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ORIGINAL ARTICLE

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A rat model of severe VWD by elimination of the VWF gene using CRISPR/Cas9

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Abstract

Background: Von Willebrand Disease (VWD) is the most common inherited bleeding disorder, caused by quantitative and qualitative changes in von Willebrand factor (VWF). The biology of VWD, studied in canine, porcine, and murine models, differ in species-specific biology of VWF and the amenability to experimental manipulations such as phlebotomy. The factor VIII (FVIII) levels in these models are higher than in humans with type 3 VWD, suggesting functional differences between FVIII and VWF.

Objectives

To develop a VWF knock out ($VWF^{-/-}$) rat by excision of all 52 exons of the VWF locus.

Methods: The entire VWF gene was eliminated in Sprague-Dawley (Crl:SD) rats via CRISPR/Cas9-mediated gene editing. VWF antigen (VWF:Ag), VWF propeptide, and VWF collagen IV binding (VWF:CB4) levels were determined by ELISA assays and FVIII chromogenic activity (FVIII:C) levels by chromogenic FVIII assays. Lateral tail veins were transected to measure bleeding time. $VWF^{-/-}$ rats were infused with $FVIII^{-/-}$ rat platelet poor plasma (PPP) to determine response of plasma FVIII.

Results: Breeding of VWF ± rats yielded $VWF^{-/-}$ offspring at normal Mendelian ratios. VWF:Ag, VWF propeptide, VWF:CB4, and FVIII:C plasma levels were undetectable in $VWF^{-/-}$ rats. $VWF^{-/-}$ rats bled longer and more than $VWF^{+/-}$ and $VWF^{+/+}$ rats when challenged. Transfusion of FVIII-deficient platelet-poor plasma induced a rapid rise in endogenous FVIII:C in $VWF^{-/-}$ rats.

Conclusion: This rat model of severe VWD due to elimination of the entire VWF gene recapitulates the severe secondary deficiency of FVIII seen in human type 3 VWD and facilitates the study of VWF and FVIII and their interactions.

KEYWORDS

CRISPR, factor VIII, rat model, severe von Willebrand disease, von Willebrand factor

Essentials

- The biology of von Willebrand disease (VWD) has been studied in canine, porcine, and murine models.
- We developed a rat model of severe VWD by CRISPR/Cas9 with total elimination of the von Willebrand factor (VWF) gene.
- We found absent VWF antigen, VWF propeptide, VWF collagen 4 binding, factor VIII (FVIII) chromogenic activity, and increased bleeding when challenged.
- This novel rat model facilitates the study of VWF and FVIII function and their interaction.

1 | INTRODUCTION

Von Willebrand disease (VWD) is a common inherited bleeding disorder in humans. The causes of VWD are abnormalities in the quantity or functional quality of von Willebrand factor (VWF). Severe or type 3 VWD in humans is characterized by having undetectable levels of VWF protein and activity as well as very low levels of factor VIII (FVIII) clotting factors (1-9 IU/dL) and is caused by a homozygous deficiency of VWF.[1] The prevalence of type 3 VWD is estimated as 1.38 per million population in North America.[1] Clinical manifestations of type 3 VWD include frequent epistaxis, ecchymoses, intracranial hemorrhage, prolonged bleeding after trauma or surgery, excessive menorrhagia or postpartum hemorrhage in women, and spontaneous joint and muscle bleeds.[2]

VWF has several key functions in hemostasis. One of these functions is to stabilize FVIII in circulation by FVIII binding to the D′ and D3 regions of VWF. VWF's interaction with FVIII is necessary to prevent the rapid clearance of FVIII from the circulation.[3] Humans with type 3 VWD have undetectable levels of VWF and consequently very low levels of FVIII by virtue of the rapid clearance of FVIII in the absence of VWF. VWF also has 2 distinct binding sites for collagen in VWF's A1 (collagen IV and VI) and A3 (collagen I and III) domains[4,5] that mediate VWF binding to exposed subendothelial collagen, whereas the interaction of platelet glycoprotein Ib-α (GPIbα) complex with the VWF A1 domain facilitates the capture of circulating platelets at sites of injury.[6]

In animal models, the function of VWF has mainly been studied in canine, porcine, and murine models of severe VWD.[7] Canine VWD models have similar clinical symptoms to humans with mucosal and cutaneous bleeding,[8] but in contrast to humans, normal dogs lack platelet VWF.[9] The FVIII activity levels in the type 3 VWD canine models varies from 15% to 50% of normal dog VWF.[7] The type 3 VWD porcine model exhibits residual levels of VWF but mimics human disease with respect to bleeding time and distribution of VWF in endothelial cells and platelets,[10] yet FVIII activity remains at about 30% of normal pig VWF.[6] Further limitations of the porcine model include the logistic requirements for the housing of large numbers of animals, and genetic heterogeneity of the breeding stock. The mouse model of type 3 VWD was generated by insertional disruption of intron 5 of the mouse VWF gene, shows undetectable residual VWF, and closely mimics laboratory findings presented in human disease.[11] Like the canine and dog models of type 3 VWD, the FVIII activity level of the type 3 murine model is 20% of normal mouse VWF.[7] While $VWF^{-/-}$ mice are easily maintained and exhibit a homogeneous genetic background, their small size limits the spectrum of analytical and experimental interventions.

In the current study, we developed and characterized a novel rat model of severe type 3 VWD by CRISPR/Cas9-mediated elimination of all 52 exons of the rat VWF locus. Furthermore, the heterozygous rats exhibit an intermediate phenotype as seen in humans as well.

2 | METHODS

2.1 | Generation of VWF knockout rat

Studies were approved by the Animal Care and Use Committee of the Medical College of Wisconsin. The VWF knockout (KO) (SD-*Vwf*^{em1Mcwi}) rat or $VWF^{-/-}$ was developed by CRISPR/Cas9-mediated elimination of the entire VWF gene. CRISPR target sequences flanking exon 1 and 52, respectively were identified using the MIT CRISPR Design Tool (Zhang Lab, Cambridge, MA, USA).[12] Paired oligonucleotides (upstream exon 1:5′-CCTCGGTAATTAAGAGAAGGCC-3′; downstream exon 52:5′-CCCTCCTGGGCCCACAATAAAGG-3′) were synthesized by Thermo Fisher (Waltham, MA, USA), annealed, and cloned into the pX459 plasmid at the *BbsI* site.[13] The 2 plasmids containing 5′ and 3′ CRISPR guide sequences, respectively, were coinjected into 1 cell-stage Sprague-Dawley (Crl:SD) rat embryos. Resulting offspring were screened for the desired VWF gene deletion by PCR analysis of genomic DNA (gDNA) purified from white blood cell (WBC) pellets using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The 5′ end PCR product of the VWF gene (502 bp) was identified using VWF 5′ F primer (ttcatacctgtgggtgacgg) and VWF 5′ R primer (ctggatggatctgctcaggc). The 3′ end PCR product of the VWF gene (450 bp) was identified using VWF 3′ F primer (cggcagactcctactgctac) and the VWF 3′ R primer (ctgatttccagagcccacct). The deletion PCR product of the region flanking the VWF gene (673 bp) was identified using the VWF 5′ F primer and VWF 3′ R primer (Figure 1). Founder animals were mated with a wild-type (WT) SD rats to produce heterozygous offspring that were used to generate homozygous offspring with no expression of VWF.

2.2 | Blood draws and preparation of plasma

Whole blood (0.5 mL) was obtained from the ventral tail artery at 6 to 10 weeks of age using a 25G needle and 1-mL syringe containing 4% sodium citrate (10% of final volume). Whole blood was centrifuged at 2300 *g* for 10 minutes. The plasma was then recentrifuged

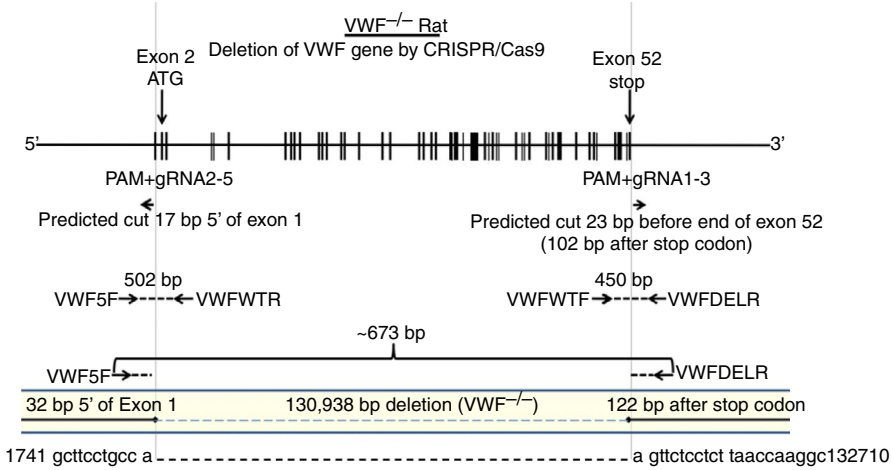

FIGURE 1 Deletion of von Willebrand factor (VWF) gene by CRISPR/Cas9. Two guide RNAs (gRNA) with protospacer-adjacent motif (PAM) were designed to cut at the 5′ and 3′ end to flank the entire coding sequence of the VWF gene. The 5′ end of the VWF gene (502 bp) was identified using the VWF 5′ F primer (VWF5F) and VWF 5′ R primer (VWFWTR). The 3′ end of the VWF gene (450 bp) was identified using the VWF 3′ F primer (VWFWTF) and VWF 3′ R primer (VWFDELR). The deletion of the regions flanking the VWF gene PCR product (673 bp) was identified using the VWF 5′ F primer (VWF5F) and the VWF 3′ R primer (VWFDELR). Rat reference sequence is from NCBI RefSeq, Accession NW 001 084 832

at 9300 *g* for 10 minutes to obtain platelet-poor plasma (PPP). PPP was aliquoted and frozen at −80˚C or used immediately for assays.

2.3 | ELISA

Rat VWF antigen (VWF:Ag) levels were measured by ELISA. The reference standard for all assays was a pool of plasma obtained from nine Sprague-Dawley wild-type rats and frozen in small aliquots. Immulon 1B plates (Thermo Scientific, Waltham, MA, USA) were coated with anti-VWF capture antibody 344.1 (Blood Research Institute, Milwaukee, WI, USA) diluted to 2.5 µg/mL in carbonate coating buffer (CCB; 0.015M $NaCO_3$ and 0.035M $NaHCO_3$, pH 9.5). Plates were blocked with 1% BSA in phosphate-buffered saline (PBS). Rat VWF samples and standard were diluted in the same blocking buffer as above, loaded 50 µL in triplicate on the ELISA plate, and incubated for 1 hour at room temperature. Detection of bound VWF was measured using a biotinylated polyclonal anti-VWF antibody (Dako, Dako Denmark) diluted to 2 µg/mL in blocking buffer.

Rat VWF propeptide was measured by ELISA using antibodies directed against the propeptide region for both capture and detection. Capture of rat propeptide used monoclonal anti-VWF propeptide antibody 349.3 (Blood Research Institute, Milwaukee, WI, USA) and detection used biotinylated monoclonal anti-VWF propeptide antibody 450.3 (Blood Research Institute, Milwaukee, WI, USA). To generate a rat-specific VWF propeptide antibody, VWF−/− mice were immunized with nickel purified recombinant rat VWF propeptide as previously described.[14] Clone 450.3 was selected based on strongest reaction with rat VWF propeptide, both from recombinant VWF expression in cell culture and rat plasma.

Rat VWF binding to human collagen IV (VWF:CB4) was measured by coating human type IV collagen (Southern Biotech, Birmingham, AL, USA) diluted to 1 µg/mL in PBS on a maleic anhydride plate (Thermo Scientific, Waltham, MA, USA).[5] Plates were blocked with 1% BSA in PBS. Rat VWF samples and standard were diluted in the same blocking buffer as above, loaded 50 µL in triplicate on the ELISA plate, and incubated for 1 hour at room temperature. Detection of bound VWF was measured using a biotinylated polyclonal anti-VWF antibody (Dako, Dako Denmark) diluted to 2 µg/mL in blocking buffer.

For analysis of VWF:Ag in platelets, platelets were isolated from citrated whole blood and diluted using modified Tyrode's buffer (20 mM 4-[2-hydroxyethyl]-1-piperazineethane sulfonic acid [HEPES], pH 7.4, 137 mM NaCl, 3.8 mM $NaHCO_3$, 2.5 mM KCl, 0.36 mM $NaH_2PO_4H_20$, 5.5 mM glucose, 0.25% BSA, 1 mM $MgCl_2$) and 50 ng/mL prostaglandin E1 (Sigma-Aldrich, St Louis, MO, USA). Blood samples were diluted with modified Tyrode's buffer and centrifuged at 200 *g* for 2 minutes at room temperature to collect platelet-rich plasma (PRP). PRP was counted for platelets and was then transferred to a fresh 1.5-mL tube and spun down at 900 *g* for 20 minutes to obtain a platelet pellet. PBS containing 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate was added to the platelet pellet for lysis. Samples were then spun at 16 100 *g* at 4˚C for 10 minutes to obtain cleared lysates.

2.4 | Gel electrophoresis

Multimer distribution was examined by gel electrophoresis as previously described[15] using a polyclonal anti-VWF antibody described above.

2.5 | FVIII chromogenic assay

Plasma FVIII in $VWF^{-/-}$ rats was determined by FVIII chromogenic activity assay (FVIII:C) using a COATEST SP4 FVIII kit (Chromogenix, Bedford, MA, USA). Plasma was diluted in 1x

COATEST buffer. The reference standard used dilutions of a pool of plasma from Sprague-Dawley WT rat controls as described above. 25 µL of diluted sample and standard plasmas were loaded in duplicate on Immulon 1B plates (Thermo Scientific, Waltham, MA, USA). Factor IXa, factor X, phospholipid, and $CaCl_2$ were added to each well and incubated at 37˚C for 10 minutes. Chromogenic factor Xa substrate S-2765 was added after incubation and read kinetically at 405 nm [16].

2.6 | Transected lateral tail vein bleeding assay

To assess the bleeding phenotype, 20- to 24-week-old anesthetized rats (both male and female) were placed in a device that allowed us to measure the location where the diameter of the tail was 6.4 mm. The tail was marked and then placed in a second device that allowed us to make a cut across the lateral tail vein that was 2.0 mm in depth. After the lateral tail vein was transected, it was placed in 45 mL of prewarmed 37˚C distilled water in a 50-mL graduated cylinder for 10 minutes, causing red blood cell (RBC) lysis as bleeding occurred. Bleeding time was recorded when the stream of blood was no longer visible. To determine the amount of blood lost, hemoglobin was quantified from RBC lysis by pipetting 50 µL of the collected blood in distilled water into the wells of an Immulon 1B plate (Thermo Scientific, Waltham, MA, USA) and the absorbance was measured at 575 nm. Dilutions of pooled fresh whole blood from 4 WT rats were prepared in distilled water as a standard curve.

2.7 | Transfusion with FVIII-deficient PPP

Whole blood was collected into 4% citrate (10% of final volume) using a 25G butterfly needle from the inferior vena cava of Sprague-Dawley $FVIII^{-/-}$ rats.[17] PPP was prepared as described above. About 10 mL/kg of pooled FVIII-deficient PPP was infused into the lateral tail vein of $VWF^{-/-}$ rats. Sequential blood draws (0.2 mL) in each rat from the tail artery for analysis of VWF:Ag and FVIII:C were obtained at the following time points: 0, 0.5, 2, 6, 12, 24, 36, and 48 hours.

2.8 | Recombinant human VWF infusion

Recombinant human VWF (Vonvendi; Shire, Lexington, MA, USA) was infused at 100 to 200 U/kg in the lateral tail vein of $VWF^{-/-}$ rats. Sequential blood draws (0.2 mL) in each rat from the tail artery for analysis of VWF:Ag and FVIII:C were obtained at the following time points: 0, 0.5, 1, 2, 4, 6, 12, and 24 hours.

2.9 | Statistical analysis

Differences between rat genotypes were examined using a Mann-Whitney test for continuous or ordinal variables. Given the presence of few outliers, a Mann-Whitney test was chosen over a t-test since a Mann-Whitney test is more conservative. A chi-square or a Fisher's exact test was performed for categorical variables. Analysis was performed using GraphPad Prism (version 7.0d; GraphPad Software, La Jolla, CA, USA). Pharmacokinetic parameters were estimated using noncompartmental theory. Area under the plasma concentration–time curve from zero to the last observed time point were estimated. The 95% confidence interval was estimated based on t-distribution. The software used was an R program PK.

3 | RESULTS

3.1 | Generation of VWF KO rats

Of 17 pups born, 11 carried insertions/deletions at the predicted 5′ and/or 3′ guide RNA target site, as judged from surveyor assays. Complete VWF gene deletion of exons 1 to 52, in 1 allele, was detected in 3 of the founders by the presence of the predicted 673-bp PCR product (Figure 1). Sequencing across the deletion boundaries showed elimination of 8 nucleotides upstream of the protospacer-adjacent motif (PAM) sequence (CCT) of the 5′ guide RNA sequence, and 3 nucleotides downstream of the PAM sequence (AGG) of the 3′ guide RNA sequence, resulting in deletion of a 130,938 bp. The deletion did not generate new translational start sites, and translational stop codons in all 3 reading frames were retained within 64 nucleotides downstream of the deletion. Intercrosses of heterozygous $VWF^{+/-}$ rats produced $VWF^{-/-}$ progeny in normal Mendelian ratios (25.3% $VWF^{+/+}$, 48.3% $VWF^{+/-}$, and 26.4% $VWF^{-/-}$; Table 1). Likewise, homozygous ($VWF^{-/-}$ × $VWF^{-/-}$) breeding produced normal sized $VWF^{-/-}$ litters, averaging 8 pups. $VWF^{-/-}$ rats survived beyond 1 year without the need for VWF replacement. No spontaneous joint or mucocutaneous bleeding was observed. This indicates that $VWF^{-/-}$ rats have normal fecundity and fertility, and that complete VWF deficiency is compatible with embryonic and postnatal development, as well as long-term survival in the absence of severe spontaneous bleeding events.

3.2 | VWF and FVIII assays

There was a significant difference in plasma VWF:Ag between all genotypes ($P < 0.0001$) with a mean of 94.7 ± 17.6 U/dL in $VWF^{+/+}$

TABLE 1 Progeny from $VWF^{+/-}$ × $VWF^{+/-}$ breeding results in normal Mendelian frequencies

Genotype	WT ($VWF^{+/+}$)	Heterozygous ($VWF^{+/-}$)	Homozygous ($VWF^{-/-}$)	Males: Females
Total	23	44	24	43:47
Expected	25%	50%	25%	1:1
Actual	25.3%	48.3%	26.4%	1:1.1

Note: $P = 0.95$.

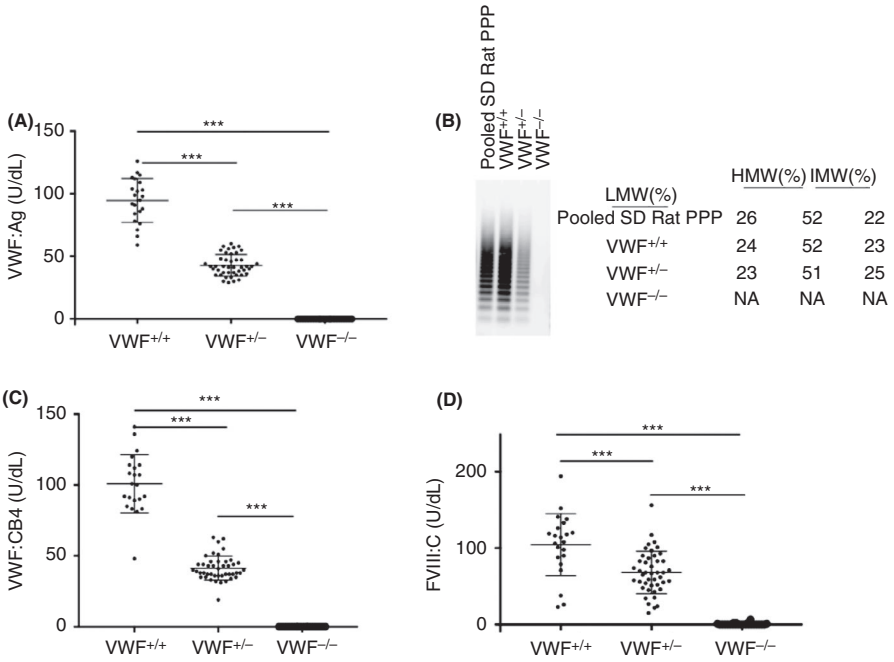

FIGURE 2 Rats with full length deletion of the von Willebrand factor (VWF) gene have undetectable VWF antigen, VWF multimers, collagen IV binding, and factor VIII activity. Blood draws (0.5 mL) were obtained from the tail artery of 6- to 10-week-old Sprague-Dawley rats to obtain platelet poor plasma. (A) VWF antigen (VWF:Ag) is not detectable in $VWF^{-/-}$ rats and reduced in $VWF^{+/-}$ rats when compared to $VWF^{+/+}$ rats. (B) VWF multimers are absent in $VWF^{-/-}$ rats and reduced concentration in in VWF+/– rats when compared to $VWF^{+/+}$ rats. (C) VWF collagen 4 binding (VWF:CB4) is undetectable in $VWF^{-/-}$ rats and reduced in VWF+/– rats. (D) FVIII activity (FVIII:C) levels are undetectable in $VWF^{-/-}$ rats (LOD 2U/dL) and reduced in $VWF^{+/-}$ rats when compared to VWF+/+ rats. VWF:Ag and VWF:CB4 were determined by ELISA assay using anti-VWF antibodies. VWF multimer distribution was examined by gel electrophoresis using polyclonal anti-VWF antibody. FVIII:C was determined by COATEST FVIII assay. All assays were compared to normal pooled Sprague-Dawley rat platelet-poor plasma. The Mann-Whitney test was used to compare genotypes. Error bars denote 1 standard deviation. N ≥ 22 for each genotype. *P<0.05, **P<0.01, ***P<0.001

rats, 42.7 ± 8.5 U/dL in $VWF^{+/-}$ rats, and undetectable levels in $VWF^{-/-}$ rats (Figure 2A), with a limit of detection (LOD) of < 1 U/dL. VWF:Ag was present in the platelet lysates of $VWF^{+/+}$ rats (9.45 U 10^{10} platelets) but was undetectable in platelets from $VWF^{-/-}$ rats. VWF multimers were absent in the $VWF^{-/-}$ rats and reduced in intensity in $VWF^{+/-}$ rats as compared to WT rats (Figure 2B). VWF propeptide was undetectable in $VWF^{-/-}$ rats. There was also a significant difference in VWF:CB4 between all genotypes (P < 0.00) with a mean of 100.8 ± 20.6 U/dL in $VWF^{+/+}$ rats, 41.3 ± 8.5 U/dL in $VWF^{+/-}$ rats, and undetectable levels in $VWF^{-/-}$ rats (Figure 2C) with LOD < 1 U/dL. There was a significant difference in FVIII:C between all genotypes (P < 0.00) with a mean of 104.5 ± 40.5 U/dL in $VWF^{+/+}$ rats (range, 23-194 U/dL), 68.3 ± 27.8 U/dL in $VWF^{+/-}$ rats (range, 15-156 U/dL), and undetectable levels in $VWF^{-/-}$ rats (limit of detection [LOD], 2 U/dL; Figure 2D). Thus, similar to human type 3 VWD, VWF $rats^{-/-}$ exhibit a profound secondary deficiency of plasma FVIII, with substantial variability of FVIII:C in WT and heterozygous rats.

3.3 | Transected lateral tail vein bleeding assay

After lateral tail vein template transection, all $VWF^{-/-}$ rats bled for > 10 minutes, while $VWF^{+/+}$ rats bled for a mean of 1.9 ± 0.8 min and $VWF^{+/-}$ rats bled for a mean of 2.3 ± 0.4 minutes (P < 0.01; Figure 3A). Blood loss measurement demonstrated that $VWF^{-/-}$ rats lost greater quantities of blood loss than $VWF^{+/+}$ rats and $VWF^{+/-}$ rats (P < 0.01). Blood loss mean for $VWF^{-/-}$ rats was 5.1 ± 1.7 mL, in $VWF^{+/-}$ rats was 1.1 ± 0.9 mL, and in $VWF^{+/+}$ rats was 0.4 ± 0.6 mL (Figure 3B). These data documented that $VWF^{-/-}$ rats exhibited increased bleeding time and blood loss following injury.

3.4 | Endogenous production of FVIII in VWF–/– rats

In order to ascertain rat VWF chaperone function for FVIII to prevent rapid clearance of endogenous FVIII, we infused completely FVIII-deficient rat PPP containing 118 to 145 U/dL of VWF:Ag into $VWF^{-/-}$ rats and examined normalization of FVIII levels. Infusion of 10 mL/kg of FVIII-deficient rat PPP caused a rise in VWF:Ag at 30 minutes (31.6 ± 13.5 U/dL), with a half-life of approximately 7 hours. FVIII:C was detectable at about 30 minutes (6.7 ± 0.9 U/dL), peaked at 6 hours (27.3 ± 5.2 U/dL), and only small amounts persisted by hour 36. The half-life of FVIII:C was measured at approximately 6 hours (Figure 4), and therefore is consistent with rapid clearance being the cause of decreased FVIII in $VWF^{-/-}$ rats.

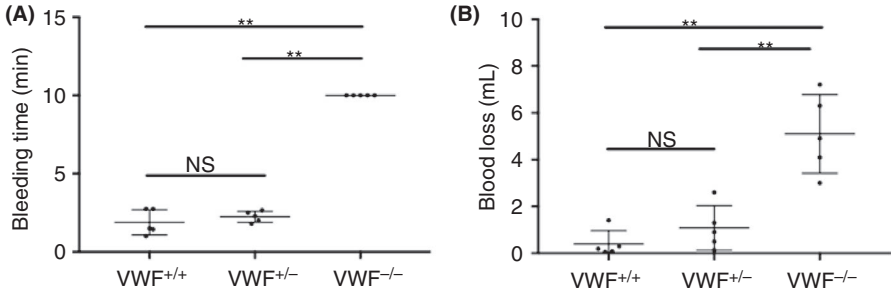

FIGURE 3 Increased bleeding in von Willebrand factor (VWF)$^{-/-}$ rats after lateral tail vein transection. Anesthetized 20- to 24-week-old Sprague-Dawley rats' lateral tail veins were transected at a diameter of 6.4 mm and a depth of 2.0 mm using a template. (A) Measurement of cessation of bleeding over time, and (B) amount of blood loss in mL, for wild-type (VWF$^{+/+}$), heterozygous (VWF$^{+/-\pm}$), and homozygous VWF deficient (VWF$^{-/-}$) rats by tail bleeding assay. VWF$^{-/-}$ rats bled longer and lost greater quantities of blood when compared to VWF$^{\pm}$ and VWF$^{+/+}$ rats. Fisher's exact test was used to compare bleeding times of VWF$^{-/-}$ rats to VWF$^{\pm}$ and VWF$^{+/+}$ rats. Mann-Whitney test was used to compare blood loss in all genotypes. Error bars denote 1 standard deviation. N ≥ 5 for each genotype. Results were pooled from 5 independent experiments. *P<0.05, **P<0.01, ***P<0.001. P = 0.22 for blood loss when VWF +/- is compared to VWF +/+ and P = 0.63 for bleeding time when VWF +/- is compared to VWF +/+

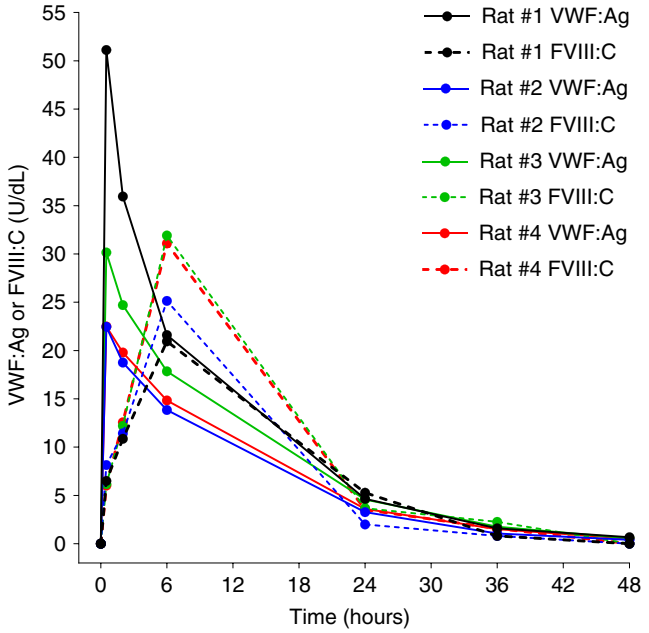

FIGURE 4 Exogenous rat von Willebrand factor (VWF) increases endogenous factor VIII (FVIII) in VWF$^{-/-}$ rats. Levels of von Willebrand factor antigen (VWF:Ag) and FVIII chromogenic activity (FVIII:C) over time in hours after 10 mL/kg of FVIII-deficient platelet-poor plasma was infused into 4 homozygous VWF$^{-/-}$ rats. The average measured half-life of VWF was approximately 7 hours, and the half-life of FVIII:C was approximately 6 hours. This demonstrates that rat VWF increases rat FVIII in the VWF$^{-/-}$ rats

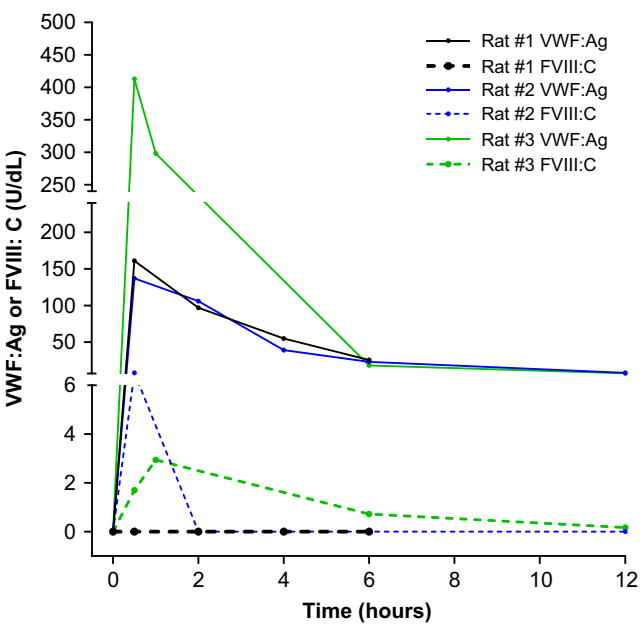

FIGURE 5 Exogenous recombinant human von Willebrand factor (VWF) does not increase endogenous rat factor VIII (FVIII) in VWF$^{-/-}$ rats. Levels of von Willebrand factor antigen (VWF:Ag) and FVIII chromogenic activity (FVIII:C) over time in hours after 100-200 U/kg of VWF infused into three VWF$^{-/-}$ rats. Rats 1 and 2 received 100 units/kg, and rat 3 received 200 units/kg. The average measured half-life of VWF was approximately 2 hours. FVIII:C was initially increased at minimal amounts in 2 of the 3 rats, but then remained undetectable at all time points. Human VWF, unlike rat VWF, does not increase rat FVIII, suggesting that human VWF does not bind rat FVIII

3.5 | Interaction of human VWF with rat FVIII

Recombinant human VWF was infused into VWF$^{-/-}$ rats to examine the interaction of human VWF with rat FVIII. After infusion of 100 U/kg of recombinant VWF (Vonvendi), we observed a rise in VWF:Ag at 30 minutes (149 ± 17 U/dL). After infusion of 200 U/kg of Vonvendi, we observed a rise in VWF:Ag at 30 minutes (413 U/dL). The VWF:Ag half-life was approximately 2 hours. FVIII:C was initially increased at minimal amounts in 2 of the 3 rats but then remained undetectable at all time points (Figure 5), indicating defective chaperone function of human VWF for rat FVIII.

4 | DISCUSSION

In this study, we established a novel rat model of severe VWD by eliminating the full 52 exons of the VWF gene locus using

CRISPR/CAS9-mediated gene editing in SD rats. The deletion of the entire VWF gene in this rat model resulted in absent VWF:Ag, VWF:CB4, and FVIII:C, as well as a bleeding phenotype when challenged. VWF deficiency in rats did not appear to adversely affect intra-uterine development, long-term survival, or fertility of male or female $VWF^{-/-}$ rats. Remarkably, $VWF^{-/-}$ females delivered pups without overt bleeding complications in either the mother or her pups. Likewise, no spontaneous, overt bleeding events were observed in joints (evident by discoloration and/or swelling) or other anatomical locations. Occasionally, some weanlings (4-6 weeks of age) exhibited minor bleeding following ear biopsies performed to obtain tissue samples for genotyping that required intervention (application of styptic powder), indicative of impaired hemostasis. This notion was ascertained in animals subjected to experimental laceration of the lateral tail vein, where 5 of 5 animals tested were unable to contain bleeding. These findings showed that $VWF^{-/-}$ SD rats, as other species, exhibit a profound defect in primary hemostasis that predisposes to severe bleeding following injury.

The most significant difference between $VWF^{-/-}$ SD rats and already existing animal models of type 3 VWD are the very low levels of plasma FVIII. Residual circulating FVIII levels are approximately 20% in the $VWF^{-/-}$ mouse,[7] 15% to 50% in the canine model, and approximately 30% in the $VWF^{-/-}$ porcine model. In the $VWF^{-/-}$ SD rat, FVIII levels were below the LOD of 1.6%, closely resembling the levels of 1% to 5% measured in patients with type 3 VWD.

Given that more than half of SD rats with complete FVIII deficiency ($FVIII^{-/-}$) have been reported to develop spontaneous joint bleeding requiring therapeutic intervention,[17] the very low FVIII levels in the $VWF^{-/-}$ SD rat nevertheless seem sufficient to sustain normal hemostasis in the absence of major challenges. Humans with severe VWD not only are prone to spontaneous mucocutaneous bleeds but also may develop soft tissue bleeds and hemarthroses, which have been attributed to low FVIII levels. We therefore suspect that the conspicuous absence of spontaneous bleeding episodes in type 3 VWD animal models predominantly reflects a higher threshold for the minimum FVIII level required for sustaining near-normal hemostasis in humans. On the other hand, future studies will be required to determine whether additional factors such as increased physical activity, age, or hormone replacement therapy may trigger bleeding episodes in the $VWF^{-/-}$ rat even in the absence of experimentally provoked injury.

Patients with type 3 VWD who are infused with completely deficient FVIII plasma (as a source of VWF) show a progressive increase of FVIII in the circulation followed by a decay phase as the infused VWF is cleared from the circulation. For example, in 8 human subjects infused with human VWF without FVIII, the FVIII:C plasma levels increased progressively with a peak at 24 hours after infusion followed by a gradual decay.[18] Likewise, infusion of human recombinant VWF into human subjects with severe VWD revealed a VWF:Ag half-life of 21.9 hours, and a substantial stabilization of endogenous FVIII:C, peaking at 24 hours after infusion.[19] In the canine model of severe VWD, infusion of human recombinant VWF resulted in a VWF:Ag half-life of 21.6 hours in one dog and 22.1 hours in a second dog with sustained increases in endogenous FVIII levels.[8] In our previous study, we showed that FVIII levels in mouse plasma could be restored up to 800 mU/mL when human recombinant VWF was infused into VWF (null) mice either by intravenous or intraperitoneal administration.[20] Although these data were not directly from a VWF/FVIII binding assay, it indicated that human VWF can bind to mouse FVIII and stabilize endogenous murine FVIII in plasma of VWF (null) mice. In marked contrast, infusion of high doses of human recombinant VWF in the $VWF^{-/-}$ rats resulted in quick clearance of VWF:Ag with a half-life of approximately 2 hours and an initial small rise in endogenous rat FVIII. We find there is 1 to 3 IU of FVIII per 100 IU of VWF, so the small rise in FVIII is probably due to minimal amounts of rat FVIII and residual FVIII in the human recombinant VWF. Since infusion of exogenous rat VWF present in rat $FVIII^{-/-}$ PPP leads to measurable amounts of endogenous FVIII with the FVIII:C peaking at 6 hours in the $VWF^{-/-}$ SD rat, the discrepancy between the SD rat on the one hand and humans and other species on the other hand in all likelihood reflects a species-specific inability of human VWF to interact with rat FVIII. This feature might be exploited to study the interaction of human FVIII and VWF in an in vivo setting without interference by endogenous factors.

In summary, we have established a novel rat model of severe type 3 VWD that resembles human VWD with very low/absent VWF:Ag and minimal plasma FVIII. A second unique feature of this model is the complete elimination of the VWF gene, precluding the synthesis of any VWF-related peptide that could potentially trigger the production of alloantibodies, as suggested in humans carrying large but not complete deletions of their VWF gene.[21] The $VWF^{-/-}$ SD rat is amenable to a host of experimental manipulations that are difficult or near impossible to perform in other species, either for technical or logistic limitations. In addition, the large number of currently available rat genetic resources[22] should enable studies toward the identification and validation of genetic factors affecting bleeding severity and other modifying parameters of VWD.

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RELATIONSHIP DISCLOSURES

The authors report nothing to disclose.

AUTHOR CONTRIBUTIONS

JG designed and performed the research, analyzed the data, and wrote the manuscript. VHF designed the research, analyzed the data, and edited the manuscript. SLH performed the research and edited the manuscript. SAF and JGM performed the research and edited the manuscript. MZ designed and performed the research and edited the manuscript. AMG and HW designed the research and edited the manuscript. QS designed the research and edited the manuscript. RRM designed the research, analyzed the data, and edited the manuscript.

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REFERENCES

1. Nichols WL, Hultin MB, James AH, Manco-Johnson MJ, Montgomery RR, Ortel TL, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). Haemophilia. 2008;14:171–232.
2. Lak M, Peyvandi F, Mannucci PM. Clinical manifestations and complications of childbirth and replacement therapy in 385 Iranian patients with type 3 von Willebrand disease. Br J Haematol. 2000;111:1236–9.
3. Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem. 1998;67:395–424.
4. Pareti FI, Niiya K, McPherson JM, Ruggeri ZM. Isolation and characterization of two domains of human von Willebrand factor that interact with fibrillar collagen types I and III. J Biol Chem. 1987;262:13835–41.
5. Flood VH, Schlauderaff AC, Haberichter SL, Slobodianuk TL, Jacobi PM, Bellissimo DB, et al. RG and others. Crucial role for the VWF A1 domain in binding to type IV collagen. Blood. 2015;125:2297–304.
6. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. Blood. 1983;61:99–110.
7. Denis CV, Wagner DD. Insights from von Willebrand disease animal models. Cell Mol Life Sci. 1999;56:977–90.
8. Schwarz HP, Dorner F, Mitterer A, Mundt W, Schlokat U, Pichler L, et al. Evaluation of recombinant von Willebrand factor in a canine model of von Willebrand disease. Haemophilia. 1998;4(Suppl 3):53–62.
9. Sanders WE, Reddick RL, Nichols TC, Brinkhous KM, Read MS. Thrombotic thrombocytopenia induced in dogs and pigs. The role of plasma and platelet vWF in animal models of thrombotic thrombocytopenic purpura. Arterioscler Thromb Vasc Biol. 1995;15:793–800.
10. Wu QY, Drouet L, Carrier JL, Rothschild C, Berard M, Rouault C, et al. Differential distribution of von Willebrand factor in endothelial cells. Comparison between normal pigs and pigs with von Willebrand disease. Arteriosclerosis. 1987;7:47–54.
11. Denis C, Methia N, Frenette PS, Rayburn H, Ullman-Culleré M, Hynes RO, et al. A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA. 1998;95:9524–9.
12. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013;8:2281–308.
13. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339:819–23.
14. Rugger ZM, De Marco L, Gatti L, Bader R, Montgomery RR. Platelets have more than one binding site for von Willebrand factor. J Clin Invest. 1983;72:1–12.
15. Flood VH, Gill JC, Friedman KD, Christopherson PA, Jacobi PM, Hoffmann RG, et al. Collagen binding provides a sensitive screen for variant von Willebrand disease. Clin Chem. 2013;59:684–91.
16. Rosenberg JB, Foster PA, Kaufman RJ, Vokac EA, Moussalli M, Kroner PA, et al. Intracellular trafficking of factor VIII to von Willebrand factor storage granules. J Clin Invest. 1998;101:613–24.
17. Nielsen LN, Wiinberg B, Häger M, Holmberg HL, Hansen JJ, Roepstorff K, et al. A novel F8–/– rat as a translational model of human hemophilia A. J Thromb Haemost. 2014;12:1274–82.
18. Menache D, Aronson DL, Darr F, Montgomery RR, Gill JC, Kessler CM, et al. AR and others. Pharmacokinetics of von Willebrand factor and factor VIIIC in patients with severe von Willebrand disease (type 3 VWD): estimation of the rate of factor VIIIC synthesis. Cooperative Study Groups. Br J Haematol. 1996;94:740–5.
19. Gill JC, Castaman G, Windyga J, Kouides P, Ragni M, Leebeek FWG, et al. Pavlova BG and others. Hemostatic efficacy, safety, and pharmacokinetics of a recombinant von Willebrand factor in severe von Willebrand disease. Blood. 2015;126:2038–46.
20. Shi Q, Kuether EL, Schroeder JA, Fahs SA, Montgomery RR. Intravascular recovery of VWF and FVIII following intraperitoneal injection and differences from intravenous and subcutaneous injection in mice. Haemophilia. 2012;18:639–46.
21. Shelton-Inloes BB, Chehab FF, Mannucci PM, Federici AB, Sadler JE. Gene deletions correlate with the development of alloantibodies in von Willebrand disease. J Clin Invest. 1987;79:1459–65.
22. Laulederkind SJF, Hayman GT, Wang SJ, Hoffman MJ, Smith JR, Bolton ER, et al. Rat genome databases, repositories, and tools. Methods Mol Biol. 2019;2018:71–96.

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