Generation of Safe and Therapeutically Effective Human Induced Pluripotent Stem Cell-Derived Hepatocyte-Like Cells for Regenerative Medicine

Kazuo Takayama,[1–3*] Naoki Akita,[1,3*] Natsumi Mimura,[3] Rina Akahira,[4] Yukimasa Taniguchi,[5] Makoto Ikeda,[6] Fuminori Sakurai,[1,7] Osamu Ohara,[6] Tomohiro Morio,[8] Kiyotoshi Sekiguchi,[5] and Hiroyuki Mizuguchi[1,3,9]

Hepatocyte-like cells (HLCs) differentiated from human induced pluripotent stem (iPS) cells are expected to be applied for regenerative medicine. In this study, we attempted to generate safe and therapeutically effective human iPS-HLCs for hepatocyte transplantation. First, human iPS-HLCs were generated from a human leukocyte antigen-homozygous donor on the assumption that the allogenic transplantation might be carried out. Highly efficient hepatocyte differentiation was performed under a feeder-free condition using human recombinant laminin 111, laminin 511, and type IV collagen. The percentage of asialoglycoprotein receptor 1-positive cells was greater than 80%, while the percentage of residual undifferentiated cells was approximately 0.003%. In addition, no teratoma formation was observed even at 16 weeks after human iPS-HLC transplantation. Furthermore, harmful genetic somatic single-nucleotide substitutions were not observed during the hepatocyte differentiation process. We also developed a cryopreservation protocol for hepatoblast-like cells without negatively affecting their hepatocyte differentiation potential by programming the freezing temperature. To evaluate the therapeutic potential of human iPS-HLCs, these cells (1×10^6 cells/mouse) were intrasplenically transplanted into acute liver injury mice treated with 3 mL/kg CCl_4 only once and chronic liver injury mice treated with 0.6 mL/kg CCl_4 twice weekly for 8 weeks. By human iPS-HLC transplantation, the survival rate of the acute liver injury mice was significantly increased and the liver fibrosis level of chronic liver injury mice was significantly decreased. *Conclusion*: We were able to generate safe and therapeutically effective human iPS-HLCs for hepatocyte transplantation. (*Hepatology Communications* 2017;1:1058-1069)

Introduction

Orthotopic liver transplantation and hepatocyte transplantation are effective treatments against chronic liver failure, acute liver failure, and hereditary liver diseases.[1,2] However, the shortage of donor livers and hepatocytes is a serious problem. Therefore, hepatocyte-like cells (HLCs) differentiated from human induced pluripotent stem (iPS) cells, which have the potential to self-replicate and differentiate into almost all types of cells, would be an attractive cell source.[3] Several groups, including us, have developed hepatocyte differentiation technologies from human iPS cells and have demonstrated the therapeutic potential of human iPS-HLCs against liver failure by using mice models of liver failure.[4–7] However, the clinical application of human iPS-HLCs has not been realized because the safety of human iPS-

Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; CiRA, Center for iPS Cell Research and Application; CYP3A4, cytochrome P450, subfamily 3, polypeptide A4; ES, embryonic stem; HBC, hepatoblast-like cell; HGF, hepatocyte growth factor; HLA, human leukocyte antigen; HLA-homo, human leukocyte antigen homozygous; HLC, hepatocyte-like cell; HLC-CD, hepatocyte-like cells generated by current hepatocyte differentiation method; HLC-CNV, hepatocyte-like cells generated by conventional hepatocyte differentiation method; iPS, induced pluripotent stem; LN111-E8, LN511-E8, recombinant laminin-111 or 511 E8 fragment; NOG, NOD.Cg-PrkdcscidIl2rgtm1Sug/ShiJic; PHH, primary human hepatocyte.

Received June 28, 2017; accepted September 19, 2017.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1111/full.

Supported by K-CONNEX, established by the Human Resource Development Program for Science and Technology (to K.T.); the Project for Technological Development, Research Center Network for Realization of Regenerative Medicine of the Japan Agency for Medical Research and Development (to H.M., K.S., O.O., and T.M.); and the Naito Foundation (to H.M.).

**These authors contributed equally to this work.*

HLC transplantation has not been sufficiently verified. Therefore, a method to generate human iPS-HLCs that are not only therapeutically effective but also safe is urgently needed.

The major concerns of the clinical application of iPS cell derivatives (including hepatocytes) are teratoma formation, oncogenesis, and immune rejection.[8] To address these concerns, measuring the rate of residual undifferentiated cells and evaluating the risk of teratoma formation by transplanting iPS cell derivatives into immunodeficient mice are necessary steps.[9] It is also necessary to determine whether harmful genetic mutations arise during the reprogramming and directed differentiation. Although autologous transplantation of iPS cell derivatives is preferable to avoid transplant rejection, the customized preparation of iPS cell derivatives for individual patients is time consuming and expensive. For this reason, allogenic transplantation using human iPS cells from a separate donor is eagerly anticipated. In human leukocyte antigen (HLA)-mismatched transplantation, graft versus host reaction and transplant rejection will occur with high probability. Therefore, with the goal of preventing transplant rejection, human iPS cells were established from an HLA-homozygous donor (a donor who received the same HLA from both parents; this describes 2%-4% of the Japanese population) for allogenic transplantation at the Center for iPS Cell Research and Application (CiRA), Kyoto University. It is necessary to generate human iPS-HLCs from an HLA-homozygous donor to perform transplantation in a large number of patients with liver failure. In addition, a hepatocyte differentiation protocol with minimal or no use of serum, Matrigel, or feeder cells is also needed to avoid unexpected adverse events.

In this study, HLA homozygous human iPS cells (HLA-homo iPS cells), which were provided from the CiRA, were differentiated into HLCs without using feeder cells, Matrigel, or serum. The risk of teratoma formation and oncogenesis was evaluated. To evaluate the therapeutic effects of human iPS-HLCs, we transplanted these cells into acute and chronic liver-failure model mice. Our aim was to generate safe and

View this article online at wileyonlinelibrary.com.
DOI 10.1002/Hep4.1111

Potential conflict of interest: Dr. Sekiguchi is employed, owns stock, has intellectual property rights, and has received grants from Matrixome, Inc. Dr. Taniguchi is a project leader for Matrixome, Inc. The other authors have declared no conflict of interest.

ARTICLE INFORMATION:

From the [1]Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [2]PRESTO, Japan Science and Technology Agency, Saitama, Japan; [3]Laboratory of Hepatocyte Regulation, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan; [4]ReproCELL Inc, Kanagawa, Japan; [5]Institute for Protein Research, Osaka University, Osaka, Japan; [6]Department of Technology Development, Kazusa DNA Research Institute, Chiba, Japan; [7]Laboratory of Regulatory Sciences for Oligonucleotide Therapeutics, Clinical Drug Development Project, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [8]Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University, Tokyo, Japan; [9]Global Center for Medical Engineering and Informatics, Osaka University, Osaka, Japan.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

For general correspondence:
Hiroyuki Mizuguchi, Ph.D.
Laboratory of Biochemistry and Molecular Biology
Graduate School of Pharmaceutical Sciences, Osaka University
1-6 Yamadaoka, Suita
Osaka 565-0871, Japan
E-mail: mizuguch@phs.osaka-u.ac.jp
Tel: +81-6-6879-8185
or

For recombinant laminin products:
Kiyotoshi Sekiguchi, Ph.D.
Division of Matrixome Research and Application
Institute for Protein Research, Osaka University
3-2 Yamadaoka, Suita
Osaka 565-0871, Japan
E-mail: sekiguch@protein.osaka-u.ac.jp
Tel: +81-6-6105-5935

therapeutically effective human iPS-HLCs that have the potential to be applied in clinical applications.

Materials and Methods

STUDY APPROVAL

This study was approved by the ethics committees of Osaka University and the National Institutes of Biomedical Innovation, Health, and Nutrition. All experiments were performed in accordance with relevant guidelines and regulations and with the approval of Osaka University and the National Institutes of Biomedical Innovation, Health, and Nutrition.

HEPATOCYTE TRANSPLANTATION FOR ACUTE LIVER-FAILURE MICE

NOD.Cg-PrkdcscidIl2rgtm1Sug/ShiJic (NOG) mice (Central Institute for Experimental Animals)[10] were intraperitoneally infused with 3 mL/kg CCl_4 (Wako) 1 day before transplantation. To obtain single-cell suspension of human iPS-HLCs for the transplantation, human iPS-HLCs were treated with a mixture of 1 mg/mL dispase (Roche) and 1 mg/mL collagenase (SERVA Electrophoresis GmbH) for 30 minutes. Recipient mice were anesthetized with isoflurane (Pfizer) and injected with 1×10^6 viable human iPS-HLCs through a small left-flank incision into the inferior splenic pole. Hepatocyte Culture Medium (Lonza) was used as the injection vehicle. After hepatocyte transplantation, the spleen was tied with sutures tight enough to achieve hemostasis.

Results

CHARACTERIZATION OF HLA-Homo iPS CELLS

We used HLA-homo human iPS cells that had been established at CiRA, Kyoto University. To generate human iPS cells, peripheral blood mononuclear cells were collected from a healthy donor and were transfected with four plasmids (pCE-hSK, pCE-hUL, pCE-hOCT4, and pCE-mp53DD).[11] The human iPS cell line Ff-l01s01 was generated from an HLA-homozygous donor. The human iPS cell line 1383D6 was established from a non-HLA-homozygous donor, using the same materials and methods as for Ff-l01s01. To assess the quality of human iPS cells, nine tests were performed (Supporting Fig. S1A) at CiRA. Donor information is summarized in Supporting Fig. S1B, and the morphology of these iPS cells is shown in Supporting Fig. S2A. We examined whether there was a difference in cellular morphology between the feeder-free iPS culture method and the on-feeder iPS culture method (Supporting Fig. S2B). In the case of the former, human iPS cells were cultured with AK03 medium, which consists solely of refined substances completely free of animal-derived components, on a human recombinant laminin (LN)511-E8 fragment (LN511E8). For the on-feeder iPS culture method, human iPS cells were cultured with ReproStem medium, which contains animal-derived components, on mouse embryonic fibroblasts. The human iPS cells were cultured for five passages under each condition. There were no significant differences in the gene expression levels of pluripotent markers (*octamer-binding transcription factor 3/4* [*OCT3/4*] and *homeobox protein NANOG* [*NANOG*] or definitive endoderm markers (*forkhead box A2* [*FOXA2*] and *sex determining region Y-box 17* [*SOX17*]) between the feeder-free and on-feeder culture method.

HEPATOBLAST DIFFERENTIATION FROM HUMAN iPS CELLS

In our previous reports, Matrigel was used for hepatoblast differentiation. However, human iPS cells, which were cultured on LN511-E8, hardly adhered to Matrigel. Interestingly, we found that the gene expression levels of collagen receptors (*integrins α1, α2, α4, α10*, and *α11*) were lower in the culture on LN511-E8 than in the culture on mouse embryonic fibroblasts (Supporting Fig. S3). This suggests that human iPS cells, which were cultured on LN511-E8, are attachable to laminin rather than collagen. Therefore, we decided to use laminins for hepatoblast differentiation. Human iPS cells (1383D6) cultured on LN511-E8 were dissociated and plated onto a mixture of LN511-E8 and LN111-E8 because human pluripotent stem cells can be maintained on LN511-E8[12] and we had reported that hepatoblast-like cells (HBCs) could be maintained on LN111.[12-14] Human iPS cells were precultured on laminins and differentiated into HBCs by definitive endoderm cells (Supporting Fig. S4A). The cell viability (Supporting Fig. S4B) and gene expression levels of pluripotent markers (*OCT3/4* and *NANOG*) (Supporting Fig. S4C) in human iPS cells cultured on LN511-E8 were similar to those of human iPS cells cultured on a mixture of LN511-E8 and

LN111-E8. After hepatoblast differentiation, the gene expression levels of hepatoblast markers (*alpha-fetoprotein* [*AFP*] and *hepatocyte nuclear factor 4* [*HNF4a*]) (Supporting Fig. S4D) and the percentage of AFP-positive cells (Supporting Fig. S4E) in HBCs, which were cultured on a mixture of LN511-E8 and LN111-E8 at a ratio of 1:3, were the highest as compared with other culture condition. These results suggest that the maintenance of human iPS cells and hepatoblast differentiation can be accomplished on a mixture of LN511-E8 and LN111-E8 at a ratio of 1:3.

PURIFICATION OF HBCs AND ELIMINATION OF RESIDUAL UNDIFFERENTIATED CELLS

To purify human iPS cell-derived HBCs (human iPS-HBCs), human iPS cell-derived cells were dissociated and plated onto various laminin isoforms. By using LN111 or LN111-E8, the percentage of AFP-positive cells reached almost 100% (Supporting Fig. S5A). In addition, LN111-E8 could maintain the AFP-positive cells at a lower concentration (0.5 $\mu g/cm^2$) compared to LN111 (4 $\mu g/cm^2$) (Supporting Fig. S5B). These results suggest that human iPS-HBCs could be purified by using LN111-E8. To estimate the percentage of residual undifferentiated cells in human iPS-HBCs, the percentage of Tral-60-positive cells in the nonpurified or purified iPS-HBCs was measured (Supporting Fig. S5C) and was 12.2% in the nonpurified iPS-HBCs versus 2.7% in the purified iPS-HBCs (Supporting Fig. S5D). However, because not all Tra1-60-positive cells have proliferative activity as pluripotent stem cells, a colony formation assay was performed (Supporting Fig. S5E) to strictly estimate the number of residual undifferentiated cells. The Tra1-60-positive cells in nonpurified or purified iPS-HBCs were sorted and cultured with AK03 medium on LN511-E8 for 10 days. The percentage of residual undifferentiated cells in purified iPS-HBCs was approximately 0.003% versus approximately 0.385% in nonpurified iPS-HBCs. The results indicated that the percentage of residual undifferentiated cells was reduced more than 100 times by the purification process using LN111-E8.

HEPATOCYTE DIFFERENTIATION FROM HBCs

To differentiate HLCs from human iPS-HBCs, human iPS-HBCs were sequentially treated with hepatocyte growth factor (HGF) and oncostatin M (OsM). During the hepatocyte maturation process, HLCs were overlaid with type I or IV collagen (Supporting Fig. S6A). The gene expression levels of hepatocyte markers (*alpha-1 antitrypsin* [*AAT*] and *albumin* [*ALB*]) in human iPS-HLCs overlaid with thin layer of type IV collagen were similar to those in primary human hepatocytes (PHHs) (Supporting Fig. S6B). In addition, the percentage of hepatocyte marker (ALB and asialoglycoprotein receptor 1)-positive cells was more than 80% (Supporting Fig. S6C). These results suggest that functional human iPS-HLCs were efficiently generated by overlaying with thin type IV collagen.

CHARACTERIZATION OF HUMAN iPS-HLCs

Our current hepatocyte differentiation method is schematically shown in Fig. 1A. After hepatocyte differentiation, the morphology of the HLCs was similar to that of the PHHs, which were polygonal with distinct round binuclei (Fig. 1B). Human iPS-HLCs were stained for cytoplasmic glycogen using the periodic acid–Schiff staining procedure, suggesting that human iPS-HLCs could store glycogen (Fig. 1B). We also confirmed that human iPS-HLCs were positive for cytochrome P450 family 3 subfamily A member 4 (CYP3A4) and ALB by immunostaining (Fig. 1B). Next, we compared our current hepatocyte differentiation method with our previous hepatocyte differentiation method using mouse embryonic fibroblasts, serum, and Matrigel.[15] There was no significant difference in the gene expression levels of hepatocyte markers (Fig. 1C), ALB (Fig. 1D, left), or urea (Fig. 1D, right) secretion capacities between human iPS-HLCs generated by our current hepatocyte differentiation method (HLC-CD) and human iPS-HLCs generated by our conventional hepatocyte differentiation method (HLC-CNV). In addition to the ALB and urea secretion capacities, there was no significant difference in the activities of CYP1A2 and CYP3A4 (Fig. 1E). To examine the induction potencies of CYP1A2, CYP2B6, and CYP3A4, we treated HLC-CNV and HLC-CD with omeprazole, phenobarbital, and rifampicin, which are known to induce CYP1A2, CYP2B6, and CYP3A4, respectively (Fig. 1F). The induction potency of the CYPs in HLC-CD was similar to that of the CYPs in HLC-CNV. These results suggest that our current hepatocyte differentiation method could efficiently generate functional human

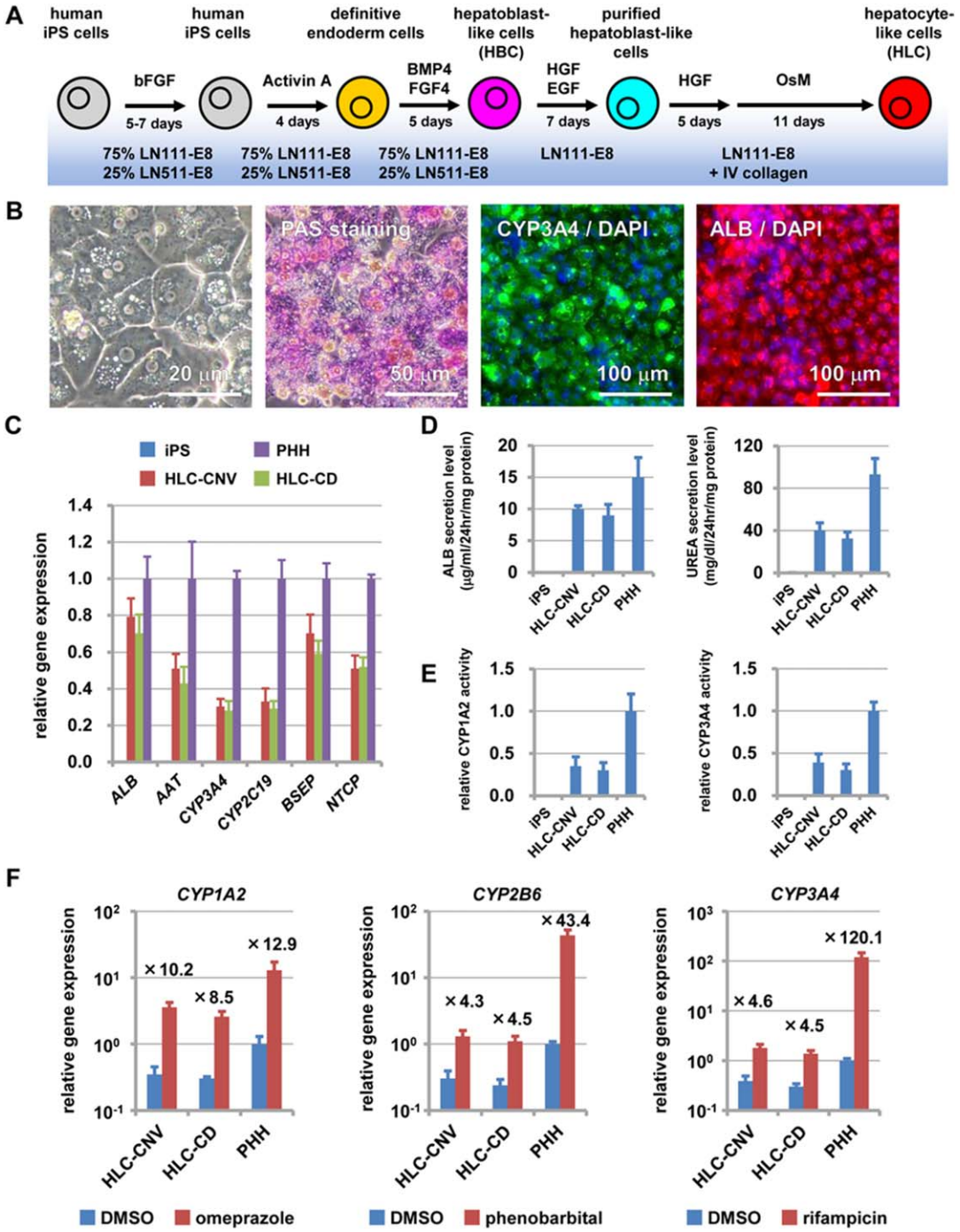

FIG. 1. Evaluation of hepatocyte functionality of human iPS cell-derived HLCs. (A) The procedure for differentiation of human iPS cells (1383D6) into HLCs is presented schematically. Details of the hepatic differentiation procedure are described in the Materials and Methods. (**B**) A phase image of the HLCs is shown. Glycogen storage in the HLCs was confirmed by PAS staining. The expression of the hepatocyte markers (CYP3A4 and ALB) was examined by immunohistochemistry on day 32 of the differentiation. Nuclei were counterstained with DAPI (blue). (**C**) The gene expression levels of hepatocyte markers (*ALB, AAT, CYP3A4, CYP2C9, BSEP*, and *NTCP*) in undifferentiated human iPS cells, HLC-CNV (mouse embryonic fibroblasts, Matrigel, and serum were used[15]), HLC-CD, and PHHs were examined by real-time RT-PCR. The gene expression levels in the PHHs were taken as 1.0. (**D**) The amounts of ALB (left) and urea (right) secretion were examined in undifferentiated human iPS cells, HLC-CNV, HLC-CD, and PHHs. (**E**) CYP1A2 (left) and 3A4 (right) activity levels were also examined. (**F**) To examine CYP1A2 (left), 2B6 (middle), and 3A4 (right) induction potency, the cells were treated with 50 μM omeprazole for 24 hours, 500 μM phenobarbital for 48 hours, or 20 μM rifampicin for 48 hours; these agents are known to induce CYP1A2, 2B6, and 3A4, respectively. The gene expression levels of the *CYPs* were measured by real-time RT-PCR. Controls were treated with DMSO (final concentration 0.1%). The gene expression levels in the DMSO-treated PHHs were taken as 1.0. All data are represented as means ± SEM (n = 3). Abbreviations: *AAT, alpha-1 antitrypsin*; BMP4, bone morphogenetic protein 4; *BSEP, bile salt export pump*; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FGF, fibroblast growth factor; *NTCP, sodium/taurocholate co-transporting polypeptide*; OsM, *oncostatin M*; PAS, periodic acid–Schiff; RT-PCR, reverse-transcription polymerase chain reaction.

iPS-HLCs as effectively as our conventional hepatocyte differentiation method. To confirm the robustness of our hepatocyte differentiation method, four human iPS cell lines (1231A3, Ff-l01s01, Ff-l01, and Ff-l14) were differentiated into HLCs (Supporting Fig. S7). Similar results were obtained by using other iPS cell lines.

CRYOPRESERVATION OF HBCs

We next attempted to develop a cryopreservation protocol for HBCs. HBC cryopreservation would allow us to store a stock of HBCs so that we could avoid having to differentiate large amounts of cells at the same time. To strictly control the temperature of the freezing cells, we used the program Deep Freezer (PDF300) and found that the cell recovery rate was highest in the thermal management of "condition 3" (Fig. 2A). In addition, the cell recovery rate was highest by using the cryopreservation medium "Bambanker" (Fig. 2B). We also found that the percentage of AFP-positive cells largely depended on cell seeding density (Fig. 2C). The percentage of AFP-positive cells was almost 100% in the high-density cell-culture condition (1.25×10^5 cells/cm^2). The final procedure for freezing human iPS-HBCs is shown in Fig. 2D. The cell recovery rate after cryopreservation was 78.7% (Fig. 2E). In addition, to examine the effect of cryopreservation on hepatocyte differentiation capacity, the nonfrozen iPS-HBCs and frozen iPS-HBCs were differentiated into HLCs. The gene expression levels of hepatocyte markers (*ALB, AAT, CYP3A4*, and *CYP2C9*) in human iPS-HLCs differentiated from the frozen iPS-HBCs were similar to those in human iPS-HLCs differentiated from the nonfrozen iPS-HBCs (Fig. 2F). Moreover, similar results were obtained by using an HLA-homo iPS cell line (Ff-l01s01) (Supporting Fig. S8). These results suggest that human iPS-HBCs could be cryopreserved without negatively affecting their hepatocyte differentiation potential.

SAFETY ASSESSMENT OF HLCs

To assess the safety of human iPS-HLCs, we first performed a teratoma formation assay (Fig. 3A). Immunodeficient mice were subcutaneously transplanted with undifferentiated human iPS cells (1×10^5 cells/NOG mouse), human iPS cell-derived purified HBCs (1×10^5 or 1×10^7 cells/NOG mouse), and human iPS cell-derived HLCs (1×10^5 or 1×10^7 cells/NOG mouse). Teratoma formation was observed at 4 weeks after transplantation in the mice transplanted with undifferentiated human iPS cells. However, no teratoma formation was observed even at 16 weeks after transplantation in the mice transplanted with human iPS cell-derived purified HBCs and human iPS-HLCs. In addition, no teratoma formation was observed in the mice transplanted with Tra1-60-positive cells in purified HBCs (2.7×10^5, 2.7×10^6, or 2.7×10^7 cells/NOG mouse) (Supporting Fig. S5F). Next, we examined whether a cancer-related somatic mutation was introduced during hepatoblast and/or hepatocyte differentiation by performing targeted sequencing with a comprehensive cancer panel (Fig. 3B). The genomic sequences of 596 cancer-related genes in human iPS-HBCs, purified iPS-HBCs, and human iPS-HLCs were compared with that of undifferentiated human iPS cells. To minimize the number of false-positive substitution calls, three independent bioinformatics tools (LoFreq, MuTech, and VarScan2) were collectively used because the somatic variant call with either bioinformatics tool has a considerably high false-positive rate.[16,17] Thus, we considered that single substitutions detected by all three bioinformatics tools would be likely candidates for somatic variations newly generated during differentiation; however, we did not observe any likely candidate for somatic variation in the 596 cancer-related genes during the hepatoblast and hepatocyte differentiation (Fig. 3B). To further characterize human iPS-HBCs and iPS-HLCs, RNA sequencing analysis was performed in undifferentiated human iPS cells, human iPS-HBCs, purified iPS-HBCs, and iPS-HLCs (Fig. 3C,D). Messenger RNA expression profiles of pluripotent markers in human iPS-HBCs, purified iPS-HBCs, and iPS-HLCs were largely different from those of undifferentiated human iPS cells (Fig. 3C). In addition, human iPS cells obtained liver-specific gene expression patterns during the hepatocyte differentiation process (Fig. 3D). Taken together, these results show that human iPS-HBCs and iPS-HCLs have a low risk of teratoma formation and oncogenesis.

TRANSPLANTATION OF HLA-homo iPS CELL-DERIVED HLCs RESCUED MICE FROM ACUTE LIVER INJURIES

To evaluate the therapeutic potential of human iPS-HLCs, these cells were transplanted into mice with acute or chronic liver injury. As a mouse model of acute liver injury, we used immunodeficient mice

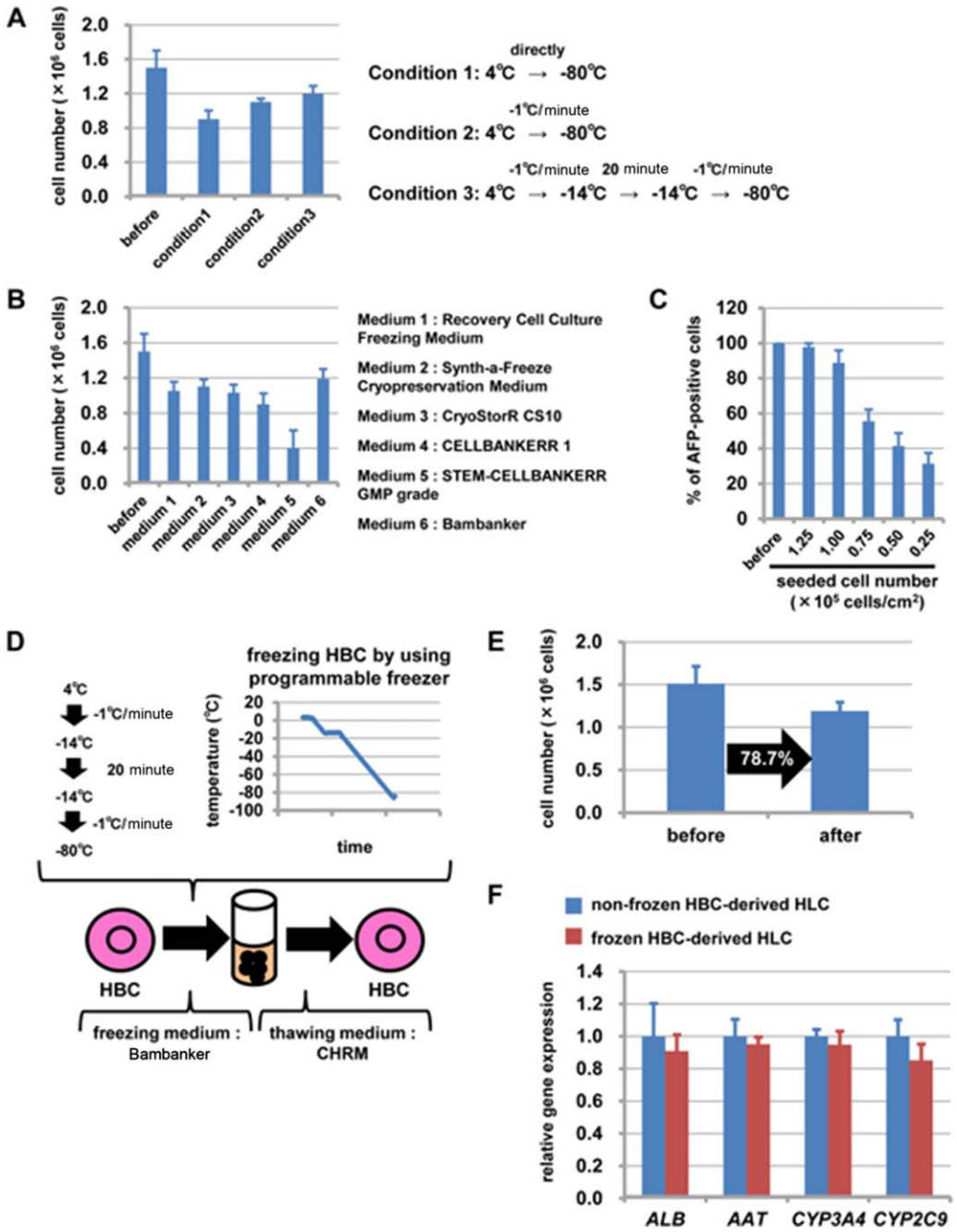

FIG. 2. Cryopreservation of human iPS cell-derived HBCs. (A) Human iPS cell (1383D6)-derived HBCs were cryopreserved under three different conditions. We counted the number of viable cells before and after cryopreservation. (**B**) HBCs were cryopreserved by using six different cryopreservation media. We counted the number of viable cells before and after cryopreservation. (**C**) Frozen HBCs were seeded at various cell densities. We used FACS analysis to determine the percentage of AFP-positive cells. (**D**) The procedure for freezing human iPS cell (1383D6)-derived HBCs is shown. Program Deep Freezer (PDF300) and cell cryopreservation solution (Bambanker) were used. (**E**) Viable cells were counted before and after cryopreservation. (**F**) Nonfrozen HBCs and frozen HBCs were differentiated into HLCs. The gene expression levels of hepatocyte markers (*ALB, AAT, CYP3A4*, and *CYP2C9*) were examined by real-time RT-PCR. The gene expression level in nonfrozen HBC-derived HLCs was taken as 1.0. All data are represented as means ± SEM (n = 3). Abbreviations: *AAT, alpha-1 antitrypsin*; CHRM, cryopreserved hepatocyte recovery medium; FACS, fluorescence-activated cell sorting; RT-PCR, reverse-transcription polymerase chain reaction.

A

cell type	4 week (%)	16 week (%)
iPS 1×10^5 cells	5/5 (100)	-
HBC 1×10^5 cells	0/7 (0)	0/6 (0)
HBC 1×10^7 cells	0/8 (0)	0/6 (0)
HLC 1×10^5 cells	0/10 (0)	0/10 (0)
HLC 1×10^7 cells	0/10 (0)	0/10 (0)

B

	LoFreq	MuTect	VarScan	LoFreq & MuTect	LoFreq & VarScan	MuTect & VarScan	LoFreq & MuTect & VarScan
iPS vs HBC	52	5	33	0	3	0	0
iPS vs purified-HBC	51	13	24	0	5	1	0
iPS vs HLC	742	11	23	6	1	1	0

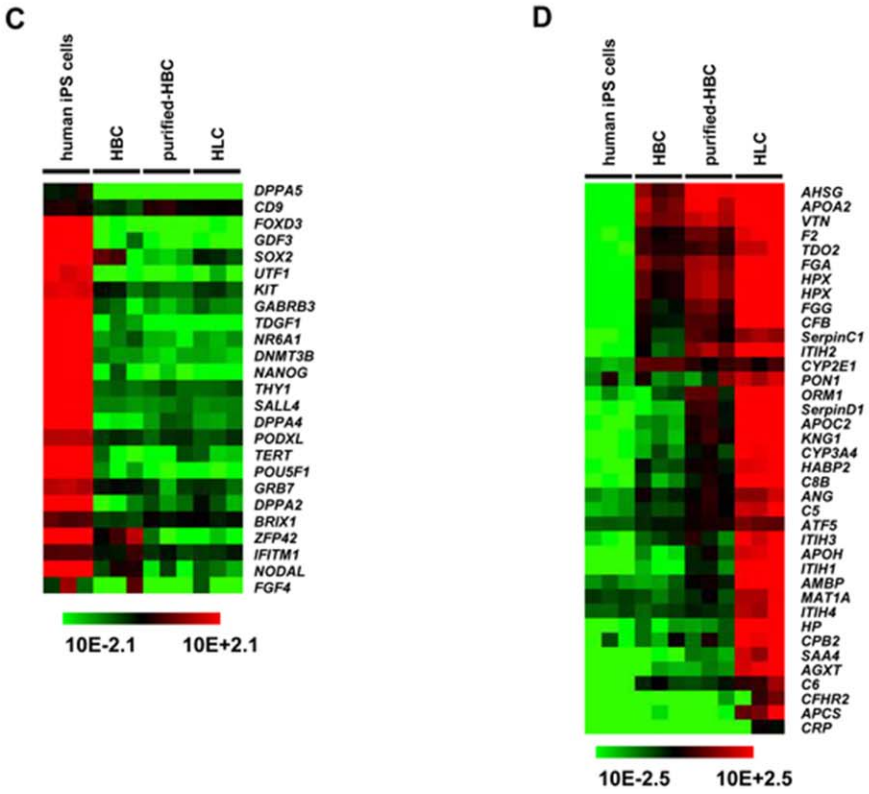

FIG. 3. Safety assessment of human iPS cell-derived HLCs. (A) To perform the teratoma assay, undifferentiated human iPS cells (1×10^5 cells/NOG mouse), human iPS cell (1383D6)-derived purified HBCs (1×10^5 or 1×10^7 cells/NOG mouse), and human iPS cell-derived HLCs (1×10^5 or 1×10^7 cells/NOG mouse) were subcutaneously transplanted. At 4 or 16 weeks after transplantation, we determined the presence or absence of teratomas. Human iPS cells (Ff-l01s01) were used in the teratoma assay. (**B**) The genomic sequence of human iPS cell (1383D6)-derived HBCs and HLCs was examined by targeted sequencing with a comprehensive cancer panel. The genomic sequences of 596 cancer-related genes of human iPS cell-derived HBCs, purified HBCs, and HLCs were compared with that of undifferentiated human iPS cells. To identify likely single-nucleotide substitutions that were newly generated in differentiated cells, three independent somatic variant callers (LoFreq, MuTech, and VarScan2) were collectively used to reduce the number of false-positive identifications.[16,17] (C,D) RNA sequencing was performed in undifferentiated human iPS cells, human iPS cell-derived HBCs, purified HBCs, and HLCs. The messenger RNA expression profiles of (**C**) pluripotent markers and (**D**) hepatocyte markers are shown.

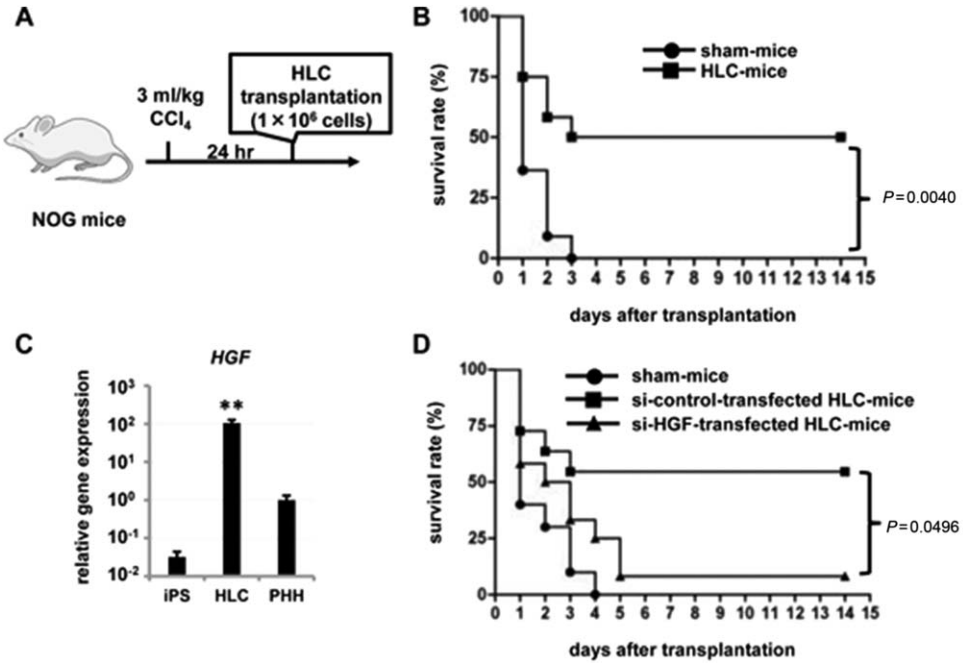

FIG. 4. Transplantation of human iPS cell-derived HLCs rescued mice from acute liver injuries. (A) Human iPS cell (Ff-l01s01)-derived HLCs were intrasplenically transplanted into mice that had been intraperitoneally inoculated with 3 mL/kg CCl_4 1 day before transplantation. (**B**) The survival rates of the mice after HLC transplantation (HLC-mice, square; n = 11) and sham operation (sham-mice, circle; n = 12) were examined. (**C**) The gene expression levels of *HGF* in undifferentiated human iPS cells (iPS), human iPS cell-derived HLCs (HLC), and PHHs were measured by real-time RT-PCR. On the *y* axis, the gene expression level in PHHs was taken as 1.0. Data are represented as means ±SEM (n=3). **, $P<0.01$. (**D**) The HLCs, which were transfected with si-control or si-HGF, were transplanted into the mice that had been intraperitoneally inoculated with 3 mL/kg CCl_4 1 day before transplantation. The survival rate of the mice after transplantation with si-control-transfected HLCs (si-control-transfected HLC-mice, squares; n = 11), si-HGF-transfected HLCs (si-HGF-transfected HLC-mice, triangles; n = 12), or sham operation (sham-mice, circles; n = 10) was examined; si-control versus si-HGF, $P = 0.0496$; si-control versus sham-mice, $P = 0.0092$; si-HGF versus sham-mice, $P = 0.1181$. Abbreviations: RT-PC, reverse-transcription polymerase chain reaction; si, small-interfering RNA.

(NOG mice) that were intraperitoneally inoculated with 3 mL/kg CCl_4 1 day before transplantation (Fig. 4A). The survival rate of the mice after the iPS-HLC transplantation (HLC-mice) was increased compared with that of the mice after the sham operation (sham-mice) (Fig. 4B). We examined therapeutic mechanisms in the transplantation of human iPS-HLCs into the acute liver injury model. Because we have found that human iPS-HLCs secrete HGF,[4] the gene expression levels of *HGF* in undifferentiated human iPS cells, human iPS-HLCs, and PHHs were measured (Fig. 4C). The HGF expression levels in human iPS-HLCs were significantly higher than those in PHHs. To examine whether the therapeutic effects of iPS-HLCs mediate HGF, HGF-knockdown human iPS-HLCs were transplanted into acute liver injury mice (Fig. 4D). The survival rate in the mice transplanted with human HGF small-interfering RNA (si-HGF)-transfected human iPS-HLCs was significantly decreased compared with the mice transplanted with small-interfering control-transfected human iPS-HLCs. These results suggest that the therapeutic effects of human iPS-HLCs were mainly explained by HGF secretion.

TRANSPLANTATION OF HLA-homo iPS CELL-DERIVED HLCs RESCUED MICE FROM CHRONIC LIVER INJURIES

As chronic liver injury model mice, we used immunodeficient mice (nude mice) that were intraperitoneally inoculated with 0.6 mL/kg CCl_4 for 8 weeks (twice weekly) before transplantation (Supporting Fig. S9A). The gene expression levels of liver fibrosis

markers (*alpha-S-adenosyl methionine* [*αSAM*], *desmin, neurotrophin receptor p75* [*p75NTR*], *collagen type 1 alpha 1* [*COL1A1*], *matrix metalloproteinase 2* [*MMP2*], *tissue inhibitor of metalloproteinase 1* [*TIMP1*], *familial amyloid polyneuropathy* [*FAP*]) in the mice liver of the HLC-mice were significantly lower than those of the sham-mice (Supporting Fig. S9B). Consistently, Sirius Red staining showed that the degree of progression of liver fibrosis was decreased by iPS-HLC transplantation (Supporting Fig. S9C, D). In intrasplenic hepatocyte transplantation, spleen is sometimes removed after the transplantation to avoid the effects of engrafted cells in the spleen. Thus, we removed the spleen after hepatocyte transplantation. The therapeutic effects of human iPS-HLCs in the mice transplanted with human iPS-HLCs followed by spleen removal (spleen-removed mice) were similar to those in the HLC-mice (Fig. S9B-D). This result suggests that the therapeutic effects of HLCs do not depend on the residual cells in the spleen. Consistently, human ALB-positive cells were not observed in the spleen, although a human ALB-positive cell cluster was observed around the portal vein of the transplanted mice (Supporting Fig. S9E). To evaluate the hepatic functionality of engrafted cells, human ALB serum levels in the transplanted mice were examined (Supporting Fig. S9F). Human ALB serum levels in the HLC-mice (approximately 350 ng/mL) were significantly higher than that in the sham-mice. Taken together, human iPS-HLCs have therapeutic potential against chronic liver injuries.

Discussion

In this study, we showed that functional HLCs were efficiently differentiated from HLA-homo iPS cells without using feeder cells, Matrigel, and serum. The risk of teratoma formation of human iPS-HLCs was extremely low. In addition, genomic stability was confirmed during the hepatocyte differentiation procedure. Finally, human iPS-HLCs were shown to have therapeutic potential against both acute and chronic liver injuries.

It is known that undifferentiated human embryonic stem (ES)/iPS cells can be maintained on a human LN511- or LN521-coated dish but not on a human LN111-coated dish.[13,18] We have reported that HBCs differentiated from human pluripotent stem cells could be maintained by using LN111.[14] It is also known that human ES cells could be more efficiently differentiated into HLCs on a mixture of LN521 and LN111 (ratio of 1:3) than on Matrigel or pure LN521.[19] Therefore, our finding that human iPS cells could be efficiently differentiated to HBCs on a mixture of LN111-E8 and LN511-E8 (ratio of 3:1) seems reasonable. In this study, we used recombinant E8 fragments of laminins (LN-E8), which are the minimum fragments conferring integrin-binding activity, for hepatocyte differentiation. Miyazaki et al.[12] reported that the yield of recombinant proteins is much higher with LN-E8 than intact laminin. Therefore, it might be possible to reduce the cost and time involved in generating HBCs by using LN-E8 rather than intact laminins. In previous studies, Matrigel was used to promote hepatocyte differentiation.[15,20-22] We succeeded in generating functional HLCs by using an LN111-E8-coated dish and overlaying thin type IV collagen without using Matrigel. The main components of Matrigel are LN111, type IV collagen, and elastin. We consider that the conditions on Matrigel were successfully mimicked by our LN111-E8-coated dish overlaid with thin type IV collagen. Therefore, our hepatocyte differentiation protocol should be a robust method for generating human iPS-HLCs for therapeutic applications. It is, however, worth noting the finding of Baxter et al.[23] that human iPS-HLCs mimic fetal hepatocytes better than they mimic adult hepatocytes. Indeed, our human iPS-HLCs consistently expressed fetal liver markers, such as AFP and CYP3A7 (data not shown). Thus, further improvement of the hepatocyte differentiation technology is needed.

In this study, we showed that the percentage of residual undifferentiated cells could be reduced to about 0.003% by performing HBC purification using LN511-E8. Gropp et al.[9] showed that spiking of 1×10^4 undifferentiated human ES cells into 5×10^5 retinal pigment epithelium cells promoted teratoma formation. However, spiking of lower numbers of human ES cells (5×10^3, 1×10^3, and 1×10^2) did not generate teratomas. Because the contamination of up to 5×10^3 undifferentiated cells to human iPS-HLCs can also be permissive, it is theoretically calculable that 1.6×10^8 human iPS-HLCs (the percentage of undifferentiated cells is 0.00312%) can be transplanted without generating a teratoma. However, it is known that teratoma formation capacity is strongly dependent on the site of engraftment.[24] For this reason, a teratoma formation assay should be performed not only in the subcutaneous space but also in the liver.

The present study also confirmed that the genome remains stable during the hepatocyte differentiation process. Nonetheless, because mitochondrial DNA

mutations are important causes of inherited disease,[25] it will be necessary to examine the occurrence of mitochondrial DNA mutations in hepatocyte differentiation. Finally, we also presented a method for the cryopreservation of HBCs. This technique will provide the technician with adequate time to perform a quality check on the differentiated cells. Just as importantly, it will afford an abundant supply and easy shipping of human iPS-HLCs for medical applications.

In the present experiments we generated safe and therapeutically effective human iPS-HLCs from an HLA-homozygous donor. Our approach will contribute to the promotion of allogenic transplantation of human iPS-HLCs. In a future study, we would like to confirm the therapeutic potential of human iPS-HLCs in an animal model larger than mice (such as pigs or dogs) because the size of the livers would be closer to that of humans.

Acknowledgments: We thank Dr. Shinya Yamanaka (CiRA, Kyoto University), Dr. Naoko Takasu (CiRA, Kyoto University), Dr. Hiroshi Suemizu (Central Institute for Experimental Animals), Dr. Makoto Seki (Regience Co., Ltd.), and Dr. Mitsuru Inamura (ReproCELL Co., Ltd.) for their helpful discussions. We also thank Ms. Yasuko Hagihara, Ms. Ayaka Sakamoto, Ms. Junko Toga, Ms. Emiko Yagi, and Mr. Masaki Takizawa for their excellent technical support. We thank the Japanese Red Cross Society for providing peripheral blood mononuclear cells.

REFERENCES

1) Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Engl J Med 1998;338:1422-1426.
2) Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med 1996;334:693-699.
3) Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-872.
4) Nagamoto Y, Takayama K, Ohashi K, Okamoto R, Sakurai F, Tachibana M, et al. Transplantation of a human iPSC-derived hepatocyte sheet increases survival in mice with acute liver failure. J Hepatol 2016;64:1068-1075.
5) Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, et al. Vascularized and functional human liver from an iPSC-derived organ bud transplant. Nature 2013;499:481-484.
6) Liu H, Kim Y, Sharkis S, Marchionni L, Jang YY. In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. Sci Transl Med 2011;3:82ra39.
7) Asgari S, Moslem M, Bagheri-Lankarani K, Pournasr B, Miryounesi M, Baharvand H. Differentiation and transplantation of human induced pluripotent stem cell-derived hepatocyte-like cells. Stem Cell Rev 2013;9:493-504.
8) Yamanaka S. A fresh look at iPS cells. Cell 2009;137:13-17.
9) **Gropp M, Shilo V**, Vainer G, Gov M, Gil Y, Khaner H, et al. Standardization of the teratoma assay for analysis of pluripotency of human ES cells and biosafety of their differentiated progeny. PLoS One 2012;7:e45532.
10) Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 2002;100:3175-3182.
11) Okita K, Yamakawa T, Matsumura Y, Sato Y, Amano N, Watanabe A, et al. An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. Stem Cells 2013;31:458-466.
12) Miyazaki T, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M, et al. Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. Nat Commun 2012;3:1236.
13) Rodin S, Domogatskaya A, Strom S, Hansson EM, Chien KR, Inzunza J, et al. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat Biotechnol 2010;28:611-615.
14) Takayama K, Nagamoto Y, Mimura N, Tashiro K, Sakurai F, Tachibana M, et al. Long-term self-renewal of human ES/iPS-derived hepatoblast-like cells on human laminin 111-coated dishes. Stem Cell Reports 2013;1:322-335.
15) Takayama K, Morisaki Y, Kuno S, Nagamoto Y, Harada K, Furukawa N, et al. Prediction of interindividual differences in hepatic functions and drug sensitivity by using human iPS-derived hepatocytes. Proc Natl Acad Sci U S A 2014;111:16772-16777.
16) **Fang LT, Afshar PT**, Chhibber A, Mohiyuddin M, Fan Y, Mu JC, et al. An ensemble approach to accurately detect somatic mutations using SomaticSeq. Genome Biol 2015;16:197.
17) Kroigard AB, Thomassen M, Laenkholm AV, Kruse TA, Larsen MJ. Evaluation of nine somatic variant callers for detection of somatic mutations in exome and targeted deep sequencing data. PLoS One 2016;11:e0151664.
18) Rodin S, Antonsson L, Hovatta O, Tryggvason K. Monolayer culturing and cloning of human pluripotent stem cells on laminin-521-based matrices under xeno-free and chemically defined conditions. Nat Protoc 2014;9:2354-2368.
19) Cameron K, Tan R, Schmidt-Heck W, Campos G, Lyall MJ, Wang Y, et al. Recombinant laminins drive the differentiation and self-organization of hESC-derived hepatocytes. Stem Cell Reports 2015;5:1250-1262.
20) Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, et al. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 2010;51:297-305.
21) Song Z, Cai J, Liu Y, Zhao D, Yong J, Duo S, et al. Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 2009;19:1233-1242.
22) Kajiwara M, Aoi T, Okita K, Takahashi R, Inoue H, Takayama N, et al. Donor-dependent variations in hepatic differentiation from human-induced pluripotent stem cells. Proc Natl Acad Sci U S A 2012;109:12538-12543.
23) **Baxter M, Withey S, Harrison S**, Segeritz CP, Zhang F, Atkinson-Dell R, et al. Phenotypic and functional analyses show

stem cell-derived hepatocyte-like cells better mimic fetal rather than adult hepatocytes. J Hepatol 2015;62:581-589.
24) Prokhorova TA, Harkness LM, Frandsen U, Ditzel N, Schroder HD, Burns JS, et al. Teratoma formation by human embryonic stem cells is site dependent and enhanced by the presence of Matrigel. Stem Cells Dev 2009;18:47-54.
25) Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet 2005;6:389-402.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1111/full.