Citation: Molecular Therapy — Methods & Clinical Development (2014) **1,** 14042; doi:10.1038/mtm.2014.42
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ARTICLE

piggyBac-mediated phenotypic correction of factor VIII deficiency

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Hemophilia A, caused by a deficiency in factor VIII (FVIII), is the most severe inherited bleeding disorder. Hemophilia A is an attractive gene therapy candidate because even small increases in FVIII levels will positively alter the phenotype. While several vectors are under investigation, gene addition from an integrated transgene offers the possibility of long term expression. We engineered the DNA transposon-based vector, *piggyBac* (*PB*), to carry a codon-optimized B-domain deleted human FVIII cDNA. Evaluation of gene transfer efficiency in FVIII null mice demonstrated that *PB* containing the FVIII cDNA, delivered via hydrodynamic injection to immunocompetent hemophilia mice, conferred persistent gene expression, attaining mean FVIII activity of approximately 60% with 3/19 developing inhibitors. In addition to efficacious expression, a goal of gene transfer-based therapies is to develop vectors with low toxicity. To assess endoplasmic reticulum stress in hepatocytes stably expressing the transgene, we evaluated levels of ER stress markers via qPCR and found no evidence of cell stress. To evaluate phenotypic correction, a tail clip assay performed at the end of the study revealed reduced blood loss. These data demonstrate that *PB* can be used to achieve sustained FVIII expression and long-term therapeutic benefit in a mouse model.

Molecular Therapy — Methods & Clinical Development (2014) **1**, 14042; doi:10.1038/mtm.2014.42; published online 10 September 2014

INTRODUCTION

Factor VIII (FVIII) deficiency (also known as hemophilia A) is the most severe inherited bleeding disorder, affecting about 1 out of 5,000 males.[1] Although recombinant FVIII protein replacement has decreased the transmission risk of blood-borne pathogens compared to plasma derived products, this therapy is still unavailable to many patients worldwide due to its high cost and need for frequent dosing.[2] An additional severe complication of protein replacement therapy is the development of inhibitors that neutralize recombinant FVIII activity. Inhibitory antibodies occur in 20–30% of severely affected patients, and current treatment options for these patients are limited and expensive.[2] Therefore, alternative treatment approaches are needed. Successful development of gene therapy for FVIII deficiency could decrease the need for frequent factor replacement, thereby decreasing costs and mortality while improving quality of life and outcomes in hemophilia A patients.[3]

Gene transfer offers the possibility of providing lasting expression of the deficient coagulation FVIII in people affected by hemophilia A.[3] Viral and nonviral gene transfer vectors as well as cell-based therapies are currently under investigation as tools for correction of FVIII deficiency.[4–6] Notable advancements have been made for hemophilia B gene therapy, as reflected in a recent report of an adeno-associated viral (AAV) factor IX (FIX) clinical trial.[7] Hemophilia A is also an attractive gene therapy candidate because even small increases in FVIII levels are anticipated to positively modify the phenotype. In addition, FVIII gene delivery to hepatocytes abolished pre-existing inhibitory antibodies in a large animal model of hemophilia A.[8] While promising, it is important to consider, develop, and test alternative approaches to hemophilia A patient therapy.

An ideal gene transfer vector for hemophilia A should be nonimmunogenic and confer sustained expression of therapeutic levels of FVIII to correct the bleeding phenotype. Integrating nonviral vectors, such as recombinant DNA transposons, provide alternatives to viral vectors and are increasingly used as tools for gene targeting, transgenesis, and gene delivery.[4,9] In a recombinant DNA transposon vector system, such as *piggyBac* (*PB*), the transposase is supplied in trans and the transgene of interest is flanked by the transposon terminal repeats. The *PB* transposase has two catalytic functions, excision and transposition. Thus, the transposon is mobilized from a vector and inserted into a new locus via a "cut and paste" mechanism. *PB* originates from the looper moth *Trichoplusia ni* and encodes the insect transposase (*iPB*).[10] Modification of *iPB* transposase has led to a hyperactive transposase (iPB7) with increased efficiency.[11] *PB* offers some potential advantages over existing tools in this field, including its substantial carrying capacity,[12,13] which is important for delivering the relatively large FVIII cDNA and elements required for expression.

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Received 24 July 2014; accepted 24 July 2014

Using *PB*, we previously demonstrated efficient gene transfer and persistent expression of a reporter transgene in mice.[11] Here, we adapt *PB* for delivery of the FVIII gene to hepatocytes. Following hydrodynamic delivery in mice, FVIII expression driven by a liver-specific promoter in a *PB* transposon persisted at least 24 weeks (the duration of the study). In addition, 13 of 19 transposon-treated mice exhibited phenotypic correction via tail clip assay without development of inhibitors. Mice stably expressing the FVIII transgene showed no evidence of endoplasmic reticulum (ER) stress in liver tissues. These results demonstrate that *PB* confers persistent and therapeutically relevant FVIII expression in hemophilia A mice.

RESULTS

PB delivery of FVIII

FVIII is a 280 kDa glycoprotein and along with FIX forms an important complex in the amplification stage of coagulation. FVIII is comprised of six domains (Figure 1a); however, the B domain of FVIII is dispensable for procoagulant activity.[14] We engineered a *PB* transposon plasmid carrying a codon-optimized B-domain deleted (BDD) human FVIII cDNA[15] under the control of a liver-specific promoter[16] (PB-coFVIII-BDD, Figure 1b). In mammalian cells, the natural insect transposase with seven amino acid substitutions, a hyperactive transposase called iPB7 (Figure 1c), transposes with greater efficiency both *in vitro* and *in vivo* than *PB* transposase.[11] As a control, the *PB* transposase was rendered catalytically inactive by a single mutation of an aspartic acid residue (PB^{D268L}) (Figure 1c).[11]

Six- to eight-week-old C57 or 129SVJ/B6 hemophilic mice (FVIII null animals, $n = 19$) were injected hydrodynamically with 25 µg each of PB-coFVIII-BDD and iPB7 to determine the efficiency and persistence of FVIII expression. Wild-type C57 and 129SVJ/B6 animals served as positive controls. As negative controls, FVIII null animals received Lactated Ringer's solution alone (LR, $n = 12$) or co-administration of PB-coFVIII-BDD and a catalytically inactive transposase[11] (PB^{D268L}, $n = 8$).

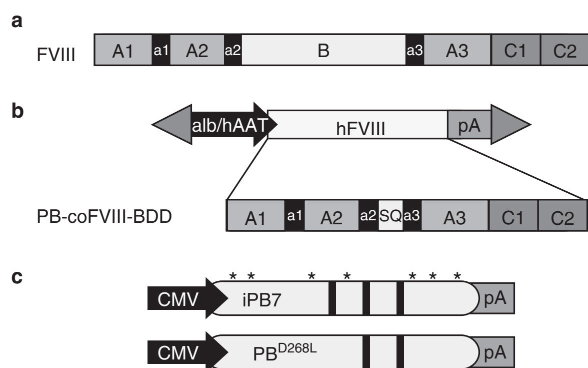

Figure 1 Schematic representation of constructs used in the study. (**a**) Full-length FVIII is a 280 kDa glycoprotein with the domain structure A1-A2-B-A3-C1-C2. (**b**) A *piggyBac* transposon construct was developed to express codon-optimized BDD human FVIII with 14 amino acids in place of the B-domain (PB-coFVIII-BDD) under the control of the liver-specific murine albumin enhancer/human alpha$_1$ anti-trypsin hybrid promoter (alb/hAAT). The promoter and transgene are flanked by identical 13 bp inverted terminal repeats. The backbone, pXLBacII, has been previously reported.[11] (**c**) *piggyBac* transposase constructs. The cytomegalovirus promoter-driven hyperactive transposase (iPB7) or inactive tranposase (PB^{D268L}) was inserted into the mammalian expression vector pcDNA3.1. Asterisks indicate sites of amino acid substitutions from wild-type transposase (I30V, S103P, G165S, M282V, S509G, N570S, N538K); black bars indicate catalytic residues D268, D346, and D447;[47,48] pA indicates polyadenylation signal from SV40.

FVIII delivery via *PB* improves survival in hemophilic mice

Previous descriptions of the hemophilia A mouse models indicated a normal lifespan and lethal bleeding with procedures such as tail clip.[17] However, we observed a shortened lifespan in hemophilia A mice receiving LR or inactive transposase after hydrodynamic tail-vein injection, an intervention known to cause liver trauma.[18] All mice receiving PB-coFVIII-BDD + iPB7 survived for the duration of the study (either 16 or 24 weeks). In contrast, only 8 of 12 mice receiving LR (67%) and 5 of 8 mice receiving inactive transposase (62.5%) survived for the duration of the study (Figure 2, $P = 0.02$). To determine if the hydrodynamic tail-vein injection altered the survival of animals injected with LR or inactive transposase, a group of untreated animals were included. These animals demonstrated an overall survival of 52% which was not statistically different from the groups receiving LR or inactive transposase. These data suggest that the hemophilia A mouse may have a shortened lifespan and FVIII expression improved outcomes after gene delivery in these mice.

PB confers persistent FVIII expression in hemophilia A mice

We next evaluated the persistence of FVIII expression after transposon delivery. Three days after delivery, animals receiving PB-coFVIII-BDD + iPB7 or PB-coFVIII-BDD + inactive transposase demonstrated FVIII levels greater than 50%. By 2 weeks after injection, mice receiving PB-coFVIII-BDD + inactive transposase had FVIII activity of 0% similar to mice that received LR ($P = 0.5$). Mice receiving LR alone had undetectable levels of FVIII (0%) throughout the study. Animals receiving PB-coFVIII-BDD + iPB7 had significantly higher FVIII activity than mice receiving LR alone ($P < 0.001$) or animals receiving inactive transposase ($P < 0.05$, Figure 3a). FVIII levels in the transposon + iPB7 treated animals were similar to wild type ($P = 0.9$, Figure 3a) and more importantly, this activity was sustained for the study duration. At 24 weeks after injection, PB-coFVIII-BDD + iPB7 treated mice averaged FVIII activity of 57% compared to pooled human plasma.

In addition to an activity assay, FVIII protein was measured via an ELISA-based antigen assay. Similar to the FVIII activity results, mice treated with PB-coFVIII-BDD + iPB7 had a significant increase in FVIII antigen compared to LR- or PB^{D268L}-treated mice ($P = 0.002$, Figure 3b). Together, these data demonstrate long-term *PB*-mediated expression of FVIII. QPCR analysis was used to determine transgene copy number per genome. DNA from the livers of PB-coFVIII-BDD +

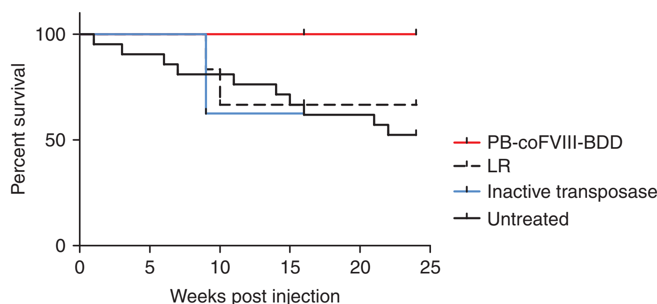

Figure 2 *piggyBac* gene transfer improved survival *in vivo*. Twenty-five microgram transposon with the liver-specific FVIII expression cassette in a 1:1 ratio with hyperactive or inactive transposase or Lactated Ringer's (LR) alone were delivered hydrodynamically to 6- to 8-week-old FVIII null mice and survival monitored. Mice injected with iPB7 ($n = 19$) had significantly improved survival compared to those injected with Lactated Ringer's alone or inactive transposase (*$P < 0.02$, log-rank (Mantel-Cox) test). Uninjected animals ($n = 21$) demonstrated a similar survival to those injected with LR ($n = 12$) or inactive transposase ($n = 8$).

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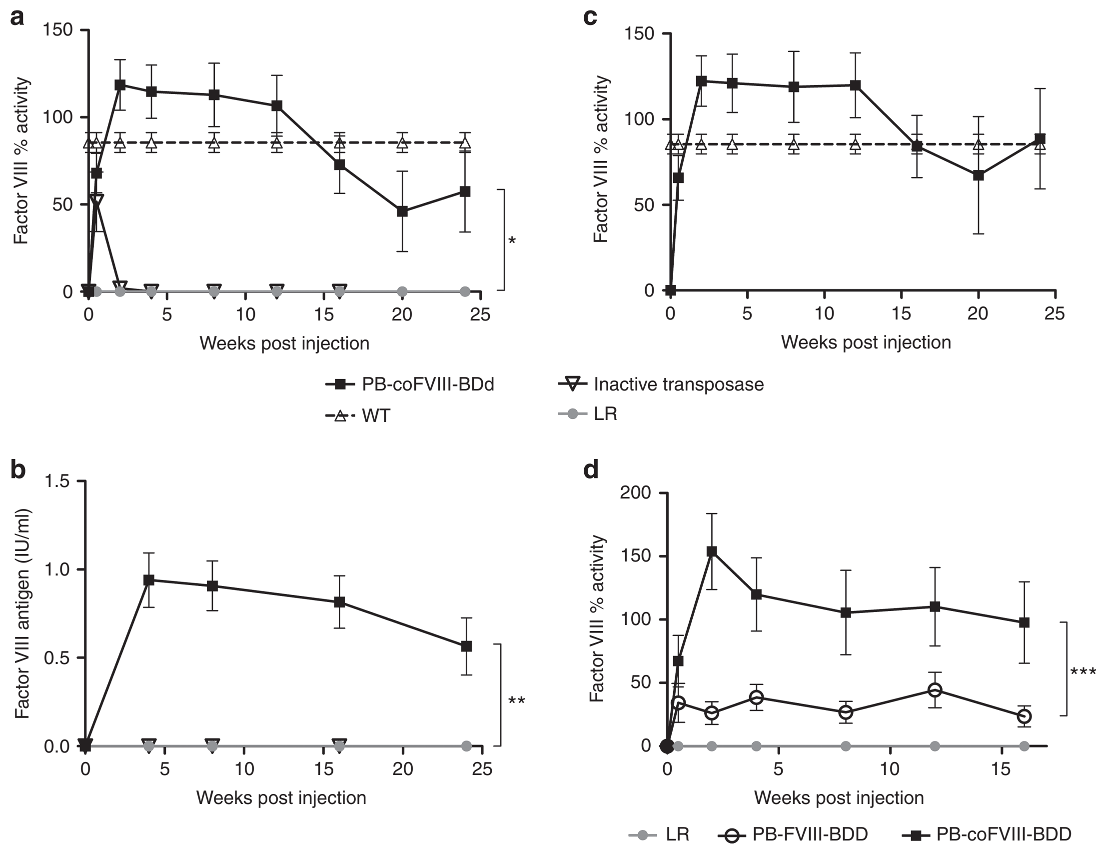

Figure 3 Hyperactive *piggyBac* transposase-mediated FVIII expression persists *in vivo*. Twenty-five microgram PB-coFVIII-BDD transposon was given in a 1:1 ratio with either iPB7 (box, $n = 19$) or inactive transposase (inverted triangle, PB^{D268L}, $n = 8$) to FVIII null mice. DNA was prepared in 2 ml Lactated Ringer's solution (LR) and delivered hydrodynamically to 6- to 8-week-old FVIII null mice. Results for LR-treated FVIII null mice (shaded circle, $n = 12$) and wild-type mice (triangle, $n = 15$) are also indicated. (**a**) FVIII activity and (**b**) antigen were measured using the Coamatic activity assay or enzyme-linked immunosorbent assay (ELISA) respectively. Points indicate means ± SE. $^{*}P < 0.001$ and $^{**}P = 0.002$, both measured via a one-way analysis of variance. (**c**) Mice treated with PB-coFVIII-BDD + iPB7 were assessed for inhibitor development. Three mice revealed low-titer inhibitors. All other mice had Bethesda titers of <0.5. (Bethesda titers >0.5 are clinically relevant.) This graph represents FVIII activity without these three mice and reveals FVIII levels indistinguishable from wild-type mice. (**d**) Twenty-five microgram PB-coFVIII-BDD transposon (box, $n = 8$) or PB-FVIII-BDD (open circle, $n = 9$) was given in a 1:1 ratio with iPB7 to FVIII null mice as described above. Results for LR-treated FVIII null mice (filled circle, $n = 7$) are also indicated. FVIII activity was measured using the Coamatic activity assay. Points indicate means ± SE. $^{***}P < 0.001$, measured via a one-way analysis of variance.

iPB7 treated animals was extracted and copy number determined. Treated animals exhibited a mean of 8.9 copies/genome ± 1.9 (mean ± SE), with a range of 0–30.2 copies/genome.

To demonstrate that the codon-optimization conferred higher expression, we evaluated the persistence of FVIII expression after delivery of iPB7 with PB-coFVIII-BDD or the non-codon-optimized PB-FVIII-BDD. Both constructs have identical amino acid sequences as previously reported.[15] Mice treated with PB-coFVIII-BDD + iPB7 conferred a 3–4 fold higher expression of FVIII throughout the 16 weeks of the study ($P < 0.001$, Figure 3d).

Low level inhibitors in only a few treated animals

Development of anti-FVIII antibodies (neutralizing antibodies, also called inhibitors) is a major complication for patients receiving recombinant protein therapy.[2] We investigated whether FVIII inhibitors developed after delivery of PB-coFVIII-BDD using the *PB* transposon system at the end of the study (16 or 24 weeks after injection). We looked for FVIII inhibitors by Bethesda assay, the standard quantitative clinical assay for FVIII inhibitors. One Bethesda Unit (BU) is the amount of inhibitor that will result in 50% residual FVIII activity during a given incubation period. An inhibitor titer greater than 5 BU is a high-titer inhibitor and is clinically significant. The Bethesda assay revealed three mice with low-titer inhibitors. One mouse had an inhibitor of 2.5 BU and two mice had Bethesda titers of 1.2 BU. All remaining mice (16 of 19) had Bethesda titers <0.5, indicating no detectable inhibitors. FVIII activity was plotted for the mice receiving PB-coFVIII-BDD excluding the mice with low titer inhibitors and revealed FVIII levels indistinguishable from wild-type mice (Figure 3c). Mice treated with PB-coFVIII BDD + inactive transposase did not develop antibodies to FVIII. Only mice exhibiting a positive Bethesda titer demonstrated IgG antibodies to FVIII (data not shown), indicating that no non-neutralizing antibodies were detected.

BDD FVIII treated mice lack hepatic ER stress

Previous studies in mice suggest that short term expression of human BDD FVIII results in hepatocyte stress following plasmid-mediated gene transfer.[19] We therefore asked whether short- or long-term FVIII expression via *PB* induced this cellular pathology. ER stress markers, including binding immunoglobulin protein (BiP), the UPR-regulated CCAAT/enhancer-binding protein homologous protein (CHOP), and ER degradation-enhancing α-mannosidase-like protein (EDEM), are elevated after initiation of the unfolded protein response (UPR).[19–21] In comparing the abundance of these

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transcripts at 1 day and 16–24 weeks after gene transfer, we found no evidence of stress in animals receiving PB-coFVIII-BDD and no significant difference between experimental groups and sham controls at either time point (Figure 4).

Phenotypic correction in hemophilia A mice after delivery of *PB* transposon carrying FVIII + iPB7

Hemophilia A mice demonstrate significant bleeding after a tail clip.[17] To evaluate functional correction of the bleeding phenotype, blood loss following a tail clip was measured at the time of sacrifice. Thirteen of sixteen mice receiving the PB-coFVIII-BDD transposon + iPB7 and without inhibitors revealed partial to full correction as determined by two metrics (Figure 5). Total blood loss was significantly less in mice receiving the PB-coFVIII BDD vector + iPB7 (n = 16) compared to LR-treated (n = 8) or inactive-transposase-treated (n = 5) hemophilia A littermate mice ($P < 0.05$). Mice demonstrating FVIII antibodies (n = 3) had similar results to LR-treated mice demonstrating the neutralizing effect of the inhibitors. There was no significant difference between wild-type mice and mice receiving the PB-coFVIII-BDD transposon + iPB7 without inhibitors. Therefore, the *PB* transposon vector system conferred partial to full correction of the bleeding phenotype in most animals.

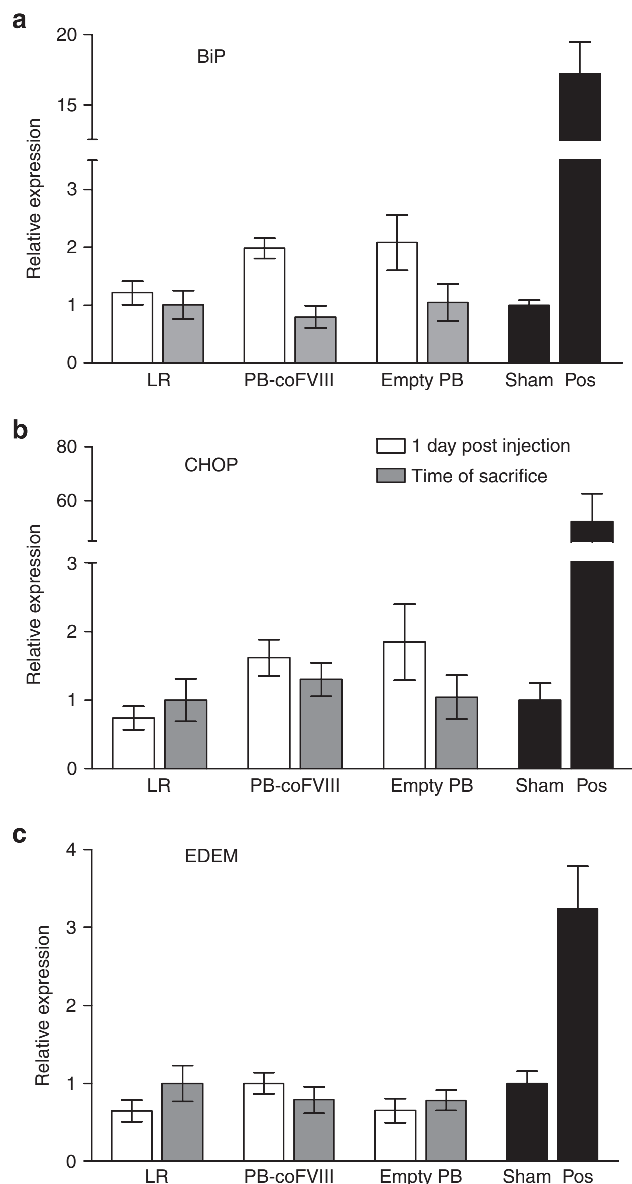

Figure 4 No evidence of liver ER stress. At 16 (n = 4) or 24 (n = 5) weeks after injection (time of sacrifice), liver tissue was assessed for signs of the UPR and ER stress. Gene expression levels for (**a**) BiP, (**b**) CHOP, and (**c**) EDEM were assessed by qPCR. PB-coFVIII indicates mice treated with PB-coFVIII-BDD transposon and iPB7 (n = 9 total). Positive control mice (n = 3) received tunicamycin. FVIII null animals represent both C57 and 129SVJ/B6 backgrounds (n = 3 each). Vector alone group received PB transposon without the FVIII transgene (n = 4). Columns indicate means ± SE.

DISCUSSION

Here, we report the first use of the *PB* transposon for therapeutic *in vivo* gene transfer of FVIII in a hemophilia A mouse model. We demonstrate corrective levels of FVIII expression in hemophilia A mice to 57% compared to pooled human plasma. In addition, FVIII activity and antigen levels persisted for the duration of the study and no elevations of ER stress markers were detected in any of the treatment groups. Remarkably, only 3 of 19 mice developed low-titer inhibitors to FVIII. FVIII levels achieved in the treatment group were predicted to eliminate the hemophilia A bleeding phenotype. Indeed, 13 of 19 mice receiving the *PB* transposon containing the FVIII gene + iPB7 demonstrated phenotypic correction in a tail clip

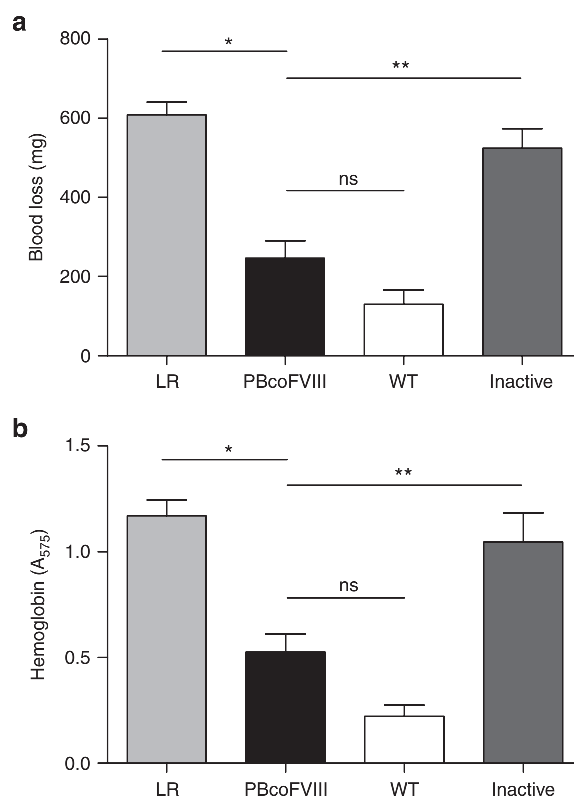

Figure 5 Phenotypic correction of hemophilia A in a mouse model. At 16 or 24 weeks after injection (time of sacrifice), functional correction was observed in mice treated with the PB-coFVIII-BDD transposon and the hyperactive transposase. LR, Lactated Ringer's treated FVIII null mice; coFVIII-BDD, mice treated with PB-coFVIII-BDD + iPB7; WT, wild-type mice; Inactive, mice treated with PB-coFVIII-BDD + inactive transposase. (**a**) Blood loss was quantified by measuring the weight of blood collected in normal saline. (**b**) Hemoglobin was quantified via absorbance. Columns indicate means ± SE. *$P < 0.0001$ measured via a one-way analysis of variance. **$P < 0.05$ measured via *t*-test. ns indicates $P > 0.05$.

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bleeding assay. These data support the therapeutic utility of the *PB* transposon system.

The most severe and challenging complication of protein replacement therapy is the development of inhibitors that neutralize FVIII activity.[2] Interestingly, only 3 of 19 *PB*-mediated, FVIII-treated mice developed low-titer inhibitors. This low incidence of inhibitory FVIII antibodies following *PB* gene transfer was unanticipated and intriguing. Further study is needed to understand the mechanisms underlying this observation and whether it represents a form of tolerance. Of note, immunosuppression,[22] B-cell depletion,[23] and microRNA regulation[24] were not necessary to maintain therapeutic expression of FVIII following *PB* gene transfer.

The *PB* vector system combines multiple attractive features, including long-term expression[11] of therapeutic FVIII levels. We also note improved survival in the PB-coFVIII-BDD treated group compared to control mice in addition to minimal inhibitor development in immunocompetent hemophilia A mice. Our data differ from prior reports using non-viral vectors and viral vectors to express FVIII in this animal model. Previously, gamma retroviral vectors have been used for hematopoietic stem cell directed delivery[25] and hepatocyte delivery[26] of FVIII with varied results of expression. The recognized risk of insertional mutagenesis presented by oncoretroviral vectors[27] has led to pursuit of alternative approaches. Lentiviral vectors have shown varied success for FVIII transgene delivery and expression in neonatal mice,[15] and in adult mice,[16,24] after transplantation of hematopoietic stem cells,[28] and after macrophage depletion.[24] The *PB* vector system achieved therapeutic levels of FVIII following delivery to 6- to 8-week-old FVIII null immunocompetent mice.

Non-viral vectors have also been studied for FVIII gene transfer. When *Sleeping Beauty*, a DNA transposon, was delivered to hepatocytes, Largaespada and coworkers discovered that animals required tolerization with neonatal infusion of FVIII protein prior to delivery of the FVIII transgene for sufficient and sustained FVIII expression.[29] Importantly, in our *PB* model, no immunotolerization was required for long-term FVIII expression in hepatocytes. We hypothesize that these differences may be in part due to the use of a tissue-specific promoter, codon-optimization of the transgene, or cell types in which FVIII was expressed.

The large carrying capacity[12,13] of the *PB* vector system is an appealing feature for gene therapy strategies for hemophilia A since FVIII is encoded by a large cDNA (total 7.055 kb). Although the B domain is not required for activity of the FVIII protein, as is evident from our studies and others,[14] addition of the B domain may be important for interaction with chaperone proteins,[30] reduction of proteolysis,[31] and intracellular trafficking.[32] Given the efficient integration observed with *PB* cassettes up to 100 kb,[12] addition of a full or partial B domain is possible. Future studies will compare the efficacy and safety of delivering FVIII with a full or partial B-domain.

A limitation of *PB* is the inefficiency of plasmid delivery *in vivo*. Hydrodynamic tail-vein injection is a method to efficiently deliver plasmid DNA to hepatocytes, but is currently limited to small animal models.[33] While groups are working to develop this delivery method for large animal models and eventually humans,[34] hydrodynamic delivery has yet to be translated to the clinic. Although local hydrodynamic delivery,[35] ultrasound delivery,[36] and *ex vivo* applications[37] of plasmid-based systems are possible, alternative strategies to deliver PB are available. These include hybrid vectors, such as packaging a transposon in an integration-defective lentiviral vector[38,39] or an adenoviral vector.[40,41] Hausl *et al*. used a hybrid adenoviral/*Sleeping Beauty* vector to deliver FIX to hepatocytes. However, their approach required Flp-mediated recombination for excision of the *Sleeping Beauty* transgene to occur.[40] Future experiments are necessary to develop effective, scalable delivery methods for the PB system.

A goal of gene transfer based therapies is to achieve efficacious expression. Malhotra *et al.* reported that 24 hours of FVIII expression using a BDD cassette caused ER stress, reduced protein production, and apoptosis.[19] FVIII dissociation from the immunoglobulin-binding protein in an adenosine triphosphate-dependent manner is required for secretion. They also demonstrated that accumulation of unfolded FVIII protein in the ER leads to reactive oxygen species generation, further activating apoptosis.[19] These findings were reduced by inclusion of a partial B domain in the FVIII cDNA (226aa/N6[32]).[19] We investigated liver ER stress at 1 day and 16 or 24 weeks after injection to assess the short- and long-term effects of the FVIII transgene expression and found no evidence of ER stress. We examined evidence of the UPR and ER responses at early and late time points and found no significant difference between experimental groups or sham controls. The ER stress and apoptosis previously reported may be a consequence of transient and acute expression of a non-integrated transgene. Alternatively, our use of a liver-specific promoter may render more physiologic levels of expression, which may also contribute to the lack of ER stress. The end result of low ER stress with sustained expression indicates that the expressed FVIII is not intrinsically cytotoxic.

Finally, we speculate that codon optimization of the BDD FVIII cDNA may reduce cell stress. There is only 76% nucleotide identity between the codon-optimized cDNA[18] used in these studies and the non-codon-optimized FVIII BDD cassette used in Malhotra *et al*.[19] It is worth noting that the B domain spacer reported in Malhotra *et al*. is different than the one used in our studies, including 76% homology and a Lys[1644] to Thr[1644] amino acid change.[15,19] These differences may also play a role in the reduction of cell stress.

In summary, our data highlight effective long-term FVIII expression from a *PB* transposon without immunotolerization or ER stress in a mouse model of hemophilia A. This approach may also have applications for expression of other secreted proteins from hepatocytes. While these studies demonstrate the efficacy of *PB* in a rodent disease model, further studies are warranted to address the long-term safety concerns of this non-viral integrating vector in large animal models of hemophilia.

MATERIALS AND METHODS

Plasmid constructs

iPB7 and PB^{D268L} were constructed as described previously.[11] To clone PB-coFVIII-BDD, a *PB* transposon plasmid, pXLBacII-MCS,[11] a mammalian expression vector containing a multiple cloning sequence driven by the murine albumin enhancer/human alpha$_1$ anti-trypsin hybrid promoter,[16] was linearized using *Pac*I and *Nru*I. The pXLBacII_cassette[42] carries the minimal PB sequences of 308 bp and 238 bp of the 5′ and 3′ ends, respectively.[43] *Pac*I and *Nru*I flanking restriction sites were introduced into the codon-optimized human FVIII cDNA (from the previously reported codon-optimized FVIII containing 14 amino acids in place of the B-domain,[15] a gift from John McVey) by PCR amplification using Phusion (New England Bio Labs, Ipswich, MA) and the primer pair (AAA-TTA-ATT-AAA-TGC-AGA-TCG-AGC-TGT-CCA and AAA-TCG-CGA-TCA-GTA-CAG-ATC-CTG-GGC). The PCR product, coFVIII-BDD, was subsequently subcloned into pCR-BluntII-TOPO (Invitrogen, Grand Island, NY) and the *Pac*I/*Nru*I fragment containing the codon-optimized BDD FVIII gene was inserted into the linearized pXLBacII-MCS.

Mice and tail-vein injection

All mice for this study were housed at the University of Iowa Animal Care Facilities. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa in accordance with National Institutes of Health guidelines. Hemophilia A mice with a targeted

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deletion of exon 16 were used in these studies.[16,17] Mice of two strains were used: 129SVJ/B6 (Jackson Laboratories) and congenic C57BL/6 mice backcrossed for more than seven generations.[16] Hemizygous affected males and homozygous affected females were used. Both C57Bl/6 and 129SVJ/B6 wild-type animals served as positive controls. Twenty-five µg PB-coFVIII-BDD in a 1:1 ratio with either pcDNA3.1-iPB7 or pcDNA3.1-PB^{D268L} (Figure 1) were delivered hydrodynamically as previously described[11,44] to 6- to 8-week-old FVIII null mice. In brief, plasmid DNA was prepared in 2 ml of sterile Lactated Ringer's at room temperature. Mice (n = 6–19 in each group) were restrained and the lateral tail vein was accessed using a 27 gauge needle (Becton Dickinson, Franklin Lakes, NJ). The solution was administered over 5–7 seconds.

Blood collection

Whole blood was collected at baseline and then every 2–4 weeks after injection for 24 weeks. For plasma collection, mice were bled via the retro-orbital plexus using micro-hematocrit capillary tubes (Scientific Glass, Rockwood, TN). Blood was collected using sodium citrate as an anticoagulant at a final concentration of 0.38% (wt/vol). The blood samples were then centrifuged at 6,000*g* for 20 minutes at 4 °C for plasma collection.

FVIII activity, antigen, and inhibitor assays

FVIII activity in plasma samples was quantified using the Coamatic FVIII Chromogenic Assay (Chromogenix, Lexington, MA) following the manufacturer's directions. Samples were read at 405 nm on a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA). Pooled human plasma (George King Bio-Medical, Overland Park, KS) in serial dilutions was used to produce a standard curve for FVIII activity. Plasma from untreated FVIII null mice served as a negative control, while plasma from wild-type C57BL/6 or 129SVJ/B6 mice served as a positive control.

In addition, human FVIII antigen was measured via ELISA (Affinity Biologicals, Ancaster, ON) following manufacturer instructions. Mouse plasma was mixed 1:4, 1:8, 1:16, and 1:32 with the sample diluent provided and added to wells pre-coated with antibody to human FVIII. The absorbance was measured at wavelength 450 nm and analyzed (VersaMax Microplate Reader). In addition to the control solutions provided, plasma from untreated FVIII null mice served as a negative control, while pooled human plasma served as a positive control.

FVIII inhibitor levels were quantified by Bethesda assay as previously reported.[45] One BU is the amount of inhibitor that will result in 50% residual FVIII activity during a given incubation period. Briefly, sample mouse plasma, along with either wild-type mouse plasma or FVIII null mouse plasma in a 1:1 ratio, was incubated at 37 °C for 2 hours. Residual hFVIII activity was measured and then quantified with the chromogenic assay (Chromogenix). Serial dilutions of the 4A4 monoclonal anti-human FVIII antibody (4A4 MAb), generously provided by Pete Lollar, were used to produce a positive control for FVIII inhibitory antibodies.[46]

Tail clip assay

Functional correction of the bleeding phenotype was assessed using a tail clip assay as previously reported.[16] Mice (16 or 24 weeks after injection) were placed in a restrainer which allows access to the tail. Tails were amputated at the 3 mm thickness making the bleeds uniform in all animals. The tail was immediately submerged into normal saline solution (prewarmed at 37 °C for at least 1 hour) in a 15 ml conical tube. Bleeding was allowed for 15 minutes without intervention; the animals were then sacrificed. Blood loss was quantified by measuring the weight and hemoglobin content of blood collected in normal saline. To measure hemoglobin, samples were centrifuged to collect erythrocytes, which were then resuspended in a lysis buffer containing NH_4Cl 8.3 g l^{-1}; $KHCO_3$ 1.0 g l^{-1}; and EDTA 0.037 g l^{-1}. The absorbance was measured at wavelength 575 nm (VersaMax Microplate Reader).

Molecular studies

Liver samples were homogenized in 1 ml of Trizol reagent (Invitrogen) and RNA isolated according to the manufacturer's instructions. cDNA was generated using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative polymerase chain reaction (qPCR) protocol and primer sequences were as previously described.[20] As a positive control, wild-type mice were injected with tunicamycin 1 mg/1 kg (mouse). Positive control livers were harvested 8 hours after injection. Experimental mouse livers were harvested at the time of sacrifice. Livers from mice receiving PB-coFVIII-BDD + iPB7 or LR were collected for genomic DNA isolation using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). qPCR was used to detect copy number specific for codon-optimized human FVIII using Power SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY): coFVIII forward (5′-CAGCTCTCACCAGCACGAC-3′) and human coFVIII reverse (5′-CTCGCTGTCGGTCAGGTC-3′). A plasmid standard curve was generated from serial dilutions of PB-coFVIII-BDD plasmid. Twenty-five ng of FVIII null mice liver genomic DNA 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. Survival was analyzed using the log-rank (Mantel-Cox) test. Analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA). Results are expressed as means ± SE. A P value of <0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We are grateful for the contributions of John McVey (Thrombosis Research Institute, UK) and Kathryn Chaloner (University of Iowa). We thank Edgardo Rodriguez (University of Iowa) for critical review of the manuscript and Ashley Cooney for contributions to the copy number assay. This work was supported by the Roy J. Carver Charitable Trust (P.B.M.), R44 HL-081976 (P.B.M.), the Bayer Healthcare Early Career Investigator Award (J.M.S.), and NIH HD027748-19, the Molecular and Cellular Research to Advance Child Health (J.M.S.).

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