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Generation of Mouse Model of Hemophilia A by Introducing Novel Mutations, Using CRISPR/Nickase Gene Targeting System

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Abstract

Developing mouse models of hemophilia A has been shown to facilitate *in vivo* studies to explore the probable mechanism(s) underlying the disease and to examine the efficiency of the relevant potential therapeutics. This study aimed to knockout (KO) the *coagulation factor viii* (*fviii*) gene in NMRI mice, using CRISPR/Cas9 (D10A/nickase) system, to generate a mouse model of hemophilia A. Two single guide RNAs (sgRNAs), designed from two distinct regions on NMRI mouse *FVIII* (*mFVIII*) exon 3, were designed and inserted in the pX335 vector, expressing both sgRNAs and nickase. The recombinant construct was delivered into mouse zygotes and implanted into the pseudopregnant female mice's uterus. Mutant mice were identified by genotyping, genomic sequencing, and mFVIII activity assessment. Two separate lines of hemophilia A were obtained through interbreeding the offspring of the female mice receiving potential CRISPR-Cas9-edited zygotes. Genomic DNA analysis revealed disruptions of the *mfviii* gene reading frame through a 22-bp deletion and a 23-bp insertion in two separate founder mice. The founder mice showed all the clinical signs of hemophilia A including; excessive bleeding after injuries, and spontaneous bleeding in joints and other organs. Coagulation test data showed that mFVIII coagulation activity was significantly diminished in the mFVIII knockout ($FVIII_{KO}$) mice compared to normal mice. The CRISPR/nickase system was successfully applied to generate mouse lines with the knockout *fviii* gene. The two novel $FVIII_{KO}$ mice demonstrated all clinical symptoms of hemophilia A, which could be successfully inherited. Therefore, both of the developed $FVIII_{KO}$ mouse lines are eligible for being considered as proper mouse models of hemophilia A for *in vivo* therapeutic studies.

Keywords: CRISPR-Cas9, Factor VIII, Hemophilia A, Knockout Gene, Mouse Models

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Hemophilia A, as an X-linked recessive bleeding disorder, caused by deficiency or dysfunction of coagulation factor VIII (FVIII), is one of the most well-known hereditary disorders (1). Replacement therapy is a common treatment for hemophilia A, done by infusion of either human plasma-derived or recombinant FVIII protein (2, 3). In the continuation of the previous attempts, ongoing efforts have been made to improve hemophilia treatment approaches through the development of upgraded products, cell therapy strategies, and powerful gene delivery vectors (4, 5). In the cases of hemophilia A and other hemorrhagic diseases, animal models facilitate studying the pathophysiology and homeostasis of the blood coagulation cascade and improve the therapeutic methods. Till now, several hemophilic animal models harboring various mutations in the *fviii* gene have been developed, including rat (6, 7), pig (8), dog (9), sheep (10), mouse (11), cow (12), and a transient hemophilic rabbit (13). In the past decade, transgenic mouse models have been added to the animal models of hemophilia (14). Bi et al. (15) reported the development of a mouse line defective in the *fviii* gene and showed that the hemophilia A phenotype in the hemophilic mice is less severe than what is observed in human patients suffering from this disease. Therefore, in light of the fact that mice lacking functional FVIII are able to live a healthy life and present a grade of hemophilic phenotypes, they could be considered suitable models for studies focusing on FVIII activity restoration in hemophilia A. Two well-characterized $fviii_{KO}$ strains of mice have been previously generated by inserting a neo-expression cassette in exons 16 (E16-/- line) and 17 (E17-/- line) of the *fviii* gene (15, 16), which are currently being used extensively in hemophilia A studies (17). The classical approach for

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gene knockout was based on homologous recombination-based techniques (18, 19). With the advent of a nuclease-based method for genome editing, named clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9), it is now possible to create animal models more simply and in a considerably shorter time (20). An improvement in this technique was the development of a modified version of Cas9, namely Cas9(D10A) or Nickase, to reduce the nonspecific incisions, referred to as off-target. Nikase can only produce single-stranded breaks in the targeted genomic DNA, reducing the rate of off-targets. However, it requires two guide RNAs simultaneously, to finally introduce breaks in both DNA strands (21). This study aimed to generate an NMRI mouse model of hemophilia A by targeting mouse *fviii* (*mfviii*) exo3 and consequently knocking out the *mfviii* gene utilizing CRISPR/Nickase editing system.

Normal 4-week-old male and female NMRI mice were purchased from the Pasteur Institute of Iran and housed in standard animal rooms equipped with proper air-conditioning systems, regular 12 hour-cycles of light and dark, and free access to food and water. All animal experiments were performed in accordance with the institutional guidelines and the standard protocols approved by the Ethic (Animal Care and Use) Committee of NIGEB (IR.NIGEB.EC.1401.12.14.C).

Before designing the gRNAs, the probable genetic variations in the desired target site located in mFVIII exon 3 among NMRI mice were examined. To do this, genomic DNAs were extracted from three NMRI mice's tails according to available standard protocols. DNA samples were then used as PCR templates to amplify a 631-bp fragment including the target site in mFVIII exon 3 using a specific primer pair (Table S1, See Supplementary Online Information at www.celljournal.org). Next, PCR products were sequenced by ABI 373A Applied Biosystems automated DNA sequencer (MWG-Germany).

To design gRNAs, the sequence of the *mfviii* gene (Accession number: NC_000086) was obtained from Genbank (NCBI) and then two short guide RNA (sgRNA) oligonucleotides were designed by using the CRISPOR website (http://crispor.tefor.net/) to target parts of exon 3 of the *mfviii* gene (Table S1, See Supplementary Online Information at www.celljournal.org). Two gRNA fragments were cloned into a region flanked by restriction sites of BbsI enzyme on the pX335-U6-Chimeric_BB-CBh-hSpCas9n (hereinafter pX335) vector (Addgene ID # 42335).

To knock out the *mfviii* gene, superovulated normal female NMRI mice (4 weeks old) were mated to NMRI males, and the fertilized oocytes were collected from oviducts. The recombinant vectors were mixed at 4 ng/µl concentration and injected into the male pronucleus of the fertilized zygotes (Fig.S1, See Supplementary Online Information at www.celljournal.org). The microinjected zygotes were then incubated in M16 medium at 37°C for 1 hour. The surviving oocytes were transferred to the oviduct of pseudopregnant female NMRI mice (foster mother) at 12 hours post-coitus.

To detect the introduced CRISPR-mediated mutations in the *mfviii* gene, genomic DNA samples were isolated from finger biopsies obtained from mouse pups aged 2-3 weeks old. The *mfviii* target site of gRNAs was amplified by PCR using specific primers (Mutfviii primers) flanking a 259-bp fragment containing the target site and then visualized by agarose gel electrophoresis. The genotypes of the mice were determined by sequencing the PCR products amplified from the genomic DNAs of both normal and mutant mice, using ABI 373A Applied Biosystems automated DNA sequencer (MWG-Germany).

The coagulation activity of the mFVIII in the mouse serum was measured by the activated partial thromboplastin time (APTT) method, using the COATEST SP4 FVIII kit (Chromogenic, Bedford, MA), as previously described by Chao et al. (22). The FVIII-deficient plasma pool (HRF Inc., Raleigh, NC), needed for the FVIII assay, contained <0.4% FVIII. Normal mouse plasma was considered as a standard sample with 100% activity equal to 1 IU/ml mFVIII.

To exclude any possible polymorphic regions in the gRNA design, a 631-bp fragment of the *mfviii* gene covering exon 3 was amplified and sequenced from three randomly selected NMRI mice (Fig.S2A, B, See Supplementary Online Information at www.celljournal.org). The multiple sequence alignment data showed no nucleotide difference among the sequenced fragments (Fig.S2C, See Supplementary Online Information at www.celljournal.org). The nucleotide BLAST analysis also showed a complete match between the sequenced fragments and the recorded sequence in NCBI GenBank as the reference sequence.

In order to generate $mFVIII_{KO}$ mice, two gRNAs were designed using CRISPOR tool to target exon 3 of the *mfviii* gene. The gRNA fragments were separately cloned between the *Bsb*I sites of the pX335 vector and were confirmed by sequencing (Fig.S3A, B, See Supplementary Online Information at www.celljournal.org). The fertilized oocytes were injected with the recombinant vectors encoding both gRNAs and nickase. The surviving oocytes were transferred into the oviduct of pseudopregnant females, resulting in 67 newborns. The sequencing data of the target region on the *fviii* gene detected 7 mutant mice (Fig.1A). The mutants mainly carried deletions, as insertion was detected only in one mouse. Two out of seven *fviii*-mutant mice, including +23 and -22 founders, carried the frameshift mutations. The first founder (designated as +23) was a heterozygous female. As shown in Figure 1B (lane +/-), three distinct bands were amplified by PCR, where the bottom band corresponds to wild type, the middle band corresponds to mutant and the upper band was the hybrid of wild type and mutant strands. Sequencing of the middle band detected a 23-bp insertion in one of the *mfviii* alleles (Fig.1A). Crossing of the +23 founder with a normal male resulted in a hemophilic male in the F1 generation, demonstrated by PCR (Fig.1B). The second founder (designated as

-22) was again a female with a heterozygous pattern of *fviii* alleles (Fig.1C, lane +/). Crossing this founder with normal males resulted in the transfer of defective genes to half of the F1 males (25% of offspring) (Fig.1C, lane Y/-). The inheritances of the mutant alleles for each mutant were verified by sequencing of target regions of the F1 males (Fig.1D).

One of the most problematic symptoms, which occur in hemophilic patients is spontaneous bleeding in the joints. The hemophilia males displayed hemarthrosis (joint bleeding) occasionally in their hip and toe joints (Fig.2).

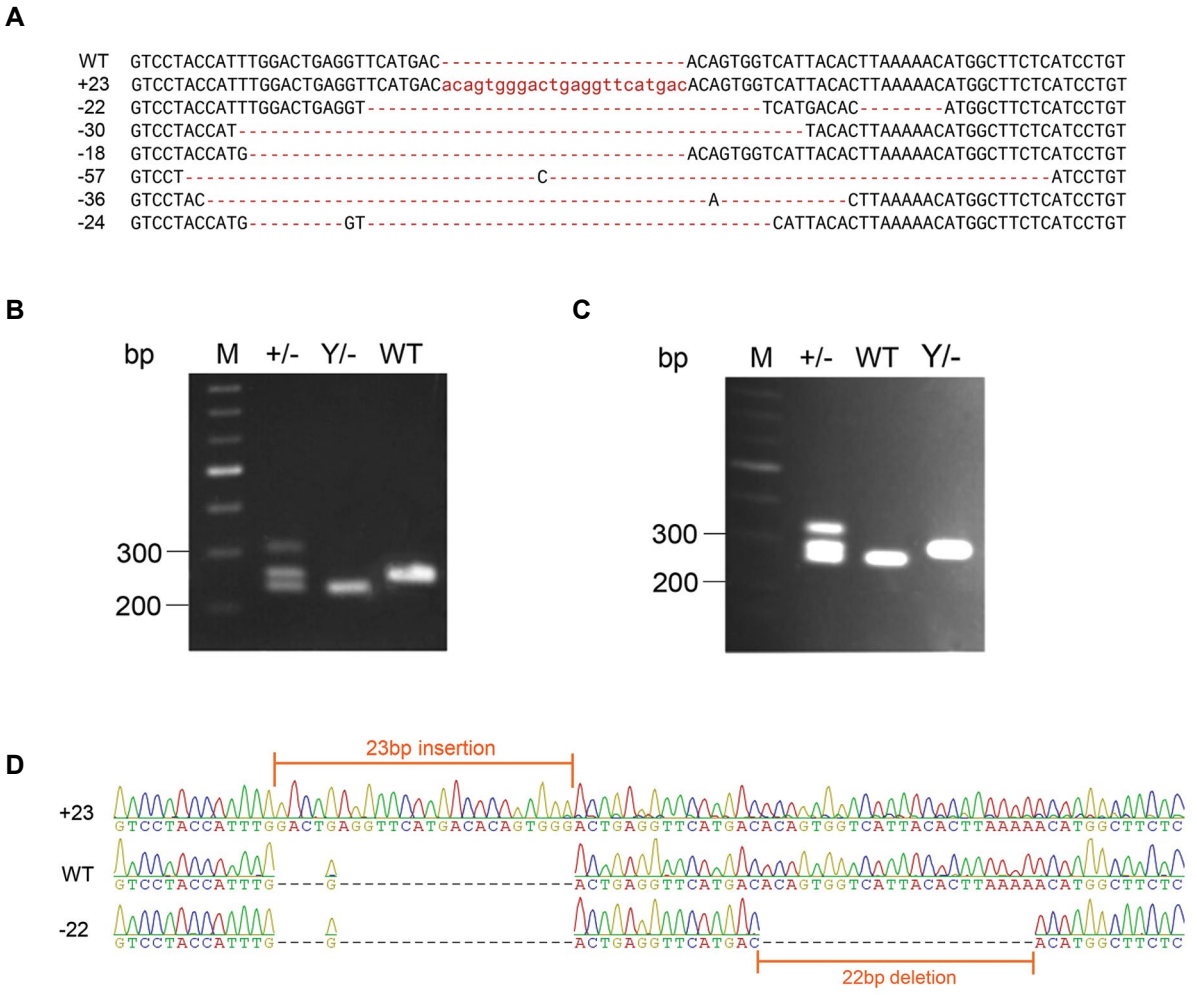

Fig.1: The genotypes of the mutant mice, determined by sequencing the PCR products from the genomic DNAs. **A.** Multiple sequence alignments of mutant mice. PCR followed by sequencing detected 7 mutants. Indel mutations were detected in the mutants. **B.** The PCR product, obtained from the genomic DNA, of the mutant mouse with a 22-bp deletion at the target site of the *mfviii* gene. **C.** The PCR product, obtained from the genomic DNA, of the mutant mouse with a 23-bp insertion at the target site of the *mfviii* gene. **D.** Genotyping of F1 generation mice from both female founders verified the inheritance of the mutant alleles in the male offspring. PCR; Polymerase chain reaction, +/-; Heterozygous female, Y/-; Hemophilic male, and WT; Wild-type mouse.

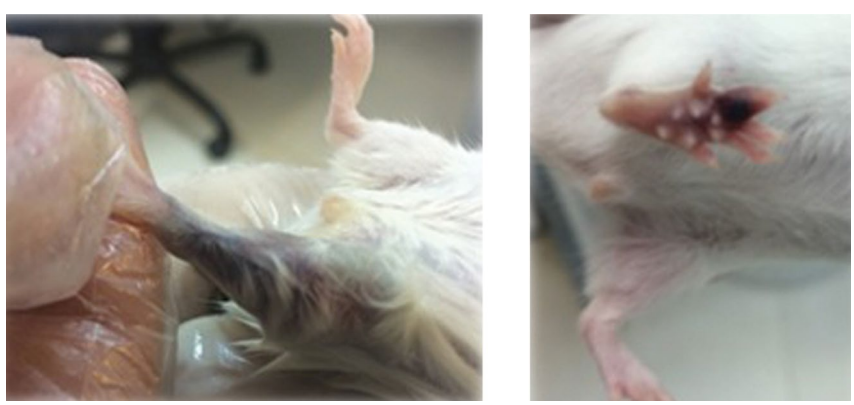

Fig.2: Hemorrhage in the joints of hemophilic males.

The effects of the mutations in the $FVIII_{KO}$ mice were first studied *in silico*. Accordingly, the computational translation of transcripts of both of the mutant alleles produced truncated polypeptides. In this regard, in the first mutant mouse the allele interrupted with a 22-bp deletion encoded a 153-amino acid polypeptide instead of the full-length protein. In the second mutant, the allele was disrupted by a 23-bp insertion and produced a defective protein of 168 amino acids. Both proteins were much shorter than the full-length protein to have a normal function (Fig.3A). The activity of the FVIII protein was experimentally assayed in the plasma of male mice using a coagulation test (Fig.3B). The results showed a significant decline in the mFVIII coagulation activities in the plasma samples derived from $FVIII_{KO}$ mice, compared to that in the normal mouse plasma.

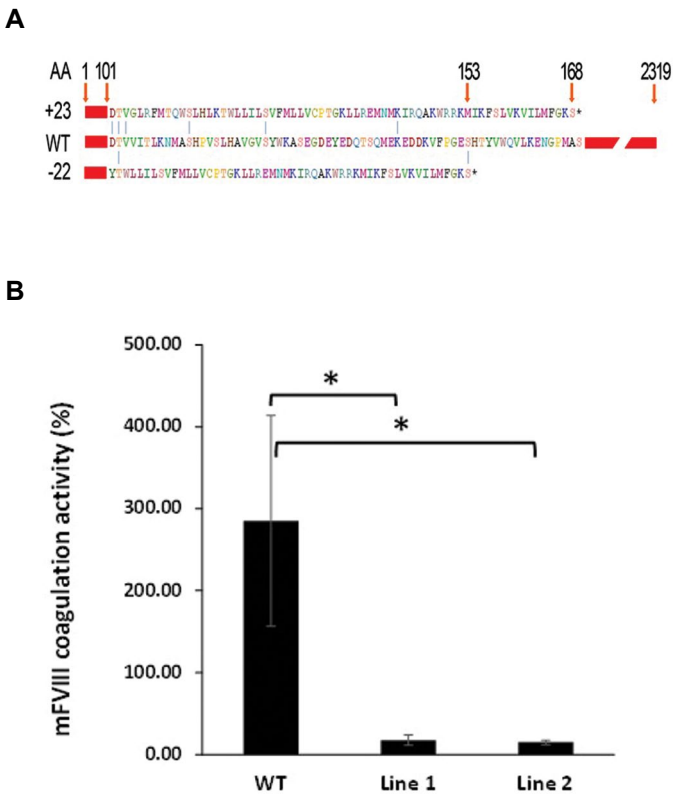

Fig.3: *In silico* and experimental evaluation of mFVIII fate as a result of mutations. **A.** *In silico* data showed that the indel mutations lead to the translation of mutant genes to truncated FVIII proteins. The 23-bp insertion resulted in a 168-aa polypeptide and the 22-bp deletion led to the production of a 153-aa protein. **B**. FVIII coagulation test (lines 1 and 2 corresponding to mutants 1 and 2, respectively) and wild type. Asterisks indicate samples that are significant differences (P<0.05), compared to wildtype.

In this work, we considered the mouse as an appropriate animal to develop a hemophilia A model. It has been known that male mice with FVIII deficiency continue to survive well and are eligible for studies dealing with FVIII correction and other in vivo therapies (15). The present hemophilia A model has been generated using CRISPR/Cas9 (nickase) tool. The previous hemophilia A mouse models, including exon 16 or exon 17 knockout models, have been generated by conventional gene targeting approaches (23). In 2016, a genetically engineered mouse model of hemophilia A was developed by complete deletion of the the *fviii* gene using the cre-loxp recombination technique (22). A recently published study reported the application of CRISPR/Cas9 in the generation of hemophilia A model via inversion of intron 22 of the *fviii* gene. In our study, the third exon of the *fviii* gene has been targeted by microinjection of all-in-one plasmids harboring both Cas9 (D10A) and sgRNAs. The mFVIII protein start site, located in exon 3, has not been already used as a target site for the generation of hemophilia A mice. When this site is disrupted, it is basically expected that the *fviii* gene to be knocked out (24). Besides, the occurrence of mutations in the *FVIII* exon 3 has been detected in several patients suffering from hemophilia A (25, 26). According to literature, the use of nickase enhances the specificity of genome editing (27). Moreover, the advantages of genome editing approaches, such as high efficiency and ease of use, in development of new animal models have been previously discussed.

Following the gRNA design and construction of the CRISPR/nickase-based gRNA expressing vectors, microinjection of the mouse fertilized oocytes with the recombinant vectors, followed by transfer of the injected oocytes into the oviduct of pseudopregnant female (recipient foster mothers) mice, successfully resulted in 67 newborns. Genotyping of the target regions of the *fviii* gene in the 67 newborns led to the detection of 7 mutant offspring. The mice bearing frameshift mutations were subjected to further analyses. The mating of two female founder mice with wild-type males resulted in mutant offspring. The results from genome analysis revealed a 22-bp deletion in the *fviii* gene in the first founder mouse and a 23-bp insertion in the other one. A coagulation test performed on the sera of the male newborn mice in the F1 generation of both founder mice showed a significant difference in the coagulation activity of FVIII compared to that in normal mice. Furthermore, our genotyping analyses revealed that the developed mice carry $fviii_{ko}$ genes and are capable of transferring the defective gene to the next generation. The stability of hemophilia A pathology has been consistently established in the consecutive generations of the two mouse models, considering both genotypic and phenotypic traits. The data achieved from the current study represents that the production of mouse models of hemophilia A using CRISPR/Cas technology is a more efficient, cost-effective, and time-saving method compared to conventional techniques. The development of hemophilia A mice in this work have provided valuable resources as non-human hemophilia A models to conduct *in vivo* studies with different therapeutic approaches, to develop new potential treatments for patients with hemophilia A.

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Authors' Contributions

A.Z., M.Sh., A.J.; Conceptualization, Methodology, and Software. A.Z, M.Sh., M.D.; Data curation, Data Analysis, and original draft preparation. A.R.-T., A.Z., M.D., F.M.; Imaging and Data collection. A.Z, M.Sh., M.D.; Writing- reviewing and Editing. All authors have read and approved the final manuscript.

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