsheim, France). Hepatocytes were then seeded on two collagen-coated 100 mm dishes using KLC-SM medium (KaLy-Cell, Plobsheim, France). The medium was replaced after 6 and 24 h. After 48 h, the medium was changed using hepatocyte basal medium (CC-3199) (Lonza, Basel, Switzerland). Thereafter, the medium was changed every two days.

Establishment of hiPSCs

We used the commercially available SeV equipping 4 types of undifferentiated genes (OCT3/4, SOX2, KLF4 and C-MYC) to establish hiPSCs using human hepatocytes. The experiment was accomplished according to the manufacturer's instructions (CytoTune-iPS 2.0; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan).

hiPSCs and iTS-L cells were established using TOKIWA-Bio SRV iPS-1 Vector, TOKIWA-Bio SRV iPS-2 Vector, TOKIWA-Bio SRV iPS-3 Vector and TOKIWA-Bio SRV iPS-4 Vector according to the manufacturer's instructions (TOKIWA-Bio Inc., Ibaraki, Japan). In brief, 1×10^5 cells were dispensed into microtubes and centrifuged (300 g × 5 min). After removing the supernatant, 10 μL of the Vector supplied in the kit was added. Another 10 μL of human hepatocytes culture medium was then added, and the solution was incubated at 37°C for 2 h. Centrifugation was repeated (300 g × 5 min), followed by washing 3 times using human hepatocyte culture medium. Culture with human hepatocytes cell medium was then started, and 2/3 volume of StemFit AK03N medium was added on days 1, 3, 5, and 7 of culture. The medium was replaced with StemFit AK03N medium on days 9, 11, and 13 after culture initiation. Cell passaging was performed from day 15 of culture.

Method for examining SeV remaining in cells after subculture

A commercially available iPS Transgene/SeV detection primer set for CytoTune-iPS 2.0 (IDT-DV0304, Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was used for detection of the transfected SeV. The protocol followed the kit instructions. The designated base sequence was amplified by PCR using the primers included in the kit.

Maintenance culture of hiPSCs

The hiPSC lines 201B7 and 15M66 were established by Shinya Yamanaka (CiRA Foundation) and obtained from the CiRA Foundation (Kyoto, Japan). To culture iPSCs, a publicly available method (CiRA_Ff-iPSC_protocol_Eng_v140310) was used (https://www.cira.kyoto-u.ac.jp/j/research/img/protocol/Ff-iPSC-culture_protocol_E_v140311.pdf). The hiPSCs lines 201B7 were established by Shinya Yamanaka (Kyoto University) and were obtained from RIKEN BioResource Center (Ibaraki, Japan). The cells were cultured on a feeder layer of MEF cells (ReproCELL Inc., Kanagawa, Japan) and seeded at 1.5 × 10^5 cells per gelatin (gelatin from bovine skin Type B, powder, BioReagent, suitable for cell culture (G9391: Sigma-Aldrich, was used for detection of the transfected SeV, MO))-coated 10-cm plate. The cells were cultured in Primate ES Cell Medium (ReproCELL Inc., Kanagawa, Japan) under 5% CO_2. For passaging, hiPSCs colonies were treated with 0.25% trypsin (27250018: ThermoFisher Scientific, Tokyo, Japan) and 0.1 mg/mL collagenase IV (17104019: ThermoFisher Scientific, Tokyo, Japan) in D-PBS(−) (11482–15: Nacalai Tesque, Kyoto, Japan) containing 20% KSR (10828028: ThermoFisher Scientific, Tokyo, Japan) and 1 mM $CaCl_2$ (036–00485: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 37°C for 5 min, followed by tapping the cultures and flushing them with a pipette. Two volumes of culture medium were added, and the detached hiPSCs clumps were broken into smaller pieces by gentle pipetting. The passages were performed using a 1:3 split ratio. For storage, hiPSCs colonies were placed into Freezing Medium for human ES/iPS Cells (DAP213) according to the manufacturer's instructions (ReproCELL, Kanagawa, Japan). To prepare seeding hiPSCs for each cell assay, the hiPSCs were first detached from the feeder layer and partially dissociated as described for maintenance passage. Next, the contaminating MEF cells were removed by incubating the

cell suspension on a gelatin-coated plate at 37°C for 2 h in Primate ES Cell Medium with 10 μM Y-27632 (257–00511: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) to ensure high purity of the hiPSCs.

Cell differentiation assays

Hepatocytes were induced to differentiate from various hiPSCs cultured to confluence in 24-well plates in Primate ES Cell Medium (ReproCELL Inc., Kanagawa, Japan) (MEF-CM) according to the methods of previous studies (Ang et al., 2018; Nakamura et al., 2012). The wells were coated with Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free (356230: Corning, NY). In this study, we induced differentiation into hepatocytes in Figure 4 according to the differentiation induction protocol described by Ang et al.

[Day 1–2]. First, the anterior primitive streak was specified using Definitive Endoderm Induction Medium A (A3062601: ThermoFisher Scientific, Tokyo, Japan) for 24 h, followed by Definitive Endoderm Induction medium B (A3062601:ThermoFisher Scientific, Tokyo, Japan) for 24 h to induce definitive endoderm (Loh et al., 2014).

[Day 3]. The definitive endoderm cells were then differentiated into posterior foregut (PFG) using 1 μM CultureSure A-83-01 (03924111: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 ng/mL Recombinant human bFGF (FGF2) (RCHEOT002: ReproCELL, Kanagawa, Japan), 30 ng/mL Bone Morphogenetic Protein 4 (truncated) (BMP-4) (02018851: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 2 μM ATRA (R0064:Tokyo Chemical Industry Co., LTD. Tokyo, Japan) or 75nM TTNPB (0761/10: Tocris Bioscience, Bristol, UK) in CDM3 medium (10% KSR (10828028: ThermoFisher Scientific, Tokyo, Japan), 0.1% PVA [Poly(vinyl Alcohol)] (P0469:Tokyo Chemical Industry Co., LTD. Tokyo, Japan), IMDM (12440053:ThermoFisher Scientific, Tokyo, Japan)/F12 (17458–65:Nacalai Tesque, Kyoto, Japan) (1:1), 1% Chemically Defined Lipid Concentrate (11905031:ThermoFisher Scientific, Tokyo, Japan) and 1% Penicillin-Streptomycin Solution (×100) (16823191: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for another 24 h.

[Day 4–6]. To further differentiate PFG to liver bud progenitors, one of two related types of differentiation conditions were used on days 4–6 of differentiation using 10 ng/mL Recombinant Human/Mouse/Rat Activin A Protein (338-AC-010: R&D Systems, Minneapolis, MN), 30 ng/mL Bone Morphogenetic Protein 4 (truncated) (BMP-4), Human, recombinant, Animal-derived-free (020–18851: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 1 mM 8-Bromo-cAMP, sodium salt (76939-46-3: R&D Systems, Minneapolis, MN) or 1 μM Forskolin (067–02191: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in CDM3 medium for 3 days (day 4 to 6).

[Day 7–8]. To further differentiate PFG to liver bud progenitors, one of two related types of differentiation conditions were used on days 7–8 of differentiation using 1 μM SB505124 (11793: Cayman Chemical, Arbor, MI), 10 ng/mL Bone Morphogenetic Protein 4 (truncated) (BMP-4) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 ng/mL Recombinant Human Oncostatin M (OSM) Protein (295-OM-010: R&D Systems, Minneapolis, MN), 10 μM Dexamethasone (047–18863: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 2 μM RO4929097 (CS-0480: ChemScene LLC, Monmouth Junction, NJ) or 10 μM DAPT (043–33581: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 μM Forskolin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 μg/mL human recombinant Insulin (093–06471: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 200 μg/mL L-Ascorbic Acid 2-phosphate (magnesium salt) (16457: Cayman Chemical, Arbor, MI) in CDM4 medium (amino acid supplements (MEM Essential Amino Acids Solution (×50) (132–15641: FUJIFILM Wako Pure Chemical Corporation) and MEM Non-essential Amino Acids Solution (×100) (139–15651: FUJIFILM Wako Pure Chemical Corporation)), 15 μg/mL Transferrin, Human, recombinant (11096-37-0: FUJIFILM Wako Pure Chemical Corporation), IMDM(12440053: ThermoFisher Scientific, Tokyo, Japan)/F12 (17458–65:Nacalai Tesque, Kyoto, Japan) (1:1), 1% Chemically Defined Lipid Concentrate (11905031:ThermoFisher Scientific, Tokyo, Japan) and 1% Penicillin-Streptomycin Solution (×100) (16823191: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

[Day 9–12]. To further differentiate PFG to liver bud progenitors, one of two related types of differentiation conditions were used on days 9–12 of differentiation using 10 ng/mL Bone Morphogenetic Protein 4 (truncated) (BMP-4) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 ng/mL Recombinant Human Oncostatin M (OSM) Protein (295-OM-010: R&D Systems, Minneapolis, MN), 10 μM Dexamethasone (047–18863: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 2 μM RO4929097 (CS-0480: ChemScene LLC,

Monmouth Junction, NJ) or 10 μM DAPT (043–33581: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 μM Forskolin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 μg/mL human recombinant Insulin (093–06471: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 200 μg/mL L-Ascorbic Acid 2-phosphate (magnesium salt) (16457: Cayman Chemical, Arbor, MI) in CDM4 medium.

[Day 13–18]. Subsequently, hiPSC-derived hepatocyte-like cells were further treated with 10 μM Dexamethasone (047–18863: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 μM Forskolin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 μg/mL human recombinant Insulin (093–06471: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 200 μg/mL L-Ascorbic Acid 2-phosphate (magnesium salt) (16457: Cayman Chemical, Arbor, MI), and 2 μM RO4929097 (CS-0480: ChemScene LLC, Monmouth Junction, NJ) or 10 μM DAPT (043–33581: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in CDM5 base medium (CDM4 basal medium, supplemented with 10% KSR (10828028: ThermoFisher Scientific, Tokyo, Japan) and without the additional amino acid-rich mixture) for 6 days (day 13 to 18).

In Figure S31, differentiation into hepatocytes was performed according to the differentiation induction protocol described by Nakamura et al.

[Day 1]. hiPSCs were induced to differentiate into endoderm (phase I) using 2mM L-Glutamine, 200 mM Solution (25030–081: ThermoFisher Scientific, Tokyo, Japan), 100 μM 2-mercaptoethanol (M3148-25ML:Sigma-Aldrich, St. Louis, MO), 1% Penicillin-Streptomycin Solution (✕100) (16823191: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 100 ng/mL Recombinant Human/Mouse/Rat Activin A Protein (338-AC-010: R&D Systems, Minneapolis, MN), 25 ng/mL Recombinant Human Wnt-3a Protein (5036-WN-010: R&D SYSTEMS, Minneapolis, MN), 0.29% Albumin, Human, recombinant expressed in plants (018–21541: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in RPMI 1640 with L-Gln, liquid medium (30264–85: Nacalai Tesque, Kyoto, Japan) for 24 h.

[Day 2]. hiPSCs were induced to differentiate into endoderm (phase II) using 1% Penicillin-Streptomycin Solution (✕100) (16823191: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 100 ng/mL Recombinant Human/Mouse/Rat Activin A Protein (338-AC-010: R&D Systems, Minneapolis, MN) in RPMI 1640 with L-Gln, liquid medium (30264–85: Nacalai Tesque, Kyoto, Japan), supplemented with 0.2% KSR (10828028: ThermoFisher Scientific, Tokyo, Japan) for 24 h.

[Day 3–7]. hiPSC-derived endoderm was further treated with 1% Penicillin-Streptomycin Solution (✕100) (16823191: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 20 ng/mL Bone Morphogenetic Protein 4 (truncated) (BMP-4) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 200 ng/mL Recombinant Human Sonic Hedgehog/Shh Protein, High Activity (8908-SH-005: R&D SYSTEMS, Minneapolis, MN), 10 ng/mL bFGF (FGF2) (ReproCELL Inc., Kanagawa, Japan) in RPMI 1640 with L-Gln, liquid medium (30264–85: Nacalai Tesque, Kyoto, Japan), supplemented with 2% KSR (10828028: ThermoFisher Scientific, Tokyo, Japan) for 5 days for the induction of hepatocytes.

[Day 8–12]. hiPSC-derived endoderm was further treated with 1% Penicillin-Streptomycin Solution (✕100) (16823191: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 20 ng/mL Recombinant Human HGF Protein (294-HG-005: R&D SYSTEMS, Minneapolis, MN), 20 ng/mL Bone Morphogenetic Protein 4 (truncated) (BMP-4) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 ng/mL bFGF (FGF2) (ReproCELL Inc., Kanagawa, Japan) in RPMI 1640 with L-Gln, liquid medium (30264–85: Nacalai Tesque, Kyoto, Japan), supplemented with 2% KSR (10828028: ThermoFisher Scientific, Tokyo, Japan) for 5 days in initial stage of hepatic maturation.

[Day 13–18(-28)]. hiPSC-derived endoderm was further treated with 1% Penicillin-Streptomycin Solution (✕100) (16823191: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 ng/mL Recombinant Human Oncostatin M (OSM) Protein (295-OM-010: R&D Systems, Minneapolis, MN), 0.1 μM Dexamethasone (047–18863: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in RPMI 1640 with L-Gln, liquid medium (30264–85: Nacalai Tesque, Kyoto, Japan), supplemented with 2% KSR (10828028: ThermoFisher Scientific, Tokyo, Japan) for 6–16 days in the latter period of hepatic maturation.

Differentiation assay to three germ layers

Cardiomyocyte differentiation. To induce differentiation to myocardial cells, hiPSCs were cultured in six-well plates in StemFit AK03N medium to confluence on a support using a PSC Cardiomyocyte Differentiation Kit, according to the manufacturer's instructions(https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0014509_psc_cardiomyocyte_diff_PI.pdf) (Thermo Fisher Scientific K.K.).

Definitive endoderm differentiation. To induce differentiation to neuroprogenitor cells, hiPSCs were cultured in six-well plates in StemFit AK03N medium to confluence. The induction of differentiation of definitive endoderm (Si-Tayeb et al., 2010) followed the previously reported protocol. In brief, endoderm differentiation was performed with media of the following composition:

   [Day 1]. Media: RPMI 1640 with GlutaMAX + B27 (-insulin) + NEAA (1%) with Activin A (100 ng/mL), BMP4 (50 ng/mL) and CHIR99021 (3μM).

   [Day 2–7]. Media: RPMI 1640 GlutaMAX + B27 (-insulin) + NEAA (1%) with Activin A (100 ng/mL) and BMP4 (50 ng/mL); Change medium daily.

   [Day 8–10]. Media: RPMI 1640 with GlutaMAX + B27 (+insulin) + NEAA (1%) with bFGF (10 ng/mL), BMP4 (50 ng/mL) and HGF (10 ng/mL); Change medium daily.

Neuroprogonitor cell differentiation. To induce differentiation to neuroprogonitor cells, hiPSCs were cultured in six-well plates in StemFit AK03N medium to confluence on a support using a PSC Neural Induction Medium, according to the manufacturer's instructions(https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fpsc_neural_induction_medium_man.pdf) (Thermo Fisher Scientific K.K.).

Mouse model

BALB/cAJcl-nu/nu male mice (age: 6 weeks; CLEA Japan, Tokyo, Japan) and C.B-17/Icr-scid/scidJcl male mice (age: 6 weeks; CLEA Japan) were maintained under controlled temperature (23 $\pm$ 2°C) and light conditions (lights on from 08:30–20:30) and fed standard rodent chow pellets (Oriental Yeast Co., Ltd., Tokyo, Japan) with *ad libitum* access to water.

METHOD DETAILS

Real time PCR

Cells were cultured in Tissue culture flask 25 cm^2 (TPP, 390026) or designated cell culture plate in Primate ES Cell Medium (ReproCELL Inc., Kanagawa, Japan) or StemFit AK03N (AJINOMOTO HEALTHY SUPPLY CO., INC., Tokyo, Japan) to approximately 80% confluence. RNA was prepared using a RNeasy Mini kit (Qiagen N.V., Hilden, Germany) or a SuperPREP II Cell Lysis & RT Kit for quantitative PCR (TOYOBO CO., LTD., Osaka, Japan) according to the manufacturer's instructions. Real-time PCR analyses were performed using a LightCycler 96 Real-Time PCR system (Roche, Basel, Switzerland) or a StepOnePlus system (Life Technologies, Carlsbad, CA, USA). The PCR protocol was as follows: (1) initial denaturation at 95°C for 10 min; (2) denaturation at 95°C for 15 s; continued denaturation of the double-stranded DNA; (3) annealing of primers at 60°C for 60 s, repeat steps (2)-(3) 40 times; (4) denaturation at 95°C for 15 s, annealing of primers at 60°C for 60 s, and denaturation at 95°C for 15 s [Melt Curve Stage]. FastStart Essential DNA Green Master (Roche) or Luna Universal qPCR Master Mix (New England Biolabs Inc., Ipswich, MA, USA) was used according to the manufacturer's instructions. For the mRNA expression analysis, a TaqMan Array 96-Well FAST Plate (Human Stem Cell Pluripotency; Applied Biosystems) was used. TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific) was used according to the manufacturer's instructions. The PCR protocol was as follows: (1) denature at 95°C for 20 s, continue denaturing the double-stranded DNA; (2) anneal primers at 60°C for 20 s, repeat steps (1)-(2) 40 times.

The expression was calculated using the ΔΔCt method. The expression of the target gene was corrected by the expression of the housekeeping gene. For the design of primers other than the primers cited in other papers, the gene names were retrieved from the US National Library of Medicine National Institutes of Health website (https://www.ncbi.nlm.nih.gov/pubmed/). The primers were designed using the Primer 3

Plus application (http://www.bioinformatics.nl/cgi-bin/primer3plus/pimer 3 plus.cgi). The primers used for PCR have been described previously (Hamasaki et al., 2012; Nakamura et al., 2012; Okita et al., 2011; Takahashi et al., 2007b). Oligonucleotides sequences are listed in Table S1.

Protein array

Stem cell-specific marker proteins were measured using the Proteome Profiler Human Pluripotent Stem Cell Array Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. In brief, the hiPSCs were seeded on a feeder layer of MEF cells (ReproCELL Inc., Kanagawa, Japan) and seeded at 1.5×10^5 cells per 10-cm plate. iPSCs were cultured in 100 mm dishes in Primate ES Cell Medium (ReproCELL Inc., Kanagawa, Japan) to ~80% confluence. To prepare sampling hiPSCs for cell assay, the hiPSCs were first detached from the feeder layer and partially dissociated as described for maintenance passage. Next, the contaminating MEF cells were removed by incubating the cell suspension on a gelatin-coated plate at 37°C for 2 h in Primate ES Cell Medium (ReproCELL Inc., Kanagawa, Japan) with 10 μM Y-27632(257–00511: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) to ensure the high purity of the hiPSCs. 1 mL cell lysate from the 100 mm dish was prepared using an EzRIPA Lysis kit according to the manufacturer's instructions (ATTO, Tokyo, Japan).

Microarray analysis

The measurement was outsourced to Takara Bio (Kusatsu, Shiga), and the GeneChip Human Genome Ul33 Plus 2.0 Array was used for the analysis. The data were subjected to a heat map analysis, cluster analysis, and principal component analysis according to the usual method using the R software program (https://www.r-project.org).

Immunofluorescence staining analysis

Hepatocytes differentiated from iPSCs were examined for EpCAM protein expressed on the cell surface using immunostaining. Immunofluorescence staining was achieved using specific antibodies for Human EpCAM aa 250 (Abcam, Cambridge, UK), goat anti-Rabbit IgG H&L (Alexa Fluor 488) (Abcam), Anti HNF-4α (H-1) (Alexa Fluor 647) (Santa Cruz Biotechnology, Inc.), and Cellstain ® - Hoechst 33342 solution (DOJINDO LABORATORIES, Kumamoto, Japan). Images were recorded using an Invitrogen™ EVOS™ FL Auto Imaging System (ThermoFisher Scientific, Tokyo, Japan) or BZ-X800 fluorescence microscope (KEYENCE CORPORATION, Osaka, Japan).

Periodic Acid Schiff (PAS) assay for glycogen storage

Glycogen storage was measured by PAS staining using a PAS staining kit (Muto Pure Chemicals, Tokyo, Japan) in accordance with the manufacturer's instructions.

Cellular uptake and release of Indocyanine Green (ICG)

ICG (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO to make a stock at 5 mg/mL and then freshly diluted in culture medium to 1 mg/mL. After incubating the cells with ICG (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, the medium with ICG was discarded and washed three times with phosphate-buffered saline, and the cellular uptake of ICG was examined by microscopy. Cells were then returned to the culture medium and incubated for 6 h for the release of cellular ICG stain.

Hepatocyte toxicity assay by D-galactosamine (D-GalN)

The cells were treated with 25 mM D-GalN for 24 h at 37 °C, and then the supernatant was collected. The measurement of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), γ-glutamyl transpeptidase (γ-GTP), leucine aminopeptidase (LAP), and isozymes of lactate dehydrogenase (LDH) in the culture medium was outsourced to SRL, Inc. (Tokyo, Japan).

Cell proliferation assay

Cell proliferation tests were performed on iPSCs created from hepatocytes and expressing hepatocyte-specific markers until the 10th passage (Shown as representative data: No. 33 and No. 41, which continued to express hepatocyte-specific markers until passage 20). On day 4 after seeding the cells in 6 wells, the cells were detached, and the number of cells was measured according to a conventional technique using

trypan blue. Thereafter, 1/16 of the iPSCs were reseeded in 6 wells, and the number of cells was measured 4 days later. This operation was repeated to perform a cell proliferation test until the 20th passage.

Western blotting

Western blotting analyses using an ATTO Products; EzRIPA Lysis kit, cPAGE Twin, myPower II 300, HorizeBLOT 2M-R, EzApply, EzStandard PrestainBlue, c-PAGEL 10%, EzRun, P plus membranes, Filter paper, EzFastBlot, EzBlock Chemi, EzTBS and EzWestBlue were performed according to the manufacturer's instructions (ATTO, Tokyo, Japan). Precision Plus Protein™ All Blue Prestained Protein Standards were used according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Blots were probed using specific antibodies for OCT3/4, Nanog, SOX2, E-Cadherin, AFP, GATA-4, HNF-3β, PDX-1, SOX17, OTX2, TP63/TP73L, Goosecoid, Snail, VEDF R2, HCG and β-actin. Images were quantified using the National Institutes of Health (NIH) Image J software program (Version 1.53; https://imagej.nih.gov/ij).

Animal care

All experimental protocols were in accordance with the guidelines for the care and use of laboratory animals set by Research Laboratory Center, Faculty of Medicine, and the Institute for Animal Experiments, Faculty of Medicine, University of the Ryukyus (Okinawa, Japan). The experimental protocol was approved by the Committee on Animal Experiments of University of the Ryukyus (permit number: R2016008). BALB/cAJcl-nu/nu male mice (age: weeks; CLEA Japan, Tokyo, Japan) and C.B-17/Icr-scid/scidJcl male mice (age: weeks; CLEA Japan, Tokyo, Japan) were maintained under controlled temperature (23 $\pm$ 2°C) and light conditions (lights on from 08:30–20:30) and fed standard rodent chow pellets (Oriental Yeast Co., Ltd., Tokyo, Japan) with *ad libitum* access to water. All efforts were made to minimize the suffering of the animals.

Teratoma formation assay

Immunodeficient male mice (age: 7 weeks; BALB/cAJcl-nu/nu (CLEA Japan, Inc., Tokyo, Japan)) and immunodeficient male mice (age: 7 weeks; C.B-17/Icr-scid/scidJcl (CLEA Japan, Inc., Tokyo, Japan)) were used for the teratoma formation assays. In brief, the teratoma formation mouse model was established by anesthetizing recipient mice with isoflurane inhalation (WAKO). For xenotransplantation of hiPSCs, 1.0×10^6 or more cells in 0.1 mL of cold Hanks' Balanced Salt Solution (HBSS) (Life Technologies) were subcutaneously injected into the shoulders and buttocks using a 22G injection needle (Terumo, Tokyo, Japan). The mice were examined daily, and tumors were extracted at 10 or 15 weeks after surgery. Teratoma samples were resected and fixed with 4% paraformaldehyde.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Student's *t*-test to compare the means of two samples. The analyses of multiple groups (i.e. more than two groups) were performed using one- and two-way ANOVAs with the StatPlus software program (AnalystSoft, Walnut, CA, USA). Statistical significance was set at *$p < 0.05$ or **$p < 0.01$ for all tests. The data shown are representative examples of two independent experiments.