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Brittany N. Chao, *NIAID*
Wallace H. Baldwin, *Emory University*
John F. Healey, *Emory University*
Ernest T. Parker, *Emory University*
Kimberly Shafer-Weaver, *NIAID*
Courtney Cox, *Emory University*
Ping Jiang, *NIAID*
Chrysi Kanellopoulou, *NIAID*
John Lollar, *Emory University*
Shannon Meeks, *Emory University*

Only first 10 authors above; see publication for full author list.

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Characterization of a genetically engineered mouse model of hemophilia A with complete deletion of the \( F8 \) gene

Brittany N. Chao\(^*\), Wallace H. Baldwin\(^†\), John F. Healey\(^†\), Ernest T. Parker\(^†\), Kimberly Shafer-Weaver, Courtney Cox\(^†\), Ping Jiang\(^*\), Chrysi Kanellopoulou\(^*\), Pete Lollar\(^†\), Shannon L. Meeks\(^†\), and Michael J. Lenardo\(^*\)

\(^*\)Laboratory of Immunology, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Bethesda, MD

\(^†\)Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta; the Department of Pediatrics, Emory University, Atlanta, GA.

Abstract

**Background**—The most important complication in hemophilia A treatment is the development of inhibitory anti-Factor VIII (FVIII) antibodies in patients after FVIII therapy. Patients with severe hemophilia who express no endogenous FVIII, i.e. cross-reacting material (CRM), have the greatest incidence of inhibitor formation. However, current mouse models of severe hemophilia A produce low levels of truncated FVIII. The lack of a corresponding mouse model hampers the study of inhibitor formation in the complete absence of FVIII protein.

**Objectives**—We aimed to generate and characterize a novel mouse model of severe hemophilia A (designated the F8\(^{TKO}\) strain) lacking the complete coding sequence of \( F8 \) and any FVIII CRM.

**Methods**—Mice were created on a C57BL/6 background using Cre-Lox recombination and characterized using \( \text{in vivo} \) bleeding assays, measurement of FVIII activity by coagulation and chromogenic assays, and anti-FVIII antibody production using ELISA.

**Results**—All \( F8 \) exonic coding regions were deleted from the genome and no \( F8 \) mRNA was detected in F8\(^{TKO}\) mice. The bleeding phenotype of F8\(^{TKO}\) mice was comparable to E16 mice by measurements of factor activity and tail snip assay. Similar levels of anti-FVIII antibody titers after recombinant FVIII injections were observed between F8\(^{TKO}\) and E16 mice.

**Conclusions**—We describe a new C57BL/6 mouse model for severe hemophilia A patients lacking CRM. These mice can be directly bred to the many C57BL/6 strains of genetically...
engineered mice making it valuable for studying the impact of a wide variety of genes on FVIII inhibitor formation on a defined genetic background.

**Keywords**
Animal model; blood coagulation factor inhibitors; factor VIII; hemophilia A; knockout mouse

**Introduction**

Hemophilia A is caused by a genetic deficiency of coagulation factor VIII (FVIII). Approximately 25-30% of patients with severe hemophilia A (<1% of FVIII activity) develop anti-FVIII antibodies. Some of these antibodies interfere with the function of FVIII in coagulation (inhibitors), which is considered to be the most serious complication of this disease due to the associated increases in morbidity, mortality, and cost of treatment. Severe hemophilia A results from intron 22 inversions, deletions/insertions, and missense, nonsense, and splice site mutations in the *F8* gene. Over fifty percent of these patients are predicted to produce no detectable FVIII protein [1, 2] and thus are prone to the development of anti-FVIII antibodies due to a lack of immunological tolerance to FVIII. The remaining severe patients are thought to produce some portion of the protein albeit with no detectable coagulation activity. Production of partial FVIII protein that can be recognized by the immune system and cause tolerance during lymphocyte ontogeny is called “cross-reacting material” (CRM). Mutation type is one of the greatest indicators of inhibitor formation risk, and those predicted to cause a complete lack of endogenous FVIII protein have the greatest incidence of inhibitors [2-6]. Rates of their development range from 57.1% in patients with large deletions to 9.5% for missense mutations [7]. In addition, inhibitors develop in 13% of patients with mild to moderate hemophilia A and as an autoimmune condition in ~1.4 people per million annually [8, 9].

The *F8* gene coding sequence is one of the largest in the human genome, spanning 26 exons over a 190 kB genomic locus. The gene encodes a 2351 amino acid protein with a predicted molecular weight of approximately 267,009 daltons before various post-translational modifications. The polypeptide undergoes further processing, including glycosylation and proteolytic cleavage to produce the mature active form in the blood.

Several animal models of hemophilia A harboring mutations in the *F8* gene have been developed, including rats [10, 11], dogs [12-14], pigs [15], sheep [16], and mice [17]. The most frequently used models are the E16 and E17 mice. These contain a neomycin cassette in exon 16 or 17, respectively, resulting in a truncated or partially deleted FVIII protein and exist in a mixed C57BL/6/S129 (E16-B6/S129) background [18]. Both display similar bleeding phenotypes [19] and show some evidence of CRM as low levels of FVIII heavy chain have been detected [18]. Therefore, the E16 and E17 strains may not completely recapitulate the immune conditions seen in CRM-negative hemophilia A patients. The goal of this project was to create a model in which all of the coding exons of the *F8* gene were removed using a Cre-Lox recombination strategy in order to produce a CRM-negative severe hemophilia A mouse.
Here, we describe the generation and characterization of a complete F8 knockout mouse, designated the Total Knockout (F8TKO) strain, in which the entire coding sequence has been deleted using a double-targeted LoxP site insertion followed by Cre-mediated deletion. Using PCR, we demonstrate that all 26 exons coding for F8 are deleted. We also carried out coagulation studies that show that the mice recapitulate the bleeding phenotype of severe hemophilia and display similar immune responses, including the formation of inhibitory antibodies to recombinant human FVIII (rFVIII). This strain is therefore a good model for patients with severe hemophilia A that are CRM-negative and could be a valuable additional tool for immunological studies and the testing of therapeutics given the complete lack of intrinsic FVIII tolerance.

Materials and methods

Materials

DMEM/F12 (11330-032), fetal bovine serum (FBS), penicillin/streptomycin, and AIM V medium were purchased from Invitrogen (Carlsbad, CA). Genotyping was done using the DNeasy Blood and Tissue Kit and HotStarTaq DNA Polymerase from Qiagen (Valencia, CA). RNA was isolated using RNeasy Mini kit from Qiagen, and cDNA was made using Superscript III cDNA Synthesis kit from Invitrogen. SYBR Green PCR Master Mix was purchased from Life Technologies (Carlsbad, CA). High binding half area ELISA plates (Costar) were purchased from Thermo Fisher Scientific (Waltham, MA). Alkaline phosphatase-conjugated goat anti-mouse IgG was purchased from Bio-Rad (Hercules, CA). Clotting times were measured using a STAtr coagulation instrument (Diagnostica Stago, Asnieres, France). Activated partial thromboplastin reagent was purchased from Trinity Biotech (Bray, Ireland). Pooled citrated normal human plasma, FVIII-deficient plasma, and FVII-deficient plasma were obtained from George King Biomedical (Overland Park, KS). Human BDD FVIII was produced in our laboratory as previously described [20]. Recombinant human full-length FVIII (Advate, Baxter, Westlake Village, CA) was a generous gift from Hemophilia of Georgia. All other materials were reagent grade or are described in the cited literature.

Generation of F8 knockout mouse and genotyping

F8TKO mice were generated by Ozgene Pty Ltd (Bentley, Australia) using a double targeting strategy as depicted in Fig 1A. C57BL/6 genomic DNA was used as a template to create two targeting vectors containing either a floxed PGK-neo selection cassette or a floxed PGK-hygro selection cassette surrounded by homology arms for exon 1 of F8 or the chromosomal region downstream of exon 26, respectively. The PGK-neo selection cassette was introduced to Bruce4 mouse embryonic stem cells [21] via electroporation, resulting in insertion of the PGK-neo selection cassette upstream of the exon 1 ATG start site via homologous recombination. Proper insertion of the cassette was verified using a restriction enzyme digest followed by Southern blotting using a Neo2 probe, as well as probes for the 5’ and 3’ homology arms. After the ES cells were confirmed to contain the Neo cassette, the PGK-hygro selection cassette was similarly inserted into the genomic DNA. Presence of the correct sequence was confirmed through Southern blots using probes for the homology arms and hygromycin cassette.
ES cells containing both target sequences were then injected into a hybrid C57BL/6 × BALB/c blastocyst. Mice containing the targeted allele were bred with OzCre mice (Ozgene), which express Cre recombinase under a PGK promoter in a C57BL/6J background, resulting in the deletion of the entire coding region of F8. This results in a frame-shift mutation with the introduction of an early stop codon. Mice containing the F8TKO allele were then bred with wildtype C57BL/6J mice to remove the Cre gene.

PCR was used to detect deletion of F8. In brief, mouse tail tissue samples were digested in lysis buffer with 50 μg of proteinase K overnight at 50°C. DNA was then extracted using isopropanol precipitation. HotstarTaq DNA polymerase (Qiagen) was used in the PCR reaction, which was run according to the manufacturer's guidelines. Two primer sets were used to distinguish between mice containing wildtype and F8TKO alleles. Set 1 (forward: 5’-GAT TCA AAC TTG TTA GGA TGC AC –3’; reverse: 5’-CAC AAA ACA GAT CTG AAA GGA TTA C –3’) produces a 130 bp band when the WT allele is present and no band when the F8TKO allele is present. Primer set 2 (forward: 5’-GAT TCA AAC TTG TTA GGA TGC AC –3’; reverse: 5’-TTT GTA AAC TTT CCC TGG TTC AAT –3’) produces a 210 bp band only where the F8TKO band is present and no band when the WT allele is present.

Mouse husbandry

Mouse colonies were established at the National Institutes of Health (NIH) and at Emory University. Heterozygous F8TKO females were bred with wild type males (NIH) or bred to homozygosity (Emory). E16 mice [17], which were originally derived from the Hoyer colony [22], were maintained on a mixed background (129S4 backcrossed to C57BL/6, and referred to as “E16-B6/S129” here), or were backcrossed into >95% C57BL/6 background (“E16-B6”) as judged by single nucleotide polymorphism (SNP) analysis (DartMouse, Lebanon, NH). All mice were housed in pathogen-free facilities. Food and water were given ad libitum. Pup survival of homozygous breeding pairs was measured by counting the number born and those that survived until weaning (28 days after birth). All other mice used were 8-12 weeks old at the beginning of experiments. All work performed at the NIH was carried out in accordance with the NIAID Animal Care and Use Committee guidelines (NIH) under the approved protocol ASP LI-47E. All work done at Emory University was performed under Protocol DAR-2001085 as approved by the Emory University Institutional Animal Care and Use Committee.

Real-time PCR

Total RNA was isolated from tissues using the RNeasy Mini kit (Qiagen) according to manufacturer’s instructions. cDNA was made using the Superscript III cDNA Synthesis kit (Invitrogen). RT-PCR was performed on the cDNA using the following primer pairs: 1) F8 exon 3-4 (Forward: 5’-AAAACATGGCTTCTCATCCTGT –3’; reverse: 5’-TGACTTTCACCAGGGAAAACCTT–3’); 2) F8 exon 11-12 (forward: 5’-GAACCAAAGCTGGTACATCACA –3’; reverse: 5’-TCAAAAACATAGCCATTGATGC–3’); 3) F8 exon 15-16 (forward: 5’-ACTGATGGCTCCTTTAGTCAGC –3’; reverse: 5’-GAGGCCTGGTTTTTGAAGTTA–3’); 4) F8 exon 25-26 (forward: 5’-
TGGAATAATAACCAGGGAGTG −3’; reverse: 5’–
CATAGGTGTGGATGAGTCCTGA– 3’); 5) FUNDC2 (forward: 5’–
TGCACTGGTTTCGTATTCCA –3’; reverse: 5’– CAATTACCAGCTGGGTTGCT–3’); and 6) ARBP (forward: 5’– CCAACTACTTCTCAAGATCATCCAAC–3’; reverse: 5’–
CATCTGCTTGGAGCCCACGTTGT–3’).

**In vivo bleeding model**

The *in vivo* bleeding model was performed as previously described [23]. Briefly anesthetized naïve mice were injected with 120 μL of either sterile saline or 180 U/kg (approx. 0.02 mg/kg) of human recombinant B-domain deleted (BDD) FVIII. Twenty minutes after the injection, 4 mm of the distal tail was transected and the proximal tail was placed in a pre-weighed tube. Forty minutes after the tail snip or at the time of death, blood loss per gram of body weight was calculated by measuring the change in tube weight.

**FVII and FVIII bioassays**

FVIII coagulant activity was measured using the activated partial thromboplastin reagent-based one-stage coagulation assay as previously described except that preincubation of FVIII-deficient plasma and aPTT reagent was conducted for 4 min at 37 °C [20]. Pooled normal human plasma referenced against a World Health Organization standard was used to calculate activity. Additionally, FVIII was measured using a chromogenic substrate assay (COATEST SP4 FVIII kit, Chromogenic, Bedford, MA) according to manufacturer’s protocol with the standard curve generated from pre-determined dilutions of pooled plasma from C57BL/6 mice. Using lower limits of absorbance in the protocol and pooled mouse plasma as the standard curve, the limit of detection was 2.28%. Results for test plasmas are reported as percentage of wild type FVIII activity.

FVII coagulant activity was measured using the prothrombin time based one-stage coagulation assay using thromboplastin (Neoplastine CL, Stago) as the activator. Pooled normal human plasma was referenced against a World Health Organization standard.

**FVIII immunization regimen**

Mice were injected retroorbitally with 2 μg of human recombinant full-length FVIII weekly for 4 weeks and then one week later given a 4 μg boost. One week following the boost, plasma was collected for anti-FVIII IgG titers.

**Anti-FVIII antibody detection by ELISA and Bethesda Assay**

Anti-FVIII IgG ELISA titers were measured as previously described [24]. Briefly, plates were coated with 1.5 μg/ml human FVIII in 20 mM Bicine, 2 mM CaCl\(_2\), pH 9 buffer and blocked with 20 mM HEPES, 0.15M NaCl, 2 mM CaCl\(_2\), 0.05% Tween-20, 0.05% sodium azide, 2.0% bovine serum albumin, pH 7.4. Murine plasma was serially diluted into blocking buffer starting at a 1/20 dilution. Antibody binding was detected with alkaline phosphatase-conjugated goat anti-mouse IgG and p-nitrophenyl-phosphate. ELISA titration curves were fitted to the four-parameter logistic equation. The dilution that produced an \(A_{405}\) of 0.3 at 20 minutes was defined as the ELISA titer.
FVIII inhibitor titers for the experiments involving patient plasma were measured using the Bethesda assay [25] with the modifications previously described [26]. Normal pooled plasma was used as the source of FVIII activity. One BU per ml is defined as the dilution of inhibitor that produces 50% inhibition of FVIII activity.

**Anti-FVIII Domain Mapping ELISA**

The domain specificity of anti-FVIII antibodies in polyclonal plasmas of E16-B6/S129 and F8TKO mice was determined by ELISA using BDD human FVIII, porcine FVIII and single human domain (SHD) human/porcine hybrid FVIII as antigens. After subtracting antibody cross-reactivity with porcine FVIII, the relative ELISA titer of a given SHD FVIII compared to human FVIII provides a measure of domain specificity.

**Statistical analysis**

Analysis for the RT-PCR was analyzed using Student’s t-test. Analyses for all other experiments were done using the Mann-Whitney U test. A p<0.05 was considered statistically significant.

**Results**

**Generation of F8TKO mice**

F8TKO mice were generated on a C57BL/6 background using a double-targeting method (Fig. 1A). First, a loxP site flanked (floxed) PGK-neomycin cassette was inserted into exon 1 in Bruce4 mouse embryonic stem cells (ES cells), which are derived from C57BL/6 mice [21]. Correctly targeted ES cell clones were then used for the incorporation of a similarly floxed PGK-hygro cassette downstream of exon 26 using homologous recombination. The presence of both insertions in ES cells was confirmed by Southern blots of genomic DNA (Fig. 1B and Supplementary Fig. 1). Since F8 is on the X chromosome and the Bruce4 ES cells used for the targeting are male, there was no need to further confirm integration of both loxP sites on the same allele. Mice were generated from double-targeted ES clones, and germline transmission was obtained. These were then bred with the Cre recombinase-expressing OzCre strain (Ozgene), resulting in excision of the selection cassettes and the entire coding sequence of F8 (Fig. 1A). We confirmed the mouse genotypes using two primer pairs: one set specific for exonic regions of F8 to detect wild type alleles and another with a forward primer for the intact portion of exon 1 and a reverse primer downstream of exon 26 to detect the knockout allele (Fig. 1C). If F8 is intact, the distance between the latter two is too great to get amplification of product; a band is only obtained if there is excision of the gene. Successful deletion of F8 in our F8TKO strain was verified in this way (Fig. 1C).

We next confirmed lack of mRNA transcripts from F8 using qRT-PCR. Because F8 is highly transcribed in the liver, we isolated mRNA and saw that none could be detected in F8TKO mice (Fig. 2A). The complete lack of mRNA, as expected from a complete gene deletion, implies that there is no FVIII protein or CRM. E16 mice also showed reduced F8 transcripts as expected. Though nonsense-mediated decay normally leads to a loss of message [27], expression levels of exons 3/4 and 11/12 were statistically higher in E16 than F8TKO mice, which could account for the small amounts of FVIII heavy chain protein.
reported in this strain [18]. Because genomic changes can affect transcription levels of neighboring genes, we also examined transcription levels of FUNDC2, a gene of unknown function at the 5’ end of the F8 locus on the opposite DNA strand (Fig. 2B). We saw that F8 deletion did not significantly affect the levels of FUNDC2 transcripts in F8TKO livers compared to E16 and WT mice (Fig. 2B), although broader expression data will be needed to confirm that the transcriptional profiles are not further altered.

**FVIII assays in F8TKO and E16-B6/S129 mice**

After the F8TKO mice were bred to homozygosity, FVIII activity was characterized. This was quantified at 0.71 U/mL (25.7% of wild-type levels) by a one-stage coagulation assay (Fig. 3A), which was no different from the 0.77 U/mL levels measured in E16 mice. FVIII activity measured by a chromogenic assay was less than the lower limit of detection for both strains (Fig. 3B). The activated partial thromboplastin time (aPTT) for undiluted mouse plasma is significantly shorter than human plasma for both normal and FVIII-deficient plasma. Previous investigation revealed that over 10-fold higher levels of factor VII (FVII) and factor V were responsible for the apparent FVIII activity detected by the aPTT based one stage assay in E16 mice [20]. We measured the FVII activity level in the F8TKO mice and found an elevated level at 9.3 U/ml that was not significantly different from that of the E16 mice (12.0 U/ml) (Fig. 3A).

**Bleeding phenotype in F8TKO mice**

Significantly fewer F8TKO pups (70.9%) survived until weaning compared to E16-B6/S129 pups (93.2%) (Mann-Whitney: p <0.01) (Fig 3C). In a tail-snip bleeding model, the amount of blood loss following tail-snip was extensive in both E16 and F8TKO mice (Fig. 3D). Both strains had similar correction of the bleeding phenotype after the infusion of 180 U/kg of human FVIII [28, 29].

**Immunogenicity of FVIII in F8TKO mice**

The immune response of F8TKO mice following 4 weekly injections of 2 μg of recombinant FVIII followed by a single 4 μg boost dose was compared to E16-B6/S129 mice as well as E16 mice that were backcrossed to a >95% C57BL/6 background through breeding with C57BL/6J animals (E16-B6). This was done to limit the confounding differences between the immunologic background of the mice, as SNP analysis (DartMouse) of the E16-B6/S129 colony revealed they were only on an approximately 70% C57BL/6 background (unpublished data). Using an ELISA titer assay for total anti-FVIII IgG, we found no significant differences between the three groups of mice with ELISA titers of 1827, 2162, and 1298 respectively for F8TKO, E16-B6/S129, and E16-B6 mice (Fig. 4A). Similarly there were no significant differences between the Bethesda inhibitor titers for these mice with median titers of 305, 253, and 410 BU/ml (Fig. 4B).

**Domain specificity of anti-FVIII antibodies in F8TKO and E16 mice**

The domain specificity of anti-FVIII IgG from plasmas of F8TKO and E16-B6/S129 mice was evaluated using human, porcine, and SHD hybrid FVIII proteins. There was wide interindividual variation with respect to relative FVIII domain titers (Table 1). Broad reactivity
against all FVIII domains except the light chain activation peptide was observed. A statistically significant increase was seen in anti-C1 antibodies in F8\textsuperscript{TKO} compared to E16-B6/S129 mice (P = 0.001, Mann-Whitney test), but no difference was detected in antibody levels against any other domain. The A2 domain was the most immunodominant domain both in E16 mice, consistent with previous results [30], and in F8\textsuperscript{TKO} mice.

Discussion

The Kazazian E16 and E17 mice have provided an invaluable resource for the study of hemophilia A for the last 20 years. In this paper, we report on a new hemophilia A mouse model in which all coding exons of FVIII have been removed resulting in a CRM-negative mouse. There are both CRM-positive and CRM-negative mutations among patients with severe hemophilia A. Inversion of intron-22, which is the causative mutation in approximately 45% of patients with severe hemophilia A, is associated with a lower incidence of inhibitor development [2]. Consistent with this, FVIII protein has been identified by immunofluorescence microscopy in hepatocytes and sinusoidal endothelial cells in liver tissue sections from three patients with the intron-22 inversion [31]. However, two independent genetic models in mice recently have demonstrated that endothelial cells but not hepatocytes produce detectable amounts of FVIII protein [32, 33], suggesting the detection of CRM-positive material in human hepatocytes may have been artifactual. However, regardless of the CRM-positive versus CRM-negative status of subpopulations of patients with severe hemophilia A, the availability of murine models for both scenarios may prove valuable as we expand our knowledge of inhibitor development.

Based on the analytical testing shown here, we have demonstrated that F8\textsuperscript{TKO} mice have a bleeding phenotype as severe as that in the most commonly used E16-B6/S129 strain. Both F8\textsuperscript{TKO} and E16-B6/S129 mice have similar factor VII and VIII activity and responses to exogenous FVIII, although fewer F8\textsuperscript{TKO} pups survive until weaning. While further studies will be required to determine the cause of this differential survival, we hypothesize that it may be caused by either a slightly more severe bleeding disorder in the F8\textsuperscript{TKO} mice or possibly behavioral differences in breeders due to strain background. In addition to bleeding phenotype, we demonstrated that injection of FVIII into F8\textsuperscript{TKO} animals showed no significant difference in the amount of anti-FVIII IgG antibodies compared to E16-B6/S129 mice. However, it is known that strain background affects immunogenicity of FVIII in mouse models [22, 34, 35] just as differences in immune regulatory genes are known to affect the immune response to FVIII in humans [36-38].

The E16 and E17 strains were originally made using 129S4/SvJae-derived J1 ES cells [17], and are commonly used as a mixed B6/129S4 background. Given these known strain-related differences, we also compared them to E16 mice on a >95% C57BL/6 background in an attempt to limit the genetic variation between the strains to the type of F8 mutation. These E16-B6 mice also showed similar anti-FVIII IgG levels and Bethesda inhibitor titers when compared with the F8\textsuperscript{TKO} mice. However, some caution must still be taken in interpreting these results as some differences seen here may nevertheless be due to strain variation. While the E16-B6 and F8\textsuperscript{TKO} mice are both on C57BL/6 backgrounds, it is known that there are genetic differences between C57BL/6 substrains [39, 40]. Though we crossed all of our
colonies to C57BL/6J mice, as our SNP analysis did not differentiate between substrains, we cannot be certain the extent to which the use of the Bruce4 ES cells in the creation of the F8TKO line or the original backcrosses from the Hoyer group [22] contribute to the genetic diversity between the lines. Nonetheless, we believe having both a CRM-positive and CRM-negative murine models of severe hemophilia A will allow for further exploration of inhibitor development.

The differences between CRM-positive and CRM-negative patients are thought to be due to the latter’s inability to present FVIII as an antigen in the thymus, which is necessary for negative selection against autoreactive T cells and central tolerance. Insertion of a neomycin cassette into F8 in the E16 and E17 strains results in truncated or partially deleted FVIII [17, 19] with low levels of FVIII heavy chain reported in plasma [18]. Therefore, these strains may retain some level of central tolerance to FVIII protein due to translation of residual F8 in the thymus, as has previously been shown in E16 and E17 liver lysates and cryoprecipitates [18]. The F8TKO strain should completely lack central tolerance as they have no F8 transcription in the thymus. The immune response to the E16-B6/S129 mice has been shown to include antibodies to all domains of FVIII [28, 30, 41]. Domain mapping of mice from this study similarly showed significant diversity, with A2 being the immunodominant domain in the F8TKO as well as E16 strains, though interestingly, there was an increase in the amount of C1 domain-specific antibodies in F8TKO compared to E16-B6/S129 mice. Given similar domain specificity between the two models, it is important to note that the CRM-positive material in the E16 mice will be from the murine FVIII sequence and the mice in this report were infused with human FVIII. Human FVIII and murine FVIII have 74% amino acid identity [42], which may limit the thymic tolerance to human FVIII sequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References


Essentials

- Anti-factor VIII (FVIII) inhibitory antibody formation is a severe complication in hemophilia A therapy.
- We genetically engineered and characterized a mouse model with complete deletion of the \( F8 \) coding region.
- \( F8^{TKO} \) mice exhibit severe hemophilia, express no detectable \( F8 \) mRNA, and produce FVIII inhibitors.
- The defined background and total lack of FVIII in \( F8^{TKO} \) mice will aid in studying FVIII inhibitor formation.
Figure 1. Generation of a new mouse model of hemophilia

(A) A representative schematic of the \( F8 \) gene with vertical boxes representing exons. Inserts 1 and 2 show the loci in which the targeting cassettes were incorporated. The wild type gene ("Gene") is shown above, with the region after correct insertion of the targeting vectors ("Targeted Gene") shown below. Gray arrows point to location in which the cassettes were inserted in the wild type locus. Cre recombinase-mediated deletion resulted in the knockout allele (\( FVIII^{\Delta} \)), in which only the 5' UTR of exon 1 remains of the \( F8 \) gene. Restriction enzyme sites are indicated, with the corresponding fragment size upon digestion.
indicated below. Diagrams were not drawn to scale. (B) Southern blots of targeted ES cells. A Neo2 probe was used in a BamHI digest of genomic DNA to probe for the 6.2 kb band indicating correct integration of vector 1 (1), and subsequently a 5′ probe was used in a Sac I digest to probe for the 6.5 kb band indicating integration of vector 2 (2). Black bars represent the location of the Southern blot probes. Notched arrows show the location and direction of primers. (C) PCR genotyping of mouse ear samples. To test for the WT allele, the WT primer set was used. To test for the knockout allele (KO), the KO primer set was used. The genotype of the mice is indicated above. WT band = 130 bp. KO band = 210 bp. Abbreviations: E1= exon 1; E26= exon 26; FUNDC2= FUN14 domain containing 2; Neo= Neomycin cassette; Hygro= hygromycin cassette; PGK= PGK-1 promoter; L=Ladder; WT= wild type ES cell; T= targeted ES cell; F= forward primer; RWT= reverse primer for the WT primer set; RTKO= reverse primer for KO primer set.

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Figure 2. The F8 gene has no detectable transcripts in F8TKO mice
(A, B) Quantification of RNA levels of several Factor VIII exons (A) or of FUN14 domain containing 2 (FUNDC2) (B), an adjacent gene, in RNA extracted from F8TKO, their WT littermates, and E16-B6/S129 mice livers (nF8=6; nFUNDC2= 4) using qPCR. Shown are mean relative expression levels ($\Delta\Delta$CT), in which values were compared to the housekeeping gene acidic ribosomal phosphoprotein P0 (ARBP) ($\Delta$CT). All samples were run in triplicate. Values were normalized to the WT level on a per plate basis with WT expression level set as 1. ***p<0.001; **p<0.01; *p<0.05; ns=non-significant (T-Test).
Figure 3. Bleeding phenotype in F8TKO mice
Factor FVII and FVIII levels in eight- to twelve-week-old E16-B6/S129, F8TKO, and C57BL/6J (Jackson) mice were compared. (A) FVIII and FVII activity measured by one-stage assays. (B) FVIII activity measured by a chromogenic assay (COATEST SP4 FVIII). (C) Survival rate of mouse litters shown as the percent of pups in each litter that survived until weaning (n_{E16-B6/S129} = 41; n_{F8TKO} = 52). Bars represent the mean and standard deviation. p<0.01 (Mann-Whitney test). (D) Murine in vivo bleeding model comparing blood loss following the injection of saline or 180 U/kg FVIII. **p<0.01; *p<0.05; ns=non-significant (Mann-Whitney Test).
Figure 4. Immunogenicity of FVIII in E16-B6/S129, E16-B6 and F8TKO mice

(A) The anti-FVIII IgG ELISA titers detected in plasma of mice infused with 4 weekly injections of FVIII followed by a boost injection. All mice were eight to twelve weeks old at the first injection. The ELISA titers were compared to the F8TKO (p=0.58 and p=0.40 respectively, Mann-Whitney). (B) The Bethesda inhibitor titers of the F8TKO were also compared with the E16-B6/S129 and E16-B6 mice and were not significantly different (p=0.53 and p=0.60, respectively, Mann-Whitney). ns= non-significant
# Table 1

Domain cross-reactivity of anti-FVIII antibodies after immunization

<table>
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<tr>
<th></th>
<th>pc_FVIII</th>
<th>hu_A1</th>
<th>hu_A2</th>
<th>hu_ap</th>
<th>hu_A3</th>
<th>hu_C1</th>
<th>hu_C2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E16-B6S129</strong> *</td>
<td>10.40 ± 3.06</td>
<td>25.93 ± 21.49</td>
<td>42.22 ± 28.95</td>
<td>2.21 ± 5.49</td>
<td>0.68 ± 13.84</td>
<td>8.62 ± 5.67</td>
<td>23.69 ± 31.81</td>
</tr>
<tr>
<td><strong>F8TKO</strong> †</td>
<td>8.76 ± 1.33</td>
<td>17.61 ± 5.79</td>
<td>41.68 ± 17.31</td>
<td>1.00 ± 3.03</td>
<td>-0.19 ± 12.71</td>
<td>24.91 ± 3.41</td>
<td>15.00 ± 14.12</td>
</tr>
</tbody>
</table>

pc_FVIII = porcine full length FVIII; hu_A1 = human FVIII domain A1; hu_A2 = human FVIII domain A2; hu_ap = human FVIII activation peptide; hu_A3 = human FVIII A3 domain; hu_C1 = human FVIII C1 domain; hu_C2 = human FVIII C2 domain.

* Measurements shown are the mean ± SD from 9 mice

† Measurements shown are the mean ± SD from 8 mice.