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ORIGINAL ARTICLE

Genetic Correction and Hepatic Differentiation of Hemophilia B-specific Human Induced Pluripotent Stem Cells[△]

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Objective To genetically correct a disease-causing point mutation in human induced pluripotent stem cells (iPSCs) derived from a hemophilia B patient.

Methods First, the disease-causing mutation was detected by sequencing the encoding area of human coagulation factor IX (*F IX*) gene. Genomic DNA was extracted from the iPSCs, and the primers were designed to amplify the eight exons of *F IX*. Next, the point mutation in those iPSCs was genetically corrected using CRISPR/Cas9 technology in the presence of a 129-nucleotide homologous repair template that contained two synonymous mutations. Then, top 8 potential off-target sites were subsequently analyzed using Sanger sequencing. Finally, the corrected clones were differentiated into hepatocyte-like cells, and the secretion of F IX was validated by immunocytochemistry and ELISA assay.

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Results The cell line bore a missense mutation in the 6th coding exon (c.676 C>T) of *F IX* gene. Correction of the point mutation was achieved *via* CRISPR/Cas9 technology *in situ* with a high efficacy at about 22% (10/45) and no off-target effects detected in the corrected iPSC clones. F IX secretion, which was further visualized by immunocytochemistry and quantified by ELISA *in vitro*, reached about 6 ng/ml on day 21 of differentiation procedure.

Conclusions Mutations in human disease-specific iPSCs could be precisely corrected by CRISPR/Cas9 technology, and corrected cells still maintained hepatic differentiation capability. Our findings might throw a light on iPSC-based personalized therapies in the clinical application, especially for hemophilia B.

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HEMOPHILIA B (HB) is an X chromosome recessive hereditary bleeding disorder, accounting for about 15% of the overall hemophilia cases.¹ It is caused by point mutations, frameshift insertions or deletions in coagulation factor IX gene (*F IX*), which phenotypically induce deficiency or malfunction of F IX, a core protein in the blood coagulation system, secreted by hepatocytes.²⁻⁴

Induced pluripotent stem cells (iPSCs) were firstly acquired from mouse embryonic or adult fibroblasts by retroviral transduction or plasmid transfection with Oct3/4, Sox2, c-Myc, and Klf4.⁵ iPSCs, which can be differentiated into three germ layers,⁶ may lead to important drug discoveries and advances in regenerative medicine.⁷ It is reported that iPSCs can be differentiated into hepatocytes,⁸ which express hepatocyte markers, including alpha-fetoprotein (AFP) and albumin (ALB), and display specific hepatic functions including ALB secretion, glycogen synthesis, and urea production.⁹ Disease specific iPSCs also hold a great potential for precisely personalized medicine.

At present, several genome editing techniques are well established, including meganucleases,¹⁰ zinc finger nucleases (ZFNs),¹¹ transcriptional activation-like effector nucleases (TALENs)¹² and Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9),¹³ *et al.* CRISPR/Cas9 system induces double-strand break (DSB) by wild type Cas9 protein at specific gene locus recognized by guide RNA. Because of its ease and simplicity, CRISPR/Cas9 now is a star technique that is transforming biological research.

In this study, to explore the potential application of CRISPR/Cas9 in HB for personalized cell-based therapies, we aimed to correct the genetic mutation in human HB specific iPSCs by CRISPR/Cas9 *via* homologous recombination. Then we analyzed the potential off-targets to assess the specificity of CRISPR technology. Furthermore, we differentiated the corrected iPSCs clones to hepatocyte-like cells and validated their F IX secreting ability.

MATERIALS AND METHODS

Cell culture

Human HB-specific iPSC line was a gift from Tao Cheng's Lab at the Institute of Hematology and Blood Diseases Hospital (Tianjin, China), which was generated from adult blood mononuclear cells derived from a HB patient by transient plasmid expression, under feeder-free and xeno-free conditions. The cells were maintained on matrigel (BD Biosciences, MA, USA) in mTeSR1 medium (STEMCELL Technologies, Vancouver, Canada), and passaged with ReleSR (STEMCELL Technologies).

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, USA).

Disease-causing point mutation screening

Genomic DNA was isolated and purified from human HB-specific iPSCs. Eight exons were amplified separately by PCR. PCR reactions were performed in the following conditions with PrimeSTAR Max DNA Polymerase (Takara, Dalian, China): 3 minutes of hot-start at 94 °C, followed by 30-35 cycles of 98 °C (10 seconds), 55 °C (5 seconds), 72 °C (10 seconds). The primers corresponding to exon 1 to 8 were designed and synthesized as shown in Table 1. PCR products were further sequenced by Sangon (Shanghai, China).

Plasmid construction and validation

The Cas9 and sgRNA plasmid PX459 was a gift from Yupo Ma's Lab at the State University of New York at Stony Brook. The sgRNAs targeting the disease-causing mutation region were designed, synthesized, annealed and ligated to PX459 following the protocol provided by Feng Zhang's Lab.¹⁴

Transfection

The iPSCs were fed with fresh mTeSR1 and 10 μmol/L

ROCK inhibitor (Sigma, St. Louis, MO, USA) for two hours before electroporation. To initiate electroporation, iPSCs were washed with phosphate-buffered saline (PBS) and further dissociated into single cells with Accutase (STEMCELL Technologies) at 37 °C for 5-8 minutes. CRISPR-sgRNA (5 µg) and homologous ssODN (5 µg) were transfected into 0.8×10^6 iPSCs using Amaxa Nucleofector II (Lonza, Basel, Switzerland) set at Program B016. Cells were reseeded into two wells of a 24-well plate and cultured in conditioned mTeSR1 with 10 µmol/L ROCK inhibitor.

Clonal screening and DNA sequencing

Puromycin (Amresco, Solon, OH, USA), at the concentration of 0.15 µmol/L, is used for selection, started at 24

hours after electroporation and incubated with iPSCs for 48 hours. Colonies were picked after additional 7 days culture, with regular daily medium change. Genomic DNA was extracted from each colony. Exon 6 was then PCR amplified and sequenced to determine if the site mutation has been corrected.

Off-target analysis

Off-target analysis was performed using an online sgRNA design tool to select potential off-target sites.¹⁵ Primers of top 8 theoretical off-target sites were designed and synthesized (Table 2). These sites were further PCR amplified and analyzed for insertion-deletions (indels) by sequencing.

Table 1. Primers for disease-causing point mutation screening

Exons	Primer sequences	Product length (bp)
Exon 1	Forward: CCACTGCCCCATTCTCTCA Reverse: TACTTACCAACCTGCGTGCT	393
Exon 2+3	Forward: CATGCCCTAAGAGAAATTGG Reverse: TGGGTTAGAGGGTTGGA CTG	639
Exon 4	Forward: TCAGGAAGACAGGAGCATCA Reverse: GAGGGAACTTTGAACCATGAG	322
Exon 5	Forward: ACCCATACATGAGTCAGTAGTTCC Reverse: GACACAGAAAGAATTGAGTTGTAG	427
Exon 6	Forward: AATACTGATGGGCCTGCTTC Reverse: ACCTGGCCTGTGTCTTGC	383
Exon 7	Forward: GCCTATTCCTGTAACCAGCAC Reverse: GACCCTTCTGCCTTTAGCC	324
Exon 8	Forward: CAATTAGGTCAGTGGTCCCAAG Reverse: GGGAAAGTGATTAGTTAGTGAGAGG	713

Table 2. Primers of the top 8 theoretical off-target sites

No.	Off-target site	Primers
OT 1	GACAGCAGTTTGGTTGTTGG GAG	Forward: gtaattcccactcctgaagaga Reverse: tggttaaaaactctcaggcagc
OT 2	GCCTCCTCTTGGGTTGTTTG GAG	Forward: ttctttagatcattttatataaaacagtgc Reverse: aatcacccattcagggtctaa
OT 3	GGCTCCCATGAGTTGTTGG CAG	Forward: tcctctaccagtgaggctac Reverse: agagtccaggctgaaccac
OT 4	CACTGCACTGGGGTTCTTGG GGG	Forward: cggacccagactcttctta Reverse: aagcttcagaggacgggtg
OT 5	GAGTGCACTGGGTGGCTGG AGG	Forward: acccagtcctcacttggtta Reverse: acagccttcagtgcagtcct
OT 6	AGCTTCATTTGGGTTGTTGG AAG	Forward: actcacctaacaagaagcttttcaa Reverse: tcctgcctccctgtcttta
OT 7	AATTTCTCTTGGGTTGTTGG GAG	Forward: tcgtatttttgtaaggctccta Reverse: aacactaatccatgagatctgaaaa
OT 8	GGCTGCACTGGGTTGTTTG AGG	Forward: tgctactgacaacaagagca Reverse: aaggacagggtgaaagagg

Hepatic differentiation

The hepatic differentiation was carried out as described by Si-Tayeb *et al*¹⁶ with minor modifications. Briefly, iPSCs were dissociated into single cells and plated onto matrigel in mTeSR1 at a high density of $2 \times 10^5/\text{cm}^2$ with 10 $\mu\text{mol/L}$ ROCK inhibitor for 16-24 hours before hepatic induction. To initiate *in vitro* differentiation, RPMI1640/B27 (Gibco, Grand Island, NY, USA) supplemented with Activin A (60 ng/ml, PEPROTECH, Rocky Hill, NJ, USA) and CHIR99021 (2 mmol/L, StemMACS, Auburn, CA, USA, only added at the first day) was used instead of mTeSR1 for 3-5 days with daily medium change. Then, cells were cultured in RPMI1640/B27 supplemented with bone morphogenetic protein 4 (BMP4, 20 ng/ml, PEPROTECH) and fibroblast growth factor 2 (FGF2, 10 ng/ml, PEPROTECH) for 5 days, followed by treatment with hepatocyte growth factor (HGF, 10 ng/ml, PEPROTECH) for another 5 days. Medium was refreshed every day. Finally, hepatocyte culture medium (HCM, Lonza, Walkersville, MD, USA), omitting epidermal growth factor (EGF), supplemented with oncostatin M (OSM, 20 ng/ml, PEPROTECH) was used for hepatic maturation during the last 5 days with medium change every other day.

Immunocytochemistry

Attached cells were fixed in cold 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.2) overnight, then washed three times with PBS (10 minutes for each time) and incubated with primary antibodies (Table 3) for 1.5 hours at room

temperature or overnight at 4°C. Primary antibodies were diluted in PBS/0.02% Na₃/3% bovine serum albumin (BSA)/0.2% Triton X-100. After washed with PBS for three times, 10 minutes each, the cells were incubated with secondary antibodies (Table 3) for 1 hour at room temperature. Cell nuclei were counterstained with Hoechst 33258 for 10 minutes at room temperature. Fluorescence signals were observed and captured using Olympus DP73 (DP73-51, Olympus, Tokyo, Japan).

ELISA

Cell culture medium was collected every other day since the stage of hepatic maturation, and centrifuged at 3000 $\times g$ for 10 minutes at 4°C to remove debris. Supernatants were further concentrated, and F IX was quantified by ELISA using human factor IX ELISA kit (Langdun, Shanghai, China). The samples could be stored at -20°C or below for up to 3 months.

RESULTS

Genetic mutation screening of human HB patient derived iPSCs

To identify the disease-causing mutation of the iPSC line, we amplified eight exons and performed Sanger sequencing. The sequencing results revealed a missense mutation in the 6th coding exon (c.676 C>T), which causes an amino acid mutation (p.Arg226Trp), and inhibits the activation of F IX protein.

Table 3. Information about primary and secondary antibodies

Antigen	Distributor	Host	Cat#	Dilution
OCT3/4	Santa Cruz	Rabbit	sc-9081	1:500
SOX17	ABCAM	Mouse	Ab84990	1:50-100
AFP	ABCAM	Rabbit	ab169552	1:100-250
ALB	R&D System	Mouse	MAB1455	8-25 $\mu\text{g/ml}$
F IX (F9)	Life science products & services	Rabbit	AB21319a	1:25-100
Fluorescein (FITC)-conjugated AffiniPure F(ab') ₂ Fragment Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch Laboratories	Donkey	715-096-150	1:50-200
Cy TM 3-conjugated AffiniPure F(ab') ₂ Fragment Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch Laboratories	Donkey	711-166-152	1:100-800

SOX17: sex determining region Y box 17; AFP: alpha-fetoprotein; ALB: albumin; FIX: coagulation factor IX.

CRISPR-sgRNA plasmid construction and homologous repair template synthesis

Based on the sequencing results, we selected four sgRNAs with theoretical minimal off-targets and distance between the cut site and the correction site using an online CRISPR design tool (Fig. 1A, 1B). Then, the four sgRNAs were cloned into PX459 plasmid with puromycin resistance (a commercial CRISPR backbone plasmid, available through Addgene) for co-expression of Cas9 and guide RNAs.

To validate the cleavage activity of the constructed CRISPR-sgRNAs, we transfected the four plasmids into HEK293T cells and detected indels mutations around the targeted loci. The mismatch based analysis showed that all four CRISPR-sgRNAs had the ability to recognize and cut the targeted sequence, and CRISPR-sgRNA2 exhibited the highest cleavage efficiency among the four (Fig. 1C).

Next, we designed a sense ssODN centered on the mutation site with homology arms of 64 nt on either side of the repair template. To avoid degrading by Cas9 after

homologous recombination, we introduced two silent mutations close to 5' end of protospacer adjacent motif (PAM) sequence in ssODN (Fig. 1D, 1E, 1F).

Genetic correction of human HB specific iPSCs

To precisely correct the disease-causing point mutation by homologous recombination, we transfected the iPSCs with CRISPR-sgRNA2 and homologous ssODN by nucleofection. Next, we enriched iPSCs that have been transfected successfully with puromycin selection for 48 hours. After about 7-day expansion, we picked drug resistant clones manually, cultured separately, and screened for mutation correction by Sanger sequencing (Fig. 2A). We finally obtained 38 clones with indels modifications at target loci out of 45 sequenced clones, with a cleavage frequency of about 84% (38/45). And 10 out of the 38 clones were identified as genetically corrected, yielding correction efficiency of 22% (10/45) (Fig. 2B). Among the 10 corrected iPSC clones, 8 were pure single cell derived clones, while Clone 4 and Clone 52 needed further purifications (Fig. 3).

Figure 1. Construction of CRISPR-sgRNA plasmids and design of the 129-nt homologous repair template.

A. Sequences of the 4 selected sgRNAs. B. Characteristics of the 4 selected sgRNAs. C. Cleavage activity validation of the 4 CRISPR-sgRNAs. D. Translation of sequences round the targeted locus. E. Codons of some interested amino acids. F. Sequence information of the homologous template.

iPSCs: induced pluripotent stem cells.

Basepairs highlighted in red indicate point mutation site or correction site; basepairs highlighted in green represent synonymous mutations; basepairs highlighted in blue indicate protospacer adjacent motif region.

For DSB in mammalian DNA, indel-forming non-homologous end joining (NHEJ) is a more frequent pathway. The 38 clones we got showed a wide range of different indels types in response to DSB introduced by CRISPR/sgrNA2. Specifically, we detected deletions, insertions and non-specific mutations in the NHEJ repair pathway. Insertions occurred here were all one-base insertions. Though deletions varied significantly in size, they are all small, no larger than 25 bps. Non-specific mutations usually occurred in company with deletions and insertions. Our results were limited by the number of cases being analyzed. More samples should be further tested to reach a general conclusion about indels distribution (Fig. 2C).

Results of off-target analysis

To address the off-target burden incurred by CRISPR/Cas9, we selected 8 genomic sites with the highest theoretical off-target potential. We amplified these regions from the original and the corrected clones, and performed direct sequencing. No indels or mutations were detected on these sites. Although off-target mutagenesis induced by CRISPR/Cas9 could not be avoided, we tried to hold the off-target effects minimal.

Hepatic differentiation of genetically corrected human iPSCs

The corrected iPSCs, cultured under feeder-free condition,

had compact colony morphologies with a high ratio of nucleus to cytoplasm and prominent nucleoli. Also they were positive for embryonic stem cell marker OCT-4 (Fig. 4A, 4B, 4C).

For derivation of hepatocytes, iPSCs were dissociated into single cells and cultured on matrigel in RPMI1640/B27 medium supplemented with Activin A. After 5 days of culture, cells expressed the definitive endoderm marker, sex determining region Y box 17 (SOX17) (Fig. 4D). For hepatic specification and proliferation, cells were cultured in RPMI1640/B27 supplemented with BMP4 and FGF2 for 5 days, followed by HGF treatment for another 5 days. At the end of this stage, cells were found to express high levels of AFP (Fig. 4E), indicating that the specified cells have committed to hepatoblast fate. Finally, the medium was replaced with HCM supplemented with OSM for additional 5 days to induce maturation. Mature hepatocyte-like cells could be recognized with the expression of ALB (Fig. 4F).

Factor IX expression in hepatic differentiated cells

During the maturation stage of differentiation, cells were found to express F IX, which could be identified by immunocytochemistry (Fig. 5A). ELISA assay was used to calculate the amount of F IX expressed in the medium. The ELISA results demonstrated that the concentration of F IX was increased along with the differentiation process, reaching about 6 ng/ml on day 21 (Fig. 5B).

Figure 2. Genetic correction of human hemophilia B specific iPSCs.

A. a schematic flowchart of correction procedure; B. summary of correction results; C. indel analysis from CRISPR cleavage and non homologous end joining.

Figure 3. Sequencing chromatograph.

A. Sequencing chromatograph of the patient derived iPSC clone. The arrow showed the missense point mutation. B. Sequencing chromatograph of the ten genetically corrected clones. Clone 4 and Clone 52 had two peaks at the mutation point, indicating these two clones were not pure.

DISCUSSION

CRISPR/Cas9 is now transforming the field of biology research because of its simplicity and efficiency in genome engineering. Cas9 would introduce DSB at the specific locus guided by guide RNA, which would trigger cellular endogenous repair machinery. When a homologous template exists, homologous recombination that repairs DSB precisely may occur.¹⁷⁻²⁰ Some recent studies showed that

CRISPR system could be injected into mice models and perform *in vivo* genome editing.²¹⁻²⁴ Guan *et al*²⁵ introduced a plasmid encoding either ssODN or a long donor DNA, along with Cas9 and the sgRNA, into mice of HB by hydrodynamic tail injection, and corrected up to 0.56% and 1.5% of hepatocytes *in vivo*, respectively. The main hurdles of *in vivo* CRISPR/Cas9 based gene therapy are delivery, low efficiency, and safety concerns caused by off-target effects.

Figure 4. Deviation of hepatocyte-like cells from genetically corrected iPSCs.

A, B, C. Compact colony morphologies, with a high ratio of nucleus to cytoplasm and prominent nucleoli, were observed. Undifferentiated iPSCs were identified by immunocytochemistry using antibody that recognized OCT3/4. D, E, F. Characteristics of hepatic differentiation procedure *in vitro*. Expression of SOX17, AFP and ALB were detected on day 5, day 15 and day 20, respectively. Scale bar = 50 μm.

Figure 5. Factor IX expressed in hepatic differentiated cells.

A. Coagulation factor IX expressed in hepatic cells derived from iPSCs. F IX was located in cytoplasm and further secreted to culture medium. Scale bar = 50 μm. B. Quantification of FIX released in culture medium by ELISA assay. *indicates $P \leq 0.01$.

Genome editing *ex vivo* achieved by CRISPR/Cas9 system on somatic stem or progenitor cells in culture is also commonly used, which enable to select and analyze corrected cells, especially for genetic disease.²⁶⁻²⁹ Here we demonstrated the correction of a missense point mutation

in human *F IX* gene *in vitro* using CRISPR/Cas9 system at a high efficiency (about 22%), while Xie *et al*²⁶ got a homologous efficiency of 23.5% using CRISPR/Cas9 and PiggyBac plasmid to correct beta-thalassemia specific iPSCs. It was reported that CRISPR/Cas9 would incur heavy

off-target effect.³⁰⁻³² In our study, however, no obvious evidence of off-target was found so far. Corrected clones in the study had not lost their pluripotency after the correction procedure, and maintained hepatic differentiation ability and F IX expression, which enable further clinical research and application. This is the first report of *in situ* genetic correction of human HB derived iPSCs. Our study may introduce a new strategy to treat HB by combining genetic correction and personalized iPSC-based cell therapy, complementing to currently used treatments, such as exogenous F IX infusion, liver transplantations,³³⁻³⁵ and gene therapies.³⁶⁻³⁷

Nevertheless, there is still a long way to clinical application. First, deep sequencing should be conducted in the future to investigate whether CRISPR/Cas9 system would incur genome-wide off-target and genomic instability. Second, studies are needed to further evaluate whether the secreted F IX could be subjected to proper post-modification, and whether genetic correction restores coagulation function. Also, improving F IX expression and looking for a proper mice model are two key steps for next research. In particular, the efficiency of orthotopic transplantation is another challenge to bring this into the clinics.

Conflict of Interest Statement

The authors have no conflict of interest to disclose.

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