Restoration of the FVIII expression by targeted gene insertion in the *FVIII* locus of the hemophilia A patient-derived iPSCs

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Directed by Professor Dong-Wook Kim

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Jin Jea Sung

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Jin Jea Sung

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Abstract

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Target-specific genome editing, using engineered nucleases zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and type II clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), is considered a promising approach to correct disease-causing mutations in various human diseases. In particular, hemophilia A can be considered an ideal target for gene modification via engineered nucleases because it is a monogenic disease caused by the mutation of coagulation factor VIII (FVIII), and a mild restoration of FVIII levels in plasma can prevent disease symptoms in patients with severe hemophilia A. In this study, we describe a universal genome correction strategy to restore FVIII expression in induced pluripotent stem cells (iPSCs) derived from patients with hemophilia A by the normal *FVIII* gene expression in patient's *FVIII* locus. We used CRISPR/Cas9 mediated homology directed repair (HDR) system to insert the B-domain deleted from of *FVIII* gene with or without the human EF1α promoter. After gene targeting, we obtained the *FVIII* gene correctly inserted in iPSC lines at a high frequency and these lines retained

pluripotency after knock-in and the neomycin resistance cassette removal. More importantly, we confirmed that endothelial cells from the gene-corrected iPSCs could generate functionally active FVIII protein from the inserted *FVIII* gene with EF1α promoter. This is the first demonstration that the *FVIII* locus is a suitable site for integration of normal *FVIII* gene and can restore FVIII expression by the EF1α promoter in endothelial cells differentiated from hemophilia A patient-derived gene-corrected iPSCs.

Key words: human induced pluripotent stem cells(hiPSCs), hemophilia A, factor VIII (FVIII), type II clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), gene correction, endothelial cells

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I. INTRODUCTION

Hemophilia A is a dominant hemophilic disorder, affecting 1 in 5000 males, caused by a deficiency in coagulation factor VIII (FVIII)[1,2]. Patients with severe cases suffer from frequent spontaneous bleeding events in various organs, including the joints and muscles that can lead to chronic musculoskeletal disabilities[3]. The major treatment for hemophilia A is supplementation of clotting factor, but this requires frequent intravenous infusions (1-3 times in a week for prophylactic treatment) and a high cost for clotting factor concentrates[4,5]. The gene therapy of hemophilia A is expected to become a therapeutic alternative to the supplement of clotting factor concentrate. In particular, hemophilia A is a feasible target for the gene therapy because increasing the plasma level of FVIII by only 1% causes therapeutic improvements in patients with severe hemophilia A[6,7].

Recently developed engineered nucleases, including zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and type II clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), are already used in gene therapy for various diseases and enable more sophisticated modification of mutated gene. Moreover, the potential for using

engineered nucleases in patient-derived induced pluripotent stem cells (iPSCs) and cell type specific differentiation techniques provides an unlimited source for future *ex vivo* cell therapy materials for autologous transplantation[8,9]. Previously, we showed that an inversion genotype can be generated or corrected in human iPSCs using TALEN[10]. We also showed that CRISPR/Cas9 can revert inversion mutations in human iPSC lines derived from patients with intron 1 or intron 22 inversions. We confirmed that transplantation of endothelial cells derived from gene-corrected iPSCs can rescue injury mortality in hemophiliac mice[11]. Others have also used TALEN to insert the exon 23-26 cDNA fragment at the deletion junction of exon 22 and intron 22 in intron 22 inversion patient-derived iPSCs and found that functionally active FVIII protein was expressed in differentiated cells from gene-corrected iPSC lines[12]. These previous corrections of intron 22 inversion in patient-derived iPSCs are crucial because intron 22 inversion is the most common mutation, occurring in almost half of patients with severe hemophilia A[13,14].

However, another half of severe hemophilia is caused by various types of mutations, including insertions, deletions, and point mutations[15]. Therefore, to correct all of these mutant types in hemophilia A patients, it is inevitable that a large array of customized sets of ZFN, TALEN, single-guide RNAs (sgRNAs) for CRISPR/Cas9, and targeting donors will be required. Another possible and universal approach is the insertion of a *FVIII* transgene into a specific site of genome. This is a more likely strategy for dealing with all *FVIII* mutant variants because the *FVIII* transgene can express the functional FVIII protein, regardless of mutant variants of hemophilia A. In this way, a single set of gene targeting engineered nuclease and *FVIII* gene donor plasmid is sufficient to address virtually all hemophilia A mutant types.

In this study, we explored the possibility of universal gene-correction strategies that the *FVIII* gene is expressed in *FVIII* locus of hemophilia A patient-derived iPSCs by using a CRISPR/Cas9 mediated knock-in. We designed two

different donor plasmids for human *FVIII* locus specific knock-in; one donor plasmid for insertion of B-domain deleted form of *FVIII* cDNA donor under the control of endogenous *FVIII* promoter after knock-in, the other for human EF1α promoter driven *FVIII* expression cassette insertion in same locus. Importantly, insertion of the *FVIII* gene driven by EF1α promoter resulted in the production of a functionally active FVIII protein from the gene-corrected iPSC line derived endothelial cells.

II. MATERIALS and METHODS

1. Cell Cultures

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) plus 1% (vol/vol) P/S. FVIII-deleted patient-derived iPSCs (CY Park submitted) and gene-corrected iPSC lines were maintained on Matrigel (Corning, Corning, NY, USA) coated cell culture plates in the STEMMACS^{TM} iPSC-brew FX (STEMMACS medium; Miltenyi Biotec, Bergisch Gladbach, Germany) medium for feeder free culture. Briefly, iPSCs were passaged as cells reached a confluency of 70–80%. For passaging, we rinsed iPSCs with Dulbecco's phosphate-buffered saline (dPBS) once, and incubated with Versene solution (Gibco, Grand Island, NY, USA) for 4–5 min. Next, we changed the Versene solution for STEMMACS medium and pipetted to dissociate the culture into small clumps. iPSC clumps were split 1:10 and reseeded on a new Matrigel-coated culture dish in STEMMACS medium supplemented with 10 μM of Y27632 (Sigma-Aldrich, St. Louis, MO, USA). The next day, the iPSC culture medium was changed to fresh STEMMACS medium without Y27632, and the medium was refreshed daily.

2. sgRNA preparation and validation

We purchased recombinant *Streptococcus pyogenes* Cas9 (SpCas9) and sgRNA expression plasmids from ToolGen (Seoul, Korea). Potential off-target sites that differed by up to three nucleotides from the sgRNA were also provided by ToolGen. To validate the cleavage activity of the sgRNA, we transfected Cas9 protein and sgRNA expression plasmid into HEK 293 cells. Three days after transfection, genomic DNA was purified with DNeasy Blood & Tissue Kits

(QIAGEN, Hilden, Germany) and applied to the T7E1 assay, as described previously[16].

3. Donor plasmid construction

We used pcDNA4/BDD-FVIII (Addgene #40135) for FVIII and EF1α-FVIII donor plasmid construction. To generate the FVIII donor plasmid, we introduced a single point mutation in the respective protospacer adjacent motif (PAM) site (C>T, 36 bp downstream from BDD-FVIII start codon) to evade cleavage by Cas9/sgRNA. Then, the cytomegalovirus (CMV) promoter of the original pcDNA4/BDD-FVIII was substituted with the 1113 bp 5'-homology arm (left arm) cloned from human genomic DNA and inserted in the MfeI/NruI site. We inserted a bovine growth hormone (bGH) polyadenylation signal and the neomycin resistance cassette flanked by *loxP* sites fused by overlapping PCR into the 3' end of *FVIII* open reading frame using the NotI/MauBI site. Afterward, a 786 bp 3'-homology arm (right arm) was cloned from human genomic DNA and inserted into the PacI/MauBI site. To generate EF1α-FVIII donor plasmid, human elongation factor 1 alpha (EF1α) promoter was inserted into the MluI/NruI site between the left arm and *FVIII* open reading frame. The DNA sequences of each donor plasmids from the 5' end of the left arm and to the 3' end of the right arm was confirmed by Sanger sequencing at Cosmogenetech (Seoul, Korea).

4. Generation of gene-corrected patient-derived iPSCs

Patient-derived iPSC colonies were pretreated 10 μM of Y27632 for 2 hr prior to electroporation. Cells were then washed once with dPBS and dissociated into single cells using TrypLE™ Select (Gibco). iPSC cells (5×10^5) were electroporated with 2 μg Cas9, 2 μg sgRNA expression vector, and 4 μg each donor

plasmids using a Neon^{R} electroporator (Invitrogen, Carlsbad, CA, USA) as previously described[10]. Transfected cells were plated onto a Matrigel-coated plate with 10 μM Y27632 for 2 days. G418 (100 μg/ml) was added to the culture medium 2 days after electroporation. After 12-14 days of G418 selection, half of the surviving colonies were manually lifted and lysed for genotype, as described previously[17]. Correctly targeted colonies were dissociated into single cells and reseeded for expansion and further analysis. To generate single cell-derived correctly targeted iPSCs, we performed additional three rounds of single colony passaging with G418 selection. After three rounds of single colony passaging and G418 selection, the correctly targeted lines underwent excision of the neomycin resistance cassette. We electroporated 2 μg pCAG-Cre:GFP vector (Addgene #13776) into 5×10^5 iPSCs and performed clonal selection without a selection drug.

5. PCR analysis of targeted *FVIII* gene knock-in

Genomic DNA was purified using DNeasy Blood & Tissue Kits (QIAGEN) according to the manufacturer's instructions. We used primer sets specific to the donor plasmid and genomic DNA sequences adjacent to the 5' and 3' ends of the integration junction. We sequenced PCR amplicons of knock-in junctions at Cosmogentech to verify their identity.

6. Analysis of indel frequency

Genomic DNA was isolated from both the patient and corrected iPSC clones using DNeasy Blood & Tissue Kits (QIAGEN). To analyze indel frequency, we amplified the off-target regions using Phusion polymerase (Thermo Fisher Scientific, MA, USA). Deep-sequencing libraries were generated from the second PCR reaction using the TruSeq HT Dual Index primers. The resulting libraries were

subjected to paired-end sequencing using MiSeq (Illumina, San Diego, CA, USA) at LAS, Inc. (Gimpo, Korea), as previously reported[18].

7. *In vitro* differentiation into three germ layers

We performed the *in vitro* 3-germ layer formation assay as previously described[10,19]. Briefly, iPSC colonies were manually dissected by glass hock and lifted using collagenase type IV (Invitrogen) to generate embryonic bodies (EBs). EBs were cultured on low-attachment cell culture dishes in 5% FBS containing EB culture medium [DMEM/F12 medium containing 4 ng/mL basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ, USA), 20% knockout serum replacement (Invitrogen), 1% nonessential amino acids (Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich)]. After 1 wk, EBs were plated onto matrigel-coated dishes and cultured for an additional 10 days for spontaneous differentiation.

8. RNA isolation, reverse transcription polymerase chain reaction (RT-PCR), and quantitative PCR (qPCR) analysis

We purified total RNA from patient-derived iPSC or iPSC-derived endothelial cells with an Easy-Spin total RNA extraction kit (Intron Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. Then, we used 1 μg purified total RNA to generate cDNA with PrimeScript™ RT Master Mix (TAKARA BIO Inc., Otsu, Japan) and performed qPCR using SYBR® Premix ExTaq™ (TAKARA BIO Inc.). mRNA levels were quantified using the CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Ct values of GAPDH were used as an endogenous reference to normalize the relative expression levels of target genes based on their Ct values. For semi-quantitative RT-PCR, we used EmeraldAmp® GT PCR Master Mix (TAKARA BIO Inc.) to amplify the target site according to the

manufacturer's instructions.

9. Differentiation of endothelial cells from iPSCs

We performed endothelial cell differentiation from iPSCs as in a previously described protocol with minor modifications[20]. Briefly, iPSCs were dissociated with Versene solution and transferred to a new matrigel-coated dish in STEMMACS medium supplemented with 10 μM Y27632. On day 0 of differentiation, iPSCs were treated with 6 μM CHIR99021 (Tocris Bioscience, Bristol, UK) in STEMdiff™ APEL™2 medium (STEMCELL technologies, Vancouver, BC, Canada) for 2 days. On day 2, CHIR99021-containing medium was changed to STEMdiff™ APEL™2 Medium with 25 ng/mL BMP4 (ProSpec, NJ, USA), 10 ng/mL bFGF (PeproTech), and 50 ng/mL VEGF-A (PeproTech) for 2 days. On day 4, cells were detached with TrypLE™ select and transferred to new culture dishes and cultured in endothelial cell growth medium-MV2 (ECGM-MV2; Promocell, Heidelberg, Germany) supplemented with 50 ng/mL VEGF-A. The ECGM-MV2 with VEGF-A was refreshed every 2 days. On day 8 of differentiation, the resulting endothelial cells were applied to the appropriate assays.

10. Immunocytochemistry

For immunofluorescent staining, we fixed cells on glass slides with a 4% paraformaldehyde solution for 10 min, washed three times with PBS, and permeabilized with PBS containing 0.1% Triton X-100 for 10 min at room temperature. After blocking in blocking buffer (PBS containing 2% bovine serum albumin) for 1 hr at room temperature, the samples were incubated with primary antibody diluted in blocking buffer at 4℃ overnight. The following primary antibodies were used: rabbit anti-OCT4 (1:200, Santa Cruz Biotechnology, Dallas,

TX, USA), mouse anti-SSEA4 (1:200, Millipore, Billerica, MA, USA), rabbit anti-NESTIN (1:1000, Millipore), goat anti-SOX17 (1:200, Santa Cruz), mouse anti-α-SMA (1:400, Sigma-Aldrich), mouse anti-CD31 (1:200, BD Biosciences, San Jose, CA, USA), rabbit anti-vWF (1:500, Millipore). After washing three times with PBS, we incubated samples with fluorescence-tagged secondary antibodies (Alexa Fluor® 488 or 594, 1:1000, Invitrogen) in PBS for 30 min at room temperature. Samples were washed again three times with PBS and mounted onto slides using 4′, 6-diamidino-2-phenylindole-containing mounting medium (Vector Laboratories, Burlingame, CA, USA). All images were captured with a fluorescence microscope (Eclipse Ti-U, Nikon Instruments Inc, Tokyo, Japan).

11. FVIII activity assay

On day 8 of differentiation, we changed the endothelial cell culture medium to phenol red free ECGM-MV2 medium with 50 ng VEGF-A. After 24 hr incubation, the supernatants were collected and concentrated 20 times using centrifugal filter units (Millipore). FVIII activity in the concentrated culture supernatant was measured using the Coamatic® Factor VIII chromogenic assay kit (Instrumentation Laboratory, Bedford, MA, USA) according to the manufacturer's instructions.

12. Statistics

All data values are expressed as the mean ± standard error of the mean (S.E.M) unless otherwise indicated. Statistical significance was estimated using Student's t test. A resulting p-value <0.01 was considered statistically significant.

III. RESULTS

1. Sequence analysis of breakpoint in the *FVIII* gene of a patient with severe hemophilia A

In this study, we used an iPSC line derived from a hemophilia A patient with a gross deletion (exon 8 - exon 22) at the *FVIII* locus. Targeted genotype PCR from intron 7 to intron 22 and Sanger sequencing analysis revealed a gross deletion of 94,172 bp from exon 8 to intron 22 at the patient's *FVIII* locus (Figure 1A, B). We identified the mRNA sequences around its deletion junction in the patient's *FVIII* locus by using mRNA transcripts from the patient-derived iPSC line. RT-PCR and Sanger sequencing analysis targeting exon 7 to exon 23 showed that partial exon 8 and 22 sequences spliced out and that exon 7 and 23 were directly linked to make a shorter version of the *FVIII* mRNA, which also generated the premature stop codon in exon 23 (Figure 2A, B).

2. Single guide RNA design and validation of nuclease activity

We hypothesized that insertion of the *FVIII* gene in exon 1 of the *FVIII* locus would express functionally active FVIII protein regardless of the mutant type. Therefore, we designed a nuclease targeting 34 bp downstream from the start codon in exon 1 of the human *FVIII* locus on chromosome X for homology directed repair (HDR) mediated knock-in (Figure 3A). Next, we tested the cleavage efficiency of the Cas9/sgRNA by transient expression of the sgRNA and Cas9 expression vector in HEK293 cells. A subsequent T7E1 analysis revealed that the Cas9/sgRNA induced indels at the target site with a frequency of 11% (Figure 3B). Sanger sequencing results also confirmed indel formation at the predicted cleavage site, including insertions and deletions (Figure 3C).

3. Donor plasmids design and construction

Next, we designed two donor plasmids to restore FVIII expression using a B-domain deleted form of *FVIII* cDNA. The first donor plasmid was designed to use an endogenous *FVIII* promoter for FVIII expression. For this donor, FVIII expression is completely depends on the endogenous transcriptional machinery of human *FVIII* locus since *FVIII* cDNA replace *FVIII* gene from transcription start codon in exon 1. For second donor plasmids, we planned to use a universal promoter for FVIII expression after donor plasmid knock-in.

We considered two important factors in donor plasmids design. First, since FVIII in donor plasmid contains the sgRNA target and PAM sequence, we mutated the one base pair in PAM site (C>T, 36 bp downstream from FVIII start codon) in coding sequence in *FVIII* cDNA, thus donor plasmids were not cleaved by the CRISPR/Cas9 (Figure 4). Second, we tried to select the most active universal promoter for the FVIII expression in iPSC derived endothelial cells. For this, we differentiate the iPSC into the endothelial cells[20] and then introduced the FVIII expression vector with cytomegalovirus (CMV), CMV early enhancer/chicken β actin (CAG) or human elongation factor-1 alpha (EF1α) promoter (Figure 5A). 2 day after transfection, we analyzed the *FVIII* transcripts in iPSC derived endothelial cells and FVIII activity from endothelial cell culture media. qPCR and FVIII activity assay results indicated that EF1α promoter was resulted highest level of *FVIII* transcripts and FVIII activity from iPSC derived endothelial cells (Figure 5B, C). Therefore, we used the human EF1α promoter for the expression of FVIII in the second donor plasmid (EF1α-FVIII donor). As a result, first donor (FVIII donor) consists of a *FVIII* cDNA with mutated PAM site, a PGK poly A signal, *loxP*-flanked neomycin-selection cassette and 5' (left)- / 3' (right)-homology arms for endogenous expression of knocked-in *FVIII* cDNA (Figure 6A). EF1α-FVIII donor has same configuration of the FVIII donor plasmid except the addition of an EF1α promoter between left homologous arm and *FVIII* cDNA (Figure 6B).

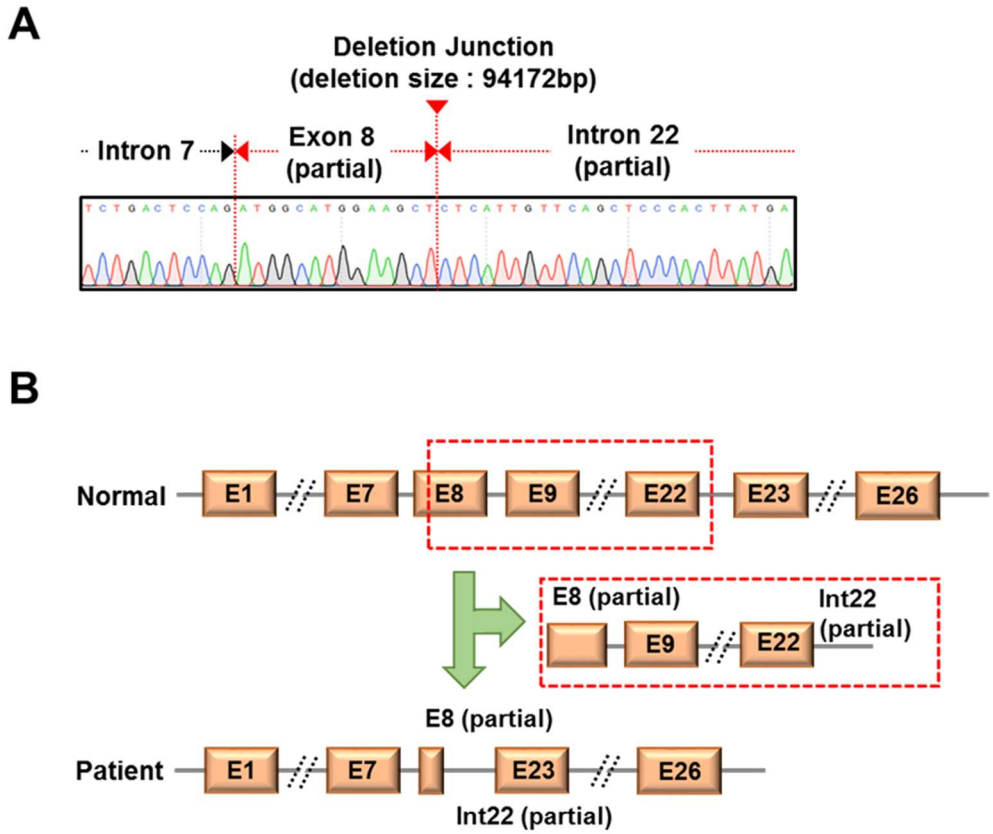


Figure 1. Breakpoint analysis of the *FVIII* locus in a hemophilia A patient with a gross deletion. (A) Sanger sequencing analysis of deletion junction between exon 8 and intron 22. The deletion junction is indicated by the red arrowhead. (B) Schematic overview of *FVIII* gene of the hemophilia A patient compared to normal *FVIII* gene.

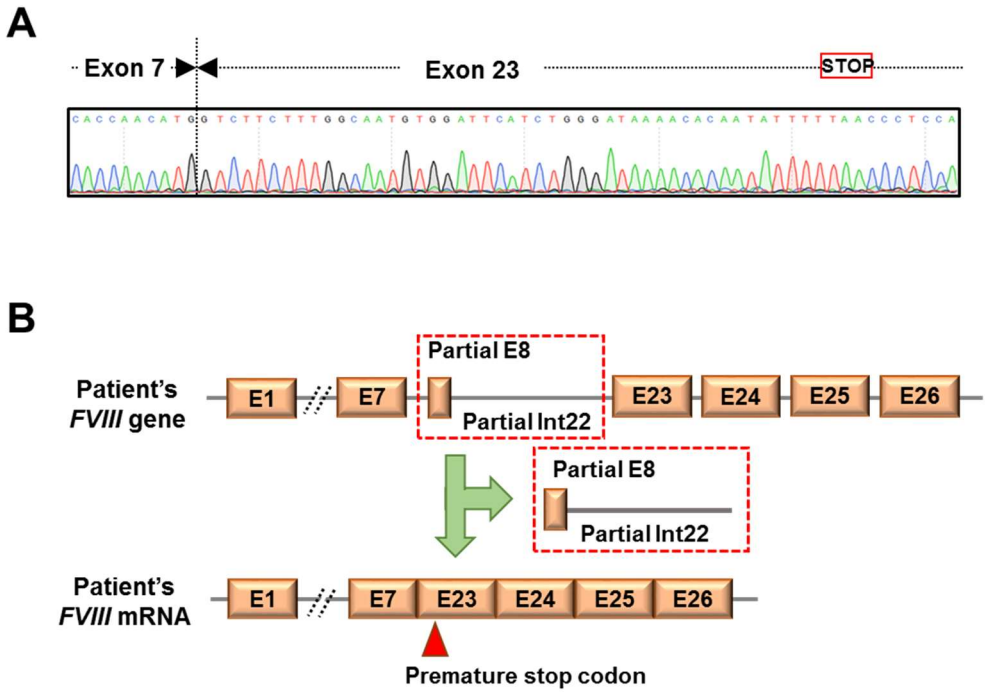


Figure 2. *FVIII* cDNA analysis in a hemophilia A patient with a gross deletion. (A) Sanger sequencing analysis showing RT-PCR amplicons of the exon 7 and exon 23 junction in FVIII transcripts from the hemophilia A patient. Premature stop codon caused by frameshift shown by red box. (B) Schematic view of *FVIII* mRNA of the hemophilia A patient.

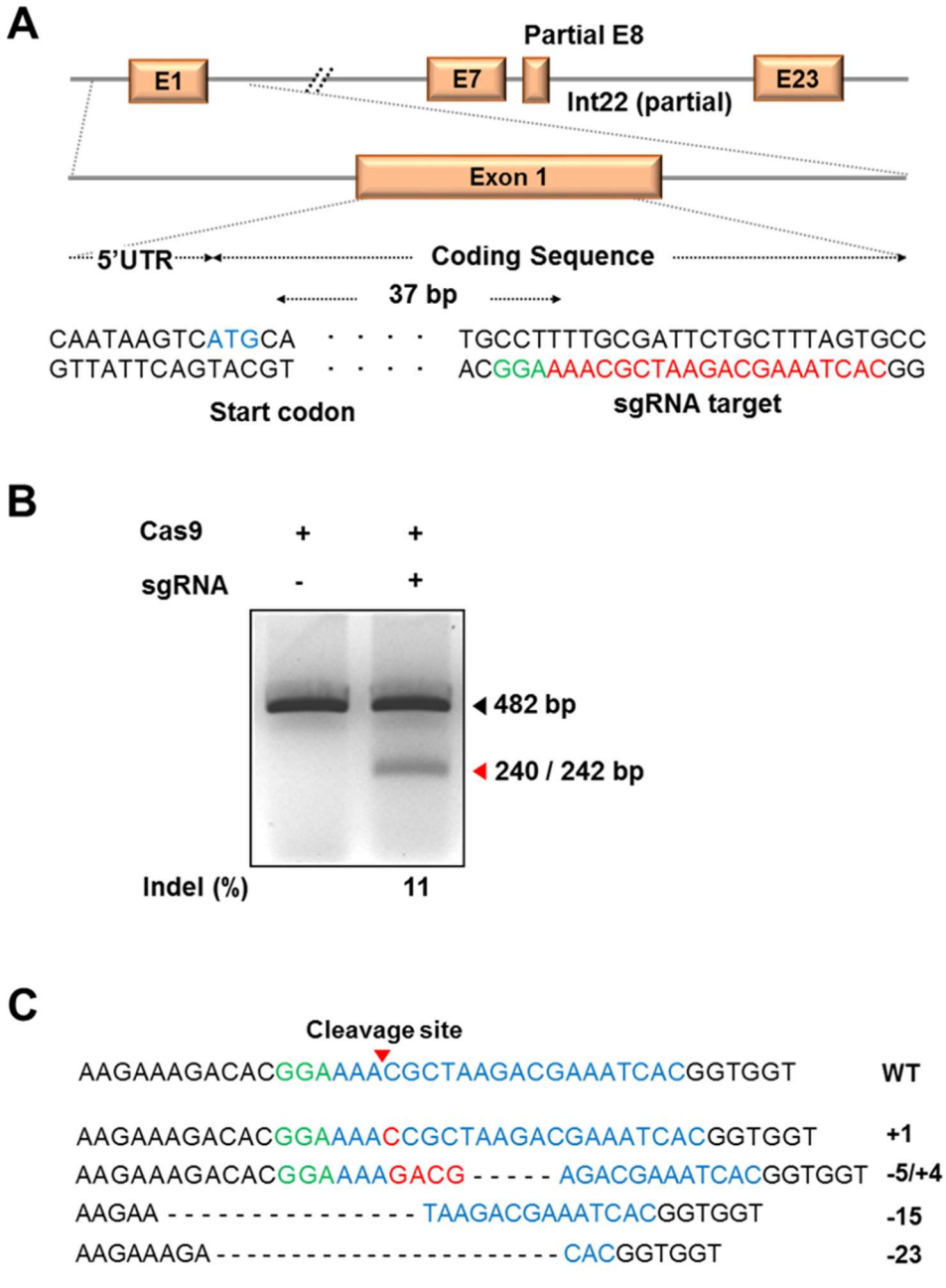


Figure 3. sgRNA design and validation of nuclease activity. (A) Schematic overview showing the sgRNA target site in *FVIII* locus. Top line depicts the *FVIII* locus with the gross deletion of exon 8 to exon 22. The DNA sequences shown

include a partial 5′ untranslated region (5′ UTR), start codon and sgRNA target site 37 bp downstream from the start codon in exon 1 of the *FVIII* locus. The start codon is shown in blue, the protospacer adjacent motif (PAM), and sgRNA target are shown in green and red. (B) Non-homologous end joining (NHEJ) frequency with the T7E1 assay. T7E1-treated PCR products amplified from genomic DNA of HEK293 cells transfected with Cas9 and sgRNA expression vector. The black arrowhead indicates on-target 482 bp PCR amplicon. The red arrowhead indicates 240 and 242 bp fragments cleaved by T7E1. (C) Indel patterns induced by the targeting of Cas9/sgRNA in HEK293 cells was analyzed by PCR amplification and Sanger sequencing. The number of bases generated by insertion (red) or removed by deletion (-) are shown on the right. The red arrowhead indicates a cleavage site. The PAM and target sequences are shown in green and blue.

Table 1. Primer set for genotyping and sgRNA validation

Primers	Sequence	Targets
F8-Int7-F	TGATTTGTGGCAAGAAGGGA	Sanger sequencing for deletion junction in FVIII deletion patient
F8-Int22-R	CCACACAGTTACAACCATATG	
F8-RT-E6-F1	GCCTGGCCTAAAATGCACAC	Sanger sequencing for exon7 – exon 23 junction in patient's *FVIII* cDNA
F8-RT-E23-R1	ATGAGTTGGGTGCAAACGGA	
F8-T7E1-F	TAAAAAGGAAGCAATCCTATCGG	On-target PCR for T7EI assay
F8-T7E1-R	TGCACACCTTACCCAGAAATG	

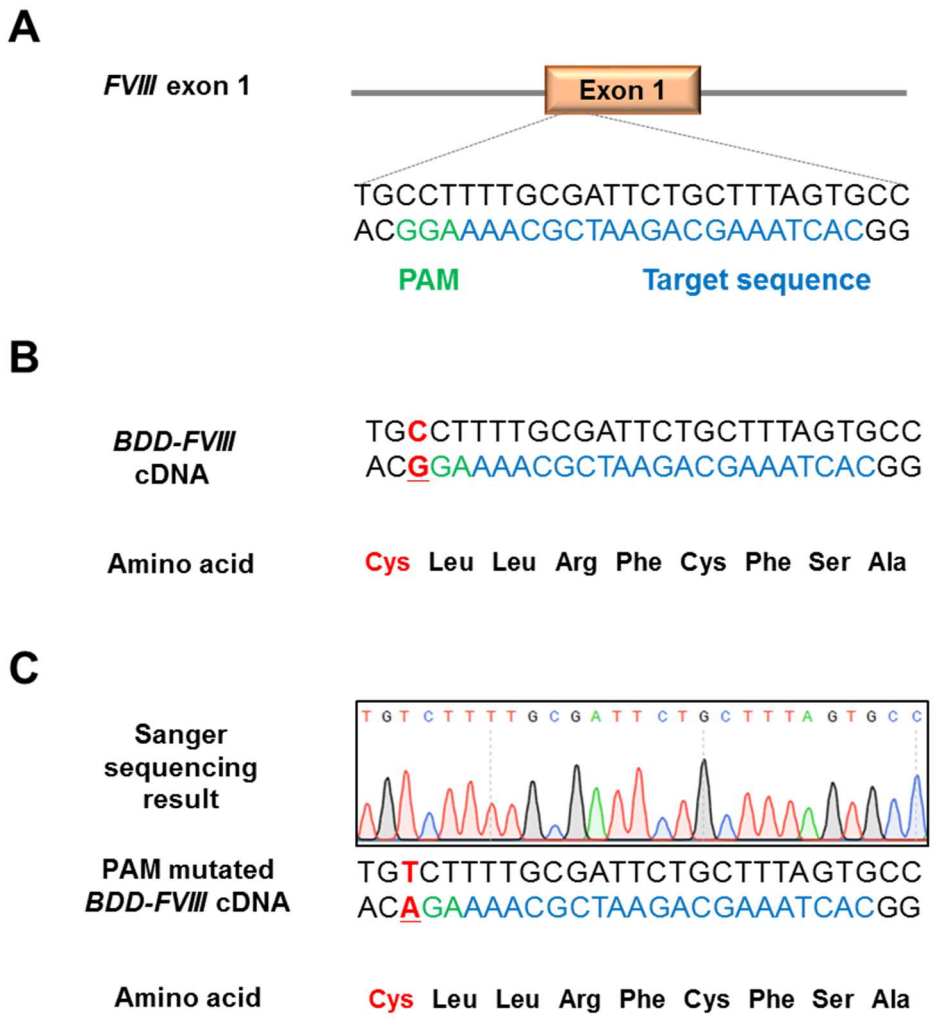


Figure 4. PAM site mutation in donor plasmids (A) sgRNA target sequence in exon 1 of *FVIII* gene. (B) The respective sgRNA target sequence in BDD-FVIII cDNA from 34 bp to 60 bp from start codon. (C) Sanger sequencing result showing single point mutation (G>A) at PAM site in donor plasmids.

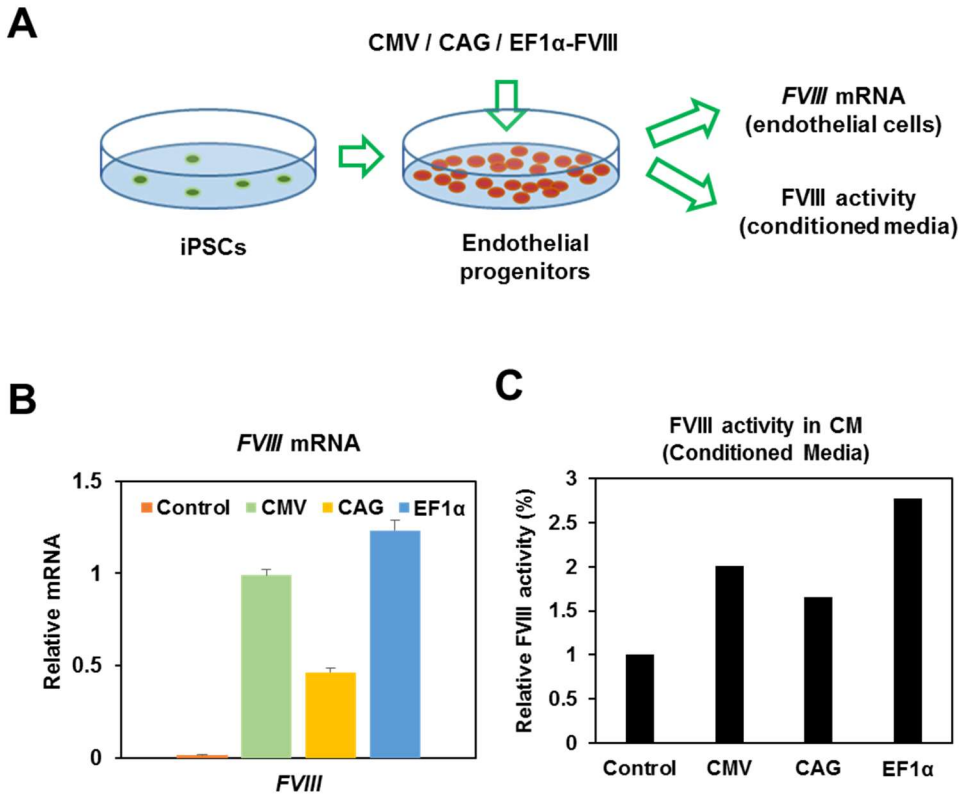


Figure 5. Analysis of promoter activity in iPSC derived endothelial cells. (A) Schematic overview of experiments. CMV, CAG or EF1α promoter driven *FVIII* cDNA transiently introduced in iPSC derived endothelial cells. Total mRNA and culture media collected from endothelial cells and analyzed. (B) Quantitative real-time PCR (qPCR) analysis of *FVIII* transcripts level from either CMV, CAG and EF1α driven FVIII expression vector introduced endothelial cells. GAPDH was used to normalize gene expression. (C) The FVIII activity of cell culture supernatants from either CMV, CAG and EF1α driven FVIII expression vector introduced endothelial cells. FVIII activity was determined in 5×10^5 endothelial cells per single detection.

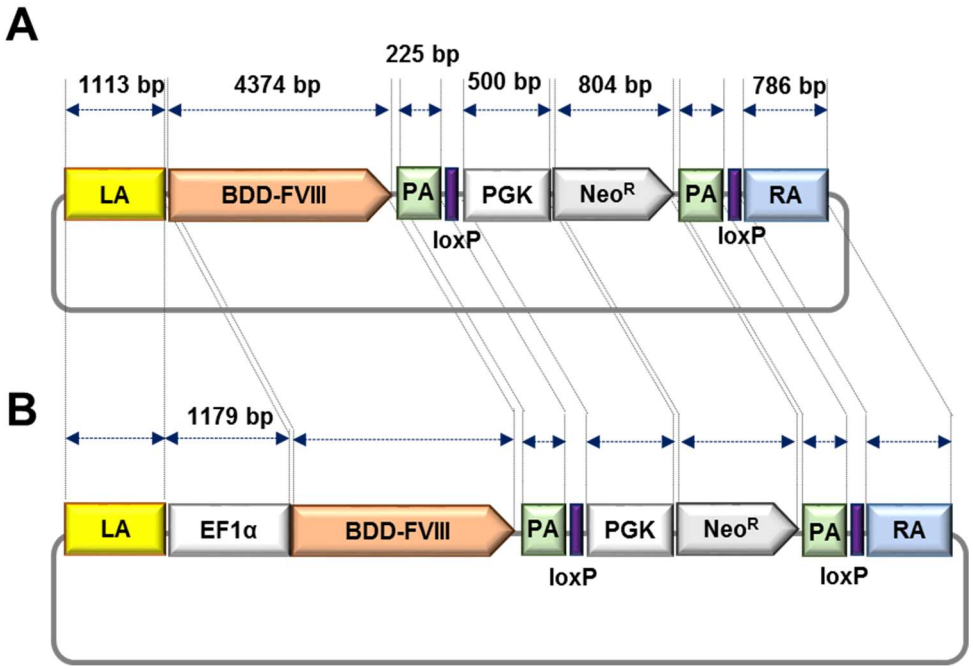


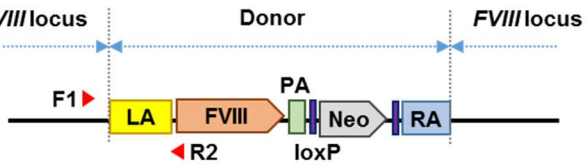
Figure 6. Donor plasmids in detailed view. (A) A schematic of the FVIII donor plasmid design. 1113 bp Left (LA) and 786 bp Right homologues arm (RA) corresponding to the cleavage site of Cas9/sgRNA in exon 1 of *FVIII* locus. BDD-FVIII, 4374 bp B-domain deleted version of FVIII cDNA; PA, 225 bp Bovine growth hormone poly A signal; PGK, 500 bp mouse phosphoglycerate kinase 1 promoter; NeoR, 804 bp Neomycin resistance gene; *loxP*, 34 bp *loxP* sequence. (B) A schematic view of EF1α-FVIII donor plasmid design. Configuration of EF1α-donor plasmid was similar to the FVIII donor plasmid except the insertion of 1179 bp EF1α promoter between left arm and 5' end of FVIII-cDNA.

4. Targeted correction by the knock-in of *FVIII* gene into the human *FVIII* locus

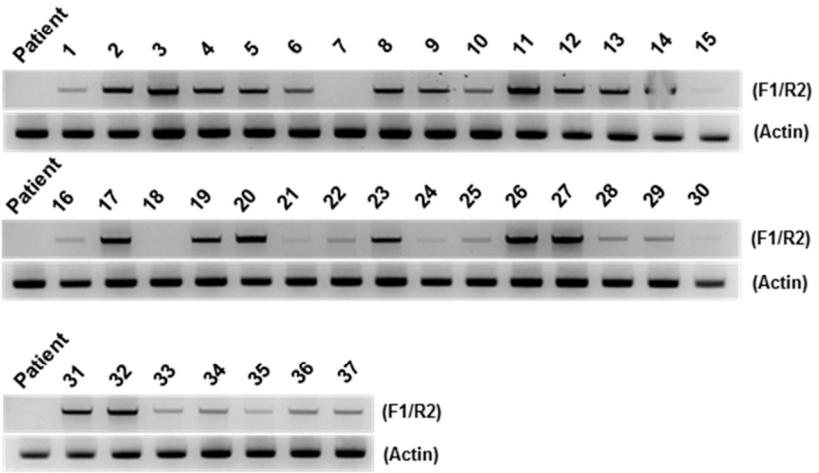
We then introduced the CRISPR/Cas9 and sgRNA expression vectors, and each donor plasmids into hemophilia A patient-derived iPSCs to insert FVIII or EF1α-FVIII donor in *FVIII* locus of the patient iPSC. After drug selection with G418, genomic DNA of survived colonies were collected for initial PCR screening to identify correctly targeted colonies. For the FVIII donor knock-in colonies, we amplified the 5' junction of knock-in site with the specific primer set F1/R2 (Figure 7A). Our initial PCR screening results shown that the FVIII donor plasmid inserted into exon 1 of the *FVIII* locus at a frequency of 94.59% (35 colonies positive/total 37 colonies) (Figure 7B, 7C). In case of EF1α-FVIII donor knocked-in colonies, we analyzed both 5' and 3' knock-in junction for initial PCR screening with specific primer set F1/R3 and F2/R1 for more precise analysis (Figure 8A). We confirmed that EF1α-FVIII donor were correctly targeted into the exon 1 of *FVIII* locus at a frequency of 81.81% (18 colonies positive/total 22 colonies) (Figure 8B, 8C). Then we obtained one clone (FVIII-KI) for FVIII donor knocked-in colonies and two clones (pFVIII-KI-1, pFVIII-KI-2) for EF1α-FVIII donor knocked-in colonies after an additional three rounds of single colony expansion.

After single colony expansion, we confirmed the target specific integration with Sanger sequencing of 5' and 3' junction of knock-in site in FVIII-KI, pFVIII-KI-1 and pFVIII-KI-2 lines (Figure 9A, B). Then all three gene-corrected iPSC lines were subjected to removal of the neomycin resistance cassette by *Cre* recombinase expression (Figure 10A). Targeted genomic DNA PCR and Sanger sequencing of the amplified PCR amplicons confirmed the complete removal of the neomycin cassette in the knock-in line after *Cre* expression (Figure 10B - D).

A



B

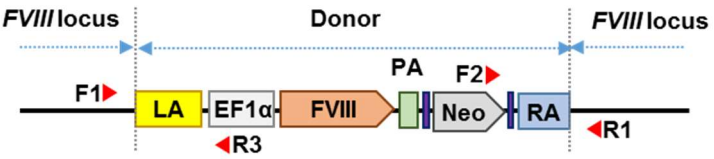


C

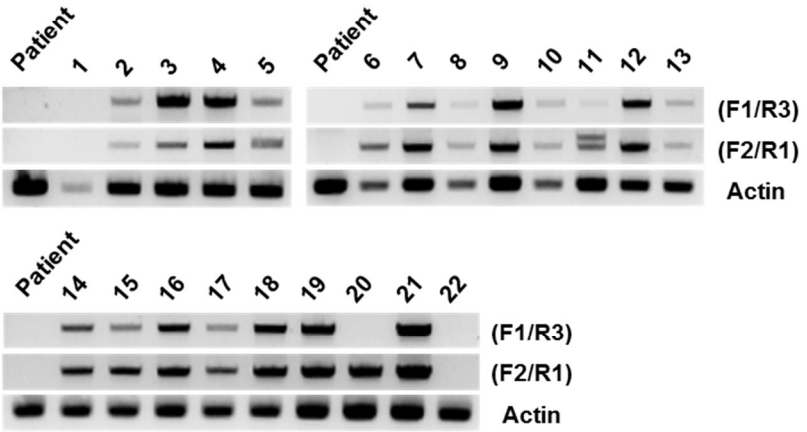
Clones picked	Targeted integrations	Targeting efficiency (%)
37	35	94.59%

Figure 7. The primary PCR screening result of FVIII donor knock-in. (A) A schematic representation indicates PCR target site for initial PCR screening after knock-in. Primers used in PCR analysis are represented by red arrowheads. (B) Genomic DNA of survived colonies (1-35) were applied to PCR screening to evaluate the site-specific integration of the FVIII donor in the *FVIII* locus by using primer sets F1/R2 (5' knock-in junction) shown in Table 2. The predicted size of PCR products are 1679 bp for F1/R2. 295 bp *β-actin* bands were used as internal reference. Genomic DNA from parental patient iPSCs was used for the control. (C) Summary of knock-in efficiency based on the primary PCR screening.

A



B



C

Clones picked	Targeted integrations	Targeting efficiency (%)
22	18	81.81%

Figure 8. The primary PCR screening result of EF1α-FVIII donor knock-in. (A) A schematic representation indicates PCR target sites for initial PCR screening after knock-in. Primers used in PCR analysis are represented by red arrowheads. (B) Genomic DNA of survived colonies (1-22) were applied to PCR screening to evaluate the site-specific integration of the EF1α-FVIII donor in the *FVIII* locus by using primer sets F1/R2 (5' knock-in junction), F2/R1 (3' Knock-in junction). The predicted size of PCR products are 1643 bp for F1/R3, 1324 bp for F2/R1. 295 bp β-actin bands were used as internal reference. Genomic DNA from parental patient iPSCs was used for the control (Patient). (C) Summary of knock-in efficiency based on the primary PCR screening.

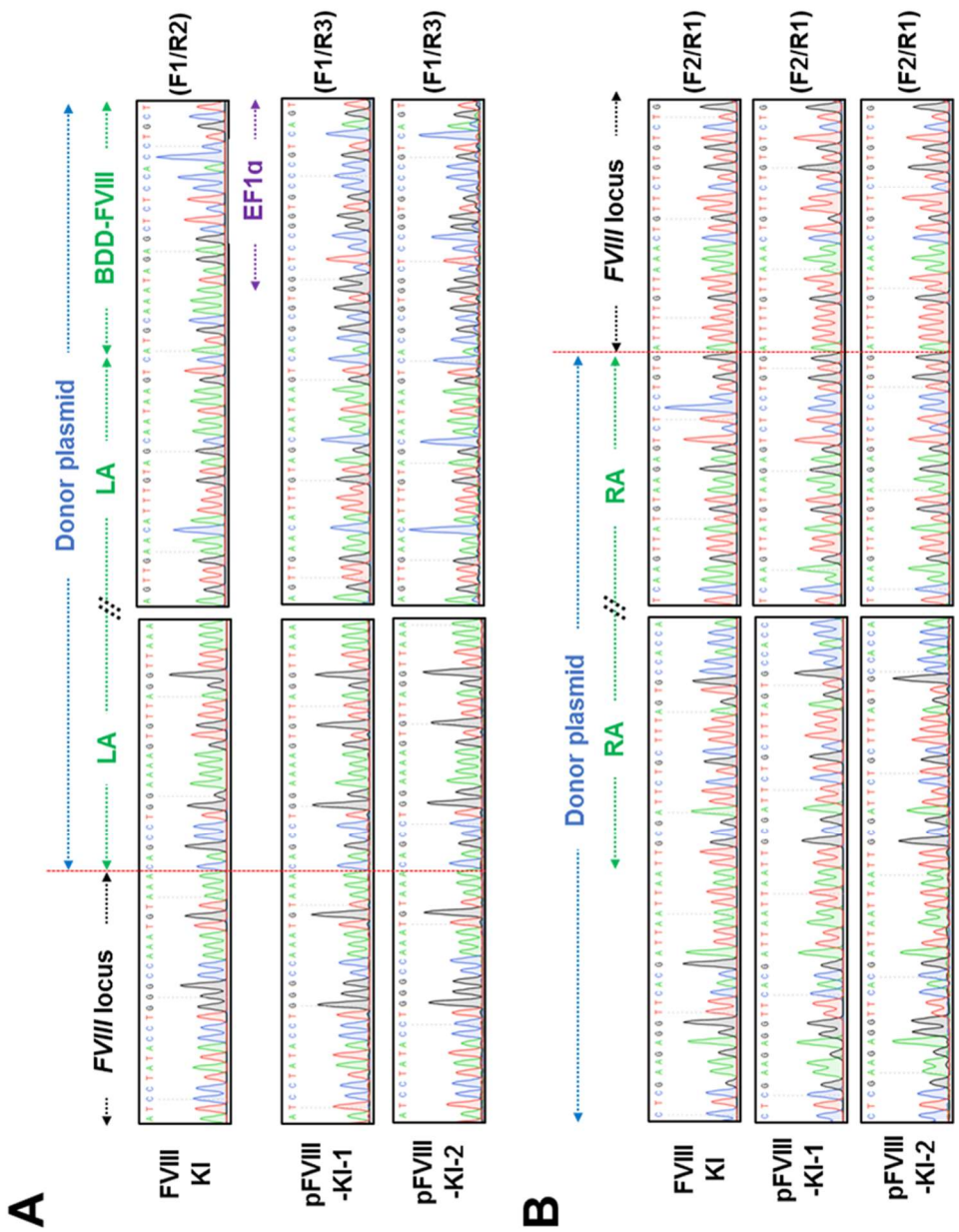


Figure 9. Sequence analysis of FVIII donor knocked-in iPSCs. (A) Partial chromatograms represent sequences at the integration junction between the left arm and exon 1 of the *FVIII* locus. 1679 bp PCR amplicon from FVIII-KI line was

generated with the F1/R2 primer set shown in Figure 7A. 1643 bp PCR amplicons from pFVIII-KI-1 and pFVIII-KI-1 lines were generated with the F1/R2 primer set shown in Figure 8A. (B) Partial chromatograms showing sequences at the integration junction between the right arm and exon 1 of the *FVIII* locus. We generated 1324 bp PCR amplicons from FVIII-KI, pFVIII-KI-1 and pFVIII-KI-2 with the F2/R1 primer set shown in Figure 8A. DNA sequence from the *FVIII* locus or donor plasmid is indicated by a black or blue dotted line with arrowhead. DNA sequence from left arm (LA), right arm (RA), and BDD-FVIII sequence is also shown by a green dotted line with arrowhead. DNA sequence from EF1α promoter (EF1α) is shown by a purple dotted line with arrowhead. The border between each arms and DNA sequence of the *FVIII* locus is indicated by red dotted lines.

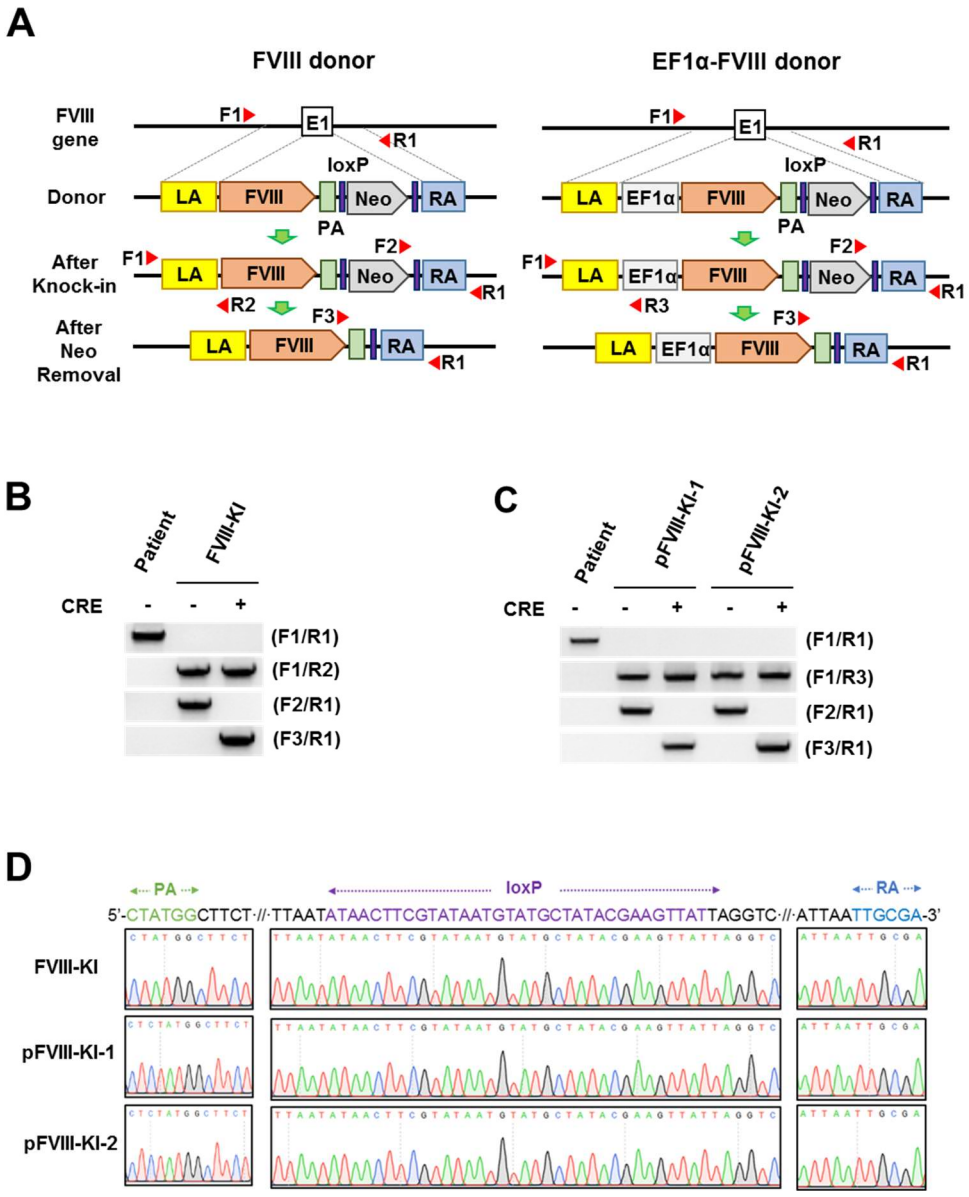


Figure 10. Site-specific integration of the *FVIII* gene at a hemophilia A patient's *FVIII* locus. (A) Schematic representations of the targeted insertion of the FVIII or EF1α-FVIII donor at the human *FVIII* locus and the excision of the neomycin resistance cassette. Top depicts exon 1 of the human *FVIII* locus, and below shows

each donor plasmids. The neomycin resistance cassette was removed by *Cre* expression after the knock-in of each donor plasmids. Primers used in PCR analysis are represented by red arrowheads. (B) Genomic PCR analysis of gene-corrected clones before (-) and after (+) Cre expression in gene-corrected line FVIII-KI. The F1/R1 primer pair was used to detect exon 1 (E1) of the *FVIII* locus. Primer F1/R2 and F2/R1 were used for detecting the knock-in junctions of the 5' and 3' ends in correctly targeted clones. The F3/R1 primer pair was used for detecting removal of the neomycin resistance cassette. Genomic DNA from parental patient iPSCs was used for the control (patient). (C) Genomic PCR analysis of pFVIII-KI-1 and pFVIII-KI-2 lines before (-) and after (+) *Cre* expression. The F1/R1 primer pair was used to detect exon 1 (E1) of the *FVIII* locus. Primer F1/R3 and F2/R1 were used for detecting the knock-in junctions of the 5' and 3' ends in correctly targeted clones. The F3/R1 primer pair was used for detecting removal of the neomycin resistance cassette. Genomic DNA from parental patient iPSCs was used for the control (patient). (D) Partial chromatograms from a 1626 bp PCR amplicon generated with F3/R1, showing the sequences around *loxP* in FVIII-K1, pFVIII-KI-1 and pFVIII-KI-2 cell lines after removal of the neomycin resistance cassette. Partial bGH poly A signal (PA), *loxP*, and partial right arm (RA) shown in green, purple and blue.

5. Pluripotency and off-target analysis of gene-corrected patient-derived iPSCs

We determined whether the gene-corrected lines remained pluripotent after gene targeting. Our quantitative real-time PCR (qPCR) results showed that gene-corrected lines expressed pluripotent marker genes *OCT4*, *SOX2,* and *LIN28* at levels similar to those of the parental hemophilia A patient-derived iPSC line (Figure 11A). We also confirmed uniform expression of OCT4 and SSEA4 in iPSC colonies by immunocytochemistry analysis (Figure 11B). *In vitro* three germ layer formation assay showed that all gene-corrected lines could be differentiated into three germ layers (Figure 12). We then sequenced off-target sites of the sgRNA in the gene-corrected iPSC clone pFVIII-KI-1. We obtained from ToolGen (Seoul, Korea) a list of potential off-target sites that differed from the on-target site by up to three nucleotides (Table 3). We selected four potential off-target sites from the list and subjected these sites to targeted deep sequencing with primer sets listed (Table 4). No significant mutations were found in all analyzed off-target sites in the corrected pFVIII-KI-1 line (Figure 13).

6. Restoration of FVIII expression in the gene-corrected iPSC-derived endothelial cells

We then asked whether endothelial cells from gene-corrected iPSC lines could restore FVIII expression. First of all, we differentiated gene-corrected FVIII-KI and pFVIII-KI-1 line into endothelial cells[23] (Figure 14A), and then examined the expression of endothelial cell markers. After 8 days of differentiation, the endothelial nature of cells was confirmed by immunocytochemistry and PCR analysis. Differentiated cells were positive for staining of the endothelial cell marker CD31 and vWF (Figure 14B). Then we used PCR analysis to evaluate the expression of the endothelial cell markers *CD31* and *vWF* in iPSC-derived endothelial cells. Our qPCR results showed no significant difference in the endothelial cell markers expression between parental patient iPSCs, gene-corrected FVIII-KI and pFVIII-

KI-1 lines (Figure 14C).

Then we used PCR analysis to evaluate the expression of *FVIII* in gene-corrected iPSC line derived endothelial cells. We used the primer set targeting from exon 7 to exon 10 to discriminate between the patients' *FVIII* and knocked-in *FVIII* mRNA. As we expected, the *FVIII* transcript was detected in the both FVIII-KI and pFVIII-KI-1 line-derived endothelial cells, as shown by both qPCR and RT-PCR analyses (Figure 15A, B). Additionally, qPCR result found out that pFVIII-KI-1 line derived endothelial cells resulted in a 7.8-fold increase in *FVIII* mRNA levels over FVIII-KI line derived endothelial cells and RT-PCR result also supported our qPCR result. (Figure 15A, B). We also confirmed by Sanger sequencing that the PCR amplicon had normal exon 7 to exon 9 sequences of *FVIII* cDNA from both gene corrected iPSC lines derived endothelial cells (Figure 15C).

Finally, we performed the FVIII activity assay to identify whether functionally active FVIII protein was secreted from gene-corrected iPSC-derived endothelial cells. We found that there were no difference in FVIII activity from the control patient iPSC and FVIII-KI line derived endothelial cells (Figure 15D). However, we confirmed a significant increase of FVIII activity (2.9-fold increase) from pFVIII-KI-1 compared to control or FVIII-KI iPSC derived endothelial cell culture supernatant (Figure 15D). Altogether, our data show that insertion of the normal *FVIII* gene with EF1α promoter into exon 1 of the mutant *FVIII* locus can generate a functional FVIII protein in iPSC-derived endothelial cells.

Table 2. Primer set for PCR screening and Sanger sequencing

Primers	Sequence	Targets
F1	CTGTCATCTCTGCATCCTTGTACC	Human *FVIII* locus, 5' and 3' Knock-in junction
R1	GGGAGCCAAACAGAAAGAACC	
F2	TCTATGGCTTCTGAGGCGGA	
R2	GGCCTTGGCTTAGCGATGT	
F3	ACAGGACCTCTACTGAGCGG	
R3	CCGTTGCGAAAAAGAACGTTCAC	
β-actin-F	TCACCCACACTGTGCCCATCTACGA	Human β-actin
β-actin-R	CAGCGGAACCGCTCATTGCCAATGG	

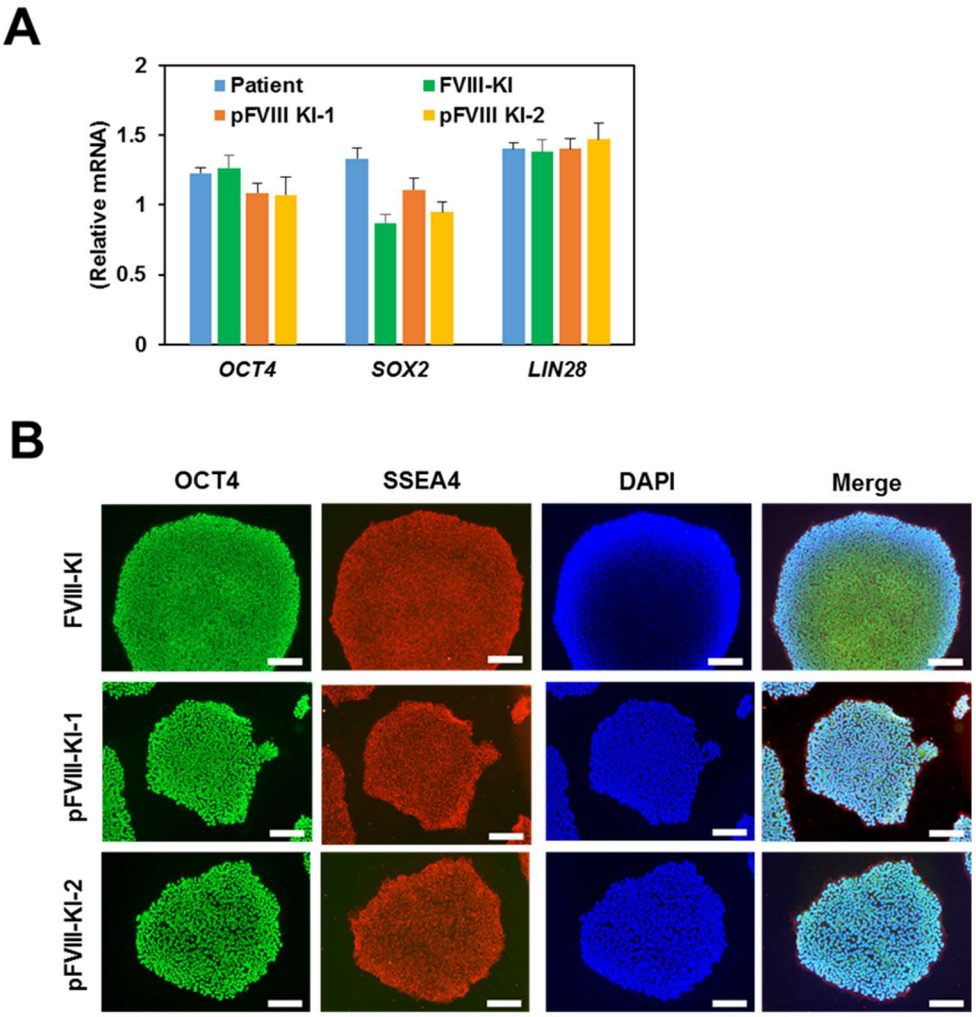


Figure 11. Pluripotency marker expression of gene-corrected iPSC lines. (A) qPCR analysis of *OCT4*, *SOX2*, and *LIN28* in parental patient and gene-corrected iPSC lines. *GAPDH* was used to normalize gene expression. (B) Immunofluorescence staining to indicate expression of the pluripotency markers OCT4 (green) and SSEA4 (red) of gene-corrected iPSC clones. Nuclei were labeled with 4′, 6-diamidino-2-phenylindole (DAPI; blue) (Scale bar, 200 μm).

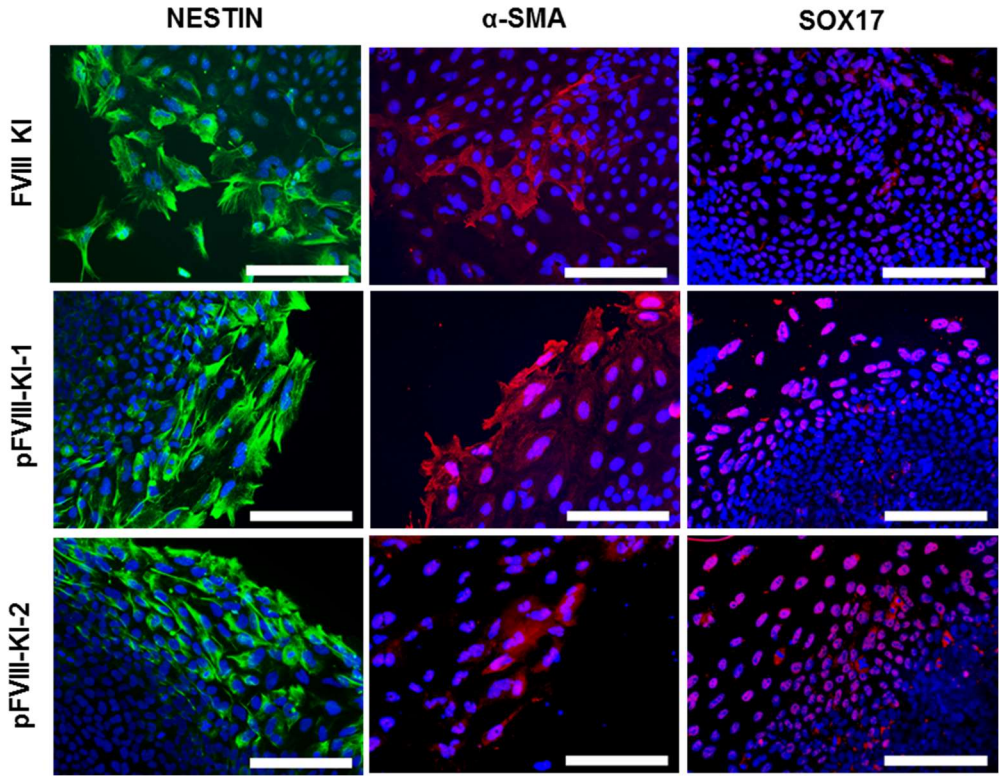
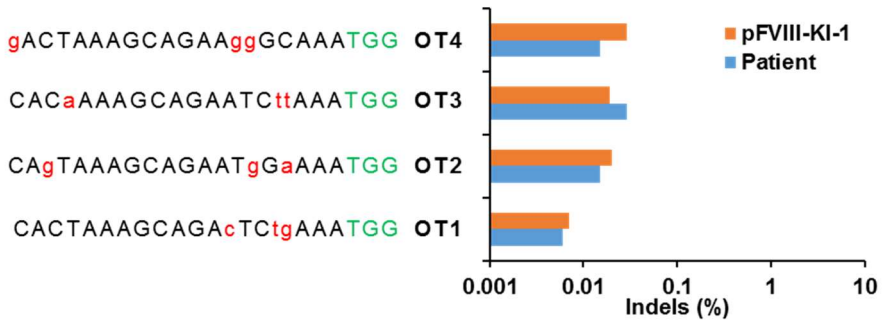


Figure 12. 3-germ layer formation assay of gene-corrected iPSC lines. Immunofluorescence staining shows the expression of marker proteins, representing ectoderm (NESTIN, green), mesoderm (α-SMA, red), and endotherm (SOX17, red). Nuclei were labeled with DAPI (blue) (Scale bar, 200 μm).

A



B

Patient	KI-1
39727	41966
30983	36549
32458	30637
31277	43207

Figure 13. Analysis of off-target sites in the gene-corrected iPSC line using targeted deep sequencing. (A) We analyzed four off-target sites among 16 total off-target sites that differed by up to three nucleotides from the targeted site in the gene-corrected iPSC line pFVIII-KI-1. Mismatched nucleotides and PAM sequences are shown in red and green. (B) The number of total reads are presented.

Table 3. Off target sites

Off targets	Target site	location	directions
OT1	CACTAAAGCAGAcTCtgAAATGG	Chr8	-
OT2	CAgTAAAGCAGAATgGaAAATGG	Chr11	-
OT3	CACaAAAGCAGAATCttAAATGG	Chr5	+
OT4	gACTAAAGCAGAAggGCAAATGG	Chr10	-
OT5	aAtTAAAGCAGAATCtCAAACAG	Chr2	-
OT6	CACTAAAGgAGAAatGCAAACAG	Chr2	-
OT7	CACTAAAGCAaAATaaCAAATAG	Chr5	+
OT8	CAaTAAAGCAGAAaCaCAAAAAG	Chr5	-
OT9	CACTggAGCAGAATaGCAAAGGG	Chr6	+
OT10	CAtTAAAGCAaAATtGCAAATGG	Chr7	-
OT11	tACTAAAtCAGAATCtCAAAGGG	Chr8	-
OT12	CACTtAAGCAaAATgGCAAAGGG	Chr9	+
OT13	CcCTAAAGCAGcATCGCAgACAG	Chr10	+
OT14	CACgAAAGaAGAATCtCAAAAGG	Chr11	+
OT15	CctTAAAGCAGAgTCGCAAAGAG	Chr12	-
OT16	aACTAcAGCAGAATaGCAAAGGG	ChrX	-

Table 4. Primer set for targeted deep sequencing

Primers	Sequence
FVIII-OT1-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCAA GTACCTTGTATTAGAACT
FVIII -OT1-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTGA CCAAAGCCTTTTTCATC
FVIII -OT2-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCACT TGAAAAATCAAAGTTCAAG
FVIII -OT2-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTGC CTTAGTAGATTAACAGCT
FVIII -OT3-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTAA TTTAGAGAAAGAGTAAAGGC
FVIII -OT3-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAA GGCTAGCTGTTAGTTCAC
FVIII -OT4-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTCTG GTAAACACTCTTTCTAC
FVIII -OT4-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAA ACCCAGCATGTAGTTCTA

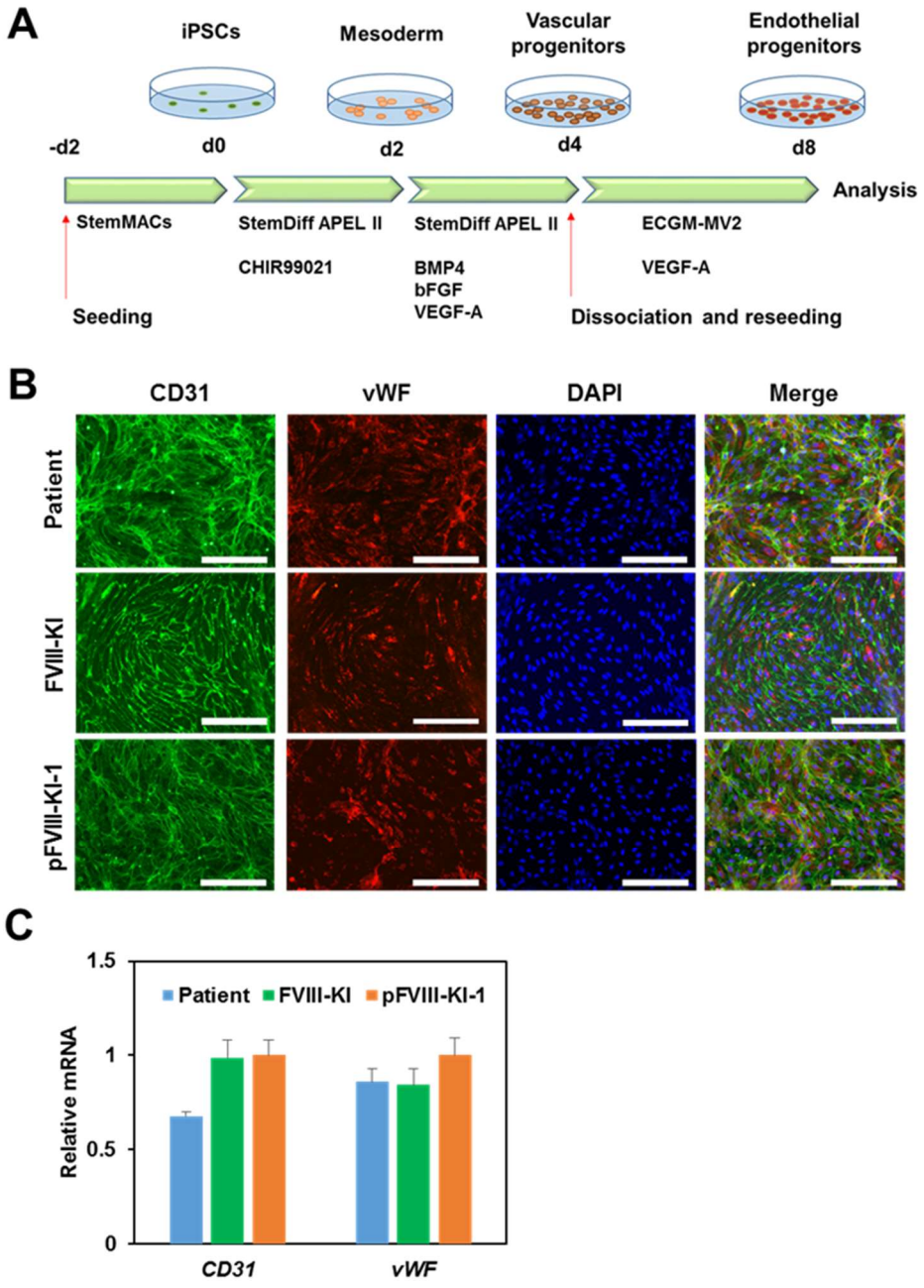


Figure 14. Endothelial cell differentiation of gene corrected iPSC lines. (A) Schematic overview of endothelial cell differentiation. (B) Immunofluorescence staining of endothelial cell markers CD31 (green) and vWF (red) differentiated from

the parental patient and gene-corrected iPSC clones. Nuclei were labeled with DAPI (blue) (Scale bar, 200 μm). (C) qPCR analysis of *CD31* and *vWF* in patient and gene-corrected iPSC line-derived endothelial cells.

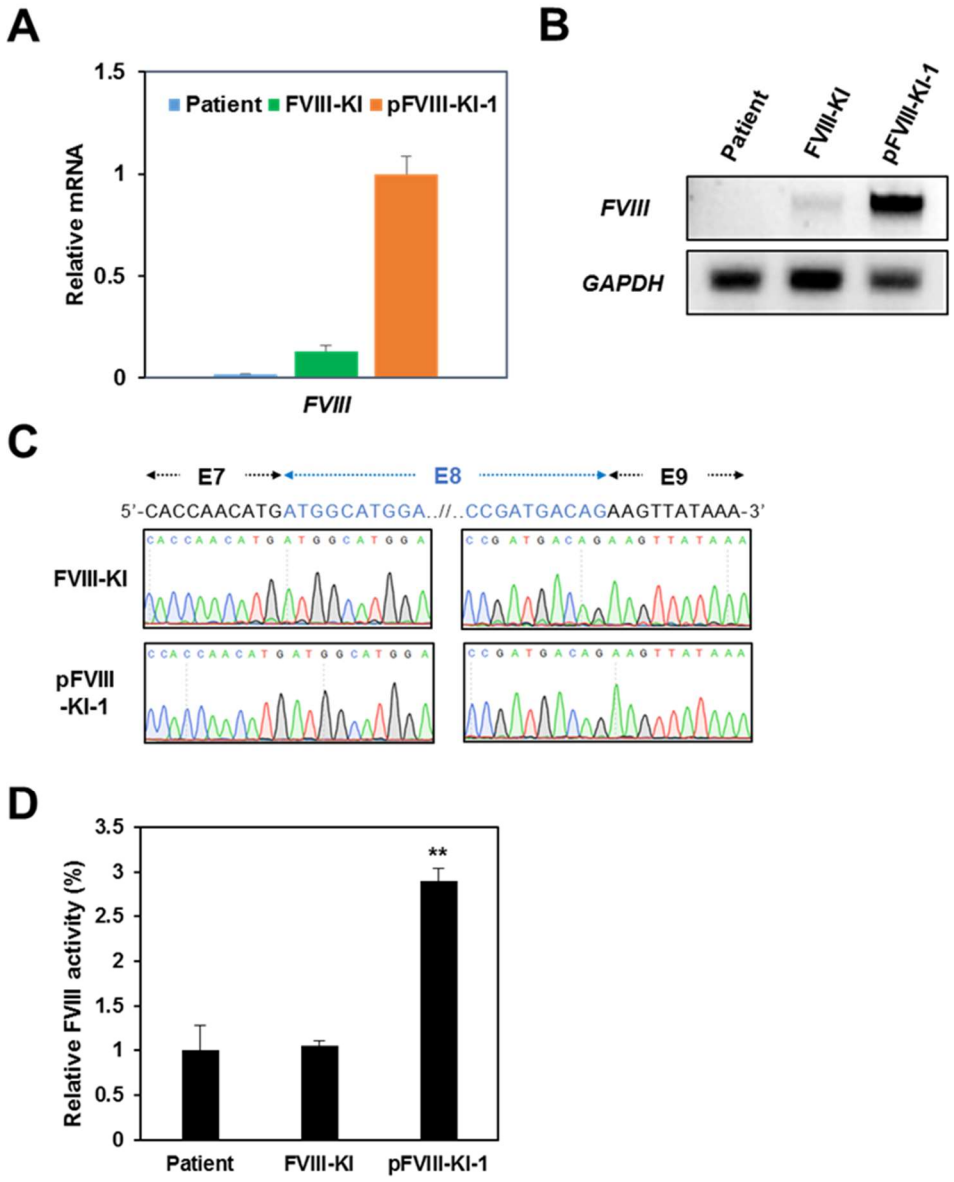


Figure 15. Restoration of FVIII expression in the gene-corrected iPSC-derived endothelial cells. (A) qPCR analysis of *FVIII* transcript in patient and gene-corrected iPSC line-derived endothelial cells. The *FVIII* transcript was amplified with primers based on exon 7 and exon 10. (B) Expression of *FVIII* and endothelial cell markers *CD31* and *vWF* were analyzed by RT-PCR. (C) Sanger sequencing

analysis of *FVIII* amplicons from gene-corrected iPSC-derived endothelial cells with restored exon 7 to exon 9 sequence. (D) The FVIII activity of cell culture supernatants from either patient or FVIII-KI line derived endothelial cells. (E) The FVIII activity of cell culture supernatants from either patient or pFVIII-KI-1-derived endothelial cells. FVIII activity was determined in 5×10^5 endothelial cells per single detection. **$p<0.01$ compared to the patient control.

Table 5. Primer set for qRT-PCR analysis

Primers	Sequence	Targets
GAPDH-F	TGCACCACCAACTGCTTAGC	qRT-PCR for pluripotency markers
GAPDH-R	GGCATGGACTGTGGTCATGAG	
OCT4-F	CCTCACTTCACTGCACTGTA	
OCT4-R	CAGGTTTTCTTTCCCTAGCT	
SOX2-F	TTCACATGTCCCAGCACTACCAGA	
SOX2-R	TCACATGTGTGAGAGGGGCAGTGT	
LIN28-F	AGCCATATGGTAGCCTCATGTCCG	
LIN28-R	TCAATTCTGTGCCTCCGGGAGCAG	
CD31-F	TGCGAATCGATCAGTGGA	qRT-PCR for Endothelial cell markers
CD31-R	ACCGGGGCTATCACCTTC	
vWF-F	TCGGGCTTCACTTACGTTCT	
vWF-R	CCTTCACTCGGACACACTCA	
F8-RT-E7–F	TCT TGT GAG GAA CCA TCG CC	qRT-PCR for F8
F8-RT-E10–R	ACA TCA GTG ATT CCG TGA GGG	

IV. DISCUSSION

In this study, we used iPSCs derived from a severe hemophilia A patient with a gross deletion of *FVIII* from exon 8 to exon 22. With this mutation, one possible approach for to restore FVIII expression might be achieved by inserting the cDNA sequence spanning exon 8 to exon 22 in the patient's *FVIII* locus. However, this approach only applies to one specific event but not for other *FVIII* mutant variants in hemophilia A. As we discussed above, we hypothesized that insertion of *FVIII* transgene into specific locus of human genome is a suitable method for the universal gene-correction to overcome this limitation. In case of hemophilia B, the second most abundant hemophilia type caused by mutation of factor IX (FIX), there have been efforts to use *FIX* locus itself to express the *FIX* gene. It is known that insertion of the *FIX* exon 2 to exon 8 sequence in intron 1 of the human *FIX* gene in a humanized hemophilia B mouse model restored the FIX expression via ZFN mediated *in vivo* gene correction[21]. Another recent report also showed that insertion of the *FIX* cDNA at exon 1 of the human *FIX* locus restored FIX expression in gene-corrected hemophilia B patient iPSC derived hepatocytes, both in *in vitro* and *in vivo* models[22].

Similar to these approaches, we designed a universal strategy to restore FVIII expression in patient-derived iPSCs. We inserted BDD-FVIII cDNA or EF1α promoter driven BDD-FVIII expression cassette inserted into exon 1 of *FVIII* locus in hemophilia A patient derived iPSC with high efficiency (94.59% for FVIII donor and 81.81% for EF1α-FVIII donor in initial PCR screening). We also checked the indel frequencies at off-target sites because unwanted mutations at off-target sites are an important risk factor in the use of engineered nucleases[23,24]. Our targeted deep sequencing data suggest that there were no significant mutations in analyzed off-target sites.

Recent findings indicate that liver sinusoidal endothelial cells are a major source of FVIII production, but other endothelial cell types, such as microvascular and lymphatic endothelial cells, can also generate the FVIII protein[25-27]. Our study

and other previous studies also show that the endothelial progenitor cells from human iPSCs could express *FVIII* mRNA and functionally active FVIII protein[11,12]. Moreover, FVIII transduced human primary endothelial cell progenitor cells are widely used for research into the *ex vivo* therapy for hemophilia A[28-30]. Therefore, we analyzed the restoration of FVIII expression in gene-corrected iPSC derived endothelial cells.

We could not detect significant elevation of FVIII activity although *FVIII* transcript was detected FVIII-KI line derived endothelial cells. However, we found out that insertion of *FVIII* cDNA with EF1α promoter resulted the restoration of both FVIII transcript and FVIII activity in pFVIII-KI-1 line derived endothelial cells. First, we observed 7.8-fold enhanced expression of *FVIII* mRNA in pFVIII-KI-1 line derived endothelial cell compare to FVIII-KI line derived endothelial cells. Second, we could see 2.9-fold elevation of FVIII activity from endothelial cells compare to patient iPSC or FVIII-KI lines derived endothelial cells. Obviously, these results indicate that EF1α promoter driven BDD-FVIII expression cassette insertion in exon 1 of *FVIII* gene is more suitable strategy for the restoration of FVIII expression rather than the BDD-FVIII cDNA expression based on the *FVIII* endogenous promoter.

The BDD-FVIII has a relatively small size (4.3kb compared to 7kb full length *FVIII* cDNA) and an enhanced expression capability compared to full length *FVIII* cDNA. Moreover, the B-domain is unnecessary for the coagulation activity of FVIII, so it is widely used in the gene therapy for hemophilia A[31-33]. However, it is also known that both B-domain deleted and full length *FVIII* cDNA have transcriptional repressor sequences that causes inefficient transcription[34,35]. Additionally, deletion of the B-domain also results in a reduced rate of FVIII secretion because it is related to the normal protein folding and efficient secretion of FVIII[33]. Especially, significant portion of the primary translated BDD-FVIII protein is misfolded and ultimately degraded[36]. Moreover, half-life of BDD-FVIII is shorter by approximately 3 hr compared with normal FVIII (approximately 12 hr)[37]. These properties of BDD-FVIII might have mildly increased FVIII activity

(2.9-fold increase) in pFVIII-KI-1 line derived endothelial cells even though we used the EF1α promoter for enhanced FVIII expression of BDD-FVIII at the human locus. Moreover, because the human *FVIII* locus is located in the X chromosome, only one copy of endogenous FVIII promoter or EF1α-driven *FVIII mRNA* transcription occurred per gene-corrected iPSC-derived endothelial cell. Therefore, we found relatively low expression of FVIII compared to the viral transduction of *FVIII* in human primary cells, which can result in multiple *FVIII* transgene insertions in one cell.

We hypothesize that using a modified coding sequence of *FVIII* with enhanced transcriptional and secretion ability might address these limitations in our future approach. Previous reports found the insertion of intron 1 of *FIX* gene into human *FVIII* cDNA or hybrid of porcine *FVIII* and human *FVIII* cDNA resulted enhanced the production or coagulant activity of FVIII[38,39]. Introducing 226 amino acids with an N-glycosylation site to the BDD form of FVIII also yielded a 10-fold increase in FVIII secretion[40]. Codon-optimized *FVIII* resulted in 29 to 44-fold enhancement of FVIII expression, and delivery of codon-optimized *FVIII* via a lentiviral vector resulted in FVIII levels in hemophilic mice that were more than 200% of those found in a normal human[41]. Although we could not use an improved version of FVIII in our experiment, the findings may provide future studies of our gene correction system with enhanced FVIII expression and secretion ability.

In this research, we provided evidences that insertion of the *FVIII* gene with an EF1α promoter at the *FVIII* locus could restore FVIII expression in endothelial cells from hemophilia A patient-derived iPSC. Although we used just one patient-derived iPSC line in this study, our gene correction strategy is applicable to a broad spectrum of *FVIII* mutations in hemophilia A because the *FVIII* gene inserted at the patient's *FVIII* locus is expressed regardless of *FVIII* mutant variation. These first proof-of-concept experiments demonstrate that the insertion of EF1α promoter with *FVIII* gene in human *FVIII* locus is a suitable strategy for the restoration of FVIII expression and provide a valuable and universal tool for future *ex vivo* cell therapy for hemophilia A.

V. CONCLUSION

The two most notable recent advances in biomedical science are the development of engineered nucleases and that of hiPSCs. Reprogramming somatic cells into a pluripotent state provides an unlimited source of potential cell therapy materials. It is already shown that combination of engineered nuclease and hemophilia A patient derived iPSCs could be applicable in *ex vivo* gene and cell therapy of hemophilia A. In this research, I provided another evidences that insertion of the *FVIII* gene with an EF1α promoter at the *FVIII* locus could restore FVIII expression in endothelial cells from hemophilia A patient-derived iPSC. These results suggest that insertion of EF1α promoter driven *FVIII* gene expression in human *FVIII* locus is a suitable universal strategy for the restoration of FVIII expression and provide a valuable tool for future ex vivo cell therapy for hemophilia A.

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ABSTRACT (IN KOREAN)

A형 혈우병 환자 유래 역분화 줄기세포에서 FVIII 위치에
유전자 삽입을 통한 혈액 응고인자 8번 단백질 발현 복구

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성 진 재

유전자가위인 ZFN, TALEN, 그리고 CRISPR/Cas9을 이용한 타겟 특이적 유전자 교정법은 인간의 유전질환을 치료할 수 있는 좋은 방법으로 여겨지고 있다. A형 혈우병은 응고인자 8번 (*FVIII*) 유전자의 돌연변이로 인해 일어나는 단일 유전자 질환으로 약간의 FVIII 단백질 양의 증가만으로도 중증 증세를 완화시킬 수 있는 특성이 있어, 유전자 교정 치료의 적절한 타겟 질환으로 볼 수 있다. 이 연구에서는 CRISPR/Cas9 유전자 가위를 이용하여, A형 혈우병 환자 유래 역분화 세포의 *FVIII* 유전자의 exon 1번에 B-domain이 제거된 *FVIII* cDNA를 cDNA 단독, 혹은 EF1α 프로모터와 함께 삽입하였다. 유전자 교정후, EF1α 프로모터와 *FVIII* cDNA를 동시에 삽입한 유전자 교정 역분화 줄기세포 유래 혈관세포에서 활성화 가능한 FVIII 단백질이 발현함을 확인할 수 있었다. 이 연구는 EF1α 프로모터에 의해 발현하는 정상 *FVIII* cDNA를 돌연변이가 있는 인간 *FVIII* 유전자 위치에 삽입하고, 이러한 시도에 의해 환자유래 역분화 줄기세포 유래 혈관세포에서 정상

FVIII를 발현할 수 있도록 할 수 있음을 보인 시도로써 그 의의가 있다고 볼 수 있다.

핵심되는 말 : 역분화 줄기세포, A형 혈우병, 혈액응고인자 8번, type II clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), 유전자 교정, 혈관내피세포

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