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Supplemental Information

Universal Correction of Blood Coagulation Factor VIII in Patient-Derived Induced Pluripotent Stem Cells Using CRISPR/Cas9

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Supplemental Figure 1

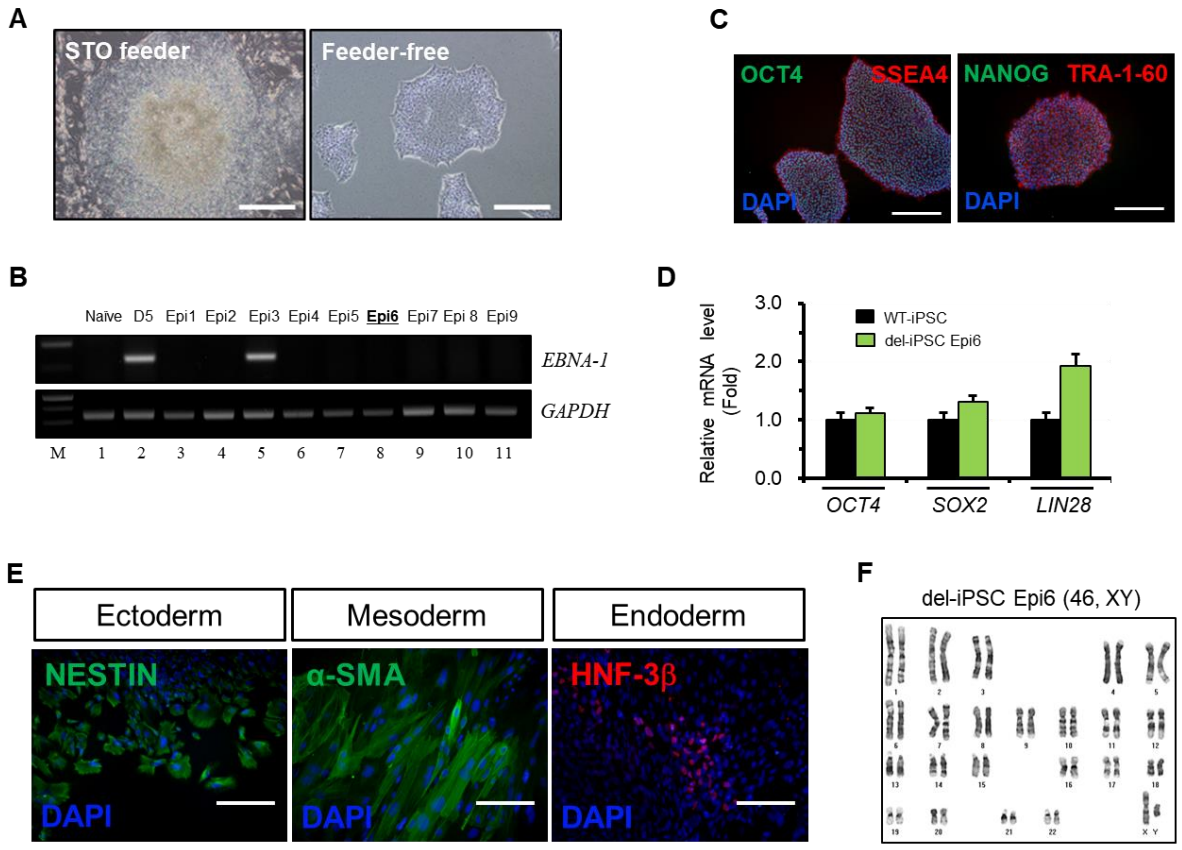

Figure S1. Generation of *FVIII* Deleted Patient-iPSCs.

(A) Human ESC-like morphology of expanded patient-iPSCs (Epi6) onto a STO feeder layer or in a feeder-free culture conditions. Scale bars, 500 μm (feeder layer) or 200 μm (feeder-free culture).

(B) Detection of episomal vector sequence (*EBNA-1*) that remained in established iPSC clones (Epi1 to Epi9). The *GAPDH* gene is used as a quality control for isolated total DNA. Total DNA isolated from the cells before (naïve) and after electroporation (day 5, D5) was used as negative and positive controls for episomal vector DNA, respectively.

(C) The expression of the pluripotency markers OCT4, NANOG, SSEA-4, and TRA-1-60 detected by immunocytochemistry. The DAPI signal indicates the total cell content in the image. Scale bar, 200 μm.

(D) The mRNA quantification of endogenous *OCT4*, *SOX2*, and *LIN28* from the indicated clones were measured by qPCR and normalized to *GAPDH* expression. Data are means ± SEM of three independent experiments.

(E) The expression of marker proteins representing ectoderm (NESTIN), mesoderm (α-

smooth muscle actin, α-SMA), and endoderm (hepatocyte nuclear factor-3β, HNF-3β). Scale bar, 100 μm. The DAPI signal indicates the total cell content in the image.
(F) G-banding analyses were performed in the Epi6 clones. Related to Figure 1.

Supplemental Figure 2

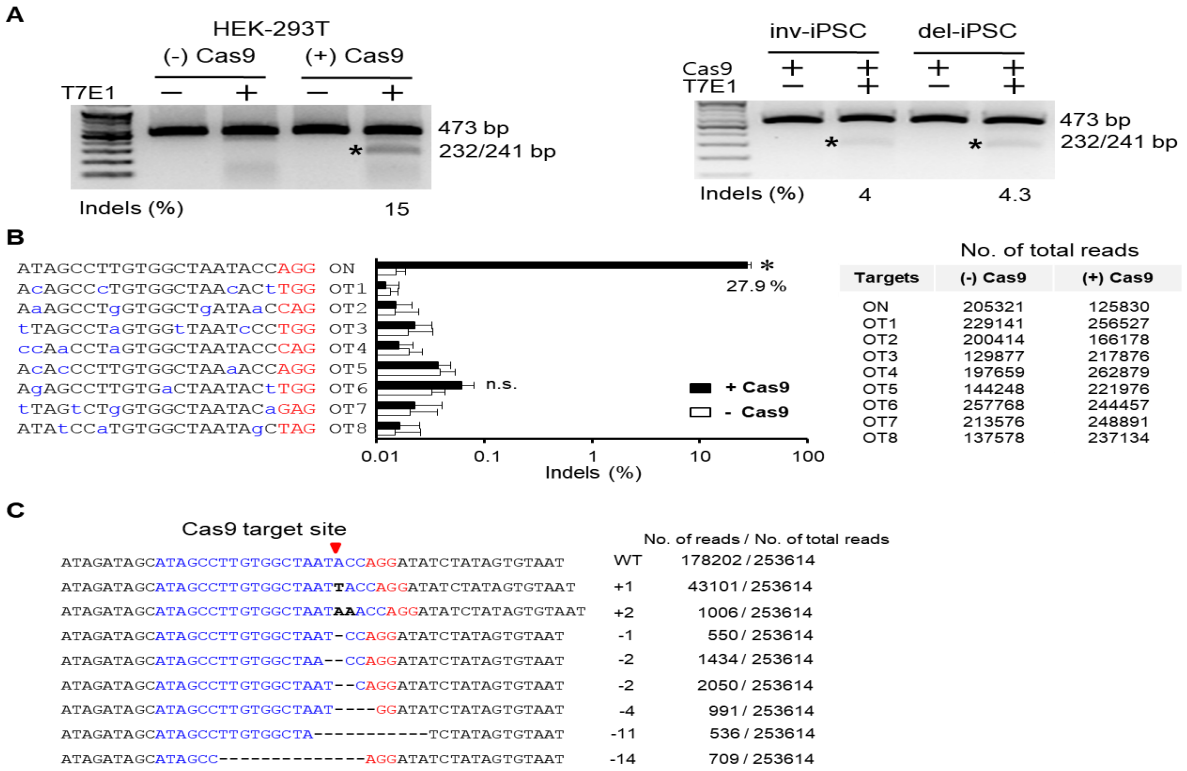

Figure S2. sgRNA design for the human *H11* locus and validation of nuclease activity at on- and off-target sites.

(A) Indels frequencies at the on-target site were analyzed in HEK-293T cells (left) and each iPSC clone (right) using the T7E1 assay. The asterisk indicates the predicted position of DNA bands cleaved by T7E1.

(B) Indels in the on- and off-target site were analyzed by targeted deep sequencing. Eight potential off-target sites in the human genome that differed from the on-target site by up to four nucleotides were examined in HEK-293T cells. Mismatched nucleotides and PAM sequences (5'-NRG-3', R = A or G) are shown in blue and in red, respectively. Data are means ± SEM of three independent experiments. The total number of reads is provided as an indicator of deep sequencing sensitivity. n.s., not significant compared with absence of Cas9; *, $p < 0.001$ compared with absence of Cas9 (Student's t-test).

(C) Data indicates the partial DNA sequences of indels that are analyzed from deep sequencing. The number of inserted or deleted bases is indicated in the right column. Red arrow indicate cleavage position in the target sequences. The number of reads with WT and each edited sequence is indicated. Related to Figure 1.

Supplemental Figure 3

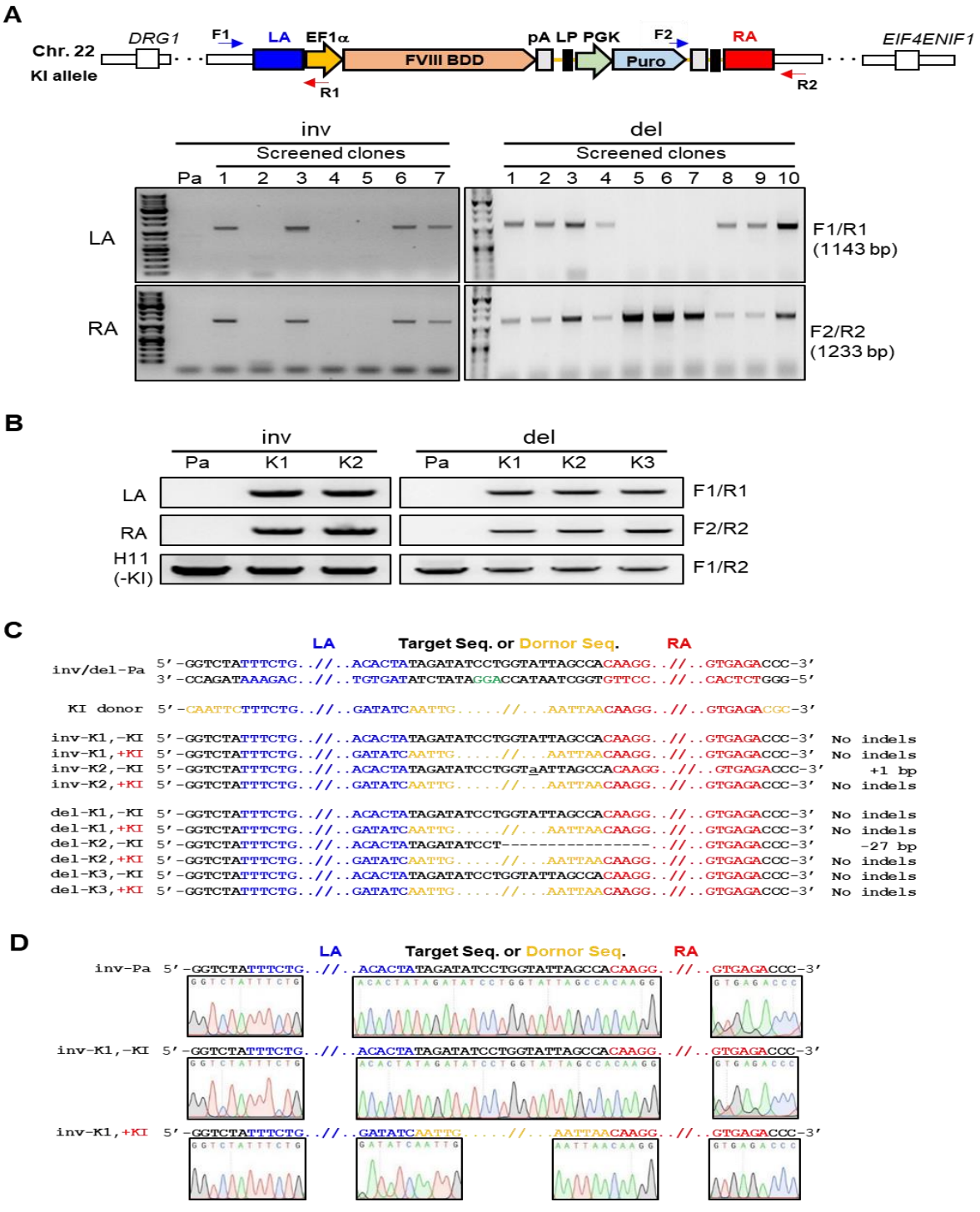

Figure S3. Targeted insertions of the *FVIII* donor DNA.

(A) Genome structure of *FVIII* inserted allele in the target site. The four specific primers used for genotyping are shown. PCR-based screening for targeted knock-in using the four specific primers listed in Table S1 (lower).

(B) PCR-based genotype analysis to confirm the targeted insertion of donor DNA in the

indicated iPSCs. The four specific primers used for genotyping are shown.

(C) The DNA sequences of knock-in junctions in the indicated iPSC clones including the template donor DNA. Underlined lowercase letter indicates inserted base. Dashes indicate deleted bases. The donor sequences are shown in deep yellow. The original genome sequences are shown in black. The left and right arm sequences are shown in blue and red, respectively. The PAM sequence is shown in green.

(D) Chromatograms showing the targeted insertion of donor DNA in the corrected clones. Related to Figure 1.

Supplemental Figure 4

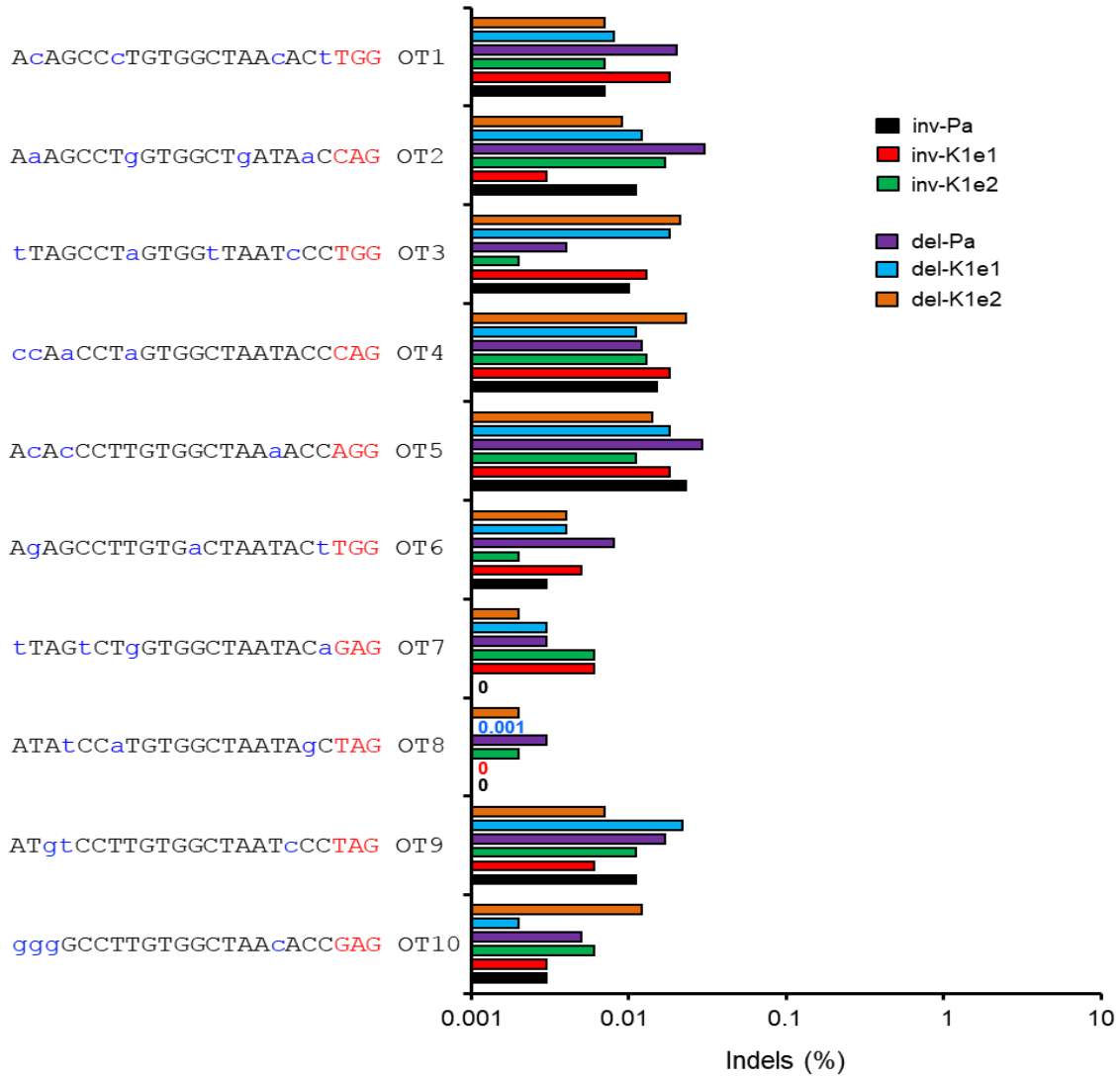

Figure S4. Off-target analyses in the corrected iPSCs by targeted deep sequencing.
Ten potential off-target sites differing by up to four nucleotides from the on-target site were examined in the corrected clones by targeted deep sequencing. Mismatched nucleotides and PAM sequences (5'-NRG-3', R = A or G) are shown in blue and in red, respectively. Related to Figures 1 and 2.

Table S1. Sequences of primers used in this study. Related to Figures 1, 2, 3, and 4.

Primer[a]	Sequence (5' to 3')	Used for the experiment of
F1	CAACCCAGTCCTCCTTACCTTGC	genotype PCR
R1	CCGTTGCGAAAAAGAACGTTCAC	genotype PCR
F2	GCCTGAGGGGATCAATTCTCTAG	genotype PCR
R2	GTTAGGCCTGTGTCAACAGTTTGG	genotype PCR
F3	GGCCCTTTCGTCTTCAAGAATTCCG	genotype PCR
H11-F	GTGAGCTAAGGAATGTGATACAG	T7E1 assay
H11-R	AGGCTGAGACAGAAGAATCGCCT	T7E1 assay
Oct4-F	CCTCACTTCACTGCACTGTA	qPCR
Oct4-R	CAGGTTTTCTTTCCCTAGCT	qPCR
Sox2-F	CCCAGCAGACTTCACATGT	qPCR
Sox2-R	CCTCCCATTTCCCTCGTTTT	qPCR
Lin28-F	AGCCAAGCCACTACATTC	qPCR
Lin28-R	AGATACGTCATTCGCACA	qPCR
DRG1-F	ACGATGAGCAGCACCTTAGC	qPCR
DRG1-R	CGACGAAGCTTAGCAAGACG	qPCR
EIF4ENIF1-F	GACCTGAAGAAGCCTCCTGCCTC	qPCR
EIF4ENIF1-R	TTCTCAGGGTCCCAGACACCATC	qPCR
GAPDH-F	TGCACCACCACCTGCTTAGC	qPCR and RT-PCR
GAPDH-R	GGCATGGACTGTGGTCATGAG	qPCR and RT-PCR
F8-exon21-F	CCGGATCAATCAATGCCTGGAG	qPCR and RT-PCR
F8-exon23-R	ATGAGTTGGGTGCAAACGGATG	qPCR and RT-PCR
F8-exon6-F	GCCTGGCCTAAAATGCACACAGTC	RT-PCR
F8-exon7-R	ATTGAGTGCACTTCAGGAGTGGTG	RT-PCR
CD31-F	GGTCAGCAGCATCGTGGTCAACATAAC	qPCR
CD31-R	TGGAGCAGGACAGGTTCAGTCTTTCA	qPCR
vWF-F	TCGGGCTTCACTTACGTTCT	qPCR
vWF-R	CCTTCACTCGGACACACTCA	qPCR
CRE-F	CTACCTGTTTTGCCGGGTCAGAAA	PCR
CRE-R	AAACTCCAGCGCGGGCCATATCTC	PCR
GAPDH-F	GAACATCATCCCTGCCTCTACTG	PCR
GAPDH-R	CAGGAAATGAGCTTGACAAAGTGG	PCR
EBNA-1-F	ATGGACGAGGACGGGGAAGA	PCR
EBNA-1-R	GCCAATGCAACTTGGACGTT	PCR

[a] Primer direction: F, forward; R, reverse

Table S2. Summary of targeting efficiency by PCR screening and Sanger sequencing. Related to Figure 1.

	iPSCs	No. of puromycine resistant colonies/ $5x10^5$ iPSCs	No. of PCR (+) colonies/ No. of screened colonies	No. of targeted KI clones by sequencing/ No. of screened clones	No. of targeted KI clones confirmed by FVIII activity/ No. of screened clones
Pre-excision of puro cassette	Inv	25	9/14 (64.3%)	2/2	2/2
	Del	37	18/27 (66.7%)	3/3	3/3
Post-excision of puro cassette	Inv	-	6/7 (85.7%)	3/3	3/3
	Del	-	5/7 (71.4%)	2/2	2/2

Table S3. Sequences of each target site and primers used in on- and off-target amplification. Related to Figures 1 and 2.

Target site	Target Seq. (5' to 3')	Forward primer Seq. (5' to 3')	Reverse primer Seq. (5' to 3')
On-target (On, Chr. 22)	ATAGCCTTGTGGCTAATACC	GATTTGTTTGAGAGAAACTACC	CCTTGAGCTTTAAAGACCCC
Off-target 1 (OT1, Chr. 17)	ACAGCCCTGTGGCTAACACT	ATGGTTAGCAGTCAACCGTG	CCACAAACAAGAGGTCATCC
Off-target 2 (OT2, Chr. 9)	AAAGCCTGGTGGCTGATAAC	CTTCACTACCATCCCCCACT	ACTTTTGACATCCATCGCCG
Off-target 3 (OT3, Chr. 6)	TTAGCCTAGTGGTTAATCCC	TTAGCACTAAGATGGACCAC	TACCTGCTTGGCACCATGTC
Off-target 4 (OT4, Chr. 20)	CCAACCTAGTGGCTAATACC	GTAGTTTTAAAATAGGAGTCAGGG	GGTTCAATAACCATTGTTCCC
Off-target 5 (OT5, Chr. 5)	ACACCCTTGTGGCTAAAACC	AGTGGACAGAATTCCCTCTG	TACACTAGGGGTCATTGAGC
Off-target 6 (OT6, Chr. 14)	AGAGCCTTGTGACTAATACT	AGGAAAGCGTCTACTGTTAG	GTGTTAAATCTAGTGTGTTGC
Off-target 7 (OT7, Chr. X)	TTAGTCTGGTGGCTAATACA	CCTTCAGAGTGGAATGCTAT	ATTAGAAGCGTCCTGGTAGG
Off-target 8 (OT8, Chr. 4)	ATATCCATGTGGCTAATAGC	CAGGCCAGACTATAGAAGTT	AAGTTCAAATGCCCAATGGG
Off-target 9 (OT9, Chr. 7)	ATGTCCTTGTGGCTAATCCC	TCTCTGGGGCTGAAACCCAA	GGAGGTCTTTGTGTCTTAGC
Off-target 10 (OT10, Chr. X)	GGGGCCTTGTGGCTAACACC	AGACCTATCAGAGAGCCTAG	TAAGTTCCCCACAGCATCTC

Table S4. Analysis of potential off-target sites with whole genome sequencing. Related to Figures 1 and 2.

Variants		del-Pa	del-K1e1	del-K1e2
All variants	Single nucleotide polymorphisms (SNPs)	3,489,220	3,565,265	3,536,560
	Small insertions and deletions (INDELs)	539,134	588,046	565,157
	Structural variations (SVs)	5,170	5,556	5,546
	Copy number variations (CNVs)	703	659	730
Filtered Variants (variants not present in the common variant database; 1000 genomes variants)	SNPs	373,168	387,401	365,403
	INDELs	333,263	375,037	303,124
Clone specific variants	SNPs	22,946	28,240	20,739
	INDELs	41,695	67,897	31,086
Candidate variants at the 2,325 potential off-target sites (by up to six nucleotides mismatches)	SNPs and INDELs	Not applicable	0	0

Supplementary experimental procedures

Cell Cultures

HEK-293T (ATCC) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. Adipose tissue-derived mesenchymal stem cells (MSCs) isolated from an *FVIII* deleted patient were cultured in DMEM (low glucose) supplemented with 10% FBS, 0.0145 g/L ascorbic acid, and 1% antibiotics on collagen type I coated plates. Human wild-type iPSC (WT-iPSCs Epi3 line) (Park et al., 2014), *FVIII* inverted patient-derived iPSCs (Pa2, intron 22 inverted iPSCs Epi5 line) (Park et al., 2015b), *FVIII* deleted patient-derived iPSCs (Epi6 line), and *FVIII* knock-in clones generated from the *FVIII* inverted or deleted iPSC clones were maintained in iPSC culture medium [DMEM/F12 medium containing 4 ng/mL basic fibroblast growth factor (bFGF; PeproTech), 20% knockout serum replacement (Invitrogen), 1% nonessential amino acids (Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma)]. In some experiments, iPSC clones were cultured in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec) according to the manufacturer's instructions for feeder-free culturing.

Preparations of Donor Plasmid and Guide RNA for SpCas9

To construct the donor plasmid, we used the pCDNA4/BDD-FVIII plasmid (www.addgene.org, no. 41035) (Peters et al., 2013) as a backbone. The *MfeI* and *MauBI* restriction sites in the backbone were used to insert 5'-homology arm (Left arm, LA) and 3'-homology arm (Right arm, RA) by In-Fusion cloning, respectively. Next, the human EF1α promoter sequence was cloned between the left arm and the *FVIII* open reading frame. Afterwards, the puromycin expression cassette flanked by *loxP* sites was inserted between the *FVIII* gene cassette and the right arm by In-Fusion cloning. After the construction of the donor plasmid, we verified the sequences of the cloned DNA by Sanger sequencing at Solgent, Inc. (Korea). The recombinant Cas9 protein derived from *Streptococcus pyogenes* (SpCas9) was purchased from ToolGen, Inc. (Korea). A 5'-GGX$_{20}$ sgRNA (5'-ATAGCCTTGTGGCTAATACC-3') for SpCas9 was transcribed *in vitro* under the control of the T7 promoter using the MEGAshortscript™ kit (Ambion) according to the manufacturer's protocol followed by purification as previously described (Kim et al., 2014).

RNA Isolation, RT-PCR, and Quantitative PCR (qPCR) Analysis

Total RNAs were purified from cells using the Easy-Spin™ Total RNA Extraction Kit (iNtRON Biotech) according to the manufacturer's instructions. The cDNAs were synthesized from 1 μg of purified total RNA using the PrimeScript™ RT Master Mix (Takara Bio). For quantification of

the mRNA levels, qPCR was performed using the SYBR® Premix Ex-Taq (Takara Bio) and a CFX96 Real-Time System (Bio-Rad). Ct values for each gene were normalized to the Ct values for *GAPDH*. Semiquantitative RT-PCR was performed using the EmeraldAmp® GT PCR Master Mix (Takara Bio). For the amplification of *FVIII* mRNA from the knock-in clones, a forward primer located in exon 21 was used in combination with a reverse primer located in exon 23. To confirm the expression of *vWF* and *CD31* mRNA, we used previously reported primer sets (Wang et al., 2007; Morita et al., 2015). The specific primer sequences are shown in Table S1.

T7E1 Assay

To validate the activity of the CRISPR/Cas9 designed for the target site, a T7E1 assay was performed as previously described (Guschin et al., 2010, Park et al., 2015a). In brief, HEK-293T cells were co-transfected with 1 μg SpCas9 plasmid and 2 μg sgRNA plasmid purchased from ToolGen, Inc. (Korea) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Four days after co-transfection, genomic DNA segments encompassing the nuclease target sequence were amplified using high fidelity PrimeSTAR® Max DNA polymerase (Takara Bio) and the specific primer set listed in Table S1. The PCR amplicon was treated with T7E1 to cut the mismatched DNA. Following agarose gel electrophoresis, the band intensities of the uncleaved and cleaved fragments were determined.

Generation of iPSCs

The *FVIII* deleted patient-iPSCs were generated from MSCs obtained from a patient with severe HA who was clinically confirmed by the Korea Hemophilia Foundation Clinic using episomal reprogramming vectors (pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL; no. 27077, 27078, and 27080 respectively; www.addgene.org) as previously reported (Okita et al., 2011). Briefly, expanded MSCs were electroporated with reprogramming vectors (1 μg each, total 3 μg) using the Neon Transfection System (Life Technologies). After being pulsed three times at a voltage of 1,650 for 10 ms, the cells were grown further in low glucose DMEM supplemented with 10% FBS on collagen type I coated plates. A week after electroporation, the cells were transferred onto mouse SIM Thioguanine/Ouabain-resistant mouse fibroblast cell line (STO) fibroblasts (ATCC) as a feeder layer. Human ESC-like iPSCs colonies were picked mechanically and further cultured for characterization. Afterwards, iPSCs were adapted in a feeder-free culture conditions for use in the *FVIII* knock-in experiments.

Three Germ Layer Differentiation

The *In vitro* differentiation of the iPSCs into three germ layers was performed as previously

described (Xu et al., 2017). The iPSCs were partially dissociated into clumps using collagenase type IV (Invitrogen). The clumps were then transferred onto low-attachment tissue plates to form embryonic bodies (EBs) and cultured in 5% FBS containing iPSC culturing medium without bFGF for a week. Next, the EBs were attached on Matrigel-coated coverslips and further cultured for 10 days to allow spontaneous differentiation of EBs into cells representing the three germ layers.

CRISPR/Cas9-Mediated Correction by *FVIII* Knock-in

Cas9 ribonucleoproteins (RNPs) and *FVIII* Knock-in donor DNA were electroporated into the patient-iPSCs as previously described (Park et al., 2015b) with slight modifications. Briefly, the SpCas9 protein (15 μg) was mixed with 20 μg of *in vitro* transcribed sgRNA and incubated for 10 min at room temperature to allow the formation of Cas9 RNPs. The patient iPSCs were pretreated with a 10 μM ROCK inhibitor (Y-27632, Sigma) for at least 2 hr before electroporation. After washing with PBS, the cells were treated with 1 × Versene solution (Life Technologies) for 3 min. Next, the cells were scraped and dissociated into approximately single cells. After centrifugation, 5×10^5 cells were electroporated with Cas9 RNPs and 3 μg donor DNA using the Neon Transfection System (Life Technologies). After being pulsed one time at a voltage of 950 for 10 ms, the cells were cultured in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec) with 10 μM Y-27632 for 1 day. Four days post-transfection, the cells were selected using 0.5 μg/mL puromycin. To isolate clonal populations of the targeted cells, we performed three rounds of passaging.

PCR Analysis and DNA Sequencing of the Knock-in Junction

Genomic DNA was isolated from the cells using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. To confirm the targeted knock-in of donor DNA into the *H11* site, the DNA fragments for each knock-in junction were amplified using the EmeraldAmp® GT PCR Master Mix (Takara Bio) and the specific primer sets listed in Table S1. Following PCR purification, the sequence of each DNA amplicon was verified by Sanger sequencing at Solgent, Inc. (Korea).

Droplet Digital PCR (ddPCR) Analysis

Genomic DNA purified from each iPSC clone was subjected to ddPCR analysis to determine the copy number of the knock-in fragment using ddPCR SuperMix (without UTP; Bio-Rad) according to the manufacturer's protocol. The ddPCR reaction mixtures were converted into approximately 20,000 droplets using the QX200 Droplet Generator (Bio-Rad) and then were transferred into a 96-well plate for thermal cycling. Following PCR, the droplets were read

using the QX200 Droplet Reader (Bio-Rad) and were analyzed using Quantasoft software (Bio-Rad). The copy numbers were determined by calculating the ratio of the target molecule concentration to that of the reference molecule using the following equation: copy number = (*A*/*B*) N_B (A: concentration of target species, B: concentration of reference species, N_B: number of copies of reference loci in the genome). The following primer sets were used for ddPCR: Intron1-F: 5'-CGGGTTAGGATGGTTGTGATG-3' and Intron1-R: 5'-ATGACGAAGAGAAGCAATGGAC-3'; F8-Exon21-F: 5'-CGGATCAATCAATGCCTGGAG-3' and F8-Exon23-R: 5'-GAAGAGTGCTGCGAATGCT-3'. The primers for human *RPP30* (Bio-Rad; dHsaCP1000485) were used as a reference.

Whole-Genome Sequencing and Variant Calling

Genomic DNA was extracted from each clone using the G-DEX™ IIc genomic DNA extraction kit (iNtRON Biotech) according to the manufacturer's protocols. To construct the sequencing library, purified DNA was randomly fragmented, followed by 5' and 3' adaptor ligation. Adaptor-ligated libraries were then PCR amplified and gel purified. The libraries were subjected to sequencing using the Illumina NovaSeq6000 at Macrogen (South Korea). The sequencing depth was 40X. The sequencing data were converted into raw data using illumine package bcl2fastq (ver. 2.20.0). Adapters are not trimmed away from the reads. The paired-end sequences generated by the NovaSeq instrument were mapped to the human genome using Isaac Aligner (ver. 01.15.02.08) and the UCSC assembly hg19 (original GRCh37 from NCBI, Feb. 2009) was used as the reference sequence. Variants were called using the Isaac Variant Caller (ver. 2.0.13) for single-nucleotide variants and small indels, Control-FREEC (ver. 6.4) for copy-number variants, and Manta (ver. 0.20.2) for structural variants. We applied bioinformatic filters to more than three million variants to discard common variations listed in a public database (1000 Genomes Project, ftp://ftp.1000genomes.ebi.ac.uk). We then focused on identification of small indels including single nucleotide variations. We also identified unique variants induced in each clone by removing variations that were found simultaneously in all clones including the patient iPSC clone, because those were not induced by the nuclease. In parallel, we searched for 2,325 potential off-target sites that differed up to six nucleotides from the on-target site using Cas-OFFinder (www.rgenome.net) (Bae et al., 2014). To identify candidate variants as off-target mutations, we then compared 2,325 potential off-target sites with specific variants found in the each corrected clone.

Differentiation into Endothelial Cells

To induce the differentiation of the iPSCs into endothelial cells (ECs), we used a previously described protocol (Harding et al., 2017) with slight modifications. Briefly, the iPSCs were

pretreated with a 10 μM ROCK inhibitor (Y-27632, Sigma) for at least 2 hr before passaging. After washing with PBS, the cells were treated with 1× Versene solution (Life Technologies) for 3 min. Next, the cells were scraped and dissociated into small clumps. After centrifugation, the clumps were seeded onto Matrigel-coated culture dishes and cultured further in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec) with 10 μM Y-27632 for 1 day. After 2 days, the culture medium was changed to STEMdiff™ APEL™ Medium (STEMCELL Technologies) with 6 μM CHIR99021 (Tocris Bioscience) for 2 days to induce the mesoderm. Afterwards, the cells were cultured in STEMdiff™ APEL™ Medium with 25 ng/mL bone morphogenetic protein 4 (BMP4, R&D Systems), 10 ng/mL bFGF (PeproTech), and 50 ng/mL vascular endothelial growth factor (VEGF, R&D Systems) for 2 days to induce vascular progenitors. Finally, the cells were cultured in EC Growth Medium MV2 (ECGM-MV2, PromoCell) with 50 ng/mL VEGF to generate endothelial progenitors, followed by media changes every 2 days.

Immunofluorescence Staining and Karyotype Analysis

Immunofluorescence staining was performed as previously described (Park et al., 2015a). Briefly, cells were fixed in a 4% paraformaldehyde solution containing 0.2% Triton X-100 for 10 min at room temperature (RT), washed three times with PBS, and incubated in blocking solution containing 5% normal goat serum and 2% bovine serum albumin for 1 hr at RT. Next, the cells were incubated with the primary antibodies for 2 hr at RT. The following primary antibodies were used: anti-OCT4 (Santa Cruz, SC9081), anti-NANOG (Abcam, AB21624), anti-SSEA-4 (Millipore, MAB4304), anti-TRA-1-60 (Millipore, MAB4360), anti-NESTIN (Millipore, MAB5326), anti-α-SMA (Sigma, A5228), anti-HNF-3β (Santa Cruz, SC6554), anti-CD31 (BD Bioscience, 555444), and anti-vWF (Millipore, AB7356). Afterwards, the cells were washed twice with PBS and incubated with fluorescence-conjugated secondary antibodies (Alexa Fluor® 488 or 594; Invitrogen) for 1 hr at RT. The cells were then washed again with a PBS containing 0.1% Tween 20 and mounted onto coverslips using a mounting medium. For the visualization of nuclei, 4′, 6-Diamidino-2-Phenylindole (DAPI; Vector Laboratories) was used. The images were captured and analyzed using an Olympus IX71 fluorescence microscope. For karyotype analysis, the chromosomes isolated from each iPSC clone were stained with Giemsa for G-banding analysis and analyzed using the Chromosome Image Processing System at GenDix Inc. (Korea).

Measurement of FVIII Activity

To measure FVIII activity, culture supernatants were concentrated 20-fold using an Amicon® Ultra-4 centrifugal filter (Millipore). The FVIII activities were measured in the culture

supernatants using the commercially available Coamatic® Factor VIII chromogenic assay kit (Instrumentation Laboratory) according to the manufacturer's instructions. The FVIII activity measurements were performed in a 96-well microplate and the absorbance at 405 nm was determined by an endpoint reading using a microplate reader (Molecular Devices). A standard curve was prepared by diluting the human calibration plasma (Instrumentation Laboratory).

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