CRISPR/Cas9-mediated knockin of human factor IX into swine factor IX locus effectively alleviates bleeding in hemophilia B pigs

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Supplementary Information

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Supplementary Methods

Plasmid construction

pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid #42230). First, two paired oligonucleotides were designed based on porcine *F9* sequences. Each pair of oligonucleotides formed a double strand by annealing. The mixture, which contained a pair of oligonucleotides and 10× standard Taq buffer (NEB, Beijing, China), was boiled for five minutes in boiling water and then incubated in the water until the temperature decreased to 30°C. The pX330 plasmid was digested with BbsI and ligated with annealed oligonucleotides to construct sgRNA-expressing vectors.

The 5′ and 3′ homology arms were PCR-amplified from porcine genomic DNA using Taq DNA polymerase (TIANGEN, KT205). The coding sequence of human *F9* was produced by PCR from liver cDNA. The homology arms, human *F9* CDS and pLB vector were assembled together to generate the donor plasmid. The detailed sequences are provided in Supplementary Table 4.

Cell culture and selection

Thirty-three-day-old fetuses were separated from Large White pigs, and primary porcine fetal fibroblasts (PFFs) were isolated from these fetuses. After removing the head, tail, limb bones, and viscera, these fetuses were cut into small pieces. The samples were digested for 4-6 h in culture medium containing 20% fetal bovine serum (Gibco), 12.5 μg/ml DNase I (2,000 U/mg, Sigma, St. Louis, MO), 200 U/ml collagenase IV (type IV, 260 U/mg, Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Gibco). The cells were resuspended and cultured in 10-cm cell culture dishes until the cell density reached 90%. The isolated cells were frozen in fetal bovine serum

containing 10% dimethyl sulfoxide. After recovery, the cells were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 15% fetal bovine serum and maintained at 39°C in a humidified atmosphere containing 5% CO2.

Approximately 3 × 10^6 PFFs and the corresponding plasmids (KO: 20 μg of the Cas9-sgRNA1 targeting vector and 20 μg of the Cas9-sgRNA2 targeting vector; KI: 20 μg of the Cas9-sgRNA1 targeting vector, 20 μg of the Cas9-sgRNA2 targeting vector and 30 μg of the donor vector) were suspended in 300 μL of Opti-MEM (Gibco, Grand Island, New York, USA). The mixture was electroporated by using the manufacturer's specified parameters with a BTX-ECM 2001 instrument (Harvard Bioscience, Holliston, MA).

To acquire single-cell clones by limited dilution, the electroporated cells were inoculated into fifteen 10-cm culture dishes 72 h post transfection. The average cell inoculation density was 3,500 cells/dish. After 9 days of culture, single-cell clones were selected and expanded in 24-well plates. After a confluence of 80% or more was reached, 20% of each cell clone was digested and lysed with 10 μl of NP40 lysis buffer for 1 h at 56°C, followed by 10 min at 95°C for PCR screening. In addition to using primers that identified the knockout positive clones, we also performed a 5′-junction and 3′-junction PCR to test cell clones for the site-specific insertion of human *F9* CDS. All PCR products were visualized on 1.0% agarose gels and sequenced. These primers are listed in Supplementary Table 5.

Quantitative real-time PCR and western blot analysis

For quantitative real-time PCR, the livers of the two-month-old cloned pigs were harvested after euthanasia for RNA extraction. Total RNA was isolated using TRIzol A+ reagent (Tiangen, China)

according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 ug of total RNA using a FastQuant RT Kit (Tiangen, China), and the samples were analyzed with a Quant qRT-PCR kit (Tiangen, China) with gene expression primers for porcine *F9*, human *F9* and porcine *GAPDH*. The sequences of the primers are listed in Supplementary Table 6.

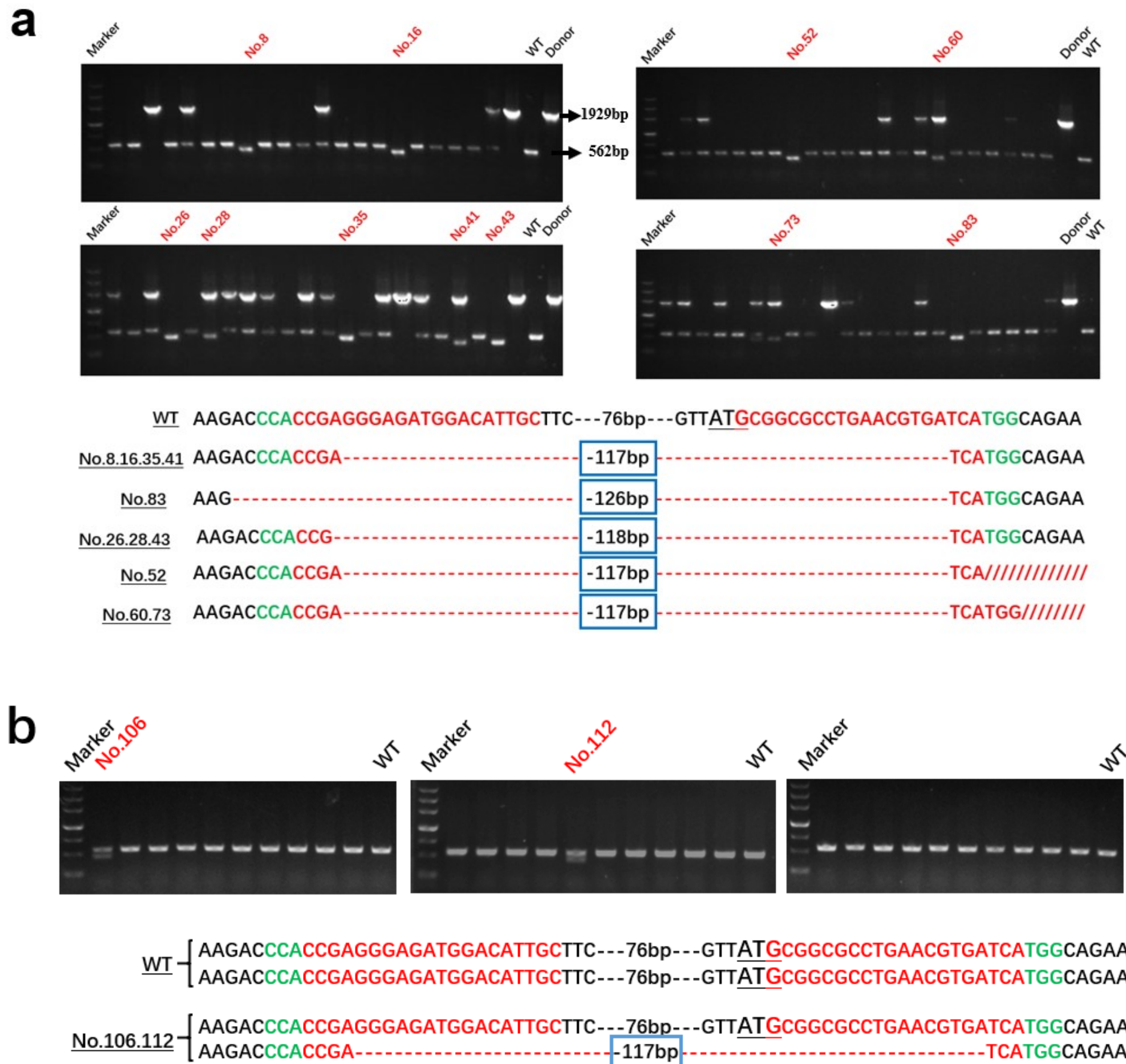
For western blot analysis, we collected the livers in cold phosphate-buffered saline (PBS) and lysed the livers with lysis buffer (Beyotime, China) containing a 100× phosphatase inhibitor and 1 mM PMSF for 1 h on ice. Protein concentrations were determined using an enhanced bicinchoninic acid (BCA) protein assay kit (Beyotime, China). Sixty micrograms of each protein sample were separated by 10% SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane and subsequently blocked for 2 h at room temperature in 5% nonfat milk in TBST. The membranes were incubated with primary antibodies (antibodies against Factor IX (ab97619, Abcam, UK) and GAPDH (60004-1-Ig, Proteintech, USA), 1:1000 dilution) overnight at 4°C, washed with TBST three times and then incubated for 1 h at room temperature with the appropriate secondary antibodies (1:1000 dilution). After three washes with TBST, the bands could be detected with ECL-Plus Western blotting reagent (Beyotime, Haimen, China).

Blood sampling and blood analysis

The precaval venous blood was collected from 35-day-old pigs and placed into serum, citrate (3.2%) and EDTA tubes. Plasma was taken and placed at 4°C after centrifugation (3,000 rpm, 15 min), and the detections were completed within 2 hours. The activity of coagulation factor IX, the activated partial thromboplastin time (APTT) and the prothrombin time (PT) were measured at Jilin University First Hospital clinical laboratory. FIX:C was detected by one stage clotting assay with

coagulation factor IX-deficient human plasma. Normal human plasma was used as the standard for the test. The coagulation factor IX activity in porcine plasma was expressed as the percentage of the coagulation factor activity in normal pooled plasma. And the concentration of human FIX in KI pig plasma was also detected by a Human Factor IX Elisa Kit (ab188393, Abcam, UK). A full-automatic blood analysis on EDTA blood and biochemical analysis (total protein, albumin) in serum were performed at Animal Hospital of Jilin University.

Supplementary Figures

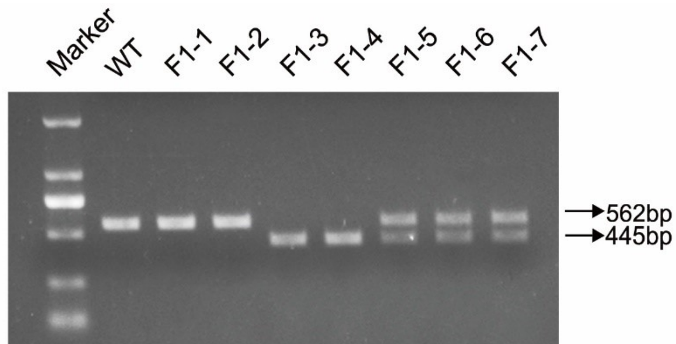


Supplementary Figure 1: Single-cell clones harboring porcine *F9* deletions identified via PCR

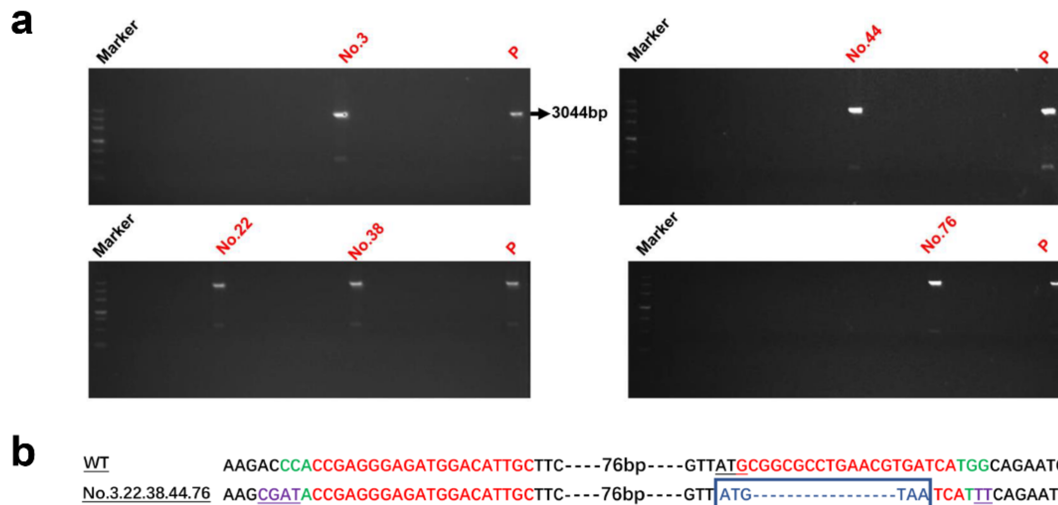
(a) Male cell clones were identified using the primer pair F1/R1. The positions of the primers are shown in Figure 1A. The electrophoresis results (88 samples) showed that the lysis solution from a single colony could produce a sufficient genomic DNA template for PCR. WT, wild-type control; Marker, Marker III. The exact genotype of 11 male positive clones was determined by Sanger sequencing. The PAMs are marked in green.

(b) Female cell clones were identified in the same way. As *F9* is located on the X chromosome, the

female cells have two alleles. The electrophoresis results (30 samples) showed that two heterozygous cell clones were screened.



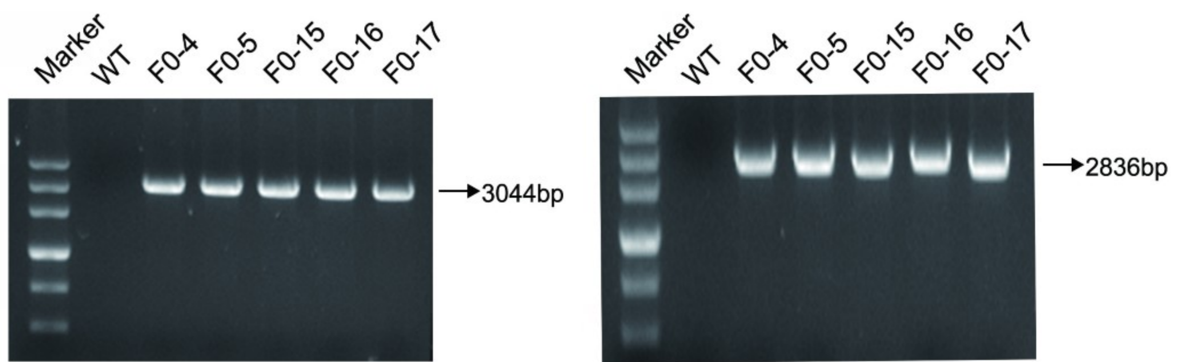
Supplementary Figure 2: PCR analysis of factor IX of seven F1 pigs.



Supplementary Figure 3: Single-cell clones carrying human *F9* identified via PCR

(a) The primer pair F2/R2 was used to identify the insertion of human *F9*. The positions of the primers are shown in Figure 1D. The electrophoresis results (92 samples) showed that the site-specific integration of human *F9* occurred in 5 clones. P, positive control; Marker, Marker III.

(b) The exact genotypes of 5 positive cell clones were determined by Sanger sequencing. The insertion of *hF9* is framed in blue.



Supplementary Figure 4: The primer pairs (F2/R2 and F3/R3) were used to identify the site-specific integration of *hF9.* The positions of the primers are shown in Figure 1D.

Supplementary Tables

Supplementary Table 1: Overview of the histopathology scoring system assessing severity of synovitis, cartilage degradation.

	0	1	2	3
Synovitis (HE)	No changes	Increased number of lining cell layers and/or slight proliferation of subsynovial tissue	Increased number of lining cell layers. Moderate proliferation of subsynovial tissue and/or infiltration of few inflammatory cells	Increased number of lining cell layers. Massive proliferation of subsynovial tissue and/or infiltration of large numbers of inflammatory cells
Cartilage degradation (Safranin O)	No cartilage degradation	Loss of proteoglycan and/or chondrocytes in the superficial layer (above the tidemark) without structural changes	Loss of proteoglycan and chondrocytes extending through the calcified layer of cartilage, and/or fibrillations without loss of cartilage	Erosions extending to the calcified cartilage

Supplementary Table 2: Summary of embryo transfer for the generation of gene-targeted pigs.

Surrogate ID	No. embryos transferred	No. pigs born	No. pigs positive	No. pigs alive
160	200	2	2	2
162	200	1	1	1
164	200	-	-	-
224	250	5	5	2
226	215	-	-	-
228	215	-	-	-
230	250	-	-	-
232	250	4	4	3
234	200	-	-	-
166	200	-	-	-
168	215	2	2	2
170	200	-	-	-
236	200	-	-	-
238	200	-	-	-
240	200	3	3	3

The upper part of the table shows the embryo transfer for the generation of *pF9* KO pigs. The male positive clones were transferred into surrogates (160, 162, 164, 224, 226, 228) and the female heterozygous clones were transferred into surrogates (230, 232, 234).

The low part shows the embryo transfer for the generation of *hF9* KI pigs.

Supplementary Table 3: Records of spontaneous bleeding events for 8 weeks.

(**WT**: F1-1, F1-2; **KO**: F0-2, F0-6, F0-10, F1-3, F1-4; **KI**: F0-15, F0-16, F0-17)

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
F0-2	1 (right ankle)	0	1 (right ankle)	0	2 (right knee; muscle)	-	-	-
F0-6	0	1 (left toe)	0	3 (muscle; right knee; right ankle)	0	1 (right ankle)	2 (right knee; right ankle)	0
F0-10	0	0	0	1 (left ankle)	0	2 (right elbow; left knee)	1 (nose)	1 (right ankle)
F1-3	0	1 (left knee)	1 (left elbow)	0	0	1 (nose)	0	1 (right knee)
F1-4	0	0	1 (right ankle)	1 (left ankle)	1 (eye)	-	-	-
F0-15	0	0	0	1 (left ankle)	2 (left ankle; left elbow)	0	0	0
F0-16	0	0	0	0	1 (left toe)	0	1 (right ankle)	0
F0-17	0	0	0	0	0	0	0	0
F1-1	0	0	0	0	0	0	0	0
F1-2	0	0	0	0	0	0	0	0

Supplementary Table 4: Primers for constructing the donor plasmid and the corresponding sequences.

5' homology arm	1-1-F	CTCGAGTTTTTCAGCAAGATCCACGGGTCCTACTCAGGTTC
	1-1-R	GTCCATCTCCCTCGGTATCGCTTTGGGCTGCTTCCAGTG
	1-2-F	GATACCGAGGGAGATGGACATTG
	1-2-R	AACCTCTGCTAGTAGATTGTGCAAG
Human *F9* CDS	2-F	CAATCTACTAGCAGAGGTTATGCAGCGCGTGAACATGATC
	2-R	GCCCAGTGATTCTGAAATGATTAAGTGAGCTTTGTTTTTTCCTTAATC
3' homology arm	3-F	TTTCAGAATCACTGGGCCTC
	3-R	GGAGATCTTCTAGAAAGATCTATTTCTTGGGCTGCTCCTG

Supplementary Table 5: Knockout- and knockin-specific primers and the corresponding sequences.

Knockout	F1	TCCCTGACAAGGATAAGA
	R1	ACCAGTATTTACCAACCC
Knockin	F2	CTGGCAATACTAGACCCT
	R2	ATCTTCTCCACCAACAAC
	F3	CTGTGGAGGCTCTATCGT
	R3	GTGTTTGCCCAGGTAATG

Supplementary Table 6: Quantitative real-time PCR primers and the corresponding sequences.

porcine *F9*	up1	CTGTGGCGGTTCCATCATTA
	down1	GTTCTGTCTCCTCGGTGTTATAC
human *F9*	up2	CTGTGGAGGCTCTATCGTTAAT
	down2	GCTCTGTATGTTCTGTCTCCTC
porcine *GAPDH*	up3	CCTTCCGTGTCCCTACTGCCAAC
	down3	GACGCCTGCTTCACCACCTTCT