Title

CRISPR/Cas9-mediated gene correction in hemophilia B patient-derived iPSCs

Running Title

Gene correction in hemophilia B-iPSCs

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Abstract

Introduction: The clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is an efficient genome editing tool that holds potential for gene therapy. Here, we report an application of this system for gene repair in hemophilia B (HB) using induced pluripotent stem cells (iPSCs).

Material and Methods: We prepared targeting plasmids with homology arms containing corrected sequences to repair an in-frame deletion in exon 2 of the factor IX (*F9*) gene, and transfected patient-derived iPSCs with the Cas9 nuclease and a guide RNA expression vector. To validate the expression of corrected *F9*, we attempted to induce the differentiation of iPSCs towards hepatocyte-like cells (HLCs) *in vitro*.

Results: We successfully repaired a disease-causing mutation in HB in patient-derived iPSCs. The transcription product of corrected *F9* was confirmed in HLCs differentiated from gene-corrected iPSCs.

Conclusion: Although further research should be undertaken to obtain completely functional hepatocytes with secretion of the coagulation factor IX, our study provides a proof-of-principle for HB gene therapy using the CRISPR/Cas9 system.

Keywords: Clustered regularly interspaced short palindromic repeats, CRISPR-associated proteins, gene editing, hemophilia B, induced pluripotent stem cells

Introduction

Hemophilia B (HB) is an inherited, X-linked recessive bleeding disorder, with an incidence of 1 in 30,000 male births. HB occurs due to a deficiency in or absence of the functional coagulation factor IX (FIX), caused by mutations in the factor IX (*F9*) gene located on chromosome Xq27.1. About 80% of severe HB is caused by missense, nonsense, or frameshift mutations in *F9* [1].

HB is well suited for gene therapy, and viral vectors such as adeno-associated viral (AAV) vector have been reported by several groups [2, 3]. However, there have been some potential limitations in its use for replacing current therapies. In particular, adverse events such as the host humoral and cellular immune response against the viral vector remain major concerns.

To overcome the limitations of conventional strategies based on exogenous gene transfer using viral vectors, genome editing technologies have been developed, enabling the precise modification of genome sequences in many organisms, including mammals. Recently, the clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system has emerged as a new genome editing

technology [4-6]. The CRISPR/Cas9 system has been shown to efficiently mediate genome editing in a broad range of cell types, including human induced pluripotent stem cells (iPSCs). Patient-derived iPSCs may represent an ideal autologous cell source, avoiding the risk of immune rejection. But, iPSCs established from patients with monogenic diseases, including hemophilia, would still carry disease-causing mutations. Therefore, the precise correction of genes in patient-derived iPSCs using genome editing tools holds great promise for stem cell-based gene therapies [7, 8].

Recently, there have been several reports of CRISPR/Cas9-based genome editing in HB patients. Ramaswamy et al. reported CRISPR/Cas9-based genomic correction of HB patient-derived iPSCs, in which universal knock-in of *F9* cDNA into exon 1 of the resident *F9* gene and a point correction of disease causing mutation were performed [9]. Lyu et al. demonstrated that human full-length *F9* cDNA was inserted into the AAVS1 locus using the CRISPR/Cas9 system [10].

In this study, we generated iPSCs from a patient with HB carrying an in-frame deletion in exon 2 of *F9*, and achieved a successful site-specific gene correction through homology-dependent repair (HDR) using the CRISPR/Cas9 system.

Materials and Methods

Cell culture

All cells were maintained at 37°C and 5% CO_2 in a humidified incubator. Human iPSC line 201B7 (Riken BioResource Center, Tsukuba, Japan) and patient-derived iPSCs were maintained on cell culture plates coated with iMatrix-511 (0.5 μg/cm^2; Nippi, Inc., Tokyo, Japan) in StemFit medium (Ajinomoto Co., Inc., Tokyo, Japan), as described previously [11]. Passaging was performed via dissociation into single cells using Accutase (Innovative Cell Technologies, Inc., San Diego, CA, USA), which enabled rapid cell detachment, a single-cell suspension, and high viability in sensitive cell types such as iPSCs. For prevention of dissociation-induced apoptosis of iPSCs, Y-27632 Rho-associated kinase (ROCK) inhibitor (Wako Pure Chemical, Osaka, Japan) was added to the medium at 10 μM only on the same day, after passaging.

Generating iPSCs from peripheral blood mononuclear cells

Informed consent was obtained from patients in accordance with the Helsinki Declaration, and the study was approved by the Ethics Committee of the Kurume University School of Medicine.

Peripheral blood mononuclear cells (PBMCs; 1×10^6) were cultured in StemPro-34 SFM complete medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 2 mM l-alanyl-l-glutamine (Wako Pure Chemical), 100 ng/mL stem cell factor (SCF), 100 ng/mL FMS-like tyrosine kinase 3 (FLT3)-ligand, 20 ng/mL thrombopoietin (TPO), and 10 ng/mL interleukin 6 (IL-6) (Invitrogen). After 4 days, the reprogramming of PBMCs into iPSCs was induced using the CytoTune-iPS 2.0 Sendai Reprogramming kit (DNAVEC Corp., Ibaraki, Japan) in accordance with the manufacturer's protocol. After 14–21 days, colonies with iPSC morphology were picked and transferred to a novel, efficient feeder-free culture system, in which there is no need for the feeder cell preparation, which requires significant time, and iPSCs can be stably passaged for long periods by dissociating the cells into single cells under xeno-free medium, StemFit AK02N (Ajinomoto, Tokyo, Japan).

Isolation of genomic DNA and PCR

Genomic DNA was extracted from the cells using NucleoSpin Tissue (Macherey-Nagel, Düren, Germany). On PCR screening of successful knock-in clones, genomic DNA was isolated using SDS/Proteinase K. Unless otherwise specified, PCR was

performed using the KOD-Plus-Neo polymerase (Toyobo Co., Ltd., Osaka, Japan). PCR programs and primer sets used in each experiment are described in Table SI, SII. Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI, USA) was used for purification of PCR products from gel.

Vector construction

The human codon-optimized *Streptococcus pyogenes* Cas9 (SpCas9) gene was cloned from hCas9 vector (a gift from George Church, Addgene plasmid #41815) (Mali *et al*, 2013) and inserted into the SpCas9 expression vector. Target guide sequences, with 3′-ends followed by the NGG protospacer-adjacent motif sequence for SpCas9 recognition, were selected from intron 1 of *F9*. The first base of the guide was changed to guanine for optimal expression from the human U6 promoter. Synthetic oligonucleotides corresponding to 20-nucleotide guide sequences (Table SIII) were ligated into the single-guide RNA (sgRNA) expression vector [6]. The targeting vector, with homology arms (1.0 kbp in length) containing the corrected sequence, was constructed. The monomeric Kusabira-Orange (mKO) fused to a 2A self-cleaving peptide, followed by the puromycin-N-acetyltransferase cassette, was used as the

positive selection marker. This was flanked by mutated lox sites (lox66 and lox71), for selectable cassette excision by Cre-recombinase. The thymidine kinase (tk) gene was used as a negative selection marker.

CRISPR/Cas9-mediated genome editing in HT-1080 cells and evaluation of genome editing efficiency

HT-1080 cells were transfected with 0.3 μg Cas9 vector and 0.3 μg individual sgRNA vectors, using Lipofectamine 3000 (Invitrogen). Genome editing efficiency was evaluated using Surveyor Mutation Detection Kit (IDT, Coralville, IA, USA). The genomic region containing the CRISPR target site was amplified by PCR, subsequently assayed in accordance with the manufacturer's instructions.

CRISPR/Cas9-mediated HDR in iPSCs

Nucleofection was performed using the 4D-Nucleofector X unit and P3 Primary Cell 4D-Nucleofector X kit (Lonza, Basel, Switzerland). iPSCs were pretreated with 10 μM Y-27632 for 2 h prior to nucleofection. After single-cell dissociation, 2×10^5 iPSCs were resuspended in 20 μL of P3 Primary Cell Solution (Lonza), supplemented with 0.8 μg of SpCas9, 0.8 μg of sgRNA, or 0.8 μg of the targeting vector. Cells were

immediately nucleofected using program CA137 (Lonza), and seeded onto iMatrix-511-coated (0.5 μg/cm^2) 96-well culture plates in StemFit medium supplemented with 10 μM Y-27632. From day 3 after nucleofection, 0.5 μg/mL puromycin (Sigma Aldrich, St Louis, MO, USA) and 2 μM ganciclovir (Tokyo Chemical Industry, Tokyo, Japan) were added to the medium daily for 7 days. For drug-resistant clones, the targeted knock-in events were further checked by PCR screening**.**

Southern blot analysis

Genomic DNA was digested with *Eco*RI-HF (New England Biolabs, Beverly, MA, USA). Digested genomic DNA (10 μg) was electrophoresed in 1.0% agarose gel and transferred to a Hybond N+ nylon membrane (Amersham Biosciences, Little Chalfont, UK). Southern hybridization was performed with AlkPhos Direct Labelling Reagents (Amersham Bioscience) in accordance with the manufacturer's instructions. The hybridized probe was detected with the CDP-Star Detection Reagent (Amersham Biosciences), followed by analysis using the LAS-4000 mini luminescent image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Cre/loxP-mediated excision of the selection cassette

To excise the loxP-flanked selection cassette, 1 μg of LV-Cre plasmid DNA expressing Cre recombinase (Addgene plasmid #12106) was nucleofected into targeted iPSCs. The mKO-negative iPSC clones were screened by PCR for a successful Cre/loxP-mediated excision, using the same primer sets as for the Surveyor assay.

In vitro hepatocyte-like cells differentiation

iPSCs were differentiated into hepatocyte-like cells (HLCs) using the Cellartis DE differentiation kit (Takara Bio Europe AB, Gothenburg, Sweden). To promote endoderm induction, *FOXA2* and *HNF1A* were transduced using a lentiviral system, at the phase of definitive endoderm (DE) induction. At the hepatic specification/maturation phase, *C/EBPα*, *ATF5*, and *PROX1* were sequentially overexpressed with the same system. Vitamin K1 (50 ng/mL; Wako Pure Chemical) dissolved in DMSO (Sigma Aldrich) was added to hepatocyte maintenance medium. The final concentration of DMSO in culture medium was 0.2% (v/v).

Quantitative real-time PCR analysis for hepatocyte-specific markers and F9 mRNA

Quantitative real-time PCR (qPCR) assay was performed on a StepOnePlus real-time

PCR system (Applied Biosystems, Carlsbad, CA, USA). To analyze the relative expression of liver-specific genes, a SYBR green-based qPCR was carried out in a reaction mixture containing 500 nM each of the forward and reverse primers (Table SIV), cDNA corresponding to 2 ng of total RNA, and THUNDERBIRD SYBR qPCR mix (Toyobo), a final volume of 20 μL. Relative expression levels were calculated using the ΔΔCt quantification method. The expression level of each gene was normalized to that of *GAPDH* as an internal standard. To determine the *F9* transcript copy number, the probe-based qPCR approach was applied. The qPCR reaction mix contained 2× PrimeTime Gene Expression Master Mix (IDT), cDNA corresponding to 20 ng of total RNA, 900 nM of each primer, 200 nM probe, and nuclease-free water to a final volume of 20 μL. The experiment was performed in triplicate. For the absolute quantification of *F9* mRNA, a standard curve was generated based on a serial dilution of its PCR products, containing a known number of molecules. By using the standard curve, the copy number of *F9* cDNA was measured. For normalization, we used *GAPDH* as an internal standard.

Enzyme-linked immunosorbent assay (ELISA)

FIX antigen levels in culture medium and cell lysate were measured using an AssayMax Human Factor IX ELISA kit (AssayPro, St. Charles, MO, USA). The minimum detectable dose of factor IX was 0.63 ng/mL. Hepatocyte maintenance medium collected from day 25 to day 31 after induction of HLCs was concentrated 100-fold using a 30,000 MWCO concentrator (Vivaspin Turbo 15, Sartorius, UK). Total cell lysates were prepared using CelLytic M (Sigma Aldrich) containing protease inhibitor cocktail (Sigma Aldrich).

Results

Generation and characterization of patient-derived iPSCs

The patient was a 63-year-old man with severe HB caused by an in-frame deletion (227/229delAAG) in exon 2 of *F9*. His FIX activity was less than 1% (normal range: 70–130%) and antigen levels measured 2.3 μg/mL (normal average: 5.0 μg/mL). Three weeks after transduction from his PBMCs, iPSC colonies with typical human embryonic stem cell (ESC)-like morphology and alkaline phosphatase expression emerged (Fig S1A and S1B). These patient-derived iPSC clones expressed pluripotent marker genes such as *POU5F1*, *SOX2*, *MYC*, *KLF4*, *NANOG*, *GDF3*, *REX1*, and *DMNT3*, as verified by end-point RT-PCR (Fig S1C). In addition, genomic DNA analysis confirmed that these iPSC clones presented the disease-specific genotype of their parental somatic cells (Fig S1D). For targeted gene repair of patient-derived iPSCs, we selected the most highly proliferative iPSC clone that formed flat colonies with sharp edges and possessed high plating efficiency among these iPSC clones.

CRISPR/Cas9-mediated gene editing at specific gene loci

We constructed the sgRNA for inducing double-strand breaks (DSBs) near the mutation

site to promote gene repair via HDR. A total of four guide sequences were selected in the intron 1 region of *F9*. To test whether these sgRNAs can achieve targeted cleavage of mammalian chromosomes, each sgRNA expression vector was co-transfected into HT-1080 cells along with a vector encoding a codon-optimized SpCas9 protein. Using the Surveyor nuclease assay, we evaluated the ability of each Cas9-sgRNA complex to generate indels in HT-1080 cells through the induction of DSB and subsequent non-homologous end joining (NHEJ). The percentage of indel frequencies induced by sgRNAs1, 2, 3, and 4 were 3.8%, 7.5%, 8.5%, and 8.7%, respectively. To perform CRISPR/Cas9-mediated HDR in patient-derived iPSCs, we selected the most efficient sgRNA (sgRNA4), containing a targeting sequence located 292 bp upstream of the mutation site (Fig 1).

Targeted gene correction in iPSCs derived from a patient with HB

To correct the in-frame deletion in exon 2 of *F9*, we prepared a targeting construct with homology arms containing the corrected sequence. The homology arms were designed to flank the SpCas9 cleavage site (Fig 1). Next, 2×10^5 iPSC cells at passage 20 were co-transfected with SpCas9, the sgRNA, and the targeting vector by nucleofection.

Eight undifferentiated puromycin-resistant ganciclovir-insensitive iPSC colonies were manually selected, and genomic DNA isolated from these clones was used for screening of targeted integration events. We identified three successful knock-in clones by use of primer sets located outside the homology arm in combination with primers specific for the targeting construct at the 5' and 3' ends (Fig 2A). For each clone, the corrected sequence was confirmed. We verified the correct integration by Southern blot analysis, indicating that no random integration had occurred (Fig 2B). The sequencing results showed that the in-frame deletion in exon 2 was corrected in all three clones (Fig 2C). To assess the possibility of CRISPR/Cas9-mediated mutagenesis at off-target sites, we analyzed a total of ten potential off-target sites for sgRNA4 (Table SV). The potential off-target sites were predicted using CRISPR Design (http://crispr.mit.edu) and were PCR amplified. In comparison to uncorrected-iPS, sequence analysis showed that candidate loci were intact in the corrected clones. To excise the selection cassette flanked by two mutant loxP sites, we next transfected the knock-in clones with the Cre expression plasmid. The iPSC clones post-excision were identified as mKO-negative colonies that retained typical iPSC morphology (Fig 3A). The excision of the transgene

in these iPSC clones was confirmed by genomic PCR with primer sets used in the Surveyor assay, and the 724-bp PCR products indicating successful excision were obtained in all three clones (Fig 3B). The recombination of two mutant loxP sites into a double-mutant lox72 site was confirmed by sequencing (Fig 3C).

The expression of corrected F9 in HLCs differentiated from gene-corrected iPSC

To confirm the expression of corrected *F9*, gene-corrected iPSC clone was differentiated into HLCs (Fig S2A). The resultant cells induced via DE (Fig S2B) exhibited typical HLC morphology (Fig S2C), and most of these cells were positive for ALB, as demonstrated by immunofluorescence staining (Fig S2D). Compared with the levels in undifferentiated patient-derived iPSCs, the mRNA expression levels of liver-specific genes such as *AFP*, *ALB*, *FOXA2*, and *HNF4A* in iPSC-derived HLCs were approximately 35000-, 5900-, 4.7-, and 9.6-fold higher (Fig S3). We evaluated the expression of *F9* by qPCR in HLCs. Although *F9* mRNA could not be quantified in undifferentiated iPSC line 201B7, patient-derived uncorrected iPSCs, or corrected iPSCs, equivalent *F9* mRNA could be detected in iPSC line 201B7-derived,

uncorrected iPSC-derived, and corrected PSC-derived HLCs (Fig 4A). We also analyzed the cDNA sequence of corrected and uncorrected *F9* to confirm the integrity of transcription. The results showed that the transcription product contained only the corrected sequence, without contamination of the uncorrected sequence (Fig 4B). While exogenous lox72 site remained in intron 1 of gene-corrected *F9* genome, mRNA was firmly spliced between exon 1 and exon 2 (Fig 4B). The concentrations of FIX antigen in the culture supernatant of iPSC line 201B7-derived, uncorrected iPSC-derived, and corrected PSC-derived HLCs were below the detectable level (<0.63 ng/mL). The culture supernatant obtained after 2 days of culture with Vitamin K1 was concentrated 100-fold, but FIX antigen was not obtained in the concentrate. The FIX antigen was also not detected in the cell lysate of those iPSCs-derived HLCs.

Discussion

Promising results on gene therapy using AAV vector for HB have been reported [2, 3]. AAV vector is capable of exogenous gene transfer to non-proliferating cells such as hepatocytes, and long-term target gene expression is feasible. AAV vectors are predominantly maintained episomally; therefore, cell division will dilute the AAV genome, resulting in the loss of therapeutic expression[12]. Thus, in pediatric patients, because of high hepatocyte cellular turnover, therapeutic efficacy may not be durable. AAV vectors are immunogenic, and re-challenges of AAV vectors seem not to be feasible. AAV vectors are considered as nonintegrating vectors, and an unknown but small amount of the cassette integrates into the genomes, with a potential risk of genotoxicity at insertion site [13]. The recent advent of genome editing technologies such as the CRISPR/Cas9 system has enabled the site-specific precise modification of the genome to achieve a therapeutic effect. Unlike AAV vector, the CRISPR/Cas9 system can be applied to proliferating cells such as iPSCs without the loss of genetic modifications along with cell division. The technology has been expected as a new gene therapy method in regenerative medicine for blood

disorders, including hemophilia A [14]. An advantage of patient-derived iPSCs is that they are not immunogenic. The iPSCs come from a single patient and are sent back to the same patient after in-vitro genetic engineering and/or differentiation, avoiding the problem of immunologic rejection. In contrast to in-vivo gene editing, this approach allows for the selection and removal of unintended mutagenesis. Furthermore, hepatocytes from gene-edited iPSCs of an HB patient[10] and hepatic progenitor-like cells from gene-edited mouse embryonal stem cells[15] were reported to survive and secret FIX after being transplanted in mice via splenic injection. Thus, gene-edited iPSCs are promising therapeutic options for HB therapy, with potentials of greater durability and better safety profile compared with gene therapy using AAV vector for HB.

In our study, we assessed the applicability of this system to gene therapy in HB-derived iPSCs carrying an in-frame deletion in exon 2 of *F9* and performed a successful gene correction. Our findings support that the CRISPR/Cas9 system-based gene correction can be applied not only to point mutation, but also to an in-frame deletion mutation in *F9*.

In construction of the CRISPR/Cas9 system for gene correction of exon 2 of *F9*, we selected four candidate target sequences in intron 1 to avoid disruption of exon sequence. We designed each sgRNA and achieved efficient genome editing in human somatic cells at all target sites. We succeeded in performing a gene correction in iPSCs derived from a patient with HB through the precise knock-in of the targeting vector. The efficiency of successful integration events was consistent with previous reports [16, 17], in which targeted integration events reached 10-20 events per million in human iPSCs.

We confirmed the expression of corrected *F9* mRNA in HLCs differentiated from gene-corrected iPSC clone. In our differentiation protocol for HLCs using Cellartis DE differentiation kit, several transcriptional factors were transduced to enhance HLC differentiation [18, 19]. These approaches are based on the hypothesis that the induction of key transcription factors to mimic the *in vivo* hepatic development is required for full HLC specification. In accordance with previous reports, the expression of characteristic hepatocyte marker genes, such as *ALB*, increased in iPSC-derived HLCs. However, we could not detect the FIX antigen in the culture supernatants of iPSC-derived HLCs owing to low expression level of *F9* mRNA.

Recently, Ramaswamy et al. reported successful CRISPR/Cas9-based gene correction of disease-causing point mutation in HB patient-derived iPSCs genome, demonstrating FIX antigens in iPSC-derived HLCs and the culture supernatant, with incomplete differentiation of iPSC to hepatocytes. They used an in vitro hepatic differentiation protocol that closely recapitulates key developmental events during hepatogenesis [9]. Thus, our differentiation protocol for HLCs using Cellartis DE differentiation kit with transduced several transcriptional factors needs more improvement to obtain functional FIX.

Apart from the difference in culture conditions, several factors should be considered as reasons for the failure to detect the FIX antigen. First, the propensity of iPSCs for hepatic differentiation has been reported to depend on donor differences. Kajiwara et al. reported that those variations in hepatic differentiation from iPSCs were largely attributable to donor differences, rather than to the types of the original cells [20]. Unfortunately, for studying patient-derived iPSCs, as in our experiment, donor selection is not an option. Second, for hepatic differentiation from iPSCs, incubation with a mixture of human endothelial and mesenchymal stem cells seems to be necessary

to recapitulate what naturally occurs during early organ development. Takebe et al. also described the importance of cellular interactions during organogenesis and that of hemodynamic stimulation during maturation [21]. Our experiment tried to induce hepatic differentiation by forced expression of key liver-enriched transcription factors alone. Incubation of iPSCs with endothelial and mesenchymal stem cells seems to be worth investigation. Third, the epigenetic state may affect *F9* expression. In a study investigating hepatic differentiation in mouse ESCs, Cao et al. demonstrated that treatment with sodium butyrate (SB), bone morphogenetic proteins (BMPs), and FGFs in hepatic progenitor cells is critical for increasing the mRNA and protein levels of coagulation factors VIII and IX [22]. They speculated that treatment with SB, a histone deacetylase inhibitor, triggered epigenetic changes in ESCs, increasing cellular response to the bFGF- and BMP4-mediated induction of endoderm- and hepatocyte-specific gene expression. Epigenetic modification was not investigated in our experiment, and should be investigated in the future to achieve the induction of FIX secretion from iPSC-derived HLCs.

In summary, we demonstrated that the CRISPR/Cas9 system can be used to

repair an in-frame deletion mutation in *F9*, and succeeded in the hepatic differentiation of iPSCs. We could confirm the correctly spliced transcriptional product from corrected *F9*; however, further research should be undertaken to obtain completely functional hepatocytes from gene-corrected iPSCs as a potential cellular source for the treatment of HB. Despite several hurdles, our study provides a proof-of-principle for the gene therapy of HB using the CRISPR/Cas9 system.

Author contributions

All authors have fulfilled the conditions required for authorship. S. Morishige contributed to all experiments in the collection and interpretation of the data, and wrote the manuscript. S. Mizuno contributed to the study concept and the study design and supervised the research. H. Ozawa performed experiments and analyzed and interpreted the data. T. Nakamura performed experiments and analyzed data. T. Okamura, A. Mazahery, and K. Yamamura supervised experiments. K. Nagafuji critically revised the manuscript. All authors discussed the results and approved the final version of the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplemental materials and methods

Figure S1. Generation of iPSCs from peripheral blood mononuclear cells of a hemophilia B patient.

Figure S2. Differentiation of patient-derived iPSCs into HLCs.

Figure S3. Relative quantification of hepatocyte-related genes in iPSC-derived HLCs using real time-PCR.

1 **Table SI.** PCR programs

2 **Table SII.** Primer sets for PCR analysis.

3 **Table SIII.** Oligonucleotides for generating the sgRNA expression vector.

4 **Table SIV.** Primer sets and probe for qPCR analysis.

5 **Table SV.** Off-target candidates for sgRNA4.

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Figure legends

Fig 1. Schematic overview of the gene-targeting strategy for correcting the mutant allele.

After the CRISPR/Cas9-mediated knock-in of the targeting construct into the genomic locus, the successful knock-in clones were identified by PCR screening and Southern blot analysis. The CAG-mKO-2A-puro cassette flanked by two mutant loxP sites (lox66 and lox71) was removed by Cre/lox site-specific recombination, resulting in the replacement of the whole cassette by the lox72 sequence. Blue arrows denote the PCR primer sets for detecting the successful knock-in events, and the red bar indicates the specific DNA probe used for Southern blot analysis. DSB, double-strand break; HSV-TK, herpes simplex virus thymidine kinase gene.

Fig 2. Gene correction of the *F9* gene in patient-derived iPSCs.

(A) *Locus analysis for detecting the precise knock-in event at the corrected site.* To evaluate site-specific integration by HDR, genomic DNA extracted from eight puromycin-resistant clones were PCR amplified using primer sets flanking the homology arms. The expected sizes of the PCR products were 1697 (top) and 1345 bp

(bottom). NC indicates the negative control, using genomic DNA extracted from patient-derived iPSCs as the PCR template. StyI-digested lambda DNA was used as a molecular marker (lane M).

(B) *Southern blot analysis of knock-in clones.*

*Eco*RI-HF-digested genomic DNA derived from the PCR-positive clones was electrophoresed in 1% agarose gel and transferred to a Hybond N+ nylon membrane, followed by hybridization with the selection cassette-specific DNA probe. The size of expected DNA fragments was 2209 bp (black arrowheads). StyI-digested lambda DNA was used as a molecular marker (lane M). The black line indicates the boundary between the agarose gel and a chemiluminogram. DNA derived from uncorrected iPSC was used as the negative control (NC). Brightness and contrast were adjusted.

(C) *Sequence analysis of PCR products.*

PCR products amplified using primer sets flanking the homology arms were subjected to sequencing analysis to confirm the correction of the disease-causing mutation. Corrected sequence is marked with red box.

Fig 3. Excision of the selectable marker gene.

(A) *Cre-mediated excision of the loxP-flanked selection marker from a successfully targeted iPSC clone.*

Phase-contrast and fluorescence (mKO) images of an iPSC colony before (a, c) and after (b, d) Cre-mediated excision of the CAG-mKO-2A-puro cassette (200× magnification).

(B) *Successful removal of the selection cassette.*

The successful Cre-mediated excision was verified by PCR analysis using primer sets used in the Surveyor assay. The expected size of the PCR products was 724 bp. A 100-bp DNA ladder was used as a molecular marker (lane M).

(C) *Recombination of two mutant loxP sites.*

The replacement of the whole cassette by the lox72 sequence was confirmed by sequencing of the 724-bp PCR products. The lox72 sequence is marked with red box.

Fig 4. Quantitative real-time PCR for *F9* mRNA and cDNA sequence analysis.

A) *Absolute quantification of F9 mRNA in iPSC-derived HLCs using real time-PCR.*

The copy number of *F9* mRNA was quantified using a standard curve. Data represent the averages of three independent experiments ± SD. GAPDH was used as internal control. *F9* mRNA could not be quantified in undifferentiated iPSC line 201B7, patient-derived uncorrected iPSCs or corrected iPSCs, equivalent *F9* mRNA could be detected in iPSC line 201B7-derived, uncorrected iPSC-derived, and corrected PSC-derived HLCs.

B) *Sequence analysis of cDNA encoding F9.*

PCR-amplified *F9* cDNA was subjected to sequencing analysis to confirm the correction of the transcription product. Deletion site before gene correction is indicated by red arrowhead (top). Corrected sequence is marked with red box (middle) and predicted exon junction in corrected clone is depicted by red bar (bottom).

Fig. 1

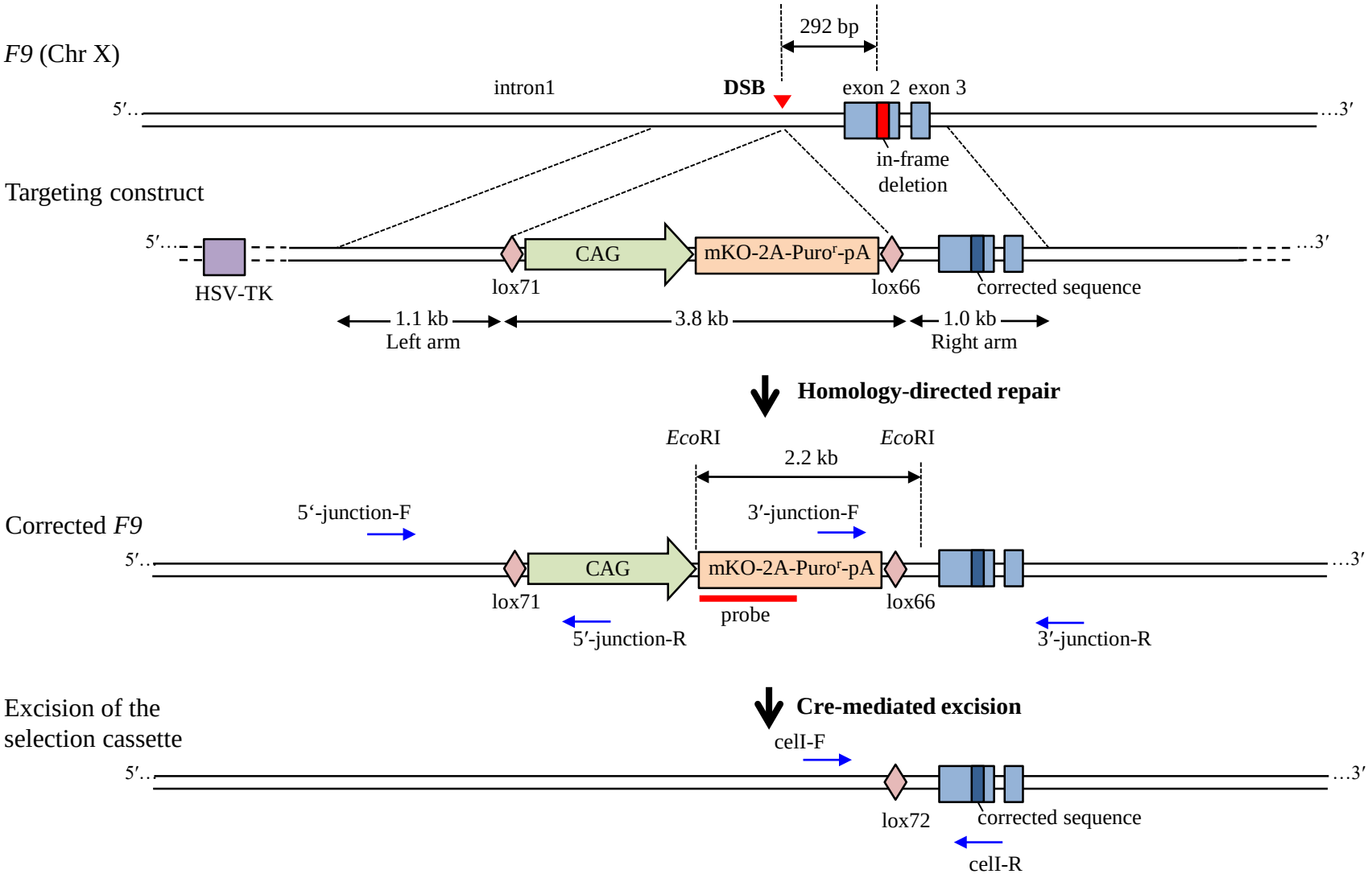

F9 (Chr X)
292 bp
intron1
DSB
exon 2
exon 3
5′…
…3′
in-frame deletion
Targeting construct
CAG
mKO-2A-Puro^r-pA
HSV-TK
lox71
lox66
corrected sequence
1.1 kb
Left arm
3.8 kb
1.0 kb
Right arm
Homology-directed repair
EcoRI
EcoRI
2.2 kb
Corrected F9
5‘-junction-F
3′-junction-F
probe
5′-junction-R
3′-junction-R
Excision of the selection cassette
Cre-mediated excision
celI-F
lox72
celI-R

Fig. 2

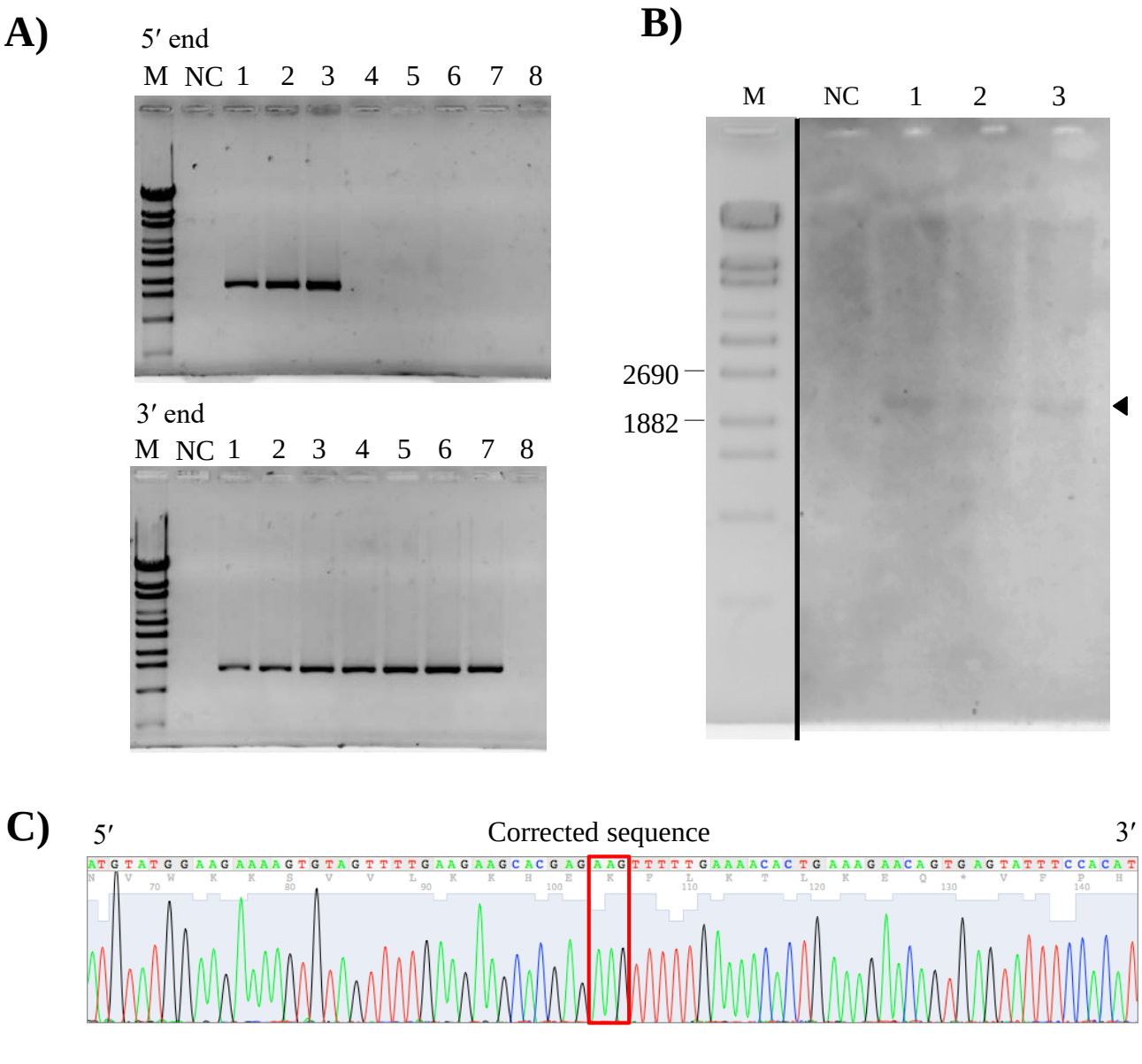

A)
5′ end
M NC 1 2 3 4 5 6 7 8
3′ end
M NC 1 2 3 4 5 6 7 8
B)
M NC 1 2 3
2690
1882
C)
5′
Corrected sequence
3′

Fig. 3

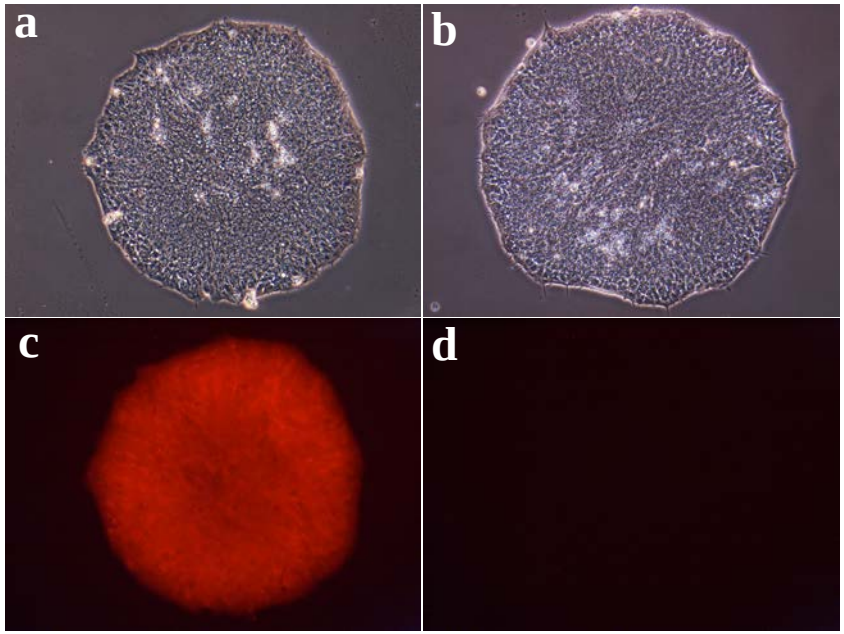
A)

iPSC clone before Cre-mediated excision

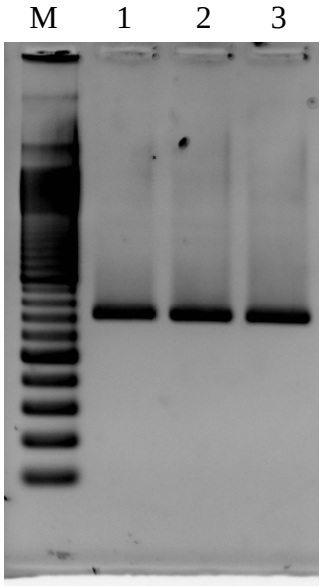
iPSC clone after Cre-mediated excision

Phase

mKO



B)



C)

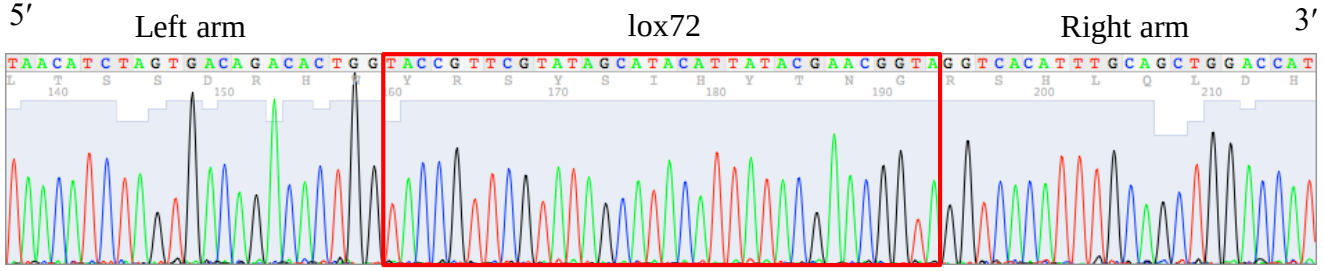
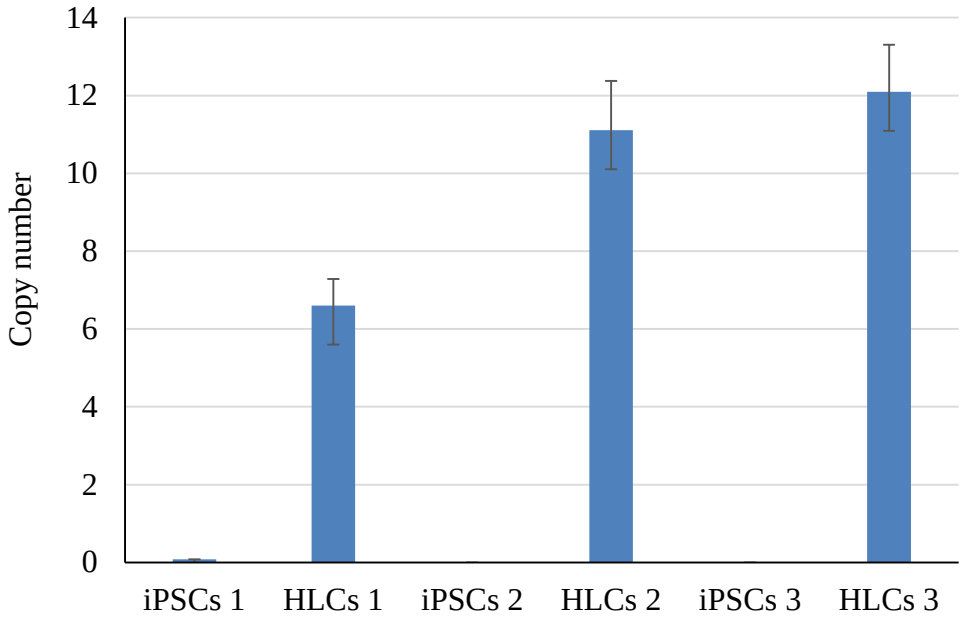


Fig. 4

A)



iPSCs 1: 201B7 iPSCs
HLCs 1: HLCs-derived from 201B7 iPSCs
iPSCs 2: Uncorrected iPSCs
HLCs 2: HLCs-derived from uncorrected iPSCs
iPSCs 3: Corrected iPSCs
HLCs 3: HLCs-derived from corrected iPSCs

B)