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Minimal Essential Human Factor VIII Alterations Enhance Secretion and Gene Therapy Efficiency

Wenjing Cao,[1,8] Biao Dong,[1,8] Franziska Horling,[2] Jenni A. Firrman,[3] Johannes Lengler,[2] Matthias Klugmann,[2] Maurus de la Rosa,[2] Wenman Wu,[1] Qizhao Wang,[1] Hongying Wei,[1] Andrea R. Moore,[1] Sean A. Roberts,[1] Carmen J. Booth,[4] Werner Hoellriegl,[2] Dong Li,[1] Barbara Konkle,[5] Carol Miao,[6] Birgit M. Reipert,[2] Friedrich Scheiflinger,[2] Hanspeter Rottensteiner,[2] and Weidong Xiao[7]

[1]Sol Sherry Thrombosis Research Center, Temple University Medical School, 3400 North Broad Street, Philadelphia, PA, 19140, USA; [2]Drug Discovery Austria, Baxalta Innovations GmbH (now part of Takeda), Donau-City Str. 7, Vienna 1220, Austria; [3]Dairy and Functional Foods Research Unit, ARS, USDA, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA; [4]Department of Comparative Medicine, Yale University School of Medicine, 310 Cedar St., BML 330, New Haven, CT 06510, USA; [5]Seattle Children's Research Institute, University of Washington, 1900 9th Ave, Seattle, WA 98195, USA; [6]Department of Medicine/Hematology, University of Washington, 1900 9th Ave, Seattle, WA 98195, USA; [7]Herman B Wells Center for Pediatric Research, Indiana University, Indianapolis, IN 46202, USA

One important limitation for achieving therapeutic expression of human factor VIII (FVIII) in hemophilia A gene therapy is inefficient secretion of the FVIII protein. Substitution of five amino acids in the A1 domain of human FVIII with the corresponding porcine FVIII residues generated a secretion-enhanced human FVIII variant termed B-domain-deleted (BDD)-FVIII-X5 that resulted in 8-fold higher FVIII activity levels in the supernatant of an *in vitro* cell-based assay system than seen with unmodified human BDD-FVIII. Analysis of purified recombinant BDD-FVIII-X5 and BDD-FVIII revealed similar specific activities for both proteins, indicating that the effect of the X5 alteration is confined to increased FVIII secretion. Intravenous delivery in FVIII-deficient mice of liver-targeted adeno-associated virus (AAV) vectors designed to express BDD-FVIII-X5 or BDD-FVIII achieved substantially higher plasma FVIII activity levels for BDD-FVIII-X5, even when highly efficient codon-optimized *F8* nucleotide sequences were employed. A comprehensive immunogenicity assessment using *in vitro* stimulation assays and various *in vivo* preclinical models of hemophilia A demonstrated that the BDD-FVIII-X5 variant does not exhibit an increased immunogenicity risk compared to BDD-FVIII. In conclusion, BDD-FVIII-X5 is an effective FVIII variant molecule that can be further developed for use in gene- and protein-based therapeutics for patients with hemophilia A.

INTRODUCTION

Hemophilia A (HA) is an X-linked, recessive bleeding disorder caused by deleterious mutations in the *F8* gene.[1–6] Current treatment for HA predominantly relies on human FVIII (hFVIII) protein replacement therapy (PRT) administered through relatively frequent intravenous infusions and is, in a subgroup of patients with severe HA, complicated by the development of inhibitory antibodies against the exogenously delivered FVIII.[6,7] Gene therapy, on the other hand, offers the prospect of a functional cure with a single therapeutic dose and might have, in addition to providing substantial and constantly present FVIII levels, a tolerizing effect, which might reduce the risk of inhibitor development.[8–12]

The results from gene therapy clinical trials using adeno-associated virus (AAV) to treat hemophilia B are encouraging,[13,14] and the recent release of preliminary data for an AAV5-FVIII human clinical trial demonstrates sustained FVIII activity with no significant adverse events reported.[6,15,16] More recently however, three years of follow-up data also identified a decline in FVIII expression levels over time, suggesting that this therapy may not last lifelong[17] and causing the US Food and Drug Administration (FDA) to ask for two years of data from the phase 3 trial to show substantial evidence of a durable effect.[18]

HA gene therapy using the AAV vector system is complicated by three key issues. One challenge is the size of the *BDD-F8* cDNA, which is around 4.4 kb in length, bringing the final vector size close to the canonical 4.7 kb AAV genome. Small and efficient promoters are therefore essential for packaging and delivering FVIII using AAV vectors, since little room is left for the addition of genetic elements such as promoters and enhancers to increase transgenic

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[8]These authors contributed equally

Correspondence: Hanspeter Rottensteiner, PhD, Baxalta Innovations GmbH (now part of Takeda), Donau-City Str. 7, Vienna 1220, Austria.
E-mail: hanspeter.rottensteiner@evotec.com
Correspondence: Weidong Xiao, Herman B Wells Center for Pediatric Research, Indiana University, Indianapolis, IN 46202, USA.
E-mail: xiaow@iu.edu

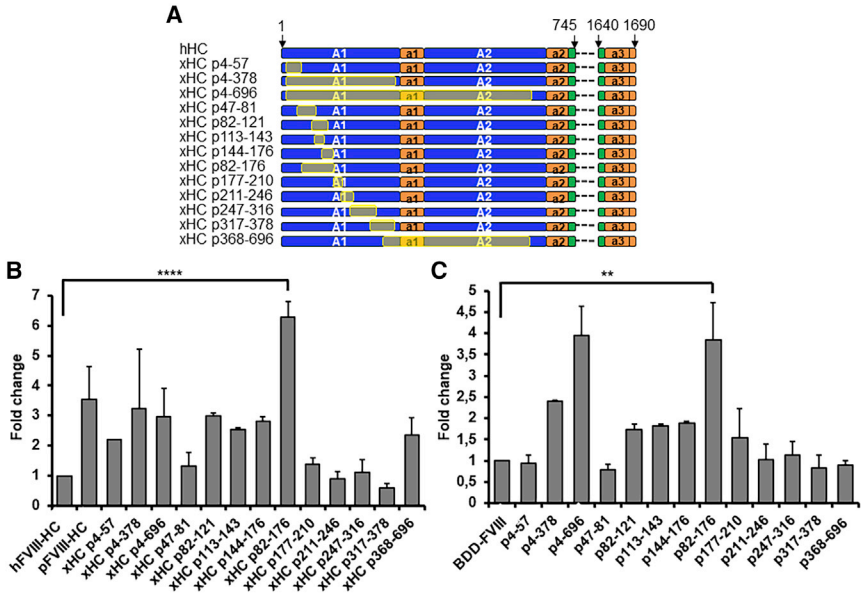


Figure 1. Identification of the Key Secretion Determinants in the Heavy Chain of Porcine FVIII

(A) Schematic illustration of hFVIII heavy chain (HC) constructs with corresponding region from pFVIII. The HC construct was based on the human HC. The porcine amino acids are shaded in yellow. (B) Influence of pFVIII amino acid regions on human FVIII HC expression. Expression constructs shown in (A) were transfected into HEK293 cells along with a human LC-encoding expression plasmid, and FVIII activity in the media was measured by 1-stage coagulation assay at 24 h post transfection. FVIII activities are shown as changes in fold as compared to human HC. (C) Influence of pFVIII amino acid regions on hBDD-FVIII function. The porcine amino acid regions as illustrated in (A) were used to replace the corresponding regions in hBDD-FVIII. hBDD-FVIII variants were transfected into HEK293 cells, and the FVIII activity in the media was measured by 1-stage coagulation assay at 24 h post transfection. FVIII activities are shown as changes in fold as compared to hBDD-FVIII. $^{**}p < 0.01$; $^{****}p < 0.0001$.

expression.[15,19–22] The second limitation is inefficient secretion of FVIII, likely caused by a slow folding process of the factor in the endoplasmic reticulum. Third, upon ectopic overexpression of FVIII, an unfolded protein stress response may be triggered,[23,24] the consequences of which are not fully understood.

Efforts to increase therapeutic FVIII activity levels include codon optimization of the *F8* gene, engineering FVIII to have a higher specific activity, and modulating the FVIII secretory pathway by influencing the interactions between FVIII and immunoglobin binding protein (BiP), calnexin, and calreticulin.[15,25–29] Addition of non-human sequences may foster protein expression levels but at the same time might increase the potential risk for unwanted anti-drug antibody formation.[30,31]

Porcine FVIII (pFVIII), which shares approximately 65% sequence identity with hFVIII (excluding the B-domain), was shown in transfection studies and by preclinical *ex vivo* gene therapy approaches to be expressed 10- to 100-fold more efficiently than hFVIII.[32–35] This effect could be narrowed down by demonstrating that a porcine/human BDD-FVIII hybrid construct termed ET3i containing the porcine A1 and A3 domains retained the higher expression levels of pFVIII in various expression systems.[34]

We therefore hypothesized that a subset of amino acids in pFVIII, and likely in domains A1 and/or A3, may facilitate its enhanced secretion. By implementing a systematic screening strategy, five key amino acids in pFVIII were identified (X5), which, when introduced into the backbone of hBDD-FVIII, resulted in a highly efficiently secreted protein. Our study demonstrated that this protein, called BDD-FVIII-X5, could be the basis for a promising gene therapy strategy in which an effective FVIII variant with enhanced secretion properties may help to substantially reduce the vector dose for treating HA patients.

RESULTS

Identification of the Secretion-Enhancing Amino Acid Residues in the HC of pFVIII

Initial testing *in vitro* demonstrated that replacement of the human FVIII heavy chain (hHC) with porcine heavy chain (pHC) resulted in enhanced secretion of BDD-FVIII (data not shown), in line with previous reports.[34] However, swapping of hHC with pHC would result in a large number of amino acid substitutions with potentially negative immunological consequences.[30] We therefore designed a strategy to narrow down the region within pHC that is responsible for enhanced hFVIII secretion. Because the isolated hHC is particularly inefficiently secreted as compared to full-length FVIII or BDD-FVIII, we elected to use hHC as the template molecule for the screening assays.[25,29] A full set of hHC variants containing corresponding regions of pFVIII were generated in the A1 and A2 domains (Figure 1A). *In vitro* testing using dual chain delivery[29,36–38] of the human light chain (hLC) with the hHC variants showed that of the 13 FVIII-HC constructs tested, those containing porcine amino acids 82–176 (construct xHC p82–176) exhibited the highest procoagulant activity (Figure 1B). Similar substitutions in human BDD-FVIII confirmed this observation (Figure 1C). An amino acid sequence alignment revealed that within amino acids 82–176 there are 10 amino acids that differ between pFVIII and hFVIII: I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T, and L152P (Figure S1). Therefore, we designated xHC p82–176 as HC-X10 in the following experiments.

We next determined if all 10 porcine amino acids are required to enhance hFVIII secretion by adopting a negative selection strategy.

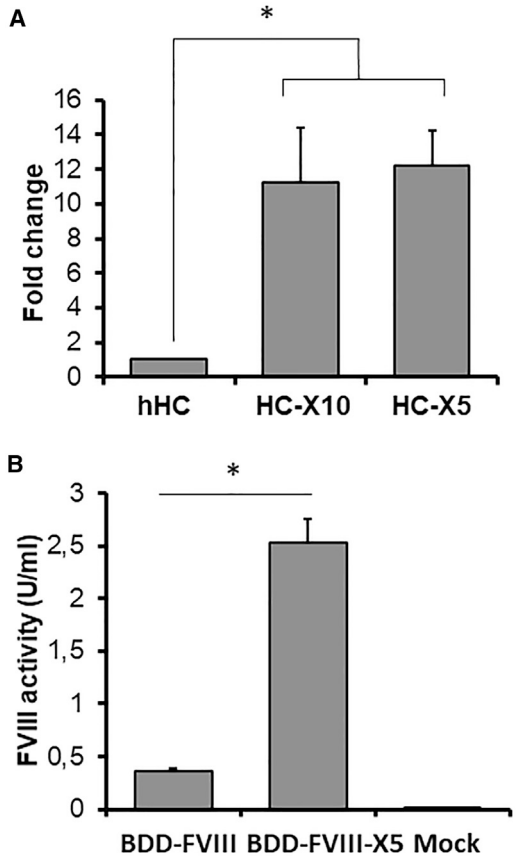


Figure 2. Five Amino Acids (X5) in the A1 Domain Are Responsible for Enhanced FVIII Secretion

(A) The HC expression constructs HC-X10, HC-X5, and hHC were co-expressed with hLC in BHK cells. FVIII activity was determined by a 1-stage coagulation assay. Activities are shown as changes in fold as compared to hHC. (B) BDD-FVIII or BDD-FVIII-X5 constructs were expressed in BHK cells. FVIII activity was determined by a 1-stage coagulation assay. Statistical comparison was performed between groups using a 2-tailed Student's t test; *$p < 0.0001$.

In this strategy, each of the 10 porcine amino acids in the HC-X10 hybrid molecule was replaced with its human counterpart, with the assumption that a significant change would reduce the efficiency of HC-X10 secretion. The negative selection results identified five porcine-to-human amino acid exchanges (V86I, S108A, K132G, T147M, and P152L) that reduced HC-X10 performance by at least 20% (Figure S2), suggesting that these 5 amino acids may play a key role in facilitating FVIII secretion. This hypothesis was tested by constructing HC-X5, a hHC containing these 5 porcine amino acids.

Testing this construct *in vitro* revealed that delivery of either HC-X5 or HC-X10 in conjunction with hLC resulted in an approximately 10-fold increase in FVIII activity (Figure 2A). While both HC-X5 and HC-X10 had significantly higher FVIII activity compared to hHC, they were not significantly different from each other, indicating that the 5 positions selected are indeed the key porcine amino acid residues facilitating FVIII secretion. We next evaluated whether the observed effect is also seen in the context of a human BDD-FVIII molecule that had been engineered to contain the X5 alteration. Testing *in vitro* revealed an 8-fold increase in FVIII clotting activity compared to BDD-FVIII (Figure 2B). Taken together, these results provided evidence that the X5 amino acid alteration had transferred improved secretion properties of pFVIII to hFVIII, as it was able to enhance the levels of secreted hFVIII activity *in vitro*.

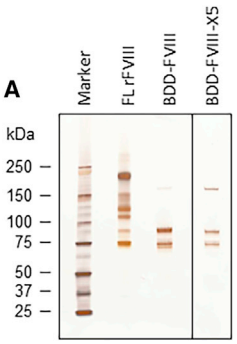
A pulse-chase experiment was used to verify that the increased FVIII activity measured in the medium is due to the increased secretion of BDD-FVIII-X5. Following labeling of the newly translated proteins with ^{35}S-methionine and ^{35}S-cysteine, the secreted BDD-FVIII variants were immunoprecipitated using a FVIII-specific antibody. More BDD-FVIII-X5 was indeed detected in the medium compared to BDD-FVIII (Figures S3A and S3B), indicating that the X5 alteration led to more efficient FVIII secretion.

The results so far indicated that the X5 variant improves secretion of hFVIII but left open the possibility that it also affects FVIII's cofactor activity, causing an increased specific activity of the X5 variant. We therefore produced recombinant BDD-FVIII and the X5 variant thereof using a Chinese hamster ovary (CHO)-production cell line. A silver gel of the purified material revealed for both proteins the expected pattern of distinct bands for the HC and the LC, as well as a tiny band of single-chain BDD-FVIII, caused by an incomplete intracellular processing in CHO cells (Figure 3A). Whether the increased intensity of this band for BDD-FVIII-X5 points toward a fraction of the protein being secreted as single chain remains to be addressed in further studies. Importantly, the specific activity of the X5 variant was similar to that of BDD-FVIII (Figure 3B), indicating that the biochemical and biophysical functionality of BDD-FVIII-X5 is not compromised and that the sole effect of the X5 alteration is to enable efficient secretion of BDD-FVIII.

Delivery of AAV Carrying BDD-FVIII-X5 Produced High Levels of FVIII in a HA Murine Model

To demonstrate suitability of BDD-FVIII-X5 for AAV delivery, we generated an AAV8-BDD-FVIII-X5 vector in which the *BDD-F8* gene was under the control of a minimal mouse liver-specific transthyretin (TTR) promoter/enhancer assembly[39] (Figure 4A). AAV8-BDD-FVIII-X5 was administered to immunocompetent HA mice at a high dose (4×10^{11} vector genomes [vg]/mouse, ~1.6×10^{13} vg/kg) and a low dose (1×10^{11} vg/mouse, ~4×10^{12} vg/kg), and blood plasma samples were collected over the course of 22 weeks.

The FVIII expression as measured by a one-stage coagulation assay (activated partial thromboplastin time [aPTT]) reached its peak level at week 4 post-injection for the high-dose group (Figure 4B), and at 6 weeks post-injection for the low-dose group (Figure 4C). At high dosage, mice receiving AAV8-BDD-FVIII expressed around 0.4 U/mL FVIII, whereas mice receiving AAV8-BDD-FVIII-X5 expressed ~10–12 U/mL FVIII (Figure 4B). At the low dose, the peak level of FVIII expression was 0.2 U/mL FVIII, and that of AAV8-BDD-FVIII-X5 was over 2 U/mL FVIII (Figure 4C). Over the 22 weeks



B

Method	BDD-FVIII	BDD-FVIII-X5 Batch A	BDD-FVIII-X5 Batch B
Total protein $_{UV280nm}$ mg/ml	0.64	0.55	0.52
FVIII chromogenic activity IU/ml	4572	4703	4501
Specific activity (chrom/protein) IU/mg	**7144**	**8489**	**8423**

Figure 3. The X5 Alteration Does Not Affect the Specific Activity of BDD-FVIII

(A and B) Recombinant BDD-FVIII and BDD-FVIII-X5 were produced in CHO cells and purified to apparent homogeneity. The X5 alteration gave rise to a comparable pattern of protein fragments (A) and showed a specific activity that was similar to that of BDD-FVIII (B). Note that the image of the silver gel was processed to only show relevant lanes. FL rFVIII, full-length recombinant FVIII (Advate).

tracked, mice receiving the AAV8-BDD-FVIII-X5 vector consistently exhibited a high level of FVIII activity.

The X5 Alteration Confers Also Improved Secretion when Introduced into Codon-Optimized BDD-FVIII Nucleotide Sequences

Since the wild-type nucleotide sequence of human *F8* is poorly expressed, current HA gene therapy vectors critically depend on codon-optimized sequences to increase the expression of FVIII to therapeutically relevant levels. It was therefore important to test whether the observed benefit of the X5 alteration would be diminished or even lost when introduced to a codon-optimized sequence background. We chose three codon-optimized *BDD-F8* sequences, all of which give rise to improved expression compared to the wild-type nucleotide sequence but to a different extent. Specifically, CO1 codon optimization was based on the nucleotide sequence published by Ward et al.,[28] CO2 was a nucleotide sequence designed using the Web-based GeneArt algorithm, and CO3 was designed in Takeda. The three nucleotide sequences and the corresponding X5 variants were cloned into a liver-specific expression cassette and AAV8-based vectors were generated. Vectors were then administered to FVIII knockout (KO) mice at a dose of 4×10^{12} vg/kg body weight (corresponding to approximately 1×10^{11} vg/mouse) and plasma samples collected for up to 8 weeks. Resulting FVIII activity levels were consistently higher for the respective X5 variants at all time points measured (Figure 5). There was also no trend seen toward a diminishing improvement of the X5 alteration with increasing expression caused by the codon-optimized sequences; even with sequence CO3, showing a 25-fold higher expression than the non-codon-optimized wild-type sequence, X5 gave rise to a 7-fold increase in FVIII plasma levels.

To exclude that the observed difference in FVIII levels was due to preferential transduction by the X5 variants, we analyzed the livers of representative animals treated with CO3 and CO3-X5, the pair of vectors causing the highest FVIII expression levels, for vector copy numbers (VCNs) and *F8* mRNA levels (i.e., transduction efficiency). Similar VCNs and mRNA levels were obtained for the two groups (Table S1), indicating that the difference in measured FVIII activity levels in plasma was indeed due to a more efficient secretion of the BDD-FVIII-X5 variant.

To confirm the biologic potency of BDD-FVIII-X5, a mouse tail-tip bleeding assay was performed. Administration of CO3-X5 to FVIII KO mice resulted in a trend for reduced blood loss over 60 min for the lowest dose tested (4.5×10^{11} vg/kg) compared to the buffer-

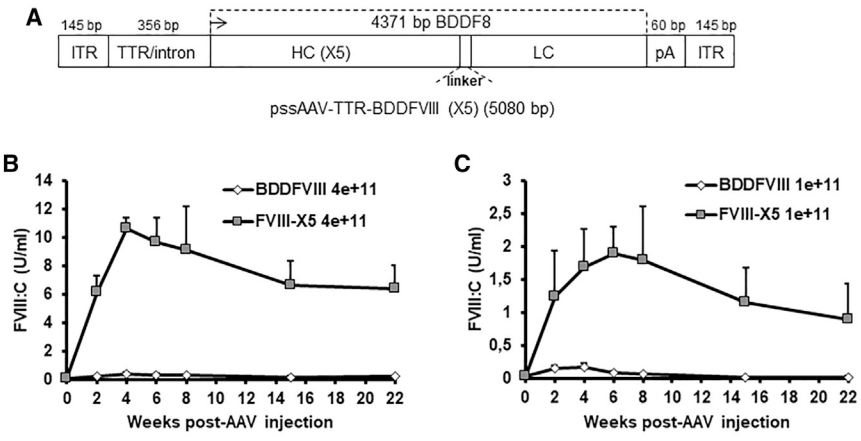


Figure 4. *In Vivo* Performance of AAV8-Vectorized BDD-FVIII-X5 Using the Human Wild Type (WT) FVIII Nucleotide Sequence

(A) Schematic representation of the vector design of AAV8-BDD-FVIII and AAV8-BDD-FVIII-X5. (B and C) Vectors were injected into hemophilia A (HA) mice (C57BL6/SvJ129S) at (B) a high dose of 4×10^{11} vg/mouse (n = 4) and (C) a low dose of 1×10^{11} vg/mouse (n = 5). The FVIII activity levels in plasma were determined by a 1-stage coagulation assay. ITR, inverted terminal repeats; TTR, transthyretin promoter; pA, PolyA tail; vg, vector genomes.

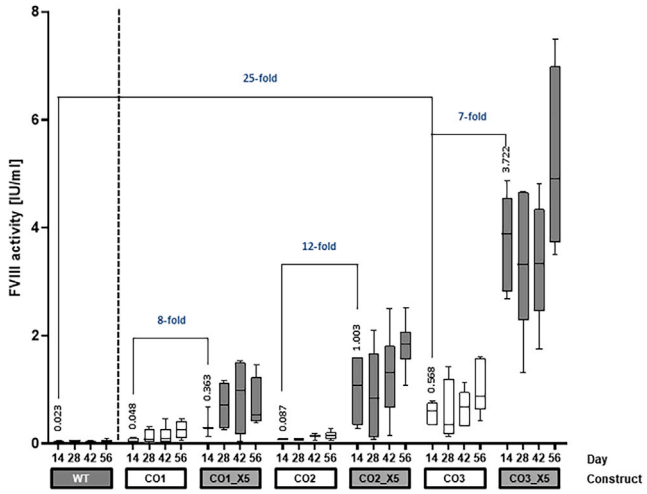


Figure 5. *In Vivo* Performance of AAV8-Vectorized BDD-FVIII-X5 Using Codon-Optimized hFVIII Nucleotide Sequences

Three codon-optimized variants of BDD-FVIII (CO1, CO2, and CO3) were engineered to contain the X5 alteration (CO1_X5, CO2_X5, and CO3_X5). FVIII knockout mice (B6;129S4-*F8*^{tm2Kaz}) were injected with 4×10^{12} vg/kg body weight of WT BDD-FVIII, the codon-optimized BDD-FVIII, or the codon-optimized BDD-FVIII containing the X5 variant. Activity was measured over the course of 56 days for each group. Data are presented in a box-and-whisker diagram, indicating the median and the variability outside the upper and lower quartiles. For day 14, mean FVIII activity levels, activity increases resulting from codon optimization (WT versus CO3) and the X5 alteration (CO1 versus CO1_X5, CO2 versus CO2_X5, CO3 versus CO3_X5) are also shown. The X5-containing variants gave rise to consistently higher FVIII activity levels than the corresponding BDD-FVIII sequences.

treated group and was statistically significant ($p < 0.001$) for the two higher doses (1.84×10^{12} and 4.5×10^{12} vg/kg; Figure S4).

The X5 Alteration Does Not Show Any Signs of Increased Immunogenicity

Since development of anti-FVIII antibodies is a major complication in current treatment of HA, we addressed the immunogenic potential of BDD-FVIII-X5 relative to BDD-FVIII by performing a comprehensive immunogenicity risk evaluation. For the *in vivo* analysis, three different murine models of HA were used. In addition to the E17 FVIII KO strain (E17), we evaluated E17 mice harboring either a hFVIII transgene, which makes them more tolerant to exogenous hFVIII,[40] or the human HLA DRB1*1501 MHC class II haplotype, which is a risk factor for the development of FVIII inhibitors.[41] FVIII binding antibodies were measured after 8 weekly tail vein injections of 1 μg of purified recombinant BDD-FVIII-X5 and BDD-FVIII. As shown in Figures 6A–6C, we did not observe significant differences in anti-FVIII antibody responses in any of the above-mentioned strains.

We further assessed the protein's immunogenicity risk by using a human PBMC-based *in vitro* stimulation assay, which allows the detection of potential anti-BDD-FVIII-X5-specific T cell responses with high sensitivity (Figure 6D). Dendritic cells from 20 representative donors were loaded with the respective peptide pools and co-cultured with autologous $CD4^+$ T cells for six days to allow expansion of potential BDD-FVIII- or BDD-FVIII-X5-specific T cells. After co-culture, T cells were re-stimulated with fresh peptide-loaded antigen-presenting cells from the same donors for 18 h and secreted interferon γ (IFNγ) levels measured as marker for T cell activation. Significant differences in IFNγ levels between BDD-FVIII and BDD-FVIII-X5 were not observed, indicating that the BDD-FVIII-X5-specific peptide sequences did not provoke an increase in T cell activation (Figure 6E). Together, these preclinical data support the conclusion that the X5 alteration does not pose an additional immunogenic risk to BDD-FVIII.

No Evidence for X5-Specific Inhibitory Antibody Formation in HA Rats

To further evaluate whether inhibitory antibodies could potentially develop against X5-specific sequences in BDD-FVIII-X5, we designed an experiment as outlined in Figure 7A. FVIII inhibitors were raised in two WAG/RijYcb HA rats by administration of an AAV8 vector designed to express human BDD-FVIII-X5. The resulting rat plasma, which had a Bethesda titer of 60–110 BU/mL, was depleted of inhibitory antibodies against human wild-type FVIII by biotinylated FVIII, mock-treated with biotinylated BSA, or left untreated. When a BDD-FVIII-X5 concentrate was incubated with these three plasma samples as well as reference control plasma from non-immunized rats, no inhibition of FVIII activity was seen for the plasma that had been pre-treated with biotinylated FVIII, whereas non-treated plasma and plasma treated with biotinylated BSA caused a reduction in FVIII activity (Figure 7B). This result was interpreted to mean that detectable inhibitors specifically directed against sequences containing the 5 porcine amino acids of X5 did not develop in the WAG/RijYcb HA rats, as these would have remained in the supernatant of the FVIII-depleted plasma and been effective against BDD-FVIII-X5.

DISCUSSION

Inefficient FVIII secretion remains one of the major limitations for HA gene therapy using AAV vector delivery.[42] Previous efforts to increase FVIII secretion and expression primarily focused on utilizing codon optimization and reported mechanisms that enhance FVIII secretion.[28,31,43] The well-described fact that pFVIII secretes substantially more efficiently than hFVIII provided a unique opportunity to design new BDD-FVIII variants. The combination of positive and negative screenings allowed us to create BDD-FVIII-X5, which is a human BDD-FVIII that harbors only 5 amino acids from pFVIII yet retains enhanced secretion properties (Figure 2). At the same time, the BDD-FVIII-X5 variant proved fully functional, demonstrated by a purified recombinant version having a similar specific activity as the Refacto-like BDD-FVIII (Figure 3).

Efficient secretion of BDD-FVIII-X5 was evident in *in vitro* and *in vivo* systems. In a murine HA model, an AAV vector carrying the *BDD-F8-X5* gene resulted in substantially enhanced expression when compared to native *BDD-F8*. In the initial experiment, the native human *F8* coding sequence was used (Figure 4). Nevertheless,

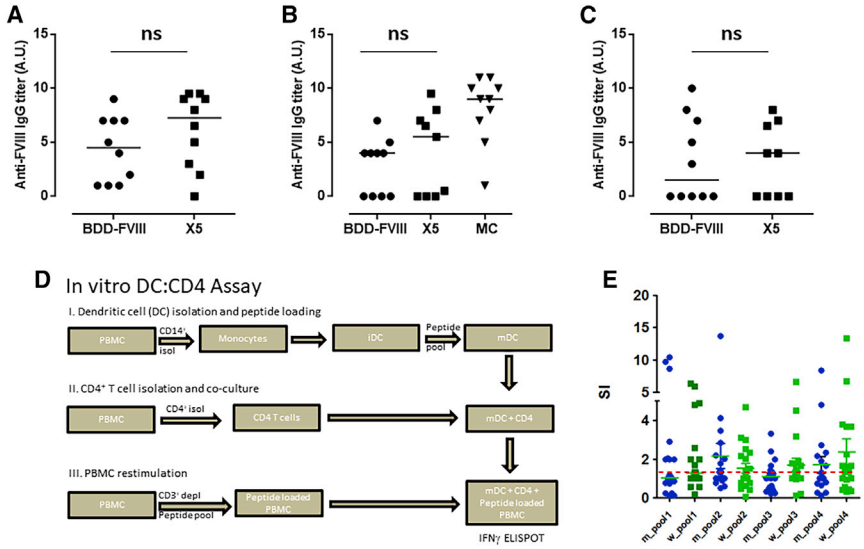


Figure 6. Comparative Immunogenicity Analysis of BDD-FVIII-X5 and BDD-FVIII

(A–C) *In vivo* evaluation using three different murine models of HA. FVIII binding antibodies were measured after 8 weekly tail vein injections of 1 μg of BDD-FVIII and BDD-FVIII-X5 (X5) in E17 mice (A), tolerant E17 mice harboring a hFVIII transgene (B), and E17 mice harboring the human HLA DRB1*1501 MHC class II haplotype (C). Groups were considered significantly different for the anti-FVIII antibody response at a p value < 0.05, using the non-parametric Mann-Whitney U test. MC, model control (an immunogenic FVIII control preparation that is capable of mounting a FVIII antibody response in the tolerant E17 mouse model); ns, non-significant. (D and E) PBMC-based *in vitro* stimulation assay. (D) Flow chart of the assay. CD14 positive monocytes were isolated from 20 donors, loaded with BDD-FVIII or BDD-FVIII-X5-specific peptides, and differentiated into dendritic cells (DCs). In parallel, CD4 cells were isolated from the same donors and co-cultured for six days with the DCs. To boost potential anti-FVIII T cell responses, cells were re-stimulated with fresh peptide-loaded, T cell (CD3)-depleted PBMCs for 18 h. (E) IFNγ secretion of different donors after 18 h of BDD-FVIII and BDD-FVIII-X5 peptide stimulation measured by ELISPOT. The values are represented as stimulation indexes (SIs) relative to the DMSO control. The 15-mer peptide pools used spanned the entire amino acid substitutions of BDD-FVIII-X5 (m_pool1-4) and the corresponding BDD-FVIII WT sequences (w_pool1-4). Significant differences in IFNγ levels between BDD-FVIII and BDD-FVIII-X5 were not observed.

FVIII codon optimization was reported to greatly improve FVIII expression,[2,28] and in fact all vectors currently being used in clinical HA gene therapy trials rely on codon-optimized *F8* sequences. Since *F8* codon optimization is expected to improve transcription/translation but not necessarily to facilitate FVIII secretion per se, we anticipated that BDD-FVIII-X5 could have an additive effect on codon-optimized *BDD-F8*. As shown in Figure 5, incorporation of the X5 alteration into various codon-optimized *BDD-F8* sequences indeed improved BDD-FVIII expression further, indicating that X5's positive effect on expression is downstream of protein translation.

Our approach aimed to identify porcine-specific amino acid residues in FVIII's HC that confer enhanced secretion but left open whether introduction of additional residues out of pFVIII's LC would further enhance hFVIII levels. Since an ancillary role in secretion was reported for the porcine ap-A3 domains,[34] BDD-FVIII-X5's secretion efficiency may not reach that of porcine BDD-FVIII. Another limitation of the study is the lack of expression data for BDD-FVIII-X5 in non-human primates, which are thought to be more predictive to the situation in humans. Another bio-engineered FVIII variant that is being tested in gene therapy is FVIII-V3, which has introduced a peptide sequence with 6 potential N-glycosylation sites instead of the B domain.[31] This variant resulted in a 3-fold improvement in BDD-FVIII plasma levels in hemophilic mice and is now evaluated clinically in a phase 1/2 study. While the molecular basis for this improved expression is not entirely clear, it will be interesting to explore in future work whether introduction of the V3 peptide into BDD-FVIII-X5 would result in even higher FVIII plasma levels.

Development of a gene therapy product enabling robust and substantial improvement in efficacy is particularly relevant in light of the observed vector dose-dependent signs of liver inflammation and anti-capsid immune responses. Various clinical hemophilia gene therapy trials using different AAV capsids (AAV2, AAV8, AAVrh10, and LK03) showed such signs of hepatotoxicity already at a dose of 2×10^{12} vg/kg.[13,15,44,45] Although the evoked responses can be usually managed quite well by temporal steroid administration (or even prevented when given prophylactically), improved hemophilia gene therapy approaches should render immune suppression dispensable. This may be achieved by the selection of capsids with a strong tropism for human hepatocytes such as AAV3 or NP40,[46,47] thereby allowing for lower dosing.

In the context of HA, the biological properties of BDD-FVIII-X5 also appear highly valuable in keeping vector-mediated hepatotoxicity under control. Expression of the efficiently secreted BDD-FVIII-X5 variant might in part compensate for the need to express the *BDD-F8* transgene at very high levels, which is challenging because of the size restriction imposed on the choice of the promoter due to the size of the *BDD-F8* transgene (4.4 kb) being almost as large as the wild-type AAV vector genome. Perhaps even more importantly, BDD-FVIII-X5 might help reduce the potential risk of triggering an unfolded protein response in the transduced hepatocytes,[23] thereby increasing the likelihood of sustained FVIII expression. Studies are underway to specifically address whether ER stress markers are differentially induced upon heterologous expression of BDD-FVIII and BDD-FVIII-X5.

We have addressed the potential concern of BDD-FVIII-X5 immunogenicity in a carefully designed comparative study using an *in vitro* stimulation assay and three mouse models, two of which were specifically developed for preclinical evaluation of FVIII

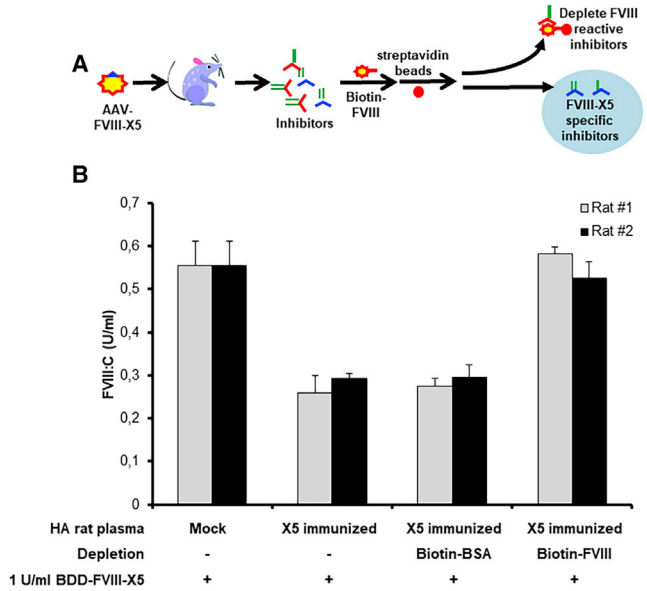


Figure 7. FVIII-X5 Did Not Form Neo-antigenic Epitopes

(A) Flow chart of depleting FVIII-specific inhibitors. Polyclonal antibodies raised in rats were incubated with biotin-labeled regular hFVIII (biotin-FVIII) and removed by streptavidin beads. The remaining inhibitors should be BDD-FVIII-X5 specific. (B) Inhibitors from (A) in different stages of the depletion process were measured for BDD-FVIII-X5-specific inhibitor activities. Mock HA rat plasma (non-immunized by BDD-FVIII-X5), immunized HA rat plasma, immunized HA rat plasma depleted with biotin-FVIII, or biotin-BSA (control) was pre-incubated with a BDD-FVIII-X5 concentrate, and FVIII activity was measured by a coagulation assay.

immunogenicity risks. The transgenic hFVIII mouse model is particularly suitable to study mechanisms causing break of immune tolerance against hFVIII. To date, patients in hemophilia gene therapy clinical studies have been previously treated and are thereby not expected to develop anti-FVIII antibodies to gene therapy products. The existing data from ongoing clinical studies indeed support this assumption. However, there is a potential risk that previously treated patients might experience an immune response against a FVIII variant. As compared to the ET3 porcine-human BDD-FVIII hybrid that contains more than 150 pFVIII amino acids,[33,48] BDD-FVIII-X5 is a hFVIII harboring only 5 amino acids from pFVIII (I86V, A108S, G132K, M147T, and L152P), all of which are located in the A1 domain. This domain has not been reported as a major immunogenic region, even after extensive analysis of human antibodies against human FVIII.[48–51] In our evaluation, BDD-FVIII-X5 did not induce an increased immunogenic risk in any of the mouse models (Figures 6A–6C). The PBMC re-stimulation data (Figure 6E) further support the conclusion that the X5 alteration may not increase the immunogenic risk in previously treated hemophilia patients. Finally, a complementary assessment in rats suggested that neo-antigenic epitopes are limited by introduction of the five porcine amino acids (Figure 7).

Although we have been comprehensive in our evaluation, the design of our studies did not address whether continuous expression of BDD-FVIII-X5 (or BDD-FVIII for that matter) will lead to an induction of immune tolerance to hFVIII. Also, we have not exposed larger animal models to BDD-FVIII-X5. Their immune system may respond to the X5 alteration differently than that of rodents, possibly mimicking the human situation more closely. However, larger animal models, such as non-human primates, are not transgenic for hFVIII and hence not tolerant even to native hFVIII due to differences in their FVIII sequence. The immune response against BDD-FVIII-X5 in such animals could therefore be altered because of their high propensity to mount an anti-FVIII immune response against hFVIII even without the X5 insertion. We therefore believe that our selected models are valid in terms of predictability of immunogenic outcomes in humans. At the same time, one should avoid over-interpretation of the data given the caveats inherent to all *in vitro* and *in vivo* animal models.

Taken together, we conclude that BDD-FVIII-X5 has great potential as a leading candidate for a more efficient and better tolerated gene therapy for patients with HA.

MATERIALS AND METHODS

FVIII Expression Plasmids and Mutagenesis

pFVIII was synthesized by GenScript based on Doering et al.[34,35] BDD-FVIII and FVIII HC and LC have been described previously.[29,36] FVIII expression constructs for testing in cultured cells were driven by a Cytomegalovirus enhancer with a human β-actin promoter (CB). All hybrid human and pFVIII variants including desired point mutations were confirmed by DNA sequencing.

For the *in vivo* expression of BDD-FVIII (B domain replaced by the 14-amino acid SQ linker sequence) and BDD-FVIII-X5, the human wild-type coding sequence and three codon-optimized sequences (CO1, CO2, and CO3) as well as the corresponding BDD-FVIII-X5 sequences (CO1-X5, CO2-X5, and CO3-X5) were synthesized and cloned into an AAV expression cassette containing a liver-specific murine transthyretin promoter/enhancer combination.[39]

Tissue Culture and Transfection

For evaluating FVIII plasmid performance *in vitro*, transfected baby hamster kidney (BHK) cells or human embryonic kidney (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were grown for 6–12 h in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and then replaced with Ham's F-10 Nutrient Mix (GIBCO, Grand Island, NY, USA) medium with 2% heat-inactivated fetal bovine serum. The media were collected 24 h later, and the activity levels of FVIII in the media were measured. All experiments were performed in triplicate.

AAV Vector Preparation

Recombinant AAV8 vectors were produced by a triple plasmid co-transfection method as described previously.[29,36] AAV vectors were purified by two rounds of cesium chloride gradient ultracentrifugation followed by an extensive buffer exchange against PBS with 5% D-sorbitol. Alternatively, cell pellets of 1.2 L cultures were processed

using one round of iodixanol gradient centrifugation followed by an anion-exchange polishing step. Quantification of the vector genome was done by inverted terminal repeat (ITR)-specific qPCR.[52]

Animal Procedures

Exon 16-disrupted FVIII KO (HA) mice (B6;129S-*F8*^{tm1Kaz}/J) were obtained from Haig Kazazian (University of Pennsylvania), and exon 17-disrupted FVIII KO mice (B6;129S4-*F8*^{tm2Kaz}) were bred at Charles River (Sulzfeld, Germany). Wag/RijYcb HA *Rattus norvegicus* rats (strain designation: WAG-F8^{m1Ycb}) with a spontaneous mutation in the A1 domain (Leu176Pro) were obtained from Yale University.[53]

Male mice of around 8 weeks of age and a body weight of about 25 g were either dosed per animal or per kg body weight. The dose per mouse as used in Figure 4 thus translates into an approximately 40-fold higher dose per kg body weight. Mouse whole blood samples were collected by retro-orbital bleeding. Rat blood samples were collected through tail vein draw. The tail-tip bleeding assay was performed using exon 17-disrupted FVIII KO mice (B6;129S4-*F8*^{tm2Kaz}) as described previously.[54] All animal studies complied with national laws governing animal experimentation and were approved by the animal care and use committees of the respective institutions.

Quantitative Analysis of FVIII Activity

Biologically active FVIII in media and plasma was measured using an aPTT-based 1-stage coagulation assay as previously described.[55] FVIII activities of variants CO1-CO3 and CO1-X5-CO3-X5 in mouse plasma were determined by a FVIII chromogenic assay according to the supplier's instruction (Technoclone, Vienna, Austria). Animals with detectable anti-FVIII antibodies (measured by a total immunoglobulin [Ig] ELISA) were not considered for calculation of mean FVIII activities. All experiments were performed in triplicate.

Generation of Recombinant BDD-FVIII and BDD-FVIII-X5 Protein

Both recombinant proteins (BDD-FVIII and BDD-FVIII-X5) were similarly produced in CHO cells and purified to apparent homogeneity using standard procedures. The preparations were assayed for total protein by UV absorbance at 280 nm and FVIII activity by a chromogenic assay (Coatest SP4 FVIII, Chromogenix, Austria). Proteins were separated on a 3%–8% Tris-Acetat (NuPAGE Novex, Life Technologies, Austria) gel and subjected to silver staining with a SilverQuest kit (Novex, Life Technologies) according to the manufacturers' instructions.

Pulse-Chase Analysis of FVIII Secretion

FVIII secretion dynamics were tracked using a pulse-chase procedure.[56] Briefly, BHK cells were transiently transfected with BDD-FVIII expression plasmids and incubated for 2 h with labeling medium containing 200 μCi ^{35}S-cysteine and 200 μCi ^{35}S-methionine/mL. After stopping of the labeling reaction with an excess of cysteine and methionine, media samples were collected after 0 min, 30 min, and 4 h and labeled FVIII immuno-precipitated using biotinylated GMA-8015 and streptavidin agarose. Isolated FVIII was separated by SDS-PAGE and subjected to radioactive exposure analysis as described.[53]

Immunogenicity Risk Assessments

Male exon 17-disrupted FVIII KO mice, E17 hFVIII transgenic mice,[40] and HLA-DRB1*1501 E17 hemophilic mice[41] were used for comparative immunogenicity studies. Mice received weekly tail vein injections of 1 μg of BDD-FVIII or BDD-FVIII-X5 for a total of 8 weeks. The E17 human transgenic FVIII mouse line was additionally challenged with a modified FVIII protein known to break tolerance.[40] Blood was collected by retro-orbital bleeding at week 6 or by cardiac puncture one week after final FVIII administration. Resulting plasma samples were analyzed for anti-FVIII binding antibodies by direct ELISA as described.[40] Studies were analyzed by using non-parametric Mann-Whitney U test to compare statistical significance; a p value < 0.05 was considered significant.

Human peripheral blood mononuclear cell (PBMC) re-stimulation assays were performed at Lonza using 20 individual donors representing a global population distribution of major histocompatibility complex (MHC) class II haplotypes (HLA-DRB1) and peptide pools (15-mer) spanning the X5 amino acid substitutions (m_pool) or corresponding wild-type peptide sequences (w_pool). The CEFT (Cytomegalovirus, Epstein-Barr virus, Flu-virus, and Tetanus toxin) peptide pool (0.5 μM) was used as positive control. In brief, autologous CD4 T cells were incubated with peptide-loaded monocyte-derived dendritic cells from the same donor for 6 days. On day 6, preloaded CD3-depleted PBMCs from the same donor were added and release of IFNγ expression measured after 18 h of incubation using ELISPOT (enzyme-linked immune absorbent spot). The values are represented as stimulation indexes (SIs) relative to the DMSO control. As positive control, the benchmark CEFT peptide pool (0.5 μM) was used, which induced significant CD4+ T cell activation in all donors tested. For statistical analysis, a modified-distribution free resampling (DFR_{eq}) was utilized. DFR_{eq} permutation resampling allows for a maximum false-positive rating of 5% ($p \leq 0.05$) and a minimum of 1% ($p \leq 0.01$).

FVIII Inhibitor/Neutralizing Antibody Assay

Plasma anti-FVIII IgGs were quantified by ELISA.[57] FVIII inhibitor titer was determined by modified Bethesda assay.[57] A FVIII monoclonal antibody (GMA-8021) with defined titers was used as standard. Rat antibodies against BDD-FVIII-X5 were generated by injecting an AAV8-FVIII-X5 vector into HA rats. Inhibitor-containing plasma was collected, hFVIII-specific antibodies were absorbed by an excess of biotinylated hFVIII, and the resulting biotin-FVIII-inhibitor complexes were removed by streptavidin agarose. Biotinylated BSA was used as non-binding control. Resulting plasma samples were used to assay for X5-specific FVIII inhibitors using BDD-FVIII-X5 as target.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.10.013.

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AUTHOR CONTRIBUTIONS

W.C., B.D., F.H., J.L., B.M.R., H.R., and W.X. designed research and analyzed data; W.C., B.D., J.A.F, A.R.M., Q.W., W.W., H.W., and S.A.R. performed experiments; W.C, C.J.B., J.A.F., H.R., and W.X. wrote the paper. D.L., C.J.B., B.K., M.K., M.d.l.R., F.S., and C.M. contributed key reagents and helpful comments and discussion.

DECLARATION OF INTERESTS

A patent application has been submitted by W.X., B.D., and W.C. for the FVIII-X5 (application number W02014209942A1). W.X., B.D., and W.C. hold equity in Ivygen. J. L. is a full-time employee of Baxalta Innovations GmbH, now part of Takeda. F.H., M. K., M.d.l.R., W. H., B.M.R., F. S., and H.R. were employees of Baxalta Innovations GmbH, now part of Takeda, at the time of the study.

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