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Supplementary Materials for

In vivo delivery of CRISPR-Cas9 using lipid nanoparticles enables antithrombin gene editing for sustainable hemophilia A and B therapy

Jeong Pil Han, MinJeong Kim, Beom Seok Choi, Jeong Hyeon Lee, Geon Seong Lee, Michaela Jeong, Yeji Lee, Eun-Ah Kim, Hye-Kyung Oh, Nanyeong Go, Hyerim Lee, Kyu Jun Lee, Un Gi Kim, Jae Young Lee, Seokjoong Kim, Jun Chang, Hyukjin Lee*, Dong Woo Song*, Su Cheong Yeom*

*Corresponding author. Email: hyukjin@ewha.ac.kr (H.L.); dw.song@toolgen.com (D.W.S.); scyeom@snu.ac.kr (Y.S.C.)

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This PDF file includes:

Fig. S1.

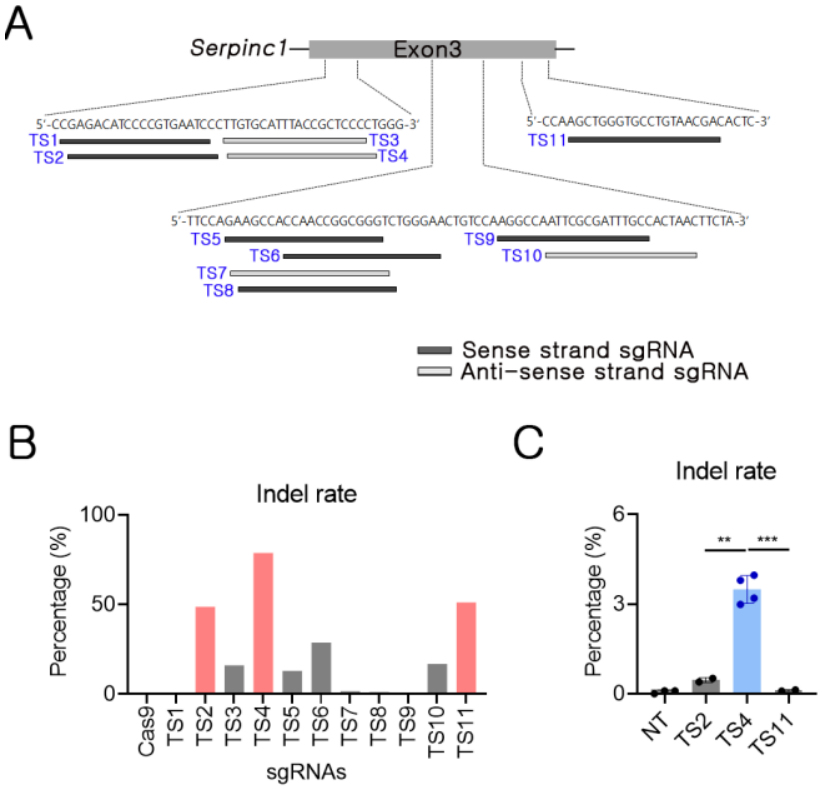

Fig. S1. Screening of sgRNAs targeting the *Serpinc1* gene in C2C12 cells. **(A)** Target sites (TS) and sequences surrounding the exon 3 of mouse *Serpinc1* gene. TSs located on the sense or anti-sense strand are indicated in black or gray boxes, respectively. (**B)** Indel frequencies induced by the tested sgRNAs. Frequencies were measured by targeted deep sequencing in the C2C12 cell line. **(C)** LNP-mediated activity test of the three selected sgRNAs in mouse primary hepatocyte. Indel frequencies were measured by targeted deep sequencing after 2 days of the treatment. NT indicates no treatment. Data were presented as mean ± SEM. **, $p < 0.01$; ***, $p < 0.001$.

Fig. S2.

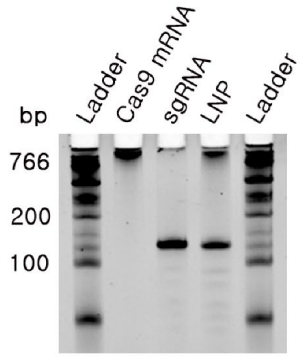

Fig. S2. Gel electrophoresis assay of LNPs encapsulating Cas9 mRNA/sgRNA in a 1:1 ratio by weight. After PAGE gel analysis, intact band intensities were quantified with Image Lab software. LNPs were formulated in 7 mM citrate buffer, and most of the highly modified sgRNAs were not encapsulated. (mRNA 100% → 13%, sgRNA 100% → 70%).

Fig. S3.

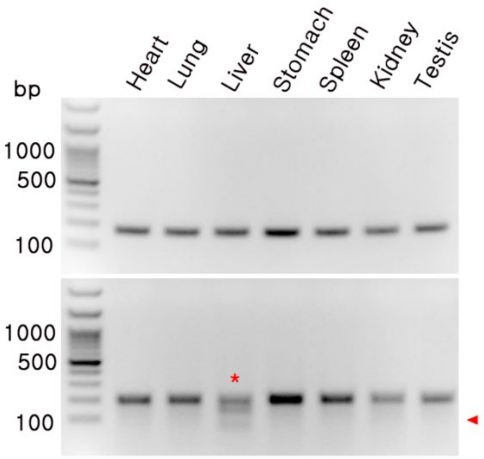

Fig. S3. Identification of indels using T7E1 analysis in various tissues. The LNP-CRISPR-mAT developed detectable indels only in the liver tissue.

Fig. S4.

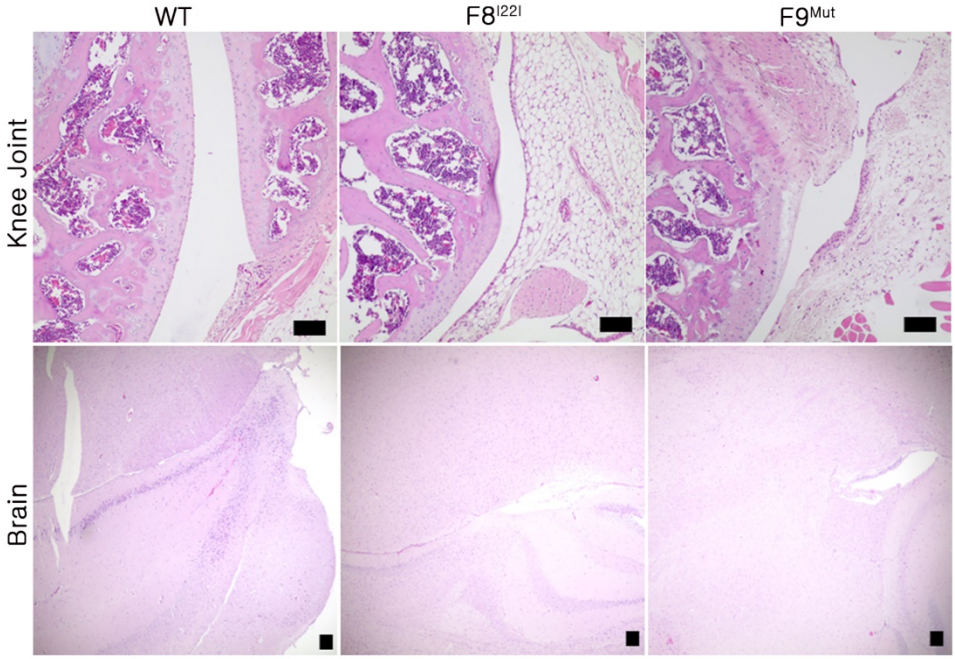

Fig. S4. Histological analysis of the knee joint and brain was conducted on the WT, $F8^{I22I}$, and $F9^{Mut}$ mice. The articular cavity and cerebrum were examined, but no bleeding lesions were detected in the mice. Scale bar = 100 μm.

Fig. S5.

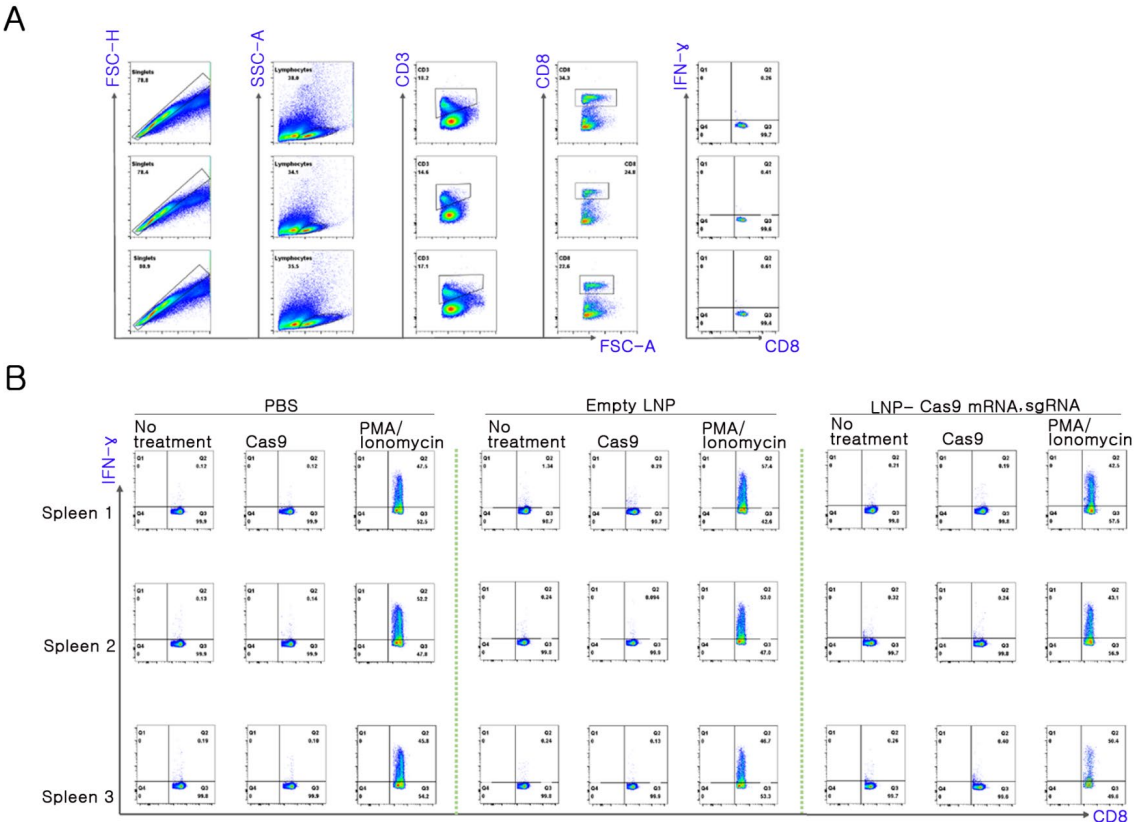

Fig. S5. Flow cytometry showing gating strategy and the analysis of IFN-γ expression in CD8+ T cells stimulated with Cas9 protein. **(A)** CD3+ antibody was used for T cells, then CD8+ was used for CD8+ T cells. Gates for IFN-γ expressing CD8+ T cells were drawn based on PBS injected control mice (n=3). **(B)** IFN-γ expression in CD8+ T cells was not detected in mice (n=3).

Fig. S6.

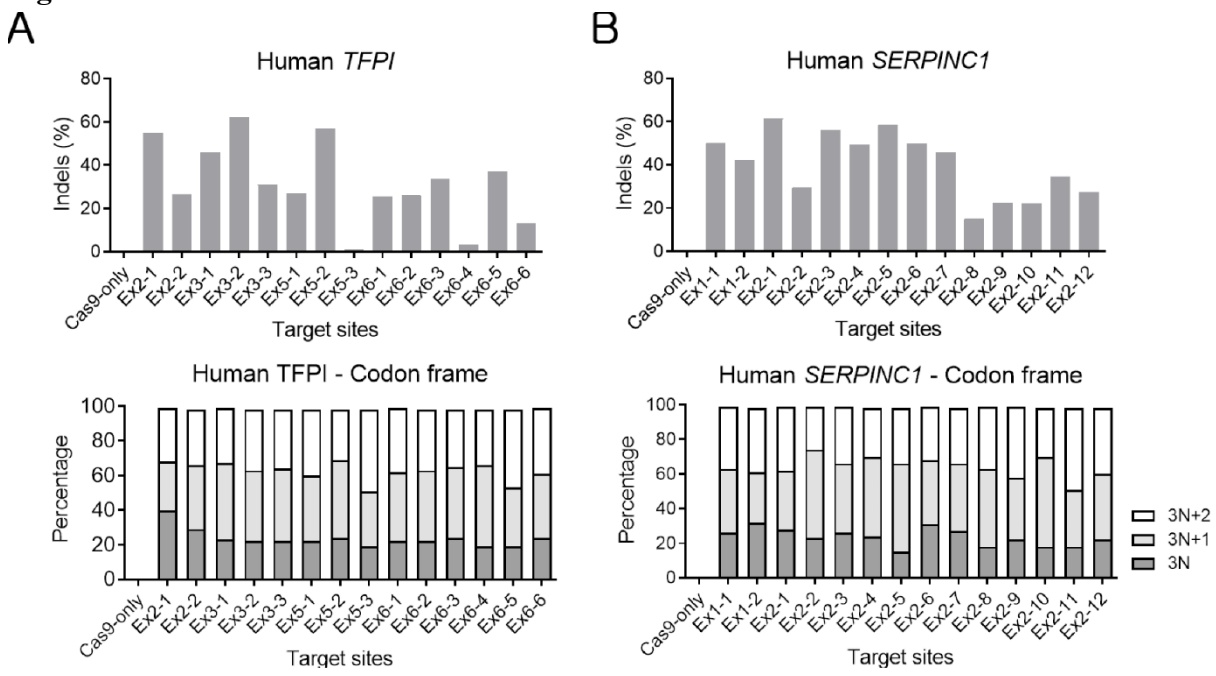

Fig. S6. Indel frequencies and codon frame patterns at human *TFPI* **(A)** and *SERPINC1* **(B)** target sites in the Jurkat cell line. Frequencies were measured by targeted deep sequencing (upper panel), and percent values of the in-frame (3N) and out-of-frame (3N+1 and 3N+2) indels were analyzed (lower panel) at each TS.

Fig. S7.

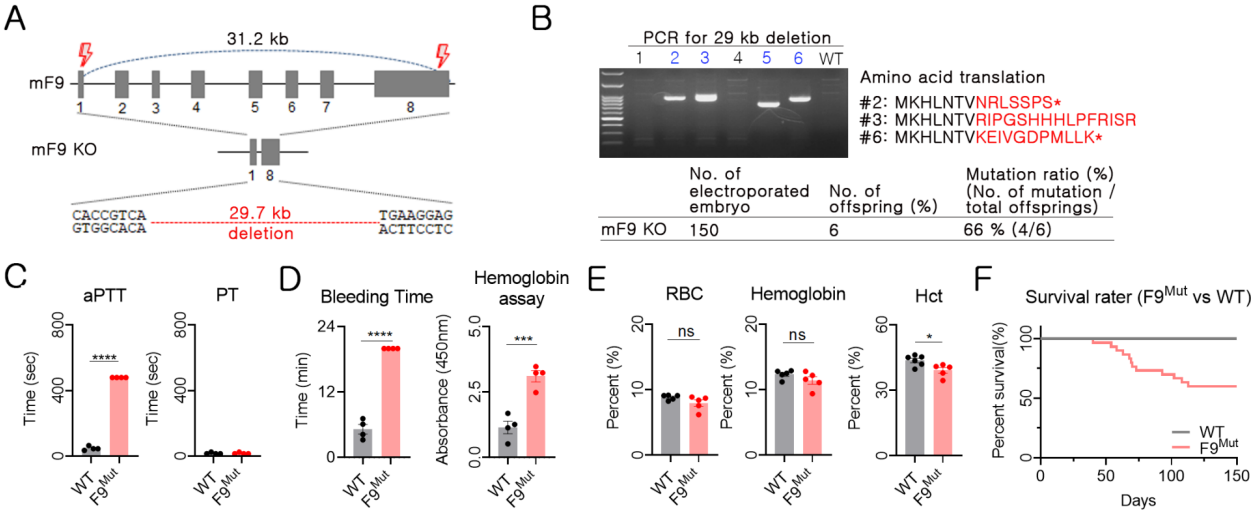

Fig. S7. Generation of Factor IX (F9) knock-out (KO) mouse (F9mut) and phenotype analysis. **(A)** Brief strategy for generation of F9Mut mice and the expected deletion. Two double-stranded DNA breakages (DSBs) were induced using CRISPR/Cas9 in exons 1 and 8 (Thunder symbol: DSB site, gray box: exon). **(B)** Genotyping of the produced F9Mut pups using polymerase chain reaction and Sanger sequencing-mediated translation prediction (Blue digits: KO pups, red letters: translated amino acid residues, *: stop codon). **(C)** Partial thromboplastin time (PT) and activated partial thromboplastin time (aPTT). **(D)** In vivo bleeding (wild-type [WT]: n = 4, F9Mut: n = 4), showing coagulation activity. **(E)** Complete blood count (WT: n = 5, F9Mut: n = 5). Each dot represents data from an individual mouse. Data for **C**, **D** and **E** are presented as mean ± standard error of mean, calculated using unpaired Student's t-test. (ns : not significant, * : $p < 0.05$, *** : $p < 0.001$, ****: $p < 0.0001$) **(F)** Survival rate in WT and F9Mut mice for 5 months after birth (WT: n = 29, F9Mut: n = 30), statistically analyzed using the Mantel-Cox test.

Fig. S8.

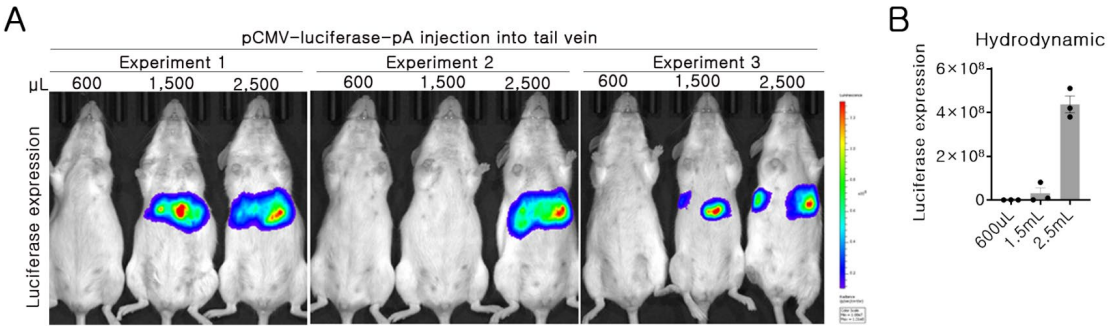

Fig. S8. 20ug of luciferase plasmid (pCMV-Luciferase-pA) was prepared with a total volume of 600μL, 1,500μL, and 2,500μL solution and injected into the tail vein. A bioluminescence signal was measured. **(A)** Image for in vivo bioluminescence signal detection. **(B)** Quantification of bioluminescence signal.

Table S1.

Targets	Chr	Location	Sequence(5' to 3')	Related genes
ON(TS4)	Chr1	160989494	TGTGCATTTACCGCTCCCCTGGG	*Serpinc1*
Off1	Chr4	115922991	TGTACATTCACCTCTCCCCTTGG	Intron(*Dmbx1*)
Off2	Chr7	80080647	TGTGCACTTACCGAACCCCTGGG	Intron(*Zfp710*)
Off3	Chr9	42792265	TATGCATTTACTGCTCACCTGGG	Intron(Grik4)
Off4	Chr10	53752760	TGTGCATTTTCTGCTCCCTTAAG	Intergenic
Off5	Chr14	59818497	TGTGCATTTAATGCTCCCCATAG	Intron(*Atp8a2*)
Off6	Chr18	53480031	TGAGCATTTACCGCCTCCCTCAG	Intron(*Prdm6*)
Off7	Chr18	86859014	TGTGCATTTACAGTTCCCATGGG	Intergenic
Di-Off1	Chr4	115922998	TGTACATTCACCTCTCCCCTTGGG	Intergenic(*dmbx1*)
Di-Off2	Chr9	56223771	G-GC-TCTCCGCACCGGACCCGGT CCCGAC-GGG	Intergenic*(peak1)*
Di-Off3	Chr10	69926267	TATGCAAATACCCCTCCCCTTGG	Intergenic*(ank3)*

Table S1. Off-target sites. Off1 – Off7 were selected by in-silico based methods and Di-Off1 – Di-Off3 were found in the Digenoe-seq analysis. In the Digenome-seq, the on-target site showed the highest cleavage score (ON, 166.5; Di-Off1, 5.3; Di-Off2, 9.7; Di-Off3, 11.9)

Table S2.

Target gene		Sequence (5'-3')	Product size
mAT On (TS4)	F	5'-AGGAATAAGACTGTGGTGGTC-3'	279bp
	R	5'-TTGGCCTTGGACAGTTCCCAG-3'	
mAT Off1	F	5'-GGCATTGAGTACTGATGACTGC-3'	178bp
	R	5'-AGAATAAGTACTGGAGCGTCTG-3'	
mAT Off2	F	5'-GGCAGAGTCAGTCAGGAGG-3'	202bp
	R	5'-GTAGTTGTAGGCGGTCTAGAAC-3'	
mAT Off3	F	5'-CTCCCAAACCAGGCCTTGGC-3'	216bp
	R	5'-TTCTTGTCTTGAGGCTGCTGC-3'	
mAT Off4	F	5'-CAGTACTTCAGTAGTAGACCTTCC-3'	220bp
	R	5'-CAAGATATCTCTGGCTGTTCTC-3'	
mAT Off5	F	5'-AGTAGTATGGATGGGGACTGG-3'	219bp
	R	5'-GTTGCAGGTGGATGCAGG-3'	
mAT Off6	F	5'-GCTCCAAGATTTCATGAGTTCAG-3'	231bp
	R	5'-CCATTAGGCTGGAGCCTGG-3'	
mAT Off7	F	5'-GCAAAACGTGTAGGCTTGC-3'	150bp
	R	5'-AAGAGTCTCAACACAGTGCAC-3'	
mAT Di-Off1	F	5'- ACTTGCTTTGGGCATTGAGT -3'	165bp
	R	5'- GGGATTGGAACATGACCGTA-3'	
mAT Di-Off2	F	5'- ACGTGCGTTCAACGTGACGG -3'	172bp
	R	5'- GAAGGAGCCTCGGTTGGCC-3'	
mAT Di-Off3	F	5'- GTACTCCCCAACCAGTCGTG -3'	190bp
	R	5'- TCCAAATGTAAAACAGTAAACAGAT CA-3'	

Table S2. Primer information used in this study