Molecular Therapy
Original Article

Gene Therapy with BMN 270 Results in Therapeutic Levels of FVIII in Mice and Primates and Normalization of Bleeding in Hemophilic Mice

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Hemophilia A is an X-linked bleeding disorder caused by mutations in the gene encoding the factor VIII (FVIII) coagulation protein. Bleeding episodes in patients are reduced by prophylactic therapy or treated acutely using recombinant or plasma-derived FVIII. We have made an adeno-associated virus 5 vector containing a B domain-deleted (BDD) FVIII gene (BMN 270) with a liver-specific promoter. BMN 270 injected into hemophilic mice resulted in a dose-dependent expression of BDD FVIII protein and a corresponding correction of bleeding time and blood loss. At the highest dose tested, complete correction was achieved. Similar corrections in bleeding were observed at approximately the same plasma levels of FVIII protein produced either endogenously by BMN 270 or following exogenous administration of recombinant BDD FVIII. No evidence of liver dysfunction or hepatocyte endoplasmic reticulum stress was observed. Comparable doses in primates produced similar levels of circulating FVIII. These preclinical data support evaluation of BMN 270 in hemophilia A patients.

INTRODUCTION

Hemophilia A and B are X chromosome-linked congenital clotting disorders caused by deficient activity of either blood coagulation factor VIII (FVIII, type A) or factor IX (FIX, type B).[1–3] Both factors are essential for blood coagulation, and affected individuals can have a severe, moderate, or mild form of disease, defined by factor plasma levels of 1% or less, 2%–5% and 5%–40%, respectively. Hemophilia A is more common and affects approximately 80% of hemophilia patients, with a prevalence of 1 in 5,000 male live births.[4] Hemophilia B affects about 20% of patients, with a prevalence of 1 in 30,000 male live births. The severe form of hemophilia A or B is characterized clinically by spontaneous musculoskeletal and soft tissue bleeding, as well as the inability to achieve hemostasis after trauma unless concentrates of clotting factor are infused.[1–3] The current treatment of choice is prophylactic administration of recombinant or plasma-derived clotting FVIII or FIX. However, injections are required frequently (two or three times per week) and may be burdensome and painful, affecting some patients' adherence to treatment.[5]

The use of gene therapy to treat hemophilia has been under investigation for the past two decades, and although early preclinical studies in dogs and mice were promising, clinical trials using an adeno-associated virus (AAV) 2-FIX construct failed to demonstrate therapeutic levels of FIX when injected intramuscularly.[6,7] Although subsequent trials with intravascular delivery did achieve therapeutic FIX plasma levels, they were not sustained because of an apparent immune response to the transduced hepatocytes.[8,9]

In 2011, Nathwani et al.[10–12] reported successful treatment of hemophilia B patients by gene therapy with a single intravenous (i.v.) infusion of self-complementary AAV8 vector containing a human FIX (hFIX) transgene. After treatment, two-thirds of the patients in this ongoing study discontinued prophylactic FIX and remained free of spontaneous hemorrhage. The remaining patients increased the interval between preventive injections of FIX, and clinical improvement persisted at the 3-year follow-up period without adverse events from therapy.[11]

More recently, Spark Therapeutics reported a mean steady-state FIX activity of ~29% in ten patients dosed with a novel AAV vector with 12 weeks of follow-up.[13,14] UniQure reported a mean FIX expression level of 5.2% in the lower dose cohort with an AAV5 vector during 1 year of follow-up and 6.9% in the higher dose cohort during 26 weeks of follow-up.[15,16] Finally, Shire reported patients with transient FIX activity >50%, but only two patients had persistent FIX expression at 1 year, and one patient continued to have sustained 20% FIX activity after 2.5 years of follow-up.[17–19] These results not only represent landmarks in the field of gene therapy but also suggest that a similar treatment for hemophilia A could be developed using AAV technology.[20,21]

Hemophilia A gene therapy has proved more challenging because of the size of the FVIII gene (the coding region is approximately 7.0

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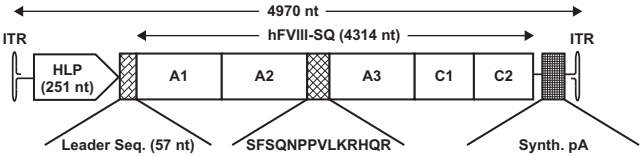

Figure 1. BMN 270 Construct

A schematic illustrating the recombinant adeno-associated virus AAV5-hFVIII-SQ vector sequence, which includes double-stranded inverted terminal repeats (ITRs) at its 5′ and 3′ ends, and single-stranded DNA encoding a hybrid human liver-specific promoter (HLP), a B domain-deleted (BDD) human factor VIII (hFVIII) cDNA, and a synthetic polyadenylation signal (Synth. pA). The hFVIII A2 and A3 domains are linked by DNA encoding a 14 amino acid (SQ) sequence from the B domain. The SQ sequence contains a furin cleavage site (RHQR).

kb, well beyond the packaging capacity of AAV capsids) and the poor cellular expression efficiency of full-length FVIII protein. Attempts to overcome these obstacles include B domain modification or deletion, codon optimization, the use of mini-promotors, oversized vectors, dual vectors, elimination of furin cleavage sites, and the introduction of glycosylation sites.[7,22–24] Using these approaches, therapeutic levels of FVIII were observed in preclinical models.[24] We incorporated some of these features into BMN 270, a replication-incompetent AAV5 vector containing a 4.97 kb genome encoding a codon-optimized B domain-deleted (BDD) human coagulation FVIII under the control of a small, liver-specific promoter (HLP) (Figure 1). In addition, the FVIII A2 and A3 domains are linked by DNA encoding a 14-amino acid (SQ) sequence from the B domain, and the SQ sequence contains a furin cleavage site (hFVIII-SQ).[24] Here, we describe the expression of therapeutic levels of hFVIII-SQ in mice and cynomolgus monkeys following a single i.v. dose of BMN 270 and the correction of the bleeding phenotype in a double-knockout (DKO) mouse model of hemophilia.

RESULTS

Production of AAV vectors to support clinical use requires both scalability and reproducibility.[25] Two systems currently in widespread use to produce clinical grade vectors are DNA transfection in mammalian HEK293 cells or the more readily scalable Sf9/baculovirus insect cell system.[26] Using a recombination activating gene 2 knockout ($RAG2^{-/-}$) mouse model that lacks the capability of mounting a B or T cell-mediated immune response to foreign proteins, we compared different AAV5-FVIII vectors[24] that were produced either in the Sf9/baculovirus system or in the HEK293 system. Plasma human FVIII (hFVIII) protein levels were measured 5 or 10 weeks following a single i.v. administration of vectors at 2e13 vg/kg. No significant differences in plasma hFVIII levels were shown between the two systems (Figure S1). On the basis of the comparability of the material produced in these two systems and the potential to scale the sf9/baculo system for clinical use, the sf9/baculovirus-produced construct (BMN 270) was chosen to evaluate further in preclinical studies.

Administration of BMN 270 to Mice Results in Dose-Dependent Production of Functional hFVIII-SQ and a Continued Increase in Plasma hFVIII-SQ Protein Levels over 13 Weeks Post-dosing

Dose Response in Mice

In early studies conducted with BMN 270 in wild-type (WT) mice, sporadic formation of anti-hFVIII antibodies was detected beyond 4 weeks post-dosing (data not shown). Therefore, in order to study long-term pharmacodynamics effects of BMN 270, DKO mice with mutations in both endogenous mouse coagulation FVIII and RAG2 were bred to provide a mouse model of hemophilia A without the capability of mounting a B or T cell-mediated immune response to foreign proteins. Experiments were conducted in DKO mice to determine the dose range of BMN 270 required for hFVIII-SQ protein expression. BMN 270, at doses spanning 2e10 to 2e14 vg/kg, or vehicle was administered as a bolus injection via the tail vein. Eight weeks after injection, hFVIII-SQ protein and activity levels in mouse plasma were assessed and livers were collected to measure hFVIII-SQ transgene (DNA) levels and RNA production. No hFVIII-SQ DNA, RNA, or protein was detected in vehicle-treated mice or the two lower dose groups (2e10 and 2e11 vg/kg). Following BMN 270 doses of 2e12, 2e13, and 2e14, hFVIII-SQ DNA was detected with a mean of 4.66×10^{-2}, 1.29×10^{-1}, and 2.41×10^{1} vg/cell, respectively (Figure 2A). Over the same dose range, hFVIII-SQ RNA was detected with a mean of 3.91×10^{4}, 2.26×10^{5}, and 1.32×10^{7} copies/μg RNA, respectively. The levels of RNA correlated with levels of DNA. Plasma hFVIII-SQ protein levels were 6.9, 46.8, and 355.0 ng/mL, respectively (Figure 2B). Plasma hFVIII-SQ activity, measured by a chromogenic factor X (FX) activation assay, correlated with plasma hFVIII-SQ protein levels (4.69%, 23.5%, and 287% of normal FVIII) (Figure 2B).

In order to more finely titrate the dose response of BMN 270 administration over the range of doses projected to produce >5% of normal levels of hFVIII-SQ, plasma protein and activity levels, as well as liver DNA and RNA levels, were measured in $RAG2^{-/-}$ mice (n = 10) 5 weeks after administration of BMN 270 at doses of 6e12, 2e13, and 6e13 vg/kg. Increasing doses of BMN 270 led to increasing values in all parameters measured. hFVIII-SQ DNA and RNA measured in homogenized liver tissue at this 5-week time point increased fairly linearly over this dose range (Figure 2C). Plasma hFVIII-SQ protein and activity levels correlated very closely and, similarly to liver hFVIII-SQ RNA and DNA, exhibited a dose response over the tested dose range. Plasma hFVIII-SQ protein levels increased from 8.8 to 64 to 273 ng/mL, respectively, for doses of 6e12, 2e13, and 6e13 vg/kg. Plasma hFVIII-SQ activity increased from 4.9% to 53% to 299% of normal FVIII (Figure 2D).

Kinetics of Transgene Expression

The time course of transgene expression was studied in male DKO mice (n = 10/group) given a single BMN 270 i.v. administration of either 6e12 or 6e13 vg/kg by measuring plasma hFVIII-SQ protein levels and activity and liver DNA and RNA at 4 and 13 weeks post-dose. Four weeks post-administration, only two of the ten mice dosed with 6e12 vg/kg had detectable levels of hFVIII-SQ protein in plasma.

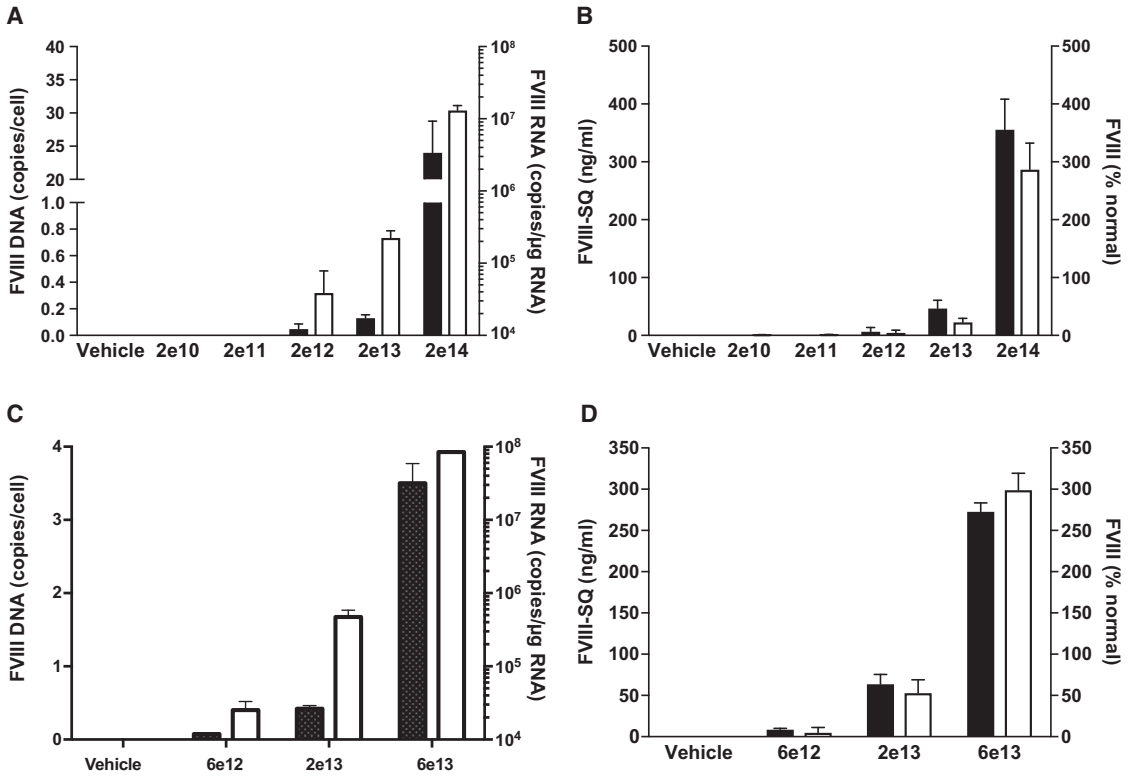

Figure 2. Evaluation of Dose Response of BMN 270 Treatment

(A) FVIII transgene (DNA; left y axis, black bars) and transcript (RNA; right y axis, white bars) copy numbers in liver homogenates from DKO mice 8 weeks following a single tail vein administration of vehicle or BMN 270 at 2e10, 2e11, 2e12, 2e13, or 2e14 vg/kg. Results are mean ± SEM (n = 10/group). (B) hFVIII-SQ protein (left y axis; black bars) and activity (right y axis; white bars) levels in DKO mouse plasma 8 weeks following a single tail vein administration of vehicle or BMN 270 at 2e10, 2e11, 2e12, 2e13, or 2e14 vg/kg. Results are mean ± SEM (n = 10/group). (C) FVIII transgene (DNA; left y axis, black bars) and transcript (RNA; right y axis, white bars) copy numbers in liver homogenate from RAG2$^{-/-}$ mice at 5 weeks following a single tail vein administration of vehicle or BMN 270 at 6e12, 2e13, or 6e13 vg/kg. Results are mean ± SEM (n = 10/group). (D) hFVIII-SQ protein (left y axis, black bars) and activity (right y axis, white bars) levels in RAG2$^{-/-}$ mouse plasma at 5 weeks following a single tail vein administration of vehicle or BMN 270 at 6e12, 2e13, or 6e13 vg/kg. Results are mean ± SEM (n = 10/group). FVIII, human coagulation factor VIII; hFVIII-SQ, human B domain-deleted FVIII; RAG2, recombination activating gene 2; DKO, Rag2 X FVIII double-knockout.

By 13 weeks post-dose, neither protein nor activity was measurable in this low-dose group. However, in mice given 6e13 vg/kg, hFVIII-SQ protein levels were a mean of 180 ng/mL at 4 weeks and increased by 2-fold at 13 weeks (Figure 3A). In WT mice given 6e13 vg/kg of BMN 270, hFVIII-SQ protein levels were a mean of 167 ng/mL at 4 weeks post-dosing (Figure S2), demonstrating that transduction efficiency is comparable between WT and DKO mice. FVIII activity mirrored hFVIII-SQ protein levels (Figure 3A). hFVIII-SQ DNA (Figure 3B) was detected in liver tissue at both 4 and 13 weeks post-dose, with levels higher following the 6e13 dose than the 6e12 dose. DNA levels in both groups were 3- to 5-fold lower by 13 weeks post-dose. Although liver hFVIII-SQ DNA in both dose groups fell between weeks 4 and 13, liver hFVIII-SQ RNA rose over this time frame (Figure 3B). The RNA levels correlated well with the levels of plasma hFVIII-SQ protein. Because FVIII expression levels increased between 4 and 13 weeks post-BMN 270 dosing, a longer term study over a 6-month period was conducted in DKO mice to determine when hFVIII-SQ protein produced from BMN 270 reached steady-state. Groups of six or seven mice were treated with BMN 270 at either 2e13 or 6e13 vg/kg and sacrificed at 12 and 24 weeks post-dosing. The levels of plasma hFVIII-SQ protein and FVIII activity at 12 weeks post-dosing were comparable with those measured at 13 weeks in previous experiments (Figure 3A) and 24 weeks post-dosing (Figure 3C), suggesting that hFVIII-SQ production from BMN 270 reached steady state by 12 weeks post-dosing.

hFVIII-SQ Protein Produced from BMN 270 in Mice Is the Same Size as Xyntha (Recombinant hFVIII-SQ)

Western blot analysis was used to demonstrate that the hFVIII-SQ expressed in BMN 270-treated mice was of the same size as recombinant hFVIII-SQ protein (rhFVIII-SQ) (Figure 4). Plasma samples from individual DKO mice treated either with BMN 270 or vehicle, or WT mouse plasma spiked with Xyntha rhFVIII-SQ, were resolved by denaturing, reducing SDS-PAGE followed by western blotting. In western blots stained with antibodies against either hFVIII-SQ light chain (Figure 4, left) or hFVIII-SQ heavy chain (Figure 4, right), a

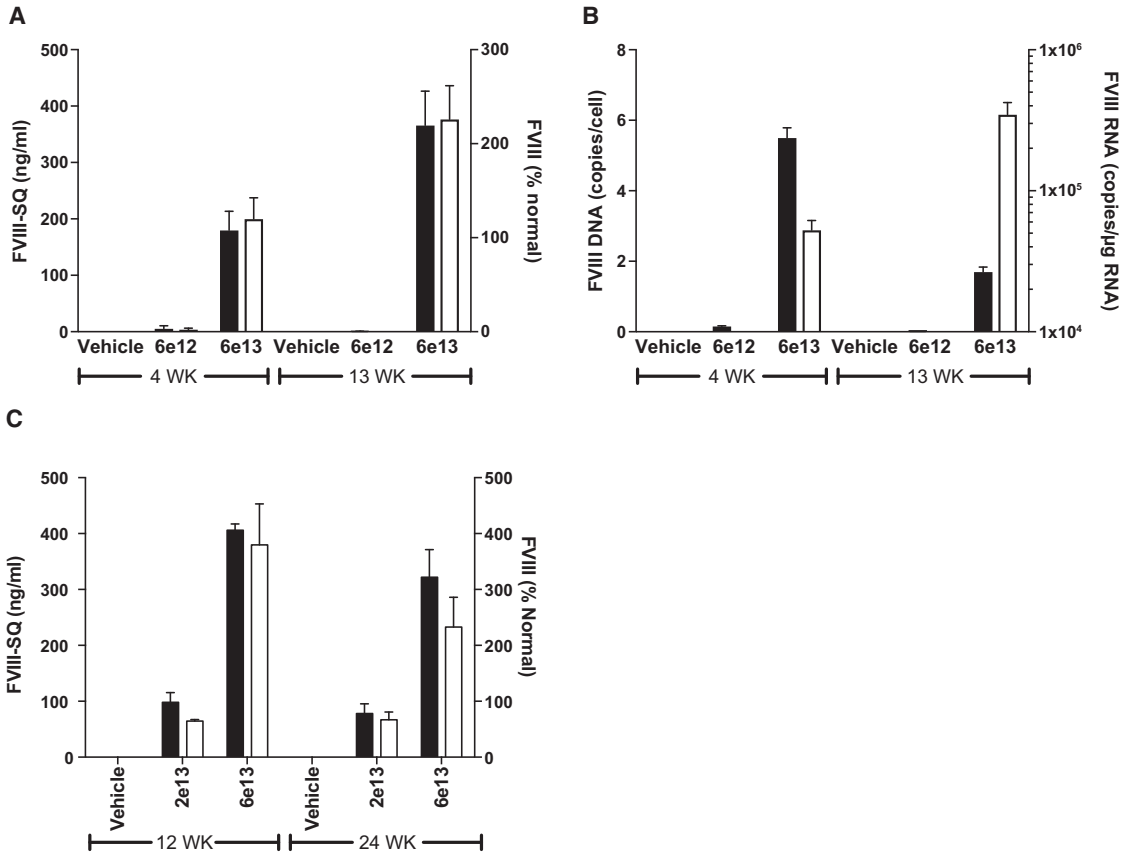

Figure 3. Evaluation of the Kinetics of FVIII Transgene Expression

Groups of 10 DKO mice were treated with vehicle or BMN 270 at 6e12, or 6e13 vg/kg and levels of (A) hFVIII-SQ protein (left y axis; black bars) and activity (right y axis; white bars) in mouse plasma and (B) FVIII transgene (DNA; left y axis, black bars) and transcript (RNA; right y axis, white bars) copy numbers in liver homogenates, 4 and 13 weeks post-dosing. Groups of six or seven DKO mice were treated with vehicle or BMN 270 at 2e13 or 6e13 vg/kg, and levels of (C) hFVIII-SQ protein (left y axis; black bars) and activity (right y axis; white bars) were determined in mouse plasma 12 and 24 weeks post-dosing. Results are mean ± SEM.

single band corresponding to each chain was observed. The light-chain and heavy-chain bands migrated at an identical position as in WT mouse plasma samples spiked with Xyntha. No bands were observed in WT mouse plasma (containing endogenous mouse FVIII), or in individual plasma samples from vehicle group indicating the specificity of the antibodies for detection of hFVIII heavy and light chains.

Tail Bleeding Time Assay Demonstrates Functionality of Expressed hFVIII-SQ

A tail bleeding time test was conducted in DKO mice to demonstrate that circulating hFVIII-SQ protein, produced 8 weeks after administration of BMN 270, could correct the bleeding phenotype of FVIII-deficient mice in a manner similar to treatment with Xyntha (Figure 5). DKO mice treated with vehicle had a mean blood loss and bleeding time of 0.741 g and 28.96 min, respectively. WT mice receiving vehicle had a mean blood loss of 0.040 g and bleeding time of 5.11 min. Mice receiving BMN 270 at 2e13 vg/kg showed significantly reduced blood loss ($p < 0.001$ versus DKO + vehicle, $p < 0.001$ versus WT) and bleeding time ($p < 0.0001$ versus DKO + vehicle, $p < 0.01$ versus WT). Treatment with BMN 270 at a dose of 1e14 vg/kg corrected blood loss and bleeding times to WT levels (0.104 g [$p = 0.192$ versus WT, $p < 0.0001$ versus DKO + vehicle] and 5.58 min [$p = 0.847$ versus WT, $p < 0.0001$ versus DKO + vehicle], respectively). DKO mice treated with an i.v. bolus of 50 IU/kg of Xyntha 5 min prior to tail transection had a mean blood loss of 0.492 g and bleeding time of 18.14 min, which were not significantly different from mice receiving BMN 270 at 2e13 vg/kg. DKO mice receiving 200 IU/kg of Xyntha had a mean blood loss of 0.134 g and bleeding time of 4.29 min, which were not significantly different from mice receiving BMN 270 at 1e14 vg/kg.

BMN 270 Producing Therapeutic Levels of hFVIII-SQ Protein Does Not Induce Endoplasmic Reticulum Stress or Apoptosis in Mouse Liver

Expression and secretion of WT and BDD hFVIII in transgenic mouse models have been shown to be less efficient compared with proteins expressed from FVIII constructs with truncated B domains

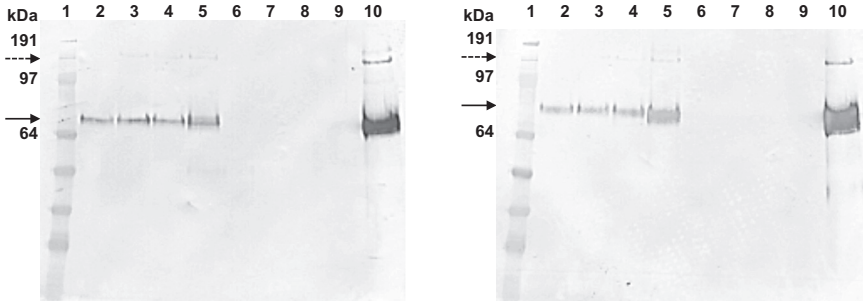

Figure 4. Western Blot Analysis of Mouse Plasma Using Anti-human Factor VIII Antibodies

Plasma samples were obtained from DKO mice (n = 3/group) treated with BMN270 (lanes 2–4) or vehicle (lanes 7–9). Samples were enriched using anti-factor VIII polyclonal antibodies, resolved under denaturing and reducing conditions and probed using anti-factor VIII light chain-specific (left) or heavy chain-specific (right) monoclonal antibodies. Solid arrows denote the individual heavy or light chains, and dotted arrows denote the intact hFVIII-SQ. Reference positive controls were Xyntha recombinant hFVIII-SQ spiked in buffer (lane 10) and in wild-type mouse plasma (lane 5). Unspiked wild-type mouse plasma was the negative control (lane 6).

that are highly glycosylated.[24,27] Malhotra et al.[27] reported that newly synthesized WT and BDD hFVIII proteins accumulate in the endoplasmic reticulum (ER) lumen, activate an acute unfolded protein response (UPR) leading to ER stress, and induce apoptosis in mice hydrodynamically injected through tail veins with plasmids encoding hFVIII genes. In addition, DNA encoding BDD hFVIII packaged in AAV8 capsids has been shown to transiently induce cellular stress in mouse liver.[28,29] Therefore, retention of hFVIII-SQ and induction of ER stress in liver was evaluated at BMN 270 doses that achieve therapeutic circulating levels of FVIII.

Therapeutic levels of circulating hFVIII-SQ protein (64 ng/mL; 83% normal FVIII activity) were achieved 5 weeks after dosing in Rag2$^{-/-}$ mice treated with 2e13 vg/kg of BMN 270 (Figure 2D). No retention of hFVIII-SQ was detected in liver by ELISA or immunofluorescence (IF) (Figures 6A and 6B, respectively). Mice dosed with BMN 270 at 6e13 vg/kg produced supraphysiological levels of circulating hFVIII-SQ (273 ng/mL, 329% normal FVIII activity; Figure 2D), and a total of ~200 ng of hFVIII-SQ was detected in homogenized whole liver (Figure 6A). The total amount of hFVIII-SQ produced in these mice was calculated by summation of the total circulating hFVIII-SQ protein (multiplying the concentration [ng/mL] of circulating hFVIII-SQ by 2, the estimated total blood volume of the mouse) and the total liver hFVIII-SQ protein. The majority of the total hFVIII-SQ produced in the 6e13 vg/kg-dosed mice was detected in the circulation (~72%), and the secreted hFVIII-SQ was functional (Figure 2D). In mice with hFVIII-SQ protein detected in the liver, 12.4 ± 3.4% of liver hepatocytes stained positive and exhibited a peri-central vein pattern of expression (Figure 6B), consistent with the pattern observed in mice injected with other AAV capsids delivering constructs driven by constitutively active or hepatocyte-specific promoters.[30–32]

Because retention of hFVIII-SQ was observed in some hepatocytes following the 6e13 vg/kg dose of BMN 270 to RAG2$^{-/-}$ mice, markers of the UPR were evaluated in liver homogenate and formalin-fixed sections. No significant change in levels of glucose regulated protein 78 (Grp78), spliced Xbox binding protein (XBP-1), activating transcription factor 6 (ATF6), and CCAAT/-enhancer-binding protein homologous protein (CHOP) was detected in liver homogenates collected 5, 12, and 24 weeks post-dosing with BMN 270 (Figures 6C–6E, S3A, and S3B). Elevated expression of molecular chaperones was observed in Rag2$^{-/-}$ mice after treatment with another AAV5-FVIII-SQ construct driven by a stronger promoter (data not shown), demonstrating that this strain of mice is capable of mounting a UPR. However, because hFVIII-SQ retention was shown to be in ~12% of hepatocytes in the peri-central vein region, it is possible that an increase in ER stress markers was not detected in liver homogenate because of dilution of signals from the rest of liver cells not staining positive for hFVIII-SQ protein. IF co-staining of hFVIII-SQ and Grp78 was carried out to evaluate this possibility. No elevated Grp78 staining was observed in the peri-central hepatocytes that stained positive for hFVIII-SQ (Figure 6F).

Active caspase-3 levels in liver protein extracts were measured as a marker of apoptosis in liver, and levels of liver enzymes in plasma was assessed. There was no increase in active caspase-3 in mice dosed with BMN 270 that produced either normal or supraphysiological levels of hFVIII-SQ (Figure 6G). There was no significant increase in plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels compared with vehicle-treated mice (Figures S4A and S4B). In a separate experiment, DKO mice dosed with BMN 270 at doses up to 2e14 vg/kg (Figures 3A and 3B) had no increase in liver active caspase-3 or plasma AST and ALT 8 weeks post-dosing (Figures S5A–S5C).

Therapeutic Levels of hFVIII-SQ in Nonhuman Primates following Peripheral Vein Administration of BMN 270

Previous studies using a self-complementary AAV8 vector to express hFIX have shown that levels of circulating hFIX decrease dramatically from mouse to rhesus monkey (6-fold drop) and from monkey to human (~80-fold drop) when given at the 2e12 vg/kg dose.[10,11,33] Therefore, hFVIII-SQ expression levels were evaluated in nonhuman primates (NHPs) following BMN 270 administration at doses that produced therapeutic levels of circulating hFVIII-SQ in mice (Figure 2D). Male cynomolgus monkeys were screened for antibodies and neutralizing factors against AAV5 and only monkeys screened negative for both tests were enrolled in the study.[34] Groups of two cynomolgus male monkeys were injected with either 1.0e13 or 3.6e13 vg/kg of BMN 270 via the peripheral vein. Weekly plasma

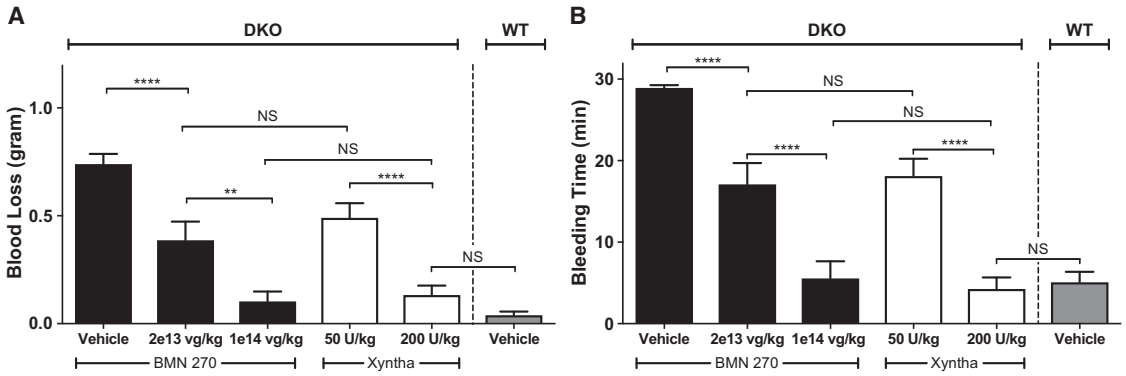

Figure 5. Evaluation of the Efficacy of BMN 270 Treatment in Hemophilia A Mice by Tail Bleeding Model

Blood loss (A) and bleeding time (B) in DKO mice 8 weeks following a single tail vein administration of vehicle or BMN 270 (2e13 or 1e14 vg/kg; black bars), compared with DKO mice treated with a single tail vein injection of rhFVIII (Xyntha) (white bars), administered 5 min prior to testing, compared with wild-type (WT) mice given a tail vein injection of vehicle 5 min prior to testing. Results are shown as mean ± SEM (n = 20/group). Multiple comparisons of mean values to detect significant differences among groups were performed by ANOVA followed by a subsequent Tukey test for repeated-measures. Statistically significant differences between compared groups are indicated: **$p < 0.01$, ****$p < 0.0001$; NS, not significant.

samples collected from 1 week pre-dose to 8 weeks post-dose were analyzed for circulating hFVIII-SQ levels. Monkeys dosed with BMN 270 produced plasma levels of hFVIII-SQ in a dose-dependent manner, achieving therapeutic levels (Table 1). These hFVIII-SQ levels were similar to those produced in mice treated with similar doses (Figures 2B and 2D). Circulating hFVIII-SQ protein was detected at 1 week post-dose, peaked between 3 and 5 weeks (Figure 7), and then declined, consistent with expression kinetics observed in monkeys given recombinant AAV8 vectors expressing codon-optimized hFVIII variants without immune modulatory treatment.[24] Indeed, anti-hFVIII antibodies were detected in three of four monkeys (1002, 2001, and 2002). NHP subject 1001 had no detectable anti-FVIII antibody titer but still had appreciable hFVIII-SQ protein levels at 8 weeks post-dosing. All monkeys developed anti-AAV5 antibodies post-dosing, and no elevation of plasma ALT or AST levels was observed throughout the study.

DISCUSSION

The use of AAV vectors for gene complementation in hemophilia has been the focus of considerable research, from preclinical studies to clinical proof-of-concept trials.[35] The primary focus has been on hemophilia B, in which the FIX protein is smaller and more readily expressed by hepatocytes. An early clinical trial using AAV2 FIX vectors administered directly into the hepatic artery resulted in circulating levels of FIX up to 12% of normal,[8] establishing that gene transfer to the liver using an AAV delivery system could result in expression of functional protein into the circulation. These FIX levels would have been sufficient to provide significant clinical benefit had they persisted. Unfortunately, FIX expression was lost after approximately 2 months, along with a concomitant asymptomatic rise in liver transaminases. This was postulated to be due to a cytotoxic T cell (CTL) response directed to the AAV2 capsid proteins, leading to the destruction of transduced hepatocytes.[8,9] More recently, Nathwani et al.[10,11] demonstrated prolonged expression of FIX in patients treated with a single i.v. dose of a codon-optimized, self-complementary FIX gene packaged in an AAV8 capsid. Although liver transaminase increases were observed in some patients, treatment with prednisolone halted further transaminase increases and reduced the loss of FIX expression. In this trial, dose-dependent expression of FIX between 1% and 7% of normal levels was achieved and resulted in discontinuation of FIX protein infusions for some patients or a significant reduction in frequency of use in others, for more than 4 years. Other groups are pursuing a similar approach but using a naturally occurring FIX variant with a higher specific activity to try to further boost functional circulating FIX levels.[13,17]

Using AAV technology to treat hemophilia A, which requires delivery of the FVIII gene, is more challenging than FIX for several reasons. The size of the full-length gene is beyond the packaging capabilities of AAV, and cellular expression and secretion of FVIII following transfection or transduction are less efficient compared with FIX. Several versions of FVIII protein have been tested in cellular systems to improve expression levels, while still retaining coagulation cofactor activity. The recombinant forms of FVIII protein that are available for clinical use frequently have a deleted or modified B domain that improves folding and secretion in cellular systems.[24] Nevertheless, AAV constructs containing modified FVIII cDNAs have been designed and shown to be functional in preclinical models.[24,27,36] McIntosh et al.[24] compared various FVIII constructs in mice and rhesus monkeys and were able to achieve clinically meaningful FVIII expression and activity that also corrected prolonged bleeding time in a mouse model of hemophilia A.[24] Various parameters were systematically tested, and it was concluded that the best results in mice were obtained with an AAV8 vector using a hybrid liver-specific promotor (HLP) to drive expression of a codon-optimized FVIII gene, with a highly glycosylated, truncated B-domain (V3). Although some of these features, including codon optimization and the use of the HLP, were incorporated into

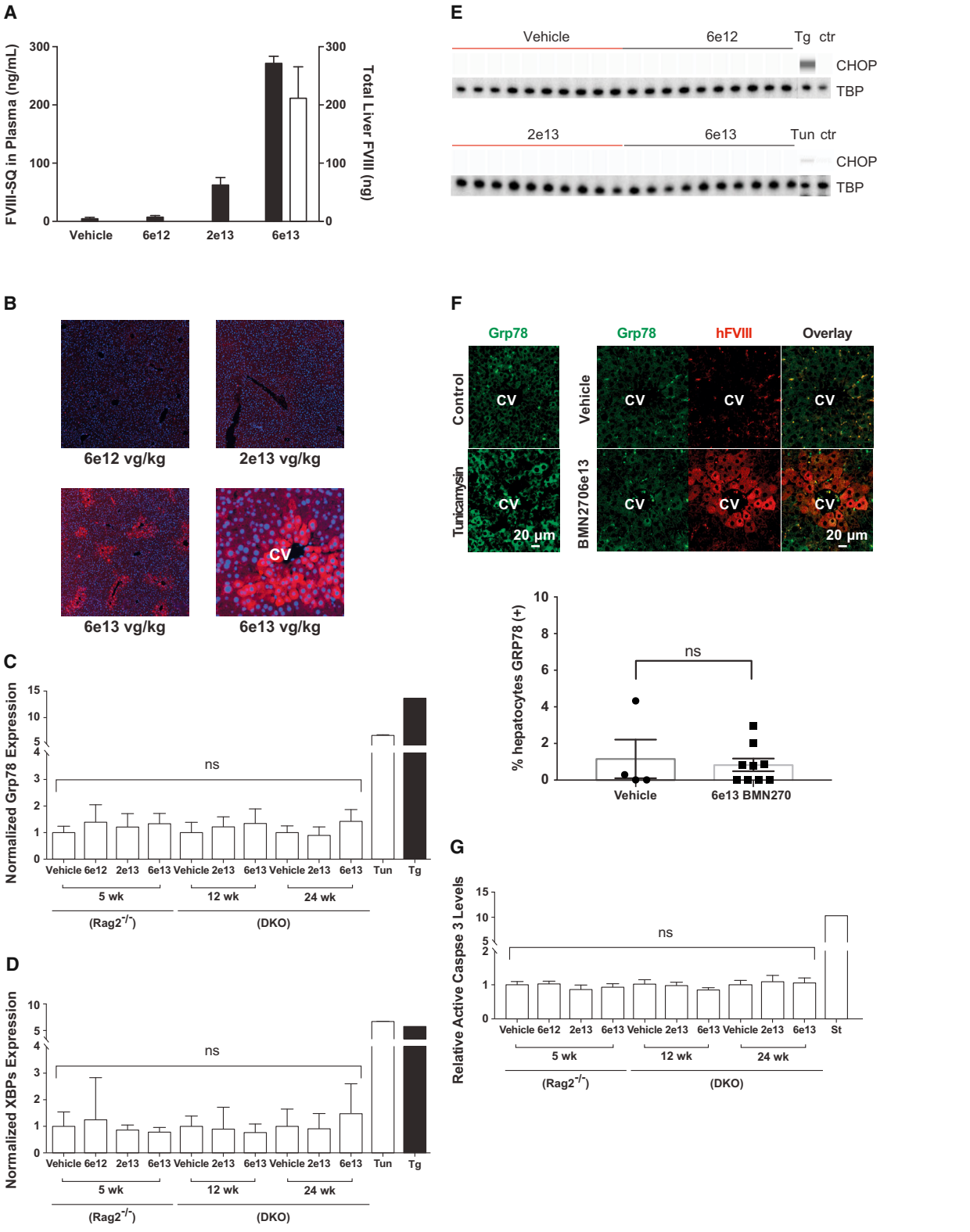

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Table 1. Peak Circulating Levels of hFVIII-SQ Protein in Monkeys Dosed with BMN 270

Dose of BMN 270 (vg/kg)	Animal ID	Peak Plasma FVIII-SQ Level (ng/mL)
1.0e13	1001	22.8
	1002	4.8
3.6e13	2001	41.3
	2002	45.6

The study was designed to dose two groups of male cynomolgus monkeys (n = 2/group) with BMN 270 at two vector levels (1e13 and 3.6e13 vg/kg).

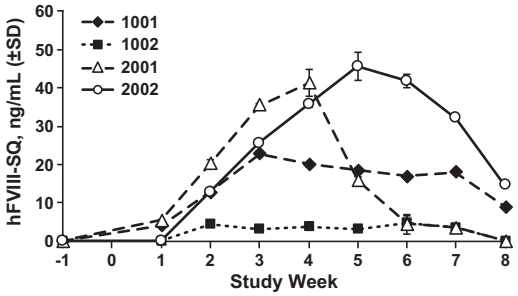

Figure 7. Evaluation of AAV5-hFVIII-SQ Expression in Nonhuman Primates
Comparison of circulating hFVIII-SQ levels expressed from BMN 270 in cynomolgus monkeys. Vectors were administered via peripheral vein in groups of two male cynomolgus monkeys at low dose (1001 and 1002 at 1.0e13 vg/kg) or high dose (2001 and 2002 at 3.6e13 vg/kg). Weekly plasma samples were collected from 1 week prior to and up to 8 weeks post-dosing (at necropsy) and analyzed for hFVIII-SQ levels. Error bars are SD.

BMN 270, we chose to use BDD FVIII and AAV5 capsids for several reasons. First, FVIII protein produced from BMN 270 has a similar amino acid sequence to that of BDD recombinant proteins, such as Xyntha, a commonly used recombinant FVIII replacement product that has been demonstrated to be safe and well tolerated without increases in the frequency of neutralizing antibodies against FVIII compared with full-length hFVIII protein products.[37] Therefore, patients will have been exposed to the BDD form of FVIII during FVIII protein therapy, reducing the likelihood of a novel immune response to the same protein produced endogenously following AAV gene therapy. Second, the decision to use the AAV5 vector to deliver the FVIII gene was based on efficacy and safety considerations. Previously, transduction efficiency in humans using AAV2 vectors has been impaired by pre-existing immunity. AAV2 is endemic in humans with neutralizing antibodies and/or other factors against AAV2 found in more than 70% of individuals.[8] Furthermore, it has been shown that pre-existing antibody titers in excess of 1:10 substantially diminished AAV8-based hepatocyte transduction in rhesus monkeys.[38] Therefore, efficacious AAV-based gene therapy for hemophilia patients may require a vector system that is not subjected to pre-existing neutralizing factors against the specific AAV capsids. The prevalence of different AAV serotypes has been shown to vary with geographic location and age.[39–41] Several groups reported the highest neutralizing factor seroprevalences were observed for AAV2 (10%–60%) and AAV1 (3%–67.7%), while the lowest were observed for AAV8 (1%–19%) and AAV5 (3.2%–9%) in adult populations in developed countries, while higher prevalences for AAV2 (96.6%), AAV8 (82%), and AAV5 (40.2%) were observed in normal Chinese subjects.[40,42–45] In the Chinese study, low titers were detected in more than 80% of AAV5-seropositive subjects.[42] Li et al.[41] reported higher AAV5 neutralizing antibody (NAb) prevalence, at 25.8%, in pediatric patients with hemophilia but suggested that these NAbs were detected because of partial cross-reactivity of AAV2-directed NAb. Therefore, AAV5 may allow successful treatment of more patients. Third, it is well established that AAV5 capsids result in lower liver transduction and transgene expression in mice compared with AAV8.[24,46] In previous studies using a self-complementary AAV8 vector to express hFIX, a significant drop in expression levels was observed when comparing mice with primates treated at similar dose levels.[10,33] A further drop was observed from primate to human with the same AAV8 construct.[10–12] Our data with BMN 270 showed that the drop in FVIII expression is less than 2-fold from mice to primates, suggesting better species scaling for AAV5 capsids. Recently, results from BMN 270 phase I/II human clinical trial showed that mean FVIII levels plateaued at 20 weeks at 104% of normal hFVIII,[47] while in the mouse experiments, it reached steady-state levels of FVIII of 380% at 12 weeks (Figure 3C). This results in approximately 3.7-fold drop of FVIII expression of BMN 270 from mouse to human.

Figure 6. Human FVIII-SQ Expression in BMN 270-Transduced Hepatocytes Exhibited Peri-central Vein Pattern and Did Not Induce ER Stress in Mouse Liver
Groups of 10 RAG2$^{-/-}$ mice were injected with vehicle or 6e12, 2e13, or 6e13 vg/kg of BMN 270 through tail vein injection. After 5 weeks, plasma and liver tissues were isolated for analysis. (A) Human FVIII-SQ protein concentration in plasma samples (black bars) and total amount of FVIII-SQ protein in liver homogenates (white bar) were measured by ELISA. The total amount of FVIII-SQ in the liver was back-calculated on the basis of protein concentration of the homogenate and total liver weight. (B) Mouse liver tissue sections were analyzed for immunolocalization of hFVIII-SQ protein. Red, human FVIII-SQ; blue, DAPI. CV, central vein. Groups of 10 Rag2$^{-/-}$ mice were injected with vehicle or 6e12, 2e13, or 6e13 vg/kg of BMN 270 and taken down 5 weeks post-dosing, or groups of six or seven DKO mice were injected with vehicle or 2e13 or 6e13 vg/kg of BMN 270 12 or 24 weeks post-dosing. (C) Grp78 and (D) spliced XBP-1 mRNA in mouse liver tissues were analyzed using reverse transcription followed by droplet digital PCR. Tun, liver from tunicamycin treated mouse; Tg, cells treated with thapsigargin, as positive controls for ER stress induction. The values were normalized to GAPDH levels and expressed in fold relative to vehicle (for BMN 270 treated) or negative controls (for Tun or Tg). (E) Western blot analysis of mouse liver tissue samples for detection of CHOP and loading control TATA-box binding protein (TBP). (F) Immunofluorescence staining of hFVIII and Grp78, as well as overlaid images around central vein (CV) region in liver sections of Rag2$^{-/-}$ mice treated with vehicle or 6e13 vg/kg of BMN 270 and taken down 5 weeks post-dosing. Scale bar, 20 μM. Quantitative analysis of percent hepatocytes with signals above endogenous levels of Grp78. (G) Active caspase-3 levels were measured in mouse liver tissue extracts by ELISA. All bar graph values represent mean ± SEM. Multiple comparisons of mean values to detect significant differences among groups were performed by ANOVA followed by a subsequent Tukey test for repeated-measures. ns, not significant.

In vitro and *in vivo* studies have shown that overexpression of FVIII can result in a UPR or cellular stress response related to the ER, which can ultimately lead to apoptosis.[27] It was important, therefore, to determine if the balance of promotor strength, codon optimization, and choice of FVIII construct would result in clinically meaningful plasma levels of FVIII without inducing a cellular stress response *in vivo*. Zolotukhin et al.[29] described transient induction of hepatocyte ER stress by AAV8-BDD-FVIII administration is mouse strain dependent. Specifically, 129/BL6-F8$^{-/r}$ mice showed no evidence of ER stress, while there was clear induction of ER stress in C57BL/6 mice. Although the background of Rag2$^{-/-}$ mice is B6.129S6-Rag2^{tm1Fwa}, ER stress induction was observed in this strain of mice when they were treated with an AAV5-FVIII-SQ construct driven by a stronger promoter (data not shown). Our data showed no evidence of increased cellular stress response even at BMN 270 doses that produced supraphysiological plasma levels of FVIII.[28,29]

In summary, our findings demonstrate that a dose-dependent expression of hFVIII was produced in mice and primates following administration of BMN 270. The FVIII levels that were achieved following a single administration of BMN 270 normalized the bleeding phenotype in hemophilic mice. The FVIII produced was similar in size to exogenously administered BDD recombinant protein, and equally effective at correcting the prolonged bleeding time. Even at supraphysiological levels of expression, there was no evidence of hepatocyte stress. BMN270 is currently being investigated as a potential therapy for patients with severe hemophilia A.[48]

MATERIALS AND METHODS

Construct and AAV Vector

The BMN 270 (AAV5-hFVIII-SQ) vector is a replication-incompetent AAV5 vector containing the hFVIII-SQ expression cassette flanked by AAV2 inverted terminal repeats (ITRs) at its 5′ and 3′ ends. The expression cassette sequence is composed of a hybrid human liver-specific promoter (HLP), a codon-optimized human BDD coagulation FVIII cDNA, and a synthetic polyadenylation signal (Synth pA) (U.S. Patent No. 9,504,762) (Figure 1). The WT B domain is replaced by a 14-amino acid "SQ" linker sequence such that the protein produced is identical to the rhFVIII product Xyntha. The codons of the entire coding region are optimized for expression in human cells.[49] The hFVIII-SQ DNA was packaged into AAV5 viral particles by using either a "triple transfection" process in 293 cells or Sf9 host cells infected with helper and vector baculovirus.[25]

Mouse Studies

All *in vivo* mouse experimentation was performed in accordance with institutional guidelines under protocols approved by the Institutional Animal Care and Use Committee of the Buck Institute.

RAG2/FVIII DKO Mice

The FVIII$^{-/-}$ mouse (B6;129S-F8^{tm1Kaz}/J; Jackson Laboratories) is homozygous for a targeted, X chromosome-linked mutant allele.[50] Homozygous females and carrier males have less than 1% of normal mouse FVIII activity, exhibit prolonged clotting times, and recapitulate the bleeding phenotype of human hemophilia A. The RAG2$^{-/-}$ mouse (B6.129S6-Rag2^{tm1Fwa}; Taconic) contains a disruption of the recombination activating gene 2 (RAG2), which prevents homozygous mice from initiating V(D)J rearrangement and generating mature T or B lymphocytes,[51] minimizing the chance of antibody production against foreign protein. For these studies, RAG2$^{-/-}$ mice, RAG2$^{-/-}$ X FVIII$^{-/-}$ DKO mice, or WT mice (C57BL/6J; Jackson Laboratories) were used as the test animals. DKO mice were bred in house by breeding male RAG2$^{-/-}$ mice with female FVIII$^{-/-}$ mice to generate the first filial (F1) generation of heterozygotes. Mating F1 mice yielded mice homozygous for both mutations at a frequency approximating one in eight offspring. Male and female DKO mice were mated to each other to stably propagate the line.

Administration of Vectors

In these studies, only male mice were used to avoid possible gender differences in rAAV gene transfer and because hemophilia A is primarily a male disease.[52] All animals were 8–9 weeks old at the time of dosing, except for a group of 16-week-old mice serving as rhFVIII-positive controls in the bleeding time study. AAV5-hFVIII-SQ vector, prepared in vehicle (0.001% pluronic F-68 in Dulbecco's PBS), rhFVIII (Xyntha; Wyeth BioPharma), or PBS was administered to mice via single i.v. bolus tail vein injection (4 μL/g body weight). At termination, mice were deeply anesthetized with inhaled isoflurane and exsanguinated via cardiac puncture. Blood was collected with sodium citrate anticoagulant (0.38% final concentration). Following cervical dislocation, liver samples were harvested and half snap frozen for biochemical and half fixed in 10% formalin for immunohistochemical analysis.

Tail Bleeding Model

The tail bleeding study was performed as described previously.[53,54] Eight weeks after treatment with either BMN 270 vector or vehicle (10 mM sodium phosphate, 140 mM NaCl, 2% mannitol, 0.2% pluronic F-68 [pH 7.4]), each DKO mouse was anesthetized with 2.5%–3% inhaled isoflurane and remained anesthetized throughout the experiment. Body temperature was maintained by placing the mouse on a temperature-controlled heating pad. The mouse tail was immersed in 37°C saline for 10 min to standardize the local blood circulation. The tail was then removed from the warmed saline and transected 6 mm from the tip using a disposable surgical blade. After transection, the tail was immediately placed in a pre-weighed 15 mL conical tube filled with 37°C saline for 30 min. Tubes were re-weighed at the end of the 30-minute period, and blood loss was quantified as the difference in pre- and post-test weights. The time (minutes) to cessation of blood flow was also noted. For mice in which bleeding never stopped, 30 min was the cutoff time, and that value was used for statistical analysis. To compare the effect of the gene therapy to treatment with rhFVIII, Xyntha was administered at a dose of either 50 or 200 IU/kg by tail vein injection in two groups of 16-week-old DKO mice, and bleeding times were assessed 5 min after injection.

NHP Study

For NHP studies, male cynomolgus monkeys were received from Charles River Laboratories (CRL) and housed in the CRL facility in

Reno, Nevada. All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the CRL facility. The animals were 3.9–4.3 years old and weighed between 2.8 and 3.5 kg at the time of initiation of dosing. All monkeys were prescreened for total antibodies and neutralizing factors against AAV5. Monkeys screened negative in both tests were enrolled in the study.[34] Groups of two monkeys were dosed with BMN 270 at two dose levels (low, 1.0e13 vg/kg; and high, 3.6e13 vg/kg) into the saphenous vein. Dosing solution concentrations were determined by quantitative real-time PCR as described to determine doses given. Blood samples were collected by venipuncture from all animals 1 week prior to vector administration and at weekly intervals after dosing until the time of necropsy at 8 weeks after dosing. The blood samples were gently mixed with 3.8% sodium citrate at a 9:1 ratio and centrifuged. The resulting plasma was analyzed for hFVIII-SQ protein using an ELISA specific for hFVIII, anti-hFVIII, and AAV5 antibodies as well as for AST and ALT levels.

Detection of hFVIII-SQ Protein in Mouse or Monkey Plasma

Levels of human BDD FVIII-SQ protein in sodium citrate-anticoagulated plasma samples from mice and monkeys were measured using a sandwich ELISA or a bridging electrochemiluminescent assay (ECLA). Both assays used the same human-specific anti-FVIII capture (GMA-8023; Green Mountain Antibodies) and detection (Affinity Biologics) antibody pairs to specifically measure hFVIII and not endogenous FVIII from the test species (mouse or cynomolgus monkeys).

hFVIII was measured in plasma collected from DKO mice using the bridging ECLA. The monoclonal antibody (mAb) to the A2 domain of hFVIII was conjugated to biotin, and the sheep polyclonal antibody to hFVIII was conjugated to a ruthenium tag. Samples diluted 1:10 in blocking buffer were incubated with the conjugated reagents for 1 hr at ambient temperature before being transferred on a blocked MesoScale Discovery (MSD) streptavidin plate. After 1 hr incubation on the MSD plate to capture the hFVIII-antibody complexes via the biotin label, the plate was washed, and MSD Read Buffer containing the substrate tripropylamine (TPA) was added to the plate. In the presence of TPA, ruthenium produces an electrochemiluminescent (ECL) signal when voltage is applied to the wells of the plate. The signals detected on the MSD Sector Imager 2400 were proportional to the levels of hFVIII-SQ protein in the samples, and the concentrations were extrapolated from a standard curve. The 11-point standard curve was prepared by spiking in clinical grade recombinant BDD hFVIII (Xyntha) in pooled murine plasma with the range of 0.588–603 ng/mL.

hFVIII was measured in RAG2$^{-/-}$ mouse and cynomolgus monkey plasma or mouse liver lysate using a sandwich ELISA method. Briefly, high-binding black polypropylene plates were coated with 4 μg/mL of anti-FVIII (domain A2) antibodies. Samples were diluted 1:10 in diluent buffer and incubated for approximately 2 hr at ambient temperature. hFVIII was detected by the addition of sheep anti-FVIII antibodies conjugated to horseradish peroxidase (HRP) and incubated at ambient temperature for 1 hr. After the final wash, QuantaBlu substrate solution was used for detection. The relative fluorescent units detected on the Molecular Device M2.0E instrument were proportional to the levels of hFVIII-SQ protein in the samples, and the concentrations were extrapolated from a standard curve as described above. The ten-point standard curve was prepared by spiking in clinical grade recombinant BDD hFVIII (Xyntha) in pooled monkey plasma with the range of 0.588–301 ng/mL.

Measurement of FX Activation as a Pharmacodynamic Activity Readout of hFVIII-SQ in Mouse Plasma

Because FVIII serves as a cofactor for the enzyme factor IXa in its activation of the zymogen FX, a commercial FXa chromogenic-based kit (Chromogenix) was used to measure hFVIII activity in sodium citrate-anticoagulated murine plasma samples. The chromogenic FXa assay indirectly measures the total FVIII activity resulting from both murine endogenous FVIII and hFVIII-SQ produced from BMN 270 in the RAG2$^{-/-}$ animals. In contrast, the assay measures FX activity resulting solely from hFVIII-SQ activity produced following administration of BMN 270 in the DKO animals, which lack endogenous FVIII.

Briefly, during the first stage of the assay, an excess of tenase complex factors (purified bovine FIXa, FX, phospholipids, and calcium) except FVIII was added to plasma samples at the minimum required dilution (MRD) of 1:320 (RAG2$^{-/-}$) or 1:16 (DKO) in buffer. The mixture was then incubated at 37°C for 2 min to allow tenase complex stabilization. During the second stage, the FXa chromogenic substrate S-2765 and synthetic thrombin inhibitor I-2581 were then added to the mixture, followed by 14 min of incubation at 37°C. The FXa reaction generated chromophore pNA and was stopped by addition of 20% acetic acid. The color intensity, which was linearly proportional to the amount of FVIII present in the samples, was detected by light absorbance at 405 nm with a SpectraMax M2.0E instrument. The levels of FX activity in the samples were extrapolated from a standard curve. A seven-point standard curve was generated by spiking reference normal human plasma (George King Bio-medical) in FVIII-deficient or murine plasma (DKO) with the range of 1.31%–84% normal hFVIII activity.

Western Blot

Anti-FVIII antibodies were obtained from Green Mountain Antibodies or Affinity Biologicals. FVIII heavy and light chains were detected via prior enrichment of plasma samples using chain-specific mAbs. In each case, mAbs were dissolved in buffer (50 mM sodium carbonate [pH 9.6]) at final concentration of 2 μg/mL, and 50 μL was added to each well of Nunc Maxisorp black plates. Following incubation at 4°C for 16 hur, wells were washed with Tris-buffered saline containing 0.05% Tween-20 (TBST). Individual wells were blocked with green diluent (250 μL/well; Affinity Biologicals) at 37°C for 90 min. Mouse plasma samples (50 μL) from DKO animals treated with vehicle or BMN 270 at 6e13 vg/kg, or normal mouse plasma samples spiked with Xyntha (500 ng/mL), were diluted with equal volume of green diluent and 100 μL mixture was added to

individual wells. The mixture was incubated at room temperature for 90 min, followed by washing the wells with TBST. Lithium dodecyl sulfate (LDS) buffer (30 μL/well; Invitrogen) was added and incubated at 37°C for 10 min. Fifteen microliters of LDS was removed from each well, mixed with an equal volume of water, and 30 μL was resolved by denaturing reducing PAGE. Following transfer to nitrocellulose membrane, hFVIII-SQ heavy and light chain was detected by incubating sequentially with anti-hFVIII polyclonal or mAbs (1.0 μg/mL) and appropriate secondary conjugated alkaline phosphatase (0.25 μg/mL; Jackson ImmunoResearch). Membranes were developed using colorimetric precipitating alkaline phosphatase substrate (WesternBlue; Promega) and imaged.

Dose Concentration Analysis

Dose concentration analyses (DCA) were performed using TaqMan Probe-based qPCR to detect BMN 270 hFVIII-SQ vector genomes in dosing solutions collected from each non-clinical study. Boiled dosing samples were serially diluted and subjected to qPCR reactions consisted of the identical hFVIII-SQ-specific probe and primer set used to quantify vector genome present in liver samples. Twenty-five microliter reactions were set up with TaqMan Universal PCR Mix, 200 nM of forward and reverse primers, 100 nM of florescent probes targeting hFVIII-SQ transgene, and 10 μL of serially diluted dosing solutions. The PCR mixture was loaded into 96-well PCR plates and analyzed using an ABI Prism 7900TM Sequence Detection System. Following an initial denaturation step at 95°C for 10 min, 40 thermal cycles at 95°C for 15 s and 53°C for 1 min was performed. Fluorescence at 483–533 nm was used to detect amplicon corresponding to hFVIII-SQ vector genome. Cycle threshold (Ct) values, defined as the number of PCR cycles required for the fluorescent signal to cross the background threshold, were calculated for hFVIII-SQ amplification in each reaction. The vector genome copies present in each sample were extrapolated from the corresponding standard curves. The eight-point standard curve was generated by spiking in linearized and purified hFVIII-SQ plasmid in buffer with the range of 9.03–9.03e7 copies per reaction.

Real-Time qPCR to Detect hFVIII-SQ DNA or RNA in Mouse Liver

Total DNA and RNA were extracted from ~25 mg of monkey or mouse liver segments using the RNA/DNA/Protein All Prep kit from QIAGEN following the manufacturer's instructions. Two micrograms of total RNA was reverse-transcribed to generate first-strand cDNA using SuperScript VILO MasterMix from Invitrogen. Twenty microliter PCR reactions were set up with 2 × iQ Multiplex Powermix (Bio-Rad Laboratories), 300 nM of forward and reverse primers, and 150 nM of fluorescent probes targeting hFVIII with 100 ng of cDNA template or genomic DNA. The sequences for the primers and probe were as follows: forward primer: 5′-ATGCACAGC ATCAATGGCTA-3′; reverse primer 5′-CCATCTTGTGCTTGA AGGTG-3′; and probe FAM-CCTGAGCATTGGGGCCCAGA-BHQ1. Triplicate PCR reactions per sample were loaded into white 384-well PCR plates (Roche) and run on a Roche Light Cycler 480 II. Following denaturation at 95°C for 2 min, 50 thermal cycles were performed at 95°C for 3 s and at 60°C for 20 s. Fluorescent signals at 483–533 nm were used to detect the hFVIII amplicon. Cycle cross-point values, defined as the number of PCR cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level), were calculated in each reaction by using Light Cycler 480 software version 1.5.1. A standard curve ranging from 9 to 9e7 copies per reaction was run along with all samples tested. Linear regression was used to calculate the copies per well in each sample.

hFVIII Immunofluorescent Staining

Formalin-fixed paraffin-embedded RAG2$^{-/-}$ mouse livers were sectioned at 5 μm and collected on Superfrost Plus slides. Slides were deparaffinized and rehydrated in a series of graded ethanols. Antigen retrieval was performed at 95°C for 32 min using Ventana Discovery CC1 antigen retrieval solution (catalog no. 950-500). Sections were blocked in 2% NDS, 0.1% BSA, and 0.3% Triton in 1× TBS for 45 min at room temperature. Anti-hFVIII antibody (ab139391; Abcam) was diluted 1:1,000 in Ventana Reaction Buffer (catalog no. 950-300), and slides were incubated overnight at 4°C. Slides were washed in 1× TBS. Donkey anti-sheep 555 antibody (A21436; Life Technologies) was diluted 1:500 in Ventana Reaction Buffer, and sections were incubated for 1 hr at room temperature. Slides were washed in 1× TBS, counterstained with DAPI and mounted with Fluoromount G. Slides were imaged on a Zeiss Axio Scan.Z1 using a Plan-Apochromat 20×/0.8 objective equipped with a Hamamatsu Orca Flash camera. One whole section of liver was acquired per animal, and two regions were randomly selected, one from a pericapsular region and one more central region, for export and image analysis. Total hFVIII hepatocytes were counted using a custom ImageJ macro.[55]

ER Stress Assessment

Droplet Digital PCR Assay to Detect Grp78 and sXBP1

To determine the expression levels of ER stress markers, sXBP1 (the spliced form of XBP1 generated in response to ER stresses), and GRP78 (BiP), in mouse liver tissue samples, droplet digital PCR assay was carried out using a QX200 Droplet digital PCR system (ddPCR; Bio-Rad). Each ddPCR assay reaction (20 μL) contained 1× EvaGreen ddPCR Supermix (Bio-Rad), 125 nM gene-specific primers, and diluted cDNA (1/4 dilution for sXBP1, 1/20 dilution for GAPDH and GRP78). Droplets were generated from the reaction mix and QX200 Droplet Generation Oil for EvaGreen (Bio-Rad) using a QX200 Droplet Generator (Bio-Rad). After the droplet generation, droplets were gently transferred to 96-well PCR plates, which were subsequently sealed using a PX1 PCR plate sealer (Bio-Rad). PCR was performed in a C1000 Touch Thermal Cycler (Bio-Rad) with the following cycling conditions: 1 × (95°C, 10 min), 40 × (95°C, 30 s; 58°C, 1 min), and 1 × (4°C, 5 min; 90°C, 5 min; 4°C, hold) with 2°C/s ramp rate. Following PCR, the samples were analyzed using a QX200 Droplet Reader (Bio-Rad). Target mRNA transcript concentrations (copies per microliter) were calculated using the Poisson statistics in the QuantaSoft software (Bio-Rad). sXBP1, and GRP78 primers for ddPCR assay (Eurofins) were designed as described.[56] GAPDH primers were purchased from QIAGEN, and the expression

levels of GAPDH were used to normalize expression data obtained from sXBP-1 and GRP78 analysis.

Detection of the Proapoptotic Protein CHOP in Liver Total Protein Extract

RAG2$^{-/-}$ and DKO mouse liver protein samples at 20 μg/lane were analyzed using Wes instrument, an automated capillary immunoblot system (ProteinSimple), and the 12–230 kDa mouse master kit according to the manufacturer's instruction. As a positive control, 3 μg/lane of total protein extract from thapsigargin-treated cells was analyzed in parallel. A mouse monoclonal anti-CHOP primary antibody (MA1-250; Thermo Fisher Scientific) was used at a dilution of 1:100. Wes data analysis was performed with the Compass software (ProteinSimple).

Immunofluorescence Staining of hFVIII and Grp78

Liver tissue was immersion fixed in formalin, embedded in paraffin, and sectioned in 5-μm-thick sections onto Superfrost Plus slides. Sections were immunostained with antibodies against hFVIII (1:500, ab139391; Abcam) and BiP/GRP78 (1:200, C50B12; Cell Signaling Technology). Anti-hFVIII antibody was detected using donkey anti-sheep IgG (H+L) cross-adsorbed secondary antibody conjugated to Alexa Fluor 488 (1:250, A-11015; Thermo Fisher Scientific). Anti-GRP78 antibody was detected using donkey anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody conjugated with Alexa Fluor 555 (1:500, A-31572; Thermo Fisher Scientific). Whole sections were scanned on a Zeiss Axio Scan.Z1 using a 20× Plan-Apo objective with 0.75 NA. Five images per animal per group (with regions of interest selected around central veins) were analyzed to quantify the number of cells with elevated GRP78 signals. Because GRP78 is endogenously expressed in all hepatocytes, this quantification was designed to detect GRP78 IF signal above the endogenous level. Specifically, using liver sections from vehicle and tunicamycin-treated mice, we set the threshold for the GRP78 channel such that cells in the vehicle-treated control were negative, while cells in the tunicamycin-treated control were positive.[57] Using these settings, the percentage of total hepatocytes with GRP78 IF signal above the set threshold in the vehicle- and BMN 270-treated groups was quantified.

Analysis of Liver Apoptosis

One hundred fifty micrograms of total mouse liver protein was used from each sample to measure levels of active caspase-3 using cleaved caspase-3 (Asp175) ELISA kits from R&D Systems.

Statistical Analyses

Two-tailed Student's *t* tests and one-way ANOVA with Newman-Keuls post-test were performed with GraphPad Prism 6.04 (GraphPad Software) or Excel version 14.5.9 (Microsoft). A p value less than 0.05 was considered to indicate statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods and five figures and can be found with this article online at https://doi.org/10.1016/j.ymthe.2017.12.009.

AUTHOR CONTRIBUTIONS

Conceptualization, S. Bunting, L.T., P.C., and B.C.; Methodology, L.Z., S. Bullens, S.F., A.B., R.M., and D.H.; Investigation, L.Z., L.X., R.M., S.F., K.S., B.Y., B.H., C.-R.S., and N.G.; Resources, S.L. and P.C.; Writing – Original Draft, S. Bunting, L.Z., S. Bullens, and S.F.; Writing – Review & Editing, S. Bunting, S. Bullens, S.F., P.C., B.C., and G.V.; Supervision, S. Bunting, S. Bullens, S.F., D.H., A.B., C.O., B.C., and G.V.

CONFLICTS OF INTEREST

S. Bunting, L.Z., L.X., S. Bullens, R.M., S.F., K.S., D.H., B.Y., B.H., C.-R.S., N.G., C.O., A.B., P.C., S.L., G.V., and B.C. are employees of BioMarin Pharmaceutical. L.T. was an employee of BioMarin Pharmaceutical.

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