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Generation of a gene edited hemophilia A patient-derived iPSC cell line, YCMi001-B-1, by targeted insertion of coagulation factor *FVIII* using CRISPR/Cas9

Jin Jea Sung[a,b,c,1], Sanghyun Park[a,c,1], Sang-Hwi Choi[a,c], Jongwan Kim[d], Myung Soo Cho[d], Dong-Wook Kim[a,b,c,*]

[a] Department of Physiology, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, South Korea
[b] Severance Biomedical Research Institute, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, South Korea
[c] Brain Korea 21 PLUS Program for Medical Science, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, South Korea
[d] S.Biomedics Co., Ltd, 28 Seongsui-ro, 26-gil, Seongdong-gu, Seoul 04797, South Korea

A B S T R A C T

Hemophilia A is an ideal target for cell or gene therapy because a mild increase in coagulation factor VIII (FVIII) improves symptoms in patients with severe hemophilia A. In this study, we used CRISPR/Cas9 to insert *FVIII* cDNA into exon 1 of the mutant *FVIII* locus in induced pluripotent stem cells (iPSCs) from a hemophilia A patient. This gene-modified YCMi001-B-1 line maintained its pluripotency, formed all three germ layers, and had a normal karyotype. In addition, *FVIII* expression was confirmed in YCMi001-B-1-derived endothelial cells.

1. Resources table

Unique stem cell line identifier	YCMi001-B-1
Alternative name(s) of stem cell line	FVIII-KI
Institution	Yonsei University, College of Medicine, Seoul, Korea
Contact information of distributor	Dong-Wook Kim, dwkim2@yuhs.ac
Type of cell line	iPSC
Origin	Human
Additional origin info	*Age: 44* *Sex: Male* *Ethnicity if known: Asian (Korean)*
Cell Source	Del-iPSC-Epi6 (Park et al., 2019)
Clonality	Clonal
Method of reprogramming	N/A
Genetic Modification	Yes
Type of Modification	Transgene knock-in
Associated disease	Hemophilia A
Gene/locus	FVIII/Xq28.1
Method of modification	CRISPR/Cas9
Name of transgene or resistance	BDD FVIII-bGH-loxP
Inducible/constitutive system	N/A
Date archived/stock date	2017/3
Cell line repository/bank	https://hpscreg.eu/cell-line/YCMi001-B-1
Ethical approval	Ethical committee: Yonsei university health system, Severance Hospital, Institutional review board approval number: #4-2012-0028

2. Resource utility

The YCMi001-B-1 line was generated by target-specific knock-in of the B-domain deleted form of *FVIII* cDNA into the *FVIII* locus of iPSCs from a hemophilia A patient. The YCMi001-B-1 line can be used for autologous cell therapy in Hemophilia A patients.

3. Resource details

Hemophilia A is caused by mutations in FVIII that inhibit coagulation (Mannucci and Tuddenham, 2001). Previously, we generated an induced pluripotent stem cell (iPSC) line, Del-iPSC-Epi6, using adipose tissue-derived mesenchymal stem cells obtained from a hemophilia A patient with a gross deletion of exons 8–22 in *FVIII* locus (Park et al., 2019). In this study, we inserted the B-domain deleted form of *FVIII* (BDD-FVIII) cDNA into exon 1 of *FVIII* locus in the Del-iPSC-Epi6 line using CRISRP/Cas9 to restore *FVIII* expression. BDD-*FVIII* cDNA does not contain a part of Exon 14 (~ 2.7 kb), and thus has a relatively small size (~ 4.3 kb) which makes it easier for gene delivery and an enhanced expression capability compared to the full-length *FVIII* cDNA (~ 7 kb),

* Corresponding author at: Department of Physiology, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, South Korea.
E-mail address: dwkim2@yuhs.ac (D.-W. Kim).
[1] These authors contributed equally to this work.

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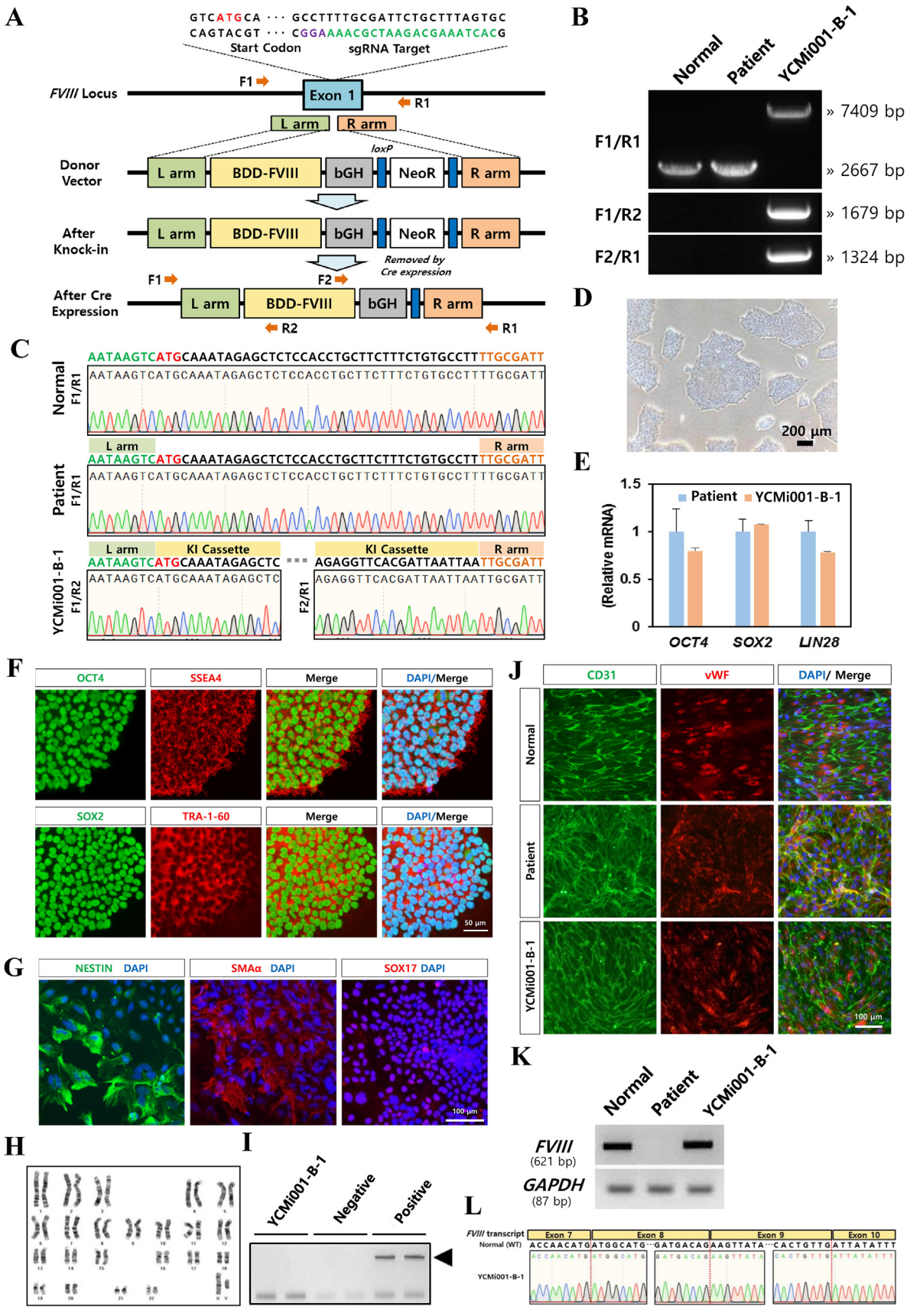

Fig. 1. Characterization of YCMi001-B-1 iPSC line.

without affecting coagulation activity of FVIII (Mannucci and Tuddenham, 2001). Thus, BDD-*FVIII* is widely used in gene therapy for hemophilia A.

A schematic representation of the guide RNA target site and the knock-in strategy to integrate the donor plasmid into the *FVIII* locus is shown (Fig. 1A). In a previous study, we evaluated the cutting efficiency of Cas9 using a single guide RNA (sgRNA) designed to cleave exon 1 of the *FVIII* locus (Fig. 1A) (Sung et al., 2019). pcDNA4/BDD-*FVIII* (Addgene #40135) was used to construct the *FVIII* cDNA donor plasmid. The human cytomegalovirus immediate early enhancer and the promoter of pcDNA4/BDD-*FVIII* were substituted with the 5′ left homology arm (L arm) and a neomycin resistance cassette (NeoR) flanked by two *loxP* sequences ligated to the 3′ end of the bovine growth hormone (bGH) polyadenylation signal. The 3′ right homology arm (R arm) was attached to the 3′ end of NeoR (Fig. 1A).

After electroporation with Cas9, sgRNA, and the *FVIII* cDNA donor plasmids and following G418 selection, NeoR in the gene-corrected iPSC lines was removed by Cre recombinase expression. To determine if the correct gene was targeted, cells were screened by PCR amplification for the Cas9 target site (F1/R1) and the 5′ (F1/R2) and 3′ (F2/R1) knock-in junctions (Fig. 1B). PCR analysis using a F1/R1 primer set confirmed that the YCMi001-B-1 clone demonstrated hemizygous insertion of the donor vector at *FVIII* locus (Fig. 1B, upper panel) and following sanger sequencing of the PCR amplicons revealed that the donor plasmid incorporated correctly into exon 1 of the *FVIII* locus (Fig. 1C).

Investigation of cells in bright field showed that the gene-modified YCMi001-B-1 line maintained typical iPSC morphology (Fig. 1D). Real-time PCR analysis showed that expression of pluripotent markers (*OCT4, SOX2, LIN28*) was similar in YCMi001-B-1 cells and in parental iPSCs from the patient (Fig. 1E). Immunocytochemistry of YCMi001-B-1 cells and colonies also confirmed uniform expression of pluripotent markers (OCT4, SOX2, SSEA4, TRA-1-60) (Fig. 1F). The *in vitro* 3-germ layer assay (ectoderm; NESTIN, mesoderm; α-SMA, endoderm; SOX17) showed that the YCMi001-B-1 line differentiated normally (Fig. 1G). Additionally, YCMi001-B-1 exhibited a normal 46, XY karyotype with no detectable abnormalities and no trace of mycoplasma contamination (Fig. 1H, I). Short tandem repeat (STR) analysis also showed that the YCMi001-B-1 iPSCs and parental iPSCs were genetically identical (Table 1).

Endothelial differentiation ability and knocked-in FVIII expression was evaluated in the YCMi001-B-1 line as previously described (Harding et al., 2017; Sung et al., 2019). Immunocytochemistry showed that the YCMi001-B-1 line could differentiate into CD31- and vWF-positive endothelial cells similar to the normal iPSC and the parental iPSC line (Fig. 1J). Furthermore, PCR amplicons corresponding to exons 7–10 of the *FVIII* transcript (621 bp) were detected in endothelial cells from the normal iPSCs and YCMi001-B-1, but were not detected in the derivative from the patient iPSCs (Fig. 1K, upper panel). However, the PCR products targeted to *GAPDH* (87 bp) as a control were detected in derivatives from all three cell lines (Fig. 1K, bottom panel). Sanger sequencing also revealed that *FVIII* transcripts in YCMi001-B-1 derived endothelial cells had normal exon 7–10 sequences of *FVIII* cDNA (Fig. 1L). These results indicated that YCMi001-B-1 restored *FVIII* mRNA expression via gene correction.

4. Materials and methods

4.1. Cell cultures

iPSCs derived from a male healthy (normal) donor and a male Hemophilia A patient with a gross deletion (Park et al., 2019) and the YCMi001-B-1 line were cultured in STEMMACS™ iPSC-brew FX (STEMMACS medium; Miltenyi Biotec) in Matrigel (Corning)-coated dishes.

4.2. Generation of gene-corrected patient-derived iPSCs

Cas9 and sgRNA were purchased from ToolGen, Inc., Korea. For electroporation, Y-27632-pretreated patient-derived iPSCs were dissociated into single cells using TrypLE™ Select. Two micrograms of Cas9, 2 μg sgRNA expression vector, and 4 μg donor plasmid were introduced into 5×10^5 iPSC cells using a Neon^R electroporator (Invitrogen). The cells were reseeded in a Matrigel-coated dish and cultured in STEMMACS medium supplemented with G418 for 2 d. Then, 2 μg pCAG-Cre:GFP (Addgene #13776) was electroporated into 5×10^5 surviving iPSCs to remove NeoR. Genomic DNAs from iPSCs were purified using the DNeasy Blood & Tissue Kit (QIAGEN) and PCR amplification was performed using EmeraldAmp® GT PCR Master Mix (TAKARA BIO Inc.) and primers listed in Table 2.

4.3. RNA extraction, cDNA synthesis, and real-time RT-PCR

Total RNA from cells was extracted using the Easy-Spin® Total RNA Extraction kit (iNtRON Biotechnology) according to the manufacturer's instructions. Total RNA was converted to cDNA using PrimeScript™ RT Master Mix (TAKARA BIO Inc.). Transcript levels were evaluated via real-time RT-PCR (RT-qPCR) using SYBR® Premix ExTaq™ (TAKARA BIO Inc.) and the CFX96 Real-Time System (Bio-Rad). GAPDH was used as the internal control. PrimeSTAR® Max DNA polymerase (TAKARA BIO Inc.) was used to amplify the target site for semi-quantitative RT-PCR. Primer sequences are listed in Table 2.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: Normal	Fig. 1 panel D
Phenotype	Qualitative analysis *Immunocytochemistry*	Positive for pluripotency markers including OCT4, SOX2, SSEA4, and TRA-1–60	Fig. 1 panel F
	Quantitative analysis *RT-qPCR*	Positive for pluripotency markers including OCT4, SOX2, LIN28	Fig. 1 panel E
Genotype	Karyotype (G-banding) and resolution	46, XY Resolution 550	Fig. 1 panel H
Identity	Microsatellite PCR (mPCR) OR	N/A	
	STR analysis	18 sites were tested and all matched	Available with the authors
Mutation analysis	Sequencing	Hemizygous	Fig. 1 panel B
	Southern Blot OR WGS	N/A	
Microbiology and virology	Mycoplasma	Mycoplasma testing via PCR. Negative	Fig. 1 panel I
Differentiation potential	Spontaneous differentiation	NESTIN (ectoderm); SOX17 (endoderm); α-SMA (mesoderm)	Fig. 1 panel G
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry Antibody		Dilution	Company cat # and RRID
Pluripotency markers	Rabbit anti-OCT4	1:200	Santa Cruz, cat #sc-9081, RRID:AB_2167703
	Rabbit anti-SOX2	1:200	Millipore, cat # AB5603 RRID:AB_304980
	Mouse anti-TRA-1-60	1:100	Millipore, cat #MAB4360 RRID:AB_2277963
	Mouse anti-SSEA4	1:200	Millipore, cat #MAB4304 RRID:AB_177629
Differentiation markers	Rabbit anti-NESTIN	1:1000	Millipore, cat #ABD69 RRID:AB_2744681
	Mouse anti-α-SMA	1:400	Sigma-Aldrich, cat #A5228 RRID:AB_262054
	Goat anti-SOX17	1:200	Santa Cruz, cat#sc-17356 RRID:AB_2195655
	Mouse anti-CD31	1:200	BD Pharmingen, cat#555444 RRID:AB_395837
	Rabbit anti-vWF	1:500	Millipore, cat#AB7356 RRID:AB_92216
Secondary antibodies	Donkey anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 488	1:1000	Invitrogen, cat #A-21206 RRID:AB_2535792
	Donkey anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 488	1:1000	Invitrogen, cat #A-21202 RRID:AB_141607
	Donkey anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 594	1:1000	Invitrogen, cat #A-21203 RRID:AB_141633
	Donkey anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 594	1:1000	Invitrogen, cat #A-21207 RRID:AB_141637
	Donkey anti-goat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 594	1:1000	Invitrogen, cat #A-11058 RRID:AB_2534105
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Genotyping	Knock-in site (F1/R1: 2667 bp or 7409 bp)	CTGTCATCTCTGCATCCTTGTACC/GGGAGCCAAACAGAAAGAACC	
	5′ Knock-in junction (F1/R2 : 1679 bp)	CTGTCATCTCTGCATCCTTGTACC/GGCCTTGGCTTAGCGATGT	
	3′ Knock-in junction (F2/R1 : 1324 bp)	ACAGGACCTCTACTGAGCGG/GGGAGCCAAACAGAAAGAACC	
Pluripotency markers (qPCR)	*OCT4* (164 bp)	CCTCACTTCACTGCACTGTA/CAGGTTTTCTTTCCCTAGCT	
	SOX2 (80 bp)	TTCACATGTCCCAGCACTACCAGA/TCACATGTGTGAGAGGGGCAGTGT	
	LIN28 (129 bp)	AGCCATATGGTAGCCTCATGTCCG/TCAATTCTGTGCCTCCGGGAGCAG	
FVIII (qPCR)	*FVIII* Exon 7 to Exon 10 (621 bp)	TCTTGTGAGGAACCATCGCC/ACATCAGTGATTCCGTGAGGG	
House-keeping gene (qPCR)	*GAPDH* (87 bp)	TGCACCACCAACTGCTTAGC/GGCATGGACTGTGGTCATGAG	

4.4. Immunocytochemistry

Cells were fixed with a 4% paraformaldehyde for 10 min, permeabilized with PBS containing 0.1% Triton X-100 for 10 min at room temperature (RT) and then blocked with PBS containing 2% BSA for 1 h at RT. After blocking, cells were incubated with primary antibody overnight at 4 ˚C, washed, and incubated with secondary antibody for 30 min at RT (Table 2). Cells were mounted onto slides using mounting medium containing 4′, 6-diamidino-2-phenylindole (Vector Laboratories). All images were captured with a fluorescence microscope (Nikon Instruments Inc).

4.5. Differentiation into three germ layer cell types and endothelial cells

For the *in vitro* 3-germ layer assay, iPSC colonies were lifted and embryonic bodies (EBs) were generated. EBs were maintained on low-attachment plates in 5% FBS containing EB culture medium [DMEM/F12 medium containing bFGF (PeproTech), 20% knockout serum replacement (Invitrogen), 1% nonessential amino acids (Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich)]. After 7 d, EBs were plated onto matrigel-coated dishes and maintained for an additional 10 d to allow for spontaneous differentiation. iPSCs were also differentiated into endothelial cells as described previously (Harding et al., 2017; Sung et al., 2019).

4.6. Karyotyping, mycoplasma testing and STR analysis

Standard G banding of metaphase spreads was performed at GenDix, Inc., Korea. Cells were checked for the absence of mycoplasma contamination at Cosmogenetech, Korea. YCMi001-B-1 and parental iPSCs were sent to the Cosmogenetech (Korea) for STR analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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