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Factor VIII delivered by hematopoietic stem cell-derived B cells corrects the phenotype of hemophilia A mice

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Summary

The main impediments to clinical application of hematopoietic stem cell (HSC) gene therapy for treatment of hemophilia A are the bone marrow transplant-related risks and the potential for insertional mutagenesis caused by retroviral vectors. To circumvent these limitations, we have adapted a nonmyeloablative conditioning regimen and directed factor VIII (FVIII) protein synthesis to B lineage cells using an insulated lentiviral vector containing an immunoglobulin heavy chain enhancer-promoter. Transplantation of lentiviral vector-modified HSCs resulted in therapeutic levels of FVIII in the circulation of all transplanted mice for the duration of the study (6 months). Immunostaining of spleen cells showed that the majority of FVIII was synthesized by B220⁺ B cells and CD138⁺ plasma cells. Subsequent challenge with recombinant FVIII elicited at most a minor anti-FVIII antibody response, demonstrating induction of immune hyporesponsiveness. All transplant recipients exhibited clot formation and survived tail clipping, indicating correction of their hemophilic phenotype. Therapeutic levels of FVIII could be transferred to secondary recipients by bone marrow transplantation, confirming gene transfer into long-term repopulating HSCs. Moreover, short-term therapeutic FVIII levels could also be achieved in secondary recipients by adoptive transfer of HSC-derived splenic B cells. Our findings support pursuit of B cell-directed protein delivery as a potential clinical approach to treat hemophilia A and other disorders correctable by systemically distributed proteins.

Keywords

Hemophilia A; animal models; hematopoietic stem cell gene therapy; B cell-targeted transgene expression; bioengineered factor VIII

Introduction

Hemophilia A is a severe congenital bleeding disorder caused by deficiency in coagulation factor VIII (FVIII) (1). The disease is characterized by easy bruising and repeated bleedings into joints and muscles leading to chronic disabling arthropathy, although potentially fatal intracranial hemorrhage or retroperitoneal hematoma following trauma or surgery may also occur. There is currently no cure for hemophilia A and the mainstay of treatment is FVIII replacement therapy by infusion of plasma-derived or recombinant FVIII at the time of bleeding (2, 3). In addition to being inconvenient and expensive, a serious problem

associated with current protein replacement therapies is the development of inhibitory antibodies to FVIII (FVIII inhibitors) in ~25% of severe hemophiliacs, rendering the patients unresponsive to further FVIII protein infusions (4).

Hemophilia A remains an attractive candidate for somatic cell gene therapy as systemic distribution of FVIII secreted from engrafted genetically-modified cells may correct the bleeding disorder (5-7). However, the clinical success of hemophilia A gene therapy requires overcoming a number of obstacles, necessitating development of a strategy that will achieve stable therapeutic FVIII expression and induce immune tolerance to the FVIII neoantigen while minimizing the risks of vector insertional mutagenesis or transplant-related complications (8-11). We recently reported correction of murine hemophilia A following nonmyeloablative transplantation of hematopoietic stem cells (HSCs) engineered to express an enhanced human FVIII variant (eFVIII)—containing a combination of A1 domain point mutations (L303E/F309S) and an extended partial B domain for improved secretion (12) plus A2 domain mutations (R484A/R489A/P492A) for reduced immunogenicity (13)—using a safety-augmented retroviral vector (11). In this study, we report the use of an insulated simian immunodeficiency virus (SIV)-derived lentiviral vector as a safer alternative to gammaretroviral vectors (10, 14, 15), and the transcriptional targeting of eFVIII expression to B lymphocytes.

While resting B cells synthesize small amounts of cell-surface immunoglobulin (Ig) receptors, upon antigen stimulation, the activated B cells increase their secretory apparatus to accommodate synthesis of thousands of Ig molecules per second, which are efficiently assembled and secreted from their terminally differentiated plasma cell progeny (16). Each day an average adult produces approximately three grams of antibodies. Utilization of the plasma cell high efficiency protein synthetic machinery may allow production of sufficient amounts of transgenic proteins to engender a therapeutic effect following delivery of small numbers of genetically-modified HSCs via a “mini-transplant” procedure (17). Other studies have demonstrated that tolerance to FVIII inhibitors could be induced by treatment with B cells expressing the A2 or C2 domains of FVIII (18, 19). We hypothesized, therefore, that by directing eFVIII transgene expression to plasma cells we could employ their highly specialized secretory apparatus towards systemic delivery of therapeutic and perhaps also tolerogenic FVIII (20, 21). Moreover, incorporation of enhancer-promoter sequences that are inactive in HSCs in combination with the insulated lentiviral vector backbone should reduce the potential for insertional oncogenic transformation of these susceptible target cells (10, 22-26).

Materials and methods

Vector construction

The self-inactivating (SIN) simian immunodeficiency virus (SIV_{mac1A11})-derived lentiviral vector FB.SIV-MSCV-GFP, and the eFVIII transgene have been described previously (10, 11). The FB.SIV-E μ P μ -GFP vector was generated by removing the internal MSCV LTR enhancer-promoter from the FB.SIV-MSCV-GFP vector and replacing it with the mouse Ig heavy chain enhancer (E μ) and its flanking matrix attachment regions (992-bp *Xba*I fragment from pRSP-6 plasmid) linked to the mouse Ig heavy chain promoter (P μ) (600-bp *Bam*HI fragment from pH16 plasmid) using blunt-end ligations (27-29). The FB.SIV-MSCV-eFVIII and FB.SIV-E μ P μ -eFVIII vectors were generated by first cloning the 5.1-kb *Nco*I/*Not*I fragment containing the eFVIII cDNA from pRL-eFVIII into *Sac*II/*Not*I-digested FB.SIV-MSCV-GFP and FB.SIV-E μ P μ -GFP vectors, respectively (*Nco*I and *Sac*II ends were blunted), replacing the green fluorescent protein (GFP) coding sequence. Subsequently, a 1.8-kb *Xho*I/*Xho*I fragment containing the PGK-GFP expression cassette from the MSCV-GW vector (30) was cloned downstream of eFVIII at the *Not*I site (blunt-

end ligation). All vectors contain the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) for increased vector RNA 3' end formation.

Cell lines

Human 293T/17 embryonic kidney cells (American Type Culture Collection, Manassas, VA, USA; No. CRL-11 268) were grown in Dulbecco's modified Eagle medium (Mediatech, Manassas, VA, USA) supplemented with 4.5 g/L glucose, 4 mM L-glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin and 10% heat-inactivated fetal bovine serum (FBS; Cambrex BioScience Walkersville, Walkersville, MD, USA). Mouse Sp2/0-Ag14 B cell hybridoma cells (ATCC No. CRL-1581), human Jurkat T cell acute lymphoblastic leukemia cells (ATCC No. TIB-152), human HL-60 promyelocytic leukemia cells (ATCC No. CCL-240) and human K-562 erythroleukemia cells (ATCC No. CCL-243) were cultured in Iscove's modified Dulbecco's medium (Mediatech) plus 10% FBS, 4 mM L-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin. All cells were maintained at 37°C in a humidified incubator containing 5% CO₂.

FVIII-deficient mice

Animals used in this study were derived from FVIII exon 16-disrupted hemophilia A mice (B6;129S4-*F8^{tm1Kaz}*) (31) that were backcrossed to the BALB/c strain for more than 10 generations (32). Breeding pairs of these BALB/c-congenic C.129S4(B6)-*F8^{tm1Kaz}* mice were generously provided by David Lillicrap (Queen's University, Kingston, ON, Canada). Male or female mice aged 8 to 12 weeks were used as bone marrow (BM) transplant donors and recipients. All animal procedures were carried out in accordance with our Institutional Animal Care and Use Committee guidelines.

Production of lentiviral vector particles

Vesicular stomatitis virus (VSV)-G pseudotyped SIV vector particles were generated by transient transfection of 293T/17 cells with vector and packaging (pCAG-SIVgprre and pCAG4-RTR-SIV) plasmids (provided by Arthur Nienhuis, St. Jude Children's Research Hospital, Memphis, TN, USA) plus the VSV-G envelope plasmid pMD.G as described previously (8, 10, 33). To determine vector titers, an aliquot of each vector preparation was thawed and serial dilutions added in the presence of 6 µg/ml polybrene (Sigma-Aldrich, St Louis, MO, USA) to 2×10^5 NIH3T3 or Sp2/0 cells which had been seeded in 12-well plates 8 hours earlier. Fresh medium was added after 4 hours of transduction, and 72 hours later the relative end-point vector titers were determined by flow cytometric analysis of GFP fluorescence using a FACSCalibur analyzer (BD Biosciences, San Jose, CA, USA) as described previously (8, 33, 34). When required, high-titer vector stocks were prepared by ultracentrifugation (45,000g, 90 min).

Transduction of cell lines with lentiviral vector particles

The various hematopoietic cell lines were stably transduced by spinoculation at a multiplicity of infection (MOI) of 5, cultured for 10 days, sorted on a FACSaria instrument (BD Biosciences) where indicated, and analyzed for GFP expression as described (8, 11, 33).

Isolation, transduction and transplantation of Stem Cell Antigen-1⁺ (Sca-1⁺) HSCs

BM cells were harvested from hemophilia A mice at 6 to 8 weeks of age and used to enrich for Sca-1⁺ HSCs (35). Sca-1⁺ cell isolation was performed by positive immunomagnetic bead selection according to a protocol provided by the manufacturer (Anti-Sca-1 MicroBead Kit; Miltenyi Biotec, Auburn, CA, USA). BM processing, transduction, and GFP expression analysis were carried out as described in detail previously (10, 11, 36). Sca-1⁺ cells were

cytokine prestimulated for two days and stably transduced by spinoculation at an MOI of 5 for 2 consecutive days. Recipient mice received a nonmyeloablative chemotherapy conditioning regimen with busulfan (BU; MP Biomedicals, Solon, OH, USA) plus transient immunosuppression by anti (mouse)-thymocyte serum (ATS) treatment (Inter-Cell Technologies, Jupiter, FL, USA), as described previously (11, 35). ATS is analogous to anti-human thymocyte globulin which is used clinically to enhance transplantation tolerance following allogeneic BM and organ transplantation. Conditioned mice received 1×10^5 transduced Sca-1⁺ cells via tail vein injection on day 0. Transduction and engraftment efficiencies were analyzed by flow cytometry (for GFP expression) and Southern blot analysis (using a GFP-specific probe). Representative mice were killed at 24 weeks after transplantation and their BM cells (2×10^7 cells) were injected into 2-3 lethally irradiated (800 cGy) secondary transplant recipients each. Both primary and secondary recipients were evaluated for phenotypic correction by tail clipping at 24 weeks after primary transplantation and 16 weeks after secondary transplantation, respectively (11, 36).

Adoptive transfer of B cells

B cell transfers were performed by tail vein injection of 1×10^7 spleen cells from primary donors into sublethally irradiated (550 cG), nonmyeloablative (BU-ATS-), bortezomib- (LC Laboratories, Woburn, MA, USA), or bortezomib-ATS- conditioned recipient mice (37, 38). Bortezomib was administered i.p. at 0.75 mg/kg on days -2 and -1.

FVIII analysis

Peripheral blood was obtained from the retroorbital plexus in 1/10 volume of 0.1 M sodium citrate (2.94%) and centrifuged at 2,000g for 10 min, the plasma was frozen on dry ice immediately and then stored at -80°C for future analysis. The red blood cells were then lysed and the remaining nucleated cells were analyzed for expression of GFP by flow cytometry (11, 36, 39). FVIII antigen levels in murine plasma were measured as described previously (11, 39) using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cedarlane Labs, Burlington, ON, Canada). Factor VIII activity was determined as described previously (11, 36, 39) by a chromogenic assay (COATEST VIII:C/4; DiaPharma Group). All samples were measured in triplicate and the means calculated. Standard curves were generated by diluting reconstituted, normal pooled human plasma (Calibration plasma; DiaPharma Group, West Chester, OH, USA). FVIII immunizations and total anti-FVIII IgG levels were determined by ELISA as described previously (11, 36, 39). Intracellular staining for FVIII by flow cytometry was performed using the Cytofix/Cytoperm kit according to the manufacturer's suggestions (BD Biosciences) with a biotinylated mouse anti-human FVIII primary antibody (Cedarlane Labs, Burlington, ON, Canada) in combination with cell surface staining using APC-conjugated rat anti-mouse CD138 (Syndecan-1; clone 281-2; BD Pharmingen, San Diego, CA) and CD45R (B220; clone RA3-6B2, eBioscience, San Diego, CA, USA) antibodies.

Immunophenotyping of peripheral blood cells expressing GFP

Peripheral blood was collected from FB.SIV-MSCV-GFP and FB.SIV-E μ P μ -GFP vector-treated and control mice and used for immunophenotyping as described previously (39), using allophycocyanin (APC) anti-mouse Ly-6G (Gr-1; clone RB6-8C5), CD45R (B220; clone RA3-6B2), CD90.2 (Thy1.2; clone 53-2.1), and CD11b (Mac-1A; clone M1/70). Corresponding APC-conjugated rat IgG_{2a,k} or IgG_{2b,k} isotype controls were included in each analysis (all antibodies were purchased from eBioscience).

Nucleic acid analysis

Southern blot analysis was performed as described previously (33, 36). Briefly, genomic DNA was extracted using the GenElute Mammalian Genomic DNA Kit (Sigma-Aldrich). Genomic DNA (10 µg) was digested with *EcoRI* and *SfiI* (New England Biolabs, Beverly, MA, USA), separated through 0.8% agarose gels, and transferred to Hybond-N⁺ membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were fixed by exposure to UV light and hybridized with a ³²P-labeled randomly primed 1.4-kb *EcoRI/SfiI* fragment of FB.SIV-MSCV-GFP which was used as a GFP-specific probe. Digital images were acquired using a Storm 860 PhosphorImager and radioactivity was quantitated using ImageQuant software (Amersham Biosciences). As a control for equal loading of DNA, each blot was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

Statistical analysis

Data from experiments are expressed as mean values ± SD. The Student's paired *t* test was used to compare differences between the indicated groups. *P* < 0.05 was considered significant.

Results

Generation of an EµPµ enhancer-promoter-containing lentiviral vector and evaluation of B cell-specific transgene expression *in vitro*

The gene therapy vectors used in this study were derived from the previously described SIN FB.SIV-MSCV-GFP lentiviral vector containing a GFP transgene, which is a safety-augmented derivative harboring a novel 77-bp enhancer-blocking element in its deleted 3' U3 region (designated FB for FII/BEAD-A) (10). In an attempt to develop a B cell-restricted vector, the constitutively active MSCV LTR (murine stem cell virus long terminal repeat) enhancer-promoter driving GFP expression was replaced with B lineage-specific transcriptional regulatory elements from the mouse Ig heavy chain enhancer (Eµ) linked to the Ig heavy chain promoter (Pµ) (27, 28). In addition, the matrix attachment regions, which flank the Eµ sequences in the Ig heavy chain locus, were included for increased probability of persistent, position-independent B lineage-specific transgene expression (Figure 1A) (29).

B cell lineage restriction was initially evaluated in a series of hematopoietic cell lines—B-lymphoid (Sp2/0), T-lymphoid (Jurkat), myeloid (HL-60) and erythroid (K-562)—using GFP as reporter. In order to ensure comparable vector copy numbers, the cell lines were transduced at similar MOI. As seen in Figure 1B, while all cell lines tested expressed high levels of GFP when transduced with the ubiquitously expressed FB.SIV-MSCV-GFP vector, only the Sp2/0 B cell line expressed significant levels of GFP from the FB.SIV-EµPµ-GFP vector, documenting selective permissivity of the EµPµ enhancer-promoter for the B cell lineage.

The EµPµ enhancer-promoter directs B cell-specific transgene expression *in vivo*

To evaluate activity of the FB.SIV-EµPµ-GFP vector in HSCs and early hematopoietic precursors, Sca-1⁺ BM cells were isolated and transduced with FB.SIV-EµPµ-GFP and FB.SIV-MSCV-GFP vectors. Although Southern blot analysis demonstrated similar copy numbers, no GFP expression could be detected in the *ex vivo* expanded FB.SIV-EµPµ-GFP-transduced cell cultures whereas nearly 60% of cells expressed the GFP marker from the FB.SIV-MSCV-GFP vector after short-term (1 week) culture (Figure 2A).

The *in vivo* lineage specificity of the FB.SIV-EµPµ-GFP vector was next examined by transplanting 1×10^5 transduced Sca-1⁺ BM cells into recipient mice that had been conditioned with a nonmyeloablative preparative regimen involving busulfan (BU) plus the

immunosuppressive agent anti-thymocyte serum (ATS) ($n = 4$). At 12 weeks post-transplantation, GFP expression in B cells, T cells and myeloid cells of peripheral blood was analyzed by flow cytometry (Figure 2B). In sharp contrast to the FB.SIV-MSCV-GFP vector, which was found to be expressed in all hematopoietic lineages, high-level expression of the FB.SIV-E μ P μ -GFP vector was observed only in cells expressing the B220 B cell marker. Likewise, the vast majority of GFP expression was associated with B220⁺ cells in spleen and BM (Figure 2C), confirming B lineage-restricted activity of the E μ P μ enhancer-promoter *in vivo*.

Phenotypic correction of hemophilia A mice transplanted with FB.SIV-E μ P μ -eFVIII-transduced Sca-1⁺ BM cells following nonmyeloablative conditioning

Based on the above results, we constructed the FB.SIV-E μ P μ -eFVIII and FB.SIV-MSCV-eFVIII lentiviral vectors in which the eFVIII cDNA was placed downstream of the E μ P μ enhancer-promoter and MSCV LTR, respectively (Figure 1A). To facilitate quantitative comparisons, a constitutive GFP expression cassette (PGK-GFP) was inserted into both vectors. Initially, Sp2/0 cells (transformed equivalents of plasma cells) were transduced and sorted for similar levels of GFP. As seen in Figure 1C, high levels of eFVIII were detected in the supernatants of both FB.SIV-E μ P μ -eFVIII- and FB.SIV-MSCV-eFVIII-transduced Sp2/0 cells (1.5 ± 0.2 and 1.4 ± 0.2 IU/ml/ 10^6 cells/24 hours, respectively). These results indicated that the FB.SIV-E μ P μ -eFVIII vector directs eFVIII expression at levels comparable to those achieved from the strong MSCV LTR enhancer-promoter in differentiated B cells, and demonstrated the ability of these cells to efficiently secrete eFVIII.

To investigate the utility of the FB.SIV-E μ P μ -eFVIII vector in correcting the hemophilic phenotype, HSCs (Sca-1⁺ BM cells) from hemophilia A mice were transduced with FB.SIV-E μ P μ -eFVIII and FB.SIV-MSCV-eFVIII vectors, and 1×10^5 total cells were transplanted into BU-ATS-conditioned recipient mice. MOIs were chosen such that ~10% of the target cells would be transduced with either vector. The low transduction efficiency made it unlikely that any transduced cell would carry more than one vector copy. GFP expression and plasma eFVIII levels were measured for 24 weeks after transplantation (Figure 3A). At this time, $4.4 \pm 1.6\%$ and $4.0 \pm 1.5\%$ GFP⁺ peripheral blood cells were detected in FB.SIV-E μ P μ -eFVIII- and FB.SIV-MSCV-eFVIII-treated mice, respectively ($n = 9$ mice per cohort). The plasma eFVIII levels in the FB.SIV-MSCV-eFVIII-treated animals were 170 ± 55 mIU/ml (17% of normal). More important, therapeutic eFVIII levels were also detected in the plasma of the FB.SIV-E μ P μ -eFVIII-treated mice (225 ± 75 mIU/ml; 23% of normal). Irrespective of the vector, all hemophilia A mice treated by eFVIII gene therapy exhibited clot formation and survived tail clipping, indicating correction of their hemophilic phenotype. Plasma samples collected at the time of tail clipping were analyzed for FVIII activity by a chromogenic (COATEST) assay. We detected 194 ± 53 mIU/mL and 158 ± 41 mIU/mL FVIII activity in the mice that received transplants of FB.SIV-E μ P μ -eFVIII- and FB.SIV-MSCV-eFVIII-transduced Sca-1⁺ BM cells, respectively (Table 1).

To determine whether the FB.SIV-E μ P μ -eFVIII vector was directing B cell-specific eFVIII expression *in vivo*, we analyzed intracellular FVIII expression in total spleen cells costained for expression of either the B220 B cell marker or the CD138 plasma cell marker. In the representative example presented in Figure 3B, ~11% of the spleen cells expressed eFVIII, with the majority produced by CD138⁺ plasma cells (8-9%) and the remainder detected in B220⁺ cells (2-3%).

Transfer of therapeutic eFVIII levels to secondary hemophilia A recipients

To confirm gene transfer into long-term repopulating HSCs, serial transplants were performed using 2×10^7 total BM cells from primary recipients into lethally irradiated (800 cGy) secondary recipients. Therapeutic levels of eFVIII expression were achieved in all secondary recipients of FB.SIV-E μ P μ -eFVIII-transduced BM cells (155 ± 55 mIU/ml) 12 weeks after transplantation (Figure 4A); all of these mice survived the tail clip assay (Table 1).

To investigate whether phenotypic correction could also be adoptively transferred by B cells, 1×10^7 total spleen cells from the primary FB.SIV-E μ P μ -eFVIII-corrected hemophilia A mice were infused into conditioned secondary hosts and GFP expression and plasma eFVIII levels were measured for 4 weeks after transfer (Figure 4B). We compared several nonmyeloablative conditioning regimens in order to facilitate adoptive transfer of B cells (37, 38). In particular, we were interested in determining the effects of bortezomib which efficiently depletes plasma cells, including long-lived ones (38). For up to 4 weeks post-transfer (the last time point analyzed), sublethally irradiated (500 cGy) and BU-ATS-conditioned recipients exhibited between 2-3% GFP⁺ peripheral blood cells. Interestingly, mice conditioned by bortezomib treatment alone had significantly lower engraftment levels ($\sim 0.5\%$ GFP⁺ peripheral blood cells). By comparison, $\sim 2\%$ GFP⁺ peripheral blood cells could be detected in mice conditioned by bortezomib treatment plus transient immunosuppression with ATS (Figure 4B). Although small amounts of eFVIII (~ 40 mIU/mL) could be detected in mice conditioned with bortezomib alone, none of these animals survived the tail clip assay. Remarkably, however, therapeutic levels of eFVIII were detected in the peripheral blood of mice conditioned with sublethal irradiation or BU-ATS as well as with bortezomib-ATS (90, 105, and 140 mIU/mL, respectively) at the 4-week time point; except for one mouse in the BU-ATS group, all of these animals exhibited clot formation and survived the tail clip assay. Combined, these results indicated that short-term therapeutic FVIII levels could also be achieved in secondary recipients by adoptive transfer of HSC-derived splenic B cells following nonmyeloablative conditioning. Importantly, complete depletion of plasma cells was not necessary to achieve a therapeutic effect (Figure 4B) (38).

Analysis of the anti-FVIII immune response in corrected hemophilia A transplant recipients

Sustained expression of eFVIII in the plasma of all immunocompetent hemophilia A BM transplant recipients for up to 24 weeks after transplantation indicated lack of stimulation of a potent inhibitory immune response against the eFVIII protein. To directly confirm this, we measured human FVIII-specific antibodies (IgG fraction) in the plasma of the mice by ELISA at the 24-week time point. All mice had less than 0.5 μ g/mL anti-FVIII IgG antibodies in their plasma, similar to naive hemophilia A mice (Figure 5). To directly assess whether B cell-specific expression of FVIII was tolerogenic in the context of the BU-ATS nonmyeloablative conditioning regimen, we attempted to break tolerance by actively immunizing selected mice from the stable FB.SIV-E μ P μ -eFVIII ($n = 3$) and FB.SIV-MSCV-eFVIII ($n = 4$) transplant recipients using purified human FVIII (Figure 5). While strong anti-FVIII humoral immune responses were elicited in immunized naive hemophilia A mice ($n = 5$), with an average anti-FVIII IgG antibody titer of $1,022 \pm 222$ μ g/mL, hemophilia A mice corrected by FB.SIV-E μ P μ -eFVIII or FB.SIV-MSCV-eFVIII gene transfer developed significantly attenuated anti-FVIII antibody titers (144 ± 90 and 93 ± 67 , respectively; $P < 0.001$).

Discussion

All forms of medical treatment are accompanied by some risk of unwanted side effects. Nonetheless, irrespective of therapeutic efficacy, an HSC-based FVIII gene transfer approach toward hemophilia A treatment will only become clinically acceptable if there is a low risk-to-benefit ratio (5-7). Besides reducing the frequency of multiple integration events per cell, methods that minimize transcriptional activation of neighboring oncogenes at vector insertion sites should therefore prove beneficial (8-11). In this context, accumulated data indicate that lentiviral vectors are less genotoxic in direct comparisons to similarly-configured gammaretroviral vectors in both *in vitro* assays and tumor-prone mouse models (10, 14, 15). Additionally, we previously demonstrated that the hematopoietic transforming activity of the SIV-MSCV lentiviral vector backbone could be virtually eliminated—to background levels under the experimental conditions used—by incorporation of the novel 77-bp FB enhancer blocking element in the deleted U3 region of the 3' LTR (10). In other work, we previously reported correction of the bleeding disorder in hemophilic mice by HSC-based ubiquitous FVIII transgene expression (11, 36, 39). It is, however, anticipated that additional protection could be gained by eliminating transgene expression in HSCs completely. In this regard, several recent studies have demonstrated the feasibility of long-term expression of therapeutic levels of human FVIII or factor IX in HSC-derived platelets or erythrocytes, although not all such strategies have led to induction of immune tolerance (23-26). To engineer an enhancer-promoter element that mediates specific and efficient B cell-directed transgene expression, while inactive in HSCs, we constructed the EμPμ transcriptional regulatory element. Because insertional mutagenesis still remains a concern for inadequately insulated lentiviral vectors (40), we incorporated it within an FB-insulated SIV lentiviral platform. Notably, when the FB.SIV-EμPμ-GFP vector was used to transduce Sca-1⁺ HSCs, the EμPμ enhancer-promoter was silenced and became active *in vivo* only in B lineage cells, recapitulating the B cell specificity demonstrated by *in vitro* studies with a series of hematopoietic cell lines. Moreover, following transplantation of FB.SIV-EμPμ-eFVIII-transduced Sca-1⁺ HSCs, only B lineage cells in the spleen (B220⁺ B lymphocytes and CD138⁺ plasma cells) of hemophilia A transplant recipients were found to produce intracellular eFVIII.

The development of neutralizing antibodies to FVIII is one of the most serious complications of FVIII protein therapy, and patients with these antibodies could benefit from gene therapy strategies with the potential to induce immune tolerance to FVIII (4). The association between donor-host hematopoietic chimerism and tolerance induction has been well-recognized (41). More recently, gene therapy approaches have been proposed to similarly achieve tolerance to transgenes through “molecular chimerism” (42). However, the morbidity and mortality associated with myeloablative conditioning strategies commonly employed make such regimens unacceptable for nonmalignant hematologic disorders. The future application of this gene therapy approach as a viable treatment option for hemophilia A patients necessitates implementation of a nonmyeloablative conditioning regimen to achieve this goal. Importantly, proof-of-principle that nonmyeloablative induction of hematopoietic chimerism can be used to induce allograft tolerance in the clinical setting has been demonstrated (43). In agreement with our previous study (11), we show here that engraftment levels as low as 2% in transplanted mice were sufficient to achieve therapeutic levels of eFVIII and induction of eFVIII-specific peripheral immune hyporesponsiveness using a nonmyeloablative conditioning regimen. The unique feature of the present study is the ability to do so while restricting eFVIII transgene expression to the B cell lineage and utilizing plasma cells, which are highly specialized terminally-differentiated secretory cells (16), to systemically deliver eFVIII.

An alternative to the HSC-based molecular chimerism strategy to induce tolerance involves context-dependent FVIII presentation by antigen presenting cells, such as tolerogenic B cells (18, 19) or immature dendritic cells (44, 45). Our approach herein was inspired by previous studies where specific humoral and cellular tolerance to human FVIII could be induced in both naive and FVIII-primed hemophilia A mice by transplantation of activated B cell blasts expressing IgG heavy chain constructs fused with either the A2 or the C2 domains of FVIII (18). In our study, sustained expression of eFVIII in the plasma of all immunocompetent hemophilia A BM transplant recipients and absence of FVIII-specific antibodies indicated lack of stimulation of a potent immune response to the eFVIII protein. Furthermore, when we immunized the transplanted mice with purified human FVIII, compared to control hemophilia A mice which developed a vigorous anti-FVIII antibody response, FB.SIV-E μ P μ -eFVIII corrected mice in addition to FB.SIV-MSCV-eFVIII transplanted mice exhibited a minimal anti-FVIII response. We therefore conclude that immune hyporesponsiveness was established in BM chimeras expressing eFVIII under the transcriptional control of the B cell-specific E μ P μ enhancer-promoter in a nonmyeloablative transplant setting.

In summary, our results support further pursuit of B cell-mediated systemic protein delivery as a novel strategy to correct hemophilia A and potentially other deficiencies that can be treated by enzyme replacement therapy (although, admittedly, perhaps not hemophilia B since factor IX requires efficient γ -carboxylation for functional activity (46)). It will be informative to extend this work in mice with preexisting FVIII inhibitors and to compare the efficacy of eFVIII in this context with new codon-optimized B domain-deleted FVIII cDNA sequences (47). Moreover, it will be important to determine in future experiments whether adoptive transfer of FVIII-expressing B cells (Figure 4B) can be exploited by directly targeting memory B cells and modulating their activity to further enhance the safety and utility of this approach (48, 49). Along these lines, an additional safety feature that could be implemented for clinical application would be to incorporate a conditional suicide gene into the vector—such as the inducible caspase 9 gene that is currently being evaluated in a Phase I clinical trial of adoptively transferred T cells (50)—to eliminate the genetically-modified cells if necessary.

What is known about this topic?

- Expression of bioengineered FVIII transgenes has resulted in disease correction in mouse hemophilia A models following myeloablative and nonmyeloablative transplantation of genetically-modified HSCs.
- HSC gene therapy has been used recently to successfully treat patients with severe combined immunodeficiency disease, Wiskott-Aldrich syndrome, β -thalassaemia and X-linked adrenoleukodystrophy.
- Safety issues concerning the risk of retroviral insertional mutagenesis demand the continued development of safer gene-delivery systems for HSC gene therapy.

What does this paper add?

- An insulated lentiviral vector based on simian immunodeficiency virus was developed as a safer alternative to gammaretroviral vectors for FVIII gene delivery and expression. Incorporation of enhancer-promoter sequences that are inactive in HSCs in combination with the insulated lentiviral vector backbone should further enhance the safety of HSC therapeutic gene transfer.
- An Ig heavy chain enhancer-promoter was utilized to direct FVIII expression to B lineage cells, employing the highly specialized plasma cell secretory apparatus to

systemically distribute bioengineered FVIII. This strategy allowed therapeutic FVIII levels to be achieved in the circulation of immunocompetent hemophilia A mice for the duration of the study following transplantation of small numbers of genetically-modified HSCs under a nonmyeloablative conditioning regimen.

- The results of this feasibility study suggest further exploration of B lineage-specific FVIII gene expression as a potential clinical approach to treat hemophilia A.

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Figure 1. Schematic representation and *in vitro* analysis of B cell-specific SIV-derived lentiviral vectors

(A) Schematic representation of the FB.SIV-EμPμ-GFP, FB.SIV-MSCV-GFP, FB.SIV-EμPμ-eFVIII, and FB.SIV-MSCV-eFVIII SIV_{mac1A11}-based SIN lentiviral vectors. Plasmid forms of the vectors are illustrated. All vectors included a GFP transgene under the transcriptional control of an internal EμPμ promoter, an internal MSCV LTR promoter, or an internal human PGK promoter. Arrows depict initiation and direction of transcription. Other abbreviations: CMV, cytomegalovirus immediate early enhancer-promoter; cPPT, central polypurine tract; RRE, Rev-responsive element; eFVIII, glycosylation facilitated secretion-enhanced human FVIII gene with reduced immunogenicity; W (WPRE), woodchuck hepatitis virus posttranscriptional regulatory element; ΔU3, deletion in the U3 region of the 3' LTR; FB, FII enhancer-blocking component of the chicken β-globin 5'HS4 insulator and the BEAD-A homologous region from the human T-cell receptor α/δ BEAD-I insulator; and B, bovine growth hormone poly(A) signal. Transfer of the deletion in the U3 region of the 3' LTR to the 5' LTR after reverse transcription self-inactivates the vector as denoted by the dashed arrow with an X through it. Vector titers (TU/ml; transducing units/ml) were measured on Sp2/0 B cells and are shown for triplicate determinations (right). (B) *In vitro* transgene expression from FB.SIV-EμPμ-GFP and FB.SIV-MSCV-GFP vectors. Sp2/0 (B cells), Jurkat (T cells), HL-60 (myeloid cells) and K-562 (erythroid cells) were transduced once at MOI 5 and evaluated for GFP expression (mean fluorescence intensity [MFI]) by flow cytometry 10 days later. (C) *In vitro* eFVIII expression from FB.SIV-EμPμ-eFVIII and FB.SIV-MSCV-eFVIII vectors in stably transduced Sp2/0 B cells. Sp2/0 cells were transduced once at MOI 5 and sorted for GFP expression after 10 days. FVIII levels were determined in 24-hour culture supernatants per 1×10^6 cells (top). Data presented are the mean values of several independent experiments ($n = 3$) $\pm$ SDs. The corresponding flow cytometric histograms of GFP fluorescence 1 week after cell sorting are shown (bottom). The percentages of GFP-expressing Sp2/0 cells are indicated (10,000 total events collected).

Figure 2. Analysis of B lineage-specific vector expression in *ex vivo* cultured Sca-1⁺ cells and in BM transplant recipients

(A) Flow cytometric analysis of GFP transgene expression in Sca-1⁺ BM cell-initiated cultures. The percentages of GFP⁺ cells are indicated 1 week after mock transduction (a) or transduction with the FB.SIV-EμPμ-eFVIII (b) and FB.SIV-MSCV-eFVIII (c) vectors (10,000 total events collected). Below is a Southern blot analysis of genomic DNA isolated from the corresponding cell cultures showing similar copy numbers of integrated FB.SIV-EμPμ-eFVIII and FB.SIV-MSCV-eFVIII vectors. DNA (10 μg) was digested with *Eco*RI plus *Sfi*I, and sequentially hybridized with a GFP probe (top panel) and a GAPDH probe (bottom panel). A 1.4-kb GFP gene-containing fragment originating from each vector (GFP) and the endogenous GAPDH gene (GAPDH) are indicated. (B) Flow cytometric analysis of GFP transgene expression in various lineages in the peripheral blood cells of mice (4 per group) 14 weeks after transplantation with 1×10^5 FB.SIV-EμPμ-eFVIII- or FB.SIV-MSCV-eFVIII-transduced Sca-1⁺ HSCs. Representative analyses are shown (30,000 total events collected). (C) Flow-cytometric analysis of GFP transgene expression in BM and spleen after transplantation with FB.SIV-EμPμ-GFP transduced Sca-1⁺ HSCs. The results shown are representative of two independent experiments (30,000 total events collected).

Figure 3. B cell-specific eFVIII expression analysis in transplanted hemophilia A mice following nonmyeloablative conditioning

(A) *Top*, Shown is a representative flow cytometric analysis of GFP transgene expression in an *ex vivo* cultured sample of Sca-1⁺ BM cells 1 week after transduction with the FB.SIV-EμPμ-eFVIII and FB.SIV-MSCV-eFVIII vectors. The percentages of GFP⁺ cells are indicated (10,000 total events collected). *Middle*, The percentage of GFP⁺ peripheral blood cells in BU-ATS-conditioned hemophilia A mice that received FB.SIV-EμPμ-eFVIII- or FB.SIV-MSCV-eFVIII-transduced Sca-1⁺ BM cells as determined by quantitative flow cytometric analysis at various time points after transplantation. Error bars represent the SEM. *Bottom*, Human eFVIII levels in the plasma of hemophilia A mice that received Sca-1⁺ BM cells transduced with FB.SIV-EμPμ-eFVIII or FB.SIV-MSCV-eFVIII presented as mean values $\pm$ SDs at various time points after transplantation (n = 9 mice per cohort). Data presented are the mean values of 2 independent experiments. (B) Intracellular staining for eFVIII in spleens of mice transplanted with FB.SIV-EμPμ-eFVIII-transduced Sca-1⁺ cells. Spleen cells were analyzed by flow cytometry for intracellular eFVIII staining using a mouse anti-hFVIII antibody, the B220 B cell surface marker or the CD138 plasma cell marker. Spleen cells from a naive hemophilia A mouse were used as negative control (top). Representative analyses are shown.

Figure 4. Transfer of BM and B cells from corrected hemophilia A mice into secondary recipients

(A) eFVIII expression levels in secondary hemophilia A BM transplant recipients. To ascertain gene transfer in long-term repopulating HSCs, secondary transplants were performed using BM cells from representative primary recipients collected at 20 weeks after transplantation. Peripheral blood cells from secondary transplant recipients of FB.SIV-E μ P μ -eFVIII-transduced primary BM cells were analyzed by flow cytometry after 12 weeks (left). eFVIII levels in the plasma of the same secondary transplant recipients (right). Mice were conditioned by lethal (800 cGy) total body irradiation before transplantation with BM cells (2×10^7 cells) from primary corrected hemophilia A mice. (B) Phenotypic correction by B cell transfer. Several conditioning regimens were compared for engraftment of transferred B cells. Spleen cells (1×10^7) from the primary FB.SIV-E μ P μ -eFVIII-transduced BM transplant recipients were injected into sublethally irradiated (500 cGy), BU-ATS-, bortezomib (Bz)-, or Bz-ATS- conditioned recipient mice; GFP expression and plasma eFVIII levels were measured after 4 weeks. Shown on the bottom are the corresponding average plasma eFVIII levels and the tail clip survival test results. Data presented are the mean values of two independent experiments $\pm$ SDs. *Significantly higher levels ($P < 0.05$).

Figure 5. Analysis of the anti-FVIII immune response in corrected hemophilia A transplant recipients

Total FVIII-specific IgG was measured in the plasma of BU-ATS-conditioned FB.SIV-EµPµ-eFVIII- (n = 9) and FB.SIV-MSCV-eFVIII- (n = 8) corrected hemophilia A mice at 24 weeks posttransplant by ELISA before any FVIII injections and after five intravenous challenges with recombinant FVIII. Naive (n = 5) hemophilia A mice were included as controls. The values are reported as average $\pm$ SD. *Significantly lower levels ($P < 0.001$).

Table 1

Phenotypic correction of hemophilia A mice

Mouse	Conditioning	Vector	Weeks after transplantation	%GFP	FVIII, mIU/mL	Survival
Primaries						
1	BU-ATS	FB.SIV-EμPμ-eFVIII	24	5.5	208	+
2	BU-ATS	FB.SIV-EμPμ-eFVIII	24	2.7	162	+
3	BU-ATS	FB.SIV-EμPμ-eFVIII	24	4.4	191	+
4	BU-ATS	FB.SIV-EμPμ-eFVIII	24	4.1	144	+
5	BU-ATS	FB.SIV-EμPμ-eFVIII	24	3.1	223	+
6	BU-ATS	FB.SIV-EμPμ-eFVIII	24	4.4	261	+
7	BU-ATS	FB.SIV-EμPμ-eFVIII	24	4.4	159	+
8	BU-ATS	FB.SIV-EμPμ-eFVIII	24	7.9	277	+
9	BU-ATS	FB.SIV-EμPμ-eFVIII	24	3.2	123	+
10	BU-ATS	FB.SIV-MSCV-eFVIII	24	4.8	156	+
11	BU-ATS	FB.SIV-MSCV-eFVIII	24	7.5	182	+
12	BU-ATS	FB.SIV-MSCV-eFVIII	24	3.1	112	+
13	BU-ATS	FB.SIV-MSCV-eFVIII	24	4.7	201	+
14	BU-ATS	FB.SIV-MSCV-eFVIII	24	2	133	+
15	BU-ATS	FB.SIV-MSCV-eFVIII	24	3.5	171	+
16	BU-ATS	FB.SIV-MSCV-eFVIII	24	3.6	155	+
17	BU-ATS	FB.SIV-MSCV-eFVIII	24	3.1	94	+
18	BU-ATS	FB.SIV-MSCV-eFVIII	24	4.3	221	+
Secondaries (BM transplant)						
1	800cGy	FB.SIV-EμPμ-eFVIII	16	3.4	156	+
2	800cGy	FB.SIV-EμPμ-eFVIII	16	2.3	139	+
3	800cGy	FB.SIV-EμPμ-eFVIII	16	2.6	150	+
Secondaries (B cell transfer)						
1	500cGy	FB.SIV-EμPμ-eFVIII	4	2.8	84	+
2	500cGy	FB.SIV-EμPμ-eFVIII	4	1.2	96	+
3	BU-ATS	FB.SIV-EμPμ-eFVIII	4	1.1	63	-

Mouse	Conditioning	Vector	Weeks after transplantation	%GFP	FVIII, mIU/mL	Survival
4	BU-ATS	FB.SIV-EμPμ-eFVIII	4	2	148	+
5	BZ-ATS	FB.SIV-EμPμ-eFVIII	4	4.2	165	+
6	BZ-ATS	FB.SIV-EμPμ-eFVIII	4	2	115	+
7	BZ	FB.SIV-EμPμ-eFVIII	4	0.3	52	-
8	BZ	FB.SIV-EμPμ-eFVIII	4	0.6	24	-
Controls						
Hemophilia A mice						
Wild type mice						
ATS, anti-thymocyte serum						
BU, busulfan						
Bz, bortezomib						
						0/5
						5/5