Identification of Key Coagulation Activity Determining Elements in Canine Factor VIII

Jenni Firrman,1,2 Qizhao Wang,3 Wenman Wu,3 Biao Dong,3 Wenjing Cao,3 Andrea Rossi Moore,3 Sean Roberts,3 Barbara A. Konkle,4,5 Carol Miao,5 LinShu Liu,2 Dong Li,6 and Weidong Xiao1,2,3

1Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, PA, USA; 2Dairy and Functional Foods Research Unit, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA, USA; 3Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA, USA; 4Bloodworks Northwest, Seattle, WA, USA; 5Department of Medicine, University of Washington, Seattle, WA, USA; 6Shanghai Tongji Hospital, Tongji University, Shanghai, P.R. China

It is well known that canine factor VIII (cFVIII) has a higher specific activity than does human FVIII (hFVIII), and it has been previously demonstrated that cFVIII light chain is able to enhance hFVIII activity. The goal of this study was to first determine which amino acids in cFVIII light chain were responsible for enhancing hFVIII activity, and second to use these amino acids to develop a hFVIII variant with enhanced functional activity. We systematically screened segments of cFVIII light chain by testing an array of human-canine light chain hybrids and found that canine amino acids 1857–2147 were key to this enhancement. Each canine amino acid within this span was screened individually using a negative selection method, which led to the identification of 12 aa (JF12) in the FVIII light chain that could enhance activity. Substitution of the corresponding 12 aa into hFVIII (hFVIIIJF12BDD) elevated the specific activity profile in vitro. Furthermore, hFVIIIJF12BDD expressed an in vivo-displayed increased coagulation activity compared to wild-type, while maintaining normal secretion efficiency. In conclusion, we identified the amino acids in cFVIII that are the key determinants for higher specific activity and may be the basis for future development of therapeutic treatments for hemophilia A.

INTRODUCTION

Hemophilia A (HA) is an X-linked genetic disorder caused by pathogenic variants in the F8 gene resulting in the production of either no factor FVIII (FVIII) protein or a nonfunctional or dysfunctional FVIII protein.1–5 Currently, the standard treatment of HA relies on the prophylactic intravenous (i.v.) infusion of recombinant or plasma-derived FVIII protein.5,6 While this replacement treatment corrects the abnormal bleeding phenotype, it is life-long and time-consuming7 and is estimated to cost from $150,000 to $300,000 per patient per year in the United States.7 Therefore, the development of a FVIII protein with increased activity would be valuable and could potentially enhance the quality of life for HA patients.

The concept that a more effective FVIII protein could be developed came from the observation that multiple FVIII orthologs have superior clotting profiles compared to human FVIII (hFVIII).8–10 FVIII protein from pigs, dogs, mice, and monkeys has been tested and was revealed to function appropriately in the human clotting cascade and have the ability to bind human von Willebrand factor (vWF),10 yet also display different biochemical profiles. For example, recombinant ovine FVIII with the B domain deleted has a greater specific activity, and longer half-life following activation, compared to its human counterpart.10–12 Porcine FVIII has been demonstrated to secrete 10- to 100-fold more efficiently compared to hFVIII,11,12 and recently a recombinant porcine FVIII was approved for the treatment of acquired HA.13 Recombinant canine FVIII (cFVIIIIBDD) has a higher specific activity compared to its human counterpart.14,15 However, the direct use of these orthologs in normal patients, without inhibitors, is considered disadvantageous due to the possibility of an immune response. Since the etiology of inhibitor development is unclear,16 changes to amino acid sequence and protein structure are avoided. Therefore, determining the amino acids responsible for the beneficial properties of these orthologs would be valuable, in terms of developing a modified hFVIII construct that has increased coagulation activity.

Previously, it was reported that cFVIIIIBDD is 3- to 7-fold more active compared to B domain-deleted hFVIII (hFVIIIIBDD).11,14 The observed increase in specific activity was predominantly due to the canine light chain (cLC) sequence,11 which was confirmed in vitro, and from dual delivery of AAV carrying FVIII human heavy chain (hHC) and cLC to HA mice in vivo.11 In the current study, the amino acids in cFVIII LC were systematically evaluated for their ability to enhance hFVIII activity. To begin, human and canine hybrid constructs containing different spans of canine and human amino acid sequences were generated in order to ascertain the portion of cLC that is responsible for the observed increase in FVIII activity. Next, the canine amino acids within this region were individually analyzed for their ability to enhance hFVIII activity using a process of negative selection.
selection. This led to the identification of a unique 12-aa sequence that was incorporated into hFVIII. The resulting variant, hFVIII-BDDJF12, exhibited enhanced FVIII specific activity in vitro and in vivo. Based on these results, hFVIII-BDDJF12 has potential for use in both protein replacement therapy and gene therapy.

RESULTS

Canine Amino Acids 1857–2047 Enhance FVIII Activity In Vitro and In Vivo

Preliminary testing in vitro confirmed that the cLC was able to increase hFVIII activity (Figure S1). Prompted by this observation, we set out to determine which amino acids in the cLC increased FVIII activity. This was accomplished using eight sets of primers designed based on areas of shared nucleotide sequence between hLC and cLC (Figure S2) to create 33 human/canine hybrid constructs (Table S1) that contained different portions of human and canine amino acid sequences. Constructs were expressed in vitro through transfection of HEK293 cells using a dual-chain delivery method,17 tested for activity using a one-stage activated partial thromboplastin time (APTT) assay, and protein was measured using an ELISA detecting hHC (Figures S3A and S3B).

Based on these results, the specific activity was calculated by comparing construct activity (U/mL FVIII determined by APTT) to protein amount (ng of hHC/mL quantified by ELISA) (Figure 1). Only two constructs were identified that had activity similar to that of cLC, constructs hLC[1652–1688;1857–2332cLC] and hLC[1857–2147cLC]. Since both of these constructs contained canine amino acid sequences from amino acids 1857–2147, this region of canine sequence was considered positively correlated with enhanced activity, and construct hLC[1857–2147cLC] was selected for further studies (Figure 1B).

Next, the activity of hLC[1857–2147cLC] was tested in vivo through hydrodynamic injection of HA mice with plasmid DNA coding for hHC and either cLC, or hLC[1857–2147cLC]. Results found that mice injected with hHC and either cLC or hLC[1857–2147cLC] had significantly higher coagulation activity compared to mice injected with hHC and hLC, as determined by a one-stage APTT assay, yet there was no significant difference in protein amount (Figures 2A and 2B). The calculated specific activity was significantly higher for mice injected with hHC and either cLC or hLC[1857–2147cLC] compared to mice injected with hHC and hLC (Figure 2C). There was no significant difference between cLC and hLC[1857–2147cLC] for any of the analyses performed.

Identification of Key Amino Acids in hLC[1857–2147cLC] through Negative Selection

Alignment of amino acid sequences for hLC[1857–2147cLC] and hLC revealed a 26-aa difference (Figure S4). It was hypothesized that not all of these amino acids were necessary to enhance FVIII activity. Based on this supposition, each canine amino acid in construct hLC[1857–2147cLC] was evaluated individually. This was accomplished using a process of negative selection, in which mutant constructs were designed to carry the hLC[1857–2147cLC] sequence with one amino acid switched back to the original human amino acid (Table S3). If the amino acid contributed significantly to the increase in FVIII activity, removing it should result in a decrease in activity. Each mutant was tested in vitro through co-transfection of HEK293 cells with hHC, and activity was determined using a one-stage APTT assay and calculated as percent hLC[1857–2147cLC] activity (Figure 3). In this experiment, protein amount was not measured because previous testing demonstrated that hLC[1857–2147cLC] did not alter protein levels (Figure S3). The majority of mutants tested (n = 13) fell within the range of 70%–100% hLC[18547–2147cLC] activity. However, 10 mutants demonstrated less than 70% activity, which indicated that canine amino acid was essential for increasing FVIII activity. Based on these results, the hLC-JF10 construct was designed as a hLC that contained these 10 canine aa (Figure S5).

Development of a hFVIII Variant with Enhanced FVIII Activity

During the cloning process for hLC-JF10, potential constructs were screened for activity using a one-stage APTT assay. One clone was
found to have activity superior to that of cLC and hLC[1857–2147cLC] (Figure S6). Sequencing of this clone revealed two exogenous mutations in the C1 domain, S2157N and R2159H, that were neither human nor canine amino acids (Figure S7). These were unexpected and random mutations, which produced intriguing results. This hLC construct that had 10 canine aa and 2 exogenous aa was named hLC-JF12. In order to determine whether the 2 exogenous aa had an impact on activity, hLC-X2 was engineered as a hLC construct that carried only the 2 exogenous aa mutations (Table 1; Table S5).

Testing in vitro expectedly revealed that cLC, hLC[1857–2147cLC], and hLC-JF10 had significantly higher coagulation activity compared to hLC, but none of these could achieve the same level of activity as hLC-JF12 (Figure 4A). Interestingly, hLC-JF12 also resulted in a significant increase in protein amount, where other constructs did not significantly affect protein levels compared to hLC (Figure 4B). The specific activity was calculated for each construct, which demonstrated that cLC, hLC[1857–2147cLC], hLC-JF10, and hLC-JF12 all had a significantly higher specific activity compared to hLC; however, hLC-JF12 had the highest activity of all of these variants (Figure 4C). Construct hLC-X2 was not significantly different from hLC in any of the assays. Based on these results it was evident that without the X2 mutations, hLC-JF10 was unable to achieve activity to the same level as hLC-JF12; however, the X2 mutations alone did not confer any changes to activity. Taken together, it was concluded that all 12 aa changes were required for the superior activity.

Next, the hLC-JF12 construct was tested in vivo using an AAV vector for delivery. The hHC, hLC, cLC, and hLC-JF12 sequences were packaged into single-stranded AAV8 vectors and delivered along with an AAV8 vector containing hHC via tail vein injection to 6-week-old male CD4KO/HA mice, and blood was harvested via retro-orbital eye bleeding for 12 weeks. The results of a one-stage APTT test demonstrated that both the AAV8-cLC and AAV8-JF12 constructs were able to significantly increase coagulation activity at 2, 4, and 6 weeks post-injection (Figure 5A). At 8 and 12 weeks post-injection, the AAV8-cLC and AAV8-JF12 groups had higher coagulation activity, but this did not reach significance due to variation. An ELISA detecting hHC antigen demonstrated that there was no significant difference in the amount of protein present in any group (Figure 5B). An ELISA detecting hLC confirmed that there was no significant difference in protein amounts between the hLC and the hLC-JF12 groups (Figure 5C).

Single-Stranded FVIII Carrying the JF12 Modifications Enhances Coagulation Activity In Vitro and In Vivo

Single-stranded hFVIII-BDD constructs were engineered to contain either the JF12 mutations (hFVIII-JF12BDD) or the canine amino acids 1857–2147 (hFVIIIhLC[1857–2147cLC]BDD). In vitro testing revealed that the hFVIIIhLC[1857–2147cLC]BDD construct increased coagulation activity by ~2.8-fold, while the JF12 modification resulted in an ~8.8-fold increase in coagulation activity (Figure 6A). For single-chain delivery, both hFVIII[1857–2147cLC] and hFVIIIJF12BDD had a significant increase in protein amount as measured by ELISA, although hFVIIIJF12BDD was significantly higher than both hFVIII-BDD and hFVIII[1857–2147cLC]BDD (Figure 6B). Both hFVIII[1857–2147cLC]BDD and hFVIIIJF12BDD had significantly higher specific activity compared to hFVIII-BDD, but they were not significantly different from each other (Figure 6C). Next, hFVIIIJF12BDD and the hFVIII-BDD constructs were packaged into single-stranded AAV8 vectors, resulting in AAV8-hFVIII-JF12BDD and AAV8-hFVIII-BDD. These vectors were delivered to CD4KO/HA mice via tail vein injection, and blood was harvested through retro-orbital eye bleeding during the course of 12 weeks. The results of a one-stage APTT test demonstrated that the AAV8-hFVIII-JF12BDD construct produced a significantly higher amount of coagulation activity compared to AAV8-hFVIII-BDD for every time point tested (Figure 6D). An ELISA detecting hHC demonstrated that, except for week 2 after injection, there was no significant increase in protein amount for AAV8-hFVIII-JF12BDD compared to AAV8-hFVIII-BDD (Figure 6E). This indicates that the JF12 modifications produce a FVIII protein with higher coagulation activity and have no effect on protein secretion.
The hFVIIIJF12BDD Protein Had Increased Specific Activity and Generated FXa More Robustly Compared to hFVIIIBDD

In order to gain insight into how the JF12 modifications increased FVIII specific activity, protein was partially purified using a SP Sepharose high-performance strong cation exchange column. The results of a western blot indicated that there were no differences in the banding patterns between hFVIIIBDD and hFVIIIJF12BDD, which demonstrated that the JF12 modifications do not affect chain size or heterodimer formation (Figure S8A). Next, the specific activity of hFVIIIJF12BDD was determined by comparing the amount of protein, quantified by ELISA, to the activity produced by a one-stage APTT assay. The results showed that the hFVIIIJF12BDD protein produced a specific activity of 39,153.69 U/mg compared to hFVIIIBDD, which only had a specific activity of 6,247.92 U/mg (Figure S8B). These results verified the in vivo observation that the JF12 modifications increase protein specific activity.

Finally, a FXa generation test demonstrated that the hFVIIIJF12BDD protein was able to convert FX to FXa more efficiently compared to hFVIIIBDD (Figure S8C). Using a Michaelis-Menten kinetics analysis, the $K_m$ (substrate concentration at one-half the maximum velocity) and $V_{max}$ (maximum velocity) for hFVIIIJF12BDD and hFVIIIBDD were determined. The $K_m$ for hFVIIIJF12BDD was calculated at 24.29, compared to 29.14 for hFVIIIBDD, showing that the tenase complex formed from hFVIIIJF12BDD binds to FX with a higher affinity (Figure S8D). The $V_{max}$ for hFVIIIJF12BDD was 11.93, which is lower compared to hFVIIIBDD, with a $V_{max}$ of 15.33 (Figure S8D). This indicated that the hFVIIIJF12BDD construct converts FX to FXa at a quicker rate.

DISCUSSION

It is highly desirable to improve the hFVIII coagulation profile through bioengineering, and a number of FVIII variants with altered properties have been previously described.18–22 Herein, we present the first study to systemically identify key amino acids that may improve hFVIII specific activity utilizing cFVIII as the starting template, which has been documented for its higher specific activity. This strategy avoids the use of random mutations that may disrupt the structure entirely and focuses on the differences that may confer the better properties.
In order to determine how these 12 aa functioned to enhance activity, protein was partially purified and analyzed. The results of testing using this protein found that the JF12 modifications do not affect FVIII structure. Functional testing found that the hFVIII(JF12BBDD) protein has a higher specific activity compared to hFVIII(BDD), validating the hypothesis that the JF12 modifications increase FVIII specific activity. Basic functional testing was performed in order to try and explain the observed phenomenon and found that the hFVIII(JF12BBDD) protein was able to convert FX to FXa more efficiently compared to hFVIII(BDD) by binding to FX with a higher affinity and converting FX to FXa at a quicker rate. However, the recorded increases were not enough to fully explain the enhancing effects of the JF12 modifications. Previously it was reported that changes to the FVIII C1 spike (amino acids 2158–2159) may also alter protein interactions and cofactor activity. In this study, we identified that a total of 12 aa changes are necessary for enhanced specific activity. The results suggested that coordinated action of these amino acids is necessary for the improvement. While this variant provides new insight into the structure and function of factor VIII, the potential of this variant for therapeutic use may be explored under special situations. Note that whether these modifications affect antigenicity and converting FX to FXa differently compared to hFVIII(BDD) may also alter protein interactions and cofactor activity. These analyses, and further functional testing, are required to detail the pharmacokinetics of the hFVIII(JF12BBDD) protein and to understand the mechanism behind the enhanced activity to a fuller extent.

In this study, we identified that a total of 12 aa changes are necessary for enhanced specific activity. The results suggested that coordinated action of these amino acids is necessary for the improvement. While this variant provides new insight into the structure and function of factor VIII, the potential of this variant for therapeutic use may be explored under special situations. Note that whether these modifications affect antigenicity and converting FX to FXa differently compared to hFVIII(BDD) may also alter protein interactions and cofactor activity. These analyses, and further functional testing, are required to detail the pharmacokinetics of the hFVIII(JF12BBDD) protein and to understand the mechanism behind the enhanced activity to a fuller extent.
inhibitor formation or any other immunological properties will require further evaluation.

MATERIALS AND METHODS

Engineering Human/Canine Hybrids and Mutant Constructs

All human/canine hybrid constructs and single-chain constructs were engineered using primers (Integrated DNA Technologies) that annealed to areas of a shared nucleotide sequence. The list of hybrid constructs can be found in Table S1, and primers used are found in Tables S2 and S3. The hLC[1857–2147cLC] mutants and hLC-J10 and hLC-X2 were engineered using primers containing the nucleotide sequences of interest; primer sequences can be found in Tables S4 and S5. Using the specified primers, constructs were cloned according to the following protocol: PCR amplification (Biometra thermocycler) was run for 35 cycles using either Vent polymerase (New England Biolabs) or Taq polymerase (Sigma-Aldrich). Fragments were mixed and inserted into an adeno-associated virus (AAV) plasmid backbone using a Ligation kit (New England Biolabs) or Taq polymerase (Sigma-Aldrich). Fragments were mixed and inserted into an adeno-associated virus (AAV) plasmid backbone using a Ligation kit (New England Biolabs).

For the initial screening process, hybrids were engineered as LC constructs that were tested in conjugation with the hHC using the dual-chain delivery method. In this way, each hybrid LC was tested using the dual-chain delivery strategy. Blood was harvested via retro-orbital bleeding during the course of 3 months. Statistical analysis was performed using a two-tailed Student’s t test, and differences were considered significant when p < 0.05. *p < 0.05, for differences between AAV8-cLC or AAV8-JF12 and AAV8-hLC. Error bars represent standard deviation. (A) U of FVIII/mL produced by each hybrid, determined via an APTT assay. (B) ng of hHC antigen/mL detected by ELISA for each hybrid construct. (C) ng of LC antigen/mL detected by ELISA for hLC- and hLC-JF12-injected groups.

Cellular Transfection Method

HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin antibiotics (Invitrogen), and maintained at 37°C, supplemented with 5% CO₂. To test constructs, cells at 80%–100% confluency were transiently transfected using PolyJet (SignaGen Laboratories). For dual-chain transfection, LC and HC plasmids were transfected using a 1:2 ratio. Transfection was maintained for 16 h, after which cells were washed with Ham’s FXIII media (Gibco), followed by the addition of a total of 400 μL of Ham’s FXIII media, supplemented with 2% heat-inactivated FBS.

AAV Vector Preparation

AAV vectors were serotype 8 and produced following the well-described triple-plasmid transfection method.31,32 After purification by cesium chloride gradient ultracentrifugation, they were buffer exchanged against PBS with 5% D-sorbitol. Vector purity and genome titers were determined by silver staining (Pierce silver stain kit) and qPCR using primers targeting the FVIII HC.

Partial Protein Purification

Protein was produced by transfection of HEK293 cells using PolyJet reagent. After transfection, media were collected and filtered using a 0.2-μm Whatman filter. 2-(N-morpholino)ethanesulfonic acid (MES) was added to a final concentration of 20 mM, and pH was adjusted to 6.0. The protein was partially purified using a SP Sepharose high-performance strong cation exchange column (HiTrap SP HP; GE Healthcare) pre-equilibrated with equilibration/washing buffer (10 mM MES, 20 mM CaCl₂, 200 mM NaCl, 0.01% Tween 80, pH 6.0). The column was washed with a 5-column vol of washing buffer, and protein was eluted using a linear increase of sodium concentration up to 1 M. The elution was monitored using a ÄKTAFPLC buffer, and protein was eluted using a linear increase of sodium concentration up to 1 M. The elution was monitored using a ÄKTAFPLC system (GE Healthcare), and fractions with the highest clotting activity, determined by a one-stage APTT clotting assay, were pooled together and concentrated by an Amicon concentrator (molecular weight cut-off of 50,000 Da) (Millipore). The purified protein was visualized using SDS-PAGE electrophoresis.

In Vivo Testing of Constructs

C57BL6/129sv/HA and CD4KO/HA mice were bred and maintained in a pathogen-free environment supplied with a normal...
Hydrodynamic injections were performed on 12-week-old C57BL6/129sv/HA mice by delivering 150 mg of plasmid DNA in a total volume of 2 mL of 0.9% NaCl solution via the tail vein within 10 s. After 48 h, mice were sacrificed following standard euthanasia procedures, and blood was harvested via the hepatic artery and collected into 0.4% sodium citrate solution. AAV vector was diluted in 0.9% NaCl solution to a total volume of 200 µL (1–4 × 10¹¹ vector particles [vp]/mouse) and injected via the tail vein of 6- to 8-week-old CD4KO/HA mice. Following injections, blood was harvested every 2–4 weeks via retro-orbital eye bleeding and collected in 0.4% sodium citrate.

**FVIII Clotting Activity and Quantification of FVIII Protein by ELISA**

FVIII clotting activity was determined for each hybrid tested using a one-stage APTT assay. In short, 50 µL of sample, 50 µL of FVIII-deficient plasma (Haematologic Technologies), and 50 µL of APTT reagent (PTT Automate 5; Diagnostica Stago) were mixed in a standard APTT cuvette (Diagnostica Stago) and incubated for 170 s at 37°C. Afterward, 50 µL of 25 mM CaCl₂ was abruptly added, and the time until clot formation was measured. FVIII protein Kogenate (Bayer Healthcare) was used as the standard. An ELISA was used to quantify FVIII antigen. Briefly, plates were coated with hHC antigen GMA-8016 or human LC antigen GMA-8018 (Green Mountain Antibodies), stored overnight at 4°C, and then replaced with 3% BSA in phosphate-buffered saline with Tween 20 (PBST) and incubated. Then, wells were washed with PBST, and secondary antibody was added; HC was GMA-8015 biotinylated and LC was GMA-8022 biotinylated. After the secondary antibodies were removed, wells were washed with PBST, and horseradish peroxidase was added. Then, wells were washed and developed using a KPL tetramethylbenzidine (TMB) microwell peroxidase substrate system. The plate was read at 450 nm using a Molecular Devices Thermomax microplate reader. Kogenate FS (Bayer Healthcare) was used as the standard.

**Western Blot Analysis**

Protein samples were combined with NuPage dye containing 50 mM DTT, loaded onto an 8% SDS-PAGE gel, and run for 1 h at 180 V. Then, the protein was transferred to a nitrocellulose membrane. After transfer, the membrane was washed in PBST, and 3% non-fat milk in PBST was added. After 1 h, the membrane was washed and incubated with a polyclonal sheep anti-hFVIII antibody (Haematologic...
Technologies). Next, the membrane was washed and incubated with a IRDye 800CW-conjugated donkey anti-sheep IgG (immunoglobulin G) (H+L) (Rockland). An Odyssey infrared imaging system (LI-COR Biosciences) was used to scan the membrane.

### Protein Activity

The specific activity was determined using the following steps: the hFVIII-BDD and hFVIII-JF12-BDD proteins were purified in the manner described above. An ELISA was performed to quantify final FVIII protein using antibodies for the hHC antigen. After quantification, protein was diluted to a concentration of 100 ng/mL in HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer supplemented with 1% BSA. A one-stage APTT assay was used to determine coagulation activity. The specific activity was calculated as U of APTT activity per mg of protein (U/mg). A FXa generation assay was included in reaction mixture for 1 min to allow for the tenase complex to form. The FXa generation reaction was started by adding different concentrations of FX at 37°C, and the reaction was terminated 2 min later by 10 mM EDTA. The FXa generated was measured by its hydrolysis of chromogenic substrate S2765 (350 μM). The rate of absorbance change was calculated with a standard curve generated with serially diluted FXα. The rate of FXα generation was plotted against FX concentration and curve was fitted with GraphPad software using the Michaelis-Menten equation to obtain Km and Vmax.

### Statistical Analysis

Statistical analysis was performed using a two-tailed Student t test, based on the comparison of the same variable between two groups in an experiment. The difference between groups was considered significant when the p value was less 0.05 and not significant when the p value was more than 0.05. Error bars for each graph were derived using standard deviation from the average of at least three points.

### References

1. Bowen, D.J. (2002). Haemophilia A and haemophilia B: molecular insights. MJ, Mol. Pathol. 55, 127–144.
2. Castaldo, G., D’Argenio, V., Nardiolli, P., Zarailli, F., Sanna, V., Rocino, A., Coppola, A., Di Minno, G., and Salvatore, F. (2007). Haemophilia A: molecular insights. Clin. Chem. Lab. Med. 45, 450–461.
3. Doering, C.B., Healey, J.F., Parker, E.T., Barrow, R.T., and Lollar, P. (2002). High level expression of recombinant porcine coagulation factor VIII. J. Biol. Chem. 277, 38345–38349.
4. Caffir, L.A., and Kempston, C.L. (2017). Current and emerging factor VIII replacement products for haemophilia A. Ther. Adv. Hematol. 8, 303–313.
5. Delignat, S., Peyron, I., El Ghazaly, M., V Kaveri, S., Rohde, J., Mueller, F., and Lacroix-Desmazes, S. (2018). Biochemical characterization and immunogenicity of Neureight, a recombinant full-length factor VIII produced by fed-batch process in disposable bioreactors. Cell. Immunol. 331, 22–29.
6. Oldenburg, J. (2015). Optimal treatment strategies for hemophilia: achievements and limitations of current prophylactic regimens. Blood 125, 2038–2044.
7. Armstrong, E.P., Malone, D.C., Krishnan, S., and Wessler, M.J. (2014). Costs and utilization of haemophilia A and B patients with and without inhibitors. J. Med. Econ. 17, 798–802.
8. Zakas, P.M., Gangadharan, B., Almeida-Porada, G., Porada, C.D., Spencer, H.T., and Doering, C.B. (2012). Development and characterization of recombinant ovine coagulation factor VIII. PLoS ONE 7, e49841.
9. Miao, H.Z., Sirachainan, N., Palmer, L., Kucab, P., Cunningham, M.A., Kaufman, R.J., and Pipe, S.W. (2004). Bioengineering of coagulation factor VIII for improved secretion. Blood 103, 3412–3419.
10. Zakas, P.M., Vanijcharoenkarn, K., Markowitz, R.C., Meeks, S.L., and Doering, C.B. (2015). Expanding the ortholog approach for hemophilia treatment complicated by factor VIII inhibitors. J. Thromb. Haemost. 13, 72–81.
11. Wang, Q., Dong, B., Firman, J., Wu, W., Roberts, S., Moore, A.R., Liu, L.S., Chin, M.P., Diao, Y., Kost, J., and Xiao, W. (2016). Evaluation of the biological differences of canine and human factor VIII in gene delivery: implications in human hemophilia treatment. Gene Ther. 23, 597–605.
12. Roberts, S.A., Dong, B., Firman, J.A., Moore, A.R., Sang, N., and Xiao, W. (2011). Engineering factor VIII for hemophilia gene therapy. J. Genet. Syndr. Gene Ther.
13. Tarantino, M.D., Cuker, A., Hardesty, B., Roberis, J.C., and Sholezbeg, M. (2017). Recombinant porcine sequence factor VIII (rFVIII) for acquired haemophilia A: practical clinical experience of its use in seven patients. Haemostasis 23, 25–32.
14. Sabatino, D.E., Fregua, C.F., Toso, R., Santos, A., Merricks, E.P., Kazazian, H.H., Jr., Nichols, T.C., Camire, R.M., and Arruda, V.R. (2009). Recombinant canine B-domain-deleted FVIII exhibits high specific activity and is safe in the canine hemophilia A model. Blood 114, 4562–4565.
15. Staber, J.M., Poliipeter, M.J., Anderson, C.G., Burasciano, M., Cooney, A.L., Sinn, P.L., Rutkowski, D.T., Raschke, W.C., and McCray, P.B. (2017). Long-term correction of hemophilia A mice following lentiviral mediated delivery of an optimized canine factor VIII gene. J. Gene Ther. 24, 742–748.
16. Lai, J.D., and Lillicrap, D. (2017). Factor VIII inhibitors: advances in basic and translational science. Int. J. Lab. Hematol. 39 (Suppl 1), 6–13.
17. Burton, M., Nakai, H., Colosi, P., Cunningham, J., Mitchell, R., and Couto, L. (1999). Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. Proc. Natl. Acad. Sci. USA 96, 12725–12730.
18. Mancuso, M.E., and Santagostino, E. (2017). Outcome of clinical trials with new extended half-life FVIIIIX concentrates. J. Clin. Med. 6, E39.
19. Meunier, S., Alamelu, J., Ehrenforth, S., Hanabusa, H., Abdul Karim, F., Kavakli, K., Khodaei, M., Staber, J., Stasylysh, G., Yee, D.L., and Ragelien, L. (2017). Safety and efficacy of a glycopefylated rFVIII (turoctocog alpha pegol, N8-GP) in paediatric patients with severe haemophilia A. Thromb. Haemost. 117, 1705–1713.
20. Jimenez-Yuste, V., Lejniczce, S., Klamroth, R., Suzuki, T., Santagostino, E., Karim, F.A., Saugstrup, T., and Mes, J. (2015). The pharmacokinetics of a B-domain truncated recombinant factor VIII, turoctocog alfa (NovoEight®), in patients with hemophilia A. J. Thromb. Haemost. 13, 370–379.
21. Shetopal, S.A., Hao, J.L., Karnaukhova, E., Liang, Y., Ovanesov, M.V., Lin, M., Kurasawa, J.H., Lee, T.K., Mcvey, J.H., and Sarafanov, A.G. (2017). Expression and characterization of a codon-optimized blood coagulation factor VIII. J. Thromb. Haemost. 15, 709–720.

22. Nguyen, G.N., George, L.A., Siner, J.L., Davidson, R.J., Zander, C.B., Zheng, X.L., Arruda, V.R., Camire, R.M., and Sabatino, D.E. (2017). Novel factor VIII variants with a modified furin cleavage site improve the efficacy of gene therapy for hemophilia A. J. Thromb. Haemost. 15, 110–121.

23. Bloem, E., van den Biggelaar, M., Wroblewska, A., Voorberg, J., Faber, J.H., Kjalke, M., Stennicke, H.R., Mertens, K., and Meijer, A.B. (2013). Factor VIII C1 domain spikes 2092–2093 and 2158–2159 comprise regions that modulate cofactor function and cellular uptake. J. Biol. Chem. 288, 29670–29679.

24. Chiu, P.L., Bou-Assaf, G.M., Chhabra, E.S., Chambers, M.G., Peters, R.T., Kulman, J.D., and Walz, T. (2015). Mapping the interaction between factor VIII and von Willebrand factor by electron microscopy and mass spectrometry. Blood 126, 935–938.

25. Mazurkiewicz-Pisarek, A., Płucienniczak, G., Ciach, T., and Płucienniczak, A. (2016). The factor VIII protein and its function. Acta Biochim. Pol. 63, 11–16.

26. Zhang, A.H., Yoon, J., Kim, Y.C., and Scott, D.W. (2018). Targeting antigen-specific B cells using antigen-expressing transduced regulatory T cells. J. Immunol. 201, 1434–1441.

27. Parvathaneni, K., and Scott, D.W. (2018). Engineered FVIII-expressing cytotoxic T cells target and kill FVIII-specific B cells in vitro and in vivo. Blood Adv. 2, 2332–2340.

28. Gangadharan, B., Ing, M., Delignat, S., Peyron, I., Teyssandier, M., Kaveti, S.V., and Lacroix-Desmazes, S. (2017). The C1 and C2 domains of blood coagulation factor VIII mediate its endocytosis by dendritic cells. Haematologica 102, 271–281.

29. Peyron, I., Hartholt, R.B., Pedró-Cos, L., van Alphen, F., Brinke, A.T., Lardy, N., Meijer, A.B., and Voorberg, J. (2018). Comparative profiling of HLA-DR and HLA-DQ associated factor VIII peptides presented by monocyte-derived dendritic cells. Haematologica 103, 172–178.

30. Steinitz, K.N., van Helden, P.M., Binder, B., Wraith, D.C., Unterthurner, S., Hermann, C., Schuster, M., Ahmad, R.U., Weiller, M., Lubich, C., et al. (2012). CD4+ T-cell epitopes associated with antibody responses after intravenously and subcutaneously applied human FVIII in humanized hemophilic E17 HLA-DRB1*1501 mice. Blood 119, 4073–4082.

31. Wright, J.F. (2009). Transient transfection methods for clinical adeno-associated viral vector production. Hum. Gene Ther. 20, 698–706.

32. Wang, Q., Dong, B., Firrman, J., Roberts, S., Moore, A.R., Cao, W., Diao, Y., Kapranov, P., Xu, R., and Xiao, W. (2014). Efficient production of dual recombinant adeno-associated viral vectors for factor VIII delivery. Hum. Gene Ther. Methods 25, 261–268.