inducer of fetal hepatic cells in the presence of glucocorticoid [8,9]. Differentiation from human ES and iPS cells toward mature hepatocyte-like cells is induced by sequential addition of cytokines or transfection of genes involved in embryonic liver organogenesis [10,11,12,13]. However, it is still difficult to obtain large numbers of highly functional hepatocytes from human iPS cells. In this regard, differentiation from human iPS cells toward highly proliferative hepatic progenitor cells will provide a method to obtain large quantities of hepatocytic cells.

Because differentiation from iPS cells toward hepatic lineage cells mimics *in vivo* step-wise developmental processes, human iPS cell-derived hepatic progenitor-like cells (HPCs) might exist at an appropriate time point during similar *in vitro* differentiation steps. Endodermal progenitor cells were established from human pluripotent cells, and these cells can differentiate into several endodermal lineage cells, such as pancreatic β cells, hepatocytes, and intestinal epithelial cells [14]. It has been recently shown that hepatic progenitor cells can be isolated from differentiated human ES cells using the cell surface marker N-cadherin [15]. However, methods for effective purification and cultivation of human iPS-derived HPCs have not been well established. We previously found that CD13 and CD133 are mouse hepatoblast-specific cell surface markers during the early and middle (E 9.5–14.5) stages of fetal development [16,17]. Mouse $CD13^{+}CD133^{+}$ liver cells in the middle stage of fetal development express hepatic genes and differentiate into hepatocytic cells and cholangiocytic cells *in vitro* and *in vivo*. However, it remains unknown whether these mouse hepatoblast-specific markers are common to human hepatic progenitor cells. In addition, we previously reported that mouse embryonic fibroblasts (MEFs) have the ability to support *in vitro* proliferation of mouse hepatoblasts and hepatic gene expression [17]. In this study, because MEFs can be substituted for non-parenchymal cells in the liver, human iPS cell-derived HPCs were co-cultured with MEFs. Taken together, our data demonstrate that HPCs from human iPS cells can be highly purified using cell surface markers CD13 and CD133. Further investigation revealed that human iPS cell-derived HPCs exhibit a long-term proliferative potential and maintain bipotent differentiation toward hepatocytic cells and cholangiocytic cells.

Materials and Methods

Human iPS and ES cells and other-types of cells

A human iPS cell line, TkDA3-4, was established from human dermal fibroblasts (Cell Applications, Inc., San Diego, CA) as described previously [18]. Human ES cells (KhES-3) were obtained from the Institute for Frontier Medical Sciences, Kyoto University (Kyoto, Japan), with approval for human ES cell use granted by the Ministry of Education, Culture, Sports, Science, and Technology of Japan [19]. The Review Board of the Institute of Medical Science, The University of Tokyo, approved this research. The entire study was conducted in accordance with the Declaration of Helsinki. Human ES cells and human iPS cells were maintained according to previously described standard methods [18]. Briefly, they were cultured on irradiated mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium/F-12 medium (DMEM/F-12; Sigma, St. Louis, MO) supplemented with 0.1 mM nonessential amino acids (Invitrogen, Carlsbad, CA), 1× penicillin streptomycin glutamine (Sigma), 20% knockout serum replacement (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), and 5 ng/ml recombinant human basic fibroblast growth factor (basic FGF; Wako Pure Chem., Osaka, Japan). These cells were maintained on mitomycin C (Wako Pure Chem.)-treated MEFs. ES and iPS cells were passaged every 5 days to maintain them in an undifferentiated state.

HepG2 (RCB1886) cells were provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cryopreserved human hepatocytes (Lot HEP187242; BioPredic International, Rennes, France) were used.

Preparation of MEFs

E13.5 ICR or C57BL/6-Tg (CAG-EGFP) mouse embryos (Nihon SLC, Shizuoka, Japan) were dissected, and the head and internal organs were completely removed. The torso was minced and dissociated in 0.05% trypsin-EDTA (Sigma) for 30 min. After washing with phosphate-buffered saline (PBS; Sigma), cells were expanded in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1× penicillin streptomycin glutamine. MEFs were treated with mitomycin C at 37°C for 2 h and used as feeder cells. Animal experiments were performed with approval of the Institutional Animal Care and Use Committee of the Institute of Medical Science, the University of Tokyo (permit number: PA09-21) and the Institutional Animal Care and Use Committee of Tokai University (permit number: 122047).

Differentiation from human iPS cells toward the hepatic lineage and isolation of HPCs

For hepatic lineage cell differentiation, semi-confluent human iPS cells were cultured in RPMI 1640 (Sigma) containing 2% B27 supplement (Invitrogen). Cells were stimulated with 100 ng/ml recombinant human activin A (PeproTech, Rocky, NJ) at day 0–4, 10 ng/ml basic FGF and 20 ng/ml recombinant human BMP-4 (PeproTech) at day 5–8, and 40 ng/ml recombinant human HGF (PeproTech) at day 9–12. Cells were cultured at 37°C in a 10% O_2 incubator at day 0–4 and in 5% O_2 at day 5–12.

HPCs were isolated from human iPS cells stimulated with cytokines. After 12 days of culture, cells were trypsinized using 0.05% trypsin-EDTA (Sigma). Trypsinized cells were washed with PBS containing 3% FBS, and then incubated with antibodies against cell surface proteins (shown in **Table S1**) for 1 h at 4°C. After washing with PBS containing 3% FBS and staining the dead cells with propidium iodide, the cells were analyzed and sorted using a MoFlo™ fluorescence-activated cell sorter (Dako, Glostrup, Denmark) and a FACSAria cell sorter (Becton Dickinson, Franklin Lakes, NJ).

HPC culture and passaging

Mitomycin C-treated MEFs (2×10^5 cells/well) were plated onto 0.1% gelatin (derived from porcine skin; Sigma)-coated 12-well plates the day before sorting. $CD13^{high}CD133^{+}$ cells were sorted onto MEF feeder layers plated at a low density (280 cells per cm^2). The standard culture medium was a 1:1 mixture of H-CFU-C medium and DMEM. H-CFU-C medium consisted of DMEM/F-12 supplemented with 10% FBS (Nichirei Biosciences, Tokyo, Japan), 1× Insulin-Transferrin-Selenium X (Invitrogen), 10 mM nicotinamide (Sigma), 10^{-7} M dexamethasone (Sigma), 2.5 mM HEPES buffer solution (Invitrogen), 1× penicillin streptomycin glutamine, and 1× nonessential amino acids. For expansion, the cells were cultured in standard medium supplemented with 0.25 μM A-83-01 (Tocris Bioscience, Bristol, United Kingdom), 10 μM Y-27632 (Wako Pure Chem.), 40 ng/ml HGF, and 20 ng/ml recombinant human epidermal growth factor (EGF; PeproTech). The medium was replaced every 3 days. After 10–12 days of culture, colonies formed by individual human iPS cell-derived $CD13^{high}CD133^{+}$ cells were trypsinized using 0.05% trypsin-EDTA and replated onto freshly plated MEFs.

Isolation of secondary CD13$^+$ and/or CD133$^+$ cells from human iPS cell-derived HPCs

After differentiation of human iPS cells induced by cytokines, CD13^{high}CD133$^+$ cells were cultured on MEFs derived from C57BL/6-Tg (CAG-EGFP) mice. After 12 days of culture, HPC colonies were trypsinized and stained with PE- and APC-conjugated antibodies against human CD13 and CD133, respectively. CD13$^+$ and/or CD133$^+$ cells in the GFP-negative fraction were sorted using a MoFlo^{TM} fluorescence-activated cell sorter. Sorted cells were seeded onto freshly plated MEFs, and the colony forming activities of these cells were analyzed.

Immunocytochemistry

Cultured cells were washed with PBS and fixed with 4% paraformaldehyde/PBS. After three washes with PBS, cells were permeabilized with 0.25% Triton X-100 (Sigma)/PBS for 10 min, washed with PBS, and incubated with 5% donkey serum (Millipore, Bedford, MA) in PBS for 1 h at room temperature. The cells were then incubated with diluted primary antibodies either overnight at 4°C. Primary antibodies are listed in **Table S1**. The cells were washed with PBS several times, and then incubated with diluted secondary antibodies for 40 min at room temperature. Then, the cells were washed with PBS and their nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma). Normal goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA), negative control mouse IgG1 (Dako), and normal rabbit IgG (Santa Cruz Biotechnology) were used as negative controls for the appropriate antibodies. Colonies were imaged under a Carl Zeiss Axio Observer Z1 using AxioVision version 4.8 software (Carl Zeiss, Jena, Germany). α-fetoprotein (AFP)- and hepatocyte nuclear factor 4α (HNF4α)-positive colonies were counted using an ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, MA).

Induction of mature hepatocytic functional genes by spheroid formation

Colonies derived from CD13^{high}CD133$^+$ cells were passaged and trypsinized with 0.05% trypsin-EDTA, washed in DMEM containing 10% FBS, and counted. The culture medium for spheroid formation was DMEM supplemented with 10% FBS, 1× nonessential amino acids, 1× penicillin streptomycin glutamine, and 10^{-7} M dexamethasone, with or without 20 ng/ml recombinant human OSM (R&D Systems, Minneapolis, MN). Individual drops (40 µl) containing 1×10^4 cells were plated on the inside of lids of 100-mm dishes containing PBS (to avoid desiccation). After 3 days of culture, the spheroids were collected and analyzed.

Real-time RT-PCR analysis

For analysis of hepatic functional gene expressions, total RNA was extracted from human iPS cells, colonies derived from CD13^{high}CD133$^+$ cells, and spheroids using TRIzol (Invitrogen). First-strand cDNA synthesized using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) was used as a template for PCR amplification. For semiquantitative RT-PCR, cDNA samples were normalized by the number of hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA copies. The Universal Library (Roche Diagnostics, Basel, Switzerland) was used for quantitative RT-PCR assays of AFP, catechol-O-methyltransferase (COMT), chemokine (C-X-C motif) receptor 4 (CXCR4), cytochrome P450 (CYP) 3A4, CYP3A7, CYP7A1, epoxide hydrolase 1, microsomal (xenobiotic) (EPHX1), flavin containing monooxygenase 5 (FMO5), goosecoid homeobox (GSC), hematopoietically expressed homeobox (hHex), hepatocyte nuclear factor (HNF) 3β, HNF4α, HPRT1, monoamine oxidase A (MAOA), MAOB, Mix paired-like homeobox (MIXL1), one cut homeobox 1 (ONECUT1), Sox17, sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1 (SULT1A1), SULT1A2, and T transcripts. The primer sequences and probe numbers for each gene are shown in Table S2.

Induction of cholangiocytic cyst formation by human iPS cell-derived CD13^{high}CD133$^+$ cells

Colonies derived from CD13^{high}CD133$^+$ cells were passaged and trypsinized using 0.05% trypsin-EDTA, washed in DMEM containing 10% FBS, and then counted. The cells were then combined with an extracellular matrix gel consisting of a mixture of 40% collagen type-I (Nitta Gelatin, Osaka, Japan) and 40% Matrigel (BD Biosciences, Bedford, MA), and cultured in 24-well culture plates (1500 cells/50 µl extracellular matrix gel/well). After the 10 min incubation, culture medium was added, followed by incubation for 10–12 days with medium changes every 3 days. The culture medium was a 1:1 mixture of H-CFU-C medium and DMEM/F-12 supplemented with 2% B27 supplement, 0.25 µM A-83-01, 10 µM Y-27632, 20 ng/ml EGF, 40 ng/ml HGF, 40 ng/ml recombinant human Wnt-3a (R&D Systems), and 100 ng/ml recombinant human R-spondin 1 (PeproTech). Cysts in gels were stained according to previously described methods [20], and analyzed under a LSM700 confocal microscope (Carl Zeiss). The antibodies are listed in Table S1.

Albumin secretion assay

To detect human albumin secretion, colonies from human iPS cell-derived HPCs (3rd culture) were cultured for a long time (19 days). These cells were differentiated by cell-cell interactions. The differentiated cells and HepG2 cells (standard control) were incubated for 3 days in hepatocyte differentiation medium. The hepatocyte differentiation medium was DMEM supplemented with 10% FBS, 1× nonessential amino acids, 1× penicillin streptomycin glutamine, 10^{-7} M dexamethasone, and 1% dimethyl sulfoxide (Sigma). Conditioned medium was saved and debris was removed by centrifugation. Human albumin was detected using a Human Albumin ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's protocol.

Statistics

Calculations of statistically significant differences between samples using the Student's two-tailed t test as well as the standard deviation (SD) were performed using Microsoft Excel 2007 software.

Results

Differentiation from human iPS cells toward hepatic lineage cells

Human ES cells and iPS cells have been reported to differentiate into hepatocytes using a stage-wise process that mimics developmental processes [10,11]. We speculated that human iPS cell-derived hepatic lineage cell cultures contained HPCs induced by several cytokines such as activin A, basic FGF, BMP-4, and HGF. For hepatic lineage cell differentiation, TkDA3-4 human iPS cells were seeded onto mitomycin C-treated MEFs and stimulated with the cytokines shown in **Figure 1A**. After 12 days of culture, expression of fetal hepatocytic markers (AFP and HNF4α) were observed in cells stimulated with cytokines (**Figure 1B**). We then tried to isolate HPCs from hepatic lineage

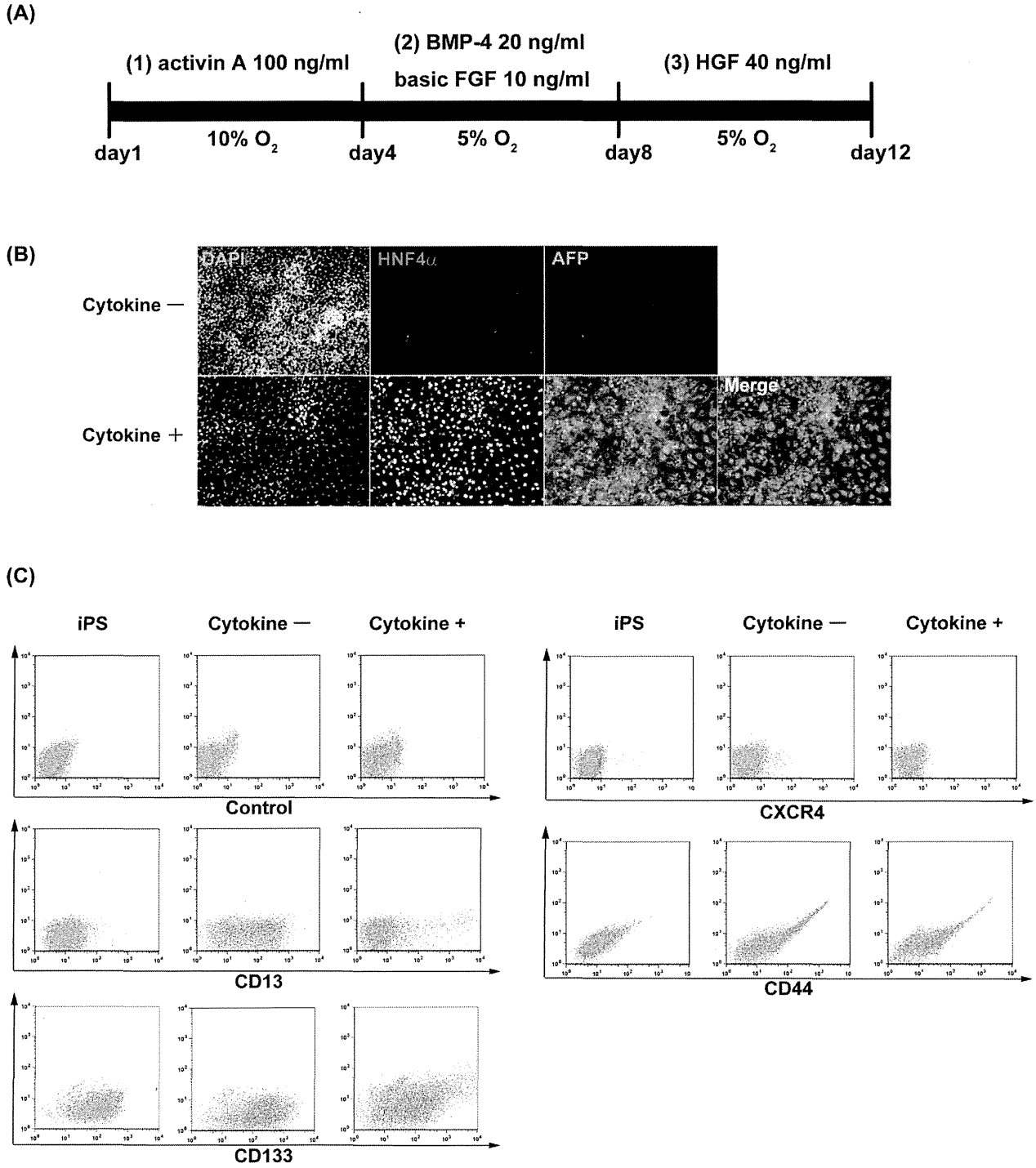

Figure 1. Differentiation from human iPS cells toward hepatic lineage cells. (**A**) Schematic of the experimental procedure. Human iPS cells were sequentially stimulated with various cytokines: (1) activin A, (2) basic FGF and BMP-4, and (3) HGF. The cells were cultured in 10% O_2 for days 0–4 and 5% O_2 for days 5–12. (**B**) After 12 days of culture with or without cytokines, cells were stained with antibodies against AFP and HNF4α. Nuclei were counterstained with DAPI. (**C**) Expression of cell surface markers in human iPS cell-derived hepatic lineage cells.
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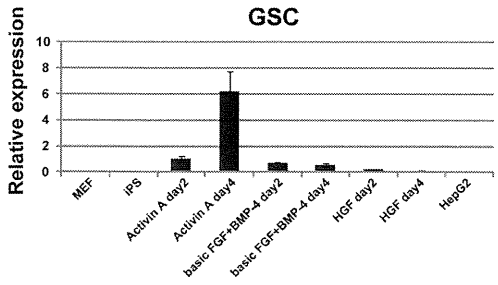
cell cultures. As shown previously, several cell surface markers, such as CD34, CD44, CD49f, CD56, CD117, CD326, and CXCR4, have been reported to be expressed in mouse and human endodermal and hepatic progenitor cells, and fetal hepatic cells [21,22,23,24,25,26]. We recently found that CD13 and CD133 are specific cell surface markers for mouse hepatoblasts [16,17]. To characterize human iPS cell-derived hepatic lineage cells, undifferentiated human iPS cells and cells cultured with or without cytokines were analyzed with antibodies against hepatic progenitor cell-related surface markers by flow cytometry. CD13 was barely expressed in human iPS cells, but was induced during the 12 days of culture without cytokines, indicating that spontaneous differen-


GSC
Relative expression
MEF
iPS
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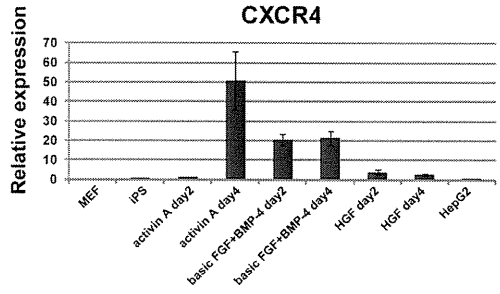
Activin A day4
basic FGF+BMP-4 day2
basic FGF+BMP-4 day4
HGF day2
HGF day4
HepG2


CXCR4
Relative expression
MEF
iPS
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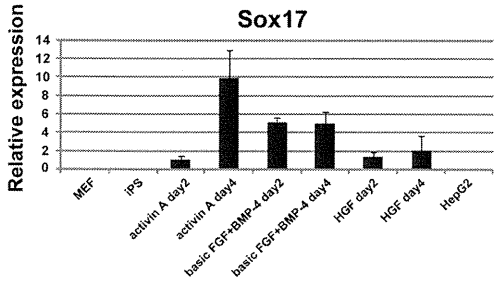
activin A day4
basic FGF+BMP-4 day2
basic FGF+BMP-4 day4
HGF day2
HGF day4
HepG2


Sox17
Relative expression
MEF
iPS
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activin A day4
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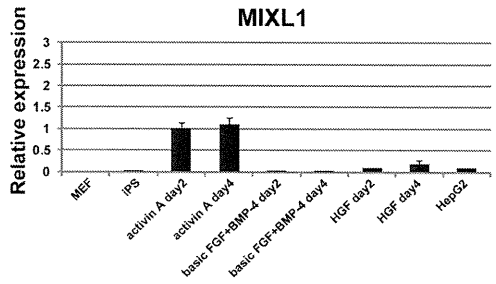
basic FGF+BMP-4 day4
HGF day2
HGF day4
HepG2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Relative expression
MEF
iPS
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activin A day4
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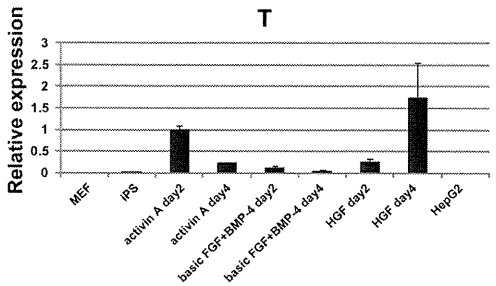
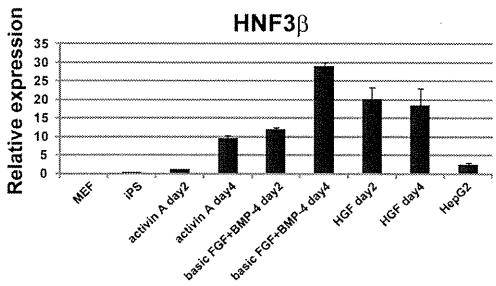
basic FGF+BMP-4 day4
HGF day2
HGF day4
HepG2


T
Relative expression
MEF
iPS
activin A day2
activin A day4
basic FGF+BMP-4 day2
basic FGF+BMP-4 day4
HGF day2
HGF day4
HepG2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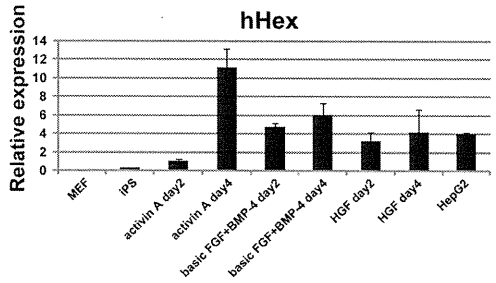
HGF day4
HepG2


hHex
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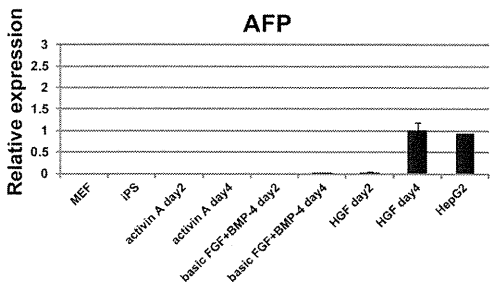
HGF day2
HGF day4
HepG2


AFP
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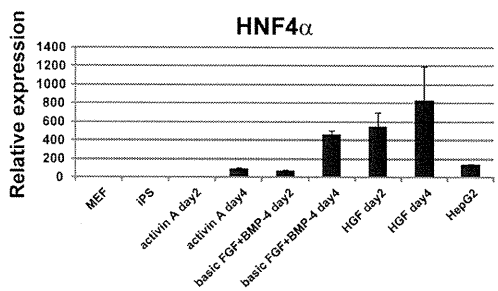
HGF day4
HepG2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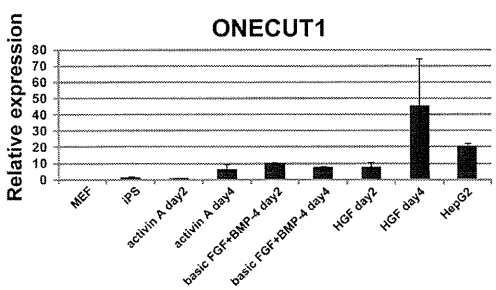

ONECUT1
Relative expression
MEF
iPS
activin A day2
activin A day4
basic FGF+BMP-4 day2
basic FGF+BMP-4 day4
HGF day2
HGF day4
HepG2

Figure 2. Expressions of undifferentiated and differentiated cell markers in human iPS cell-derived cells. The expressions of marker genes for mesodermal and endodermal cells (GSC, CXCR4, Sox17, MIXL1, T, HNF3β, and hHex) and hepatic cells (AFP, HNF4α, and ONECUT1) were examined in normal human iPS cells (iPS) and differentiated iPS cells. For quantitative PCR analyses, mRNAs were purified from MEF, HepG2 cells, normal iPS cells, and iPS cells stimulated with activin A on days 2 and 4, basic FGF and BMP-4 on days 2 and 4, and HGF on days 2 and 4. The results are represented as the mean colony counts ± SD (triplicate samples).
doi:10.1371/journal.pone.0067541.g002

tiation of human iPS cells generated $CD13^{mid}$ cells. Interestingly, $CD13^{high}$ cells were only detected in cultures stimulated with cytokines and expressed CD133, another hepatoblast surface marker (**Figure 1C** and **Figure S1A**). Moreover, $CD13^{high}CD133^{+}$ cells expressed other hepatic cell surface markers, CD49f and CD326 (**Figure S1A**, and data not shown). In contrast, expression of CD34 and CD56 was barely detected in $CD13^{high}CD133^{+}$ cells (**Figure S1B**). Expression of CD44 and CXCR4 were not significantly changed by the addition of cytokines (**Figure 1C**).

Expressions of differentiation marker genes during differentiation of human iPS cells

When differentiation of human pluripotent stem cells into endodermal cells was induced by addition of activin A, several differentiation marker genes were transiently upregulated [26]. We analyzed the expressions of mesodermal and endodermal marker genes (GSC, CXCR4, Sox17, MIXL1, T, HNF3β, and hHex) and hepatocytic marker genes (AFP, HNF4α, and ONECUT1) (**Figure 2**). The hepatocytic marker gene expressions were highly increased when iPS cells were stimulated by HGF. We found that most cells derived from human iPS cells differentiated into AFP^{+} and $HNF4\alpha^{+}$ hepatocytic cells during our differentiation steps, although several cell clusters that could not differentiate into the hepatic lineage remained (**Figure S2A**). The pluripotency maker Oct3/4 was not expressed in the AFP^{+} hepatocytic cells at the end of the differentiation steps (**Figure S2B**). Part of AFP^{-} undifferentiated cell clusters expressed T (**Figure S2C**), indicating that expression of T was detected by RT-PCR at the end of the differentiation. In contrast, the expressions of other mesodermal and endodermal marker genes (GSC, CXCR4, Sox17, and MIXL1) were transiently upregulated at the endodermal differentiation stage (by stimulation with activin A) and decreased during hepatocytic differentiation. These results suggested that the iPS cells differentiated into hepatocytic cells through the definitive endodermal pathway.

Purification and *in vitro* culture of human iPS cell-derived $CD13^{high}CD133^{+}$ cells

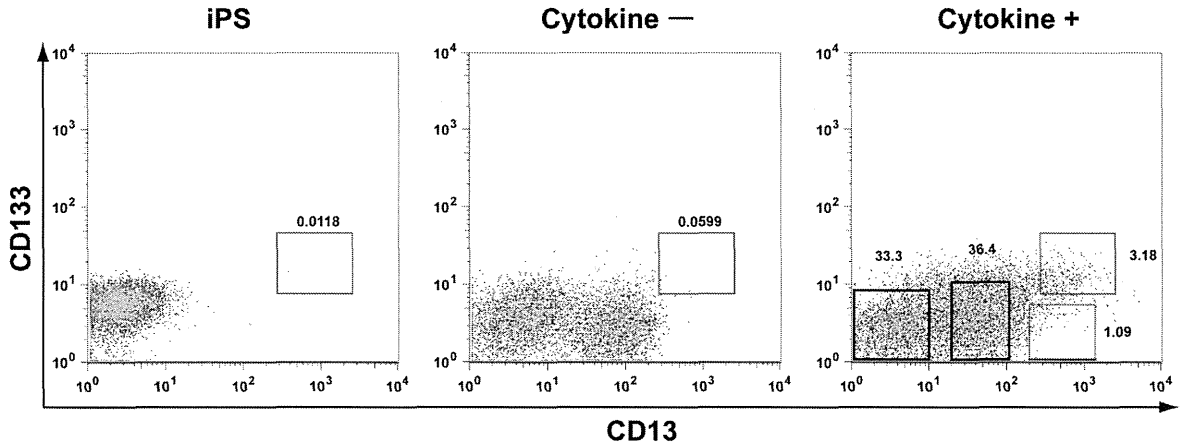
Next, we analyzed the characteristics of human iPS cell-derived cells expressing hepatic progenitor cell surface markers, CD13 and CD133. During fetal liver development, hepatic progenitor cells interact with non-parenchymal cells such as mesenchymal cells, fibroblasts, mesothelial cells and endothelial cells [27,28,29,30]. We recently established a co-culture system consisting of mouse early fetal hepatic progenitor cells and mesenchymal feeder cells [17]. Cell-cell interactions with mesenchymal cells are required for effective expansion of early fetal hepatic progenitor cells *in vitro*. Thus, we sorted $CD13^{high}CD133^{+}$ cells derived from human iPS cell cultures stimulated with cytokines (**Figure 3A**), and cultured the sorted cells on MEFs in H-CFU-C medium supplemented with EGF and HGF. After 12 days of culture, single $CD13^{high}CD133^{+}$ cells produced colonies expressing both AFP and HNF4α (**Figure 3B**). These results suggest that the $CD13^{high}CD133^{+}$ fraction contains HPCs. In this culture system, the addition of an ALK inhibitor, A-83-01, was required for effective expansion of $CD13^{high}CD133^{+}$ cells (**Figure 3C**). The combination of A-83-01 and Y-27632 significantly increased the number of large colonies derived from $CD13^{high}CD133^{+}$ cells. Based on these results, we used standard culture medium supplemented with EGF, HGF, A-83-01 and Y-27632 for expansion of human iPS cell-derived HPCs. To determine which fraction contained HPCs, we sorted the cultured cells into $CD13^{-}$, $CD13^{mid}$, $CD13^{high}CD133^{-}$, and $CD13^{high}CD133^{+}$ fractions, and the cells were plated individually onto MEFs. After these cells were cultured, large single cell-derived colonies containing over 100 cells were obtained and counted. As shown in **Figure 3D**, we found that the $CD13^{high}CD133^{+}$ fraction contained many HPCs and formed large colonies expressing AFP and HNF4α.

Long-term *in vitro* expansion of human iPS cell-derived HPCs

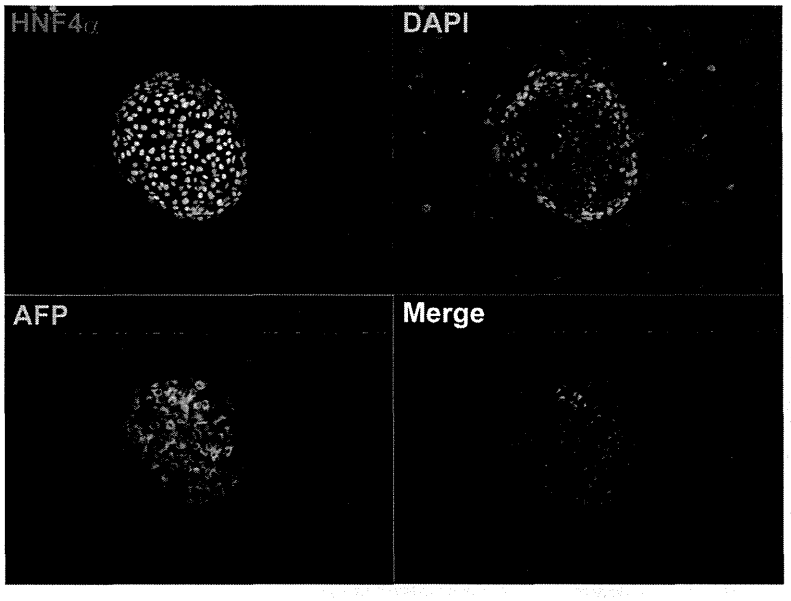
One of the most important characteristics of hepatic stem/progenitor cells is a high proliferative ability. To analyze whether human iPS cell-derived HPCs could proliferate in long-term cultures *in vitro*, colonies derived from $CD13^{high}CD133^{+}$ cells were trypsinized and replated onto new feeder cells. The number of cells was counted at each replating step. The cells continued to proliferate for more than 1 month (**Figure 4A**). After the 4th passage, the $HNF4\alpha^{+}$ human iPS cell-derived HPC colonies expressed the proliferation marker Ki67 (**Figure 4B**). In contrast, these HPC colonies did not express the pluripotency marker Oct3/4, which is expressed in human iPS cells (**Figure 4B and C**). Next, we analyzed the expression of hepatocytic and cholangiocytic marker genes in human iPS cell-derived HPCs at each replating step. Colonies were stained with specific antibodies against AFP, HNF3β, HNF4α and cytokeratin 7 (CK7). Expression of the endodermal marker, HNF3β, and hepatocytic markers, AFP and HNF4α, was observed in primary colonies and maintained during *in vitro* expansion (**Figure 5A**). In particular, human iPS cell-derived HPCs were able to proliferate for more than 3 months and still expressed the hepatocytic markers HNF4α and AFP (**Figure S3A and B**). Cholangiocytic marker CK7 was not expressed in primary colonies but was induced after several passages. A small number of cells in colonies of the 1st culture expressed albumin, a mature hepatocytic marker gene (**Figure 5B**). $CD13^{high}CD133^{+}$ cells derived from human iPS cells could expand *in vitro* over a long term while maintaining hepatocytic and cholangiocytic marker gene expression, indicating that these cells have hepatic progenitor-like potentials.

As shown above, $CD13^{high}CD133^{+}$ cells spontaneously differentiated into CK7-positive cholangiocyte-like cells AFP and HNF4α-negative non-hepatocytic cells during passaging. To determine the cell fraction containing self-renewing cells after serial passaging, human iPS cell-derived HPC colonies at each replating step were analyzed using antibodies against CD13 and CD133 (**Figure 6**). In addition to the $CD13^{+}CD133^{+}$ fraction, $CD13^{-}CD133^{+}$, $CD13^{+}CD133^{-}$ and $CD13^{-}CD133^{-}$ fractions were found in cultures derived from human iPS cell-derived $CD13^{high}CD133^{+}$ cells, indicating that HPCs spontaneously differentiated and lost expression of CD13 and CD133. We analyzed the colony-forming activities of these fractions at replating steps. Cells at each replating step were sorted and cultured on MEFs for 12 days, and then HNF4α-positive colonies

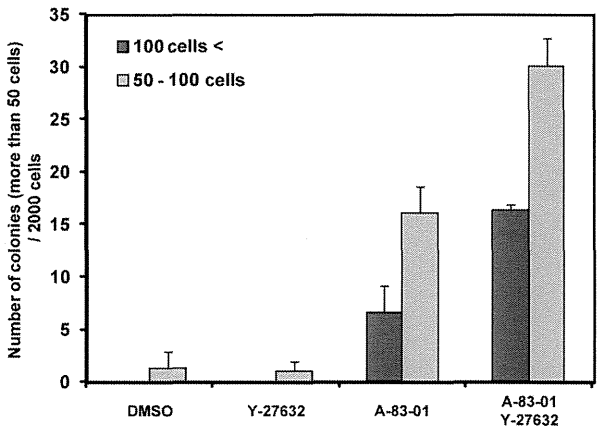
(A)


iPS
Cytokine —
Cytokine +
CD133
CD13
0.0118
0.0599
33.3
36.4
3.18
1.09

(B)


HNF4α
DAPI
AFP
Merge

(C)


100 cells <
50 - 100 cells
Number of colonies (more than 50 cells) / 2000 cells
DMSO
Y-27632
A-83-01
A-83-01 Y-27632

(D)

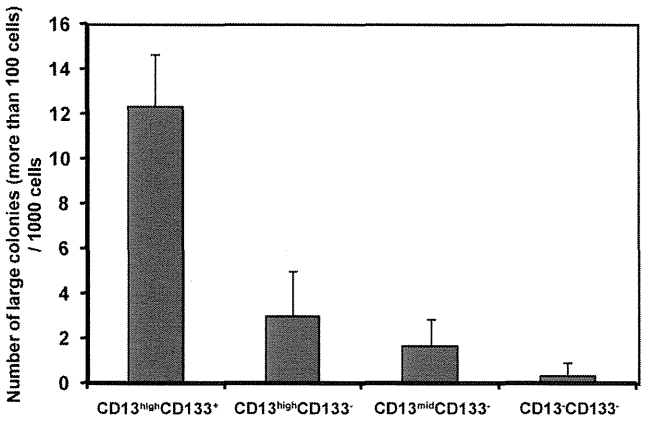

Number of large colonies (more than 100 cells) / 1000 cells
CD13highCD133+
CD13highCD133-
CD13midCD133-
CD13-CD133-

Figure 3. Isolation of HPCs from human iPS cell-derived hepatic lineage cells. (**A**) Expression of hepatic progenitor markers in undifferentiated human iPS cells and differentiated cells. After 12 days of culture with or without cytokines, cells were stained with antibodies against CD13 and CD133, and then analyzed by flow cytometry. Ratios of $CD13^{high}CD133^{+}$ cells are shown. (**B**) Representative images of a colony derived from a single $CD13^{high}CD133^{+}$ cell. Colonies were stained with antibodies against AFP and HNF4α. Nuclei were counterstained with DAPI. (**C**) Culture condition of the human iPS cell-derived hepatic progenitor colony assay. $CD13^{high}CD133^{+}$ cells were sorted and cultured on MEFs in the presence or absence of A-83-01 (ALK inhibitor) and Y-27632 (ROCK inhibitor). Results are represented as the mean colony count ± SD (triplicate samples). (**D**) $CD13^{-}CD133^{-}$, CD13 weakly single positive, $CD13^{mid}$ single positive and $CD13^{high}CD133^{+}$ cells were sorted onto MEFs. The cells were cultured in standard culture media in the presence of A-83-01 and Y-27632. Large colonies (containing more than 100 cells) derived from individual sorted cells were counted. Results are represented as the mean colony count ± SD (triplicate samples).
doi:10.1371/journal.pone.0067541.g003

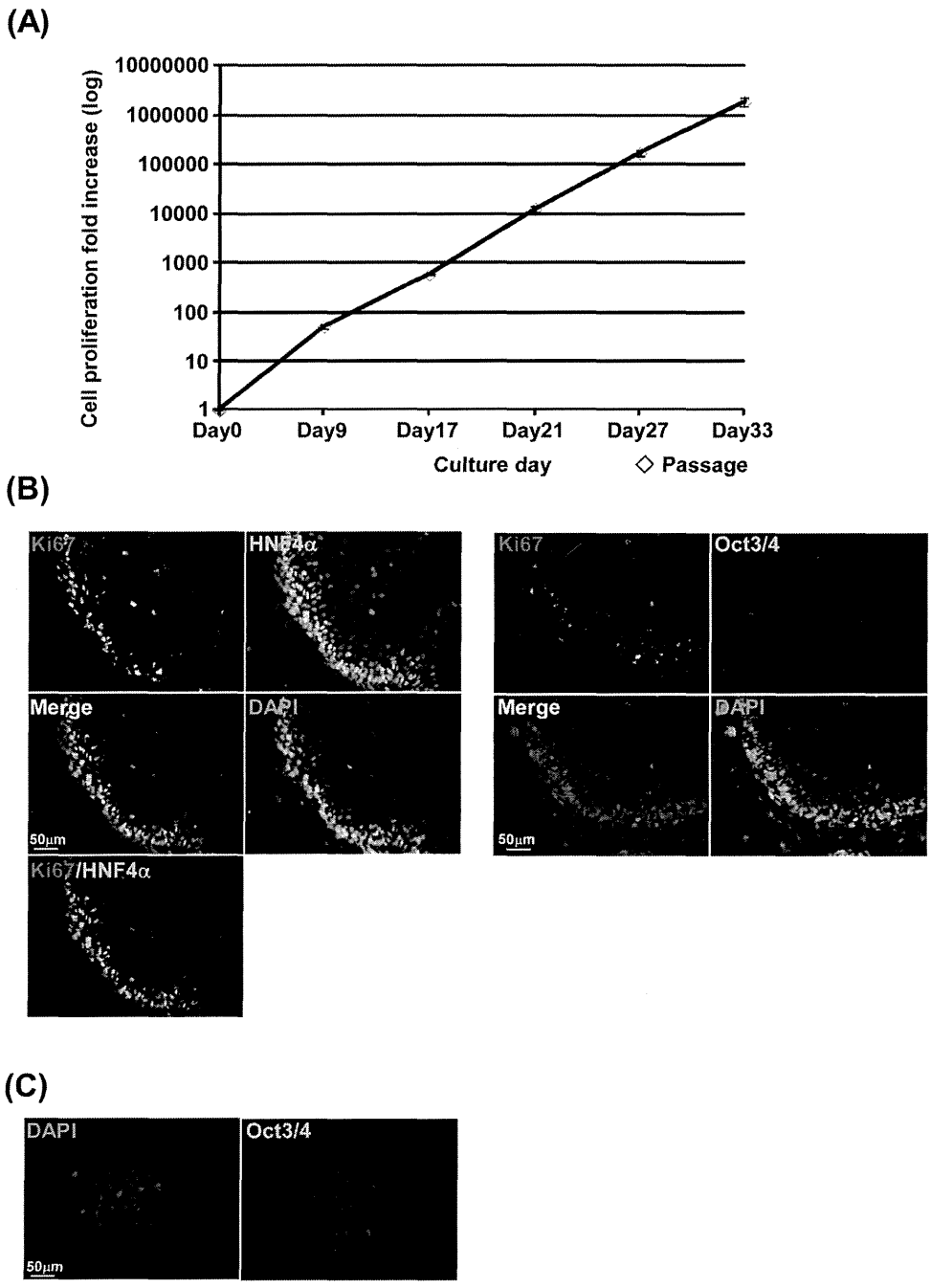


Figure 4. *In vitro* long-term expansion of $CD13^{high}CD133^{+}$ cells. (**A**) Colonies derived from $CD13^{high}CD133^{+}$ cells were trypsinized and replated onto MEFs. The number of cells was counted at each replating step. The cells continued to proliferate for more than 1 month. A representative growth curve is shown. Similar results were obtained in two independent experiments. (**B**) Expression of the proliferation marker Ki67 in human iPS cell-derived hepatocytic colonies. After the 4th passage, the colonies were stained with antibodies against Ki67, HNF4α, and Oct3/4. (**C**) Human iPS cells were stained with an antibody against Oct3/4.
doi:10.1371/journal.pone.0067541.g004

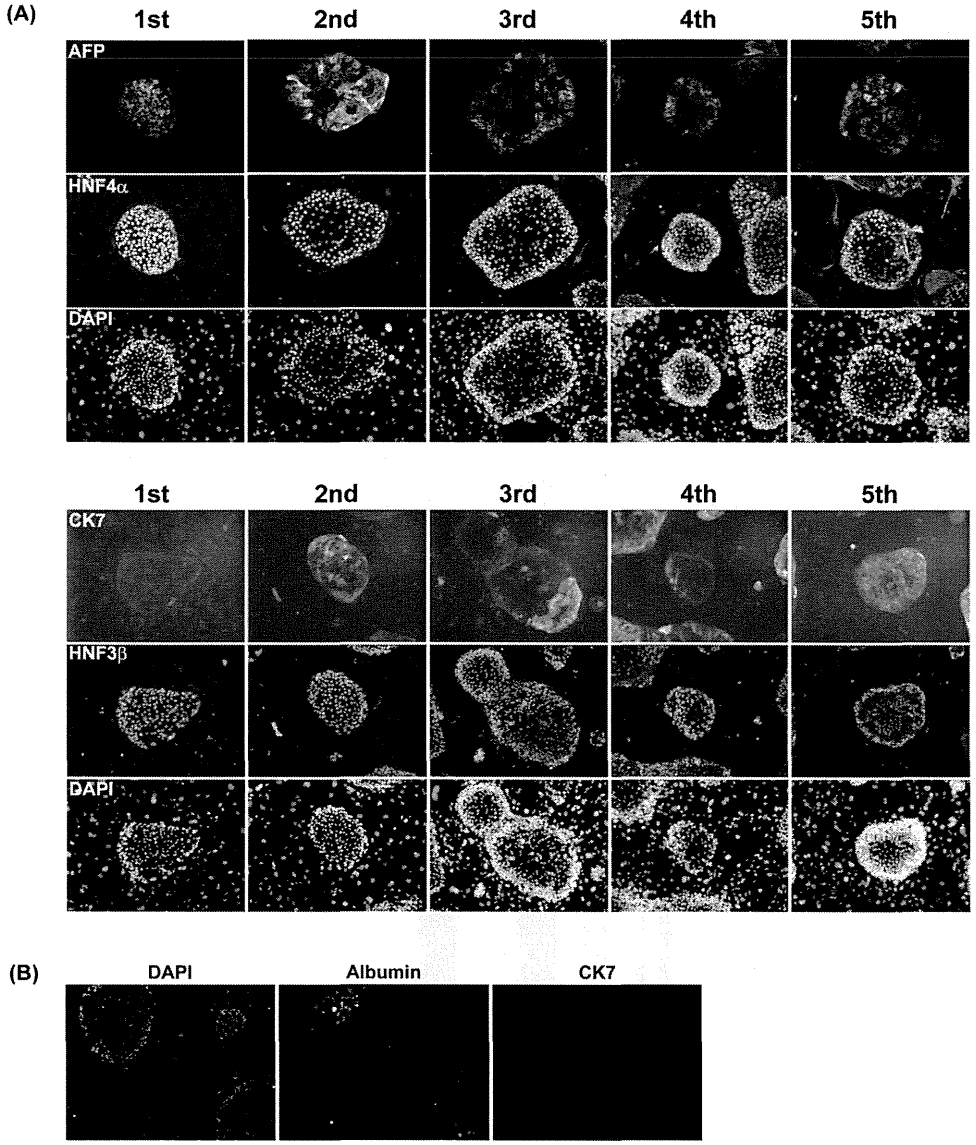


Figure 5. Expression of hepatocytic and cholangiocytic marker genes in human iPS cell-derived hepatic progenitor-like colonies. (**A**) Expression of hepatocytic and cholangiocytic markers during *in vitro* expansion. Colonies derived from $CD13^{high}CD133^{+}$ cells were trypsinized and replated onto MEFs. Cells were fixed at each replating step. An endodermal marker (HNF3β), hepatocytic markers (AFP and HNF4α) and a cholangiocytic marker (CK7) were stained with specific antibodies. Nuclei were counterstained with DAPI. (**B**) Expression of albumin in colonies derived from human iPS cell-derived HPCs. Albumin but not CK7 was detected in several colonies in the 1st culture.
doi:10.1371/journal.pone.0067541.g005

derived from each fraction were counted (**Figure 6B–D**). The rate of HNF4α positive colony formation was high in cultures derived from $CD13^{+}CD133^{+}$ cells at each replating step. Interestingly, $CD13^{+}CD133^{-}$ cells after several passages also formed many HNF4α-positive colonies. These results suggest that CD13 is likely to be a useful surface marker to purify self-renewing HPCs after serial passaging. Next, we analyzed whether the $CD13^{+}$ cells among human iPS cell-derived HPCs had self-renewal potency. When human iPS cell-derived HPCs were passaged twice, the 3rd cultured-cells were stained with suitable antibodies, and $CD13^{+}$ and $CD13^{-}$ cells were sorted. After 11 days of culture, the expanded colonies (4th culture) derived from $CD13^{+}$ and $CD13^{-}$ cells were analyzed by flow cytometry (**Figure S3C**). Interestingly, the HPC colonies derived from $CD13^{+}$ cells contained many $CD13^{+}$ cells. In contrast, the HPC colonies derived from $CD13^{-}$ cells barely contained $CD13^{+}$ cells. We obtained similar results using the 5th cultured-cells. In addition, we compared the colony-forming abilities of $CD13^{+}$ and $CD13^{-}$ cells in the 5th culture. As shown in **Figure S3D**, $CD13^{+}$ cells were able to form colonies more efficiently than $CD13^{-}$ cells. These results suggested that $CD13^{+}$ cells maintained the self-renewal-like ability to proliferate for a long time.

Differentiation of human iPS cell-derived HPCs toward both mature hepatocytic and cholangiocytic cells

Hepatic progenitor cells in fetal liver development have a bipotent differentiation ability toward hepatocytes and cholangiocytes [23,31]. Therefore, we assessed whether human iPS cell-derived HPCs had the potential to differentiate into mature hepatocytic cells. To maintain and induce the functions of mature

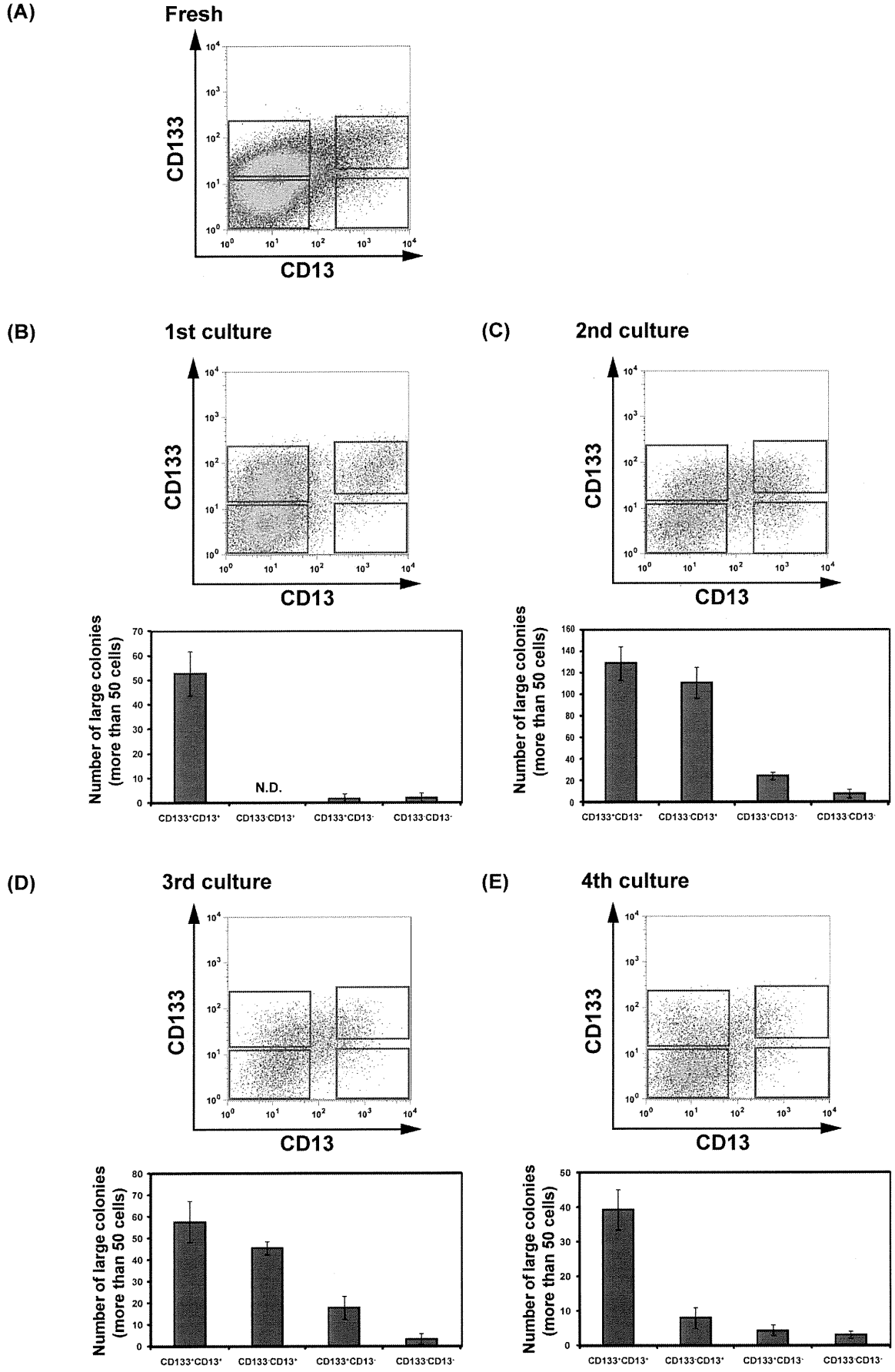

(A)
Fresh
CD133
CD13
(B)
1st culture
CD133
CD13
Number of large colonies (more than 50 cells)
N.D.
CD133+CD13+
CD133-CD13+
CD133+CD13-
CD133-CD13-
(C)
2nd culture
CD133
CD13
Number of large colonies (more than 50 cells)
CD133+CD13+
CD133-CD13+
CD133+CD13-
CD133-CD13-
(D)
3rd culture
CD133
CD13
Number of large colonies (more than 50 cells)
CD133+CD13+
CD133-CD13+
CD133+CD13-
CD133-CD13-
(E)
4th culture
CD133
CD13
Number of large colonies (more than 50 cells)
CD133+CD13+
CD133-CD13+
CD133+CD13-
CD133-CD13-

Figure 6. Changes of HPC surface marker expression in human iPS cell-derived hepatic progenitor-like colonies. (**A**) After 12 days of culture with cytokines, $CD13^{high}CD133^{+}$ cells were sorted onto GFP-MEFs. After 10–12 days, cells were trypsinized and stained with antibodies against CD13 and CD133. Expression of CD13 and CD133 was analyzed by flow cytometry. (**B–E**) Colony forming activity of $CD13^{+}CD133^{+}$, $CD13^{+}CD133^{-}$, $CD13^{-}CD133^{+}$, and $CD13^{-}CD133^{-}$ fractions of human iPS cell-derived HPCs. At every replating step, cells stained with antibodies against CD13 and CD133 were sorted onto new GFP-MEFs, and their colony-forming activity was analyzed. Results are represented as the mean colony count ± SD (duplicate samples). N.D. shows "not determined".
doi:10.1371/journal.pone.0067541.g006

hepatocytes *in vitro*, three-dimensional (3D) biological structures are known to be important [32,33,34]. For this purpose, the hanging drop method is often used to self-assemble hepatic stem/progenitor cells into 3D aggregates (spheroids). For human iPS cell-derived hepatocytic differentiation, human iPS cell-derived HPCs in the 3rd culture were trypsinized and suspended in hepatocyte medium supplemented with dexamethasone, with or without OSM, for spheroid formation (**Figure S4A**). After 3 days of spheroid culture, the expressions of hepatic functional genes in human iPS-derived cells were compared with those in primary human hepatocytes (**Figure 7**). Both phase 1 enzymes (CYP3A4, CYP3A7, CYP7A1, EPHX1, FMO5, and MAOB) and phase 2 enzymes (COMT and SULT1A2) were expressed in spheroids derived from human iPS cell-derived HPCs from the 3rd culture. Except for MAOA, the expression levels of mature hepatocyte functional marker genes in spheroids without OSM were higher than those in spheroids with OSM. The expressions of several functional genes in human iPS-derived spheroids were significantly lower than those in primary hepatocytes, whereas mature hepatocyte functional marker genes, FMO5, MAOA, MAOB, and SULT1A2, were expressed highly or at similar levels compared with a previous report on human ES and iPS cell-derived hepatocytic cells [12].

Albumin secretion is one of the most important functions of mature hepatocytes. We cultured human iPS cell-derived HPCs at a high density for a long time and induced hepatic maturation by cell-cell interactions [35]. The HPCs in the high-density culture exhibited significant expression of albumin (**Figure S4B**). Subsequently, the amounts of albumin protein in the conditioned culture media were analyzed by enzyme-linked immunosorbent assays. We compared the albumin secretion levels in these HPCs with those in HepG2 cells. As shown in **Figure S4C**, the albumin secretion levels of HPCs were higher than those of HepG2 cells, and the secreted protein levels were similar to those in a previous report on human iPS cell-derived hepatocytic cells [12]. These results suggest that human iPS cell-derived HPCs have the potential to differentiate into mature hepatocytic cells after *in vitro* expansion.

It has been reported that cholangiocytic cells form cysts with an epithelial polarity, demonstrating *in vitro* tubulogenesis in an extracellular matrix gel supplemented with cytokines [20]. We therefore examined whether human iPS cell-derived HPCs could form a cholangiocytic structure during 3D-gel culture. Human iPS cell-derived HPC colonies were trypsinized and cultured in an extracellular matrix gel supplemented with EGF, HGF, R-spondin 1, Wnt-3a, A-83-01, and Y-27632. After 12 days of culture, many epithelial cysts were formed. We analyzed the expression of F-actin, protein kinase Cζ (PKCζ), integrin α6 (CD49f) and β-catenin as markers for the apical and basolateral domains [20]. Luminal space surrounded by F-actin bundles and PKCζ was detected, and the expression of integrin α6 and β-catenin was located at the basolateral region (**Figure 8A**). Some of these epithelial cysts expressed CK7 but not expressed hepatocytic marker AFP (**Figure 8B**), suggesting that these cells could form cholangiocyte-like cyst structures with an epithelial polarity. These results indicate that human iPS cell-derived HPCs exhibit a bipotent differentiation ability toward hepatocytes and cholangiocytes.

Purification and expansion of HPCs derived from human ES cells

Differentiated human ES cells (KhES-3) toward hepatic lineage cells also contained $CD13^{high}CD133^{+}$ cells (**Figure S5A**). These human ES cell-derived $CD13^{high}CD133^{+}$ fractions formed large colonies on MEFs in H-CFU-C medium supplemented with EGF, HGF, A-83-01, and Y-27632. To analyze whether human ES cell-derived $CD13^{high}CD133^{+}$ cells could proliferate in long-term cultures *in vitro*, colonies from human ES cell-derived $CD13^{high}CD133^{+}$ cells were trypsinized and replated onto new feeder cells. Similar to the results for human iPS cells, human ES cell-derived $CD13^{high}CD133^{+}$ cells continued to proliferate for a long time (at least 1 month). $CD13^{high}CD133^{+}$-derived colonies expressed the hepatocytic markers AFP and HNF4α and the endodermal marker HNF3β (**Figure S5B**). A small number of cells in colonies of the 1st culture expressed albumin (**Figure S5C**). The proliferation marker Ki67 and HNF4α were also expressed in human ES cell-derived $CD13^{high}CD133^{+}$-derived colonies after the culture passage (**Figure S6**). These cells did not express Oct3/4. The cells had hepatic progenitor-like potential, similar to HPCs from human iPS cell-derived $CD13^{high}CD133^{+}$ cells. These results indicate that our methods are useful for expansion of HPCs derived from not only human iPS cells, but also human ES cells.

Discussion

In the present study, we showed that CD13 and CD133 are specific cell surface markers of HPCs derived from human iPS cells. When human iPS cells were stimulated with suitable cytokines, a small population of cells began to express CD13 and CD133, and could expand in long-term culture. CD13 and CD133 are specific markers of mouse fetal hepatoblasts and adult liver progenitor cells [16,17,36]. CD13 is also expressed in human liver cancer stem cells [37]. CD133 is known as an important stem cell marker because many types of cancer stem cells and somatic stem/progenitor cells, such as neural, intestine and hematopoietic stem cells, express this cell surface marker [38,39,40,41]. These results suggest that somatic and cancer stem/progenitor cells share similar cell surface proteins. A population of human iPS cells cultured without cytokines spontaneously expressed a moderate level of CD13. In contrast, $CD13^{high}CD133^{+}$ cells were only detected in cultures with appropriate cytokine stimulation. Current protocols for induction of the hepatocytic lineage from human iPS cells have been established by mimicking the events that occur during developmental stages. Such protocols contain 4 steps: (1) induction of endodermal progenitor cells by activin A, (2) hepatocytic specification by basic FGF and BMP-4, (3) differentiation to hepatic stem/progenitor-like cells by HGF, (4) maturation to hepatocytes by OSM. We purified HPCs derived from human iPS cells when these cells were cultured at step (3). It was reported that self-renewing endodermal progenitors derived from human ES cells and iPS cells were established [14]. These cells

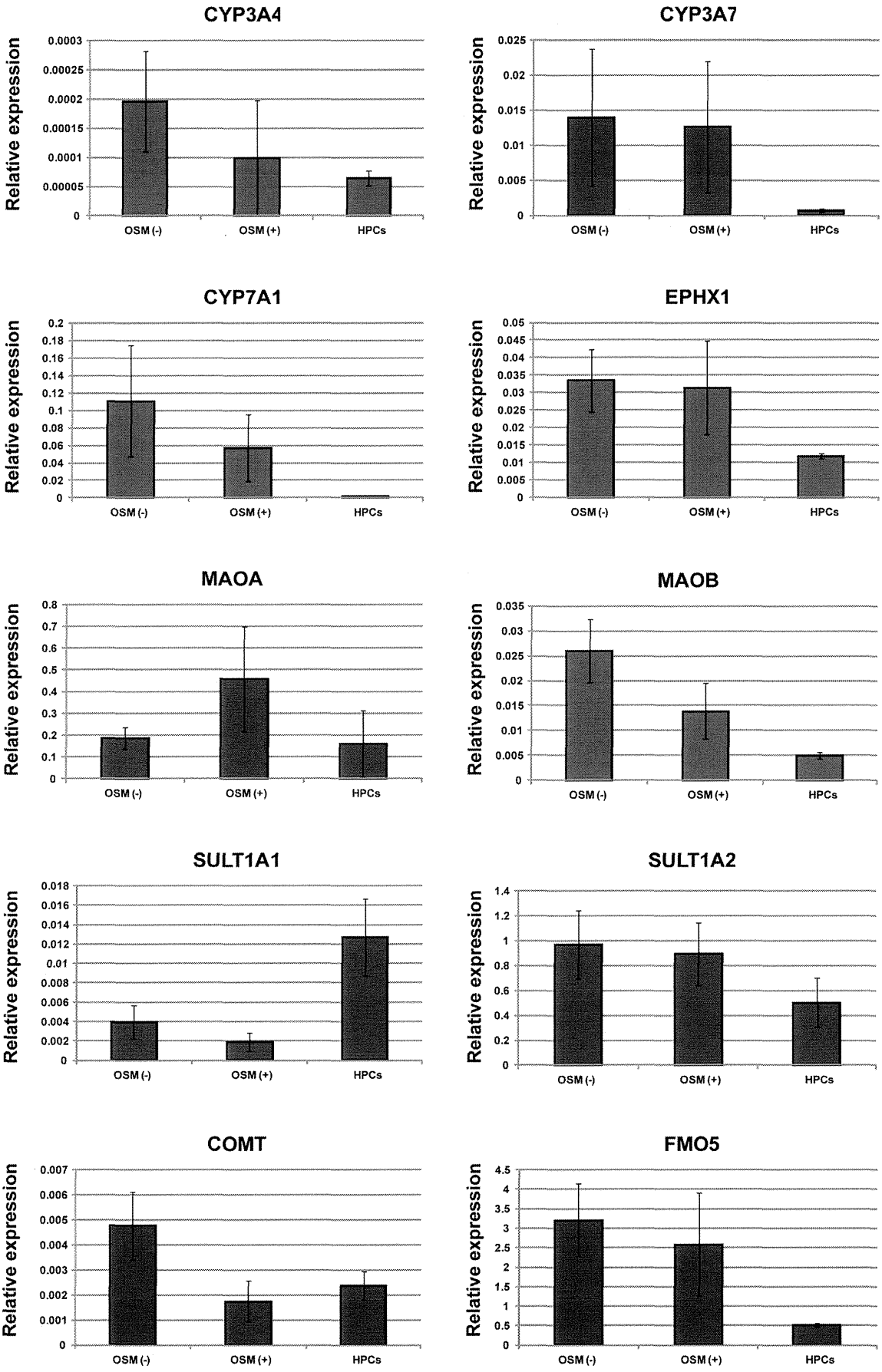

CYP3A4
Relative expression
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
OSM (-)
OSM (+)
HPCs
CYP3A7
Relative expression
0.025
0.02
0.015
0.01
0.005
0
OSM (-)
OSM (+)
HPCs
CYP7A1
Relative expression
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OSM (-)
OSM (+)
HPCs
EPHX1
Relative expression
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
OSM (-)
OSM (+)
HPCs
MAOA
Relative expression
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OSM (-)
OSM (+)
HPCs
MAOB
Relative expression
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
OSM (-)
OSM (+)
HPCs
SULT1A1
Relative expression
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
OSM (-)
OSM (+)
HPCs
SULT1A2
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OSM (-)
OSM (+)
HPCs
COMT
Relative expression
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
OSM (-)
OSM (+)
HPCs
FMO5
Relative expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
OSM (-)
OSM (+)
HPCs

Figure 7. Expressions of hepatic functional genes in differentiated HPCs. The levels of mRNAs encoding phase 1 and 2 enzymes in human iPS cell-derived HPCs from the 3rd culture, and spheroids derived from human iPS cell-derived HPCs from the 3rd culture are shown as the fold values relative to the levels in uncultured human hepatocytes. Spheroid formation was induced by hanging drop culture in the presence or absence of OSM. The results are represented as the mean colony counts ± SD (spheroid culture, n = 6; HPCs, n = 3; uncultured human hepatocytes, n = 2).
doi:10.1371/journal.pone.0067541.g007

expressed both CD117 and CXCR4, endodermal progenitor cell markers, and had multipotency to differentiate into pancreatic, hepatic, and intestinal cells. In contrast, the $CD13^{high}CD133^{+}$ HPCs in the present study expressed AFP, a primitive hepatocytic marker, but not CXCR4 (**Figure 1C** and data not shown), suggesting that our cells were committed progenitor cells. A previous study reported that N-cadherin is a cell surface marker of hepatic progenitor cells derived from human ES cells stimulated with activin A and basic FGF. However, only a few cells in N-cadherin-positive populations can form large hepatocytic colonies [15]. In contrast, the present study clearly indicates that the usage of CD13 and CD133 in combination with our culture condition is suitable for purification and expansion of HPCs.

A-83-01 and Y-27632 were important for the expansion of human iPS cell-derived HPCs. Human ES and iPS cells are not able to survive as single cells because activation of the Rho-Rock signal induces blebbing and apoptosis of these cells [42]. Survival and expansion of hepatic progenitor cells derived from early fetal and adult livers are also induced by ROCK inhibition [17,36,43]. Further studies are needed to clarify whether inhibition of ROCK acts in anti-apoptosis and/or proliferation of human iPS cell-derived HPCs. TGFβ and ALK signaling pathways are involved in regeneration and epithelial-mesenchymal transition of liver cells [44]. In this study, we found that inhibition of the ALK signal is required for colony formation of HPCs. The molecular mechanism of the ALK inhibitor regulating human iPS cell-derived HPCs remains unknown. However, activation of TGFβ-ALK signals, such as phosphorylation of Smad family proteins, might cause cell cycle arrest or epithelial-mesenchymal transition of HPCs.

It has been reported that iPS cells generated by retroviral vectors re-express Yamanaka factors in culture during differentiation of several cell types [18,45]. In our culture system, HPCs derived from human iPS cells could proliferate over a period of 1 month, while maintaining a bipotent differentiation ability. Interestingly, there was no re-activation of exogenous Yamanaka factors (Oct3/4, Klf4, Sox2, and c-Myc) during expansion of these cells, suggesting that exogenous genes (particularly c-Myc) were not involved in the proliferative ability of our HPCs (data not shown). Cell cycle-dependent kinases and their inhibitors are important regulators for the cell cycle and long-term proliferation of stem/progenitor cells. During *in vitro* expansion of mouse hepatocytic cells, up-regulation of $p19^{ARF}$ ($p14^{ARF}$ in human cells) induces cell cycle arrest and senescence [46]. In contrast, activation of $p16^{Ink4a}$ and $p14^{ARF}$ was detected during the long-term proliferation of human iPS cell-derived HPCs (data not shown). These results suggest that there may be unknown mechanisms regulating the cell cycle of these cells *in vitro*.

Analyses of human hepatic progenitor cells are difficult because of the shortage of human fetal tissue samples and culture systems.

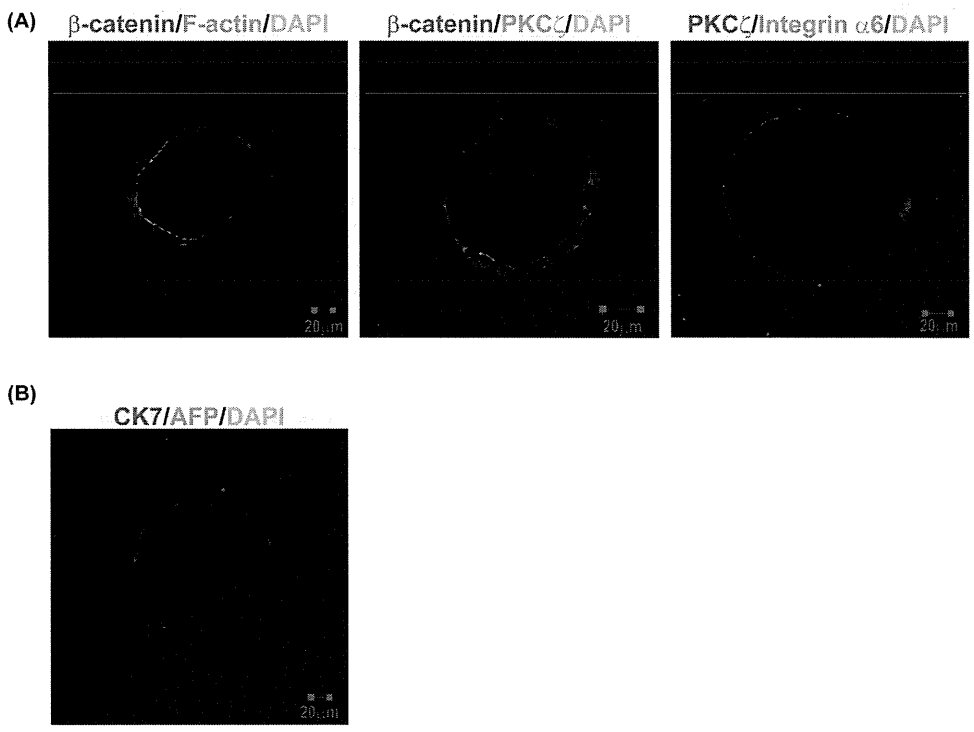


Figure 8. Cholangiocytic cyst formation of human iPS cell-derived HPCs. (**A**) Human iPS cell-derived HPC colonies were trypsinized and cultured in an extracellular matrix gel. After 10–12 days of culture, several numbers of epithelial cysts were formed. Expression of β-catenin, F-actin, integrin α6 and PKCζ was detected in cysts. (**B**) Expression of CK7 in a HPC-derived epithelial cyst. Cells were stained with antibodies against CK7 and AFP. Nuclei were counterstained with DAPI.
doi:10.1371/journal.pone.0067541.g008

In this regard, our human HPCs derived from iPS cells might be useful for the analysis of human hepatic cell development. In addition, mature hepatocytes can barely maintain a proliferative ability following cryopreservation, whereas our human iPS cell-derived HPCs have a highly proliferate ability even after cryopreservation. Thus, the *in vitro* expansion system presented here may contribute to regenerative therapies of liver diseases using functional human hepatic progenitor cells and hepatocytes.

Supporting Information

Figure S1 Expression of cell surface markers in human iPS cell-derived hepatic lineage cells. (**A**) Expression of hepatic progenitor marker CD49f in $CD13^{high}CD133^{+}$ cells. Human iPS cells were stimulated with cytokines and stained with suitable antibodies. $CD13^{high}CD133^{+}$ cells slightly expressed CD49f. (**B**) Expression of progenitor cell markers CD56 and CD34 in $CD13^{high}CD133^{+}$ cells.
(TIF)

Figure S2 Differentiation of human iPS cells into hepatic lineage cells. (**A**) Expressions of AFP (red) and HNF4α (green) in differentiated iPS cells at step (3) in Figure 1. The four fields of view are shown. Many cells have differentiated into AFP- and HNF4α-positive hepatocytic cells, although several cell clusters have not differentiated (arrowheads). (**B**) Expressions of AFP (red) and Oct3/4 (green) in differentiated iPS cells at step (3). Oct3/4 is not expressed in the AFP-positive cells. (**C**) Expressions of AFP (green) and T (red) in differentiated iPS cells at step (3). T is not expressed in the AFP-positive cells. (**A–C**) Nuclei were stained with DAPI (blue).
(TIF)

Figure S3 Long-term proliferation of human iPS cell-derived HPCs. (**A**) Representative image of colonies of long-term proliferative human iPS cell-derived HPCs. The colonies were passaged six times and cultured for a total of 90 days after the first sorting. (**B**) Expressions of hepatocytic marker genes in the long-term culture. The colonies were cultured as described for (A) and fixed with 4% PFA. AFP (red) and HNF4α (green) were stained with suitable antibodies. (**C**) After 12 days of culture with cytokines, $CD13^{high}CD133^{+}$ cells were sorted onto MEFs. After two passages, the 3rd cultured-cells were trypsinized and stained with antibodies against CD13 and CD133. $CD13^{+}$ (red) and $CD13^{-}$ (blue) cells were purified and serially cultured (4th and 5th cultured-cells). 11d culture: 11-day culture. (**D**) Expansion of $CD13^{+}$ and $CD13^{-}$ cells after long-term culture. As shown in (C), $CD13^{+}$ (red) and $CD13^{-}$ (blue) cells in the 5th-cultured cells were purified and cultured for 9 days on MEFs. The results are represented as the mean colony counts ± SD (duplicate samples).
(TIF)

Figure S4 Differentiation of human iPS cell-derived HPCs toward mature hepatocytic cells. (**A**) Schematic diagram of the experimental procedure. HPCs in the 3rd culture were dissociated with 0.05% trypsin-EDTA. Spheroids derived from HPCs were formed using hanging drop culture. (**B**) Expression of albumin in HPCs matured by cell-cell interactions. (**C**) Albumin secretion by human iPS cell-derived HPCs is identified after 3 days of culture in medium by enzyme-linked immunosorbent assays.
(TIF)

Figure S5 Purification of human ES cell-derived HPCs. (**A**) Expressions of CD13 and CD133, cell surface markers of hepatic progenitor cells, in human ES cells cultured with or without cytokines. After 12 days of culture, the cells were stained with antibodies against CD13 and CD133, and then analyzed by flow cytometry. (**B**) Expressions of hepatocytic and cholangiocytic markers during *in vitro* expansion of human ES cell-derived HPCs. Colonies derived from $CD13^{high}CD133^{+}$ cells were cultured on MEFs. The expressions of several liver markers are detected in the 1st and 2nd cultures. An endodermal marker (HNF3β), hepatocytic markers (AFP and HNF4α), and a cholangiocytic marker (CK7) were stained with specific antibodies. (**C**) Expression of albumin in colonies derived from human ES cell-derived $CD13^{high}CD133^{+}$ cells. Albumin is detected in several colonies in the 1st culture.
(TIF)

Figure S6 Proliferative ability of human ES cell-derived $CD13^{high}CD133^{+}$ cells. Expressions of a pluripotency marker (Oct3/4) and a proliferation marker (Ki67) are observed in colonies derived from human ES cell-derived $CD13^{high}CD133^{+}$ cells. Ki67-expressing proliferative cells express HNF4α in the 2nd culture. These cells do not express Oct3/4. Nuclei were counterstained with DAPI.
(TIF)

Table S1 List of antibodies used for immunostaining and flow cytometry experiments.
(DOCX)

Table S2 Lists of PCR primers for detection of human gene expression. Afp, α-fetoprotein alpha; COMT, catechol-O-methyltransferase; CXCR4, chemokine (C-X-C motif) receptor 4; CYP, cytochrome P450; EPHX1, epoxide hydrolase 1, microsomal (xenobiotic); FMO5, flavin containing monooxygenase 5; GSC, goosecoid homeobox; hHex, hematopoietically expressed homeobox; HNF, hepatocyte nuclear factor; HPRT1, hypoxanthine phosphoribosyltransferase 1; MAO, monoamine oxidase; MIXL1, Mix paired-like homeobox; ONECUT1, one cut homeobox 1; Sox17, SRY-box containing gene 17; SULT1A1, sulfotransferase family, cytosolic, 1A, phenol-preferring, member1.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: AY AK. Performed the experiments: AY KI HC AK. Analyzed the data: AY HN AK. Wrote the paper: AY HN AK.

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【肝細胞移植を用いた肝疾患の治療】

Establishment of hepatocyte transplantation for liver diseases

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Key words
hepatocytes, tissue engineering, hepatic stem/progenitor cells, fluorescence activated cell sorting

要　約

肝細胞移植療法の開発として，①肝外部位に成熟肝細胞を移植することにより小肝組織を作製する組織工学と②肝幹・前駆細胞を体外で増殖させ移植する研究を紹介する。成熟肝細胞を用いた組織工学的アプローチとして，肝外部位への成熟肝細胞移植による小肝組織の作製の報告例がある。この異所性小肝組織は本来の肝臓と類似の機能を有しており，動物実験レベルでは臨床的な有効性が示されている。一方で，成熟肝細胞は生体外での増殖が困難であり，治療に必要な大量の細胞の確保に問題がある。そこで，生体外で高い増殖能力を有する肝幹・前駆細胞を純化・増殖させる細胞生物学的アプローチが進められている。筆者らは表面抗原マーカーの探索から，胎仔または成体肝臓からの肝幹・前駆細胞の純化・培養系を構築し，さらに胎仔由来肝前駆細胞の細胞移植療法における有用性を示している。今後，臓器移植とともにこのような細胞移植による肝疾患治療が期待される。

はじめに

肝臓は，血清蛋白質の合成・アミノ酸，糖代謝・アルコール，薬物の分解など多種の代謝機能を持つ，生体のホメオスタシス維持のための重要臓器である。また肝臓は固形臓器としては稀な高い再生能力を持ち，臓器全体の70%を切除しても約1週間で元の大きさを回復する。しかし，肝炎ウイルスの感染やアルコールの過剰摂取などで肝細胞の破壊と再生を繰り返す慢性肝炎の状態が進行することで，肝線維症・肝硬変を経て肝ガンへと移行する重篤な肝疾患につながり，最終的には死に至ることも少なくない。このような肝疾患の根本的治療法として肝臓移植が挙げられるが，健常人ドナーに対する侵襲性（生体肝移植）や脳死判定等の問題（脳死肝移植）が存在する。このため，肝臓移植に替わる治療法として，細胞移植療法が期待されている。肝細胞を用いた次世代の再生医療として，①肝外部位に成熟肝細胞を移植することにより小肝組織を作製する肝組織工学や，②肝幹・前駆細胞を体外で増幅させ移植する研究などが現在行われている。（図1）

1．成熟肝細胞を用いた組織工学的アプローチ

肝臓の多様な代謝機能を人工的に再現することは非常に困難である。そこで生細胞を用いた組織工学的アプローチとして，異所性部位に肝細胞を移植し小肝組織を形成させることで，低下した肝臓の代謝機能を補完し病態を改善する試みがなされている。異所性部位として腎被膜下と皮下における研究報告について以下に概説する。

腎被膜下は豊富な血流に隣接し，腹膜からの栄養供給を受けることから，代謝活性の活発な肝細胞の生着に有利である。そこでEHSマトリックスとともに分離肝細胞を腎被膜下に注入したところ，移植した肝細胞が腎皮膜下でドナー由来の血管等を取り込み疑似的な小葉構造を再構成し，マウスの生涯を通して維持される小肝組織が形成できることが報告された[1]。一方

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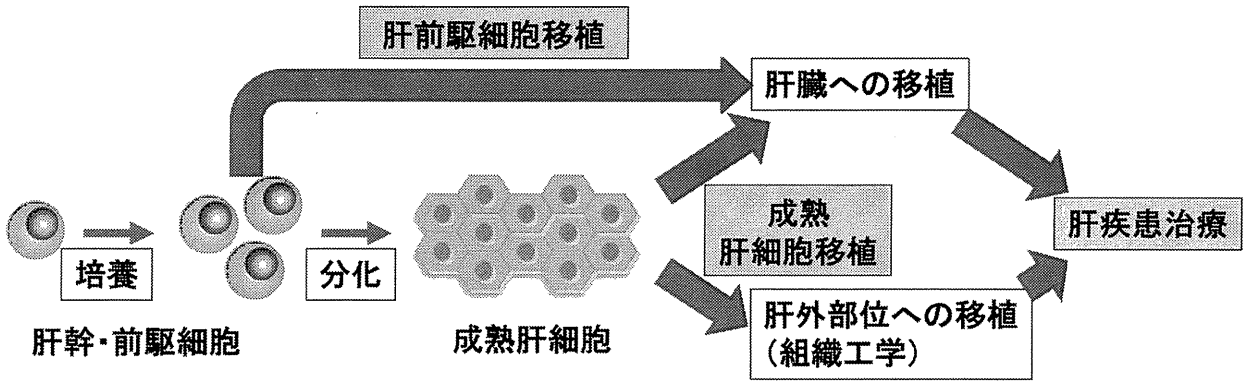

図1　次世代再生医療を目指した肝細胞移植療法
肝細胞移植療法として成熟肝細胞を用いた組織工学的アプローチと肝前駆細胞を用いた細胞生物学的アプローチなどが現在行われている。

で皮下組織は治療の侵襲度が最も小さく肝組織工学の標的部位として利点を持つものの，血流の乏しさから肝細胞の生着やその後の代謝機能の発揮に適さない。そこで，皮下の組織作成予定部位に予めbFGFの除放システムを組み込み血管を誘導した後に，EHSマトリックスとともに肝細胞を移植するという二段階法によって皮下における小肝組織の作製系が樹立された[2]。このような異所性小肝組織はアルブミンの発現や薬物代謝酵素群の誘導が認められ，肝臓の代謝機能を有する[2]。また，このような小肝組織を血友病モデルマウス腎被膜下に作製することで血液凝固因子の産生を促し，治療が可能なことが分かった[1]。

以上の結果では，自己肝臓と同等で長期機能維持可能な異所性肝臓の作製に成功したものの，本来の肝臓の三次元的な構造を誘導することは困難である。そこで，肝細胞相互の機能接着と肝細胞-血管内皮細胞の接着を制御することにより三次元構造が誘導できることを利用し，シート工学を応用した肝組織作製手法が開発されている。ポリN-イソプロピルアクリルアミドをナノメートルレベルで均一にコーティングした培養皿は32℃以上では疎水性で接着培養可能であるが，32℃以下では親水性に変化し，細胞をシート状に回収できる。これにより取得した肝細胞シートは，機能的細胞間結合が維持されており，かつ細胞外マトリックスによる細胞外微小環境を有した二次元構造を有する。そこで6FGFにより誘導した皮下の血管組織上にこのような肝細胞シートを層状に複数移植することで，肝細胞相互の機能接着と肝細胞-血管内皮細胞の接着を有する三次元的構造を形成し，より高い肝機能を発揮できることが報告されている[3]。

以上のように，成熟肝細胞の異所性移植による小肝組織の作成系が研究されている。この異所性小肝組織は本来の肝臓と類似した代謝機能を有し，また動物実験レベルでの臨床的な有効性も示されており，今後の研究によるヒトへの応用が期待されている。

2．肝幹・前駆細胞を用いた細胞生物学的アプローチ

成熟肝細胞の障害肝臓への移植，また先に示した異所性肝構造形成による肝機能向上による肝疾患治療の有用性が検証される一方で，分離した成熟肝細胞は生体外では増殖が困難であり必要とされる大量の成熟肝細胞のソースが問題となっている。そこで，生体外でも高い増殖能力を有する肝幹・前駆細胞に着目し，この肝幹・前駆細胞を純化・増殖させる細胞生物学的アプローチも進められている。ここでは肝幹・前駆細胞の純化・増幅系を確立するための基礎研究，および肝幹・前駆細胞を用いた細胞移植療法への応用について概説する。

生体内では，胎児期および成体肝臓における肝幹・前駆細胞の存在やその性状が報告されている。肝芽細胞は，胎児期の肝臓に存在する高い増殖性と肝細胞・胆管上皮細胞への二方向の分化能を持つ肝前駆細胞である。肝芽細胞を標識する表面抗原マーカーとして，Delta-like 1 homolog（Dlk1）やLiv2などが報告されている[4,5]。Dlk1は，胎児期の肝前駆細胞を用いたシグナルシークエンストラップ法によって同定された表面

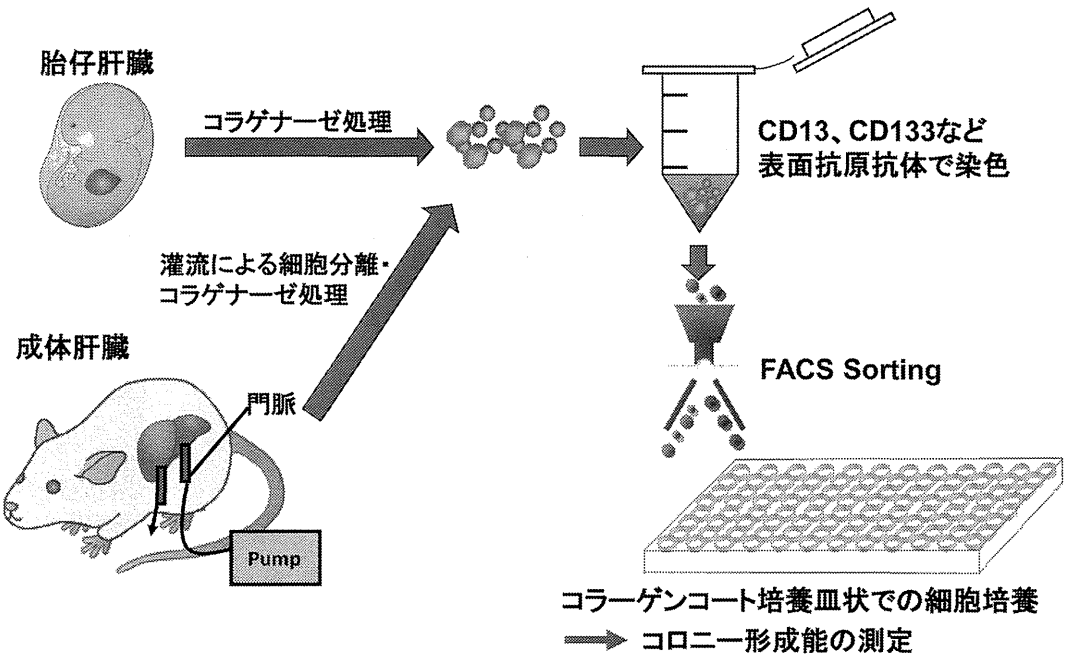

図２　FACS を用いた肝幹・前駆細胞の分離と培養

胎仔肝臓または成体肝臓より細胞を取得し，CD13，CD133 などの表面抗原抗体で染色後 FACS により分離する。分離した細胞はコラーゲンコート培養皿状で培養し，コロニー形成能を測定する。

抗原タンパク質であり，幼弱な肝細胞に発現する一方で肝発生の進行に伴いその発現が低下・消失する[4]。

また，筆者らは表面抗原抗体を用いた網羅的なスクリーニングによって，CD13 等の新規の肝芽細胞マーカーを同定している[6]。胎児肝由来 CD13 陽性細胞は，高増殖性とアルブミン陽性の肝細胞系およびサイトケラチン（CK）19 陽性の二方向性の分化能をもつ。また，肝障害モデルマウスへ CD13 陽性細胞を移植することで，アルブミン陽性の成熟肝細胞としてレシピエント肝臓に生着することが分かった。以上の結果から，肝幹・前駆細胞のマーカーとして CD13 の有用性が示された。

このように胎生期における肝幹・前駆細胞の同定が進んでいる一方で，正常の成体肝臓での肝幹・前駆細胞については不明な点が多い。肝臓の一部を切除した際の肝再生などでは成熟肝細胞の肥大と一過性の増殖により再生を行い，肝幹・前駆細胞は関与していない[7]。しかし，重篤な肝障害等で成熟肝細胞が増殖できない状態では，成体肝臓の門脈領域周囲に Oval cell とよばれる細胞が出現し肝再生に重要な役割を果たしていることが分かった[8]。Oval cell の起源は胆管か肝細胞か議論があるところであるが，Oval cell の誘導には，肝障害時に門脈周囲の Thy1 陽性細胞が産生する FGF7 が必要であることが明らかにされている[9]。さらに正常成体肝臓においても胆管周囲の Canals of Hering 領域に EpCAM 陽性の肝幹・前駆細胞が存在することが報告された[10]。筆者らは表面抗原マーカーの網羅的探索によって，正常成体肝臓由来の肝幹・前駆細胞の純化・培養系の構築を行った。その結果，マウス胎仔肝幹細胞の表面抗原マーカーである CD13 および CD133 の陽性細胞が正常成体肝臓の非実質細胞画分に存在することを見出した。CD13+CD133+ 細胞を純化しクローナルに培養した結果，アルブミンと CK19 が共に陽性で高増殖性のコロニーが複数出現した。この細胞は継代による長期培養が可能である。さらに，オンコスタチン M と細胞外マトリックスの添加により成熟肝細胞の機能遺伝子の発現を誘導できる一方で，コラーゲン包埋培養によって胆管様構造が誘導されることから，肝細胞と胆管上皮細胞への二方向への分化能を有することが分かった。以上の結果から，正常肝臓の CD13+CD133+ 細胞画分に，成体における肝幹・前駆細胞が存在することが示唆された[11]。（図2）

次に筆者らは，肝幹・前駆細胞の細胞移植療法における有用性を検討した。アポリポプロテイン E は家族性高脂血症の原因遺伝子であり，その欠損マウスはコレステロール代謝異常による血中コレステロール上昇や動脈硬化などの病態を示す。ApoE は肝細胞が主要

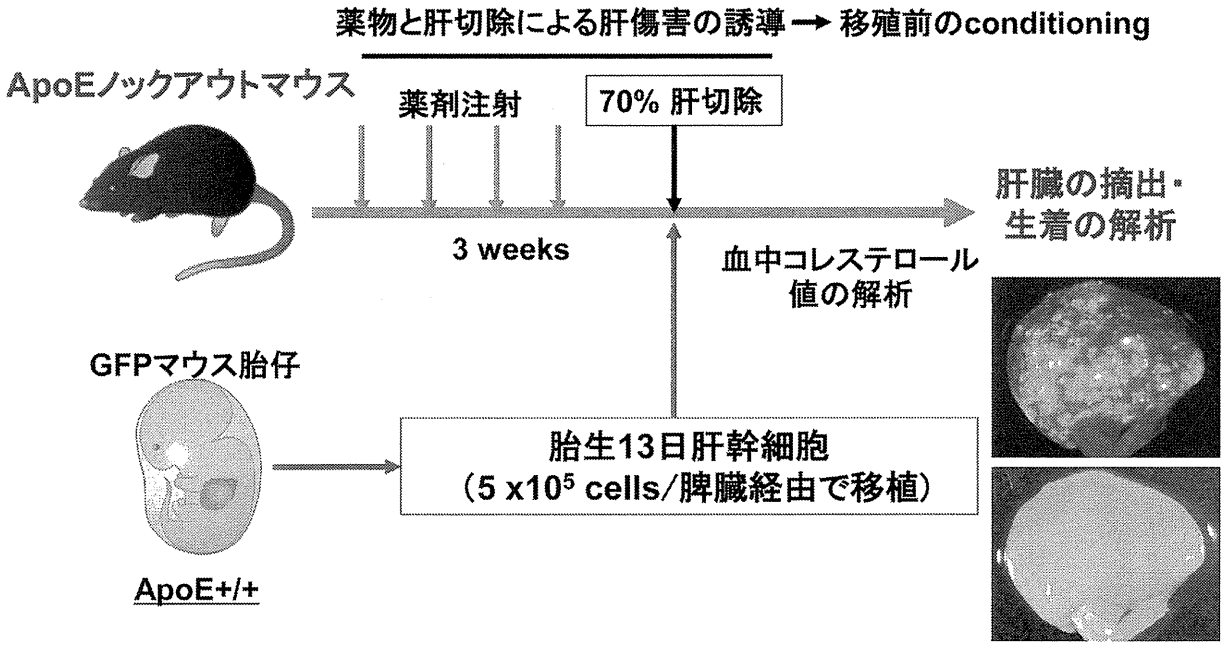

図３　肝幹細胞を使用したApoE欠損マウスの細胞移植療法

ApoE欠損マウスは肝傷害を誘導した後，GFPトランスジェニックマウス由来肝前駆細胞を脾臓経由で移植した。移植後4か月経過してもGFP陽性細胞の生着が認められた。

な産生細胞であることから，細胞移植による治療効果を期待し以下の実験を行った。胎生13日マウス（GFPトランスジェニックマウス由来）肝臓より肝前駆細胞を単離し，ApoE欠損マウス肝臓へ経脾臓的に移植した結果，移植後4か月以上でもドナー細胞の生着が認められた（図3）。このとき血中コレステロールも速やかに低下し，1年以上にわたり効果が持続した。これらのことから，移植した肝幹・前駆細胞は長期にわたり生着・機能し，疾患の治療が可能であることが示された。

おわりに

本稿では，肝臓移植を代替する肝疾患治療法として，①成熟肝細胞を用いた組織工学的アプローチ，②肝幹・前駆細胞を用いた細胞生物学的アプローチを解説した。いずれの治療法も動物実験での有効性が示されており，今後ヒトへの臨床応用が期待される。しかし，生体外に取り出した成熟肝細胞や肝幹・前駆細胞は，肝機能関連遺伝子などの発現が生体内の肝細胞と比較し低下していることが知られている。細胞移植療法の確立のために，生体外で高い肝機能を維持する，または誘導できるような培養系の構築が重要と考えられる。

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肝腫瘍に対する創薬シーズ
幹細胞マーカーSALL4の肝癌治療法における可能性

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索引用語：SALL4，幹細胞，癌幹細胞，肝癌

1 はじめに

幹細胞は自己複製能と多分化能を兼ね備えた細胞で，さまざまな臓器・組織の機能細胞の元になる組織幹細胞が同定されている．一方，癌組織でも一部のヘテロな細胞集団が癌の維持に重要であり，癌幹細胞の存在が示唆されていた．近年，白血病に加えてさまざまな固形癌においても自己複製と腫瘍形成能を有する癌幹細胞の存在が報告され，癌の階層社会の起点として発癌，浸潤，転移，再発などに密接に関与している可能性が報告されている[1]．正常幹細胞と癌幹細胞は，自己複製制御機構や表面マーカーの発現パターンが類似しており，この自己複製制御機構の破綻が癌化につながる可能性が示唆されている[2]．癌幹細胞の同定にはさまざまな細胞表面マーカーが用いられており，肝癌ではEpithelial cell adhesion molecule（EpCAM），CD133, CD90, CD44, CD24, CD13などが報告されている[3-7]．またATP-binding cassette(ABC) transporterを介したDNA染色色素Hoechst33342の排泄能が高い細胞集団side population（SP細胞）が幹細胞に近い性質を持つことが知られており，肝癌でのSP細胞の存在が確認されている[8,9]．これらの幹細胞マーカー陽性の細胞を含む肝細胞癌では予後が不良であることが報告されており[10]，癌の根治を目指すには抗癌剤感受性や浸潤転移に深く関与する癌幹細胞を標的とした治療が重要になる．

本稿では，ヒト正常肝幹細胞と肝癌における新規幹細胞マーカーSALL4の発現ならびに機能と将来的な肝癌治療法における可能性について考察する．

2 幹細胞マーカーSALL4

SALL4はショウジョウバエからヒトまで広く保存されたジンクフィンガー蛋白質であり，その遺伝子異常から生じるOkihiro症候群は，主に上肢形成不全や眼球運動制限，ま

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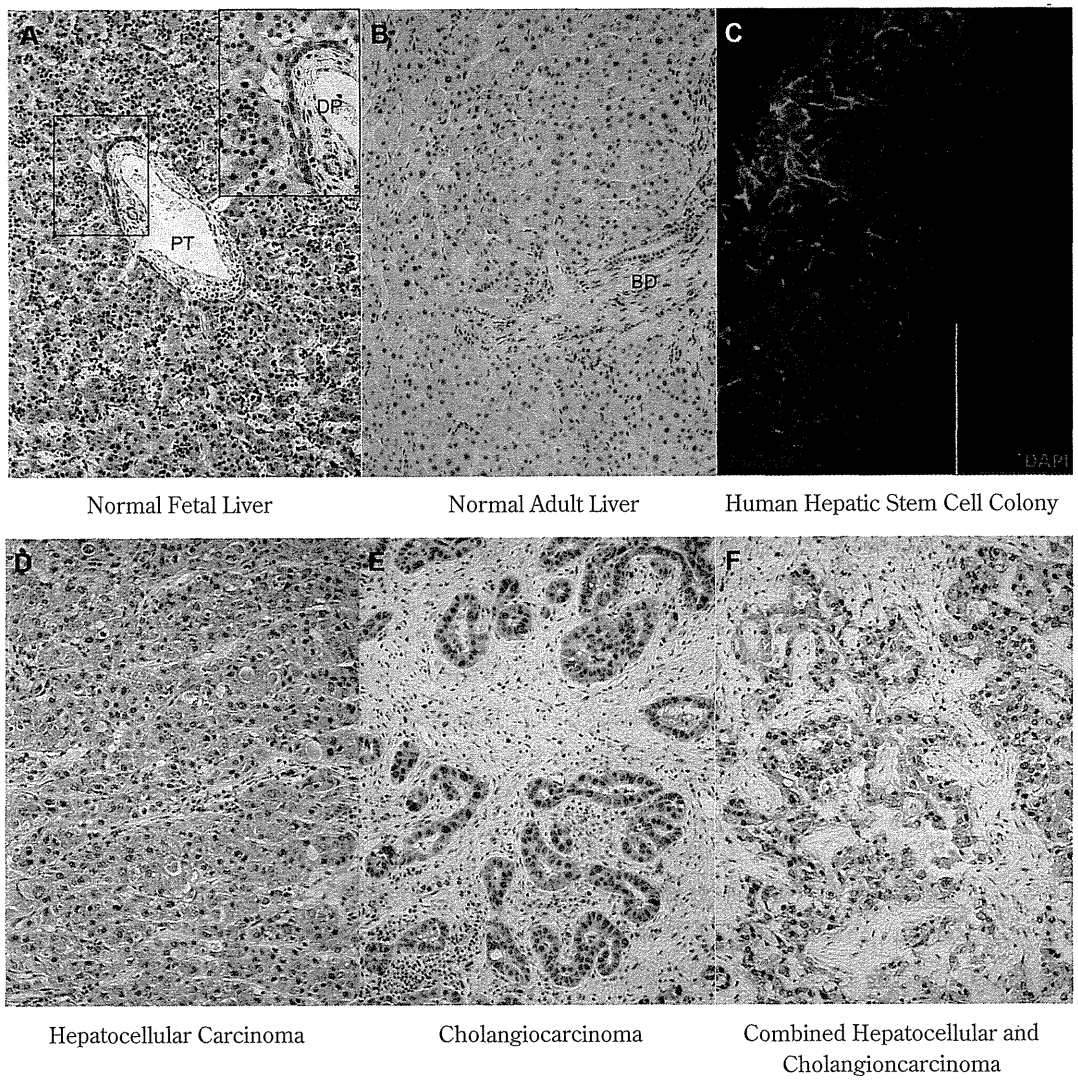

図1　ヒト正常肝組織とさまざまな肝癌組織におけるSALL4の発現

A：正常胎児肝（19週）ではductal plate（DP）を含め，肝小葉にびまん性にSALL4発現を認めるが，B：正常成体肝では全くSALL4発現がみられない，C：*in vitro*培養ヒト肝幹細胞においてSALL4はEpCAMと共発現する，D：肝細胞癌（HCC），E：胆管細胞癌（CC），F：混合型肝癌（HC-CC）でSALL4の発現が認められる．PT：portal tract, DP：ductal plate, BD：bile duct（文献21より改変引用）

た心室中隔欠損や直腸形成異常を一部に呈する[11]．Sall4ノックアウトマウスの解析では，ホモマウスは胎生致死であり，ヘテロマウスの大半は正常に生育・繁殖するものの，ヒトのOkihiro症候群に類似した心臓，直腸や脳の奇形が確認されている[12]．SALL4は核内に存在する転写因子として作用しembryonic stem細胞の機能に重要であることが知られており，NANOG, OCT3/4, SOX2と相互作用することで増殖と未分化性の維持や分化決定を制御する[12,13]．近年では体細胞からinduced pluripotent stem（iPS）細胞を誘導する際のリプログラミングファクターの一つとして報告されている[14]．したがって，SALL4