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Long-term correction of hemophilia A mice following lentiviral mediated delivery of an optimized canine factor VIII gene

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Abstract

Current therapies for hemophilia A include frequent prophylactic or on-demand intravenous factor treatments which are costly, inconvenient and may lead to inhibitor formation. Viral vector delivery of factor VIII (FVIII) cDNA has the potential to alleviate the debilitating clotting defects. Lentiviral-based vectors delivered to murine models of hemophilia A mediate phenotypic correction. However, a limitation of lentiviral-mediated FVIII delivery is inefficient transduction of target cells. Here, we engineer a feline immunodeficiency virus (FIV) -based lentiviral vector pseudotyped with the baculovirus GP64 envelope glycoprotein to mediate efficient gene transfer to mouse hepatocytes. In anticipation of future studies in FVIII-deficient dogs, we investigated the efficacy of FIV-delivered canine FVIII (cFVIII). Codon-optimization of the cFVIII sequence increased activity and decreased blood loss as compared to the native sequence. Further, we compared a standard B-domain deleted FVIII cDNA to a cDNA including 256 amino acids of the B-domain with 11 potential asparagine-linked oligosaccharide linkages. Restoring a partial B-domain resulted in modest reduction of endoplasmic reticulum (ER) stress markers. Importantly, our optimized vectors achieved wild-type levels of phenotypic correction with minimal inhibitor formation. These studies provide insights into optimal design of a therapeutically relevant gene therapy vector for a devastating bleeding disorder.

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CONFLICT OF INTEREST

CGA, MB and WCR are employees of Virogenics, Inc. Janice Staber has received honorarium from Baxalta.

AUTHOR CONTRIBUTIONS

PBM, WCR, PLS and JMS designed the experiments. JMS, MJP, CGA, MB, DTR and ALC. collected the data. JMS and DTR analyzed the data. JMS and PLS wrote the manuscript. All authors approve the submitted and final versions for publication.

INTRODUCTION

Hemophilia A is a common X-linked coagulopathy affecting ~ 1:5–10 000 males worldwide^{1,2} and caused by a deficiency in plasma FVIII protein. The resultant hemorrhages in patients with hemophilia cause significant morbidity and may be fatal. Intravenous injection of FVIII preparations purified from human plasma or produced by recombinant technology are effective in controlling bleeding episodes.² However, treatment for hemophilia A is problematic due to limited availability and high cost. In addition, several problems remain with protein replacement therapy including frequent need for injection or the devastating production of inhibitory antibodies.^{2–7}

Gene therapy is a potential therapeutic approach for the long-term correction of FVIII deficiency.^{3,4,8–16} Expression of a functional FVIII gene in somatic cells, such as hepatocytes, could provide continuous protein production. Examples of other cells targeted for hemophilia gene transfer include muscle, hematopoietic stem cells and platelets.^{17–19} A sustained level of FVIII in the bloodstream controls the bleeding diathesis preventing spontaneous bleeding and ultimately chronic joint injury. As little as 10 ng ml⁻¹ of plasma FVIII (~5% of normal levels) is sufficient to convert severe hemophilia A to a mild form.³ Indeed, recent reports from several laboratories including ours demonstrate the feasibility of gene therapy for hemophilia in animal models and human clinical trials.^{3,4,20–22} Despite these promising steps, expression of the transgene at therapeutic levels for more than 6 months has not been observed in the clinic. The lack of sufficient expression levels in patients with hemophilia A treated with previous gene transfer vectors^{23,24} indicates that further improvements of current delivery systems are needed to generate a therapeutic product. However, ongoing gene therapy trials using non-integrating AAV vectors (Spark Therapeutics and Biomarin; clinicaltrials.gov) will be informative.

We previously reported lentiviral vector mediated human FVIII expression *in vivo*.^{22,25} In addition, we demonstrated improved expression of codon-optimized human FVIII *in vivo* following hydrodynamic injection of a DNA transposon-based vector.²⁶ In this study, we focus on developing the vector system for delivery and therapeutic FVIII protein expression from an optimized cFVIII cDNA to achieve safe and long-term expression from hepatocytes. This proof-of-principle study in a relevant small animal model bridges the way to pre-clinical studies in a canine model of hemophilia A.

RESULTS AND DISCUSSION

MiniFIV efficiently transduces mouse hepatic cells *In Vivo*

Lentiviral vector titer is inversely proportional to the length of the packaged RNA genome.²⁷ To improve vector titer and efficient transduction of the partial B-domain cFVIII cDNA, we designed an FIV transgene shuttle plasmid with the minimum viral sequence required to package and integrate a transgene.²⁸ This 1.2 kb-(LTR to LTR length) backbone termed ‘miniFIV’ (Figure 1) is appreciably shorter than our previously described 2.6 kb FIV3.3 vector backbone.²² As an initial test, we made vector preparations delivering a 4.5 kb control cDNA and titered vector supernatants. The FIV3.3-based vector yielded supernatant titers of 4×10^6 TU ml⁻¹. The miniFIV-based vector yielded supernatant titers of 5×10^7 TU ml⁻¹,

suggesting that the miniFIV vector yields higher titers with large transgenes. Of note, we observed no increase in titers with mCherry vectors (711 bp cDNA). On the basis of these results, we focused our cFVIII construct design using the miniFIV vector platform. The GP64 envelope was selected for its improved hepatocyte tropism compared to VSV-G.²²

To confirm that a GP64 pseudotyped miniFIV-based vector will transduce and express a transgene of interest in mouse hepatocytes, wild-type mice were injected with miniFIV-RSV-mCherry (Figure 2a) or a buffer-only (sham) negative control (Figure 2b). Liver tissue was collected 10 days after vector delivery. In the miniFIV cohort, ~ 30% of liver cells expressed mCherry, confirming that mouse hepatic cells are transducible using this modified vector.

Codon-optimized cFVIII efficiently and persistently corrects the hemophilia phenotype

We next examined two optimization strategies of the FVIII cDNA: (1) codon optimization of cFVIII; and (2) inclusion of a partial B-domain. Codon optimization of human FVIII is a proven strategy to increase protein expression *in vivo*.^{26,29} Here, we codon-optimized canine FVIII (CO-cFVIII) and observed enhancements to FVIII activity *in vitro* (Figure 2c) and in FVIII-deficient mice (Figure 2d). Wild-type cFVIII and CO-cFVIII share 100% amino acid identity, but only ~ 78% nucleotide identity. The mechanism of improved protein production presumably involves aligning cellular tRNA abundance for a given species to the codon usage of the mRNA. However, the proprietary GenScript algorithm used also takes into consideration variables including GC content, CpG dinucleotide content, mRNA secondary structure and premature polyA sites. Our cFVIII cDNA was optimized for expression in human and canine hepatocytes. Indeed, when FVIII-deficient mice were injected with a plasmid expressing CO-cFVIII, higher FVIII activity was consistently observed compared to the activity seen in FVIII null animals injected with plasmid expressing the wild-type FVIII cDNA (Figure 2d). These results are consistent with previous reports using codon-optimized human FVIII cDNAs.^{26,29} On the basis of these results, we focused on the CO-cFVIII cDNA constructs for additional studies.

To date, all gene therapy studies performed in hemophilia A dogs with canine expression cassettes used the B-domain deleted (BDD) cDNA.³⁰ Human full-length FVIII contains 25 consensus sequences for N-linked glycosylation of which 19 reside in the B-domain. Wild-type cFVIII is similarly glycosylated, with 25 consensus sites for N-linked glycosylation in the B-domain (Figure 1) and a total of 31 in the full-length sequence. We hypothesized that the addition of a partial canine B-domain would decrease toxicity by increasing transport from the ER to Golgi, as described for human FVIII.^{26,31,32} We compared a conventional BDD cDNA and a cDNA including a partial B-domain sequence encoding 11 predicted N-linked glycosylation sites (N11) (Figure 1). To confirm functional cDNA expression, we first delivered the expression plasmids by hydrodynamic tail vein injection in FVIII null mice. Three days later, we measured plasma FVIII function by Coamatic activity assay (Figure 2d). Each vector conferred functional FVIII activity to mice at levels greater than sham controls ($P < 0.05$). Of note, both the BDD and N11 CO-cFVIII constructs achieved activity levels capable of correcting the bleeding phenotype.

We next delivered the viral vectors miniFIV-CO-cFVIII-N11 and miniFIV-CO-cFVIII-BDD to FVIII null mice via tail vein injection. At ~ 4 week intervals, blood was collected and FVIII activity quantified by Coamatic assay. Wild-type mice and sham injected FVIII null mice served as positive and negative controls, respectively. Because the miniFIV-CO-cFVIII-BDD vector titers tended to be higher, both high and low doses (only low dose was titer matched with miniFIV-CO-cFVIII-N11) were examined. Importantly, each vector achieved therapeutically relevant levels of FVIII activity ranging from 10–125% wild-type levels (Figure 3a). At a low dose, the BDD and N11 vectors resulted in similar levels of activity that persisted for the duration of the experiment. At a higher dose, the miniFIV-CO-cFVIII-BDD vector conferred wild-type levels of FVIII activity for the duration of the experiment (28 weeks) (Figures 3a, $P<0.05$ compared to lower doses).

We also measured FVIII protein levels by ELISA. At a low dose, BDD and N11 vectors conferred similar, persistent levels of FVIII protein production (Figure 3b, $P<0.05$ compared to sham injected animals). At a higher dose, miniFIV-CO-cFVIII-BDD vector achieved ~ 60% of wild-type cFVIII protein levels (as compared to pooled canine plasma) for the duration of the experiment (28 weeks) (Figure 3b, $P<0.001$ CO-cFVIII-BDD compared to sham). Together, these data demonstrate that miniFIV-CO-cFVIII persistently confers high levels of cFVIII expression in hemophilia A mice.

To determine if FVIII activity would be sustained, levels of FVIII neutralizing antibodies (also known as inhibitors) were measured once by Bethesda assay at the end of the study. Two out of five mice receiving miniFIV-CO-cFVIII-BDD and one out of six mice receiving miniFIV-CO-cFVIII-N11 had detectable inhibitors (Figure 3c). Importantly, no mouse had an inhibitor titer greater than 1.5 BU ml^{-1} . These results suggest that lentiviral gene transfer was sufficient to restore FVIII activity to hemophilic mice. Although our studies did not result in complete prevention of inhibitor formation, expression of our transgene resulted in at least a partial tolerance to FVIII. In addition, we acknowledge the possibility of a xenoprotein response to the canine product as a possible cause of the inhibitors against FVIII. We speculate that this would be less likely to occur in studies utilizing a hemophilia A dog model.

To evaluate if FVIII expression level correlated with vector copy number, livers were harvested, DNA extracted, and quantitative PCR (qPCR) analyzed to determine transgene copy number per genome. Animals receiving low dose ($1.2 \times 10^8 \text{ TU ml}^{-1}$) miniFIV-CO-cFVIII-BDD-treated animals exhibited a mean of 0.14 copies/genome ± 0.02 (mean $\pm$ s.e.) with a range of 0.12–0.15 copies/genome. Mice receiving high-dose ($6 \times 10^9 \text{ TU ml}^{-1}$) miniFIV-CO-cFVIII-BDD exhibited a mean of 0.6 copies/genome ± 0.003 (mean $\pm$ s.e.) with a range of 0.59–0.6. MiniFIV-CO-cFVIII-N11-treated animals ($1.4 \times 10^8 \text{ TU ml}^{-1}$) exhibited a mean of 0.07 copies/genome ± 0.03 (mean $\pm$ s.e.) with a range of 0.02–0.2 copies/genome. Copy number did correlate with vector dose (MiniFIV-CO-cFVIII-BDD low dose vs MiniFIV-CO-cFVIII-N11, $P = 0.24$ and MiniFIV-CO-cFVIII-BDD low vs MiniFIV-CO-cFVIII-BDD high, $P<0.01$). No correlation was observed when copy number was compared to FVIII expression in either group. Of note, we observed therapeutically relevant levels of FVIII expression with copy number of less than 1 integration per genome.

We next explored the therapeutic potential of this expression. Following the 24-week time course, blood loss in the tail transection assay was measured in the low-dose miniFIV-CO-cFVIII-N11 and miniFIV-CO-cFVIII-BDD cohorts. By weight, blood loss in both cohorts that received vector was attenuated to levels intermediate between the wild-type and sham groups of mice (Figure 4a). Using hemoglobin content as a measure of blood in the tail transection assay, the mice that received vector were indistinguishable from wild-type (Figure 4b). These results suggest that even at low-dose, miniFIV-CO-cFVIII-N11 and miniFIV-CO-cFVIII-BDD confer therapeutically relevant levels of cFVIII expression in hemophilia mice. We observed trends for improved cFVIII activity and decreased bleeding times with the CO-cFVIII-N11 as compared to CO-cFVIII-BDD.

Liver ER stress from B-domain deleted and partial B-domain CO-cFVIII

The CO-cFVIII-N11 and CO-cFVIII-BDD cDNA constructs differ by the inclusion in N11 of 256 amino acids of the B-domain which includes 11 potential sites for N-linked glycosylation (Figure 1). We asked whether inclusion of an N-linked glycosylation domain reduced expression of ER stress markers in the livers of mice transduced with miniFIV-CO-cFVIII. Previous studies suggest that expression of human BDD FVIII results in hepatocyte stress following plasmid-mediated gene transfer.³¹ Furthermore, N-linked oligosaccharides participate in protein folding interactions within the ER and may help facilitate ER-Golgi transport.

At the protein level, elevated phosphorylation of the translation initiation factor eIF2 α was uniformly observed in CO-cFVIII-BDD expressing animals (both high-dose and low-dose groups), but this response was attenuated in the N11 animals (Figure 5a). This result was mirrored in expression of the stress-responsive eIF2 α -dependent transcription factor, CHOP (UPR-regulated CCAAT/enhancer-binding protein homologous protein), which though modest, was seen in four out of five CO-cFVIII BDD animals but only one out of seven N11 animals (Figure 5a). Expression of the ER chaperone BiP, considered a hallmark of ER stress, was also elevated in the BDD samples when compared to N11 samples. Of note, PeIF2 α , CHOP and BiP expression in animals receiving CO-cFVIII-N11 were not different from uninjected controls. Mice receiving miniFIV-CO-cFVIII-BDD demonstrated significantly increased levels of BiP and PeIF2 α protein compared to sham injected and N11-injected animals (Figures 5b, $P < 0.05$). quantitative PCR with reverse transcription analysis of mRNAs did not detect differences in most ER stress-responsive genes, with the exception of *Chop*, which was more highly expressed in BDD animals than in N11 animals (Figure 5c), similar to its protein expression. Taken together, these data suggest that expression of CO-cFVIII BDD elicits a very modest ER stress response compared to animals subjected to a bona fide ER stressor (data not shown), or the related integrated stress response,³³ and that this response is attenuated in N11 animals. Because both constructs, when used at equivalent titers, confer equivalent therapeutic benefit, the degree of ER stress induced may not be therapeutically worrisome.

CONCLUSIONS

There are over 2000 known disease causing mutations in the human FVIII gene (<http://www.factorviii-db.org/>), including point mutations, genetic inversions, and deletions. Further, most mutations are unique to a single family. As such, a gene replacement strategy is far more practical than a gene-editing strategy.

The fundamental goal of this study was to use our experience to optimize a lentiviral vector for cFVIII delivery. These initial efficacy studies in mice will serve as a bridge to preclinical studies in dogs. Phenotypically, the hemophilia A dogs are a large animal model that exhibit spontaneous bleeding in soft tissues and joints and provide a more diverse immune response, making the hemophilic dog model a key step on the path toward developing new therapies. Our results suggest that miniFIV-CO-cFVIII is a suitable vector choice for preclinical studies in a hemophilia A dog model. In addition, based on previous studies and clinical trials, vector production scale-up is possible.^{34,35} The question remains: Is the BDD or N11 a better choice? Due to its shorter genome, the BDD vector produced higher titer vector; whereas, the N11 vector had marginally better activity and elicited reduced CHOP, BiP and PeIF2 α expression. For our goals, safety and persistence are the paramount considerations; to this end, integrating vectors expressing cFVIII likely have the best chance of success for long-term correction of hemophilia A dogs. In conclusion, we demonstrate the use of an optimized expression system that confers therapeutically relevant and stable cFVIII protein production.

MATERIALS AND METHODS

Construction and production of viral vectors

The miniFIV shuttle vector that minimized any non-essential viral sequence and contains no residual enhance activity was designed *in silico* using previously described FIV vector sequences.^{28,36} The vector was synthesized and sequence verified (GenScript, Piscataway, NJ, USA). Wild-type cFVIII cDNA was provided by Dr David Lillicrap. CO-cFVIII with the partial B-domain (CO-cFVIII-N11) was designed *in silico*, synthesized, and sequence verified (GenScript). Convenient restriction enzyme sites were included that allowed for removal of the partial B-domain to generate the CO-cFVIII-BDD construct (GenBank: MF158962) and directional cloning into the miniFIV shuttle vector. All cFVIII vectors include the murine albumin enhancer/human α_1 anti-trypsin hybrid promoter.²² The reporter vector, mCherry driven by the rous sarcoma virus promoter, was moved from a previously described lentiviral vector³⁷ using standard directional cloning techniques. Lentiviral vectors were produced at Virogenics, Inc (Del Mar, CA, USA). Supplemental vector preparations and titering was performed by the University of Iowa Viral Vector Core (www.medicine.uiowa.edu/vectorcore). Baculovirus (GP64)-pseudotyped FIV vector particles were generated by transient transfection, concentrated 250-fold by centrifugation, and titered using real-time PCR from samples collected 3 days after cells transduced as previously described.²⁵

***In vitro* assay**

300 ng plasmid containing either miniFIV-cFVIII-BDD or miniFIV-CO-cFVIII-BDD were transfected into Huh-7 cells (2×10^5 cells) using TransIT-LT1 reagent (Mirus, Madison, WI, USA) according to the manufacturer's instructions and cultured for three days. Supernatant was harvested and subsequently used for assays.

Mice, tail vein injection and blood collection

Mice were housed in the University of Iowa Animal Care Facilities. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa in accordance with National Institutes of Health guidelines. Congenic E16 hemophilia A mice, backcrossed at least seven generations on a C57Bl/6 background were used for these studies.^{22,26} A neomycin cassette inserted in exon 16 of genomic FVIII leads to protein truncation and the associated bleeding phenotype in these animals.³⁸ Uninjected hemophilia A and wild-type C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) served as controls.

Six-to-eight-week-old hemizygous affected males and homozygous affected females were either injected by hydrodynamic tail vein injection as previously described²⁶ or transduced with lentiviral vectors in 200 μ l on each of 2 consecutive days via tail vein.²² In brief, mice ($n = 2-7$ /group) were restrained and lateral tail vein was accessed. In experiments using the reporter gene, viral transduction was measured by the number of cells expressing mCherry compared to total number of cells. Quantification was performed using Image J software (NIH, Bethesda, MD, USA). In experiments assessing FVIII expression, three doses alternating between consecutive mice and consisting of miniFIV-CO-cFVIII-BDD (1.2×10^8 TU ml⁻¹ (low dose, three mice)), miniFIV-CO-cFVIII-BDD (6×10^9 TU ml⁻¹ (high dose, two mice)), and miniFIV-CO-cFVIII-N11 (1.4×10^8 TU ml⁻¹, seven mice) were included. Whole blood in sodium citrate (final 3.8% concentration) collected via retro-orbital plexus using micro-hematocrit capillary tubes (Scientific Glass Inc, Rockwood, TN, USA) was then centrifuged for plasma collection as previously described.²⁶

FVIII activity, antigen and inhibitor assays

Functional FVIII activity was measured via Coamatic FVIII Chromogenic Assay (Chromogenix, Lexington, MA, USA) using a standard curve of serial dilutions of normal pooled human plasma (George King Bio-Medical, Overland Park, KS, USA) and results quantified as previously reported.²⁶ Normal C57Bl/6 wild-type and untreated FVIII null mouse plasma served as positive and negative controls, respectively.

In addition, we measured cFVIII antigen utilizing a matched pair antibody set for ELISA of canine FVIII antigen (Affinity Biologicals, Ancaster, ON, Canada) according to manufacturer instructions and read at 490 nm on a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA). Values were compared to a reference curve of normal pooled canine plasma (Innovative Research, Novi, MI, USA). Plasma from untreated FVIII null mice served as a negative control.

Inhibitory antibodies to cFVIII were measured using the Nijmegen modified Bethesda Assay, as previously reported.^{26,39} Inhibitor levels are reported in Bethesda Units (BU ml⁻¹), where one BU is the amount of antibody capable of decreasing the residual FVIII coagulant activity by 50%. In short, plasma samples of lentiviral-injected mice were mixed in incremental ratios of normal canine pooled plasma and FVIII null pooled mouse plasma, followed by two hour incubation at 37 °C. These various dilution mixtures, along with positive control serial dilutions of 4A4 monoclonal anti-human FVIII antibody,^{26,40} were then measured for residual FVIII activity using chromogenic assay (Chromogenix) as above.

Tail transection assay

Tail clips were conducted at 24 or 28 weeks post injection for quantification of the bleeding phenotype as previously described.^{22,26} Accumulated blood loss over 15 min was determined by weight.²⁶ To measure hemoglobin, erythrocytes were lysed and optical density measured at 575 nm.

Liver ER stress markers

Liver samples were harvested at the time of sacrifice followed by homogenization in 1 ml of Trizol reagent (Invitrogen). RNA was isolated according to the manufacturer's instructions followed by cDNA generation using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative PCR protocol and primer sequences were as previously described.^{26,41} As a positive control, wild-type mice were injected with tunicamycin 1 mg per 1 kg (mouse) and livers were harvested 8 h post injection. Immunoblots were performed as previously described.⁴²

Copy number

Experimental mouse livers were harvested at the time of sacrifice for genomic DNA isolation using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Real-time qPCR was used to detect copy number using Power SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY, USA). Primer sets were tested for selective amplification of cFVIII and the inability to bind or amplify similar sequences within murine genomic FVIII: CO-cFVIII forward primer: 5'-GTGTGTAAAGAAGGCTCCCTGG-3'; CO-cFVIII reverse primer: 5'-GTCAGAGGCAGAGAGCCG-3'. FVIII null mice liver genomic DNA (25 ng) was added to each standard curve reaction to mimic the sample conditions.

Statistical analysis

Significant differences among groups were analyzed via *t*-test (Wilcoxon signed rank test) or by a one-way analysis of variance followed by a Tukey's multiple comparison test. Analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Results are expressed as means ± s.e. A *P*-value of <0.05 was considered statistically significant.

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Figure 1. Schematic representation of cFVIII expressing FIV vectors. Full-length cFVIII is a 280 kDa glycoprotein with the domain structure A1-A2-B-A3-C1-C2. The four canine FVIII constructs contain various lengths of the B-domain. The lengths of these partial B-domains are 136 (BDD) or 392 (N11) amino acids with 0 or 11 consensus *N*-linked glycosylation sites, respectively. In addition, both cFVIII-BDD and cFVIII-N11 constructs express codon-optimized canine FVIII (CO-cFVIII-BDD and CO-cFVIII-N11, respectively). The number of nucleotide changes from the wild-type sequence is indicated. The miniFIV constructs are under the control of the liver-specific murine albumin enhancer/human alpha₁ anti-trypsin hybrid promoter (Alb/hAAT). MiniFIV regulatory regions are not to scale: RRE, rev responsive element; SIN LTR, self-inactivating long terminal repeat; Ψ, packaging signal.

Figure 2.

MiniFIV mediates gene transfer *in vitro* and *in vivo*. Immunofluorescence analysis of liver sections from C57BL/6 mice was performed following vector delivery. (a) Mice were injected with miniFIV-RSV-mCherry (4×10^8 TU/animal) or (b) sham injected (buffer only) animals served as a negative control (each $n = 3$). Red, mCherry; blue, DAPI nuclear stain. Scale bar, 500 μ m. (c) The hepatoma cell line Huh-7 was transfected with plasmid containing cFVIII-BDD or CO-cFVIII-BDD. A mock transfected culture served as a negative control. Columns indicate means plus standard error. * $P < 0.001$, measured via one-way ANOVA. (d) 25 μ g plasmid containing cFVIII-BDD ($n = 3$), cFVIII-N11 ($n = 3$), CO-cFVIII-BDD ($n = 5$), or CO-cFVIII-N11 ($n = 5$) was administered to FVIII null mice. DNA was prepared in 2 ml Lactated Ringer's solution and delivered hydrodynamically to 6–8-week-old FVIII null mice. Results for sham FVIII null mice ($n = 5$) are also indicated. FVIII activity was measured using the Coamatic activity assay. Columns indicate means plus standard error. * $P < 0.05$, measured via one-way ANOVA.

Figure 3.

MiniFIV-mediated FVIII expression persists *in vivo*. MiniFIV-CO-cFVIII-BDD ($n = 2-3$) or miniFIV-CO-cFVIII-N11 ($n = 7$) to 6-8-week-old FVIII null mice. Results for sham-injected FVIII null mice ($n = 5$) and wild-type mice ($n = 5$) are also indicated. (a) FVIII activity and (b) antigen were measured using the Coamatic activity assay or ELISA, respectively. Points indicate means plus standard error. * $P < 0.05$ and ** $P < 0.001$, both measured via a one-way ANOVA. (c) Mice were assessed for inhibitor development via Bethesda assay. Three mice revealed low-titer inhibitors. All other mice had Bethesda titers of < 0.5 . (Bethesda titers > 0.5 are clinically relevant).

Figure 4.

Partial phenotypic correction of hemophilia A in a mouse model. At 24 (low-dose groups) or 28 weeks (high-dose group) post injection (duration of the experiment), functional correction was observed in FVIII null mice treated with the miniFIV-CO-cFVIII-BDD (combined low- and high-dose groups) and miniFIV-CO-cFVIII-N11. Wild-type (WT) mice were included as a positive control. **(a)** Blood was collected in a pre-weighed conical tube, and bleeding was allowed for 15 min without intervention. Blood in the tail transection assay was quantified by calculating the weight of blood collected in normal saline (post-blood loss weight minus pre-weight). **(b)** Hemoglobin was quantified via absorbance. Columns indicate means plus standard error. ** indicates $P < 0.001$ measured via a one-way ANOVA. * indicates $P < 0.05$ measured via one-way ANOVA.

Figure 5.

Liver ER stress partially relieved by N11. At 24 (low-dose groups) or 28 weeks (high-dose group) post injection, liver tissue was assessed for signs of the UPR and ER stress. **(a)** Protein expression levels for BiP, CHOP and Pelf2α were assessed by western blot.⁴³ Although subtle, when assessed by densitometry, BiP, CHOP and Pelf2α were increased in animals injected with the B-domain deleted construct compared to sham injected animals ($P < 0.05$); however, there was no significant difference when animals injected with N11 constructs were compared to sham-injected animals. Calnexin was used as a loading control as it controls for any nonspecific expansion of the ER and thus protein content.⁴³ **(b)** Quantification of the BiP and Pelf2α band intensity. Mice injected with miniFIV-CO-cFVIII-BDD demonstrated significant increased levels compared to sham injected and N11-injected animals ($P < 0.05$). Quantification was not performed for CHOP as no band was detected in the sham injected animals. **(c)** Gene expression levels for BiP, CHOP glucose-regulated protein 94 (GRP94), ER degradation-enhancing alpha-mannosidase-like (EDEM), X-box-binding protein 1 total (XBP1t), XBP1 spliced (XBP1s), ER protein 72 (ERp72), peroxisome proliferator-activated receptor alpha (PPARA), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) were assessed by qPCR. Columns indicate means plus standard error.