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Human iPS Cell–based Liver-like Tissue Engineering at Extrahepatic Sites in Mice as a New Cell Therapy for Hemophilia B

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Abstract

Instead of liver transplantation or liver-directed gene therapy, genetic liver diseases are expected to be treated effectively using liver tissue engineering technology. Hepatocyte-like cells (HLCs) generated from human-induced pluripotent stem (iPS) cells are an attractive unlimited cell source for liver-like tissue engineering. In this study, we attempted to show the effectiveness of human iPS cell–based liver-like tissue engineering at an extrahepatic site for treatment of hemophilia B, also called factor IX (FIX) deficiency. HLCs were transplanted under the kidney capsule where the transplanted cells could be efficiently engrafted. Ten weeks after the transplantation, human albumin (253 μg/mL) and α-1 antitrypsin (1.2 μg/mL) could be detected in the serum of transplanted mice. HLCs were transplanted under the kidney capsule of FIX-deficient mice. The clotting activities in the transplanted mice were approximately 5% of those in wild-type mice. The bleeding time in transplanted mice was shorter than that in the nontransplanted mice. Taken together, these results indicate the success in generating functional liver-like tissues under the kidney capsule by using human iPS cell–derived HLCs. We also demonstrated that the human iPS cell–based liver-like tissue engineering technology would be an effective treatment of genetic liver disease including hemophilia B.

Keywords

hepatocyte, human iPS cells, kidney capsule, hemophilia B, liver-like tissue engineering

Introduction

Instead of liver transplantation for genetic liver diseases, hepatocyte transplantation and liver tissue engineering technology is expected to be an effective treatment. It was previously reported that intraportal transplantation of hepatocytes was effective for the treatment of Crigler–Najjar syndrome and glycogen storage disease[1,2]. However, transplantable cell number via the portal vein is severely restricted because of the risk of embolism in the blood vessels[3]. Other studies reported that engraftment efficiencies of hepatocytes are restricted because transplanted hepatocytes are distributed into other organs through blood circulation[4–6]. Moreover, it was demonstrated that transplanted hepatocytes lose contact with the extracellular matrix in the initial period of transplantation, resulting in anoikis in some cases[7,8]. Thus, to solve these problems, the use of ectopic transplantation sites instead of the liver could be considered a promising alternative.

Ohashi et al. conducted intensive studies to achieve prolonged functionality of engrafted primary hepatocytes at ectopic sites, which led to the use of engineering technology for therapeutic liver tissues in mice[9–13]. Engineered liver

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tissues under the kidney capsule space were found to perform active regenerative growth at a level similar to the native liver[9]. Human liver tissues could also be engineered in mice using human primary hepatocytes isolated from human liver tissues[14]. An important question that is raised here is whether functional liver-like tissues could be engineered in mice using human hepatocyte-like cells (HLCs) differentiated from human pluripotent stem cells.

It is difficult to supply abundant human hepatocytes for transplantation. Human embryonic stem (ES) cells or human-induced pluripotent stem (iPS) cell–derived HLCs have the potential to resolve this problem because human ES/iPS cells are known to proliferate infinitely[15,16]. Importantly, as compared with human ES cells, human iPS cells have less problems in ethical and allogenic immune rejection in the transplantation setting because human iPS cells can be generated from a patient's own cells. For these reasons, human iPS-HLCs could be considered as useful cell sources in hepatocyte transplantation.

Recently, we succeeded in generating functional human iPS-HLCs via definitive endoderm cells and hepatoblast-like cells[17–21]. Hepatic gene expression levels of the human iPS-HLCs were similar to those of primary human hepatocytes (PHHs), which were cultured for 48 h. In this study, we initially examined whether the human iPS-HLCs have the capacity to engraft under the kidney capsule. We then optimized the conditions of the transplantation with human iPS-HLCs. Finally, to determine the therapeutic value of the human iPS-HLCs, we transplanted the human iPS-HLCs under the kidney capsule of hemophilia B (factor IX [FIX] deficiency) mice and then assessed the clotting activity and bleeding time in the transplanted mice.

Materials and Methods

Human ES/iPS Cells

The human ES cell lines, H9 (WiCell Research Institute, Madison, WI, USA) and KhES3 (provided by Dr. N. Nakatsuji, Kyoto University, Kyoto, Japan), were maintained on a feeder layer of mitomycin C-treated EmbryoMax Primary Mouse Embryo Fibroblasts (MEFs; Merck Millipore, Guyancourt, France) with ReproStem medium (ReproCELL) supplemented with 10 ng/mL fibroblast growth factor 2 (FGF-2; Katayama Chemical Industries, Osaka, Japan). Human ES cells were used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan and furthermore, and the study was approved by the Independent Ethics Committee.

The human iPS cell lines, Dotcom[22,23] (provided by Dr. A. Umezawa, National Center for Child Health and Development, Tokyo, Japan), HC2-14-iPS (iHC-7), and QOQ-iPS (QOQ) cells[20], were maintained on a feeder layer of mitomycin C-treated MEFs with ReproStem medium supplemented with 10 ng/mL FGF-2.

Hepatocyte Differentiation

Before the initiation of hepatocyte differentiation, human iPS cells were dissociated into clumps by using dispase (Roche Diagnostics, Mannheim, Germany) and plated onto BD Matrigel Basement Membrane Matrix Growth Factor Reduced (Becton Dickinson Mountainview, CA, USA). These cells were cultured for 3 to 4 d in the mouse embryonic fibroblast-conditioned medium. The differentiation protocol for the induction of definitive endoderm cells, hepatoblast-like cells, and human iPS-HLCs was based on our previous reports, with some modifications[20]. Briefly, in the definitive endoderm differentiation, human iPS cells were cultured with the Wnt family member 3A (WNT3A)-expressing L cell (CRL2647; American Type Culture Collection [ATCC])-conditioned Roswell Park Memorial Institute (RPMI)1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 100 ng/mL Activin A (R&D Systems, Minneapolis, MN, USA), 1% GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA), 0.2% fetal bovine serum (FBS), and 1× B-27 Supplement Minus Vitamin A (Thermo Fisher Scientific) for 4 d. For the induction of hepatoblast-like cells, the definitive endoderm cells were cultured for 5 d with RPMI 1640 medium containing 20 ng/mL bone morphogenetic protein 4 (BMP-4) (R&D Systems) and 20 ng/mL fibroblast growth factor-4 (FGF-4) (R&D Systems), 1% GlutaMAX, and 1× B-27 Supplement Minus Vitamin A. For hepatocyte differentiation, the hepatoblasts were cultured for 5 d with RPMI 1640 medium containing 20 ng/mL hepatocyte growth factor (HGF) (R&D Systems), 1% GlutaMAX, and 1× B-27 Supplement Minus Vitamin A. Finally, the cells were cultured for 11 d with the hepatic maturation medium (hepatic maturation medium consists of hepatocyte culture medium (HCM; Lonza, Basel, Switzerland, without epidermal growth factor (EGF) containing 20 ng/mL oncostatin M and 3% GlutaMAX).

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Cells or whole mouse kidneys were homogenized in ISOGENE (Nippon Gene, Tokyo, Japan) and then the total RNA was isolated according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific). Real-time RT-PCR was performed using TaqMan gene expression assays or Fast SYBR Green Master Mix using an ABI Step One Plus (all from Applied Biosystems, Foster City, CA, USA). Relative quantification was performed against a standard curve, and the values were normalized against the input determined for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The human-specific primer sequences and assay IDs used in this study are described in Supplementary Tables S1 and S2, respectively. The other human-specific primers were also designed to detect only human genes but not mouse genes.

Animals and Treatment

Animal experiments were conducted in accordance with the ethical approval of National Institute of Biomedical Innovation, Health and Nutrition and Osaka University. In all studies except for the hemophilia B mouse experiments, 10 to 20 week old thymidine kinase (TK)-NOD/Shi-scid IL2rgamma(null) (NOG) mice (Central Institute for Experimental Animals, Kawasaki, Japan)[24] were used as recipients. TK-NOG mice are severely immunodeficient mice (NOG mice) with transgenic expression of TK under the control of a liver-restricted albumin (ALB) promoter. The mice were intraperitoneally infused with 6 mg/kg ganciclovir (Sigma-Aldrich) at 8 and 6 d before the transplantation. The mice were anesthetized with isoflurane (Pfizer, New York, NY, USA) and then the transplantation procedure was started. Human ES/iPS-HLCs were suspended (2×10^6 cells/100 μL) in cold HCM and an equal volume of cold growth factor–reduced Matrigel. A total of 2×10^6 human ES/iPS-HLCs were transplanted under the kidney capsule. Because the growth factor–reduced Matrigel quickly polymerizes into a 3-dimensional gel at room temperature, all of the procedures were done at 4 °C.

In the hemophilia B mouse experiments, we used a mouse model of hemophilia B in the C57Bl/6 background, which lacks the production of functional coagulation FIX (The Jackson Laboratory, Bar Harbor, ME, USA). Human iPS-HLCs were suspended in cold HCM and an equal volume of cold growth factor–reduced Matrigel. Human iPS-HLCs (2×10^6 cells/mouse) were transplanted under the kidney capsule spaces of FIX-knockout (KO) hemophilia B (FIX-KO) mice. Tatsumi et al. previously established an appropriate perioperative management of FIX-KO mice, in which 500 μL of pooled normal mouse plasma was intraperitoneally injected[13] and after 30 min surgical procedures for human iPS-HLC transplantation or sham operation were conducted[9,12]. After the cell transplantation, the recipient mice were treated with cyclosporine (2 mg/kg) by subcutaneous injection once every other day.

Enzyme-linked Immunosorbent Assay (ELISA)

Mouse serum samples were collected periodically from the orbital plexus and analyzed by ELISA to determine their human ALB and α-1 antitrypsin (αAT) levels. ELISA kits for human ALB and αAT were purchased from Bethyl Laboratories (Montgomery, TX, USA) and DiaSorin (Saluggia, Italy), respectively. ELISA was performed according to the manufacturer's instructions. The serum ALB and αAT levels were calculated according to each standard.

Immunohistochemistry

For detection of human HLA Class 1 ABC, human ALB, human FIX, and mouse CD31, human iPS-HLC-grafted kidneys were harvested at 6 wk after the transplantation. Tissue samples were frozen in Tissue-Tek Optimal Cutting Temperature (O.C.T.). Compound (Sakura Finetek, Osaka, Japan), then sectioned at 10 μm, and fixed with 4% paraformaldehyde (PFA) for 15 min. After incubation with 0.1% Tween 20 and blocking with ImmunoBlock (DS Pharma Biomedical, Osaka, Japan), the sections were incubated overnight at 4 °C with a primary antibody, followed by incubation at 4 °C for 1 h with a secondary antibody labeled with Alexa Fluor 488 (Thermo Fisher Scientific).

For detection of human cytokeratin (CK) 19, the harvested kidneys were fixed with 4% PFA, embedded in paraffin, and sectioned at 4 μm. After deparaffinization, the sections were incubated with 0.1% Tween 20 and blocked with ImmunoBlock; they were then incubated overnight at 4 °C with a primary antibody, followed by incubation at room temperature for 1 h with a secondary antibody labeled with biotin (Vector Laboratories, Burlingame, CA, USA). Immunoreactivities were visualized with a VECTASTAIN ABC Elite kit and ImmPACT DAB Substrate (all from Vector Laboratories) according to the manufacturer's instructions. The sections were counterstained with Mayer's hematoxylin (Wako, Osaka, Japan). All the primary antibodies are listed in Supplementary Table S3.

Hematoxylin and Eosin Staining

The harvested mouse kidneys were fixed with 4% PFA, embedded in paraffin, and sectioned at 4 μm. These sections were stained with hematoxylin and eosin Y (H&E, Wako).

Elastin Staining

The harvested mouse kidneys were fixed with 4% PFA, embedded in paraffin, and sectioned at 5 μm. These sections were stained with an Elastic Stain kit (Modified Verhoff's; ScyTek Laboratories, Logan, UT, USA).

Sirius Red Staining

Harvested organs were fixed with 4% PFA, embedded in paraffin, and sectioned at 4 μm. After deparaffinization, immunohistochemistry analysis was performed using a Picrosirius Red Stain kit (Polysciences Inc, Warrington, PA, USA), according to the manufacturer's instructions for visualization of the fibrotic area.

PHHs

Three lots of cryopreserved human hepatocytes were used (lot: QOQ [VERITAS, Tokyo, Japan, donor age: 66 y], YOW [VERITAS, donor age: 78 y], and HC2-14 [XenoTech Kansas City, KS, USA, donor age: 48 y]). The vials of hepatocytes were rapidly thawed in a shaking water bath at 37 °C; the contents of each vial were emptied into prewarmed Cryopreserved Hepatocyte Recovery Medium (Thermo Fisher Scientific), and the suspension was centrifuged at 900 rpm for 10 min at room temperature. Trypan blue exclusion

testing indicated that the postthaw cell viability was approximately 92%. The hepatocytes were seeded at 1.25×10^5 cells/cm^2 in HCM (Lonza), containing 10% fetal calf serum (FCS) onto type I collagen–coated 12-well plates. Six hours after seeding, the medium was replaced with HCM. The hepatocytes used in the experiments were cultured 48 h after plating.

Measurement of the Mouse FIX Activity

The plasma FIX biological activity was quantitated by a 1-stage clotting assay based on the activated partial thrombin time using human FIX-deficient plasma (SYSMEX, Kobe, Japan). Pooled human plasma (SYSMEX) was used as the FIX activity standard. Before performing the assay, we confirmed that the FIX activity in the pooled plasma of FIX-deficient hemophilia B mice was less than 5% of normal human FIX activity, whereas that of normal mice was higher than 100%. A mouse tail-clip bleeding time assay was performed by cutting 3 mm from the tip of the mouse tail, at 2 wk after the transplantation. The mouse was transferred to a separate cage and the bleeding time was measured. If the mouse did not stop bleeding at 25 min, the wounds were cauterized to save the mouse.

Results

Optimization of the Protocol for Human ES/iPS-HLC Transplantation under the Kidney Capsule

To generate the human ES/iPS-HLCs, the human ES/iPS cells were sequentially treated with various growth factors and cytokines (Fig. 1A). In human iPS-HLCs, asialoglycoprotein receptor 1- and ALB-positive cells were 79.5% and 95.3%, respectively (Supplementary Figs. S1A and B). The gene expression levels of hepatocyte markers in human iPS-HLCs were approximately 50% of those in PHH (Fig. S1C). These results suggest that the hepatocyte differentiation was efficiently performed in this study, just as in our previous study[20]. To determine the feasibility and the therapeutic values of cell transplantation using the human ES/iPS-HLCs, these cells were transplanted into TK-NOG mice harboring liver failure induced by ganciclovir treatment. TK-NOG mice are severely immunodeficient (NOG mice) with transgenic expression of TK under the control of a liver-restricted ALB promoter[24]. The transplantation procedure is schematically shown in Fig. 1B. An image of the human ES/iPS-HLCs transplanted under the kidney capsule is shown in Fig. 1C. We attempted to determine the optimized transplantation conditions. In terms of cell number, the serum human ALB levels of mice transplanted with a larger number of human ES/iPS-HLCs (2×10^6 cells/mouse) were higher than those of mice transplanted with a smaller number of human ES/iPS-HLCs (5×10^5 and 1×10^6 cells/mouse; Fig. 1D). Because it is known that hepatic functions of human ES/iPS-HLCs are largely dependent on the donor of the human ES/iPS cell line[20], we searched for an appropriate human ES/iPS cell line for the hepatocyte transplantation (Fig. 1E). The serum human ALB levels of mice transplanted with QOQ-iPS-HLCs were higher than those of mice transplanted with other human ES/iPS-HLCs. Therefore, in the following experiments, QOQ-iPS-HLCs (2×10^6 cells/mouse) were transplanted under the kidney capsule of mice.

Characterization of the Engrafted Human iPS-HLCs under the Kidney Capsule

To examine the human liver–specific functionality of the engineered tissues, we performed a long-term engraftment experiment. The serum human ALB (Fig. 2A) and αAT (Fig. 2B) serum levels in the transplanted mice reached a plateau at 6 and 2 wk after transplantation, respectively. Ten weeks after transplantation, the serum human ALB and αAT levels in the transplanted mice were approximately 253 and 1.2 μg/mL, respectively. In addition, human FIX serum levels in the transplanted mice reached a plateau at 2 wk after transplantation (Supplementary Fig. S2). We also confirmed that the engineered hepatic tissues under the kidney capsule were positive for human ALB, human HLA Class 1 ABC, and human FIX (Fig. 2C). These results indicate that the engineered hepatic tissue stably engrafts under the kidney capsule. To further characterize the engineered hepatic tissue under the kidney capsule, the kidneys were visually observed. Human liver-like tissues with multiple layers of human iPS-HLCs were successfully constructed (Fig. 2D) without fibrotic changes (Fig. 2E) because oxygen and nutrients might be supplied throughout these vascular-like structures. Moreover, we confirmed that human CK19-positive cells were observed under the kidney capsule, indicating that bile duct–like structures were constructed in the engineered hepatic tissues (Fig. 2F). Importantly, cluster of differentiation 31 (CD31)-positive vascular-like structures were also observed at the engrafted area (Fig. 2G). These results suggest that the engineered hepatic tissue under the kidney capsule would have significant hepatic functions. However, in several mice, the transplanted kidneys were surrounded by the cysts at 10 wk after transplantation for reasons unknown (Fig. S3).

Gene Expression Analysis of Hepatocyte-Related Genes in the Transplanted Human iPS-HLCs

To examine whether the hepatic functionalities of the engineered liver-like tissues were enhanced by transplantation into TK-NOG mice, the gene expression levels of human hepatocyte-related markers in the human iPS-HLCs that were engrafted into the kidney capsule space of TK-NOG mice were compared 10 wk after transplantation (after transplantation; Fig. 3) with those used at the time in the human iPS-HLCs for transplantation (before transplantation; Fig. 3). The gene expression levels of human *ALB*, human cytochrome (*CYP*)*1A2*, human *CYP2C9*, human *CYP3A5*, human

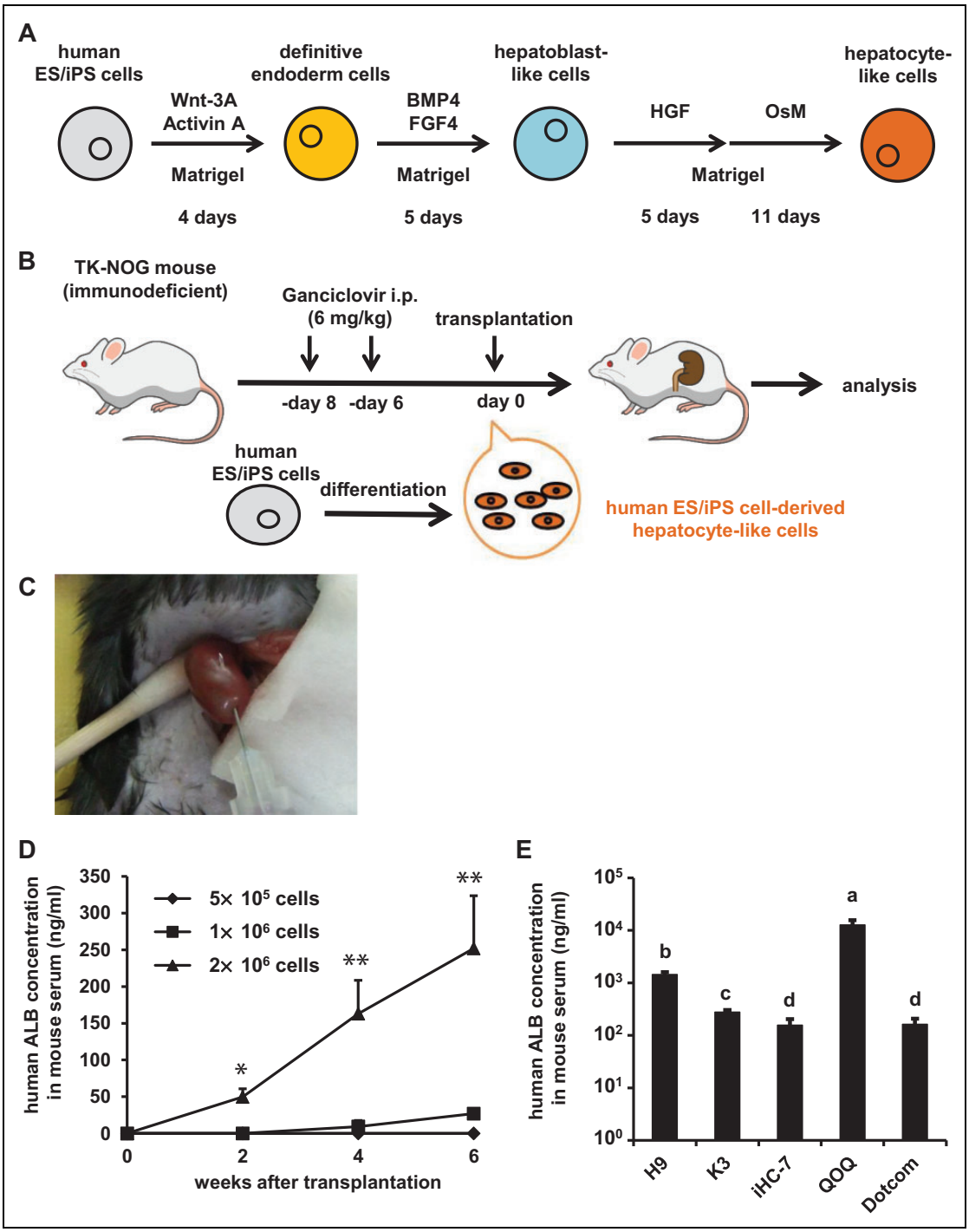

Fig. 1. Optimization of the protocol for human embryonic stem (ES)/human-induced pluripotent stem (iPS)-hepatocyte-like cell (HLC) transplantation under the kidney capsule. (A) The procedure for differentiation of human ES/iPS cells into the HLCs via definitive endoderm cells and hepatoblast-like cells. (B) Human ES/iPS-HLCs were transplanted under the kidney capsule of thymidine kinase-NOG mice after the ganciclovir-induced liver failure. (C) An image of the human ES/iPS-HLCs transplanted under the kidney capsule is shown. (D) Two, four, or six weeks after the transplantation of human iPS-HLCs (5×10^5, 1×10^6, or 2×10^6 cells/mouse), serum human albumin (ALB) levels of the recipients were measured by enzyme-linked immunosorbent assay (ELISA). Human iPS cell line, Dotcom, was used in this experiment. All data are represented as means $\pm$ *SE* ($n = 6$). Statistical analysis indicated that the human ALB levels in the group transplanted with 2×10^6 cells were significantly higher than that in the groups transplanted with 5×10^5 cells or 1×10^6 cells (*$P > 0.05$, **$P < 0.01$, two-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni's post hoc tests). (E) Four weeks after the transplantation of human ES/iPS-HLCs differentiated from various human ES (H9 and K3) and iPS (HC2-14-iPS (iHC-7), QOQ-iPS (QOQ), and Dotcom) cell lines, serum human ALB levels of the recipients were measured by ELISA. All data are represented as means $\pm$ *SE* ($n = 4$). Statistical significance was evaluated by one-way ANOVA followed by Tukey's post hoc tests to compare all groups. Groups that do not share the same letter are significantly different from each other ($P < 0.05$).

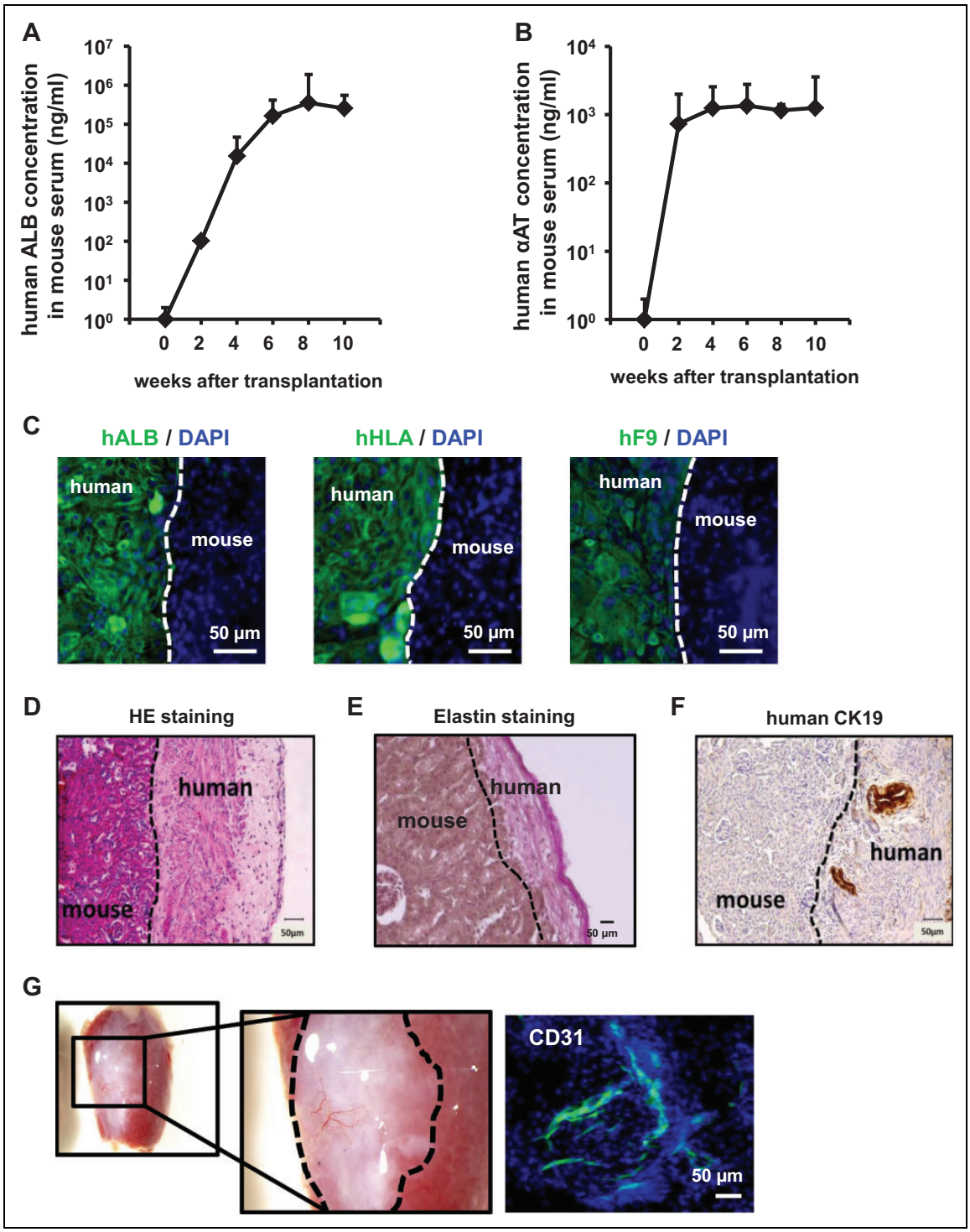

Fig. 2. Characterization of engrafted human-induced pluripotent stem (iPS)-hepatocyte-like cells (HLCs) under the kidney capsule. (A, B) Human iPS (QOQ-iPS) cells were differentiated into the HLCs, and these cells were transplanted under the kidney capsule of thymidine kinase-NOG mice. The serum human albumin (ALB) (A) and α-1 antitrypsin (B) levels of the recipients were analyzed by enzyme-linked immunosorbent assay (ELISA). All data are represented as means ± *SE* ($n = 6$). (C) Six weeks after transplantation, the kidneys of recipients were analyzed by immunohistochemical staining. Frozen sections of these kidneys were stained with antihuman ALB (green, left panel), human HLA Class 1 ABC (green, middle panel), and human factor IX (green, right panel) antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). (D, E) The kidneys of recipients were analyzed by hematoxylin and eosin (H&E) (D) and elastin (E) staining. (F) The kidneys of recipients were analyzed by immunohistochemical staining using anti-CK19 antibodies. (G) Representative photograph of the kidney that received human iPS-HLCs transplantation is shown. The dotted area indicates the transplanted human iPS-HLCs. Six weeks after transplantation, the kidneys of recipients were analyzed by immunohistochemical staining. Frozen sections of these kidneys were stained with antimouse CD31 (green antibodies. Nuclei were counterstained with DAPI (blue).

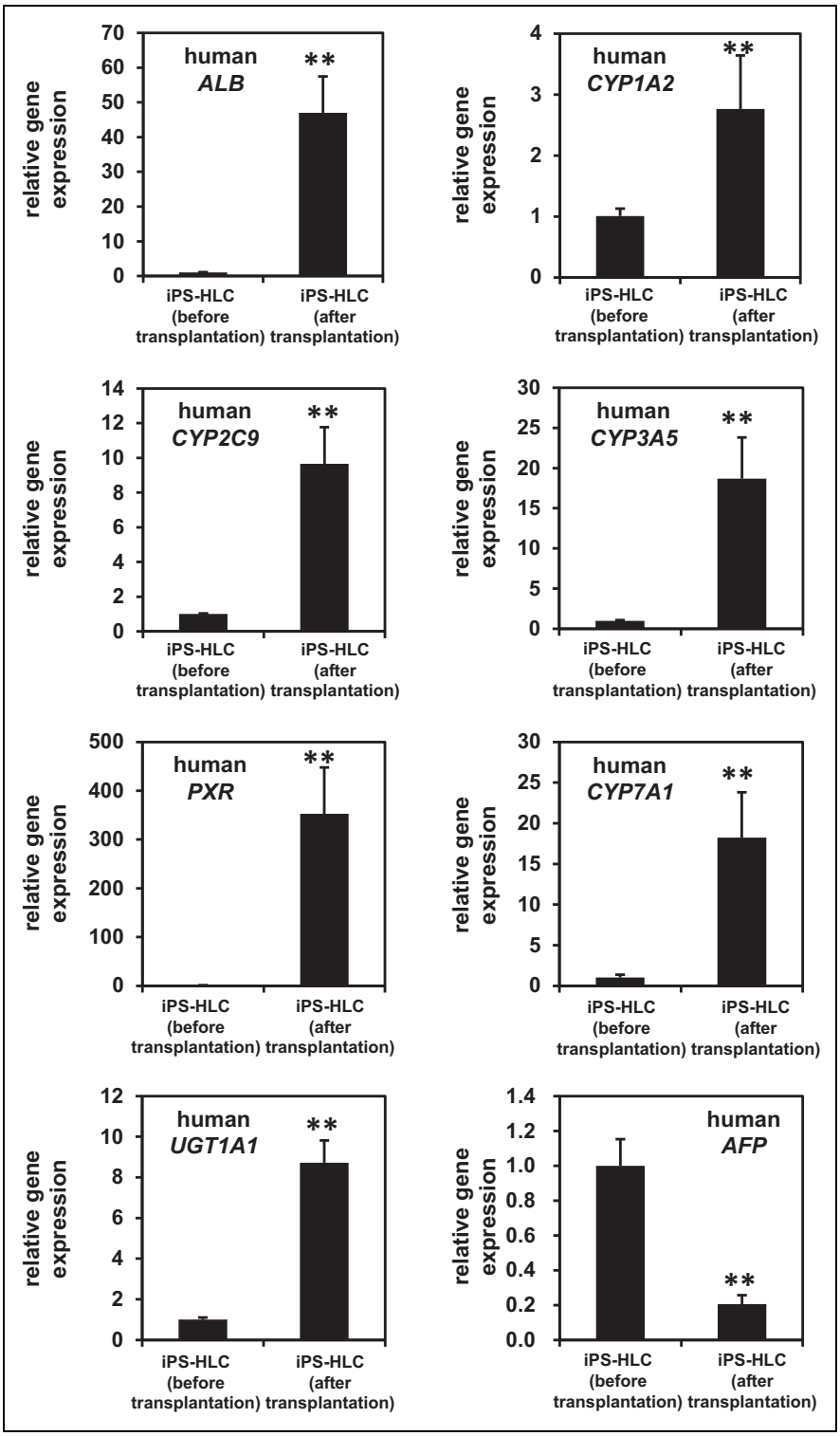

Fig. 3. The hepatocyte-related gene expression levels in the human-induced pluripotent stem (iPS)-hepatocyte-like cells (HLCs) were enhanced after the transplantation. Human iPS (QOQ-iPS) cells were differentiated into the HLCs, and these cells were transplanted under the kidney capsule of thymidine kinase-NOG mice. Ten weeks after transplantation, the gene expression levels of human hepatocyte-related markers, such as human *ALB*, human *CYP1A2*, human *CYP2C9*, human *CYP3A5*, human *PXR*, human *CYP7A1*, human *UGT1A1*, and human *AFP*, were measured in the nontransplanted (iPS-HLCs, before transplantation) and transplanted (iPS-HLCs, after transplantation) human iPS-HLCs by real-time reverse transcription–polymerase chain reaction (RT-PCR). The kidneys of recipients, rather than isolated human iPS-HLCs, were used in this analysis. The gene expression levels in the human iPS-HLCs before transplantation were taken as 1.0. All data are represented as means $\pm$ SE ($n = 5$). Unpaired two-tailed Student's *t* test indicated that the gene expression levels of AFP and others in the "iPS-HLC (after transplantation)" were significantly lower and higher than those in the "iPS-HLC (before transplantation)" group (**$P < 0.01$), respectively.

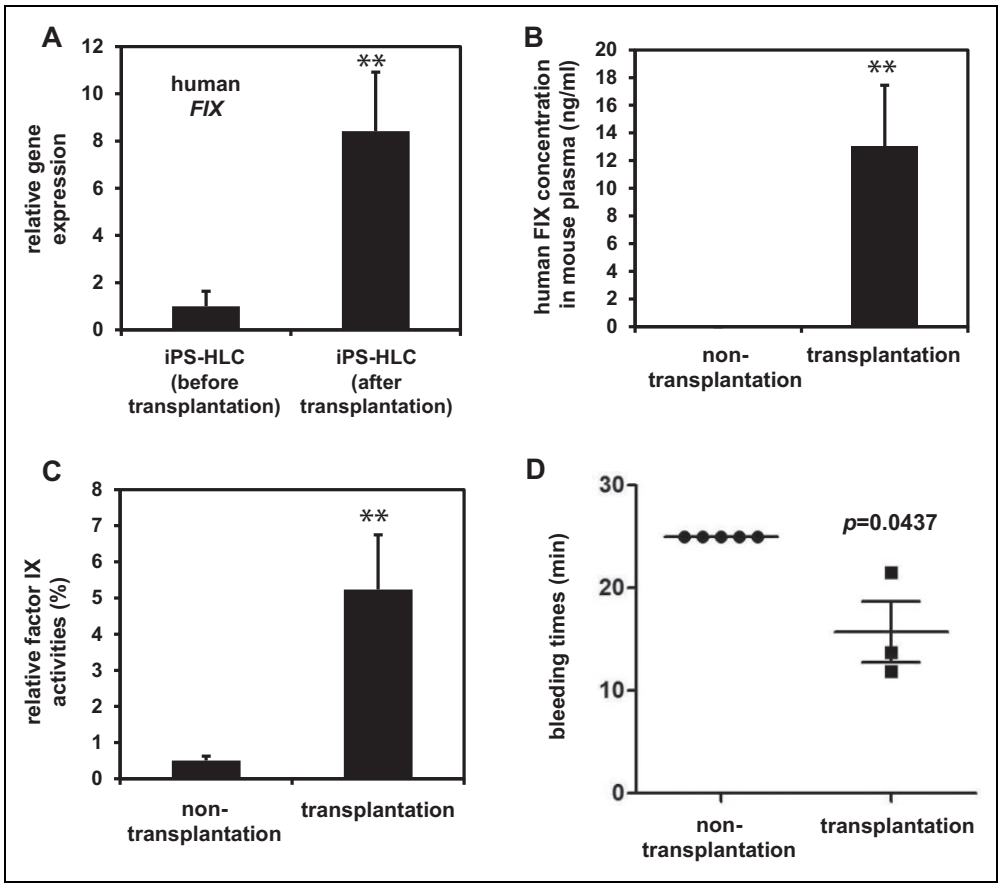

Fig. 4. Functional rescue of the factor IX (FIX)-deficient hemophilia B mice by transplanting human-induced pluripotent stem (iPS)-hepatocyte-like cells (HLCs) under the kidney capsule. Human iPS (QOQ-iPS) cells were differentiated into the HLCs and were transplanted under the kidney capsule of FIX-KO mice. (A) Two weeks after transplantation, the gene expression levels of human *FIX* were measured in the nontransplanted (iPS-HLCs, before transplantation) and transplanted (iPS-HLCs, after transplantation) human iPS-HLCs by real-time reverse transcription–polymerase chain reaction (RT-PCR). The kidneys of recipients, rather than isolated human iPS-HLCs, were used in this analysis. The gene expression levels in the human iPS-HLCs before transplantation were taken as 1.0. (B) Two weeks after the transplantation of human iPS-HLCs, plasma human FIX levels of the recipients were measured by enzyme-linked immunosorbent assay (ELISA). (C) Relative FIX activities were examined in the plasma obtained from nontransplanted and transplanted hemophilia B mice. The FIX activities in the plasma obtained from wild-type (WT) mice were taken as 100%. All data are represented as means $\pm$ *SE* ($n = 3$). In Figs. 4A–C, unpaired two-tailed Student's *t* test was performed (**$P < 0.01$). (D) Two weeks after transplantation, a mouse tail-clip bleeding time assay was performed in nontransplanted and transplanted hemophilia B mice. If the mice did not stop bleeding at 25 min, the wounds were cauterized to save the mouse. In Fig. 4D, unpaired one-tailed Student's *t* test was performed.

pregnane X receptor (*PXR*), human *CYP7A1*, and human *UDP* glucuronosyltransferase family 1 member A1 (*UGT1A1*) were drastically upregulated after the transplantation. On the other hand, the gene expression levels of human α fetoprotein (AFP, a marker for immature hepatocytes) were significantly decreased after the transplantation. These results suggest that the human iPS-HLCs proceeded with further maturation process under the kidney capsule of TK-NOG mice.

Functional Rescue of the FIX-deficient Hemophilia B Mice by Transplanting Human iPS-HLCs under the Kidney Capsule

To determine the feasibility and the therapeutic values of the liver-like tissue engineering approach in a clinically relevant model of human disease, the human iPS-HLCs were transplanted into the kidney capsule space of FIX-deficient hemophilia B mice. We confirmed that the gene expression levels of human *FIX* in the transplanted human iPS-HLCs (after transplantation) were higher than those in the nontransplanted human iPS-HLCs (before transplantation; Fig. 4A). The human FIX level in the transplanted mice was approximately 13.0 ng/mL (Fig. 4B). The FIX activity levels in the transplanted mice (approximately 5%) were significantly higher than those in the nontransplanted mice (Fig. 4C). In human patients, achievement of these levels (more than 5%) of FIX has been shown to improve a severe form of the disease to a mild one[25–27]. Next, we measured the bleeding time 2 wk after the transplantation (Fig. 4D).

The bleeding times of liver-like tissue engineered mice significantly shortened as compared with those of

the nontransplanted mice. These results indicate that human iPS cell–based liver-like tissue engineering under the kidney capsule would be an effective cell therapy for hemophilia B.

Discussion

In this study, we compared the various conditions of human iPS-HLC transplantation in order to maximize the functional volume of the engineered human liver-like tissues under the kidney capsule. We observed the highest human ALB concentration in mouse serum when we transplanted QOQ-iPS-HLCs (2×10^6 cells/mouse; Fig. 1E). In general, the number of cells that can be transplanted via the blood vessels is known to be strictly limited due to blood vessel embolism (approximately 1×10^6 cells/mouse)[3], but we confirmed that a larger number of cells can be transplanted under the kidney capsule. Interestingly, there was a difference in human ALB concentration among the human ES/iPS cell lines (Fig. 1E). Thus, to achieve much higher liver functionalities, it is important to choose the optimum human ES/iPS cell lines.

We found that the concentration of human ALB and αAT in mouse serum after the transplantation of human iPS-HLCs gradually increased (Fig. 2A and B). These results suggest that the human iPS-HLCs, which were engrafted under the kidney capsule, proliferated in response to liver regeneration signals from the TK-NOG mouse. Moreover, we observed a duct-like structure composed of human CK19-positive cells (Fig. 2G), indicating that a bile duct–like structure was spontaneously constructed in the engineered hepatic tissue. Because it is known that the transplanted human hepatocytes have the potential to differentiate into cholangiocytes[28], we considered that these human CK19-positive cells were transdifferentiated from transplanted human iPS-HLCs. Another possibility could be that some hepatoblast-like cells had differentiated into CK19-positive cholangiocytes after transplantation. This possibility was supported by our finding that a portion of the human iPS-HLCs used for transplantation was AFP positive and CYP3A7 positive (data not shown). These are thought to be hepatoblast-like cells that have bipotential differentiation capacity.

The gene expression levels of hepatic markers in the human iPS-HLCs engrafted under the kidney capsule were greatly upregulated after transplantation (Fig. 3). It is notable that the gene expression levels of *AFP* in the human iPS-HLCs significantly decreased after transplantation (Fig. 3). These results indicated that the human iPS-HLCs proceeded with the maturation process under the kidney capsule. We previously reported that human iPS-HLCs could be matured by transplanting them into the mouse liver[29]. In the case of the transplantation under the kidney capsule, the transplanted cells would not diffuse into extra-kidney tissues. Therefore, it would be easy to isolate the matured human iPS-HLCs from the kidney capsule after transplantation. The isolated mature human iPS-HLCs might be useful for drug toxicity screening in the early phase of pharmaceutical development. Moreover, it might be possible that serial transplantation of the human iPS-HLCs enhances hepatic maturation.

In this study, we demonstrated the feasibility of human iPS-HLC-based human liver-like tissue engineering technology for the hemophilia B status (Fig. 4). Therapeutic levels of FIX activities were achieved in the transplanted FIX-KO mice (Fig. 4C). This liver-like tissue engineering technology might also be useful for other liver disorders.

Conclusions

We succeeded in generating functional liver-like tissues under the kidney capsule by using human iPS-HLCs. The transplantation of human iPS-HLCs shown in this study might be useful for therapeutic tissue engineering, although improvements would still be required to achieve a clinical level of effectiveness.

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Author Contributions

Ryota Okamoto, Kazuo Takayama, and Naoki Akita contribute equally to the work.

Ethical Approval

This study was approved by and institution review board (IRB) of Osaka University and National Institute of Biomedical Innovation, Health and Nutrition.

Statement of Human and Animal Rights

All animal procedures using mice were approved by the Osaka University and National Institute of Biomedical Innovation, Health and Nutrition. All efforts were made to minimize animal suffering and to reduce the number of mice used.

Statement of Informed Consent

Statement of Informed Consent is not applicable.

Declaration of Conflicting Interests

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Supplemental Material

Supplementary material for this article is available online.

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