Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies

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Inhibitory immune response to exogenously infused factor VIII (FVIII) is a major complication in the treatment of hemophilia A. Generation of such inhibitors has the potential to disrupt gene therapy for hemophilia A. We explore what we believe to be a novel approach to overcome this shortcoming. Human B-domain–deleted FVIII (hBDDFVIII) was expressed under the control of the platelet-specific αIIb promoter in platelets of hemophilic (FVIIInull) mice to create 2bF8trans mice. The FVIII transgene product was stored in platelets and released at the site of platelet activation. In spite of the lack of FVIII in the plasma of 2bF8trans mice, the bleeding phenotype of FVIIInull mice was corrected. More importantly, the bleeding phenotype was corrected in the presence of high inhibitory antibody titers introduced into the mice by infusion or by spleen cell transfer from recombinant hBDDFVIII–immunized mice. Our results demonstrate that this approach to the targeted expression of FVIII in platelets has the potential to correct hemophilia A, even in the presence of inhibitory immune responses to infused FVIII.

Introduction

Monogenic diseases, characterized by the loss of a specific plasma protein, are currently treated by repetitive replacement therapy and are choice candidates amenable to gene therapy. Hemophilia A, a severe congenital bleeding disorder caused by the loss of clotting factor VIII (FVIII) (1), is a prototype of such monogenic diseases. Currently, hemophilia A is treated by infusion of recombinant or plasma-derived FVIII (2). However, 25–30% of patients develop antibodies (FVIII inhibitors) that selectively inactivate the clotting activity of FVIII and negate its therapeutic efficacy (3). Hemophilia A is considered a strong candidate for gene therapy because the therapeutic window is broad and even a minimal plasma level of plasma FVIII is clinically advantageous. The development of inhibitory antibodies to the FVIII transgene product in plasma remains a significant barrier to some patient candidates. Many groups have developed various strategies for directing FVIII synthesis (4–15), although inadequacies of gene delivery and expression and inhibitor formation remain clinical problems (7, 16–18).

The approach we investigated, which we believe to be novel, is based on the hypothesis that targeting the production of FVIII to a secreting cell type that acts in the immediate vicinity of sites where FVIII is needed could overcome the presence of inhibitory antibodies. Furthermore, by sequestering the FVIII, the generation of antibodies in naïve individuals might be prevented or at least rendered less relevant.

The feasibility of such an approach is supported by the fact that in plasma, VWF serves as the obligate carrier protein for FVIII and protects it from protease degradation and rapid clearance (1, 19, 20). We have previously demonstrated that coexpression of FVIII in a cell that stores VWF results in the storage, and release, of FVIII (4, 21). More specifically, directing FVIII expression to megakaryocytes results in storage of FVIII with VWF in the α-granules of platelets (22, 23). A megakaryocytic, lineage-specific promoter would direct FVIII expression only to that blood cell lineage where VWF is normally endogenously synthesized and stored (20, 24). Poncz and coworkers have reported that expressing FVIII in platelets under control of the glycoprotein Ibα (GPIbα) promoter in FVIII-deficient (FVIIInull) mice canameliorate bleeding in a FVIIInull mouse model (9). In addition to platelets, GPIbα expression has been reportedly synthesized in endothelial (25–27) and breast tumor cells (28). Thus, we chose the platelet-specific GPIbα gene promoter (the αIIb promoter) that has been demonstrated to direct megakaryocyte-specific gene transcription (22, 29–37).

In the current study, we used the αIIb promoter to direct FVIII expression and determined (a) that transgenic platelet-expressed and stored FVIII effectively protected FVIIInull mice from bleeding, (b) that this protection was transferable into FVIIInull recipients via transgenic platelet transfusion, and (c) that this therapeutic efficacy was maintained even in the presence of high-titer inhibitory antibodies to FVIII. This approach may be promising for hemophilia treatment as well as other conditions where the missing protein can be targeted directly to the site of desired activity. Furthermore, the presence of preexisting inhibitory antibodies might not be a contraindication for such an approach.

Results

Transgenic expression of FVIII in platelets. We generated transgenic mice expressing human B-domain–deleted FVIII (hBDDFVIII)
using the 7.6-kb PvuI/BsmBI insert of the human αIIb promoter–driven hBDDFVIII (2bF8) construct (Figure 1A). Germline transmission was established, and transgene-positive offspring were mated with FVIII
null mice to generate mice that express the 2bF8 transgene without expression of normal mouse FVIII (2bF8trans mice). A single 2bF8 transgene insertion site was determined by DNA Walking PCR and demonstrated that the sequence flanking the 2bF8 transgene was identical to BAC RP23-127 B9 located in the B1 band of chromosome 18. The integration site, between bases 4667 and 4668 of this BAC clone, is in a PB1D10 type repeat sequence (Figure 1A).

Demonstrating platelet-specific expression of hBDDFVIII. It has been shown that the human αIIb promoter confines transgene expression to the platelet lineage (35–37). Immunofluorescent microscopy was performed to confirm the expression of recombinant FVIII within platelets (Figure 2, A–F). FVIII protein was detected in the platelets of 2bF8trans mice and colocalized with mouse VWF (Figure 2F). EM further confirmed that FVIII is stored together with VWF in α-granules in the platelets of 2bF8trans mice (Figure 2, I–K). FVIII was absent from platelets of FVIII
null control mice, although a normal distribution of VWF was observed (Figure 2, A–C, G, and H). In contrast, FVIII was not detected in mononuclear cells from transgenic mice by confocal microscopy (Figure 2, L–Q). Similarly, FVIII activity (FVIII:C) was not detected in mononuclear cell lysates from either 2bF8trans WT, or FVIII
null mice (Figure 2R). In addition, platelet-specific expression was maintained after bone marrow transplantation (BMT) of FVIII
null mice with 2bF8trans bone marrow (see below).

Platelet localization of transgene expression and its augmentation by VWF. Functional FVIII:C was measured in mouse plasma and in platelet lysates. The FVIII:C detected in WT mouse plasma was 1.20 ± 0.02 U/ml when assayed against a recombinant hBDDFVIII (rhBDDF-VIII) standard. Plasma FVIII:C was undetectable in FVIII
null mice, as expected; however, it was also not detected in the plasma of 2bF8trans mice (Figure 3A). FVIII is normally not expressed in platelets. FVIII:C was functional when added to WT platelet lysate (Figure 3B), but was not detected in platelet lysate from either WT or FVIII
null control mice (Figure 2R and Figure 3C). However, functional FVIII:C was detected in platelet lysates from 2bF8trans mice at 0.74 ± 0.13 mU/108 platelets in heterozygous (2bF8g+/−) and 1.41 ± 0.25 mU/108 platelets in homozygous (2bF8g+/+) 2bF8trans mice (Figure 3C). Assuming the volume of plasma is 50% of whole blood and that the platelet count is 1 × 1012/ml, these amounts of platelet FVIII would correspond to approximately 1.23% and 2.35% of FVIII:C, respectively, in whole blood of normal mice. The detection limit of this assay was 0.07 mU/108 platelets. Alternatively, the stored FVIII could be measured following agonist-induced platelet activation (epinephrine, ADP, and the thrombin receptor activation peptide). The amount released, 0.61 ± 0.18 mU FVIII:C/108 platelets, was similar to the amount measured in 2bF8trans platelet lysate.

Since plasma VWF is required for sustaining normal plasma FVIII levels, we determined whether VWF was similarly required to sustain transgenic FVIII in platelets. The 2bF8 transgene was bred into VWF-deficient (VWFn/−) mice so that the 2bF8 transgene was expressed on a double-knockout background (FVIII
null/VWFn/−). The levels of platelet FVIII:C were found to be significantly decreased in 2bF8trans/VWFn/− mice compared with those of the respective 2bF8trans/VWF+/+ mice (2bF8g+/−:VWFn/−, 0.39 ± 0.18 mU/108 platelets; 2bF8g+/+:VWFn/−, 0.81 ± 0.22 mU/108 platelets; P < 0.001; Figure 3C). While platelet FVIII was expressed in the absence of VWF, the presence of the platelet VWF either enhanced storage or protected FVIII, resulting in higher levels of FVIII:C in platelets. These results demonstrate that FVIII transgene driven by the αIIb promoter is present in platelets, is functional, is enhanced by VWF, and is released following platelet activation, but does not result in measurable levels in mouse plasma.

Platelets expressing hBDDFVIII correct the bleeding phenotype of FVIII
null mice. Mice expressing the 2bF8 transgene in platelets but lacking endogenous mouse FVIII were tested for correction of the hemophilia A phenotype. To assess correction of the FVIII
null...
coagulation defect, we determined the ability to clot and survive after inducing a minor wound by tail clipping. All 18 2bF8 mice survived the tail clip test, while none of nine FVIII null mice were able to clot and survive tail clipping (Figure 3D).

In order to confirm that the benefit of 2bF8 expression resides within hematopoietic cells alone, bone marrow mononuclear cells from heterozygous 2bF8 trans mice was transplanted into lethally irradiated FVIII null mice. Three weeks after transplantation, the presence of the 2bF8 transgene in recipients was confirmed by PCR (Figure 1B). FVIII:C was not detectable in the plasma of recipients, but 0.72 ± 0.12 mU/10^8 platelets was measured in platelet lysates. There was no significant difference between the FVIII:C in platelet lysates of recipients and donors (P = 0.73; Figure 3C). Furthermore, sequential BMT into a second generation of recipients maintained similar levels of FVIII:C in their platelets as determined by platelet lysate FVIII:C assay, thereby demonstrating long-term reconstitution. All recipients (from 4 separate BMT experiments) survived tail clipping (Figure 3D). In the control group, none of 4 recipients that received bone marrow mononuclear cells from FVIII null mice had FVIII:C in their platelet lysate and none had phenotype correction.

To confirm that this beneficial effect is derived from platelets, we transfused different proportions of washed heterozygous 2bF8 trans platelets into FVIII null mice and then performed the tail clip survival test. All 7 FVIII null mice that were infused with 2bF8<sup>tg+/–</sup> platelets to achieve final levels of 30% of total platelets survived tail clipping, while 5 of 6 FVIII null mice infused with the same number of platelets from WT mice did not survive tail clipping. Infusion of FVIII null platelets had no effect on survival (Figure 3E). When lower doses (10–20%) of 2bF8<sup>tg+/–</sup> platelets were infused, some benefit was demonstrated. Of the 6 FVIII null mice infused with 2bF8<sup>tg+/–</sup> platelets to achieve final levels of 20%, 5 survived tail clipping, and 3 of 6 mice infused to 10% of total platelets survived. These data demonstrate that platelet FVIII is sufficient to correct the hemophilic A phenotype in mice. In previous studies from our laboratory, greater than 50% transduction efficiency can be demonstrated using lentivirally mediated gene therapy for platelet

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**Figure 2**
Platelet-specific expression of FVIII. (A–F) Localization of transgene protein expression was determined by immunofluorescent microscopy. Isolated platelets from FVIII<sup>null</sup> (A–C) and 2bF8<sup>trans</sup> mice (D–F) were immunostained for either human FVIII (hFVIII; A and D) or mouse VWF (B and E). The 2 images were merged in C and F, showing that in the platelets of 2bF8<sup>trans</sup> mice (F) VWF and FVIII were colocalized (yellow). (G–K) Colocalization of human FVIII and mouse VWF was confirmed by electron microscopy. Isolated platelets from FVIII<sup>null</sup> mice (G and H) and 2bF8<sup>trans</sup> mice (I–K) were immunostained for human and mouse VWF. FVIII was probed with 5 nm colloidal gold and VWF with 10 nm colloidal gold; representative gold particles are indicated by arrows and arrowheads, respectively. The results show FVIII was stored together with VWF in platelet α-granules of 2bF8<sup>trans</sup> mice (I–K). (L–Q) Confocal microscopy detected no human FVIII in mononuclear cells from transgenic mice. Isolated platelets and mononuclear cells from 2bF8<sup>trans</sup> mice were immunostained for human FVIII. Nonspecific isotype-matched primary Ab was used for background staining. (R) Quantitative evaluation of FVIII:C levels in mouse mononuclear cell lysates. Platelets and mononuclear cells were isolated and lysed in 0.5% CHAPS. FVIII:C in lysates was determined by chromogenic assay. No FVIII:C was detected in mononuclear cell lysates from either 2bF8<sup>trans</sup> or control mice (i.e., WT and FVIII<sup>null</sup> mice). FVIII:C was detected in platelet lysates from 2bF8<sup>trans</sup> mice. Scale bars: 8 μm (A–F and L–Q); 0.2 μm (G–K).
GP deficiencies (GPIIIa, ref. 37, or GPIbα, ref. 38). Thus achieving transgene expression in 30% of platelets would be obtainable through gene therapy of autologous hematopoietic stem cells.

Correction of hemophilic phenotype in FVIIInull mice occurs even in the presence of high-titer FVIII inhibitors. To explore whether platelet-derived FVIII can maintain therapeutic effectiveness in the presence of FVIII inhibitors, we used 3 strategies: (a) an acute model infusing plasma from highly immunized FVIIInull mice; (b) a chronic model using transplantation of spleen cells from immunized FVIIItrans mice; and (c) active immunization of 2bF8trans mice with rhBDDFVIII with adjuvant. The inhibitor titer was determined by a modified Bethesda assay (39) and expressed in Bethesda units (BU). Plasma inhibitor titers after infusion studies were significantly different from preimmunization levels (data not shown). All 6 immunized 2bF8trans mice survived tail clipping, and 7 of 8 mice infused to 250 BU/ml survived tail clipping (Figure 4D).

In order to determine the effect of inhibitory antibodies in 2bF8trans mice, the transgenic mice were administered plasma from immunized FVIIInull mice. Immunization of FVIIInull mice produced inhibitor titers as high as 100,000 BU/ml after 3 immunization doses of rhBDDFVIII administered with adjuvant by i.p. injection. All 16 2bF8trans mice infused to a final concentration of either 2.5 or 25 BU/ml with inhibitory plasma from immunized FVIIInull mice survived tail clipping, and 7 of 8 mice infused to 250 BU/ml survived tail clipping (Figure 4B).

In spleen cell transplantation experiments, all mice developed high titers of inhibitory antibody following reconstitution. In the presence of 5,000–50,000 BU/ml, 5 of 7 transgenic mice survived tail clipping, and 3 of 5 transgenic mice survived tail clipping in spite of an inhibitor titer greater than 50,000 BU/ml (Figure 4C). The platelet count in recipients following reconstitution (0.74 ± 0.20 × 10^9/ml; n = 8) was not significantly different from the pretransplantation platelet count (0.69 ± 0.20 × 10^9/ml; P = 0.62), despite an inhibitor titer greater than 50,000 BU/ml. This implies that circulating FVIII inhibitor antibodies in plasma do not interfere with normal platelet production or survival in these mice.

FVIII-C was not detected in the plasma of WT mice receiving spleen cells from immunized FVIIItrans mice, although their plasma FVIII-C was normal before receiving immunized spleen cells, and only 2 of 7 survived tail clipping with a titer that most (70%) transgenic mice can overcome (Figure 4C).

We followed platelet counts in 2 recipients for 6 months, and the platelet counts were not decreased, yet their inhibitor titers remained greater than 50,000 BU/ml. This implies that circulating FVIII inhibitor antibodies in plasma do not interfere with normal platelet production or survival in these mice.

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We thus demonstrated that expression of FVIII under control of the αIIb promoter maintained efficacy in hemophilia A even in the presence of high-titer inhibitors elicited through 3 different experimental approaches.

Discussion

While gene therapy for hemophilia A has been attempted in humans, therapeutic levels of FVIII have not yet been achieved (7, 17). This failure may be attributed in part to the fact that none of these approaches directed FVIII synthesis to cells that synthesize and store FVIII. What fraction of platelets optimally express FVIII? Our therapeutic approach did not provide detectable plasma FVIII. Three experiments taken together demonstrated that this therapeutic efficacy was restricted to platelet expression of FVIII: (a) confocal immunofluorescent microscopy of peripheral blood using antibodies to human FVIII demonstrated only platelet staining; (b) BMT from 2bF8trans mice into lethally irradiated FVIIInull recipients demonstrated maintenance of phenotypic correction with solely hematopoietic expression; and (c) transfusion of platelets from 2bF8trans mice into FVIIInull mice resulted in correction of the hemorrhagic phenotype, even when only a fraction of the circulating platelets contained FVIII. What fraction of platelets optimally would need to be transduced, and what is the optimal level of FVIII expression per platelet, are questions not yet answered.

While VWF is required for normal plasma levels of FVIII (20, 40), we wanted to determine its contribution to transgenic expression of FVIII in platelets. VWF storage is VWF-dependent in AtT-20 cells (4) and endothelial cells (21, 45). VWF colocalizes with FVIII when FVIII synthesis is induced in megakaryocytes (22, 23). These approaches directed FVIII synthesis to cells that synthesize and store FVIII, and thus gene therapy by constitutive synthesis and release of recombinant FVIII into plasma (i.e., specifying gene therapy to liver or endothelial cells) might not be therapeutic in these patients in the presence of such inhibitory antibodies.

We therefore wanted to develop a strategy whereby expressed FVIII is protected from these inactivating antibodies until FVIII is needed to achieve local hemostasis. Such an approach could include patients with FVIII inhibitors as candidates for gene therapy and might be a specific means of long-term treatment of these patients even in the presence of these inhibitors.

We show here that megakaryocytic expression of FVIII was achieved in a transgenic model in which FVIII production is driven by the platelet αIIb promoter. When transgenic 2bF8 platelet FVIII expression was bred into FVIIInull mice, platelet FVIII was maintained in these mice, yet plasma FVIII remained undetectable. Hemostatic efficacy was demonstrated by normalization of survival following tail clipping in 2bF8trans mice, in marked contrast to the uniform fatality observed in FVIIInull mice. This means that FVIII delivered by the 2bF8trans platelets is provided to sites where it is bioavailable to achieve local hemostatic efficacy.

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We therefore wanted to develop a strategy whereby expressed FVIII is protected from these inactivating antibodies until FVIII is needed to achieve local hemostasis. Such an approach could include patients with FVIII inhibitors as candidates for gene therapy and might be a specific means of long-term treatment of these patients even in the presence of these inhibitors.
The insertion position of the 2bF8 transgene.

- null
- trans

expression cassette for FVIII driven by a platelet-specific pro
from hemophilia A patients, transduced or transfected with an
studies are the subject of current investigation. Thus, bone mar
inhibitory antibodies were produced, platelet-expressed FVIII
stem cells can potentially provide a cure for many inherited
tial target for gene therapy. Gene transfer into hematopoietic
ferentiate into all blood lineages, they are an attractive poten
abrogating the benefit of 0.02 U/ml plasma FVIII infusion.
monstrated in Figure 4A, 100% of FVIII
48) who also have major life-threatening clinical bleeding. As dem
This strategy has the potential to offer therapeutic benefit both
ples using primers as described above.

**Table 1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>P1</td>
<td>ah83 391–365</td>
<td>5′-AGGCCCTCAGAAGCTTTCCAGTAGGA-3′</td>
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<td>P5</td>
<td>C13 3311–3392</td>
<td>5′-GAATGATAGCGGATGAAGTC-3′</td>
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<tr>
<td>M18-a7473</td>
<td>a4743–4723 mouse BAC</td>
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<td>s4536–4557 mouse BAC</td>
<td>5′-GCTGTAGGCTATCAATGAG-3′</td>
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by 27% when bred into the VWF/– background compared with a
WT background. Our studies confirmed that the presence of VWF
(VWF/– versus VWF/+) increased platelet FVIII:C levels with both 2bF8/– and 2bF8+/– mice. In the absence of platelet VWF (VWF/–
mice), ectopic platelet FVIII expression and storage persisted, but
platelet FVIII levels were only 52.7% and 57.4% of that of the levels in
2bF8/– and 2bF8+/– mice, respectively, in the presence of VWF
(Figure 3C). This implies that in platelets coexpression with VWF
is important for optimal FVIII synthesis and/or storage. In con
trast to endothelial cells, where Weibel-Palade bodies are not pres
ent in the absence of VWF (45), platelet α-granules still formed in
the absence of VWF and accounted for the continued, but subopt
imal, storage of FVIII. Furthermore, having 2 chromosome 18s
each with the same transgenic insert resulted in twice the amount
of FVIII synthesized and stored in transgenic platelets. We did not
find any difference in the specific activity of the FVIII expressed in
the presence or absence of VWF (data not shown).

While the phenotypic correction of the hemorrhagic pheno
type through ectopic expression of platelet FVIII in is itself an
important observation, it is the success of this approach even in
the presence of high-titer inhibitory antibodies that is most com
pelling. Even the development of an inhibitor titer of 2–5 BU/ml
was determined by DNA Walking PCR using DNA Walking SpeedUp kit
was generated in the Transgenic Core Facility of the Medical College of
Wisconsin and Blood Research Institute. A 7.6-kb fragment, containing
the tIb promoter, β-globin/IgG chimeric intron, hBDDFVIII, SV40 Poly A,
and a neomycin resistance cassette, was electroporated into 129S ES cells.
Colonies were selected using G418 and screened for the presence of the
transgene using the PCR strategy shown in Figure 1A and detailed in
Table 1 using primers P1–P4 specific for the 2bF8 transgene. Transgene-positive
ES cells were propagated for microinjection into blastocysts to generate
chimeric mice carrying the transgene, and 2bF8 transgene was then bred
into the exon 17 FVIII knockout background (51). Genotype determina
was performed by PCR analysis of blood-derived genomic DNA sam
ples using primers as described above.

**Insertion location analysis.** The insertion position of the 2bF8 transgene
was determined by DNA Walking PCR using DNA Walking SpeedUp kit (Seegene) per the manufactuer’s instructions. A BLAST search of Gene
Bank identified the insertion site on chromosome 18 after base 4667 of
(Table 1), flanking the putative insertion site were designed to confirm
disruption of the insertion locus in transgenic positive animals.

**Generation of transgenic mice.** Studies were approved by the Animal Care
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**Methods**

**Construction of vector.** The human FVIII cDNA used in this study has the
entire FVIII B-domain deleted and was a kind gift of R.J. Kaufman (Univer
sity of Michigan, Ann Arbor, Michigan, USA). hBDDFVIII was excised from
the pMT2 vector (50) by XhoI and SalI and used to create pIIIb-BDDFVIIIneo
vector as described in our previous study (22). Pml and Korf sites were introduced at the beginning of the tIb promoter in pIIIb-BDDFVIIIneo by PCR
mutagenesis. The resulting vector, pIIIb-BDDFVIIIneo (Pml), was used to
release the 2bF8 expression cassette for generating transgenic mice.

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disruption of the insertion locus in transgenic positive animals.

**Generation of 2bF8 homozygous and 2bF8trans-VWFnull mice.** Homozygous
2bF8trans mice were generated from heterozygous mating. Genotype was
determined by PCR using primer P5 or primers M18-a7473 and M18-s4536
(Table 1 and Figure 1). Furthermore, mating 2bF8trans mice with VWFnull
mice (20) generated 2bF8trans-VWFnull mice. VWFnull mice were identified by
means of a solid phase capture ELISA of mouse plasma, using rabbit anti
human VWF that crossreacts with murine VWF (Dako) as a capture Ab and
HRP-labeled VWF Ab for detection.

**FVIII:C assay.** FVIII:C levels in mouse plasma and platelet lysate or
releasate were quantitated by a modified FVIII chromogenic assay that we
have developed using the Coatest VIII:C/4 Kit (DiaPharm) as previously
described (21, 23). One hundred microliters of blood was collected by tail
bleeding in tubes containing 0.1 vol of 0.1 M sodium citrate. Cells were
removed by centrifugation at 960 g for 20 minutes, and the platelet poor
plasma was tested for plasma FVIII:C.

For platelet lysate or releasate assay, platelets were separated using Fico/
Lite-Platelets (Atlanta Biologicals) from 200 μl blood with 50 ng/ml of

moter, and refueled into the patient to provide long-term benefit. This could provide that patient with a self-replicating pool of stem cells that would support lifelong FVIII transgene expression in their megakaryocytes and platelets. A protected releasable
FVIII pool in platelets would reduce the bleeding diathesis not in patients with hemophilia A but also in those with hemophilia A and FVIII
inhibitory antibodies. While the latter group has not previously been thought to be candidates for FVIII gene therapy, our present studies suggest a
new approach that could be beneficial.


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prostaglandin E1 (Sigma-Aldrich). Separated platelets were washed with PBS containing 2 mM EDTA (Sigma-Aldrich) and 0.5% BSA (Invitrogen), counted with an Animal Blood Counter (Heska), and centrifuged at 960 g for 20 minutes. The platelet pellet was lysed in 200 μl of 0.5% 3-[3-chol amidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS, the zwitterionic detergent; MP Biomedicals) by vortex until suspended, incubated on ice for 10 minutes, and spun down at 20,800 g at 4°C for 10 minutes. The supernatant was then discarded for FVIII:C.

For the platelet release assay, fresh platelets were resuspended in 150 μl modified Tyrode’s buffer (20 mM HEPES, 137 mM NaCl, 13.8 mM NaHCO3, 0.36 mM NaH2PO4, 2.5 mM KCl, 1 mM MgCl2, 5.5 mM glucose, and 0.25% BSA) containing 1 mM CaCl2, 2 μM ADP (Chrono-log Corporation), and 25 μM each of epinephrine (BIO/DATA Corporation) and the murine thrombin receptor activation peptide (GYPGK-NH2, synthesized by our core laboratory), incubated at room temperature for 30 minutes, and then centrifuged at 20,800 g at 4°C for 10 minutes. The resulting supernatant was used for the assay of released FVIII:C.

Mouse plasma was diluted at 1:40 in modified Tyrode’s buffer, and 25 μl of diluted plasma, platelet releasate, or serially diluted platelet lysate were added to blocked 96-well microtiter plates in triplicate. Assay components, including FⅧa, FⅨa, CaCl2, and phospholipid were added to each well, and the plates were incubated for 10 minutes at 37°C. The chromogenic Factor Xa substrate S-2222 was added, and the plate was transferred immediately to a ThermoMax microplate reader (Molecular Devices) preset at 37°C. A standard curve was constructed by plotting known amounts of rhBDDFVIII (Refacto; Wyeth) in the appropriate buffer against Vmax (mOD/min) at 405 nm. The Vmax of each reaction was converted to units of FVIII:C (U/ml) using the instrument manufacturer’s software program (SoftMax version 2.34; Molecular Devices), and the data were averaged. Platelets from WT and FVIII–null mice served as controls in the platelet releasate or lysate assays. FVIII:C in mononuclear cell lysate was similarly determined.

Immunostaining for hBDDFVIII. Visual detection of hBDDFVIII in transgenic platelets was accomplished with immunofluorescent- or immunogold-labeled antibody and confocal laser scanning microscopy or EM. Controls (platelets from FVIII–null mice) were processed in parallel with each immunostaining assay under the same conditions.

For confocal studies, cells were spun onto glass slides, fixed using 3.7% (vol/vol) buffered formalin, permeabilized with 5% Triton X-100, and blocked in 2.5% normal goat serum in HBSS (Invitrogen) for 1 hour. Cells were incubated in primary antibody at 4°C overnight. Rabbit polyclonal antibody against human FVIII (Conan; generated by our laboratory) was directly conjugated to AlexaFluor 488 (Invitrogen) and rabbit antibody to VWF (Dako) was conjugated to AlexaFluor 568. Nonspecific isotype control antibodies served as negative controls. Cells were mounted under glass coverslips with Vectashield. Immunofluorescent detection was performed by confocal microscopy using a Leica TCS SP2 Confocal Laser Imaging System (Leica Microsystems).

For EM studies, isolated platelets were washed in modified Tyrode’s buffer and centrifuged at 960 g for 20 minutes at room temperature. The pellet was fixed in 0.25% glutaraldehyde plus 4% paraformaldehyde and 0.2% (wt/vol) picric acid in 0.1 M phosphate buffer, pH 7.3, on ice for 1 hour. The fixed cell pellets were processed using the enhanced membrane contrast technique (52) and embedded in Lowicryl K4M resin using a previously reported protocol (53). Ultrathin sections (60 nm thick) were collected on formvar/carbon-coated copper grids. All incubations were carried out on 25-μl droplets on parafilm in a humidified chamber. Sections were incubated with anti-Factor VIII mAb, 103.3, or anti-VWF polyclonal Ab (Dako) and then probed with goat anti-mouse (5 nm) and goat anti-rabbit (10 nm) colloidal gold probes, respectively. The sections were observed in a Hitachi H600 TEM (Hitachi High-Technologies) operating at 75 kV.

**Phenotyping correction analysis.** Phenotypic correction was assessed by the tail clip survival test as previously described (54–56). The tails of 8- to 16-week-old anesthetized mice were clipped at a diameter of 1.59 mm, without subsequent cautery. Clot formation and survival beyond 24 hours was used to indicate correction of the murine hemophilia A phenotype.

2bF8trans BMT. Anesthetized mice were sacrificed by cervical dislocation, and bone marrow was harvested by flushing the femurs and tibias as previously described (23). Mononuclear cells were isolated with Fico/Lite-LM (mouse; Atlanta Biologicals), washed with cold PBS containing EDTA and BSA, and resuspended in PBS (Invitrogen) for transplantation.

Six- to 8-week-old FVIII–null (recipients) were conditioned for cellular transplantation with a lethal dose of 1.100 cGy total body irradiation using a cesium irradiator (23). Twenty-four hours after irradiation, a cell dose of 8–10 x 10^7 cells from heterozygous 2bF8trans mice in a volume of 200–300 μl/mouse was infused by retroorbital injection. The recipients were analyzed after allowing 3 weeks for bone marrow reconstitution. At 4 months, a sequential BMT was performed from these transplanted mice into additional irradiated FVIII–null mice. As a control, bone marrow mononuclear cells from FVIII–null mice were transplanted into lethally irradiated littermates with the same cell dose.

**Evaluation of the efficacy of plateau-derived FVIII in the presence of anti-FVIII inhibitory antibodies.** FVIII–null mice were immunized with 200 μl of rhBDDFVIII at 600 U/kg in the presence of adjuvant (Corixa) by i.p. injection to induce anti-human FVIII antibodies. To determine the titer of inhibitors, a modified Bethesda assay was performed. Sequential dilution of immunized mouse plasma was incubated with an equal volume of 1 U/ml rhBDDFVIII at 37°C for 2 hours, and residual FVIII:C was subsequently analyzed by chromogenic assay. BU were defined by dilution of the blood plasma until 50% of the initial FVIII was neutralized (39).

In the *acute model*, mouse plasma from immunized FVIII–null mice containing FVIII inhibitory antibodies was infused into heterozygous 2bF8trans mice by retroorbital injection. One hour after infusion, blood was collected and plasma was assayed for inhibitors. The tail clip survival test was performed to determine whether the platelet-derived FVIII could still be therapeutically effective in the presence of inhibitors. Controls, FVIII–null mice that had been preinfected with mouse inhibitor plasma or control plasma were infused with 0.02 U/ml rhBDDFVIII, and tail clip was performed.

In the *chronic model*, spleen cells from immunized FVIII–null mice were transplanted into sublethally irradiated heterozygous 2bF8trans mice. Splenocytes from immunized FVIII–null mice were ground up, and red cells were lysed with Red Cell Lysing buffer (Sigma-Aldrich). Cells were washed and resuspended in PBS (Invitrogen) at 1 x 10^8 cells/ml for transplantation. Eight- to 10-week-old 2bF8trans mice were conditioned for cellular transplantation with a sublethal dose of 400 cGy total body irradiation. Twenty-four hours after irradiation, a cell dose of 2–3 x 10^7 cells/ml was infused into conditioned WT mice that contained normal mouse FVIII. Two weeks after transplantation, blood was collected, and cell number was counted with an Animal Blood Counter. Plasma was tested by inhibitor analysis, and the tail clip survival test was performed to determine phenotypic correction.

A second *chronic model* was developed and involved immunizing heterozygous 2bF8trans mice with rhBDDFVIII at 600 U/kg given with adjuvant by i.p. injection. Two weeks after immunization, blood was collected and cell number was determined with the Animal Blood Counter. Plasma was used for inhibitor analysis, and platelets were used for platelet lysate FVIII:C assay. The tail clip survival test was performed to determine phenotypic correction.

**Statistics.** All FVIII:C results are presented as mean ± SD, and the significance of differences was evaluated by 2-tailed Student’s t test. A value of *P* < 0.05 was considered statistically significant.
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FVIII inhibitors in children. 

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