Stem Cell Research & Therapy

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Development of iPSC-derived FIX-secreting hepatocyte sheet as a novel treatment tool for hemophilia B treatment

Delger Bayarsaikhan[1†], Govigerel Bayarsaikhan[1†], Jaesuk Lee[2†], Teruo Okano[3,4], Kyungsook Kim[4,5*] and Bonghee Lee[1*]

Abstract

Background Hemophilia B is an inherited disorder caused by a mutation in the FIX gene, which results in insufficient blood clotting factor IX (FIX) production from hepatocytes. Currently, there are no treatments for hemophilia B patients. The patients should be continuously administrated with clotting factor concentrates 2–3 times a month to prevent bleeding. Therefore, this study aimed to develop an engineered FIX-secreting hepatocyte sheet that can release FIX for an extended period. Within this study, the engineered FIX-secreting hepatocyte sheet was developed by integrating two core technologies, including a gene editing platform to generate FIX-secreting cells and cell sheet technology to improve cell delivery efficacy.

Methods The human FIX gene was inserted into the APOC3 site of iPSCs by CRISPR/Cas9, which secretes the target protein after differentiation into hepatocytes. FIX-secreting hepatocyte sheets were obtained by temperature-responsive polymer grafted cell culture dishes (TRCD). Immunohistochemical and functional tests were performed for hepatocyte-like cells differentiated from FIX KI-iPSCs and wild-type iPSCs (WT-iPSCs). After validating the functional activity and secretion of FIX protein, the engineered hepatocyte-like cell sheets were transplanted to NOD/SCID mice for the in vivo experiments.

Results The insertion of the human FIX gene into the APOC3 site demonstrated a significant increase in FIX secretion in hepatocyte-like cells differentiated from FIX KI-iPSCs compared with those obtained from WT-iPSCs. Among the iPSCs to hepatocyte differentiation stages, the hepatic endoderm stage was most suitable for seeding the cells on TRCD and generating cell sheets by temperature changes from 37°C to room temperature when the hepatocyte-like cells have reached maturity. The engineered FIX-secreting cell sheets showed intensive expression of the FIX proteins without losing hepatocyte morphology for 20 days. Furthermore, an in vivo study showed that engineered FIX-secreting cell sheets retained their FIX secretion functions for two weeks, whereas single-cell injected traditionally were barely detected in the experimental animals.

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Conclusions The engineered FIX-secreting cell sheets fabricated from functionally improved iPSCs with practical cell delivery tools could be a promising tool for clinically treating Hemophilia B.

Keywords CRISPR/Cas9, FIX knock-in, Hepatocytes derived from iPSCs, APOC3, Cell sheet technology, Hepatocyte differentiation

Introduction

Hemophilia B is one of the most common bleeding disorders, affecting one in 40,000 men due to a deficiency of the blood clotting factor IX (FIX). The FIX gene contains eight exons and 2,780 bp transcripts [1]. Currently, more than 3,656 mutations have been reported among hemophilia B patients, comprising point mutations, polymorphism, deletion of exons, duplication, and indel insertion. According to residual procoagulant activity, hemophilia B patients are classified into three main groups, including those with severe symptoms or FIX activity less than 1 IU/dL, moderate or FIX activity between 1 and 5 IU/dL, and mild or FIX activity between 5 and 40 IU/dL [2]. Hemophilia B patients are often diagnosed with severe diseases, suffering frequent spontaneous and recurrent joint bleeds, which can lead to further complications, such as arthritis, deformities, and an increased risk of disability and death. In addition to physical and mental burdens, patients are financially burdened by treatments that involve the administration of 2 to 3 times standard half-life FIX per week or one time of extended half-life FIXs per 2 to 3 weeks, which could show side effects, including generation of neutralizing antibodies [3, 4]. In developing countries, patients are at risk since only 20% of the global population can afford hemophilia treatment, which is restricted to costly protein replacement therapy. In addition to coagulant factors, plasma-derived factor concentrates are widely used, which presents the possibility of blood-borne transmission [5]. A further study investigates hepatocyte transplantation for its potential to treat hemophilia B due to its ability to provide FIX protein. However, the availability of donors significantly limits its application. An alternative source of functionally improved hepatocytes can be obtained by differentiating mesenchymal stem cells (MSCs), embryonic stem cells, and induced pluripotent stem cells (iPSCs) [6]. MSCs are ideal candidates for cell therapy due to their immunomodulatory properties and ease of isolation from adult tissues. ESCs have a high differentiation potential, but they have ethical concerns and are subject to immune rejection.

Among the stem cells, iPSCs were chosen for the current study due to their advantages, including pluripotency, ethical consideration, accessibility and availability, and reduced risk of immune rejection. In addition, due to the varied mutation types among hemophilia B patients, it is necessary to develop patient-specific therapies with more efficacy and reduced side effects, which can be enabled by the application of iPSCs [7–9] Altogether, these stem cell sources provide a versatile platform for generating hepatocytes capable of producing FIX, offering potential alternatives to donor-derived hepatocytes. These stem cell sources provide a versatile platform for generating hepatocytes capable of producing FIX, offering potential alternatives to donor-derived hepatocytes.

There has been an extensive advancement in genome engineering technologies along with the stem cell revolution, and the CRISPR/Cas system has been utilized more frequently than other tools, such as zinc finger nucleases and transcription activator-like effector nucleases, due to its simple structure and precise genome engineering capabilities. Class II CRISPR/Cas systems (i.e. CRISPR/Cas9 or 12) remain the most widely used systems in the world. Altogether, the advances in stem cell and gene engineering technologies hold great promise for developing new therapeutic strategies to treat a wide range of genetic disorders, thereby providing individuals with effective treatment options and quality of life [10]. CRISPR/Cas9 has demonstrated, for example, that ex vivo editing of hematopoietic stem cells, iPSCs, neural stem cells, MSCs, and muscle stem cells can be used to treat or alleviate diseases related to a variety of conditions, including sickle cell disease, anemia, X-linked severe combined immunodeficiency, hemophilia A, diabetes mellitus, neurodegenerative disorders, and acute myeloid leukemia [10]. Though genetically engineered stem cells exhibit functional improvements and compatible results with traditional approaches, their clinical translation faces several challenges, including problems with poor homing of transplanted cells, cell heterogeneity, delivery, and off-target issues in vivo [11].

To solve the challenges of cell therapy in transplantation efficacy and homogeneous therapeutic effects, it is becoming increasingly apparent that cell sheet technology as an ideal cell delivery tool is providing beneficial tools in cell-based therapy for many clinical and preclinical studies [12–18] for example, in a recent study it was found that in contrast to single-cell injection, the engineered hepatic tissue sheets have shown to increase survival rates for longer than 200 days in vivo with liver-specific functionality [19]. Cell sheet technology involves creating a sheet of interconnected cells that are entirely in contact with their cell-to-cell interactions [20–22], produced by temperature changes (from 37°C to room temperature) using poly (*N*-isopropyl acrylamide) (PIPAAm)-grafted temperature-responsive cell culture

dishes without any enzymatic treatment and scaffold [23, 24]. Further, the introduction of cell sheet technology into the tissue engineering field has mitigated the problems associated with toxicity and rejection of scaffold materials and the inability to control the retainment of injected single-cell suspensions around target tissues because cell sheet technology can transplant cells into target tissue precisely without scaffolds [23, 25]. Thus, the present study aims to contribute to the successful translation of advances in gene engineering and tissue engineering technologies into the treatment of hemophilia B disease via generating the FIX-secreting iPSCs using CRISPR/Cas9 system and producing a functional hepatocyte sheet that is capable of secreting active FIX protein in target organ for a longer period (Fig. 1).

Materials and methods

Generation of FIX KI-iPSCs

Fibroblast (Fi)-derived induced pluripotent stem cells (iPSCs) were derived from healthy individuals and produced by Korea National Stem Cell Bank (Seoul, Korea) (IMR90 cell line). The iPSCs that secrete FIX were generated by transfecting them with a CRISPR/Cas9 ribonucleoprotein system (Toolgen, Inc., Seoul, Korea) targeting the APOC3 site in chromosome 11. The FIX coding region was inserted into the APOC3 targeting donor vector by cloning technique. The cell transfection was performed by 4D nucleofector (CM-150 program) (Lonza, Basel, Switzerland) with an RNP system involving 40 μg of Cas9 protein, 10 μg of gRNA (GCGAGGGA TCGAGGCCCAAA), and 4 μg of donor DNA in 8×10^5 iPSCs. After transfection, cells were maintained for four days at 37 °C to stabilize cell condition. Single-cell-derived clonal selections were performed, and when the cell confluence reached 70%, half of the cells were harvested for PCR verification of genome integration, and half of the cells were maintained for single–cell-derived clonal selection. Then, genomic DNA was extracted using the TIANamp Genomic DNA Kit (TIANGEN, GDP304-03), followed by the manufacturer's instructions. CloneAmpTM HiFi PCR Premix (Takara; 639298) was used for PCR and the products were visualized by ethidium bromide. The PCR products were confirmed through Sanger sequencing at COSMOGENETECH (Seoul, Republic of Korea).

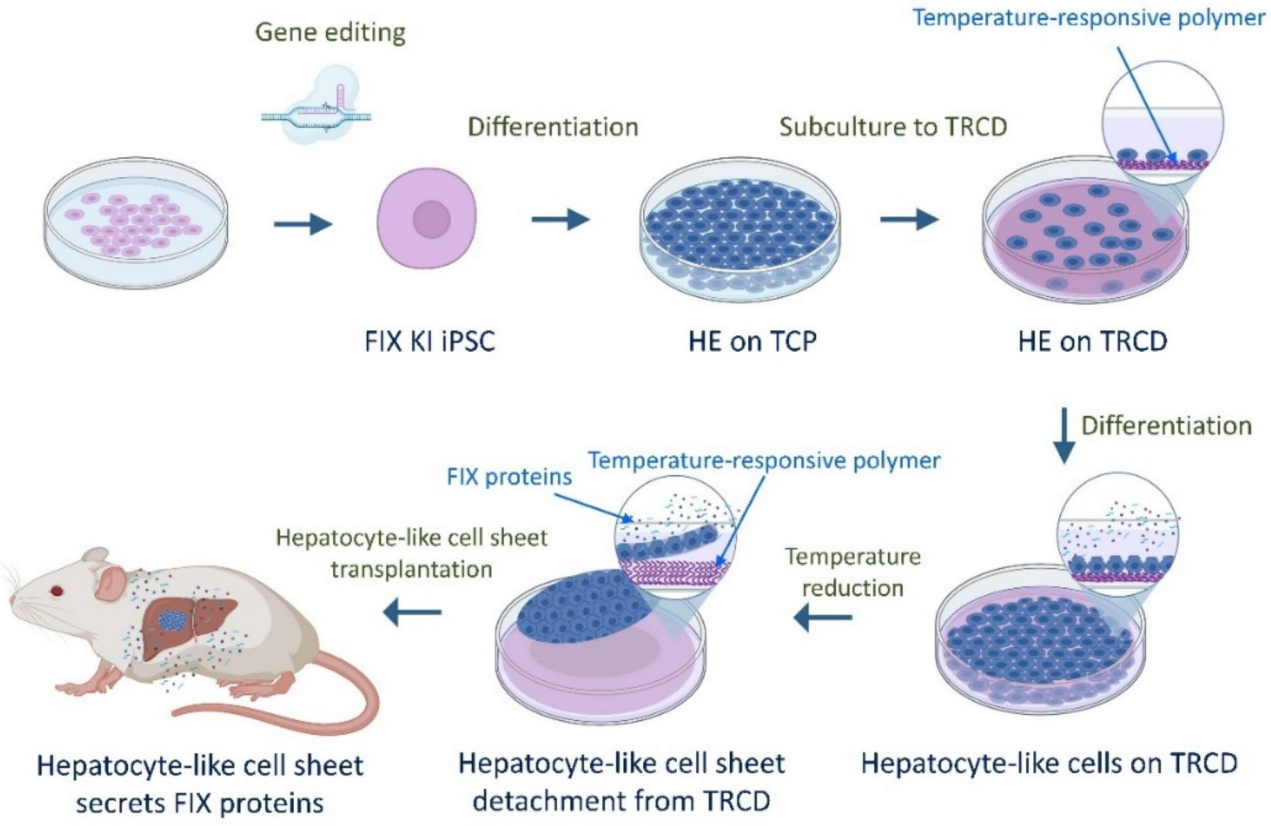


Fig. 1 The schematic illustration represents the therapeutic potential of hepatocyte-like cell sheets differentiated from FIX-KI iPSCs edited by the CRISPR/Cas system. HE: hepatic endoderm, TCP: tissue culture plate, TRCD: temperature-responsive polymer-grafted temperature-responsive cell culture dish. Temperature reduction: from 37°C to room temperature. The figure made in BioRender.com

Induced pluripotent stem cell (iPSC) culture

Cell culture wares were coated with Matrigel (Corning, Bedford, MA, USA) for 1 h. Banked iPSCs (passage 50) were seeded on Matrigel-coated cell culture ware with mTeSR^{TM}1 media (STEMCELL Technologies, Vancouver, Canada) supplemented, including 5 μM of ROCK inhibitor Y27632 (STEMCELL Technologies) at 37°C in a humidified atmosphere with 5% CO_2 for two days. Cell culture media was changed every day. Cell morphologies were observed using a Nikon eclipse Ts2 inverted microscope (Nikon, Tokyo, Japan) with NIS-Elements imaging software (Nikon, Japan). HepG2 cells were cultured in DMEM supplemented, including 10% FBS and 1% PS with 5% CO_2 as a control group. Cell culture media was changed every two days.

Animals

The animal studies were performed in 3-week-old male NOD-SCID mice (JA BIO Inc., Suwon-si, Gyeonggi-do, Korea). Animal housing and care were performed according to the Institutional Guidelines of Gachon University (IACUC No. LCDI-2023-0043). Animals were housed in groups of five per cage in standard individually ventilated cages (IVCs) under controlled environmental conditions, with a temperature of 22 ± 2 °C, humidity of 50–60%, and a 12-hour light/dark cycle. Food and water were provided ad libitum, and cage maintenance was performed regularly according to standard protocols. The Animal Care and Use Committee of Gachon University approved all animal study procedures described in this manuscript. 8 mice/groups were used in the animal study. Cell sheet group was compared with the single-cell injection group. After 2 weeks post-transplantation, the animals were euthanized in the chamber with CO_2 gas as painlessly and quickly as possible according to the Institutional Guidelines of Gachon University (IACUC No. LCDI-2023-0043). The cell sheet group prepared with the hepatocyte-like cells differentiated from FIX KI-iPSCs was compared with the single-cell injection group, prepared with the same cells as the cell sheet group in vivo study.

Immunocytochemistry and immunohistochemistry analysis

For immunocytochemistry (ICC) and immunohistochemistry (IHC), cells were fixed when they reached confluence using 4% paraformaldehyde (PFA) for 10–30 min. The tissue samples from the in vivo study were fixed using 4% PFA for three days. Non-specific binding was blocked in 0.01% Triton X-100 (Sigma-Aldrich, St. Louis, USA) containing 10% goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Cells were stained with primary antibodies: IgG control (5 μg/ml) (Invitrogen), OCT4 (1:200) (Cell Signaling, Denver, MA, USA) SOX2 (1:200) (Cell Signaling), FIX (1:100) (Abcam, Cambridge, UK), AFP (1:100) (DAKO, Glostrup Kommune, Denmark), HNF4a (1:100) (Abcam). These specimens were treated with Alexa Fluor 594-conjugated secondary antibodies (1:200) (Life Technologies, Carlsbad, CA, USA) for 1 h. Stained cells were mounted with ProLong Gold Antifade Reagent, including DAPI (Life Technologies). Immunofluorescence images were obtained using an LSM-700 confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Land Baden-Württemberg, Germany) and analyzed with ZEISS ZEN microscopy software (Carl Zeiss).

Flow cytometry analysis

iPSCs were detached using Accutase (STEMCELL Technologies) from cell culture ware for flow cytometry analysis. Cell suspensions were stained with a Fixable vility dye (Invitrogen) for 25 min. Cellss were fixed and permeabilized for 30 min in a fixation/permeabilization solution (BD Cytofix/Cytoperm kit) (BD Biosciences, San Diego, CA, USA). Cells were incubated with OCT4 (clone C30A3) (Cell signaling), SOX2 (clone D6D9) (Cell Signaling), Nanog (clone D73G4) (Cell Signaling), KLF4 (clone BG01) (R&D systems), and Fixable viability dye (Invitrogen) for 30 min and then with Alexa Fluor 566-conjugated secondary antibodies for 30 min (1:50). After incubation, cells were analyzed by Flow Cytometry (BECKMAN COULTER) (Life Sciences, Indianapolis, IN, USA). 10,000 events were counted for each analysis. Unstained cells were used for Flow cytometer calibration as a negative population. Dead cells were excluded using a fixable viability dye based on fluorescence intensity. Doublets were eliminated by distinguishing events based on SSC-H and SSC-A. After excluding dead cells and doublets, positive populations were gated and analyzed for OCT4, SOX2, KLF4, and Nanog markers expression with negative control, stained only with the viability dye and not with any markers. The gating strategy utilized for the analysis is shown in Supplemental Fig. 1.

Hepatocyte differentiation from iPSCs

Cells were seeded at 4.5×10^4 cells/cm^2 density on six-well plates in mTeSR^{TM}1, including 5 μM of ROCK inhibitor Y27632, and cultured for two days. After two days of culture, basal media was prepared with RPMI 1640 supplemented with 2% B27 (Gibco, Waltham, MA, USA) and 1% penicillin/streptomycin (PS) (Gibco). For definitive endoderm (DE) differentiation, cells were cultured in basal media supplemented with 100 ng/ml of Activin A (R&D Systems, Minneapolis, MN, USA) and 3 μM of CHIR99021 (STEMCELL Technologies) for three days. After DE differentiation, cell culture media was changed to hepatic endoderm (HE) media, which is prepared in basal media supplemented with 5 ng/ml of bFGF (R&D

Systems), 20 ng/ml of BMP4 (R&D Systems), and 0.5% DMSO (Sigma-Aldrich) for five days. Immature hepatocyte (IMH) differentiation was performed with basal media supplemented with 20 ng/ml of HGF (Peprotech, Cranbury, NJ, USA) and 0.5% DMSO for five days. Mature hepatocytes (MH) were cultured in hepatocyte culture medium bulletkit™ (Lonza) supplemented with 20 ng/ml of HGF, 20 ng/ml of Oncostatin M (R&D Systems), 100 nM of Dexamethasone (Sigma-Aldrich), and 0.5% of DMSO [26].

Engineered FIX-secreting cell sheet preparation

Cells were dissociated from cell culture ware with Accutase for 10 min after differentiating DE, HE, IMH, or MH. The dissociated cells were seeded on a 35-mm temperature-responsive cell culture dish (TRCD) (SSCW N57; Cell Sheet Tissue Engineering Regenerative Medicine Initiatives (CSTERMi), Tokyo, Japan). The cells were cultured in each stage of differentiation media until the MH stage. After 30 min incubation at room temperature, cultured MHs were spontaneously detached with gentle pipetting without any enzyme and structural damage to cells. MH sheets were used for structural and function tests. The prepared cell sheet group was compared to a single-cell group, a typical cell therapy tool.

Gene expression analysis

Total RNA from engineered cell sheets was extracted using Trizol (Invitrogen, Waltham, MA, USA) and PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocols. cDNA was prepared from 1 µg (in vitro study) or 2 µg (in vivo study) of total RNA using high-capacity cDNA reverse transcription kits (Life Technologies). qRT-PCR analysis was performed with Power SYBR Green PCR Master Mix (Life Technologies) using an Applied Biosystems Step One instrument (Applied Biosystems™, Waltham, MA, USA). Gene expression levels were assessed for the following genes in the supplemental Table 1. All primers were manufactured by Applied Biosystems. Relative gene expression levels were quantified by the comparative CT method [27]. Gene expression levels were normalized to GAPDH expression levels. Gene expression levels are relative to the iPSC group DE, HE, IMH, and MH groups for the in vitro study. For the in vivo study, gene expression levels were normalized to b-actin expression levels because the GAPDH was designed for human-specific cells [28]. Gene expression levels are relative to the single-cell group.

Protein secretion assessments

Secreted FIX amounts of hepatocyte-like cells differentiated from WT-iPSC, and FIX KI-iPSCs were measured with a supernatant of cells cultured for 24 h. In comparing experiments with engineered cell sheets versus single cells, engineered cell sheets were detached from TRCD in the MH stage by temperature changes from 37°C to RT. The detached cell sheets were transferred on six-well insert plates (SPL Lifescience, Pocheon, Gyeonggi-do, Korea). The engineered cell sheets (approximately 3×10^6 cells /a cell sheet) were cultured in MH media for 20 days. Single cells were detached from cell culture ware with Accutase in the MH stage. 3×10^6 cells were seeded on a 175 cm^2 flask to single cell condition. The single cells were cultured for five days before cell-cell junctions were formed. Supernatant media of engineered cell sheets and single cells cultured for 24 h assessed FIX and albumin protein amounts. FIX amounts secreted from engineered cell sheets and single cells were measured by FIX ELISA (Abcam) according to the manufacturer's protocols. FIX amounts were normalized to initial cell numbers and media amounts.

Indocyanine green clearance assay

Indocyanine green (ICG) is a cyanine dye to assess clinical liver functions. Differentiated hepatocyte-like cells from WT- and FIX-iPSCs (WT-iPSC/MH and FIX KI-iPSC/MH) were used for ICG assay. 1 mg/ml of ICG (Sigma-Aldrich) was dissolved in water. 1 ml of the ICG solution was added to 1 ml of cell culture media. After 2 h of incubation at 37°C, cells were washed with PBS 1X. The cells (ICG uptake) were observed by microscopy. After 6 h of incubation in cell culture media, the cells (ICG excretion) were washed with PBS 1X. The cells in precisely the same position were taken the picture after 2 h of incubation observed by microscopy. ICG excretion was quantified with the ICG-excreted cell culture media. The absorbance of the incubated cell culture media was measured at 800 nm using a microplate reader. Cell culture media without ICG was used as a control group for the quantification measurement.

Surgical procedures and cell sheet transplantation

Before the surgery, the cell sheets (mature hepatocyte stage at three days) (approximately 3×10^6 cells /a cell sheet) (cell viability ≥ 90%) were prepared with hepatocyte-like cells differentiated from FIX KI-iPSCs and detached from temperature-responsive cell culture dishes at RT. Cell numbers were calculated, and the percentage of viable cells in a cell population was demonstrated using trypan blue dye (Life Technologies) for cell viability assessment. For single-cell injection, cells (hepatocyte-like cells differentiated from FIX KI-iPSCs) were detached with collagenase P (Roche, Mannheim, Germany) for 5 min in the incubator and 10 min in the water bath. Detached cells were filtered using a cell strainer (Corning) with a 40 µm pore size to a uniform single-cell suspension and were washed with saline. Animals

were anesthetized using isoflurane. The right liver lobe was decapsulated with cotton tips. The detached hepatocyte sheets were applied on the decapsulated right liver lobe for 15 min. 1×10^6 of alive cells in 100 µl of saline were injected into the right liver lobe with a Hamilton syringe with 337 µm diameter of syringe needle (Sigma-Aldrich). The Hamilton syringe and needle were sterilized with 50 ml of 70% ethanol before injection. For the sham group, the right liver lobe was decapsulated with cotton tips, the same as the cell sheet group. All animals received ampicillin and meloxicam for two days in compliance with the IACUC protocol. The animals were sacrificed two weeks after surgery for the histological and qRT-PCR analyses.

Statistical analyses

All quantitative values are expressed as mean and standard error (SE, mean ± SE). Comparisons of two groups were tested using a two-tailed, unpaired Student's t-test. Statistical significance was defined as * $p < 0.01$. All statistical analysis was conducted on data sets of $n \geq 3$ biological replicates.

Results

Successful integration of human FIX gene into the APOC3 locus of iPSCs using CRISPR/Cas9

To integrate the human FIX genome into the APOC3 locus of iPSCs using CRISPR/Cas9, a 2 kb vector structured with left homology arm splicing acceptor– FIX–Poly A tail– the right homologous arm was designed and used (Fig. 2A - C). Followed by single-cell derived clonal selection, obtained FIX-secreting iPSCs were validated by sequencing. There was no mismatch detected among the integrated sequence of vector (Fig. 2D). In the IHC, FIX-secreting iPSCs expressed OCT4, SOX2, KLF4, and Nanog positive(pluripotency-associated markers) (Fig. 2E). Also, FIX-secreting iPSCs expressed 96.4%, 95.8%, 99.6%, 95% positive in OCT4, SOX2, KLF4, and Nanog in the FACS analysis respectively (Fig. 2F). The studies shown that pluripotency of iPSCs was not altered by vector transfection (Fig. 2E, F).

FIX Ki-iPSCs exhibit enhanced FIX gene expression and protein secretion without compromising hepatocyte differentiation capability

WT- and FIX KI-iPSCs were differentiated into definitive endoderm (DE), hepatic endoderm (HE), immature hepatocyte (IMH), and mature hepatocytes (MH) progressively (Fig. 3A). After MH stage differentiation, the cell morphologies were altered to hepatocyte likely in both WT-iPSC and FIX-KI-iPSC groups (Fig. 3B). qRT-PCR data showed similar levels of differentiated hepatocyte-specific markers (HNF4a (1.11 in WT-iPSC and 1 in FIX KI-iPSC) and AFP (1.06 in WT-iPSC and 1 in FIX KI-iPSC)) in both WT-iPSC and FIX KI-iPSC groups (Fig. 3C). The AFP levels in the HepG2 group were lower compared to those in WT-iPSC/MH and FIX KI-iPSC/MH groups because hepatocytes differentiated from iPSC resemble fetal liver cells, which exhibit elevated AFP expression. As qRT-PCR analysis provides relative data compared to the FIX-iPSC/MH group, AFP expression in HepG2 was lower than in FIX-iPSC/MH, although HepG2 also expressed the AFP gene. In FIX gene expression levels, FIX KI-iPSC group showed significantly higher FIX gene expression, compared to WT-iPSC group (2.5×10^{-5} in WT-iPSC and 1 in FIX KI-iPSC) (Fig. 3C). Furthermore, FIX protein amounts secreted from WT-iPSC and FIX KI-iPSC groups in the supernatant for 24 h were 0.19 ng/mL and 303 ng/ml (15940-fold difference). FIX KI-iPSC group secreted significantly higher levels of FIX proteins compared to the WT-iPSC group (Fig. 3D). These data indicate that the FIX gene knock-in process did not alter the hepatocyte differentiation ability of iPSCs and that the FIX gene was successfully knocked in iPSCs.

Differentiated hepatocyte-like cells expressed functional characteristics and stage-specific marker expression

Immunocytochemistry (ICC), qRT-PCR, and indocyanine green (ICG) tests were performed to assess differentiated hepatocyte characteristics. In ICC analysis, differentiated hepatocytes showed negative iPSC markers (OCT4 and SOX2) negative, and hepatocyte-specific markers (HNF4 a and AFP) were positive (Fig. 4A). qRT-PCR analysis was performed with differentiated cells in DE, HE, IMH, and MH stages in HepG2, WT-iPSC, and FIX KI-iPSC groups. FOXA2 (definitive endoderm (DE) marker), HNF4a (hepatic endoderm (HE) marker; hepatoblast), AFP (immature hepatocyte (IMH) marker; hepatocyte progenitor cell), and Albumin (Alb) (mature hepatocyte (MH) marker; hepatocyte) levels were increased respectively in the DE, HE, IMH, MH stages, compared to iPSC stage (Fig. 4B). ICG assay assessed differentiated hepatocytes' chemical uptake and secretion functionality. ICG uptake was observed in hepatocyte-like cells differentiated from WT- and FIX KI-iPSCs and in HepG2 cells as a control after 2 h of ICG incubation (Fig. 4C). After 6 h, differentiated hepatocyte-like cells excreted ICG reagents (Fig. 4C). The absorbance of ICG-extracted media from WT- iPSC/MH and FIX KI-iPSCs/MH groups exhibited significantly elevated levels compared to the control group (cell culture media without ICG) (Fig. 4C). This data suggests that FIX KI-iPSCs were successfully differentiated to hepatocytes functionally.

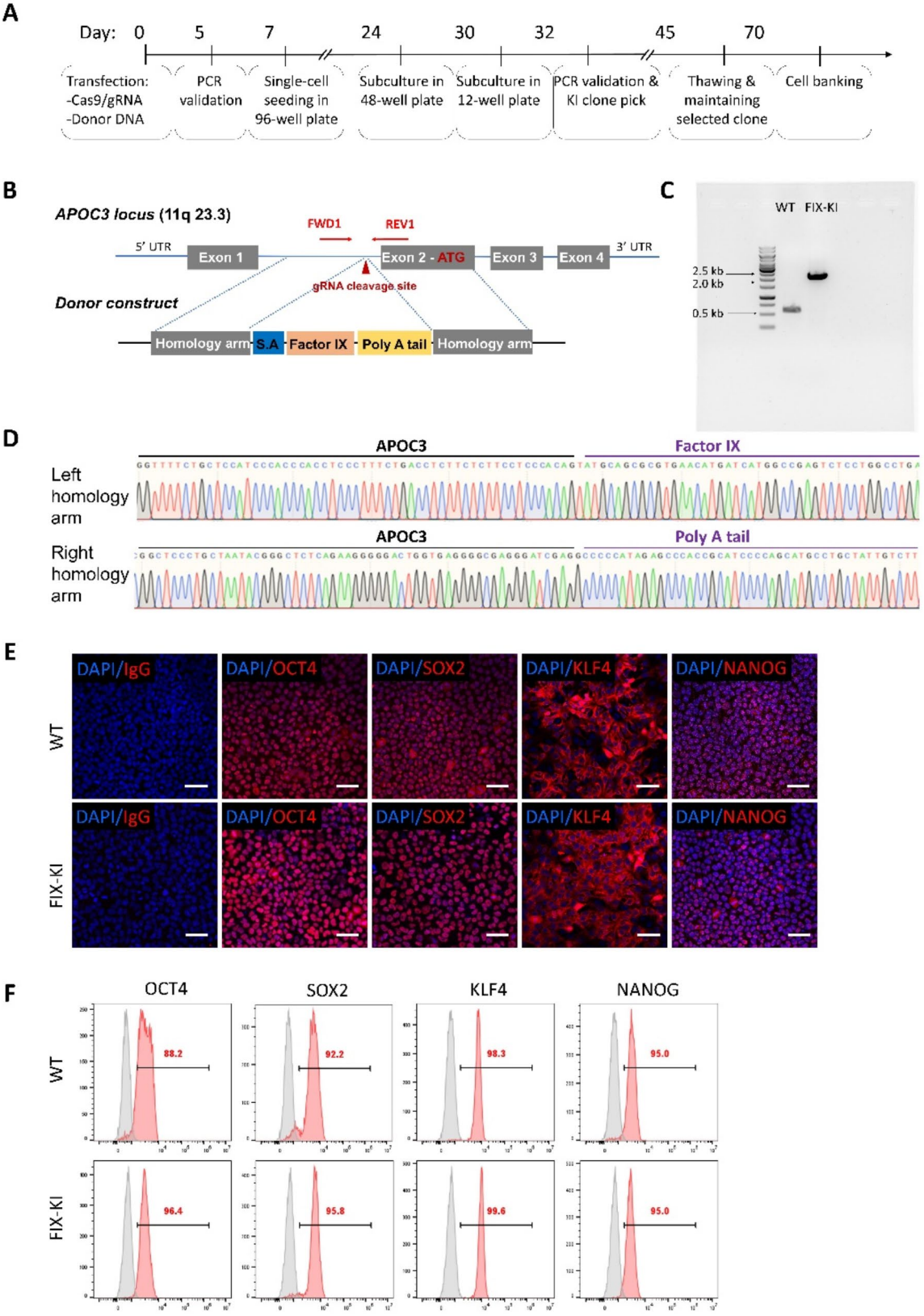


Fig. 2 Generation of coagulation FIX KI-iPS cell line. (**A**, **B**) Schematic representation of FIX KI- iPS cell line generation workflow and APOC3 targeting donor DNA construction for FIX. (**C**) PCR analysis of WT- and FIX KI-iPSCs. (Full-length gel is presented.) (**D**) Sanger sequence of FIX knock-in. (**E**, **F**) Immunohistochemistry (IHC) images and FACS analysis for OCT4, SOX2, KLF4, and Nanog (pluripotency-associated markers) of WT- and FIX KI-iPSCs. The unstained iPSCs were used as a negative population. Scale bars: 100 µm (**E**)

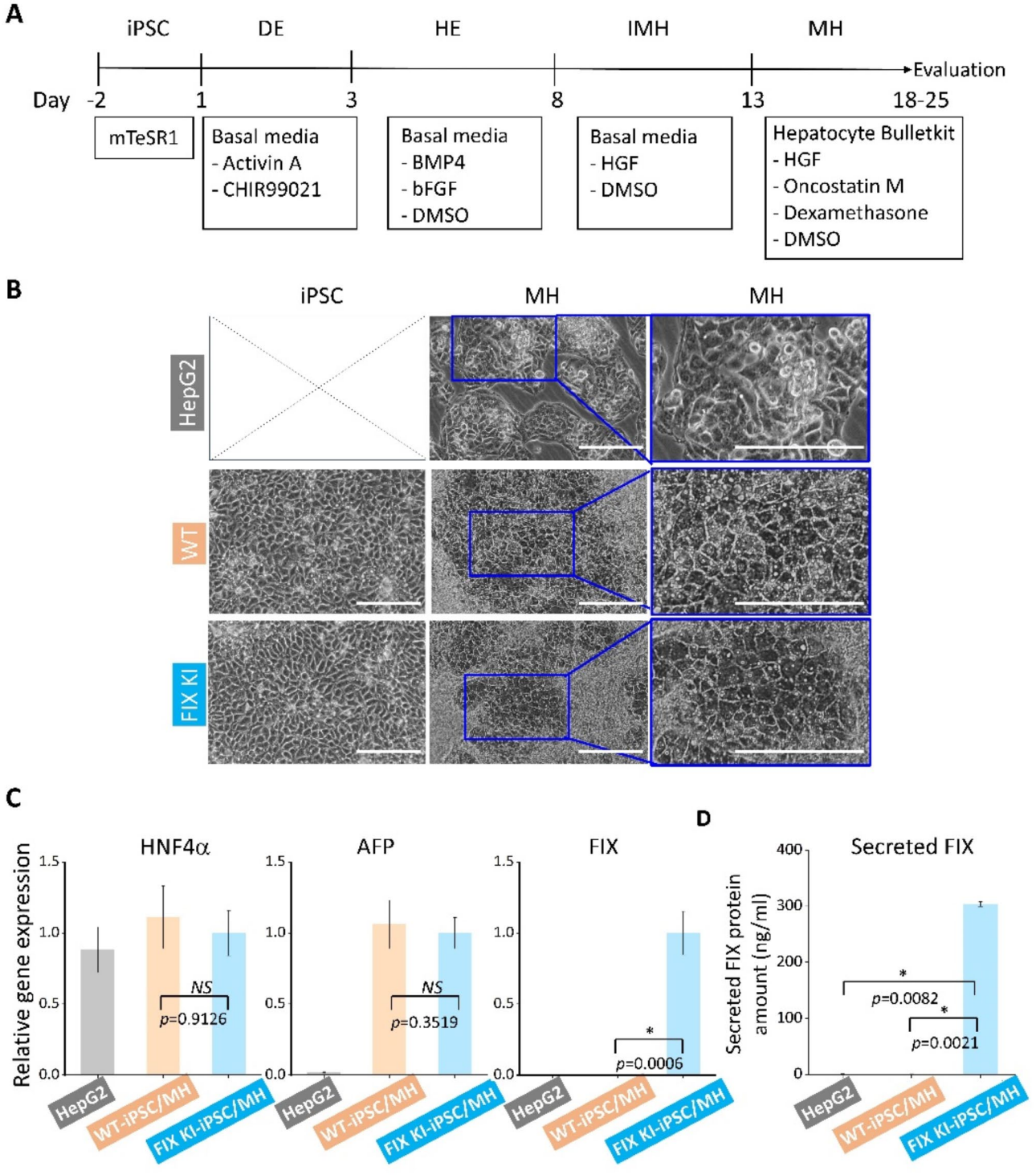


Fig. 3 Characterization of differentiated hepatocytes from WT- (WT-iPSC/MH) and FIX KI-iPSCs (FIX-iPSC/MH). (**A**) Schematic representation of the hepatocyte differentiation protocol from WT- and FIX KI-iPSCs. (**B**) Microscopic images of WT- and FIX KI-iPSC and hepatocyte-like cells differentiated from WT- (WT-iPSC/MH) and FIX KI-iPSCs (FIX KI-iPSC/MH). (**C**) qRT-PCR analysis of gene expression levels in WT-iPSC/MH and FIX-iPSC/MH groups. The gene expression levels were relative to the FIX KI-iPSC group ($n \geq 4$) as the HepG2 control group did not express the FIX gene, rendering it unsuitable for accurate comparison. (**D**) ELISA assay for FIX amounts of WT- and FIX KI-iPSC/MH groups ($n \geq 4$). HepG2 is used as a control group. Scale bars: 100 µm (**B**). *$p < 0.01$, NS = not significantly different (**C**, **D**)

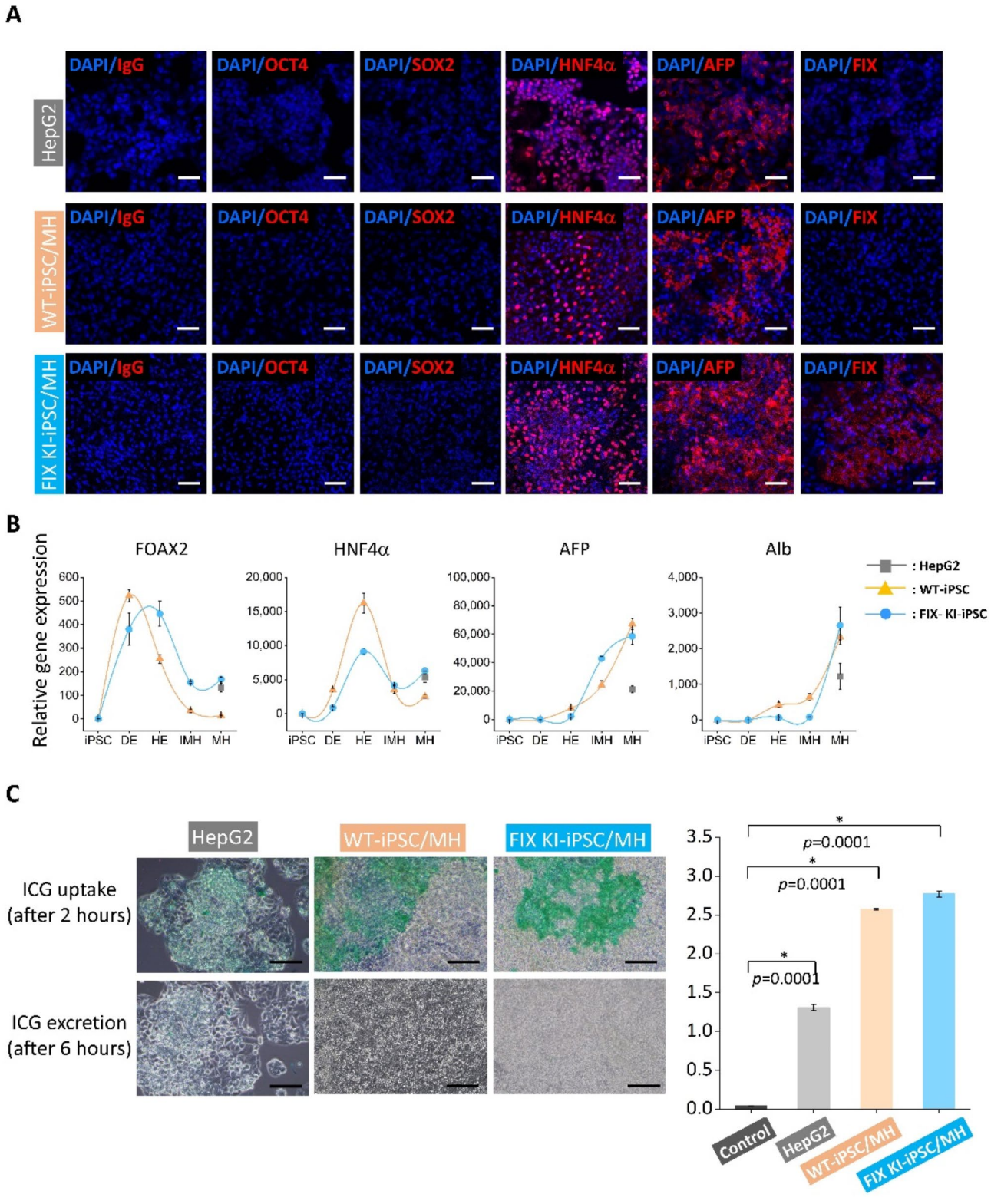


Fig. 4 Functional tests of the differentiated hepatocytes. (**A**) Immunocytochemical (ICC) analysis of WT-iPSC/MH and FIX KI-iPSC/MH groups. (**B**) qRT-PCR analysis for FOAX2 (DE marker), HNF4a (HE marker), AFP (IMH marker), and Alb (MH marker) in HepG2, WT-iPSC, and FIX KI-iPSC groups. Gene expression levels were relative to the iPSC stage before differentiation. (**C**) Indocyanine green (ICG) clearance assays for HepG2, WT-iPSC/MH, and FIX KI-iPSC/MH groups. Optical density was measured with ICG-extracted media. Cell culture media was used as a control. Scale bars: 100 μm (**A**, **C**). *$p < 0.01$

Cell sheets fabricated from hepatic endoderm stage cells exhibit superior morphology and hepatic characteristics

To fabricate hepatocyte-like cell sheets, differentiated cells were subcultured to temperature-responsive cell culture dishes (TRCD; SSCW N57) from general tissue culture plates by enzyme treatment (Accutase) in iPSC, HE and IMH stages and then cultured until mature hepatocyte stage (MH stage) (Fig. 5A). In the iPSC/MH and DE/MH groups, the cells were not attached to the temperature-responsive cell culture dishes. HE/MH group exhibited cuboidal hepatocyte-like morphologies and distinct cell-to-cell junctions. Whereas the IMH/MH group showed flattened morphologies homogeneously (Fig. 5B). This data demonstrates that cell dissociation using enzymatic treatment after the IMH stage makes hepatocyte-specific morphology lost. After MH differentiation, the cultured cells were moved to room temperature (RT) from 37°C for 30 min. Then, the hepatocyte-like cell sheets were detached from temperature-responsive cell culture dishes by pipetting. After MH cell sheet detachment, the engineered cell sheets in the HE/MH group showed thicker and bigger shapes than those in the IMH/MH group (Fig. 5C). Also, the engineered hepatocyte-like cell sheets in the HE/MH group expressed significantly higher levels of HNF4a, AFP, Alb, and FIX (hepatocyte-specific markers), compared to the IMH/MH group (Fig. 5D). This data suggests that the cells in HE stage are appropriate to prepare functional cell sheets.

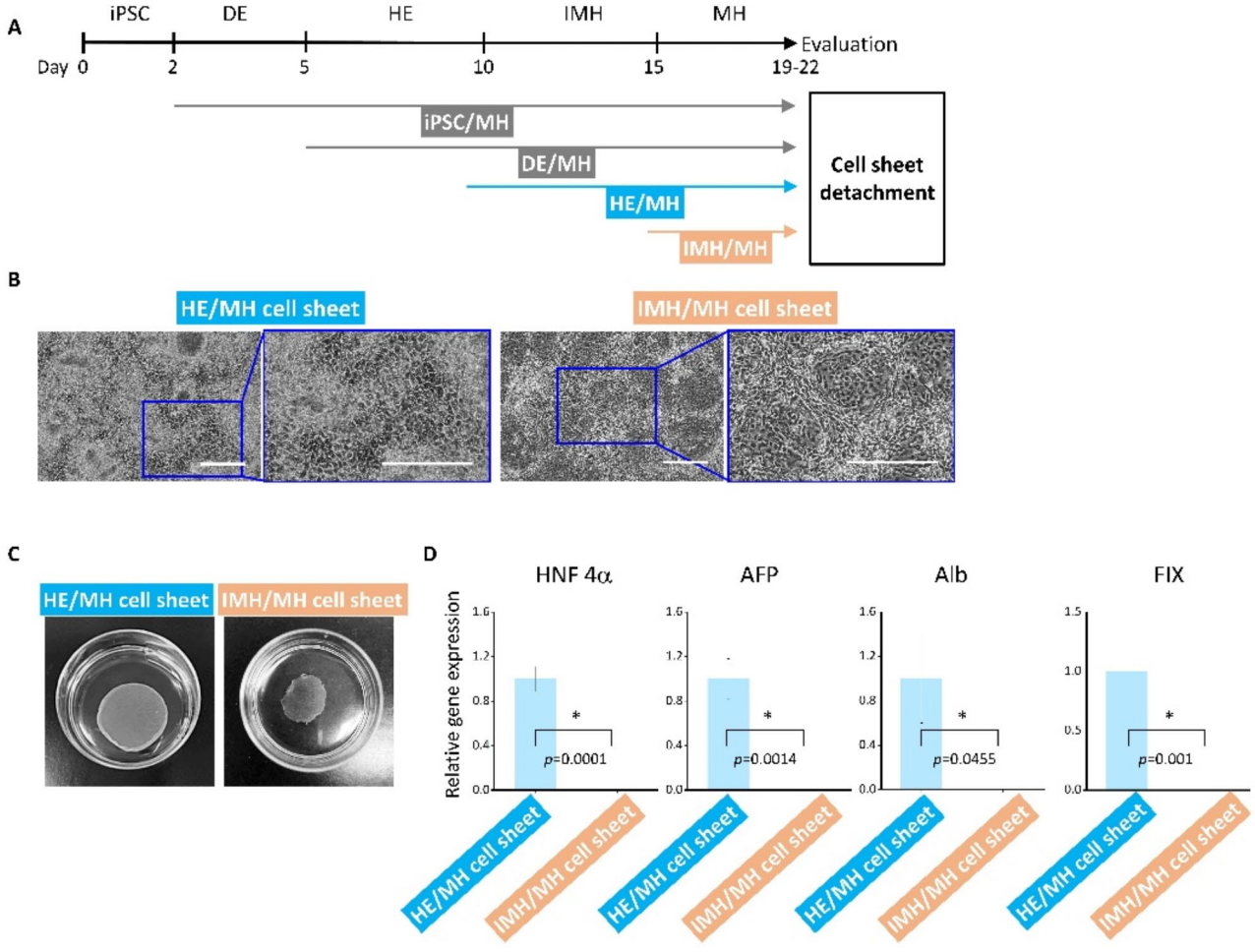
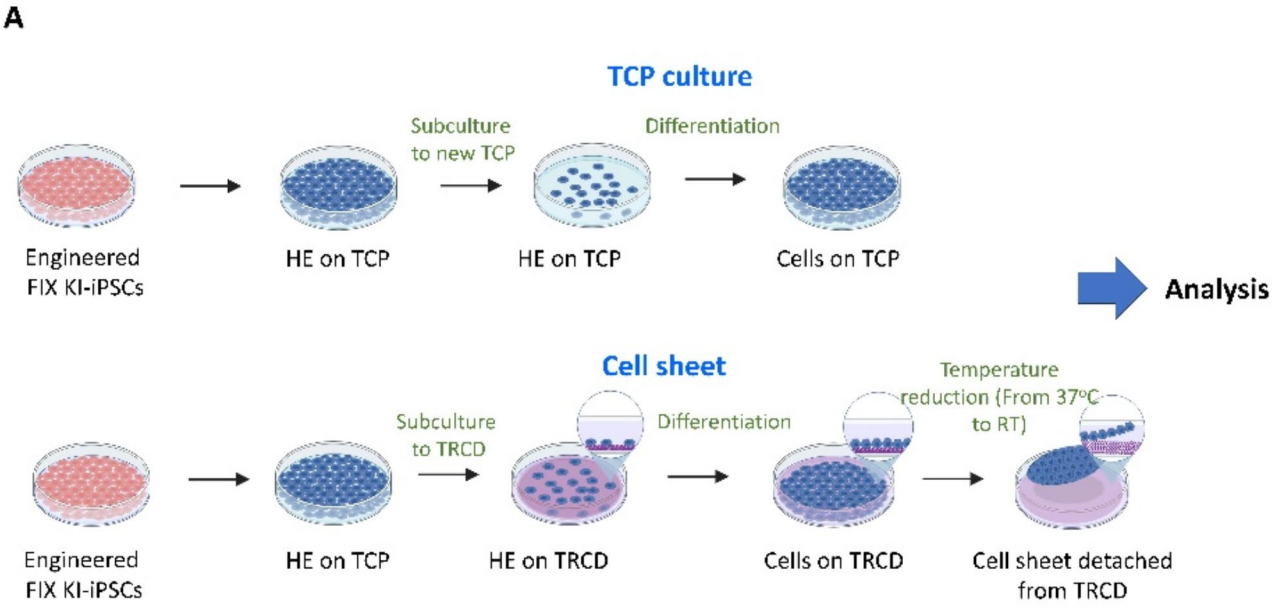
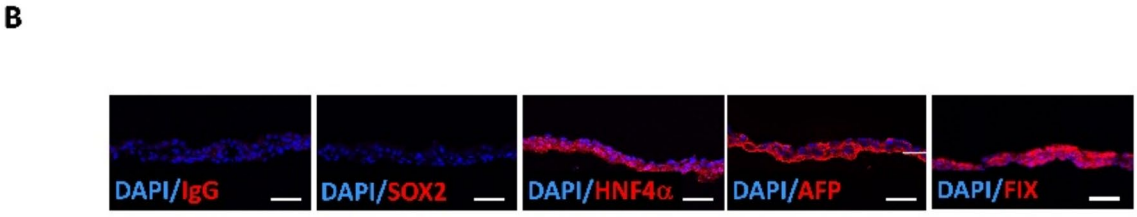


Fig. 5 Preparation of cell sheets using hepatocyte-like cells differentiated from FIX Ki-iPSCs. (**A**) Schematic representation of the timeline for hepatocyte sheet preparation. The cells were subcultured onto temperature-responsive cell culture dishes at iPSC (iPSC/MH), DE (DE/MH), HE (HE/MH), and IMH stages (IMH/MH) and then differentiated to mature hepatocyte (MH) stage to prepare hepatocyte sheets. (**B**) Morphological images of differentiated hepatocyte-like cells from HE (HE/MH) and IMH (IMH/MH) stages. (**C**) Optical images of cell sheets detached from temperature-responsive cell culture dishes (TRCD). (**D**) qRT-PCT analysis for HNF4a, AFP, and Alb of HE/MH and IMH/MH cell sheet groups. The gene expression levels were relative to the HE/MH cell sheet group ($n=4$) (early time point), allowing for a comparison of temporal changes. Scale bars: 200 µm (**B**) and 1 cm (**C**). *$p<0.01$ (**D**)





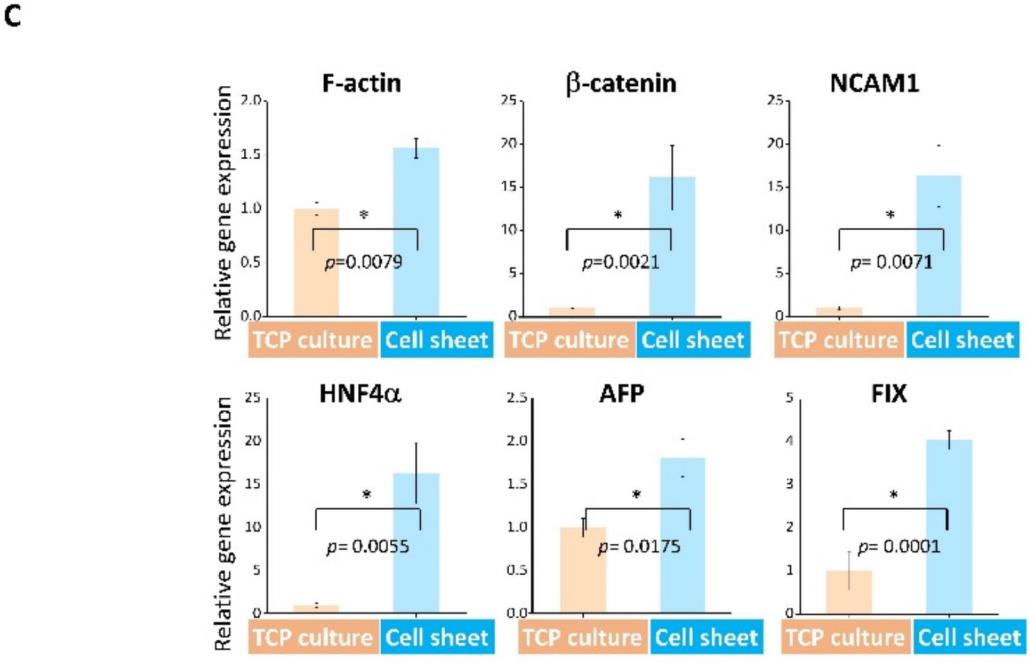


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Fig. 6 Functionality tests of engineered cell sheets. (**A**) Schematic representation of the experimental process of TCP culture and cell sheet groups. (HE: hepatic endoderm, TCP: tissue culture plate, TRCD: temperature-responsive polymer-grafted temperature-responsive cell culture dish.) (**B**) IHC images for IgG (negative control), SOX2 (iPSC marker), FIX (FIX protein marker), HNF4a, and AFP (hepatocyte-specific markers) of engineered hepatocyte sheets. (The cell viability of cell sheet ≥ 90%) (**C**) qRT-PCR analysis for F-actin (cytoskeleton structure protein marker), b-actin and NCAM1 (cell junction markers), FIX (FIX secretion protein marker), and HNF4a and AFP (hepatocyte-specific markers) using differentiated hepatocyte-like cells on tissue culture plate (TCP culture) and detached from TRCD as a sheet form (cell sheet). The gene expression levels were relative to the TCP culture cell group ($n = 4$). Scale bars: 200 µm (**B**). *$p < 0.01$ (**C**). The Fig. 6(**A**) made in BioRender.com

Engineered cell sheets exhibited enhanced hepatocyte-specific and cell adhesion protein markers compared to traditional tissue culture plastic systems

To assess hepatocyte characteristics of engineered cell sheets, H&E, IHC, and qRT-PCR assays were performed using engineered cell sheets detached from a TRCD (Fig. 6A). In IHC analysis, engineered cell sheets exhibited SOX2 (iPSC marker) negative and FIX, HNF4a, and AFP (hepatocyte markers) positive (Fig. 6B). Gene expression levels related to cell adhesion and hepatocyte characteristics were compared with cell sheet form and single-cell formula of hepatocyte-like cells differentiated from FIX Ki-iPSCs. Cell sheet formation of differentiated hepatocytes enhanced cell adhesion proteins (F-actin, b-catenin, and NCAM1). The cell sheet group with the enhanced cell adhesion proteins showed 4.6, 7329, and 10.6 times higher expression levels of hepatocyte markers (HNF4a, AFP, Alb) than the cells cultured on TCP (TCP culture group) (Fig. 6C).

Enhanced coagulation FIX protein secretion by cell sheet compared to single-cell

The differentiated hepatocytes in the single-cell group exhibited elongated cellular morphologies, distinct from typical hepatocytes (Fig. 7B).In contrast, the cell sheet group preserved hepatic morphologies for up to 20 days. The single-cell group was not maintained until day 20 because hepatocytes in the single-cell culture progressively lost their hepatic characteristics and viability due to the lack of cell-to-cell junctions, which are critical for maintaining hepatic function over an extended period. This is in contrast to the cell sheet group, where the structural integrity of the cell sheet and the preservation of cell-to-cell junctions enabled the hepatocytes to retain their functional properties for up to 20 days. Figure 7C normalized the measured FIX amounts to media volumes and cell numbers in both groups. The cell sheet groups secreted 5–11 times higher FIX proteins than the single-cell group at 1, 2, 3, 4, and 5 days post-seeding (Fig. 7C).

Transplantation of cell sheets preserves FIX secretion function in NOD-SCID mice

Cell sheets and single-cells were transplanted into the liver surface of NOD-SCID mice(Fig. 8A). No challenges were encountered while attaching the cell sheets to the liver surface. It was observed that transplanted cell sheets were fixed on the intended spot in 15 min. After single-cell injection into the liver, 2/8 mice died within 1 h after transplantation and were fixed for histological analysis (Fig. 8B). In H&E staining, some cells were observed in the liver vessels (Fig. 8C). The observed cells in liver vessels were positive in human vimentin staining (Fig. 8D). This result suggests that injected single cells accumulated into liver vessels and could block blood circulation in the liver. Transplanted cells were observed on the liver surface in the cell sheet transplantation group two weeks after transplantation. (Fig. 8E). The cell sheets on the liver surface showed positive in human-specific markers (vimentin) and FIX (Fig. 8F and G). The cell sheet group expressed a significantly higher level of the human cell GAPDH gene than the single-cell group (Fig. 8H) ($p = 0.0001$). Also, FIX gene expression levels were only detected in the cell sheet group (Fig. 8I). These data suggest that the functionality of the transplanted cell sheets was preserved for at least two weeks in vivo.

Discussion

The present study demonstrated the potential application of gene-engineered cell sheet technologies in the development of therapeutic approaches for hemophilia B diseases using three-step procedures: (i) enhancing the patient-derived iPSCs, which FIX KO-iPSCs mimicked to allow them to secrete functional active IX gene by CRISPR/Cas9 platform; (ii) differentiating the genetically engineered iPSCs to hepatocytes; and (iii) generating functionally improved cell sheet using TRCD.

Firstly, the results demonstrated that the CRISPR/Cas9 system is capable of developing an in vitro holds promising advantages for correcting genetic mutations carried by patients without affecting the pluripotency of the patient-derived stem cells, which is essential for obtaining differentiated hepatocytes for subsequent autologous transplantation of genetically modified cells. There are several in vitro and in vivo studies conducted for CRISPR/Cas9-assisted gene therapy for hemophilia B patients. However, these studies targeted the insert of the human FIX gene into AAVS1, exon 1 of the resident FIX gene, murine ROSA26 safe harbor site, swine factor IX locus, and mAlb locus using the CRISPR/Cas9 system. For example, recent studies have successfully used the CRISPR/Cas9 tool to correct mutation sites of FIX or knock in 4.9 kb of EF1a-FIX donor into AAVS1 and to insert a corrected sequence of human FIX genes into exons 2 or 1 of resident FIX genes in

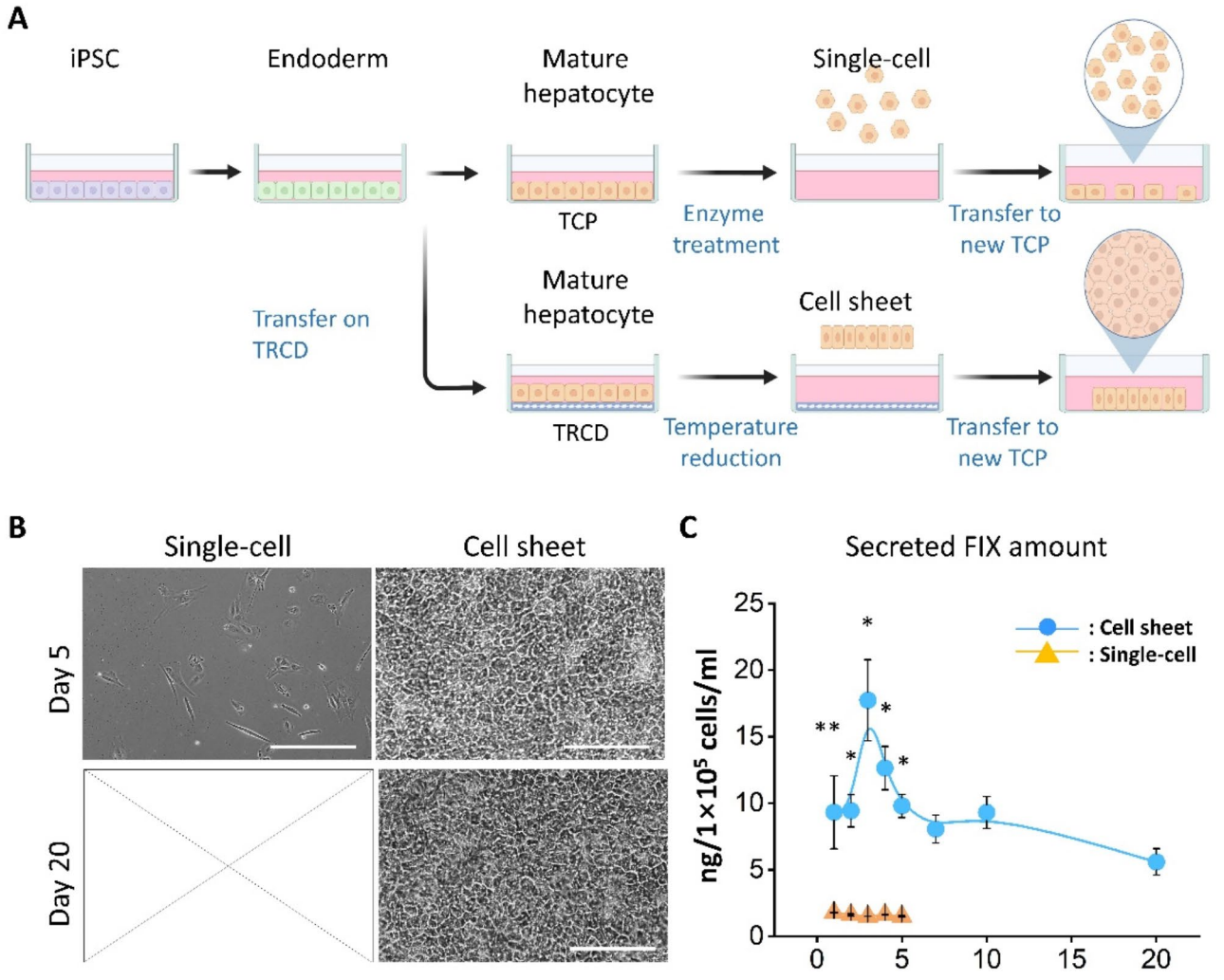

Fig. 7 FIX protein secretion of differentiated hepatocyte-like cells in single-cell formula without cell-to-cell junctions and cell sheet with cell-to-cell junction in vitro study. (**A**) Schematic representation of the protocol for FIX protein secretion test. (TCP: tissue culture plate; TRCD: temperature-responsive cell culture dish) (**B**) Morphological images of single-cell and cell sheet groups at 5 and 20 days. (**C**) ELISA analysis of FIX proteins secreted from single-cell and cell sheet groups for 5 and 20 days respectively. The statistical data is relative to the single-cell group ($p = 0.0332$ (Day 1), $p = 0.0006$ (Day 2), $p = 0.0018$ (Day 3), $p = 0.0005$ (Day 4), $p = 0.0001$ (Day 5)). Scale bars: 100 µm (**C**). $^{*}p < 0.01$ and $^{**}p < 0.05$ (**C**)

patient-derived iPSCs [29–33]. In contrast to previous studies, we designed the 2.2 kb of FIX gene insertion next to the APOC3 gene in hemophilia B- mimicked iPSCs to obtain FIX-secreting cells, suggesting the alternative site for gene-edited stem cell therapies for this disorder. A benefit of the APOC3 targeting insertion is that it could enable the transplanting cells to be standardized due to the FIX secretion levels achieved upon the generation/maturation of hepatocytes.

Secondly, hepatocytes were generated from FIX KI-iPSCs without alterations at hepatocyte differentiation; these hepatocytes showed the same hepatocytic characteristics as FIX WT- iPSCs, while FIX secretion level was significantly high in hepatocyte-like cells derived from FIX KI-iPSCs. Also, the differentiated hepatocyte-like cell showed negative results in iPSC markers, which enables it to differentiate into tumors in vivo. Furthermore, the hepatocyte-like cells derived from FIX KI-iPSC successfully did ICG uptake and clearance, which is used clinically for liver function tests. These data evidence that FIX KI-iPSC can be differentiated into hepatocytes functionally, and the differentiated hepatocytes have a low risk of tumorigenicity. Furthermore, in cells in the supernatant medium at the end of differentiation, the FIX secretion level of the hepatocytes derived from FIX-KI-iPSCs was significantly higher than the level of FIX secretion of parental iPSC-derived hepatocytes and FIX corrected cells, as reported in previous studies targeted to inserting human FIX gene at AAVS1 site and exon 2 [24, 25].

Thirdly, cell-based therapy has recently been raised to improve current medicine. However, cell-based therapy has yet to risk safety due to inconsistent treatment

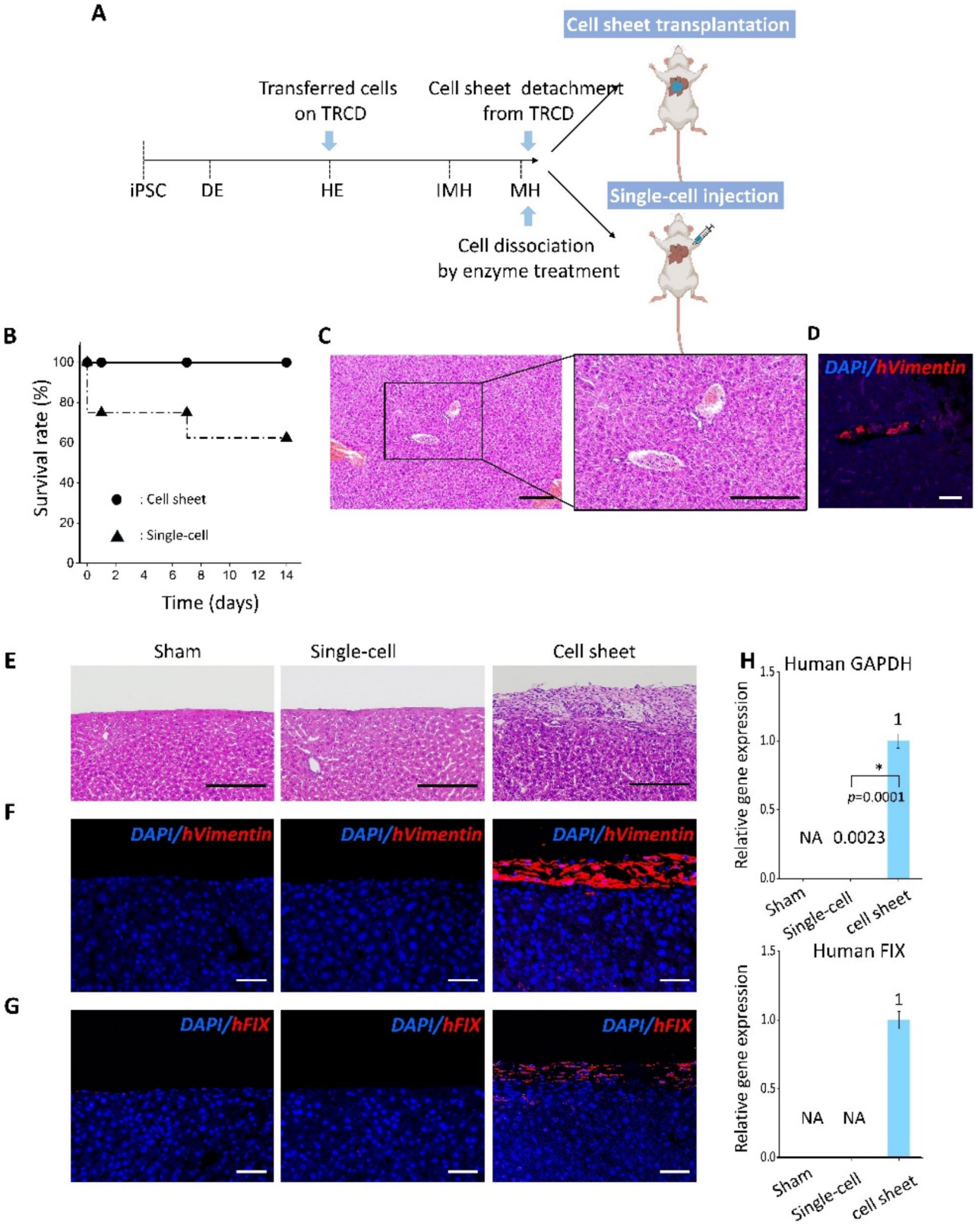

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Fig. 8 FIX secretion of differentiated hepatocyte-like cells in single-cell injection and cell sheets transplantation groups on the liver in vivo study. (**A**) Schematic representation of experimental schedule of in vivo study. (**B**) Survival rates of the mice in single-cell and cell sheet groups for 2 weeks ($n \geq 8$). (**C**, **D**) H&E and IHC staining of liver tissue in the single-cell group, which died right after single-cell injection within 1 h. (**E**) H&E staining of liver tissue at 2 weeks after transplantation. (**F**, **G**) IHC staining of human vimentin and FIX in liver tissue at 2 weeks after transplantation. (**H**, **I**) qRT-PCR analysis of human GAPDH and FIX genes with liver tissue at 2 weeks after transplantation. The gene expression levels were relative to the cell sheet group, as the sham and single-cell groups did not express detectable levels of human genes ($n = 3$). Scale bars: Scale bars: (**C**) 100 µm, (**D**) 400 µm (**E**) 100 µm, (**F**, **G**) 200 µm (**H**, **I**) NA: not applicable

effects, poor transplantation efficacy, and insufficient cellular function in single-cell injection formula. In this study, to solve the risk of current cell-based therapy, the FIX KI-iPSC sheet fabrication process was developed (Fig. 4). Engineered hepatocyte-like cell sheets were prepared using temperature-responsive cell culture dishes with temperature changes from 37°C to RT without cell adhesion protein disruption by enzyme treatment. When the engineered cell sheet detached from the temperature-responsive cell culture dishes, it shrunk about 1.75 times (19 mm) of its original size (35 mm), generating tight cell-to-cell junctions. The previous study demonstrated that cell sheet detachment altered cytoskeleton structures 3-dimensionally with shrunken cell sheet shapes [20, 34]. The altered cytoskeleton structure affected increased cell adhesion protein expressions and FIX secretion ability of cells [20, 34]. In the present study, the FIX secretion and hepatocyte-specific functions related to gene expressions were increased in cell sheet form. It could be caused by enhanced cytoskeleton structure and cell adhesion proteins through engineered cell sheet fabrication. The hepatocyte single-cell group lost their hepatic morphologies in 5 days of cultures; compared, hepatocyte-like cell sheets maintained cuboidal cell shape and precise cell junctions (hepatic morphologies) and FIX secretion ability until 20 days of cultures.

Previous in vivo studies demonstrated that cell sheet treatments show high engraftments, prolonged survival, and full functionalities. For example, it was demonstrated that transplanting FVIII transduced blood outgrowth endothelial cell sheets into mice could express FVIII protein for long periods with an estimated three to five-fold greater efficacy than the traditional method [35]. According to a study on mice, hepatocyte sheets fabricated on temperature-responsive culture dishes integrate into host tissue with laminar cell arrangement within one week of surgery. They are partially hepatectomized by the resident liver in four weeks post-surgery [36]. This study performed two methods of transplanting hepatocyte-like cells secreting FIX protein, including hepatocyte sheets and single-cell injection into NOD/SCID mice. Several advantages of hepatocyte transplantation as cell sheet form were demonstrated versus single-cell injection. For instance, due to their tight cellular connections, hepatocyte cell sheets allow transplanting at least three times more cells with a single surgery procedure. The transplanted hepatocyte sheets were also integrated with host liver cells without mechanical damage and attached to the hosts within 15 min of transplantation. There was strong attachment at the intended spot of transplantation, and most of the transplanted hepatocytes survived with good functionality and expression of human FIX protein. In contrast, in single-cell injections, mice die due to unexpected cell migration into blood vessels in the liver. Also, injected hepatocytes in the single-cell group showed low survival rates and no evidence that injected cells maintained functional activity in human FIX protein expression. Although the present study demonstrates the therapeutic potential of engineered cell sheets, the differences in cell doses between cell sheets and single-cell groups may have influenced the observed outcomes. Further studies should aim to match cell doses between treatment groups to eliminate this variable and provide a more direct comparison of treatment effects. The present study is limited by the absence of experiments on disease models to evaluate the therapeutic effects of engineered FIX-secreting cell sheets. Further study should address this limitation by incorporating disease models and clinical assessment methods for comprehensive extended–term evaluation.

Cell sheet transplantation offers distinct advantages over alternative methods such as single-cell injection and gene therapy. The procedure is minimally invasive, as the sheets are applied directly to the liver surface without sutures or fixation, reducing tissue damage. Although it may be more invasive than single-cell injection, cell sheet transplantation provides better retention of hepatic function and more controlled, localized delivery of therapeutic cells. For human applications, a non-invasive endoscopic device, successfully tested in a porcine model, allows for precise and reproducible delivery while minimizing recovery time and risks [37]. Furthermore, cell sheet technology has already been applied to human patients, demonstrating its feasibility [12–14, 16, 17]. Temperature-responsive culture dishes for creating cell sheets are well-established and can be standardized for clinical use, enhancing safety and practicality. Regarding transplantation frequency, our study demonstrated that the engineered cell sheets maintained FIX secretion for 2 weeks in vivo, suggesting sustained functionality during this period. Future studies should incorporate disease models and clinical assessments to evaluate long-term efficacy and determine optimal transplantation intervals. Compared to gene therapy, cell sheet transplantation

avoids the risks associated with viral vectors, including immune responses and off-target effects.

Conclusions

This study successfully prepared hepatocyte-like cell sheets derived from iPSCs and integrated the cell sheet technology into the genome editing platform as a translational study for hemophilia B treatment. The engineered FIX-secreting cell sheets secreted FIX proteins longer, comparing current cell therapy tools. The engineered hepatocyte sheets and a gene engineering platform developed in this study hold enormous potential to shape the new era for regenerative therapeutic approaches for hemophilia B safely and effectively.

Abbreviations

FIX	Blood clotting factor IX
WT-iPSC	Wild-type iPSCs
DE	Definitive endoderm
HE	Hepatic endoderm
IMH	Immature hepatocyte
MH	Mature hepatocytes
TRCD	Temperature-responsive cell culture dish
ICC	Immunocytochemistry
TCP	Tissue culture plate
IHC	Immunohistochemistry

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-025-04195-8.

Supplementary Material 1

Acknowledgements

We thank Hyun A Kang, Subin Lee, So Hee Han, Ji Yeon Oh, Enkmaa Lkhanva-Yondon, and Jae Won Lee for their technical support.

Author contributions

T.O. and B.L. conceived and supervised the study. K.K., D.B., G.B., and J.L. designed the experiments and supervised the project. K.K., D.B., G.B., and J. L. wrote the main manuscript text. Figures 1, 2, 3, 4, 5, 6, 7 and 8 prepared by K.K. and D.B. T.O., B.L., K.K., D.B., G.B., and J.L. reviewed and edited the manuscript.

Funding

This research was supported by the basic science research program and the development of technology based on gene editing, control, and restoration of the National Research Foundation of Korea (NRF), which is funded by the Ministry of Science and ICT (2022R1F1A1074122123178202348010 2) and the Korean government (MSIT) (No. RS-2023-00263429). This project was also conducted with the support of the Alchemist Project of the Korea Evaluation Institute of Industrial Technology (KEIT 20018560, NTIS 141584668) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea).

Data availability

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The approved project title is research about the efficacy of cell therapy on hemophilia using induced pluripotent stem cells, Gachon University Institutional Animal Care and Use Committee (IACUC), Approval# LCDI-2023-0043, June 9th, 2023. The authors declare that they have not use AI-generated work in this manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 2 May 2024 / Accepted: 29 January 2025
Published online: 23 February 2025

References

1. Goodeve AC. Hemophilia B: molecular pathogenesis and mutation analysis. J Thromb Haemost. 2015;13(7):1184–95.
2. White GC 2nd, Rosendaal F, Aledort LM, Lusher JM, Rothschild C, Ingerslev J, Factor V, Factor IXS. Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on thrombosis and Haemostasis. Thromb Haemost. 2001;85(3):560.
3. Burke T, Asghar S, O'Hara J, Chuang M, Sawyer EK, Li N. Clinical, humanistic, and economic burden of severe haemophilia B in adults receiving factor IX prophylaxis: findings from the CHESS II real-world burden of illness study in Europe. Orphanet J Rare Dis. 2021;16(1):521.
4. Zhao Y, Weyand AC, Shavit JA. Novel treatments for hemophilia through rebalancing of the coagulation cascade. Pediatr Blood Cancer. 2021;68(5):e28934.
5. Martinez-Murillo C, Quintana S, Ambriz R, Benitez H, Berges A, Collazo J, Esparza A, Pompa T, Taboada C, Zavala S, et al. An economic model of haemophilia in Mexico. Haemophilia. 2004;10(1):9–17.
6. Afshari A, Shamdani S, Uzan G, Naserian S, Azarpira N. Different approaches for transformation of mesenchymal stem cells into hepatocyte-like cells. Stem Cell Res Ther. 2020;11(1):54.
7. Liu X, Li W, Fu X, Xu Y. The immunogenicity and Immune Tolerance of Pluripotent Stem cell derivatives. Front Immunol. 2017;8:645.
8. Moradi S, Mahdizadeh H, Saric T, Kim J, Harati J, Shahsavarani H, Greber B. Moore JBt: Research and therapy with induced pluripotent stem cells (iPSCs): social, legal, and ethical considerations. Stem Cell Res Ther. 2019;10(1):341.
9. Musial-Wysocka A, Kot M, Majka M. The pros and cons of mesenchymal stem cell-based therapies. Cell Transpl. 2019;28(7):801–12.
10. Bayarsaikhan D, Bayarsaikhan G, Lee B. Recent advances in stem cells and gene editing: drug discovery and therapeutics. Prog Mol Biol Transl Sci. 2021;181:231–69.
11. Regmi S, Pathak S, Kim JO, Yong CS, Jeong JH. Mesenchymal stem cell therapy for the treatment of inflammatory diseases: challenges, opportunities, and future perspectives. Eur J Cell Biol. 2019;98(5–8):151041.
12. Iwata T, Yamato M, Washio K, Yoshida T, Tsumanuma Y, Yamada A, Onizuka S, Izumi Y, Ando T, Okano T, Ishikawa I. Periodontal regeneration with autologous periodontal ligament-derived cell sheets - a safety and efficacy study in ten patients. Regen Ther. 2018;9:38–44.
13. Kanzaki M, Takagi R, Washio K, Kokubo M, Yamato M. Bio-artificial pleura using an autologous dermal fibroblast sheet. NPJ Regen Med. 2017;2:26.
14. Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, Nagai S, Kikuchi A, Maeda N, Watanabe H, et al. Corneal reconstruction with

tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med. 2004;351(12):1187–96.
15. Ohki T, Yamato M, Ota M, Takagi R, Murakami D, Kondo M, Sasaki R, Namiki H, Okano T, Yamamoto M. Prevention of esophageal stricture after endoscopic submucosal dissection using tissue-engineered cell sheets. Gastroenterology. 2012;143(3):582–e588582.
16. Sato M, Yamato M, Mitani G, Takagaki T, Hamahashi K, Nakamura Y, Ishihara M, Matoba R, Kobayashi H, Okano T, et al. Combined surgery and chondrocyte cell-sheet transplantation improves clinical and structural outcomes in knee osteoarthritis. NPJ Regen Med. 2019;4:4.
17. Sawa Y, Miyagawa S, Sakaguchi T, Fujita T, Matsuyama A, Saito A, Shimizu T, Okano T. Tissue engineered myoblast sheets improved cardiac function sufficiently to discontinue LVAS in a patient with DCM: report of a case. Surg Today. 2012;42(2):181–4.
18. Yamamoto K, Yamato M, Morino T, Sugiyama H, Takagi R, Yaguchi Y, Okano T, Kojima H. Middle ear mucosal regeneration by tissue-engineered cell sheet transplantation. NPJ Regen Med. 2017;2:6.
19. Ohashi K, Yokoyama T, Yamato M, Kuge H, Kanehiro H, Tsutsumi M, Amanuma T, Iwata H, Yang J, Okano T, Nakajima Y. Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. Nat Med. 2007;13(7):880–5.
20. Bou-Ghannam S, Kim K, Grainger DW, Okano T. 3D cell sheet structure augments mesenchymal stem cell cytokine production. Sci Rep. 2021;11(1):8170.
21. Kim K, Ohashi K, Utoh R, Kano K, Okano T. Preserved liver-specific functions of hepatocytes in 3D co-culture with endothelial cell sheets. Biomaterials. 2012;33(5):1406–13.
22. Kim K, Utoh R, Ohashi K, Kikuchi T, Okano T. Fabrication of functional 3D hepatic tissues with polarized hepatocytes by stacking endothelial cell sheets in vitro. J Tissue Eng Regen Med. 2017;11(7):2071–80.
23. Kobayashi J, Kikuchi A, Aoyagi T, Okano T. Cell sheet tissue engineering: cell sheet preparation, harvesting/manipulation, and transplantation. J Biomed Mater Res A. 2019;107(5):955–67.
24. Okano T, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide). J Biomed Mater Res. 1993;27(10):1243–51.
25. Hannachi IE, Yamato M, Okano T. Cell sheet technology and cell patterning for biofabrication. Biofabrication. 2009;1(2):022002.
26. Hepatocyte. differentiation protocol [https://docs.abcam.com/pdf/protocols/hepatocyte-differentiation-protocol.pdf]
27. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101–8.
28. Dahn ML, Dean CA, Jo DB, Coyle KM, Marcato P. Human-specific GAPDH qRT-PCR is an accurate and sensitive method of xenograft metastasis quantification. Mol Ther Methods Clin Dev. 2021;20:398–408.
29. Lyu C, Shen J, Wang R, Gu H, Zhang J, Xue F, Liu X, Liu W, Fu R, Zhang L, et al. Targeted genome engineering in human induced pluripotent stem cells from patients with hemophilia B using the CRISPR-Cas9 system. Stem Cell Res Ther. 2018;9(1):92.
30. Morishige S, Mizuno S, Ozawa H, Nakamura T, Mazahery A, Nomura K, Seki R, Mouri F, Osaki K, Yamamura K, et al. CRISPR/Cas9-mediated gene correction in hemophilia B patient-derived iPSCs. Int J Hematol. 2020;111(2):225–33.
31. Ramaswamy S, Tonnu N, Menon T, Lewis BM, Green KT, Wampler D, Monahan PE, Verma IM. Autologous and heterologous cell therapy for Hemophilia B toward functional restoration of factor IX. Cell Rep. 2018;23(5):1565–80.
32. Stephens CJ, Lauron EJ, Kashentseva E, Lu ZH, Yokoyama WM, Curiel DT. Long-term correction of hemophilia B using adenoviral delivery of CRISPR/Cas9. J Control Release. 2019;298:128–41.
33. Wang Q, Zhong X, Li Q, Su J, Liu Y, Mo L, Deng H, Yang Y. CRISPR-Cas9-Mediated in Vivo Gene Integration at the Albumin Locus recovers Hemostasis in neonatal and adult Hemophilia B mice. Mol Ther Methods Clin Dev. 2020;18:520–31.
34. Takagi S, Ohno M, Ohashi K, Utoh R, Tatsumi K, Okano T. Cell shape regulation based on hepatocyte sheet engineering technologies. Cell Transpl. 2012;21(2–3):411–20.
35. Tatsumi K, Okano T. Hepatocyte transplantation: cell sheet technology for liver cell transplantation. Curr Transpl Rep. 2017;4(3):184–92.
36. Kano K, Yamato M, Okano T. Ectopic transplantation of hepatocyte sheets fabricated with temperature-responsive culture dishes. Hepatol Res. 2008;38(11):1140–7.
37. Maeda M, Kanai N, Kobayashi S, Hosoi T, Takagi R, Ohki T, Muragaki Y, Yamato M, Eguchi S, Fukai F, Okano T. Endoscopic cell sheet transplantation device developed by using a 3-dimensional printer and its feasibility evaluation in a porcine model. Gastrointest Endosc. 2015;82(1):147–52.

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