ADENO-ASSOCIATED VIRUS FACTOR VIII VECTORS, ASSOCIATED VIRAL PARTICLES AND THERAPEUTIC FORMULATIONS COMPRISING THE SAME

[0001] This application claims priority benefit of U.S. Provisional Patent Application No. 62/232,242 filed September 24, 2015, U.S Provisional Patent Application No. 62/323,182, filed April 15, 2016 and U.S. Provisional Application No. 62/365,544 filed July 22, 2016, which are incorporated herein by reference in their entirety.

FIELD OF INVENTION

[0002] The invention relates to adeno-associated virus (AAV) Factor VIII (FVIII) vectors, including AAV FVIII vectors with high expression activity and AAV FVIII vectors that express full-length or truncated functional FVIII protein. The invention also relates to methods of making the herein described AAV FVIII vectors, recombinant AAV FVIII virus particles comprising or expressing such vectors, associated pharmaceutical formulations comprising the same and therapeutic uses thereof.

BACKGROUND

[0003] Adeno-associated virus (AAV) is a small, replication-defective, non-enveloped animal virus that infects humans and some other primate species. Several features of AAV make this virus an attractive vehicle for delivery of therapeutic proteins by gene therapy, including, for example, that AAV is not known to cause human disease and induces a mild immune response, and that AAV vectors can infect both dividing and quiescent cells without integrating into the host cell genome. Gene therapy vectors using AAV have been successfully used in some clinical trials, for example, for the delivery of human Factor IX (FIX) to the liver for the treatment of Hemophilia B (Nathwani et al., *New Engl. J. Med.* 365:2357-2365, 2011).

[0004] AAV gene therapy vectors do have some drawbacks, however. In particular, the cloning capacity of AAV vectors is limited as a consequence of the DNA packaging capacity of the virus. The single-stranded DNA genome of wild-type AAV is about 4.7 kilobases (kb). In practice, AAV genomes of up to about 5.0 kb appear to be completely packaged, i.e., be full-length, into AAV virus particles. With the requirement that the nucleic acid genome in AAV vectors must have two AAV inverted terminal repeats (ITRs) of about 145 bases, the DNA

packaging capacity of an AAV vector is such that a maximum of about 4.4 kb of protein-coding sequence can be encapsidated.

[0005] Due to this size constraint, large therapeutic genes, i.e., those greater than about 4.4 kb in length, are generally not suitable for use in AAV vectors. One such therapeutic gene is the Factor VIII (FVIII) gene, which has an mRNA of about 7.0 kb that encodes a polypeptide of 2332 amino acids comprising, from N- to C-terminus, a 19 amino acid signal peptide, and three large domains (i.e., the heavy chain or A domain, the central or B domain, and the light chain or C domain). One strategy that had been employed to overcome the AAV vector size limitation for FVIII was to use two AAV vectors, one encoding the heavy chain or A domain, and the other encoding the light chain or C domain (see, e.g., Coutu et al., U.S. Pat. Nos. 6,221,349, 6,200,560 and 7,351,577). Another strategy to circumvent this size constraint was to generate AAV vectors encoding FVIII in which the central portion or B domain of the protein has been deleted and replaced with a 14 amino acid linker, known as the SQ sequence (Ward et al., *Blood* 117:798-807, 2011, and McIntosh et al., *Blood* 121:3335-3344, 2013).

[0006] While AAV vectors have been reported in the literature having AAV genomes of > 5.0 kb, in many of these cases the 5' or 3' ends of the encoded genes appear to be truncated (see Hirsch et al., *Molec. Ther.* 18:6-8, 2010 and Ghosh et al., *Biotech. Genet. Engin. Rev.* 24:165-178, 2007). It has been shown, however, that overlapping homologous recombination occurs in AAV infected cells between nucleic acids having 5' end truncations and 3' end truncations so that a "complete" nucleic acid encoding the large protein is generated, thereby reconstructing a functional, full-length gene.

[0007] There is a need for novel AAV vectors encoding a functional Factor VIII protein, and recombinant AAV virus particles comprising the same, useful in gene therapy approaches for the treatment of hemophilia A. As such, the present invention relates to AAV vectors that encode functionally active FVIII such that either the recombinant AAV virus encapsidates the entire nucleic acid encoding the therapeutic protein, i.e., completely packaged AAV FVIII vectors, thereby avoiding the above-mentioned problems of oversized genomes, or at least produce a functionally active Factor VIII protein, which may or may not be truncated. This invention also relates to the production of AAV FVIII vectors having high FVIII expression activity. Finally, the present invention relates to pharmaceutical formulations comprising AAV Factor VIII

vectors and/or recombinant Factor VIII AAV particles/viruses comprising any of the herein described AAV FVIII vectors, associated pharmaceutical formulations, and associated methods of administration for the treatment of hemophilia A in subjects suffering therefrom.

SUMMARY

[0007a] In one aspect, the present disclosure refers to use of a recombinant AAV5 FVIII-SQ virus in the manufacture of a medicament for treating a subject suffering from hemophilia A, wherein the medicament comprises (a) sodium phosphate dibasic at a concentration of from 0.1 mg/ml to 3 mg/ml, , sodium phosphate monobasic at a concentration of from 0.1 mg/ml to 3 mg/ml, sodium chloride at a concentration of from 1 mg/ml to 20 mg/ml, mannitol at a concentration of from 5 mg/ml to 40 mg/ml, and poloxamer 188 at a concentration of from 0.1 mg/ml to 4 mg/ml, wherein AAV5-FVIII-SQ comprises an AAV2 5′ inverted terminal repeat (ITR), a liver specific transcriptional regulatory region, a functionally active FVIII coding region, a polyadenylation sequence, an AAV2 3′ ITR, and optionally one or more introns, wherein the functionally active FVIII coding region comprises nucleotides 403-4776 of SEQ ID NO: 1 and wherein the medicament is to be administered intravenously.

[0007b] In another aspect, the present disclosure refers to a pharmaceutical formulation comprising a recombinant AAV5-FVIII-SQ virus, a buffering agent, an isotonicity agent, a bulking agent and a surfactant, wherein the formulation comprises a) sodium phosphate dibasic at a concentration of from 0.1 mg/ml to 3 mg/ml, sodium phosphate monobasic at a concentration of from 0.1 mg/ml to 3 mg/ml, sodium chloride at a concentration of from 1 mg/ml to 20 mg/ml, mannitol at a concentration of from 5 mg/ml to 40 mg/ml, and poloxamer 188 at a concentration of from 0.1 mg/ml to 4 mg/ml, wherein AAV5-FVIII-SQ comprises an AAV2 5′ inverted terminal repeat (ITR), a liver specific transcriptional regulatory region, a functionally active FVIII coding region, a polyadenylation sequence, an AAV2 3′ ITR, and optionally one or more introns, wherein the functionally active FVIII coding region comprises nucleotides 403-4776 of SEQ ID NO: 1 and wherein the formulation is to be administered intravenously,.

[0008] The present disclosure provides AAV vectors encoding functionally active FVIII (referred to herein as "AAV FVIII vectors"). The recombinant AAV vectors of the present disclosure include non-naturally occurring derivatives of the AAV virus into which nucleic acid sequences encoding a functional FVIII protein have been introduced. The genomes encoding functionally active FVIII are preferably at most 7.0 kb in length, more preferably at most 6.5 kb in length, yet more preferably at most 6.0 kb in length, yet more preferably at most 5.5 kb in length, yet more preferably at most 5.0 kb in length, with enhanced promoter function.

As used herein, a "functionally active FVIII" is a FVIII protein that has the functionality of a wild-type FVIII protein *in vitro*, when expressed in cultured cells, or *in vivo*, when expressed in cells or body tissues. This includes, for example, functionally contributing in the blood coagulation cascade and/or reducing the time that it takes for blood to clot in a subject suffering from hemophilia A. Wild-type FVIII participates in blood coagulation via the coagulation cascade, acting as a co-factor for activated FIX (FIXa) which, in the presence of calcium ions and phospholipids forms a complex that converts Factor X (FX) into activated FX (FXa). Accordingly, a functionally active FVIII can form a complex with FIXa, which can convert FX to FXa. One example of a functionally active FVIII protein is a FVIII SQ protein as described in WO 2015/038625, herein incorporated by reference.

[0009] As used herein, an "AAV vector" refers to nucleic acids, either single-stranded or double-stranded, having an AAV 5' inverted terminal repeat (ITR) sequence and an AAV 3' ITR flanking a protein-coding sequence (preferably a functional Factor VIII-encoding sequence) operably linked to transcription regulatory elements that are heterologous to the AAV viral genome, i.e., one or more promoters and/or enhancers and, optionally, a polyadenylation sequence and/or one or more introns inserted between exons of the protein-coding sequence. A single-stranded AAV vector refers to nucleic acids that are present in the genome of an AAV virus particle, and can be either the sense strand or the anti-sense strand of the nucleic acid

sequences disclosed herein. The size of such single-stranded nucleic acids is provided in bases. A double-stranded AAV vector refers to nucleic acids that are present in the DNA of plasmids, e.g., pUC19, or genome of a double-stranded virus, e.g., baculovirus, used to express or transfer the AAV vector nucleic acids. The size of such double-stranded nucleic acids in provided in base pairs (bp).

[0011] The term "inverted terminal repeat (ITR)" as used herein refers to the art-recognized regions found at the 5' and 3' termini of the AAV genome which function in cis as origins of DNA replication and as packaging signals for the viral genome. AAV ITRs, together with the AAV rep coding region, provide for efficient excision and rescue from, and integration of a nucleotide sequence interposed between two flanking ITRs into a host cell genome. Sequences of certain AAV-associated ITRs are disclosed by Yan et al., *J. Virol.* 79(1):364-379 (2005) which is herein incorporated by reference in its entirety. ITR sequences that find use herein may be full length, wild-type AAV ITRs or fragments thereof that retain functional capability, or may be sequence variants of full-length, wild-type AAV ITRs that are capable of functioning in cis as origins of replication. AAV ITRs useful in the recombinant AAV FVIII vectors of the present disclosure may derive from any known AAV serotype and, in certain preferred examples, derive from the AAV2 or AAV5 serotype.

[0012] A "transcription regulatory element" refers to nucleotide sequences of a gene involved in regulation of genetic transcription including a promoter, plus response elements, activator and enhancer sequences for binding of transcription factors to aid RNA polymerase binding and promote expression, and operator or silencer sequences to which repressor proteins bind to block RNA polymerase attachment and prevent expression. The term "liver specific transcription regulatory element" refers to a regulatory element that modulates gene expression specifically in the liver tissue. Examples of liver specific regulatory elements include, but are not limited to, the mouse thyretin promoter (mTTR), the endogenous human factor VIII promoter (F8), human alpha-1-antitrypsin promoter (hAAT) and active fragments thereof, human albumin minimal promoter, and mouse albumin promoter. Enhancers derived from liver specific transcription factor binding sites are also contemplated, such as EBP, DBP, HNF1, HNF3, HNF4, HNF6, with Enh1.

[0013] In one example, the AAV vector of the disclosure comprises a nucleic acid encoding functionally active FVIII protein having the B domain replaced by the 14 amino acid SQ sequence. The SQ sequence is disclosed in Ward et al., *Blood*, 117:798-807, 2011, McIntosh et al., *Blood* 121:3335-3344, 2013, WO 2013/186563 and WO 2015/038625. The FVIII coding region sequence may be a codon-optimized FVIII-encoding sequence (see, e.g., WO 2011/005968, published January 13, 2011, WO 2015/038625, published March 19, 2015, and McIntosh et al., *Blood* 121:3335-3344, 2013, which are incorporated herein by reference in their entirety). In a preferred example, the nucleic acid encoding the functionally active human FVIII protein of the AAV vector or recombinant AAV virus particle consists of nucleotides 403 to 4776 of SEQ ID NO:1. This sequence is herein referred to as "FVIII - SQ".

[0014] In a first example, the recombinant AAV vector of the disclosure comprises Proto 1, which is depicted schematically in Figure 2A, and comprises the nucleic acid sequence set forth in SEQ ID NO:1.

[0015] In a second example, the recombinant AAV vector of the disclosure comprises Proto 1S, which is depicted schematically in Figure 2B, and comprises the nucleic acid sequence set forth in SEQ ID NO:2.

[0016] In a third example, the recombinant AAV vector of the disclosure comprises Proto 2S, which is depicted schematically in Figure 2C, and comprises the nucleic acid sequence set forth in SEQ ID NO:3.

[0017] In a fourth example, the recombinant AAV vector of the disclosure comprises Proto 3S, which is depicted schematically in Figure 2D, and comprises the nucleic acid sequence set forth in SEQ ID NO:4.

[0018] In another example, the recombinant AAV vector of the disclosure comprises a nucleic acid encoding functional FVIII lacking the entire B domain, including the SQ sequence, and the a3 domain, which is located just N-terminal to the light chain or C domain. The FVIII coding region sequence may be a codon-optimized sequence (see, e.g., WO 2011/005968, published January 13, 2011, WO 2015/038625, published March 19, 2015, and McIntosh et al., *Blood* 121:3335-3344, 2013).

[0019] In a first example, the recombinant AAV vector of the disclosure comprises Proto 4, which is depicted schematically in Figure 3A, and comprises the nucleic acid sequence set forth in SEQ ID NO:5.

[0020] In a second example, the recombinant AAV vector of the disclosure comprises Proto 5, which is depicted schematically in Figure 3B, and comprises the nucleic acid sequence set forth in SEQ ID NO:6.

[0021] In a third example, the recombinant AAV vector of the disclosure comprises Proto 6, which is depicted schematically in Figure 3C, and comprises the nucleic acid sequence set forth in SEQ ID NO:7.

[0022] In a fourth example, the recombinant AAV vector of the disclosure comprises Proto 7, which is depicted schematically in Figure 3D, and comprises the nucleic acid sequence set forth in SEQ ID NO:8.

[0023] In other examples, the recombinant AAV vector of the disclosure comprises a nucleic acid comprising an AAV2 5' inverted terminal repeat (ITR) (which may or may not be modified as known in the art), a liver-specific transcription regulatory region, a codon-optimized functionally active FVIII coding region, optionally one or more introns, a polyadenylation sequence, and an AAV2 3' ITR (which may or may not be modified as known in the art). In a preferred example, the liver-specific transcription regulatory region comprises a shortened ApoE enhancer sequence, a 186 base human alpha anti-trypsin (hAAT) proximal promoter, including 42 bases of the 5' untranslated region (UTR), and one or more enhancers selected from the group consisting of (i) a 34 base human ApoE/C1 enhancer, (ii) a 32 base human AAT promoter distal X region and (iii) 80 additional bases of distal element of the human AAT proximal promoter; and a codon-optimized functionally active FVIII coding region encoding the FVIII - SQ variant. In another preferred example, the liver specific transcription regulatory region comprises an α1-microglobulin enhancer sequence and the 186 base human alpha anti-trypsin (AAT) proximal promoter.

[0024] In a first example, the recombinant AAV vector of the disclosure comprises Construct 100ATG comprising the nucleic acid sequence forth in SEQ ID NO:9.

[0025] In a second example, the recombinant AAV vector of the disclosure comprises Construct 100ATG bGH poly A comprising the nucleic acid sequence set forth in SEQ ID NO:10.

[0026] In a third example, the recombinant AAV vector of the disclosure comprises Construct 100ATG short bGH polyA sequence set forth in SEQ ID NO:11.

[0027] In a fourth example, the recombinant AAV vector of the disclosure comprises Construct 103ATG comprising the nucleic acid sequence forth in SEQ ID NO:12.

[0028] In a fifth example, the recombinant AAV vector of the disclosure comprises Construct 103ATG short bGH poly A comprising the nucleic acid sequence set forth in SEQ ID NO:13.

[0029] In a sixth example, the recombinant AAV vector of the disclosure comprises Construct 105ATG bGH poly A comprising the nucleic acid sequence set forth in SEQ ID NO:14.

[0030] In a seventh example, the recombinant AAV vector of the disclosure comprises Construct DC172ATG FVIII comprising the nucleic acid sequence set forth in SEQ ID NO:15.

[0031] In an eighth example, the recombinant AAV vector of the disclosure comprises Construct DC172ATG FVIII hAAT comprising the nucleic acid sequence set forth in SEQ ID NO:16.

[0032] In a ninth example, the recombinant AAV vector of the i disclosure comprises Construct DC172 2xHCR ATG FVIII comprising the nucleic acid sequence set forth in SEQ ID NO:17.

[0033] In a tenth example, the recombinant AAV vector of the disclosure comprises Construct DC172 2xHCR ATG FVIII hAAT comprising the nucleic acid sequence set forth in SEQ ID NO:18.

[0034] In an eleventh example, the recombinant AAV vector of the disclosure comprises Construct 2x SerpinA hAAT ATG FVIII comprising the nucleic acid sequence set forth in SEQ ID NO:19.

[0035] In a twelfth example, the recombinant AAV vector of the disclosure comprises Construct 2x SerpinA hAAT ATG FVIII 2x μ-globulin enhancer comprising the nucleic acid sequence set forth in SEQ ID NO:20.

[0036] In a thirteenth example, the recombinant AAV vector of the disclosure Construct 100ATG short polyA 2x μ-globulin enhancer comprising the nucleic acid sequence set forth in SEQ ID NO:21.

[0037] In a fourteenth example, the recombinant AAV vector of the disclosure comprises Construct Factor VIII-BMN001 comprising the nucleic acid sequence set forth in SEQ ID NO:22.

[0038] In a fifteenth example, the recombinant AAV vector of the disclosure comprises Construct Factor VIII-BMN002 sequence set forth in SEQ ID NO:23.

[0039] In a sixteenth example, the recombinant AAV vector of the disclosure comprises Construct 99 comprising the nucleic acid sequence set forth in SEQ ID NO:24.

[0040] In a seventeenth example, the recombinant AAV vector of the disclosure comprises Construct 100 comprising the nucleic acid sequence set forth in SEQ ID NO:25.

[0041] In an eighteenth example, the recombinant AAV vector of the disclosure comprises Construct 100 reverse orientation comprising the nucleic acid sequence set forth in SEQ ID NO:26.

[0042] In a nineteenth example, the recombinant AAV vector of the disclosure Construct 100AT comprising the nucleic acid sequence set forth in SEQ ID NO:27.

[0043] In a twentieth example, the recombinant AAV vector of the disclosure Construct 100AT 2x MG comprising the nucleic acid sequence set forth in SEQ ID NO:28.

[0044] In a twenty-first example, the recombinant AAV vector of the disclosure comprises Construct 100AT 2x MG bGH polyA comprising the nucleic acid sequence set forth in SEQ ID NO:29.

[0045] In a twenty-second example, the recombinant AAV vector of the disclosure comprises Construct 100AT 2x MG (reverse) bGH polyA comprising the nucleic acid sequence set forth in SEQ ID NO:30.

[0046] In a twenty-third example, the recombinant AAV vector of the disclosure comprises Construct 100 bGH polyA comprising the nucleic acid sequence set forth in SEQ ID NO:31.

[0047] In a twenty-fourth example, the recombinant AAV vector of the disclosure comprises Construct 100-400 comprising the nucleic acid sequence set forth in SEQ ID NO:32.

[0048] In a twenty-fifth example, the recombinant AAV vector of the disclosure comprises Construct 101 comprising the nucleic acid sequence set forth in SEQ ID NO:33.

[0049] In a twenty-sixth example, the recombinant AAV vector of the disclosure comprises Construct 102 sequence comprising the nucleic acid sequence set forth in SEQ ID NO:34.

[0050] In a twenty-seventh example, the recombinant AAV vector of the disclosure comprises Construct 103 comprising the nucleic acid sequence set forth in SEQ ID NO:35.

[0051] In a twenty-ninth example, the recombinant AAV vector of the disclosure comprises Construct 103 reverse orientation comprising the nucleic acid sequence set forth in SEQ ID NO:36.

[0052] In a thirtieth example, the recombinant AAV vector of the disclosure comprises Construct 103AT comprising the nucleic acid sequence set forth in SEQ ID NO:37.

[0053] In a thirty-first example, the recombinant AAV vector of the disclosure comprises Construct 103AT 2xMG comprising the nucleic acid sequence set forth in SEQ ID NO:38.

[0054] In a thirty-second example, the recombinant AAV vector of the disclosure comprises Construct 103AT 2xMG bGH polyA comprising the nucleic acid sequence set forth in SEQ ID NO:39.

[0055] In a thirty-third example, the recombinant AAV vector of the disclosure comprises the Construct 103 bGH polyA comprising the nucleic acid sequence set forth in SEQ ID NO:40.

[0056] In a thirty-fourth example, the recombinant AAV vector of the disclosure comprises Construct 104 comprising the nucleic acid comprising the nucleic acid sequence set forth in SEQ ID NO:41.

[0057] In a thirty-fifth example, the recombinant AAV vector of the disclosure comprises Construct 105 comprising the nucleic acid sequence set forth in SEQ ID NO:42.

[0058] In a thirty-sixth example, the recombinant AAV vector of the disclosure comprises Construct 106 comprising the nucleic acid sequence set forth in SEQ ID NO:43.

[0059] In a thirty-seventh example, the recombinant AAV vector of the disclosure comprises Construct 106AT comprising the nucleic acid sequence set forth in SEQ ID NO:44.

[0060] In a thirty-eighth example, the recombinant AAV vector of the disclosure comprises p-100 ATGB, which comprises the nucleic acid sequence set forth in SEQ ID NO:45.

[0061] In yet other examples, the present disclosure is directed to vector constructs encoding a functional Factor VIII polypeptide, wherein said constructs comprise one or more of the individual elements of the above described constructs and combinations thereof, in one or more different orientation(s). The present disclosure is also directed to the above described constructs in an opposite orientation. The present disclosure is also directed to recombinant AAV virus particles comprising the herein described AAV FVIII vectors and their use for the treatment of hemophilia A.

[0062] The AAV vectors of the disclosure in single strand form are less than about 7.0 kb in length, or is less than 6.5kb in length, or is less than 6.4 kb in length, or is less than 6.3 kb in length, or is less than 6.2 kb in length, or is less than 6.0 kb in length, or is less than 5.8 kb in length, or is less than 5.6 kb in length, or is less than 5.5 kb in length, or is less than 5.4 kb in length, or is less than 5.4 kb in length, or is less than 5.2 kb in length or is less than 5.0 kb in length. The AAV vectors of the disclosure in single strand form range from about 5.0 kb to about 6.5 kb in length, or ranges from about 4.8 kb to about 5.2 k in length, or 4.8 kb to 5.3 kb in length, or ranges from about 4.9 kb to about 5.5 kb in length, or about 4.8 kb to about 6.0 kb in length, or about 5.0 kb to 6.2 kb in length or about 5.1 kb to about 6.3 kb in length ,or about 5.2 kb to about 6.4 kb in length, or about 5.5 kb to about 6.5 kb in length.

[0063] In another example, the disclosure provides for methods of producing a recombinant adeno-associated virus (AAV) particles comprising any of the AAV vectors of the disclosure. The methods comprise the steps of culturing a cell that has been transfected with any of the AAV vectors of the disclosure (in association with various AAV cap and rep genes) and recovering recombinant AAV FVIII virus particles from the supernatant of the transfected cell.

[0064] The cells of the disclosure useful for recombinant AAV production are any cell type susceptible to baculovirus infection, including insect cells such as High Five, Sf9, Se301, SeIZD2109, SeUCR1, Sf9, Sf900+, Sf21, BTI-TN-5B1-4, MG-1, Tn368, HzAm1, BM-N, Ha2302, Hz2E5 and Ao38. Preferred mammalian cells used can be HEK293, HeLa, CHO, NS0, SP2/0, PER.C6, Vero, RD, BHK, HT 1080, A549, Cos-7, ARPE-19 and MRC-5.

[0065] The disclosure also provides for a recombinant viral particle comprising any of the AAV vectors of the disclosure or any viral particle produced by the forgoing methods of the disclosure.

[0066] An "AAV virion" or "AAV viral particle" or "AAV vector particle" or "AAV virus" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated polynucleotide AAV vector as described herein. If the particle comprises a heterologous polynucleotide (i.e. a polynucleotide other than a wild-type AAV genome such as a transgene to be delivered to a mammalian cell), it is typically referred to as an "AAV vector particle" or simply an "AAV vector". Thus, production of AAV vector particles necessarily includes production of AAV vector, as such a vector is contained within an AAV vector particle.

[0067] The disclosure also provides for cells comprising any of the AAV vectors of the disclosure, and viral particles produced by these cells of the disclosure.

[0068] In another example, the disclosure provides for methods of treating a patient suffering from hemophilia A comprising administering to the patient a therapeutically effective amount of any of the AAV vectors of the disclosure, or a viral particle of the disclosure or a viral particle produced by a method of the disclosure.

[0069] In another example, the disclosure provides for methods of increasing circulating FVIII protein levels in a subject in need thereof comprising administering to the subject any of the AAV vectors of the disclosure, or a viral particle of the disclosure or a viral particle produced by a method of the disclosure

[0070] In another example, the disclosure provides for methods for inducing the expression of FVIII protein in a subject in need thereof comprising administering to the subject any of the AAV vectors of the disclosure, or viral particles of the disclosure or a viral particle produced by a method of the disclosure.

[0071] In another example, the disclosure provides for methods for increasing FVIII protein expression in a subject in need thereof comprising administering to the subject any of the AAV vectors of the disclosure, or viral particles of the disclosure or a viral particle produced by a method of the disclosure.

[0072] The disclosure also provides for any of the methods of the disclosure further comprising the step of determining the absence or presence of anti-AAV capsid antibodies in the serum of said subject after administration of said therapeutically effective amount of said recombinant AAV FVIII virus. In addition, the disclosure provides for any of the methods of the disclosure

further comprising the step of administering an effective amount of a corticosteroid to said subject after a determination of the presence of anti-AAV capsid antibodies in the serum of said subject is made.

[0073] In a further example, the disclosure provides for a use of any of the AAV vectors of the disclosure or recombinant AAV virus particles of the disclosure for preparation of a medicament for the treatment of hemophilia A. In one example, the medicament comprises an amount of AAV vector or recombinant AAV FVIII virus particle that expresses human FVIII in an amount effective to treat hemophilia A. The disclosure also provides for any of the uses of the disclosure wherein after administration of the medicament, the absence or presence of anti-AAV capsid antibodies in the serum of the subject is determined. If the subject is determined to have anti-AAV capsid antibodies in the serum, use of an effective amount of a corticosteroid for the preparation of a medicament for the administration to the subject having anti-AAV capsid antibodies in the serum.

[0074] In another example, the disclosure provides for a composition comprising any of the AAV vectors or recombinant AAV virus particles of the disclosure for the treatment of hemophilia A. In one example, the composition comprises an amount of AAV vector or recombinant AAV virus particles that expresses human FVIII in an amount effective to treat hemophilia A. In addition, any of the compositions of the disclosure are administered with an effective amount of a corticosteroid in a subject determined to have anti-AAV capsid antibodies in the serum after administration of the composition.

[0075] In another example, the AAV vectors of the disclosure are used to produce AAV viral particles that are useful for treating a patient suffering from hemophilia A.

[0076] In another example, the disclosure provides for pharmaceutical formulations comprising recombinant FVIII-encoding AAV virus particles as described herein. More specifically, in certain examples, the present disclosure is directed to pharmaceutical formulations that comprise a recombinant AAV FVIII-encoding virus, a buffering agent, an isotonicity agent, a bulking agent and a surfactant. In particularly preferred examples, the pharmaceutical formulations of the present disclosure comprise AAV5-FVIII-SQ, p-100 ATGB or any of the other herein described vectors and/or are stable during storage at $\leq 65^{\circ}C$ for at least 2 weeks. In yet other examples of the present disclosure, the pharmaceutical formulation comprises

sodium phosphate, dibasic at a concentration of from about 0.1 mg/ml to about 3 mg/ml, sodium phosphate monobasic monohydrate at a concentration of from about 0.1 mg/ml to about 3 mg/ml, sodium chloride at a concentration of from about 1 mg/ml to about 20 mg/ml, mannitol at a concentration of from about 5 mg/ml to about 40 mg/ml, and poloxamer 188 at a concentration of from about 0.1 mg/ml to about 4 mg/ml. In a particularly preferred example, the pharmaceutical formulation of the present disclosure comprises sodium phosphate, dibasic at a concentration of about 1.42 mg/ml, sodium phosphate monobasic monohydrate at a concentration of about 1.38 mg/ml, sodium chloride at a concentration of about 8.18 mg/ml, mannitol at a concentration of about 20 mg/ml, and poloxamer 188 at a concentration of about 2 mg/ml. The pharmaceutical formulations of the present disclosure may be in liquid form and may comprise the AAV FVIII virus particle at a concentration of from about 1E12 vg/ml to about 2E14 vg/ml, more preferably at a concentration of about 2E13 vg/ml. In one example, the pharmaceutical formulations of the disclosure are useful for intravenous administration to a human suffering from hemophilia A.

[0077] The present disclosure is also directed to methods for treating a subject suffering from hemophilia A which comprise the step of administering to the subject a therapeutically effective amount of a recombinant AAV FVIII virus, which optionally may be formulated as described above. In a preferred example, the subject suffering from hemophilia A is a human. In one example, the recombinant AAV FVIII virus is AAV5-FVIII-SQ. In one example, the step of administering is accomplished by intravenous (IV) administration. In certain examples of the present disclosure, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject, preferably at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject. In certain examples, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more weeks after administration. In certain examples, the therapeutically effective amount of AAV FVIII virus administered to the subject is least 2E13 vg/kg of body weight, sometimes at least 6E13 vg/kg of body weight. In certain examples, in addition to administration of a therapeutically effective amount of AAV FVIII virus, the subject is treated either prophylactically, therapeutically, or both with a corticosteroid to prevent and/or treat any hepatotoxicity associated with administration of the

AAV FVIII virus. In one example, associated hepatotoxicity is measured by comparing baseline (i.e., pre-dosing with FVIII AAV) alanine transaminase (ALT) levels to post-treatment ALT levels, wherein an increase in ALT levels post-dosing is evidence of associated hepatotoxicity. Prophylactic corticosteroid treatment refers to the administration of a corticosteroid to prevent hepatotoxicity and/or to prevent an increase in measured ALT levels in the subject. Therapeutic corticosteroid treatment refers to the administration of a corticosteroid to reduce hepatotoxicity caused by administration of an AVV FVIII virus and/or to reduce an elevated ALT concentration in the bloodstream of the subject caused by administration of an AAV FVIII virus. In certain examples, prophylactic or therapeutic corticosteroid treatment may comprise administration of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, or more mg/day of the corticosteroid to the subject. In certain examples, prophylactic or therapeutic corticosteroid treatment of a subject may occur over a continuous period of at least about 3, 4, 5, 6, 7, 8, 9, 10 weeks, or more.

[0078] The present disclosure is also directed to a composition comprising a therapeutically effective amount of a recombinant AAV FVIII virus for use in treating a subject suffering from hemophilia A. In one example, the AAV FVIII virus is AAV5-FVIII-SQ. In another example, the AAV FVIII virus comprises the p-100 ATGB vector. The composition optionally may be formulated as described above. In certain examples, compositions comprising a therapeutically effective amount of AAV FVIII virus are administered with a composition comprising a prophylactic and/or therapeutic corticosteroid for use in preventing and/or treating any hepatotoxicity associated with administration of the AAV FVIII virus. The composition comprising a prophylactic or therapeutic corticosteroid treatment may comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, or more mg/day of the corticosteroid. In certain examples, compositions comprising a prophylactic or therapeutic corticosteroid may be administered over a continuous period of at least about 3, 4, 5, 6, 7, 8, 9, 10 weeks, or more.

[0079] The present disclosure is also directed to use of a therapeutically effective amount of recombinant AAV FVIII virus for the preparation of a medicament for the treatment of a subject suffering from hemophilia A. In certain examples, the AAVFVIII virus is AAV5-FVIII-SQ or a virus comprising the p-100 ATGB vector. The medicament optionally may be formulated as described above. In a preferred example, the subject suffering from hemophilia A is a human. In one example, the medicament is administered by intravenous (IV) administration.

In one example of the present disclosure, administration of the medicament results in expression of at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject, preferably at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject 16 weeks or more after administration. In certain examples, the medicament also comprises a prophylactic and/or therapeutic corticosteroid for the prevention and/or treatment of any hepatotoxicity associated with administration of the AAV FVIII virus. The medicament comprising a prophylactic or therapeutic corticosteroid treatment may comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, or more mg/day of the corticosteroid. In certain examples, the medicament comprising a prophylactic or therapeutic corticosteroid may be administered over a continuous period of at least about 3, 4, 5, 6, 7, 8, 9, 10 weeks, or more.

[0080] The present disclosure is also directed to methods for reducing bleeding time during a bleeding episode in a subject suffering from hemophilia A which comprise the step of administering to the subject a therapeutically effective amount of a recombinant AAV FVIII virus as described herein, which optionally may be formulated as described above. In a preferred example, the subject suffering from hemophilia A is a human. In one example, the step of administering is accomplished by intravenous (IV) administration. In certain examples, the step of administering occurs at least about 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50 weeks, or more, prior to the bleeding episode. In one example of the present disclosure, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject, preferably at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject. In certain examples, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more weeks after administration. In certain examples, in addition to administration of a therapeutically effective amount of AAV FVIII virus, the subject is treated either prophylactically, therapeutically, or both with a corticosteroid to prevent and/or treat any hepatotoxicity associated with administration of the AAV FVIII virus, as described above.

[0081] The present disclosure is also directed to a composition comprising a therapeutically effective amount of a recombinant AAV FVIII virus for use in reducing bleeding time of a bleeding episode in a subject suffering from hemophilia A. In one example, the AAVFVIII virus is AAV5-FVIII-SQ. The composition optionally may be formulated as described above.

In a preferred example, the subject suffering from hemophilia A is a human. The composition may be administered prior to the bleeding episode. In one example, the composition is administered by intravenous (IV) administration prior to the bleeding episode. In one example of the present disclosure, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject, preferably at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject. In certain examples, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more weeks after administration. In certain examples, compositions comprising a therapeutically effective amount of AAV FVIII virus for use in reducing bleeding time are administered with a composition comprising a prophylactic and/or therapeutic corticosteroid for use in preventing and/or treating any hepatotoxicity associated with administration of the AAV FVIII virus, as described above.

[0082] The disclosure also provides for any of the methods of reducing bleeding time further comprising the step of determining the absence or presence of anti-AAV capsid antibodies in the serum of said subject after administration of said therapeutically effective amount of said recombinant AAV FVIII virus. In addition, the disclosure provides for any of the methods of reducing bleeding time further comprising the step of administering an effective amount of a corticosteroid to said subject after a determination of the presence of anti-AAV capsid antibodies in the serum of said subject is made.

[0083] The present disclosure is also directed to use of a therapeutically effective amount of recombinant AAV FVIII virus for the preparation of a medicament for reducing bleeding time of a bleeding episode in a subject suffering from hemophilia A. In one example, the AAVFVIII virus is AAV5-FVIII-SQ. The medicament optionally may be formulated as described above. In a preferred example, the subject suffering from hemophilia A is a human. The medicament may be administered prior to the bleeding episode. In one example, the medicament is administered by intravenous (IV) administration prior to the bleeding episode. In one example of the presentdisclosure, administration of the medicament results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject, preferably at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject. In certain examples, administration of the medicament results in expression of at least about 1,

2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more weeks after administration. In certain examples, medicaments comprising a therapeutically effective amount of AAV FVIII virus for reducing bleeding time also comprise a prophylactic and/or therapeutic corticosteroid for preventing and/or treating any hepatotoxicity associated with administration of the AAV FVIII virus, as described above. In addition, any of the compositions of the disclosure for use in reducing bleeding time are administered with an effective amount of a corticosteroid in a subject determined to have anti-AAV capsid antibodies in the serum after administration of the composition.

[0084] The present disclosure is also directed to methods for inducing expression of a functional FVIII protein in a subject in need thereof which comprise the step of administering to the subject a recombinant AAV FVIII virus as described herein, which optionally may be formulated as described above, wherein such administration results in increased expression of functional FVIII protein or increased concentrations of functional FVIII protein in the bloodstream of the subject. In a preferred example, the subject in need is a human. In one example, the step of administering is accomplished by intravenous (IV) administration. In one example of the present disclosure, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject, preferably at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject. In certain examples, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more weeks after administration. In certain examples, in addition to administration of an AAV FVIII virus, the subject is treated either prophylactically, therapeutically, or both with a corticosteroid to prevent and/or treat any hepatotoxicity associated with administration of the AAV FVIII virus, as described above. In addition, in any of the uses of the disclosure after administration of the medicament to reduce bleeding time, the absence or presence of anti-AAV capsid antibodies in the serum of the subject is determined. If the subject is determined to have anti-AAV capsid antibodies in the serum, use of an effective amount of a corticosteroid for the preparation of a medicament for the administration to the subject having anti-AAV capsid antibodies in the serum is contemplated.

[0085] The present disclosure is also directed to methods for increasing expression of FVIII protein in a subject in need thereof which comprise the step of administering to the subject a recombinant AAV FVIII virus as described herein, which optionally may be formulated as described above, wherein such administration results in increased expression of functional FVIII protein or increased concentrations of functional FVIII protein in the bloodstream of the subject. In a preferred example, the subject in need is a human. In one example, the step of administering is accomplished by intravenous (IV) administration. In one example of the present disclosure, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject, preferably at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject. In certain examples, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more weeks after administration. In certain examples, in addition to administration of an AAV FVIII virus, the subject is treated either prophylactically, therapeutically, or both with a corticosteroid to prevent and/or treat any hepatotoxicity associated with administration of the AAV FVIII virus, as described above.

[0086] The present disclosure is also directed to a composition comprising a therapeutically effective amount of a recombinant AAV FVIII virus for use in increasing or inducing expression of FVIII protein in a subject in need thereof. In one example, the AAVFVIII virus is AAV5-FVIII-SQ. The composition optionally may be formulated as described above. In a preferred example, the subject in need is a human suffering from hemophilia A. The composition may be administered prior to the bleeding episode. In one example, the composition is administered by intravenous (IV) administration prior to the bleeding episode. In one example of the present disclosure, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject, preferably at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject. In certain examples, the step of administration results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more weeks after administration. In certain examples, compositions comprising a therapeutically effective amount of AAV FVIII virus for use in increasing or inducing expression of FVIII protein are administered with a composition

comprising a prophylactic and/or therapeutic corticosteroid for use in preventing and/or treating any hepatotoxicity associated with administration of the AAV FVIII virus, as described above.

[0087] The present disclosure is also directed to use of a therapeutically effective amount of recombinant AAV FVIII virus for the preparation of a medicament for increasing or inducing expression of FVIII protein in a subject in need. In one example, the subject in need is a human suffering from hemophilia A. In one example, the AAVFVIII virus is AAV5-FVIII-SQ. The medicament optionally may be formulated as described above. The medicament may be administered prior to the bleeding episode. In one example, the medicament is administered by intravenous (IV) administration prior to the bleeding episode. In one example of the present disclosure, administration of the medicament results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject, preferably at least about 5 IU/dl of Factor VIII protein in the bloodstream of the subject. In certain examples, administration of the medicament results in expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more IU/dl of Factor VIII protein in the bloodstream of the subject 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more weeks after administration. In certain examples, medicaments comprising a therapeutically effective amount of AAV FVIII virus for increasing or inducing expression of FVIII protein also comprise a prophylactic and/or therapeutic corticosteroid for preventing and/or treating any hepatotoxicity associated with administration of the AAV FVIII virus, as described above.

[0088] The present disclosure is also directed to a method of treating a subject suffering from hemophilia A comprising the steps of (i) determining the absence of anti-AAV capsid antibodies in the serum of said subject, and (ii) administering to said subject a therapeutically effective amount of a recombinant AAV FVIII virus.

[0089] The present disclosure is also directed to use of a therapeutically effective amount of a recombinant AAV FVIII virus for the preparation of a medicament for the treatment of a subject suffering from hemophilia A, wherein anti-AAV capsid antibodies are absent from the serum of the subject.

[0090] The present disclosure is also directed to a composition comprising a therapeutically effective amount of a recombinant AAV FVIII virus for use in treating a subject suffering from hemophilia A, wherein anti-AAV capsid antibodies are absent from the subject's serum.

[0091] The present disclosure is also directed to a method of treating a subject suffering from hemophilia A comprising the steps of (i) administering to said subject a therapeutically effective amount of a recombinant AAV FVIII virus, and (ii) after administration of said therapeutically effective amount of said recombinant AAV FVIII virus, determining the absence or presence of anti-AAV capsid antibodies in the serum of said subject. In one example, the method further comprises the step of administering an effective amount of a corticosteroid to the subject after a determination of the presence of anti-AAV capsid antibodies in the serum of said subject is made.

[0092] The present disclosure is directed to use of a therapeutically effective amount of a recombinant AAV FVIII virus for the preparation of a medicament for the treatment of hemophilia A wherein after administration of the medicament, the absence or presence of anti-AAV capsid antibodies in the serum of the subject is determined. If the subject is determined to have anti-AAV capsid antibodies in the serum, use of an effective amount of a corticosteroid for the preparation of a medicament for administration to the subject having anti-AAV capsid antibodies in the serum. The present disclosure is also directed to a composition comprising an effective amount of recombinant AAV FVIII for treatment of hemophilia A, wherein this composition is administered with an effective amount of a corticosteroid in a subject determined to have anti-AAV capsid antibodies in the serum after administration of the composition.

[0093] Other examples of the present disclosure will be evident to one skilled in the art upon reading the present patent specification.

DESCRIPTION OF DRAWINGS

[0094] Figure 1 provides a schematic of an exemplary FVIII-encoding recombinant AAV vector. From left to right, the vector comprises an AAV2 5' ITR sequence, wild-type AAV2 viral sequence, a 34 base human ApoE/C1 enhancer sequence, a 32 base human AAT promoter distal X region sequence, a 186 base human AAT promoter sequence that includes 42 bases of 5' UTR sequence, a codon-optimized human FVIII SQ sequence, a 49 base synthetic Proudfoot polyadenylation sequence, wild-type AAV2 viral sequence, and an AAV2 3'ITR sequence (see WO 2011/005968, published January 13, 2011, which is incorporated herein by reference in its entirety, and McIntosh et al., *Blood* 121:3335-3344, 2013). This vector is 5081 bases in length.

[0095] Figure 2A - Figure 2D provide schematic representations of certain recombinant AAV FVIII vectors of the present invention. (A) Proto 1, (B) Proto 1S, (C) Proto 2S and (D) Proto 3S.

[0096] Figure 3A - Figure 3D provide schematic representations of certain recombinant AAV FVIII vectors of the present invention. (A) Proto 4, (B) Proto 5, (C) Proto 6 and (D) Proto 7.

[0097] Figure 4A - Figure 4JJ provide schematic representations of certain recombinant AAV FVIII vectors of the present invention. (A) Construct 100ATG, (B) Construct 100ATG bGH polyA, (C) Construct 100ATG short bGH poly A, (D) Construct 103ATG, (E) Construct 103ATG short bGH poly A, (F) Construct 105ATG bGH polyA, (G) Construct DC172ATG FVIII, (H) Construct DC172ATG FVIII hAAT, (I) Construct DC172 2xHCR ATG FVIII, (J) Construct DC172 2xHCR ATG FVIII hAAT, (K) Construct 2x SerpinA hAAT ATG FVIII, (L) Construct 2x SerpinA hAAT ATG FVIII 2x μ-globulin enhancer, (M) Construct 100ATG short bGH poly A 2x μ-globulin enhancer, (N) Construct Factor VIII-BMN001, (O) Construct Factor VIII-BMN002, (P) Construct 99, (Q) Construct 100, (R) Construct 100 reverse orientation, (S) Construct 100AT, (T) Construct 100AT 2x MG, (U) Construct 100AT 2x MG bGH polyA, (V) Construct 100AT 2x MG (reverse) bGH poly A, (W) Construct 100 bGH poly A, (X) Construct 100-400, (Y) Construct 101, (Z) Construct 102, (AA) Construct 103, (BB) Construct 103 reverse orientation, (CC) Construct 103AT, (DD) Construct 103AT 2x MG, (EE) Construct 103AT 2x MG bGH poly A, (FF) Construct 103 bGH poly A, (GG) Construct 104, (HH) Construct 105, (II) Construct 106 and (JJ) Construct 106AT.

[0098] Figure 5 provides the results of the evaluation of the recombinant AAV FVIII Proto constructs in Rag2 mice, and demonstrates that the Proto viral constructs transduce FVIII similarly to the vector shown in Figure 1, wherein the y-axis represents ng/ml of FVIII protein determined by ELISA analysis.

[0099] Figure 6 demonstrates that various recombinant AAV FVIII constructs of the present invention induce *in vivo* expression of FVIII protein as measured in a mouse tail vein hydrodynamic injection assay.

[00100] Figure 7 demonstrates that various recombinant AAV FVIII constructs of the present invention induce in vivo expression of FVIII protein as measured in a mouse tail vein hydrodynamic injection assay.

[00101] Figure 8 demonstrates that various recombinant AAV FVIII constructs of the present invention induce in vivo expression of FVIII protein as measured in a mouse tail vein hydrodynamic injection assay.

DETAILED DESCRIPTION

[00102] The present invention provides for AAV vectors encoding functionally active FVIII, e.g., completely packaged AAV FVIII vectors or AAV FVIII vectors with high expression activity. The recombinant AAV FVIII vectors of the invention have improved transgene expression, as well as improved AAV virus production yield and simplified purification. Introducing one or more introns into the FVIII protein-coding region enhances expression. Reconfiguring the number and positioning of enhancers also enhances expression.

Exemplary AAV FVIII Vector

[00103] The exemplary recombinant AAV FVIII vector shown in Figure 1, which is described in detail in WO 2011/005968, published January 13, 2011, which is incorporated herein by reference in its entirety, and McIntosh et al., *Blood* 121:3335-3344, 2013, is an oversized, i.e., greater than 5.0 kb, AAV FVIII vector. As shown in Figure 1, this AAV FVIII vector comprises, from left to right, the AAV serotype 2 (AAV2) 5' ITR, wild-type AAV2-derived viral sequence, a 34 base human apolipoprotein E (ApoE)/C1 enhancer element, a 32 base human alpha anti-trypsin (AAT) promoter distal X region, a 186 base human AAT (hAAT) promoter, including 42 bases of 5' untranslated region (UTR) sequence, a codon-optimized human FVIII sequence in which the FVIII B domain is replaced with the 14 amino acid SQ sequence, a 49 bases synthetic Proudfoot polyadenylation sequence, wild-type AAV2-derived viral sequence, and the AAV2 3' ITR. This vector is 5081 bases in length and, as shown in WO 2011/005968, expresses functionally active FVIII both *in vitro* and *in vivo*.

Proto 1, Proto 1S, Proto 2S and Proto 3S Vectors

[00104] To avoid problems associated with over-sized AAV vectors and/or to increase the expression of a FVIII transgene from AAV vectors, the present invention provides completely packaged, smaller, i.e., less than 5.0 kb, AAV vectors encoding a functional FVIII protein. The 4970 bp nucleotide sequence of the recombinant AAV Proto 1 construct is provided in SEQ ID NO:1.

[00105] To generate the recombinant AAV FVIII vector Proto 1, sequences that were determined to be unnecessary for production of functionally active FVIII were deleted from the vector shown in Figure 1. As shown in Example 1, 111 bases of extraneous DNA were removed, including 53 bases of wild-type AAV2 viral sequence 3' to the AAV2 5' ITR, 46 bases of AAV2 viral sequence 5' to the AAV2 3' ITR, and 12 bases adjacent to the codon-optimized FVIII protein coding region. The codon-optimized FVIII SQ sequence of the vector shown in Figure 1 was also replaced by a novel, codon-optimized FVIII SQ sequence referred to herein as "FVIII-SQ". The FVIII-SQ coding sequence (bases 403-4776 of SEQ ID NO:1) was then introduced into the Proto 1 vector. The resultant Proto 1 vector is 4970 bases in length and comprises, from left to right, a modified AAV serotype 2 (AAV2) 5' ITR, a 34 base human apolipoprotein E (ApoE)/C1 enhancer element, a 32 base human alpha anti-trypsin (AAT) promoter distal X region, a 186 base hAAT promoter, including 42 bases of 5' untranslated region (UTR) sequence, a novel codon-optimized human FVIII sequence in which the FVIII B domain is replaced with the 14 amino acid SQ sequence, a 49 bases synthetic Proudfoot polyadenylation sequence, and a modified AAV2 3' ITR. When designed, it was unknown whether the Proto 1 vector would be capable of expressing functional FVIII polypeptide, either *in vitro* or *in vivo*.

[00106] To generate the AAV vector Proto 1S, 10 bases at the 3' end of the AAV2 5' ITR, and 10 bases at the 5' end of the AAV2 3'ITR, were removed from the Proto 1 vector. The resultant Proto 1S vector is 4950 bases in length. The nucleotide sequence of sequence of Proto 1S is set forth in SEQ ID NO:2.

[00107] To generate the AAV vector Proto 2S, a synthetic 100 base intron was inserted between exons 1 and 2 of the FVIII - SQ sequence in the Proto 1S vector. The 34 base ApoE/C1 enhancer and 32 base human AAT promoter distal X region was removed from upstream of the human AAT promoter and inserted into the synthetic intron in the reverse orientation (as compared to the orientation when these elements are located upstream of the human AAT promoter). The resultant Proto 2S vector is 4983 bases in length. The nucleotide sequence of sequence of Proto 2S is set forth in SEQ ID NO:3.

[00108] To generate the AAV vector Proto 3S, the human AAT promoter distal X region was removed from the Proto 2S vector, and replaced with a second copy of the 34 bases ApoE/C1

enhancer in the reverse orientation. The resultant Proto 3S vector is 4984 bases in length. The nucleotide sequence of sequence of Proto 3S is set forth in SEQ ID NO:4.

Proto 4, Proto S, Proto 6 and Proto 7 Vectors

[00109] In an attempt to further reduce the size of the AAV FVIII vectors and/or increase the expression of the FVIII transgene from the AAV vectors, the invention also provides completely packaged, smaller, i.e., less than 5.0 kb, AAV vectors encoding B domain and a3 domain deleted FVIII.

[00110] To generate the AAV vector Proto 4, the 14 amino acid SQ sequence and the a3 domain located adjacent to the C domain was removed from the Proto 1 vector. The total amount of FVIII sequence deleted is 55 amino acids or 165 bases. The resultant Proto 4 vector is 4805 bases in length. The nucleotide sequence of sequence of Proto 4 is set forth in SEQ ID NO:5.

[00111] To generate the AAV vector Proto 5, a 129 base truncated FVIII intron was inserted between exons 1 and 2 of the codon-optimized FVIII sequence in the Proto 4 vector. The resultant Proto 5 vector is 4934 bases in length. The nucleotide sequence of sequence of Proto 5 is set forth in SEQ ID NO:6.

[00112] To generate the AAV Proto 6 vector, 34 bases of the FVIII intron were replaced with a second copy of the 34 base human ApoE/C1 enhancer in the forward orientation in the Proto 5 vector. The resultant Proto 6 vector is 4934 bases in length. The nucleotide sequence of sequence of Proto 6 is set forth in SEQ ID NO:7.

[00113] To generate the AAV Proto 7 vector, 34 bases of the FVIII intron were replaced with a second copy of the 34 base human ApoE/C1 enhancer in the reverse orientation in the Proto 5 vector. The resultant Proto 7 vector is 4934 bases in length. The nucleotide sequence of sequence of Proto 7 is set forth in SEQ ID NO:8.

Additional Recombinant AAV FVIII Vectors with Improved Promoter/Enhancer Sequences

[00114] Oversized AAV vectors with strong promoters were generated to increase expression of B domain and a3 domain deleted FVIII, and these constructs were generated with modified enhancer and/or promoter sequences. In some embodiments, the AAV FVIII vectors express a

truncated functional FVIII. These constructs comprised one or more promoter and enhancer sequences such as ApoE HCR or fragments thereof, the μ-globulin enhancer or fragments thereof, the human alpha 1 antitrypsin promoter (hAAT) or fragments thereof, Serpin A enhancer or fragments thereof, the LP1 promoter enhancer or fragments thereof or macroglobulin enhancer or fragment thereof. These constructs comprise a polyadenylation sequence such as the bGH poly A sequence or the synthetic rabbit β-globin poly A sequence. In some embodiment, the constructs comprise an intron or fragments of an intron such as a hAAT intron or a human β-globin intron. In some embodiments, the recombinant AAV FVIII vectors comprise the novel codon-optimized FVIII-SQ coding sequence.

[00115] Construct 100ATG (Figure 4A) is 5511 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:9 in which bases 1-145 are a 5' AAV2 ITR, bases 160-502 are an ApoE HCR, bases 509-726 are a hAAT promoter, bases 727-910 are a modified human β-globin 2nd intron, bases 923-5296 are FVIII-SQ, bases 5305-5352 are a synthetic rabbit β-globin poly A and bases 5367-5511 are a 3' AAV2 ITR.

[00116] Construct 100ATG bGH poly A (Figure 4B) is 5688 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:10 in which bases 1-145 are a 5' AAV2 ITR, bases 160-502 are an ApoE HCR, bases 509-726 are a hAAT promoter, bases 727-910 are a modified human β-globin 2nd intron, bases 923-5296 are FVIII-SQ, bases 5305-5529 are a bGH poly A and bases 5544-5688 are a 3' AAV2 ITR.

[00117] Construct 100ATG short bGH poly A (Figure 4C) is 5613 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:11 in which bases 1-145 are a 5' AAV2 ITR, bases 160-502 are an ApoE HCR, bases 509-726 are a hAAT promoter, bases 727-910 are a modified human β-globin 2nd intron, bases 923-5296 are FVIII-SQ, bases 5305-5454 are a short bGH poly A and bases 5469-5613 are a 3' AAV2 ITR.

[00118] Construct 103ATG (Figure 4D) is 5362 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:12 in which bases 1-145 are a 5' AAV2 ITR, bases 169-344 are four copies (4x) of a 44bp ApoE repeat, bases 360-577 are a hAAT promoter, bases 578-761 are a modified human β-globin 2nd intron, bases 774-5147 are FVIII-SQ, bases 5156-5203 are a synthetic rabbit β-globin poly A and bases 5218-5362 are a 3' AAV2 ITR.

[00119] Construct 103ATG short bGH poly A (Figure 4E) is 5464 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:13 in which bases 1-145 are a 5' AAV2 ITR, bases 169-344 are four copies (4x) of a 44bp ApoE repeat, bases 360-577 are a hAAT promoter, bases 578-761 are a modified human β-globin 2nd intron, bases 774-5147 are FVIII-SQ, bases 5156-5305 are a bGH short poly A and bases 5320-5464 are a 3' AAV2 ITR.

[00120] Construct 105ATG bGH polyA (Figure 4F) is 6354 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:14 in which bases 1-145 are a 5' AAV2 ITR, bases 173-512 are two copies (2x) of a 170 bp microglobulin enhancer, bases 519-736 are a hAAT promoter, bases 737-920 are a modified human β-globin 2nd intron, bases 933-5306 are FVIII-SQ, bases 5315-5539 are a bGH poly A, bases 5546-6195 are two copies (2x) of a 325 bp ApoE HCR and bases 6210-6354 are a 3' AAV2 ITR.

[00121] Construct DC172ATG FVIII (Figure 4G) is 6308 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:15 in which bases 1-145 are a 5' AAV2 ITR, bases 160-449 are two copies (2x) of a 145 bp macroglobulin enhancer, bases 450-1347 are an 898 bp hAAT promoter, bases 1348-1531 are a modified human β-globin 2nd intron, bases 1544-5917 are FVIII-SQ, bases 5926-6149 are a bGH poly A and bases 6164-6308 are a 3' AAV2 ITR.

[00122] Construct DC172ATG FVIII hAAT (Figure 4H) is 5635 bases in length, This construct is set forth as SEQ ID NO:16 in which bases 1-145 are a 5' AAV2 ITR, bases 160-449 are two copies (2x) of a 145 bp macroglobulin enhancer, bases 457-674 are a hAAT promoter, bases 675-858 are a modified human β-globin 2nd intron, bases 871-5244 are FVIII-SQ, bases 5253-5476 are a bGH poly A and bases 5490-5635 are a 3' AAV2 ITR.

[00123] Construct DC172 2xHCR ATG FVIII (Figure 4I) is 6962 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:17 in which bases 1-145 are a 5' AAV2 ITR, bases 160-807 are two copies (2x) of a 321 bp ApoE HCR, bases 814-1103 are two copies (2x) of a 145 bp macroglobulin enhancer, bases 1104-2001 are a 898 bp hAAT promoter, bases 2002-2185 are a modified human β -globin 2nd intron, bases 2198-6571 are FVIII-SQ, bases 6580-6803 are a bGH poly A and bases 6818-6962 are a 3' AAV2 ITR.

[00124] Construct DC172 2xHCR ATG FVIII hAAT (Figure 4J) is 6289 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:18 in which bases 1-145 are a 5'

AAV2 ITR, bases 160-807 are two copies (2x) of a 321 bp ApoE HCR, bases 814-1103 are two copies (2x) of a 145 bp macroglobulin enhancer, bases 1111-1328 are a hAAT promoter, bases 1329-1512 are a modified human β -globin 2nd intron, bases 1525-5898 are FVIII-SQ, bases 5907-6130 are a bGH poly A and bases 6245-6289 are a 3' AAV2 ITR.

[00125] Construct 2x SerpinA hAAT ATG FVIII (Figure 4K) is 5430 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:19 in which bases 1-145 are a 5' AAV2 ITR, bases 168-309 are two copies (2x) of a 71 bp SerpinA enhancer, bases 326-543 are a hAAT promoter, bases 544-727 are a modified human β-globin 2nd intron, bases 740-5113 are FVIII-SQ, bases 5122-5271 are a short bGH poly A, and bases 5286-5430 are a 3'AAV2 ITR.

[00126] Construct 2x SerpinA hAAT ATG FVIII 2x μ-globulin enhancer (Figure 4L) is 5779 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:20 in which bases 1-145 are a 5' AAV2 ITR, bases 168-309 are two copies (2x) of a 71 bp SerpinA enhancer, bases 326-543 are a hAAT promoter, bases 544-727 are a modified human β -globin 2nd intron, bases 740-5113 are FVIII-SQ, bases 5122-5271 are a short bGH poly A, bases 5279-5618 are two copies (2x) of a 170 bp μ-globulin enhancer and bases 5635-5779 are a 3' AAV2 ITR.

[00127] Construct 100ATG short bGH poly A 2x μ-globulin enhancer (Figure 4M) is 5962 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:21 in which bases 1-145 are a 5' AAV2 ITR, bases 160-502 are an ApoE HCR, bases 509-726 are a hAAT promoter, bases 727-910 are a modified human β-globin 2nd intron, bases 923-5296 are FVIII-SQ, bases 5305-5454 are a short bGH poly A, bases 5462-5801 are two copies (2x) of a 170 bp microglobulin enhancer and bases 5818-5962 are a 3' AAV2 ITR.

[00128] Construct Factor VIII-BMN001 (Figure 4N) is 5919 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:22 in which bases 1-145 are a 5' AAV2 ITR, bases 160-480 are an ApoE HCR, bases 487-884 are a 398bp hAAT promoter, bases 885-1145 are a truncated hAAT intron, bases 1155-5528 are FVIII-SQ, bases 5537-5760 are a bGH poly A and bases 5775-5919 are a 3' AAV2 ITR.

[00129] Construct Factor VIII-BMN002 (Figure 4O) is 5306 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:23 in which bases 1-145 are a 5' AAV2 ITR, bases 175-705 are an LP1 promoter/enhancer, bases 718-5091 are FVIII-SQ, bases 5100-5147 are a synthetic rabbit β-globin poly A and bases 5162-5306 are a 3' AAV2 ITR.

[00130] Construct 99 (Figure 4P) is 5461 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:24 in which bases 1-145 are a 5' AAV2 ITR, bases 169-627 are an ApoE HCR/MAR, bases 634-866 are a hAAT promoter, bases 873-5246 are FVIII-SQ, bases 5255-5302 are a synthetic rabbit β-globin poly A and bases 5317-5461 are a 3' AAV2 ITR.

[00131] Construct 100 (Figure 4Q) is 5327 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:25 in which bases 1-145 are a 5' AAV2 ITR, bases 169-493 are an ApoE HCR, bases 509-726 are a hAAT promoter, bases 739-5112 are FVIII-SQ, bases 5121-5168 are a synthetic rabbit β-globin poly A and bases 5183-5327 are a 3' AAV2 ITR.

[00132] Construct 100 reverse orientation (Figure 4R) is 5309 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:26 in which bases 1-145 are a 5' AAV2 ITR, bases 160-484 are an ApoE HCR in reverse orientation, bases 491-708 are a hAAT promoter, bases 721-5094 are FVIII-SQ, bases 5103-5150 are a synthetic rabbit β-globin poly A and bases 5165-5309 are a 3' AAV2 ITR.

[00133] Construct 100AT (Figure 4S) is 5532 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:27 in which bases 1-145 are a 5' AAV2 ITR, bases 169-493 are an ApoE HCR, bases 509-726 are a hAAT promoter, bases 727-931 are a hAAT intron, bases 944-5317 are FVIII-SQ, bases 5326-5373 are a synthetic rabbit β-globin poly A and bases 5388-5532 are a 3' AAV2 ITR.

[00134] Construct 100AT 2x MG (Figure 4T) is 5877 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:28 in which bases 1-145 are a 5' AAV2 ITR, bases 169-493 are an ApoE HCR, bases 508-847 are two copies (2x) of a 170 bp μ-globulin enhancer, bases 854-1071 are a hAAT promoter, bases 1072-1276 are a hAAT intron, bases 1289-5662 are FVIII-SQ, bases 5671-5718 are a synthetic rabbit β-globin poly A and bases 5733-5877 are a 3' AAV2 ITR.

[00135] Construct 100AT 2x MG bGH poly A (Figure 4U) is 6054 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:29 in which bases 1-145 are a 5' AAV2 ITR, bases 169-493 are an ApoE HCR, bases 508-847 are two copies (2x) of a 170 bp μ-globulin enhancer, bases 854-1071 are a hAAT promoter, bases 1072-1276 are a hAAT intron, bases 1289-5662 are FVIII-SQ, bases 5671-5895 are a bGH poly A and bases 5910-6054 are a 3' AAV2 ITR.

[00136] Construct 100AT 2x MG (reverse) bGH poly A (Figure 4V) is 6054 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:30 in which bases 1-145 are a 5' AAV2 ITR, bases 169-493 are an ApoE HCR, bases 508-847 are two copies (2x) of a 170 bp μ-globulin enhancer in reverse orientation, bases 854-1071 are a hAAT promoter, bases 1072-1276 are a hAAT intron, bases 1289-5662 are FVIII-SQ, bases 5671-5895 are a bGH poly A and bases 5910-6054 are a 3' AAV2 ITR.

[00137] Construct 100 bGH poly A (Figure 4W) is 5504 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:31 in which bases 1-145 are a 5' AAV2 ITR, bases 169-493 are an ApoE HCR, bases 509-726 are a hAAT promoter, bases 739-5112 are FVIII-SQ, base pairs 5121-5345 are a bGH poly A and bases 5360-5504 are a 3' AAV2 ITR.

[00138] Construct 100-400 (Figure 4X) is 5507 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:32 in which bases 1-145 are a 5' AAV2 ITR, bases 169-493 are an ApoE HCR, bases 512-906 are a 398 bp hAAT promoter, bases 919-5292 are FVIII-SQ, bases 5301-5348 are a synthetic rabbit β-globin poly A and bases 5363-5507 are a 3' AAV2 ITR.

[00139] Construct 101 (Figure 4Y) is 5311 base in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:33 in which bases 1-145 are a 5' AAV2 ITR, bases 170-477 are two copies (2x) of a 154bp ApoE HCR, bases 493-710 are a hAAT promoter, bases 723-5096 are FVIII-SQ, bases 5105-5152 are a synthetic rabbit β-globin poly A and bases 5167-5311 are a 3' AAV2 ITR.

[00140] Construct 102 (Figure 4Z) is 5156 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:34 in which bases 1-145 are a 5' AAV2 ITR, bases 169-322 are a 154bp ApoE HCR, bases 338-555 are a hAAT promoter, bases 568-4941 are FVIII-SQ, bases 4950-4997 are a synthetic rabbit β-globin poly A and bases 5012-5156 are a 3' AAV2 ITR.

[00141] Construct 103 (Figure 4AA) is 5178 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:35 in which bases 1-145 are a 5' AAV2 ITR, bases 169-344 are four copies (4x) of a 44 bp ApoE HCR, bases 360-577 are a hAAT promoter, bases 590-4963 are FVIII-SQ, bases 4972-5019 are a synthetic rabbit β-globin poly A and bases 5034-5178 are a 3' AAV2 ITR.

[00142] Construct 103 reverse orientation (Figure 4BB) is 5160 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:36 in which bases 1-145 are a 5' AAV2 ITR, bases 160-335 are four copies (4x) of a 44 bp ApoE HCR in reverse orientation, bases 342-559 are a hAAT promoter, bases 572-4945 are FVIII-SQ, bases 4954-5001 are a synthetic rabbit β-globin poly A and bases 5016-5160 are a 3' AAV2 ITR.

[00143] Construct 103AT (Figure 4CC) is 5383 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:37 in which bases 1-145 are a 5' AAV2 ITR, bases 169-344 are four copies (4x) of a 44 bp ApoE HCR, bases 360-577 are a hAAT promoter, bases 578-782 are a hAAT intron, bases 795-4374 are FVIII-SQ, bases 5177-5224 are a synthetic rabbit β-globin poly A and bases 5239-5383 are a 3' AAV2 ITR.

[00144] Construct 103AT 2x MG (Figure 4DD) is 5728 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:38 in which bases 1-145 are a 5' AAV2 ITR, bases 169-344 are four copies (4x) of a 44 bp ApoE HCR, bases 359-698 are two copies (2x) of a 170bp μ-globulin enhancer, bases 705-922 are a hAAT promoter, bases 923-1127 are a hAAT intron, bases 1140-5513 are FVIII-SQ, bases 5522-5569 are a synthetic rabbit β-globin poly A and bases 5584-5728 are a 3' AAV2 ITR.

[00145] Construct 103AT 2x MG bGH poly A (Figure 4EE) is 5905 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:39 in which bases 1-145 are a 5' AAV2 ITR, bases 169-344 are four copies (4x) of a 44 bp ApoE HCR, bases 359-698 are two copies (2x) of a 170bp μ-globulin enhancer, bases 705-922 are a hAAT promoter, bases 923-1127 are a hAAT intron, bases 1140-5513 are FVIII-SQ, bases 5522-5746 are a synthetic rabbit β-globin poly A and bases 5761-5905 are a 5' AAV2 ITR.

[00146] Construct 103 bGH poly A (Figure 4FF) is 5355 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:40 in which bases 1-145 are a 5' AAV2 ITR, bases 169-344 are four copies (4x) of a 44 bp ApoE HCR, bases 360-577 are a hAAT promoter, bases 590-4963 are FVIII-SQ, bases 4972-5196 are a synthetic rabbit β-globin poly A and bases 5211-5355 are a 3' AAV2 ITR.

[00147] Construct 104 (Figure 4GG) is 5618 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:41 in which bases 1-145 are a 5' AAV2 ITR, bases 169-784 are four copies (4x) of a 154bp ApoE HCR, bases 800-1017 are a hAAT promoter, bases 1030-

5403 are FVIII-SQ, bases 5412-5459 are a synthetic rabbit β-globin poly A and bases 5474-5618 are a 3' AAV2 ITR.

[00148] Construct 105 (Figure 4HH) is 5993 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:42 in which bases 1-145 are a 5' AAV2 ITR, bases 173-512 are two copies (2x) of a 170 bp μ-globulin enhancer, bases 519-736 are a hAAT promoter, bases 749-5122 are FVIII-SQ, bases 5131-5178 are a synthetic rabbit β-globin poly A, bases 5185-5834 are two copies (2x) of an ApoE HCR and bases 5849-5993 are a 3' AAV2 ITR.

[00149] Construct 106 (Figure 4II) is 5337 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:43 in which bases 1-145 are a 5' AAV2 ITR, bases 173-512 are two copies (2x) of a 170 bp μ-globulin enhancer, bases 519-736 are a hAAT promoter, bases 749-5122 are FVIII-SQ, bases 5131-5178 are a synthetic rabbit β-globin poly A and bases 5193-5337 are a 3' AAV2 ITR.

[00150] Construct 106AT (Figure 4JJ) is 5542 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:44 in which bases 1-145 are a 5' AAV2 ITR, bases 173-512 are two copies (2x) of a 170 bp μ-globulin enhancer, bases 519-736 are a hAAT promoter, bases 737-941 are a hAAT intron, bases 954-5327 are FVIII-SQ, bases 5336-5383 are a synthetic rabbit β-globin poly A and bases 5398-5542 are a 3' AAV2 ITR.

[00151] Construct p-100 ATGB is 5640 bases in length. The nucleotide sequence of this construct is set forth in SEQ ID NO:45 and comprises a 5' AAV2 ITR, an ApoE HCR, a hAAT promoter, a modified human β-globin 2nd intron, an FVIII-SQ encoding sequence, a bGH poly A sequence and a 3' AAV2 ITR.

AAV Vectors

[00152] As used herein, the term "AAV" is a standard abbreviation for adeno-associated virus. Adeno-associated virus is a single-stranded DNA parvovirus that grows only in cells in which certain functions are provided by a co-infecting helper virus. There are currently thirteen serotypes of AAV that have been characterized, as shown below in Table 1. General information and reviews of AAV can be found in, for example, Carter, 1989, *Handbook of Parvoviruses*, Vol. 1, pp. 169-228, and Berns, 1990, *Virology*, pp. 1743-1764, Raven Press, (New York). However, it is fully expected that these same principles will be applicable to additional AAV

serotypes since it is well known that the various serotypes are quite closely related, both structurally and functionally, even at the genetic level. (See, for example, Blacklowe, 1988, pp. 165-174 of *Parvoviruses and Human Disease*, J. R. Pattison, ed.; and Rose, *Comprehensive Virology* 3:1-61 (1974)). For example, all AAV serotypes apparently exhibit very similar replication properties mediated by homologous rep genes; and all bear three related capsid proteins. The degree of relatedness is further suggested by heteroduplex analysis which reveals extensive cross-hybridization between serotypes along the length of the genome; and the presence of analogous self-annealing segments at the termini that correspond to "inverted terminal repeat sequences" (ITRs). The similar infectivity patterns also suggest that the replication functions in each serotype are under similar regulatory control.

[00153] An "AAV vector" as used herein refers to a vector comprising one or more polynucleotides of interest (or transgenes) that are flanked by AAV terminal repeat sequences (ITRs) and operably linked to one or more expression control elements. Such AAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been transfected with a vector encoding and expressing rep and cap gene products.

[00154] An "AAV virion" or "AAV viral particle" or "AAV vector particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated polynucleotide AAV vector. If the particle comprises a heterologous polynucleotide (i.e. a polynucleotide other than a wild-type AAV genome such as a transgene to be delivered to a mammalian cell), it is typically referred to as an "AAV vector particle" or simply an "AAV vector". Thus, production of AAV vector particle necessarily includes production of AAV vector, as such a vector is contained within an AAV vector particle.

[00155] AAV "rep" and "cap" genes are genes encoding replication and encapsidation proteins, respectively. AAV rep and cap genes have been found in all AAV serotypes examined to date, and are described herein and in the references cited. In wild-type AAV, the rep and cap genes are generally found adjacent to each other in the viral genome (i.e., they are "coupled" together as adjoining or overlapping transcriptional units), and they are generally conserved among AAV serotypes. AAV rep and cap genes are also individually and collectively referred to as "AAV packaging genes." The AAV cap genes in accordance with the present invention encode Cap proteins which are capable of packaging AAV vectors in the presence of rep and

adeno helper function and are capable of binding target cellular receptors. In some embodiments, the AAV cap gene encodes a capsid protein having an amino acid sequence derived from a particular AAV serotype, for example the serotypes shown in Table 1.

Table 1. AAV serotypes

AAV Serotype	Genbank Accession No.
AAV-1	NC_002077.1
AAV-2	NC_001401.2
AAV-3	NC_001729.1
AAV-3B	AF028705.1
AAV-4	NC_001829.1
AAV-5	NC_006152.1
AAV-6	AF028704.1
AAV-7	NC_006260.1
AAV-8	NC_006261.1
AAV-9	AX753250.1
AAV-10	AY631965.1
AAV-11	AY631966.1
AAV-12	DQ813647.1
AAV-13	EU285562.1

[00156] The AAV sequences employed for the production of AAV can be derived from the genome of any AAV serotype. Generally, the AAV serotypes have genomic sequences of significant homology at the amino acid and the nucleic acid levels, provide a similar set of genetic functions, produce virions which are essentially physically and functionally equivalent, and replicate and assemble by practically identical mechanisms. For the genomic sequence of AAV serotypes and a discussion of the genomic similarities see, for example, GenBank Accession number U89790; GenBank Accession number J01901; GenBank Accession number AF043303; GenBank Accession number AF085716; Chiorini et al., *J. Vir.* 71: 6823-33(1997); Srivastava et al., *J. Vir.* 45:555-64 (1983); Chiorini et al., *J. Vir.* 73:1309-1319 (1999); Rutledge et al., *J. Vir.* 72:309-319 (1998); and Wu et al., *J. Vir.* 74: 8635-47 (2000).

[00157] The genomic organization of all known AAV serotypes is very similar. The genome of AAV is a linear, single-stranded DNA molecule that is less than about 5,000 nucleotides (nt) in length. Inverted terminal repeats (ITRs) flank the unique coding nucleotide sequences for the non-structural replication (Rep) proteins and the structural (VP) proteins. The VP proteins form the capsid. The terminal 145 nt are self-complementary and are organized so that an

energetically stable intramolecular duplex forming a T-shaped hairpin may be formed. These hairpin structures function as an origin for viral DNA replication, serving as primers for the cellular DNA polymerase complex. The Rep genes encode the Rep proteins, Rep78, Rep68, Rep52, and Rep40. Rep78 and Rep68 are transcribed from the p5 promoter, and Rep 52 and Rep40 are transcribed from the p19 promoter. The cap genes encode the VP proteins, VP1, VP2, and VP3. The cap genes are transcribed from the p40 promoter. The ITRs employed in the vectors of the present invention may correspond to the same serotype as the associated cap genes, or may differ. In a particularly preferred embodiment, the ITRs employed in the vectors of the present invention correspond to an AAV2 serotype and the cap genes correspond to an AAV5 serotype.

[00158] In some embodiments, a nucleic acid sequence encoding an AAV capsid protein is operably linked to expression control sequences for expression in a specific cell type, such as Sf9 or HEK cells. Techniques known to one skilled in the art for expressing foreign genes in insect host cells or mammalian host cells can be used to practice the invention. Methodology for molecular engineering and expression of polypeptides in insect cells is described, for example, in Summers and Smith. 1986. *A Manual of Methods for Baculovirus Vectors and Insect Culture Procedures*, Texas Agricultural Experimental Station Bull. No. 7555, College Station, Tex.; Luckow. 1991. In Prokop et al., *Cloning and Expression of Heterologous Genes in Insect Cells with Baculovirus Vectors' Recombinant DNA Technology and Applications*, 97-152; King, L. A. and R. D. Possee, 1992, *The baculovirus expression system*, Chapman and Hall, United Kingdom; O'Reilly, D. R., L. K. Miller, V. A. Luckow, 1992, *Baculovirus Expression Vectors: A Laboratory Manual*, New York; W.H. Freeman and Richardson, C. D., 1995, *Baculovirus Expression Protocols, Methods in Molecular Biology*, volume 39; U.S. Pat. No. 4,745,051; US2003148506; and WO 03/074714. A particularly suitable promoter for transcription of a nucleotide sequence encoding an AAV capsid protein is e.g. the polyhedron promoter. However, other promoters that are active in insect cells are known in the art, e.g. the p10, p35 or IE-1 promoters and further promoters described in the above references are also contemplated.

[00159] Use of insect cells for expression of heterologous proteins is well documented, as are methods of introducing nucleic acids, such as vectors, e.g., insect-cell compatible vectors, into such cells and methods of maintaining such cells in culture. See, for example, *METHODS IN MOLECULAR BIOLOGY*, ed. Richard, Humana Press, NJ (1995); O'Reilly et al.,

BACULOVIRUS EXPRESSION VECTORS, A LABORATORY MANUAL, Oxford Univ. Press (1994); Samulski et al., *J. Vir.* 63:3822-8 (1989); Kajigaya et al., *Proc. Nat'l. Acad. Sci. USA* 88: 4646-50 (1991); Ruffing et al., *J. Vir.* 66:6922-30 (1992); Kirnbauer et al., *Vir.* 219:37-44 (1996); Zhao et al., *Vir.* 272:382-93 (2000); and Samulski et al., U.S. Pat. No. 6,204,059. In some embodiments, the nucleic acid construct encoding AAV in insect cells is an insect cell-compatible vector. An "insect cell-compatible vector" or "vector" as used herein refers to a nucleic acid molecule capable of productive transformation or transfection of an insect or insect cell. Exemplary biological vectors include plasmids, linear nucleic acid molecules, and recombinant viruses. Any vector can be employed as long as it is insect cell-compatible. The vector may integrate into the insect cells genome but the presence of the vector in the insect cell need not be permanent and transient episomal vectors are also included. The vectors can be introduced by any means known, for example by chemical treatment of the cells, electroporation, or infection. In some embodiments, the vector is a baculovirus, a viral vector, or a plasmid. In a more preferred embodiment, the vector is a baculovirus, i.e. the construct is a baculoviral vector. Baculoviral vectors and methods for their use are described in the above cited references on molecular engineering of insect cells.

[00160] Baculoviruses are enveloped DNA viruses of arthropods, two members of which are well known expression vectors for producing recombinant proteins in cell cultures. Baculoviruses have circular double-stranded genomes (80-200 kbp) which can be engineered to allow the delivery of large genomic content to specific cells. The viruses used as a vector are generally *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) or *Bombyx mori* (Bm)NPV) (Kato et al., 2010).

[00161] Baculoviruses are commonly used for the infection of insect cells for the expression of recombinant proteins. In particular, expression of heterologous genes in insects can be accomplished as described in for instance U.S. Pat. No. 4,745,051; Friesen et al (1986); EP 127,839; EP 155,476; Vlak et al (1988); Miller et al (1988); Carbonell et al (1988); Maeda et al (1985); Lebacq-Verheyden et al (1988); Smith et al (1985); Miyajima et al (1987); and Martin et al (1988). Numerous baculovirus strains and variants and corresponding permissive insect host cells that can be used for protein production are described in Luckow et al (1988), Miller et al (1986); Maeda et al (1985) and McKenna (1989).

Methods for Producing Recombinant AAVs

[00162] The present disclosure provides materials and methods for producing recombinant AAVs in insect or mammalian cells. In some embodiments, the viral construct further comprises a promoter and a restriction site downstream of the promoter to allow insertion of a polynucleotide encoding one or more proteins of interest, wherein the promoter and the restriction site are located downstream of the 5' AAV ITR and upstream of the 3' AAV ITR. In some embodiments, the viral construct further comprises a posttranscriptional regulatory element downstream of the restriction site and upstream of the 3' AAV ITR. In some embodiments, the viral construct further comprises a polynucleotide inserted at the restriction site and operably linked with the promoter, where the polynucleotide comprises the coding region of a protein of interest. As a skilled artisan will appreciate, any one of the AAV vector disclosed in the present application can be used in the method as the viral construct to produce the recombinant AAV.

[00163] In some embodiments, the helper functions are provided by one or more helper plasmids or helper viruses comprising adenoviral or baculoviral helper genes. Non-limiting examples of the adenoviral or baculoviral helper genes include, but are not limited to, E1A, E1B, E2A, E4 and VA, which can provide helper functions to AAV packaging.

[00164] Helper viruses of AAV are known in the art and include, for example, viruses from the family *Adenoviridae* and the family *Herpesviridae*. Examples of helper viruses of AAV include, but are not limited to, SAdV-13 helper virus and SAdV-13-like helper virus described in US Publication No. 20110201088 (the disclosure of which is incorporated herein by reference), helper vectors pHELP (Applied Viromics). A skilled artisan will appreciate that any helper virus or helper plasmid of AAV that can provide adequate helper function to AAV can be used herein.

[00165] In some embodiments, the AAV cap genes are present in a plasmid. The plasmid can further comprise an AAV rep gene which may or may not correspond to the same serotype as the cap genes. The cap genes and/or rep gene from any AAV serotype (including, but not limited to, AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV13 and any variants thereof) can be used herein to produce the recombinant AAV. In some embodiments, the AAV cap genes encode a capsid from serotype 1, serotype 2, serotype 4, serotype 5, serotype 6, serotype 7, serotype 8, serotype 9, serotype 10, serotype 11, serotype 12, serotype 13 or a variant thereof.

[00166] In some embodiments, the insect or mammalian cell can be transfected with the helper plasmid or helper virus, the viral construct and the plasmid encoding the AAV cap genes; and the recombinant AAV virus can be collected at various time points after co-transfection. For example, the recombinant AAV virus can be collected at about 12 hours, about 24 hours, about 36 hours, about 48 hours, about 72 hours, about 96 hours, about 120 hours, or a time between any of these two time points after the co-transfection.

[00167] Recombinant AAV can also be produced using any conventional methods known in the art suitable for producing infectious recombinant AAV. In some instances, a recombinant AAV can be produced by using an insect or mammalian cell that stably expresses some of the necessary components for AAV particle production. For example, a plasmid (or multiple plasmids) comprising AAV rep and cap genes, and a selectable marker, such as a neomycin resistance gene, can be integrated into the genome of the cell. The insect or mammalian cell can then be co-infected with a helper virus (e.g., adenovirus or baculovirus providing the helper functions) and the viral vector comprising the 5' and 3' AAV ITR (and the nucleotide sequence encoding the heterologous protein, if desired). The advantages of this method are that the cells are selectable and are suitable for large-scale production of the recombinant AAV. As another non-limiting example, adenovirus or baculovirus rather than plasmids can be used to introduce rep and cap genes into packaging cells. As yet another non-limiting example, both the viral vector containing the 5' and 3' AAV LTRs and the rep-cap genes can be stably integrated into the DNA of producer cells, and the helper functions can be provided by a wild-type adenovirus to produce the recombinant AAV.

Cell Types Used in AAV Production

[00168] The viral particles comprising the AAV vectors of the invention may be produced using any invertebrate cell type which allows for production of AAV or biologic products and which can be maintained in culture. For example, the insect cell line used can be from *Spodoptera frugiperda*, such as SF9, SF21, SF900+, drosophila cell lines, mosquito cell lines, e.g., *Aedes albopictus* derived cell lines, domestic silkworm cell lines, e.g. Bombyxmori cell lines, *Trichoplusia ni* cell lines such as High Five cells or Lepidoptera cell lines such as *Ascalapha odorata* cell lines. Preferred insect cells are cells from the insect species which are

susceptible to baculovirus infection, including High Five, Sf9, Se301, SeIZD2109, SeUCR1, Sf9, Sf900+, Sf21, BTI-TN-5B1-4, MG-1, Tn368, HzAm1, BM-N, Ha2302, Hz2E5 and Ao38.

[00169] Baculoviruses are enveloped DNA viruses of arthropods, two members of which are well known expression vectors for producing recombinant proteins in cell cultures. Baculoviruses have circular double-stranded genomes (80-200 kbp) which can be engineered to allow the delivery of large genomic content to specific cells. The viruses used as a vector are generally *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) or *Bombyx mori* (Bm-NPV) (Kato et al., 2010).

[00170] Baculoviruses are commonly used for the infection of insect cells for the expression of recombinant proteins. In particular, expression of heterologous genes in insects can be accomplished as described in for instance U.S. Pat. No. 4,745,051; Friesen et al (1986); EP 127,839; EP 155,476; Vlak et al (1988); Miller et al (1988); Carbonell et al (1988); Maeda et al (1985); Lebacq-Verheyden et al (1988); Smith et al (1985); Miyajima et al (1987); and Martin et al (1988). Numerous baculovirus strains and variants and corresponding permissive insect host cells that can be used for protein production are described in Luckow et al (1988), Miller et al (1986); Maeda et al (1985) and McKenna (1989).

[00171] In another aspect of the invention, the methods of the invention are also carried out with any mammalian cell type which allows for replication of AAV or production of biologic products, and which can be maintained in culture. Preferred mammalian cells used can be HEK293, HeLa, CHO, NS0, SP2/0, PER.C6, Vero, RD, BHK, HT 1080, A549, Cos-7, ARPE-19 and MRC-5 cells.

Testing of AAV FVIII Vectors

[00172] Assays to test the completely packaged AAV FVIII vectors of the invention include, for example, (1) transient transfection of double-stranded DNA plasmids comprising the AAV vector nucleic acids in HepG2 cells, a cell line derived from human liver to check liver-specific mRNA expression and splicing, and FVIII protein production and secretion in vitro; (2) production of AAV virions comprising the AAV FVIII vectors in HEK293 cells and baculovirus-infected insect cells; (3) evaluation of the AAV vector nucleic acids by alkaline gel analysis and replication assays; and (4) evaluation of FVIII expression, FVIII activity, and FVIII specific activity in Rag2 mice. These assays are described in greater detail in the Examples.

[00173] The completely packaged AAV FVIII vectors of the invention display at least the same expression and/or activity as the representative vector shown in Figure 1, and preferably 1.5-fold, 2-fold, 3- fold, 4-fold, or 5-fold or more expression and/or activity as compared to the vector shown in Figure 1.

[00174] The completely packaged AAV FVIII vectors of the invention have high vector yield with little or no fragmentary genome contaminants, and preferably 1.5-fold, 2-fold, 3- fold, 4-fold, or 5-fold greater vector yield as compared to the vector shown in Figure 1.

Pharmaceutical Formulations

[00175] In other embodiments, the present invention is directed to pharmaceutical formulations of FVIII AAV vectors/virions useful for administration to subjects suffering from hemophilia A. In certain aspects, the pharmaceutical formulations of the present invention are liquid formulations that comprise recombinant AAV FVIII virions produced from the vectors disclosed herein, wherein the concentration of recombinant AAV FVIII virions in the formulation may vary widely. In certain embodiments, the concentration of recombinant AAV FVIII virion in the formulation may range from 1E12 vg/ml to 2E14 vg/ml. In a particularly preferred embodiment, the concentration of recombinant AAV FVIII virion in the formulation is about 2E13 vg/ml. In another preferred embodiment, the recombinant AAV FVIII virion present in the formulation is AAV5-FVIII-SQ derived from encapsidation of the Proto 1 vector shown schematically in Figure 2A in an AAV5 capsid.

[00176] In other aspects, the AAV FVIII pharmaceutical formulation of the invention comprises one or more pharmaceutically acceptable excipients to provide the formulation with advantageous properties for storage and/or administration to subjects for the treatment of hemophilia A. In certain embodiments, the pharmaceutical formulations of the present invention are capable of being stored at $\leq 65^{o}C$ for a period of at least 2 weeks, preferably at least 4 weeks, more preferably at least 6 weeks and yet more preferably at least about 8 weeks, without detectable change in stability. In this regard, the term "stable" means that the recombinant AAV FVIII virus present in the formulation essentially retains its physical stability, chemical stability and/or biological activity during storage. In certain embodiments of the present invention, the recombinant AAV FVIII virus present in the pharmaceutical formulation retains at least about 80% of its biological activity in a human patient during storage for a determined period of time

at -65°C, more preferably at least about 85%, 90%, 95%, 98% or 99% of its biological activity in a human patient.

[00177] In certain aspects, the formulation comprising recombinant AAV FVIII virions further comprises one or more buffering agents. For example, in various aspects, the formulation of the present invention comprises sodium phosphate dibasic at a concentration of about 0.1 mg/ml to about 3 mg/ml, about 0.5 mg/ml to about 2.5 mg/ml, about 1 mg/ml to about 2 mg/ml, or about 1.4 mg/ml to about 1.6 mg/ml. In a particularly preferred embodiment, the AAV FVIII formulation of the present invention comprises about 1.42 mg/ml of sodium phosphate, dibasic (dried). Another buffering agent that may find use in the recombinant AAV FVIII formulations of the present invention is sodium phosphate, monobasic monohydrate which, in some embodiments, finds use at a concentration of from about 0.1 mg/ml to about 3 mg/ml, about 0.5 mg/ml to about 2.5 mg/ml, about 1 mg/ml to about 2 mg/ml, or about 1.3 mg/ml to about 1.5 mg/ml. In a particularly preferred embodiment, the AAV FVIII formulation of the present invention comprises about 1.38 mg/ml of sodium phosphate, monobasic monohydrate. In a yet more particularly preferred embodiment of the present invention, the recombinant AAV FVIII formulation of the present invention comprises about 1.42 mg/ml of sodium phosphate, dibasic and about 1.38 mg/ml of sodium phosphate, monobasic monohydrate.

[00178] In another aspect, the recombinant AAV FVIII formulation of the present invention may comprise one or more isotonicity agents, such as sodium chloride, preferably at a concentration of about 1 mg/ml to about 20 mg/ml, for example, about 1 mg/ml to about 10 mg/ml, about 5 mg/ml to about 15 mg/ml, or about 8 mg/ml to about 20 mg/ml. In a particularly preferred embodiment, the formulation of the present invention comprises about 8.18 mg/ml sodium chloride. Other buffering agents and isotonicity agents known in the art are suitable and may be routinely employed for use in the formulations of the present disclosure.

[00179] In another aspect, the recombinant AAV FVIII formulations of the present invention may comprise one or more bulking agents. Exemplary bulking agents include without limitation mannitol, sucrose, dextran, lactose, trehalose, and povidone (PVP K24). In certain preferred embodiments, the formulations of the present invention comprise mannitol, which may be present in an amount from about 5 mg/ml to about 40 mg/ml, or from about 10 mg/ml to about

30 mg/ml, or from about 15 mg/ml to about 25 mg/ml. In a particularly preferred embodiment, mannitol is present at a concentration of about 20 mg/ml.

[00180] In yet another aspect, the recombinant AAV FVIII formulations of the present invention may comprise one or more surfactants, which may be non-ionic surfactants. Exemplary surfactants include ionic surfactants, non-ionic surfactants, and combinations thereof. For example, the surfactant can be, without limitation, TWEEN 80 (also known as polysorbate 80, or its chemical name polyoxyethylene sorbitan monooleate), sodium dodecylsulfate, sodium stearate, ammonium lauryl sulfate, TRITON AG 98 (Rhone-Poulenc), poloxamer 407, poloxamer 188 and the like, and combinations thereof. In a particularly preferred embodiment, the formulation of the present invention comprises poloxamer 188, which may be present at a concentration of from about 0.1 mg/ml to about 4 mg/ml, or from about 0.5 mg/ml to about 3 mg/ml, from about 1 mg/ml to about 3 mg/ml, about 1.5 mg/ml to about 2.5 mg/ml, or from about 1.8 mg/ml to about 2.2 mg/ml. In a particularly preferred embodiment, poloxamer 188 is present at a concentration of about 2.0 mg/ml.

[00181] In a particular preferred embodiment of the present invention, the pharmaceutical formulation of the present invention comprises AAV5-FVIII-SQ formulated in a liquid solution that comprises about 1.42 mg/ml of sodium phosphate, dibasic, about 1.38 mg/ml of sodium phosphate, monobasic monohydrate, about 8.18 mg/ml sodium chloride, about 20 mg/ml mannitol and about 2 mg/ml poloxamer 188.

[00182] The recombinant AAV FVIII virus-containing formulations of the present disclosure are stable and can be stored for extended periods of time without an unacceptable change in quality, potency, or purity. In one aspect, the formulation is stable at a temperature of about 5°C (e.g., 2°C to 8°C) for at least 1 month, for example, at least 1 month, at least 3 months, at least 6 months, at least 12 months, at least 18 months, at least 24 months, or more. In another aspect, the formulation is stable at a temperature of less than or equal to about -20°C for at least 6 months, for example, at least 6 months, at least 12 months, at least 18 months, at least 24 months, at least 36 months, or more. In another aspect, the formulation is stable at a temperature of less than or equal to about -40°C for at least 6 months, for example, at least 6 months, at least 12 months, at least 18 months, at least 24 months, at least 36 months, or more. In another aspect, the formulation is stable at a temperature of less than or equal to about -60°C for at least 6 months,

for example, at least 6 months, at least 12 months, at least 18 months, at least 24 months, at least 36 months, or more.

Methods of Treatment

[00183] In certain embodiments, the present invention is directed to methods for treating a subject suffering from hemophilia A comprising administering to that subject a therapeutically effective amount of an AAV FVIII vector, recombinant AAV FVIII virus or a pharmaceutical composition comprising the same. In yet other embodiments, the present invention is directed to methods for reducing bleeding time during a bleeding episode in a subject suffering from hemophilia A comprising administering to that subject a therapeutically effective amount of an AAV FVIII vector, recombinant AAV FVIII virus or a pharmaceutical composition comprising the same. In this regard, a "therapeutically effective amount", in reference to the treatment of hemophilia A or for use in a method for reducing bleeding time during a bleeding episode in a subject suffering from hemophilia A, refers to an amount capable of invoking one or more of the following effects: (1) reduction, inhibition, or prevention, to some extent, of one or more of the physiological symptoms of hemophilia A including, for example, bruising, joint pain or swelling, prolonged headache, vomiting or fatigue, (2) improvement in the capability to clot blood, (3) reduction of overall bleeding time during a bleeding episode, (4) administration resulting in a measurable increase in the concentration or activity of functional FVIII protein in the plasma of a subject, and/or (5) relief, to some extent, of one or more symptoms associated with the disorder. A "therapeutically effective amount" of an AAV FVIII vector or virus or a pharmaceutical composition comprising the same for purposes of treatment as described herein may be determined empirically and in a routine manner. In certain embodiments, however, a "therapeutically effective amount" of recombinant AAV FVIII virus ranges from about 1E12 vg/kg body weight to about 1E14 vg/kg body weight, preferably from about 6E12 vg/kg body weight to about 6E13 vg/kg body weight. In a particularly preferred embodiment, a therapeutically effective amount of recombinant AAV FVIII virus is about 2E13 vg/kg body weight. In another particularly preferred embodiment, a therapeutically effective amount of recombinant AAV FVIII virus is about 6E13 vg/kg body weight.

[00184] Recombinant AAV FVIII vectors/virus of the present invention may be administered to a subject, preferably a mammalian subject, more preferably a human subject, through a variety

of known administration techniques. In a preferred embodiment, the recombinant AAV FVIII gene therapy virus is administered by intravenous injection either as a single bolus or over a prolonged time period, which may be at least about 1, 5, 10, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 or 240 minutes, or more. In a particularly preferred embodiment of the present invention, the recombinant AAV FVIII virus administered is AAV5-FVIII-SQ.

[00185] Administration of a recombinant AAV FVIII vector/virus, or pharmaceutical formulation comprising the same, of the present invention preferably results in an increase in functional FVIII protein activity in the plasma of the subject of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more IU/dl as compared to the amount of functional FVIII protein activity present in the plasma in the subject prior to administration. In certain embodiments, administration of a recombinant AAV FVIII vector/virus, or pharmaceutical formulation comprising the same, of the present invention results in the expression of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more IU/dl of functional FVIII protein activity in the plasma of the subject. In this regard, the term "IU" or "international unit" in regards to FVIII activity is a well understood and accepted term, wherein 1 IU of FVIII activity is equivalent to the quantity of FVIII in one ml of normal human plasma. FVIII activity in the plasma may be quantitatively determined by a number of well-known and accepted assays including, for example, the activated partial thromboplastin time (APPT) method (see, e.g., Miletich JP: *Activated partial thromboplastin time.* In Williams Hematology. Fifth edition. Edited by E Beutler, MA Lichtman, BA Coller, TJ Kipps. New York, McGraw-Hill, 1995, pp L85-86, Greaves and Preston, *Approach to the bleeding patient.* In Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Fourth edition. Edited by RW Colman, J Hirsh, VJ Marder, et al. Philadelphia, JB Lippincott Co, 2001, pp 1197-1234 and Olson et al, *Arch. Pathol. Lab. Med.* 122:782-798 (1998)) or chromogenic FXa assay (Harris et al., Thromb. Res. 128(6):125-129 (2011)).

[00186] In other embodiments of the present invention, bleeding time in a subject may be measured by well-known and accepted techniques including, for example, the Ivy method (see, e.g., Ivy et al., *Surg. Gynec. Obstet.* 60:781 (1935) and Ivy et al., *J. Lab. Clin. Med.* 26:1812 (1941)) or the Duke method (see, e.g., Duke et al., *JAMA* 55:1185 (1910)). A "bleeding episode" in a subject refers to an injury that results in bleeding in the subject, either externally or internally, and generally comprises the time period from injury to formation of a blood clot.

[00187] Administration of an AAV FVIII virus of the present invention may, in some cases, result in an observable degree of hepatotoxicity. Hepatotoxicity may be measured by a variety of well-known and routinely used techniques for example, measuring concentrations of certain liver-associated enzyme(s) (e.g., alanine transaminase, ALT) in the bloodstream of a subject both prior to AAV FVIII administration (i.e., baseline) and after AAV FVIII administration. An observable increase in ALT concentration after AAV FVIII administration (as compared to prior to administration) is indicative of drug-induced hepatotoxicity. In certain embodiments of the present invention, in addition to administration of a therapeutically effective amount of AAV FVIII virus, the subject may be treated either prophylactically, therapeutically, or both with a corticosteroid to prevent and/or treat any hepatotoxicity associated with administration of the AAV FVIII virus. "Prophylactic" corticosteroid treatment refers to the administration of a corticosteroid to prevent hepatotoxicity and/or to prevent an increase in measured ALT levels in the subject. "Therapeutic" corticosteroid treatment refers to the administration of a corticosteroid to reduce hepatotoxicity caused by administration of an AVV FVIII virus and/or to reduce an elevated ALT concentration in the bloodstream of the subject caused by administration of an AAV FVIII virus. In certain embodiments, prophylactic or therapeutic corticosteroid treatment may comprise administration of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, or more mg/day of the corticosteroid to the subject. In certain embodiments, prophylactic or therapeutic corticosteroid treatment of a subject may occur over a continuous period of at least about 3, 4, 5, 6, 7, 8, 9, 10 weeks, or more. Corticosteroids that find use in the methods described herein include any known or routinely-employed corticosteroid including, for example, dexamethasone, prednisone, fludrocortisone, hydrocortisone, and the like.

Detection of Anti-AAV Antibodies

[00188] To maximize the likelihood of successful liver transduction with systemic AAV-mediated Factor VIII gene transfer, prior to administration of an AAV vector in a therapeutic regimen to a human patient as described above, the prospective patient may be assessed for the presence of anti-AAV capsid antibodies that are capable of blocking cell transduction or otherwise reduce the overall efficiency of the therapeutic regimen. Such antibodies may be present in the serum of the prospective patient and may be directed against an AAV capsid of any serotype. In one embodiment, the serotype against which pre-existing antibodies are directed is AAV5.

[00189] Methods to detect pre-existing AAV immunity are well known and routinely employed in the art and include cell-based *in vitro* transduction inhibition (TI) assays, *in vivo* (e.g., in mice) TI assays, and ELISA-based detection of total anti-capsid antibodies (TAb) (see, e.g., Masat et al., *Discov. Med.* 15:379-389 (2013) and Boutin et al., *Hum. Gene Ther.* 21:704-712 (2010)). TI assays may employ host cells into which an AAV-inducible reporter vector has been previously introduced. The reporter vector may comprise an inducible reporter gene such as GFP, etc. whose expression is induced upon transduction of the host cell by an AAV virus. Anti-AAV capsid antibodies present in human serum that are capable of preventing/reducing host cell transduction would thereby reduce overall expression of the reporter gene in the system. Therefore, such assays may be employed to detect the presence of anti-AAV capsid antibodies in human serum that are capable of preventing/reducing cell transduction by the therapeutic FVIII AAV virus.

[00190] TAb assays to detect anti-AAV capsid antibodies may employ solid-phase-bound AAV capsid as a "capture agent" over which human serum is passed, thereby allowing anti-capsid antibodies present in the serum to bind to the solid-phase-bound capsid "capture agent". Once washed to remove non-specific binding, a "detection agent" may be employed to detect the presence of anti-capsid antibodies bound to the capture agent. The detection agent may be an antibody, an AAV capsid, or the like, and may be detectably-labeled to aid in detection and quantitation of bound anti-capsid antibody. In one embodiment, the detection agent is labeled with ruthenium or a ruthenium-complex that may be detected using electrochemiluminescence techniques and equipment.

[00191] The same above-described methodology may be employed to assess and detect the generation of an anti-AAV capsid immune response in a patient previously treated with a therapeutic AAV virus of interest. As such, not only may these techniques be employed to assess the presence of anti-AAV capsid antibodies prior to treatment with a therapeutic FVIII AAV virus, they may also be employed to assess and measure the induction of an immune response against the administered therapeutic FVIII AAV virus after administration. As such, the present invention contemplates methods that combine techniques for detecting anti-AAV capsid antibodies in human serum and administration of a therapeutic FVIII AAV virus for the treatment of hemophilia A, wherein the techniques for detecting anti-AAV capsid antibodies in

human serum may be performed either prior to or after administration of the therapeutic FVIII AAV virus.

[00192] Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples.

EXAMPLES

Example 1
Generation of Proto 1, Proto 1S, Proto 2S and Proto 3S Vectors

[00193] The recombinant AAV FVIII vector schematically shown in Figure 1, which is described in detail in WO 2011/005968, published January 13, 2011, which is incorporated herein by reference in its entirety, and McIntosh et al., *Blood* 121:3335-3344, 2013, is an oversized, i.e., greater than 5.0 kb, AAV vector. As shown in Figure 1, this vector comprises, from left to right, the AAV serotype 2 (AAV2) 5' ITR, wild-type AAV2 viral sequence, the 34 base human apolipoprotein E (ApoE)/C1 enhancer, the 32 base human alpha anti-trypsin (AAT) promoter distal X region, the 186 base human AAT promoter, including 42 bases of 5' untranslated region (UTR) sequence, the codon-optimized human FVIII sequence in which the B domain is replaced with the 14 amino acid SQ sequence, the 49 bases synthetic polyadenylation sequence, wild-type AAV2 viral sequence, and the AAV2 3' ITR. This vector is 5081 bases in length.

[00194] To obtain a vector that is smaller than the FVIII vector shown in Figure 1, DNA sequences believed by the inventors herein to be unnecessary for FVIII expression and/or activity, or for AAV virion production, were removed from the original vector sequence. Extraneous DNA sequence was removed, including 53 bases of AAV2 viral sequence 3' to the AAV2 5' ITR, 46 bases of AAV2 viral sequence 5' to the AAV2 3' ITR, and 11 bases adjacent to the codon-optimized FVIII SQ coding region. A novel codon-optimized, B-domain-deleted FVIII-encoding sequence possessing an SQ linker was also produced and introduced into new recombinant AAV FVIII vectors. Certain sequence changes were made to the AAV2 5' and 3' ITRs. The resultant Proto 1 vector, which is 4970 bases in length, is shown in schematic form in Figure 2A, and the complete nucleotide sequence is set forth in SEQ ID NO:1. The inventors herein have demonstrated that Proto 1 produced infectious recombinant AAV virus and encodes a functional Factor VIII polypeptide.

[00195] Sequences adjacent to the hairpin loop in the AAV2 ITR may also be dispensable in recombinant AAV vectors (see Srivastava et al., US Pat. No. 6,521,225; Wang et al., *J. Virol.* 70:1668-1677, 1996; and Wang et al., *J. Virol.* 71:3077-3082, 1997). To further reduce the size of the Proto 1 vector, 10 bases of AAV2 sequence was removed directly 3' to the hairpin loop in the AAV2 5' ITR and 10 bases of AAV2 sequence was removed directly 5' to the hairpin loop in the AAV2 3' ITR. The resultant Proto 1S vector, which is 4950 bases in length, is shown in schematic form in Figure 2B, and the sequence is set forth in SEQ ID NO:2.

[00196] In an effort to increase the expression of the FVIII SQ variant in the Proto 1S vector, a 100 base synthetic intron was inserted between exons 1 and 2 in the codon-optimized FVIII sequence. It is known that insertion of an intron possibly can result in increased level of mRNA expression in otherwise intron-less genes, such as, for example, the interferon genes.

[00197] Enhancers are defined as working in a distance- and orientation-independent manner. The 34 base ApoE/C1 enhancer works in a distance- and orientation-independent manner with respect to FVIII expression, as exemplified by its presumptive enhancer activity in U.S. Pat. No. 8,030,065 (FIX expression) and in WO 2011/005968 (FVIII expression), both of which are incorporated herein by reference in their entirety. The 32 base human AAT promoter distal X region, described in Di Simone et al., *EMBO J.* 6:2759-2766, 1987, is located within a regulatory domain that enhances expression of a heterologous promoter.

[00198] In another attempt to further increase the expression of the FVIII SQ variant in the Proto 1S vector, the synthetic intron sequence incorporated the 34 base human ApoE/C1 enhancer and 32 base human AAT promoter distal X region, which was moved from its location upstream of the human AAT promoter. These two regulatory elements were inserted in the reverse orientation with respect to their orientation in Proto 1S. The resultant Proto 2S vector, which is 4983 bases in length, is shown in schematic form in Figure 2C, and the sequence set forth in SEQ ID NO:3.

[00199] As the human AAT promoter distal X region had not previously been shown to function downstream from the transcriptional start site in an intron, this regulatory element in the Proto 2S vector was replaced with a second copy of the 34 base human ApoE/C1 enhancer in the same orientation as the first copy of the enhancer in the intron. The resultant Proto 3S vector,

which is 4985 bases in length, is shown in schematic form in Figure 2D, and the sequence is set forth in SEQ ID NO:4.

[00200] The Proto 1, Proto 1S, Proto 2S and Proto 3S vector nucleic acids were cloned into the pUC19 bacterial expression plasmid, thereby generating double-stranded forms of the AAV FVIII vectors.

Example 2

Generation of Proto 4, Proto 5, Proto 6 and Proto 7 Vectors

[00201] To further reduce the size of the Proto 1 vector and/or increase the expression of FVIII as compared to the Proto 1 vector, the a3 domain, which is located adjacent to the light chain or C domain, was deleted. The a3 domain is involved in binding to von Willenbrand Factor, but may be dispensable for functionally active FVIII in vivo.

[00202] Starting from the Proto 1 vector, the 14 amino acid SQ sequence and 41 amino acids a3 domain (corresponding to amino acids 1649-1689 of wild-type FVIII) were deleted. The resultant Proto 4 vector, which is 4805 bases in length, is shown in schematic form in Figure 3A, and the sequence is set forth in SEQ ID NO:5.

[00203] In an attempt to increase the expression of the B domain and a3 domain deleted FVIII, a 129 base, truncated FVIII intron was inserted between exons 1 and 2 in the codon-optimized FVIII sequence in the Proto 4 vector. The resultant Proto 5 vector, which is 4934 bases in length, is shown in schematic form in Figure 3B, and the sequence is set forth in SEQ ID NO:6.

[00204] In an attempt to further increase the expression of the B domain and a3 domain deleted FVIII, a second copy of the 34 base human ApoE/C1 enhancer was inserted in either the forward or reverse orientation in the Proto 5 vector. The resultant Proto 6 vector, which is 4934 bases in length and has the intronic ApoE/C1 enhancer in the forward orientation, is shown in schematic form in Figure 3C, and the sequence is set forth in SEQ ID NO:7.

[00205] The resultant Proto 7 vector, which is 4934 bases in length and has the intronic ApoE/C1 enhancer in the reverse orientation, is shown in schematic form in Figure 3D, and the sequence is set forth in SEQ ID NO:8.

[00206] The Proto 4, Proto 5, Proto 6 and Proto 7 vector nucleic acids were cloned into the pUC19 bacterial expression plasmid, thereby generating double-stranded forms of the AAV FVIII vectors.

Example 3
Assays to Test the Expression and Activity of AAV FVIII Vectors

[00207] Assays to test the recombinant AAV FVIII vectors of the invention include, for example, (1) transient transfection of double-stranded DNA plasmids comprising the AAV vector nucleic acids in HepG2 cells, a cell line derived from human liver to check liver-specific mRNA expression and splicing, and FVIII protein production and secretion in vitro; (2) production of AAV virions comprising the AAV FVIII vectors in 293 cells and baculovirus-infected insect cells; (3) evaluation of the AAV vector nucleic acids by alkaline gel analysis and replication assays; and (4) evaluation of FVIII expression, FVIII activity, and FVIII specific activity in Rag2 mice.

Transient Transfection Assays

[00208] A preliminary in vitro assay is performed to compare the FVIII expression and activity from the AAV FVIII vectors of the present invention with that from the FVIII-expressing vector shown in Figure 1. Double-stranded forms of the AAV FVIII vectors of the present invention are transiently transfected into the human liver cell line, HepG2. After transfection, for example, 24 or 48 hours later, FVIII antigen and activity in the culture supernatants is measured.

[00209] Using this assay, the FVIII activity in HepG2 cells transiently transfected with the Proto 1, Proto 1S and Proto 2S vectors was similar to the FVIII activity obtained using the FVIII vector of Figure 1, demonstrating that the Proto 1, Proto 1S and Proto 2S vectors were capable of expressing functional Factor VIII protein.

Production of AAV FVIII Virions in 293 Cells and Baculovirus-Infected Insect Cells

[00210] To demonstrate that the recombinant AAV FVIII vectors of the present invention indeed package the nucleic acids encoding FVIII, the double-stranded forms of the AAV FVIII vectors generated as described in Examples 1 and 2 are introduced into cells capable of producing AAV virions. In a first AAV virus production system, plasmids comprising the AAV FVIII vector nucleic acids in double-stranded form are co-transfected into 293 cells together with

a plasmid that expresses the AAV Cap and Rep proteins and a plasmid that expresses adenovirus helper functions needed to for AAV virion production. In a second AAV virus production system, baculovirus constructs are generated expressing the AAV FVIII vector nucleic acids and the AAV Cap and Rep proteins, and then are co-infected into insect Sf9 cells. The resultant AAV virions produced in the transiently transfected 293 cells or baculovirus-infected Sf9 cells are purified and analyzed by standard methods known in the art.

Evaluation by Alkaline Gel and Replication Assay

[00211] An alkaline gel electrophoresis assay is used to determine the size of the packaged nucleic acid. A replication center assay is used to determine which AAV FVIII vectors are packaged in an intact form by both packaging methods.

[00212] A primer extension assay is used to quantify the amount of AAV FVIII vectors nucleic acids that have complete ends, i.e., terminate at the 5‘ end of the hairpin loop in the AAV2 5’ ITR (sense strand) or 3’ ITR (anti-sense strand).

[00213] Alternatively, a PCR assay is used to determine whether the AAV FVIII vectors nucleic acids have complete ends, i.e., terminate at the 5‘ end of the hairpin loop in the AAV2 5’ ITR (sense strand) or 3’ ITR (anti-sense strand).

Evaluation in Rag2 Mice

[00214] The AAV virions produced in transiently transfected 293 cells or baculovirus-infected Sf9 cells packaged vectors are tested for FVIII expression and activity in Rag2 mice at 2e11, 2e12, and 2e13 viral genomes (vg)/kg, administered intravenously. Rag2 mice are used in this assay because FVIII expression and/or activity is/are not complicated by the presence of a host immune response to the AAV virus or human FVIII protein.

[00215] FVIII antigen is determined using an ELISA-based assay. FVIII activity is determined using a FXa activation assay and/or a coagulation assay. Using the FVIII antigen and activity assays, the FVIII specific activity is determined.

[00216] Numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the presently preferred embodiments thereof. Consequently, the only limitations which should be placed upon the scope of the invention are those which appear in the appended claims.

Example 4
Generation of Constructs with Improved Promoter/Enhancer Sequences

[00217] To generate additional recombinant AAV vectors with strong promoters that increase expression of functional FVIII, constructs were generated with modified enhancer and/or promoter sequences. In some embodiments, the constructs comprised shortened versions of the ApoE or the μ-globulin enhancers. These constructs were generated using standard DNA cloning techniques and the sequences thereof are shown in SEQ ID NOS:9-45.

Example 5
Generation of AAV Viral Particles

Generation of Recombinant Bacmid

[00218] DH10 Bac competent cells were thawed on ice. Recombinant shuttle plasmid (e.g., pFB-GFP) was added and gently mixed with the competent cells and incubated on ice for 30 minutes. The competent cells were then subjected to heat at a temperature of approximately 42°C for 30 seconds and then chilled on ice for 2 minutes. The competent cells were shocked with heat for 30 seconds at 42ºC and chilled on ice for 2 min. SOC was added to the cells and allowed to incubate at 37ºC with agitation for 4 hours to allow recombination to take place. During the incubation period, X-gal was spread onto two LB-plates (additionally containing various antibiotics (e.g., kanamycin, gentamycin and tetracycline) for transformation, is followed by IPTG.

[00219] An amount of the incubation mixture was obtained, diluted and then spread onto the two LB-plates and incubated at 37°C for approximately 30-48 hours. Several white colonies were selected from each plate and cultured overnight in LB medium containing the same combination of antibiotics provided in the LB-plates. Next, Bacmid DNA and a glycerol stock was prepared and stored at -80°C.

Purification of Recombinant Bacmid DNA

[00220] An amount of the Bacmid glycerol stock is removed and inoculated in LB medium containing the same combination of antibiotic provided in the LB-plates described above. Cultures are allowed to grow overnight at 37°C with shaking. Next, an amount of the culture is spun in a microfuge at full speed for approximately 30 seconds.

[00221] The pellets were resuspended in a resuspension buffer using a pipette followed by a lysis buffer, and the tube was inverted several times to mix the buffer and then incubated at room temperature for approximately 5 minutes. An exemplary resuspension buffer comprises 50 mM Tris-CL, pH 8.0, 10 mM EDTA and 100ug/mL RNase A. An exemplary lysis buffer comprises 200 mM NaOH and 1% SDS. An amount of precipitate buffer (e.g., a buffer comprising 3.0 M potassium acetate, pH 5.5) was slowly added and the tube was inverted several times to mix the buffer and then incubated on ice for approximately 10 minutes. The tube was centrifuged for approximately 10 minutes at full speed and the supernatant is poured into a tube containing isopropanol. The tube was inverted several times to mix the solution.

[00222] Next, the solution was centrifuged at full speed for approximately 15 minutes at room temperature and the supernatant was removed immediately after centrifuge with pipette.

[00223] An amount of 70% ethanol was added to rinse the pellet and spun again at full speed for 1 minute. The ethanol was then removed and the solution is spun again to remove trace of the ethanol. An amount of TE/EB Buffer was added to each tube and the pellet is carefully dissolved by pipette. The solution was stored at –20°C if not used immediately.

Production of P0 Stock of Recombinant Baculovirus

[00224] Sf9 cells were seeded at approximately 1 x 10^6 cells/well in a 6-well plate (or 6 x 10^6 cells in a 10-cm plate or 1.7 x 10^7 cells in a 15-cm dish) and the cells were allowed to attach for at least 1 hour before transfection.

[00225] Transfection solutions A and B are prepared as follows: Solution A: an amount of the Bacmid was diluted into an amount of serum free media without antibiotics in a 15-mL tube. Solution B: an amount of CellFectin was diluted into an amount of serum free media without antibiotics in a 15-mL tube. Solution B was added to Solution A and gently mixed by pipette approximately 3 times by pipette, and incubated at room temperature for 30 ~ 45 minutes. Next, medium from the plate was aspirated and an amount of serum free media without antibiotics was added to wash the cells. An amount of SF900II without antibiotics was added to each tube containing lipid-DNA mixtures.

[00226] The medium from the cells was aspirated, the transfection solution was added to the cells and the cells were incubated for approximately 5 hours at 28°C. The transfection solution

was removed and an amount of and serum free media + antibiotics is added, and incubated for approximately 4 days at 28°C. Media that contains the recombinant baculovirus was collected and spun for approximately 5 minutes at 1000 rpm to remove cell debris. The baculovirus was stored at 4°C under dark.

Amplification of Baculovirus (P1)

[00227] Sf9 cells were grown to approximately 4 x 10^6 cells/mL and diluted to approximately 2 x 10^6 cells/mL with fresh medium in shaking flasks. An amount of the Sf9 cells were infected with an amount of the P0 stock baculovirus. The multiplicity of infection (MOI) is approximately 0.1.

[00228] The Sf9 cells were incubated for approximately 3 days and the baculovirus was harvested. The cells were spun at 2,000 rpm for 5 minutes to pellet the cells and the supernatant was collected and stored at 4°C under dark. The titer of the baculovirus was determined according to Clontech's Rapid Titer Kit protocol.

Production of AAV using P1 Recombinant Baculoviruses

[00229] Sf9 cells were grown to about 1 x 10^7 cells/mL and diluted to about 5 x 10^6 cells/mL. An amount of the diluted Sf9 cells were infected with Bac-vector (5Moi) and Bac-helper (15Moi) for 3 days. Cell viability was assessed on the third day (approximately 50% ~ 70% dead cells are observed).

[00230] Cell pellets were harvested by centrifugation at 3000 rpm for 10 minutes. Media was removed and the cells lysed (or the cell pellets were stored at -20°C if not used immediately).

Lysis and Banding/Purification Protocol

[00231] An amount of Sf9 lysis buffer plus Benzonase is added to each cell pellet and vortexed thoroughly to resuspend the cells. The resuspended Sf9 cells were incubated on ice for approximately 10 min. to cool lysate. The lysate was sonicated for approximately 20 seconds to lyse the cells thoroughly and then incubated at 37°C for approximately 30 minutes.

[00232] An amount of 5M NaCl was added and the mixture is vortexed and then incubated for another 30 minutes at 37°C. An amount of NaCl was added to bring the salt concentration to about 500mM, vortexed and centrifuged at 8,000 rpm for 20 minutes at 15°C to produce a cleared lysate.

[00233] The cleared lysate proceeds to ultracentrifugation steps. A CsCl-gradient was prepared by adding the cleared lysate first, then an amount of 1.32g/cc and an amount of 1.55g/cc CsCl solutions through a syringe with long needle. The interface between the CsCl solutions was marked. PBS was added up to the top of the centrifuge tubes and the tubes are carefully balanced and sealed.

[00234] The tubes were centrifuged at 55,000 rpm for approximately 20 hours at 15°C. A hole was puncture on the top of each tube and the AAV band located slightly above the interface mark of the two CsCl solutions is marked.

[00235] A second CsCl centrifugation is conducted by transferring the AAV solution to centrifuge tube for 70.1 Ti rotor and an amount of CsCl solution to near top of the tube was added. The tubes were balanced and sealed. The tubes are centrifuged at 65,000rpm for approximately 20 hours and the AAV band (lower band, the higher band is empty capsids) was collected.

Example 5

Evaluation of the Constructs in Rag2 Mice

[00236] AAV virions which comprise a codon-optimized SQ FVIII-encoding gene sequence were generated using baculovirus and 293 cells using the FVIII vector of Figure 1, Proto 1, Proto 1S, Proto 2S and Proto 3S constructs. The packaging limits are about 4800bp for baculovirus and about 4950bp for 293 cells.

[00237] As shown in Figure 5, all constructs tested with truncated (T) or non-truncated (NT) genomes are capable of inducing FVIII expression. Expression of FVIII from Proto 1 was similar to the FVIII construct of Figure 1 when these AAV were made by the baculovirus system. Inclusion of the intron in Proto 2S and Proto 3S did not result in improved FVIII expression as compared to Proto 1. The FVIII vector of Figure 1 containing the AAV flanking sequences made in 293 cells were more potent than the same vector lacking the AAV sequence made in baculovirus. As a result, additional enhancers were added to Proto 1, e.g. Constructs 101, 102, 102 and 104, in an attempt to increase potency and associated FVIII expression.

Example 6
Expression and Activity of AAV FVIII Vectors with Improved Promoters/Enhancer Sequences

[00238] The expression and activity of additional recombinant AAV FVIII vectors were tested using a hydrodynamic injection protocol. Hydrodynamic delivery is a rapid method to screen the efficiency of various recombinant AAV FVIII vectors *in vivo*. Specifically, AAV FVIII plasmid DNA was generated as described above and then diluted in TransIT-QR Hydrodynamic Delivery Solution. The plasmid DNA was injected into the tail vein of 5-6 week old C57Bl/6 mice (18-25 g) at a volume determined by (mouse weight (g)/10) = 0.1 ml delivery solution). The injection time was less than 5 seconds. Plasma from each mouse was then collected 48 hours after injection and the amount of FVIII protein expressed was measured using an ELISA assay. The amount of FVIII in the plasma of the injected mouse was measured using an ELISA test and recombinant FVIII (Xyntha SQ equivalents) was used as a standard for comparison.

[00239] To investigate FVIII expression, certain recombinant AAV FVIII constructs of the present invention were tested in the hydrodynamic injection protocol to measure their ability to result in expression of functional FVIII protein *in vivo*. As shown in Figure 6, all constructs tested at a 5 μg of plasmid dose produced functional FVIII at varying levels of efficiency.

[00240] Figures 7 and 8 provide data for hydrodynamic injection for a dose of 1 μg of plasmid of various recombinant AAV FVIII constructs of the present invention. As shown in Figures 7 and 8, injection of the various constructs tested all resulted in the *in vivo* expression of FVIII protein with varying levels of efficiency.

Example 7

Analysis of AAV Virus Comprising p-100 ATGB Vector

[00241] AAV virus comprising the FVIII-SQ-encoding vector p-100 ATGB shown herein as SEQ ID NO:45 ("AAV5-p100ATGB-FVIII") were produced and evaluated for the ability to express functional FVIII-SQ protein in Rag2 mice as described in Example 5 above. More specifically, Rag2 mice were administered a single dose of either AAV5-FVIII-SQ virus or AAV5-p100ATGB-FVIII virus at a dose of either 6E12 vg/kg, 2E13 vg.kg or 6E13 vg/kg and FVIII protein concentrations were subsequently determined in the bloodstream of the mice. The

results of these analyses demonstrated that administration of the AAV5-p100ATGB-FVIII virus produced approximately a 3-fold higher level of circulating functional FVIII protein than did the AAV5-FVIII-SQ virus at the two lower doses tested. The observed difference in expression was somewhat attenuated at the highest dose tested, although even at the highest dose tested, the AAV5-p100ATGB-FVIII virus produced a higher level of circulating functional FVIII protein than did the AAV5-FVIII-SQ virus. These results demonstrate that the AAV5-p100ATGB-FVIII virus effectively transduces liver cells *in vivo* and provides for expression of high levels of functional FVIII protein.

Example 8

Studies of a Specific Recombinant FVIII AAV Vector/Virus for Hemophilia A

[00242] Hemophilia A (HA) is an X-linked recessive bleeding disorder that affects approximately 1 in 5,000 males. It is caused by deficiency in the activity of coagulation factor VIII (FVIII), an essential cofactor in the intrinsic coagulation cascade. This disorder can be either inherited, due to a new mutation or an acquired immunologic process, leading to insufficient quantities of FVIII or a dysfunctional FVIII, but all are characterized by a defective coagulation process. The clinical phenotype of HA patients is largely governed by the level of residual expression. Severe HA is classified as FVIII activity less than 1% of wild type (< 1 IU/dL), moderate disease comprises 1-5% of wild type activity (1 IU/dl - 5 IU/dl) and the mild form is 5-40% activity (5 IU/dl - 40 IU/dl). The clinical manifestations of severe HA remain frequent spontaneous bleeding episodes, predominantly in joints and soft tissues, with a substantially increased risk of death from hemorrhage when the brain is involved.

[00243] Treatment of severe HA presently consists of intravenous injection of plasma-derived or recombinant FVIII protein (rhFVIII) concentrates, both as prophylaxis 2-3 times per week, and at the time of a bleed, to prevent or control bleeding episodes, respectively. The half-life for rhFVIII (under 24 hours for most approved products) necessitates frequent infusions, and although a major advance in the treatment of HA, it remains common for severe HA patients to continue to have multiple bleeding events on treatment (mean of 1 to 7 episodes/year with prophylaxis up to 30 to 50 for on demand treatment). The consequence of multiple bleeding events is the development of an underlying pathology that contributes to debilitating multiple-joint arthropathy and substantially increased risk of death. Chemical modification (e.g. direct

conjugation of polyethylene glycol (PEG) polymers) and bioengineering of FVIII (e.g. FVIII-Fc fusion proteins) improve half-life by approximately 50%, and thus, show promise in reduced dosing and maintaining activity levels above 1% trough. However, these longer acting FVIIIs remain dependent on multiple infusions to maintain critical levels of FVIII activity in severe HA patients. There is therefore a strong unmet need for a fully preventive treatment of HA to give patients a FVIII level compatible with a normal and hemorrhage-free life.

[00244] Gene therapy offers the potential of disease-modifying therapy by continuous endogenous production of active FVIII following a single intravenous administration of a vector with the appropriate gene sequence. Hemophilia A is well suited for a gene replacement approach because clinical manifestations are attributable to the lack of a single gene product (FVIII) that circulates in minute amounts (200 ng/ml) in the plasma. Tightly regulated control of gene expression is not essential, and modest increases in the level of FVIII (any increase of the plasma level by 2 ng/ml induces an increase in activity of 1%) can ameliorate the severe form of the disease. Thus, relatively small changes in endogenous FVIII activity results in clinically relevant improvements in disease phenotype. Finally, the response to gene transduction can be assessed using validated quantitative rather than qualitative endpoints that are easily assayed using established laboratory techniques.

[00245] Several different gene transfer strategies for FVIII replacement have been evaluated, but adeno-associated viral (AAV) vectors show the greatest promise. They have an excellent and well-defined safety profile, and can direct long term transgene expression with tropism for specific tissues such as the liver (for serotypes 2, 5 and 8, among others). Indeed, an ongoing gene therapy clinical trial for a related disorder, hemophilia B, has established that stable (>36 months) expression of human factor IX at levels that are sufficient for conversion of their bleeding phenotype from severe to moderate or mild is achievable following a single peripheral vein administration of recombinant FIX AAV-8 vector. Several participants in this trial have been able to discontinue factor prophylaxis without suffering spontaneous hemorrhages, even when they undertook activities that previously resulted in bleeding. Thus, gene therapy treatment has resulted in a substantial improvement in their quality of life.

Additional Preclinical Studies

[00246] The recombinant FVIII-SQ-encoding vector Proto1 (shown herein in Figure 2A and SEQ ID NO:1) was used to produce recombinant AAV5 FVIII-SQ-encoding virus using a

baculovirus/Sf9-based expression system as described above. The virus generated (herein referred to as "AAV5-FVIII-SQ") was purified and formulated for pre-clinical animal studies in Dulbecco's phosphate buffered saline (DPBS) containing 0.001% Poloxamer 188.

[00247] The AAV5-FVIII-SQ nonclinical program was designed to elucidate the transduction, relative expression and activity of the FVIII-SQ protein and the overall safety profile of the AAV5 capsid and FVIII-SQ transgene product components of AAV5-FVIII-SQ to support a single IV administration of the recombinant virus in human patients. The nonclinical profile of AAV5-FVIII-SQ was assessed across one *in vitro* study and ten single dose studies in mice, normal wild type (WT), Rag2 -/- (B6.129S6-Rag2tm1Fwa N12) and Factor VIII -/- (B6;129S-F8tm1Kaz/J) crossed with Rag2 -/- mouse (Rag2 -/- x FVIII -/-), and cynomolgus and rhesus monkeys.

[00248] Pharmacodynamics (PD) assessment demonstrated that AAV5-FVIII-SQ gene therapy results in (i) plasma expression of the correctly sized FVIII-SQ (light and heavy chains) compared to ReFacto® (rhFVIII-SQ; marketed as ReFacto® in the EU and Xyntha® in the US) in mice, (ii) administration of AAV5-FVIII-SQ corrected the coagulopathy in a mouse model of hemophilia A, in a dose dependent fashion, similar to exogenously administered ReFacto® and (iii) the proposed clinical route of administration via IV infusion is likely to be similar to or better than bolus administration when plasma FVIII-SQ protein and activity or corresponding liver RNA and DNA levels are compared in mice.

[00249] The transient FVIII-SQ expression in non-human primates is suspected to be species-specific and not expected to occur in the clinic, as was seen in other clinical studies that have achieved stable transgene expression in human patients. Immunogenicity will be closely monitored in the clinic and the relationship to protein expression will be evaluated.

[00250] The overall nonclinical program considered the potential for toxicity due to AAV5-FVIII-SQ and its major components, AAV5 capsid and the transgene product, FVIII-SQ. FVIII-SQ has the same amino acid sequence as the marketed recombinant factor replacement treatment, ReFacto®. The design of the toxicology program was intended to characterize the toxicological profile of AAV5-FVIII-SQ including the identification of target organs, relative plasma FVIII-SQ protein and relative activity, immunogenicity and liver DNA genomes and RNA. One GLP single-dose study in normal CD-1 mice with a 4- and 13-week follow up period was conducted

with AAV5-FVIII-SQ. PD studies in Rag2 -/- x FVIII -/- mice and normal monkeys included additional toxicity parameters of histology and clinical pathology.

[00251] The nonclinical safety profile of AAV5-FVIII-SQ included expected observations of immunogenicity: (i) detection of anti-AAV5 antibodies in the plasma of all AAV5 vector treated immuno-competent animals (CD1 mouse and monkeys) and (ii) detection of anti-FVIII-SQ antibodies in immune-competent animals was observed in one mouse and several monkeys that did not correlate with FVIII expression or activity but may be a contributor in slight APTT prolongation in four monkeys given 6E12 or 6E13 vg/kg AAV5-FVIII-SQ. Antibody levels were not determined in the Rag2 -/- derived mice because they lack mature B and T lymphocytes, and are incapable of generating antibody responses. However interspecies cross reactivity of anti-FVIII-SQ antibody with monkey FVIII was not assessed, precluding firm conclusions regarding the impact of antibody on coagulation. Non-dose dependent minimal to mild kidney inflammation was observed in Rag2 -/- x FVIII -/- mice after 8-weeks with no corresponding changes in kidney clinical chemistry parameters indicating kidney dysfunction. Kidney findings were not observed in CD-1 mice after 13-weeks suggesting a strain specific response to a heterologous protein. No AAV5-FVIII-SQ-related changes in liver clinical chemistry was observed in monkey that would indicate liver dysfunction or cytotoxicity. One unscheduled euthanasia in rhesus monkey given 6E12 vg/kg on Day 14 due to body weight loss throughout the acclimation and study period, and morbidity was deemed not related to AAV5-FVIII-SQ due to persistent body weight loss and on-going colon findings. No other AAV5-FVIII-SQ-related findings, including changes in liver clinical chemistry parameters were noted in monkeys, cynomolgus or rhesus, given AAV5-FVIII-SQ.

[00252] No specific findings were associated with the FVIII-SQ transgene product other than expected immunogenicity. Because the FVIII-SQ transgene product has a final sequence that is the same as the marketed enzyme treatment, ReFacto®, no unique FVIII-specific target organs toxicity were identified.

[00253] No unique AAV5 capsid related toxicities, in addition to expected immunogenicity, were observed in the nonclinical program. Immunogenicity of the AAV capsid will be monitored in the nonclinical and clinical programs.

[00254] Both normal and disease model mice and a limited number of monkeys were utilized to establish proof of concept, evaluate potential species scaling and dose response in order to

select the FIH dose of 6E12 vg/kg. The starting dose took into consideration the overall data from the pre-clinical studies conducted in mice (normal and disease model, Rag2 -/- x FVIII -/-) and monkey. A detectable pharmacological response based on activity was observed at 6E12 vg/kg in mice and two species of monkeys. No consistent interspecies scaling was noted between the mouse and cynomolgus and rhesus monkeys that could ascertain a more precise dose recommendation. A 10-fold safety margin was based on a NOAEL of 6E13 vg/kg AAV5-FVIII-SQ in the GLP 13-week study in normal mouse at the highest dose administered. No AAV5-FVIII-SQ-related changes in clinical observations or chemistry was observed in the monkey at doses up to 6E13 vg/kg, a 10-fold safety margin after 8-weeks. Overall, no AAV5-FVIII-SQ-related findings, except expected formation of anti-AAV5 antibodies in all animals and limited formation of low titers of anti-FVIII-SQ antibodies in immune-competent animals were observed at the highest administered doses of 6E13 vg/kg in the normal mouse and monkey, respectively.

[00255] One *in vitro* and nine *in vivo* studies were conducted to evaluate the primary pharmacodynamics (PD) of AAV5-FVIII-SQ (six non-GLP mouse studies and three non-GLP monkey studies). All studies were single dose and used the intravenous (IV) route of administration. The proposed clinical route of administration is IV infusion up to 60 minutes. The majority of animals in this program were administered AAV5-FVIII-SQ via IV bolus injection, so an evaluation of the duration of administration (IV bolus versus infusion for 30 minutes) on FVIII-SQ expression was evaluated one mouse study. Two dose response studies in mouse given 2E10 to 2E14 vg/kg AAV5-FVIII-SQ established the PD relationship of FVIII-SQ protein and activity plasma concentrations including DNA and RNA expression in the liver after 8-weeks. One mouse study supported the selection of the baculovirus-infected cell line for manufacturing. One mouse study assessed plasma FVIII protein and activity along with liver DNA and RNA over 4- and 13-weeks. One mouse study evaluated bleeding time as a functional assessment of coagulation. Two monkey studies supported the selection of the vector AAV5 and the baculovirus-infected cell line for manufacturing. A third monkey study compared the PD effect of AAV5-FVIII-SQ in cynomolgus and rhesus monkey.

[00256] The PD endpoints (plasma FVIII-SQ protein and activity, liver DNA vector genomes and RNA transcription copies) were evaluated in the mouse and monkey studies. Liver DNA vector genomes and RNA transcription copies were assessed to confirm liver transduction by AAV5-FVIII-SQ. Plasma FVIII-SQ protein and activity were used as biomarkers of liver

expression of the FVIII-SQ transgene. Several toxicity endpoints were combined into one mouse study (histology) and three monkey studies (clinical pathology) to assess dose relationship across the two species.

Pharmacodynamic Assessment of AAV5-FVIII-SQ in Rag2-/- x FVIII-/- Mice

[00257] The objective of this study was to evaluate the primary PD of AAV5-FVIII-SQ over 4- and 13-weeks following a single IV administration in male Rag2-/- x FVIII-/- mice given 6E12 or 6E13 vg/kg AAV5-FVIII-SQ. PD endpoints included plasma FVIII-SQ protein and activity levels and presence of liver FVIII-SQ RNA and DNA. Sixty male Rag2-/- x FVIII-/- mice were 8-weeks of age at study initiation. Animals were randomly assigned to six groups (10/group) and were given a single IV injection via the tail vein of either vehicle, 6E12 or 6E13 vg/kg AAV5-FVIII-SQ.

[00258] Appropriate monoclonal antibodies were coated onto plates overnight at a final concentration of 2 µg/ml, GMA8023 for FVIII heavy chain, and GMA8001 for FVIII light chain. The following day, wells were blocked with green diluent, and mouse plasma samples (50ul) from Group 4 and Group 6, 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 for enrichment of FVIII heavy or light chains. Enriched plasma samples were resolved by denaturing reducing polyacrylamide gels and transferred to nitrocellulose membrane for western analysis. FVIII heavy chain was detected by sequential incubation with biotin conjugated anti-FVIII polyclonal (SAFC-APBIO, 0.5 µg/ml) and Streptavidin conjugated alkaline phosphatase (0.25 µg/ml). FVIII-SQ light chain was detected by sequential incubation with anti-FVIII monoclonal (GMA8025, 1.0 µg/ml) and Donkey anti-mouse conjugated alkaline phosphatase (0.25 µg/ml). Membranes were developed using colorimetric precipitating alkaline phosphatase substrate (WesternBlue) and imaged.

[00259] The assessment of molecular weight of AAV transgene-derived FVIII-SQ heavy and light chains of serum from animals given 6E13 vg/kg AAV5-FVIII-SQ by western blot established that that the expressed plasma FVIII-SQ heavy and light chains were of similar molecular size as rhFVIII-SQ protein. This indicates that despite a potentially truncated genome, expression of the both the heavy and light chain of FVIII-SQ was the correct size. Efficient and functional expression of dysferlin and hemophilia A factor VIII from vectors with such truncated

genomes have been demonstrated previously. The molecular weight of both chains of plasma FVIII-SQ protein were the correct size and the corresponding mice had FVIII-SQ activity.

IV Bolus and Infusion Study in Rag2-/- Mice

[00260] The objective of this study was to compare the effect of a single IV bolus or 30-minute IV infusion of 6.0E12 and 2.0E13 vg/kg on FVIII-SQ DNA and RNA in liver tissue and plasma FVIII-SQ protein and activity levels in Rag2-/- mice at 5 weeks post-dose. Sixty male Rag2-/- mice were approximately 8-weeks old at study initiation. Animals were randomly distributed into 6 groups (10 animals/group). Groups 1-3 and 4-6 were administered a single IV bolus or 30-minute IV infusion (vehicle, 6.0E12, or 2.0E13 vg/kg AAV5-FVIII-SQ) via the tail vein, respectively.

[00261] In animals given 6.0E12 vg/kg AAV5-FVIII-SQ, hFVIII-SQ vector genomes/liver cell were 5.06E-2 and 3.50E-2 in the IV infusion and slow bolus group, respectively. FVIII-SQ expression copies/µg RNA in the liver were 3.76E4 and 1.87E4 in the IV infusion and bolus groups, respectively. In animals given 2.0E13vg/kg AAV5-FVIII-SQ, DNA values were 0.342 vector genomes/cell for the infusion group and 0.316 vector genomes/cell for the bolus group. FVIII-SQ expression copies/µg RNA in the liver were 2.35E5 for the infusion group and 1.53E5 for the bolus group.

[00262] In animals given 6.0E12 vg/kg AAV5-FVIII-SQ (low dose) there was little difference in liver RNA and DNA levels or plasma FVIII-SQ protein and activity when administered IV either by bolus or 30-minute infusion. In animals given 2.0E13 vg/kg AAV5-FVIII-SQ, administration by IV infusion over 30 minutes resulted in roughly twice the FVIII-SQ protein and activity in plasma, while liver RNA and DNA levels remained similar. Based on these data, the proposed clinical administration of AAV5-FVIII-SQ via IV infusion is likely to be similar to or better than bolus administration.

Bleeding Time Evaluation in Rag2-/- x FVIII-/- Mice

[00263] The objective this study was to evaluate the functional coagulation endpoint of bleeding time 8 weeks after a single dose of AAV5-FVIII-SQ in male Rag2-/- x FVIII-/- mice, compared to wild-type mice (C57BL/6J). Additionally, the changes in bleeding time 8 weeks after AAV5-FVIII-SQ treatment were compared to results achieved in Rag2-/- x FVIII-/- mice treated with ReFacto®. One hundred male Rag2-/- x FVIII-/- mice and twenty male age-matched

C57BL/6J mice were approximately 8 weeks old at study initiation. Animals were randomly distributed into four groups (20 animals/dose) and administered a single IV injection of AAV5-FVIII-SQ via the tail vein (C57BL/6J: vehicle; Rag2-/- x FVIII-/-: vehicle, 2.0E13 or 1E14 vg/kg AAV5-FVIII-SQ).

[00264] Rag2 -/- x FVIII -/- animals given ReFacto® had dose related decrease in bleeding time and volume. In Rag2-/- x FVIII-/- animals given 50 U/kg of ReFacto® a mean blood loss of 0.49 ± 0.30 g and a mean bleeding time of 18.1 ± 9.39 min was observed. Rag2-/- x FVIII-/- mice given 200 U/kg of ReFacto® had a mean blood loss and bleeding time of 0.134 ± 0.19 g and 4.29 ± 6.16 min.

[00265] Plasma levels of ReFacto® and FVIII-SQ were similar in mice given 50 U/kg ReFacto® and 2E13 vg/kg AAV5-FVIII-SQ, respectively.

[00266] Administration of AAV5-FVIII-SQ to Rag2-/- x FVIII-/- mice resulted in a dose dependent reduction in blood loss volume and bleeding time at 8 weeks post-dose. A dose dependent reduction in blood volume loss and bleeding time was observed at 8-weeks, postdose. In animals given 1E14 vg/kg AAV5-FVIII-SQ blood loss and bleeding time was corrected to wild-type levels, comparable to the correction achieved with ReFacto® treatment. Administration of AAV5-FVIII-SQ can correct the coagulopathy in the mouse model of hemophilia A, in a dose dependent fashion, similar to exogenously administered ReFacto®.

Dose Response in Rag2-/- x FVIII-/- Mice

[00267] In Rag2-/- x FVIII-/- mice given 2E11 through 2E12 vg/kg AAV5-FVIII-SQ, no plasma FVIII-SQ protein or activity levels were detected.

[00268] In the present study, sixty male Rag2-/- x FVIII-/- mice were approximately 8 weeks old at study initiation. Animals were randomly distributed into six groups (10 animals/dose) and administered a single IV injection of AAV5-FVIII-SQ via the tail vein (vehicle, 2E12, 6E12, 2E13, 6E13 and 2E14 vg/kg AAV5-FVIII-SQ).

[00269] FVIII-SQ plasma protein levels were generally dose related in animals given ≥ 1.5E12 vg/kg AAV5-FVIII-SQ. FVIII-SQ protein levels were below the level of quantitation in animals given ≤ 1.7E11 vg/kg AAV5-FVIII-SQ. PD activity generally increased with dose and was correlated with activity. In animals given ≤ 1.8E13 vg/kg AAV5-FVIII-SQ, inter-animal

variability was observed and only a subset of animals had detectable levels of plasma FVIII-SQ and activity.

[00270] Consistent with the FVIII-SQ protein and activity levels, vector genome copies and expression copies (RNA) were observed in animals given ≥ 1.5E12 vg/kg AAV5-FVIII-SQ. Vector genome DNA copies and expression copies RNA/ μg RNA generally increased with dose.

[00271] FVIII-SQ plasma protein levels, activity levels and vector genome and RNA levels were generally dose related in Rag2-/- x FVIII-/- animals given ≥ 1.5E12 vg/kg AAV5-FVIII-SQ. In a subset of animals given 1.5E12 (two animals) or 1.8E13 vg/kg AAV5-FVIII-SQ (eight of ten animals), doses which bracket the proposed FIH clinical dose of 6.0E12 vg/kg AAV5-FVIII-SQ, activity ranged from 2.8 through 66.4% of normal. This indicates that PD activity in the clinic may be achieved at the 6.0E12 dose level because the resulting plasma FVIII-SQ protein and activity levels will likely give a more consistent response in animals.

Capsid Selection in Cynomolgus Monkeys

[00272] The objective of this study was to assess the relative activity of two capsids (AAV5.2 FVIII-SQ and AAV8.2 FVIII-SQ, i.e., AAV5 and AAV8 capsid protein, respectively, and AAV2 ITRs) with FVIII-SQ transgenes over 8 weeks when given as a single IV bolus to cynomolgous monkey. Eight male cynomolgus monkeys were 2.8 to 4.1 years old and weighed between 2.6 and 3.6 kg at the time of study initiation. All animals were prescreened for anti-AAV5 or anti-AAV8 transduction inhibition activities in comparison to immune-depleted cynomolgus monkey serum. Animals were assigned to four groups and were given either 2.0E12 or 2.0E13 vg/kg of AAV5.2-hFVIII-SQ or AAV8.2-hFVIII-SQ as a single slow bolus intravenous administration (0.5 and 5.0 mL/kg, respectively).

[00273] Administration of a single injection of AAV5.2 hFVIII-SQ and AAV8.2 hFVIII-SQ resulted in detectable levels of plasma FVIII-SQ protein levels that was well tolerated in cynomolgus monkeys given 2.0E13 vector/kg. No AAV5-FVIII-SQ related changes in liver clinical chemistry was observed, indicating no liver dysfunction was observed. The AAV5 capsid was selected for continued development.

Single Dose IV Study in Cynomolgus Monkeys

[00274] The objective of this study was to assess the relative activity of AAV5-FVIII-SQ of two manufacturing lots produced in two cell lines (Baculovirus infected sf9 insect and human 293 cells) over 8 weeks when given as a single IV administration to cynomolgous monkey. Eight naive male monkeys were 3.9 to 4.3 years of age and weighed 2.8 to 4.3 kg at treatment initiation. All animals were prescreened for anti-AAV5 antibodies and AAV5 transduction inhibition activities prior to assignment to the study. Each monkey (2/dose group) received a single slow bolus IV injection (2E13 and 6E13 vg/kg AAV5-FVIII-SQ) and was observed for eight weeks.

[00275] Relative plasma FVIII-SQ protein levels were assessed over 8-weeks. Possible AAV5-FVIII-SQ-related APTT prolongation was observed in animals with anti-FVIII antibody formation. This is a known potential immunogenicity outcome for exogenous factor replacement. No AAV5-FVIII-SQ-related changes in liver clinical chemistry was observed, indicating no liver dysfunction was observed. Plasma FVIII-SQ levels increased over three to six weeks but declined thereafter.

[00276] All animals given AAV5-FVIII-SQ expressed levels of FVIII-SQ in the plasma after Week 2 post administration. In general, FVIII-SQ levels increased over time and then decreased by Week 8. Peak levels of plasma FVIII-SQ ranged from 4.8 ng to 67.4 ng FVIII-SQ/ml.

Single Dose IV Study in Cynomolgus and Rhesus Monkeys

[00277] In cynomolgus and rhesus monkey given 6E12 and 2E13 vg/kg AAV5-FVIII-SQ, relative expression of FVIII-SQ was assessed over 6 weeks. No AAV5-FVIII-SQ-related changes in liver clinical chemistry were observed, indicating no liver dysfunction. Plasma FVIII-SQ protein levels were greater in cynomolgus monkey compared to rhesus. Plasma FVIII-SQ levels increased over four to five weeks but declined thereafter. Liver vector genome DNA was detected in all animals given AAV5-FVIII-SQ, which implied that levels of AAV5-FVIII-SQ transduction occurred in all animals. Liver FVIII-SQ RNA copies were observed in animals that expressed plasma FVIII-SQ protein. No AAV5-FVIII-SQ-related changes in liver clinical chemistry was observed in surviving monkeys, indicating no liver dysfunction was observed.

Conclusions

[00278] Overall in multiple Rag2-/- x FVIII-/-mouse studies, plasma FVIII-SQ protein and % of normal human activity appear generally proportional with dose; similarly for DNA and RNA in liver. FVIII-SQ activity and protein levels generally increased with time after a single dose of AAV5-FVIII-SQ in mouse, while RNA increased in the liver with time. Plasma FVIII-SQ protein expression and activity tended to correlate in these studies. There was high inter-animal and inter-study variability in animals given $\leq$ 6E12 vg/kg AAV5-FVIII-SQ as evidenced by plasma FVIII-SQ levels and activity. Consistent expression of plasma FVIII-SQ protein levels was observed in animals given $\geq$ 6E12 vg/kg AAV5-FVIII-SQ.

[00279] In the limited number of monkeys given 2E12 to 6E13 vg/kg AAV5-FVIII-SQ, plasma FVIII-SQ levels were detected in animals given $\geq$ 6E12 vg/mL with no detectable plasma levels observed in animals given 2E12 vg/kg.

[00280] In studies conducted in cynomolgus monkeys, expression of FVIII-SQ peaked between 3 and 5 weeks post dosing, and declined toward study end to levels that were in some cases below the limit of detection. In some instances, anti-FVIII antibodies were detected in animals prior to, or following peak FVIII-SQ levels in the plasma. However, antibody was not detected in all animals with diminished expression of FVIII-SQ, suggesting other potential mechanisms are inhibiting expression, such as cytotoxic T-Lymphocyte (CTL) mediated clearance of transduced cells, or possibly other non-specified inhibitors of expression. The transient FVIII-SQ expression in non-human primates is suspected to be species-specific and not expected to occur in the clinic.

[00281] A single IV bolus of AAV5-FVIII-SQ in the monkey resulted in measurable FVIII-SQ protein levels in plasma at the proposed clinical starting dose 6E12 vg/kg and up to 6E13 vg/kg AAV5-FVIII-SQ; administration of AAV5-FVIII-SQ in the mouse has resulted in plasma FVIII-SQ protein and activity levels consistently observed in studies over a comparable dose range. The proposed starting dose of a Phase 1/2 human clinical trial, 6E12 vg/kg AAV5-FVIII-SQ, was selected based on a 10-fold safety factor that also had a detectable plasma FVIII-SQ protein and activity level in both monkey and mice reducing the possibility of a sub-therapeutic outcome.

Dose Escalation Safety, Tolerability and Efficacy Study of AAV5-FVIII-SQ in Human Patients with Severe Hemophilia A

[00282] In the present study, recombinant FVIII AAV virions comprising the Proto1 FVIII-SQ vector of Figure 2A (SEQ ID NO:1) will be delivered to human patients by single intravenous dose. The study is designed to achieve stable, potentially life-long expression of active hFVIII in the plasma, synthesized from vector-transduced liver tissue. This clinical study is a first-in-human study designed to assess the relationship of vector dose to the augmentation of residual FVIII activity, and whether these levels are sufficient to alter the clinical phenotype. The relationship of dose to safety will be correlated to the activity of hFVIII in patients with severe HA.

[00283] The primary objectives of this study are (i) to assess the safety of a single intravenous administration of a recombinant AAV encoding human FVIII-SQ and (ii) to determine the dose of recombinant AAV encoding FVIII-SQ required to achieve expression of FVIII at or above 5% of normal activity (>5 IU/dL) at 16 weeks after infusion. The kinetics, duration and magnitude of AAV-mediated FVIII activity in individuals with hemophilia A will be determined and correlated to an appropriate dose.

[00284] Secondary objectives of this study are (i) to describe the immune response to the FVIII transgene and/or AAV capsid proteins following systemic administration of the recombinant FVIII AAV virus, (ii) to assess the impact of FVIII AAV dosing on the frequency of FVIII replacement therapy during the study and (iii) to assess the impact of dosing on the number of bleeding episodes requiring treatment during the study.

[00285] The recombinant FVIII-SQ-encoding vector Proto1 (shown herein in Figure 2A and SEQ ID NO:1) was used to produce recombinant AAV5-FVIII-SQ virus using a baculovirus/Sf9-based expression system. The AAV5-FVIII-SQ process consists of batch cell culture, harvest, purification, and formulation, resulting in formulated bulk drug substance (FBDS). The FBDS is filtered through tandem 0.2 μm sterilizing filters and collected into sterile bioprocess collection bags prior to filling. AAV5-FVIII-SQ is then aseptically prepared by filling 1.1 ml of the sterile FBDS into 2 ml cryovials and closed with sterile caps. The filled vials are then visually inspected prior to labeling, packaging and freezing at ≤ - 65°C.

Clinical AAV5-FVIII-SQ Liquid Formulation

[00286] As the AAV5-FVIII-SQ liquid formulation described above and employed for the non-/pre-clinical studies exhibited significant adsorption of the recombinant AAV to glass and plastic surfaces, work was conducted herein to develop a novel AAV5-FVIII-SQ formulation with advantageous properties for use in human clinical studies. Purified AAV5-FVIII-SQ was formulated for human clinical studies as follows.

[00287] Purified recombinant AAV5-FVIII-SQ virus was formulated at various concentrations in a liquid formulation useful for IV administration to human patients comprising 1.38 mg/ml sodium phosphate, monobasic monohydrate, 1.42 mg/ml sodium phosphate, dibasic (dried), 8.18 mg/ml sodium chloride, 20 mg/ml mannitol and 2.0 mg/ml Poloxamer 188 (Pluronic F-68), pH 7.4. In one embodiment, the concentration of recombinant AAV5-FVIII-SQ virus in the above described formulation was 2E13 vg/ml. The resulting liquid formulation is a sterile clear/colorless to pale yellow solution useful for IV infusion and, as compared to the formulation employed for the non-/pre-clinical studies described above, reduced viral adsorptive losses to binding to glass and plastic to acceptable levels. This liquid formulation proved to be stable for extended periods during storage at $\leq$ - 65°C and is employed for the human clinical studies described below.

Human Clinical Study Design

[00288] Participants in this first-in-man, dose-escalation study with severe hemophilia A will be enrolled sequentially into one of up to three cohorts according to dose level, (i) 6E12 vector genomes [vg] per kilogram of body weight, given as a single intravenous dose (iv), (ii) 2E13 vg per kilogram, iv, or (iii) 6E13 vg per kilogram, iv, followed by a 16 week post-infusion follow-up period during which safety and efficacy assessments will be taken. After the primary endpoint analysis at 16 weeks, safety and efficacy will then be assessed for approximately 5 years.

[00289] Patients will be enrolled sequentially every 3 weeks or more between cohorts. Dose escalation may occur after a single patient has been safely dosed if the resulting FVIII activity at Week 3 is < 5 IU/dL. Three weeks is expected to be the time the expression will be close to the maximum. This escalation paradigm is intended to minimize the patient numbers exposed to sub-therapeutic doses.

[00290] The starting dose was based on the expression and safety of FVIII observed in nonclinical studies of mice and monkeys. The starting dose has a significant safety margin (10-fold) from no observed adverse effect level (NOAEL) in non-human primates.

[00291] Approximately three weeks after an injection, the decision to escalate to the next dose level will be made based on the review of safety parameters and FVIII activity. If the FVIII activity is ≥5 IU/dL, then the other patients of the dose group will be enrolled without waiting for 3 weeks between patients.

[00292] Patient 1 will be dosed by intravenous perfusion with 6E12 vector genomes [vg] per kilogram of body weight. If the activity level does not reach ≥5 IU/dL at 21 days, then a higher dose (2E13 vg per kilogram) will be used for the next patient.

[00293] If the activity level does not reach ≥5 IU/dL after Patient 2, then the highest dose (6E13 vg per kilogram) will be used for the next patient.

[00294] For each dose, if the activity level reaches 5 IU/dL and if no safety issue is found, then up to four patients will receive this dose. If at any time activity levels reach 10 IU/dL or higher, no further dose escalation will take place, but additional patients will then be dosed at this dose level for a total of 6 patients per dose.

[00295] Frequent monitoring of liver enzymes will be performed on all patients in the trial. Baseline (i.e., prior to FVIII vector administration) alanine transaminase (ALT) concentrations will be determined and post-administration ALT elevations of 1.5-fold or greater will trigger therapeutic corticosteroid use. Patients may also be treated prophylactically (i.e., prior to FVIII vector administration) with corticosteroids to protect against hepatotoxicity.

Results - Patient One

[00296] Patient One was dosed by single intravenous perfusion with 6E12 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight as described above. At the time of dosing, Patient One had a circulating blood Factor VIII level of ≤ 0.5 IU/dl. Seven days after dosing, Patient One's circulating blood Factor VIII level had increased to 5.4 IU/dl and had further increased to 19.2 IU/dl 14 days post-dosing. At 21 days post-dosing, however, Patient One's circulating Factor VIII level had decreased to ≤ 0.5 IU/dl and held consistently at that level thereafter.

Results - Patient Two

[00297] As the Factor VIII activity level of Patient One was not at least 5 IU/dl on day 21 post-dosing, Patient Two was escalated to a dose by single intravenous perfusion of 2E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight as described above. At the time of dosing, Patient Two had a circulating blood Factor VIII level of $\leq$ 0.1 IU/dl. Twenty-one days after dosing, Patient Two's circulating blood Factor VIII level had increased to 0.7 IU/dl, 2.1 IU/dl at 10 weeks post-dosing, 2.4 IU/dl at 12 weeks post-dosing, 1.9 IU/dl at 16 weeks post-dosing and 2.4 IU/dl at 28 weeks post-dosing, the latter representing an at least 24-fold increase as compared to pre-dosing levels. ALT levels measured in Patient Two did not increase to 1.5-fold or greater above baseline at any point during the 28 week observation period and, as such, no corticosteroid treatment was initiated.

Results - Patient Three

[00298] Patient Three was escalated to a dose by single intravenous perfusion of 6E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight as described above. At the time of dosing, Patient Three had a circulating blood Factor VIII level of <1.0 UL/dl. Twenty-one days after dosing, Patient Three's circulating blood Factor VIII level had increased to 3.1 IU/dl, 20.8 IU/dl at 10 weeks post-dosing, 34.7 IU/dl at 12 weeks post-dosing, 56.6 IU/dl at 16 weeks post-dosing and 89.3 IU/dl at 28 weeks post-dosing, well above the concentration of Factor VIII required for satisfactory blood coagulation in humans and decrease in bleeding time during a bleeding event in the patient.

[00299] As ALT levels in Patient Three were observed to increase 1.5-fold above baseline after FVIII vector administration, the subject was treated therapeutically with corticosteroid at concentrations ranging from 5 mg/day to 60 mg/day over the continued period of observation. Therapeutic corticosteroid treatment reduced hepatotoxicity-related ALT concentration to acceptable levels without concomitant decrease in Factor VIII levels or any associated serious adverse events.

Results - Patient Four

[00300] Patient Four was dosed by single intravenous perfusion of 6E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight as described above. At the time of dosing, Patient Four had a circulating blood Factor VIII level of <1.0 UL/dl. Twenty-one days after

dosing, Patient Four's circulating blood Factor VIII level had increased to 5.6 IU/dl, 67.8 IU/dl at 10 weeks post-dosing, 89 IU/dl at 12 weeks post-dosing, >170 IU/dl at 16 weeks post-dosing and 219.2 IU/dl at 20 weeks post-dosing, well above the concentration of Factor VIII required for satisfactory blood coagulation in humans and decrease in bleeding time during a bleeding event in the patient.

[00301] Patient Four was treated prophylactically with corticosteroid at concentrations ranging from 5 mg/day to 40 mg/day over the continued period of observation. Prophylactic corticosteroid treatment maintained hepatotoxicity-related ALT concentrations at acceptable levels without concomitant decrease in Factor VIII levels or any associated serious adverse events.

Results - Patient Five

[00302] Patient Five was dosed by single intravenous perfusion of 6E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight as described above. At the time of dosing, Patient Five had a circulating blood Factor VIII level of <1.0 UL/dl. Twenty-one days after dosing, Patient Five's circulating blood Factor VIII level had increased to 2.2 IU/dl, 24.4 IU/dl at 10 weeks post-dosing, 59.4 IU/dl at 12 weeks post-dosing, 126.5 IU/dl at 16 weeks post-dosing and 271.2 IU/dl at 19 weeks post-dosing, well above the concentration of Factor VIII required for satisfactory blood coagulation in humans and decrease in bleeding time during a bleeding event in the patient.

[00303] Patient Five was treated both prophylactically and therapeutically with corticosteroid at concentrations ranging from 5 mg/day to 40 mg/day over the continued period of observation. Prophylactic and therapeutic corticosteroid treatment maintained hepatotoxicity-related ALT concentrations at acceptable levels without concomitant decrease in Factor VIII levels or any associated serious adverse events.

Results - Patient Six

[00304] Patient Six was dosed by single intravenous perfusion of 6E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight as described above. At the time of dosing, Patient Six had a circulating blood Factor VIII level of <1.0 UL/dl. Twenty-one days after dosing, Patient Six's circulating blood Factor VIII level was <1.0 IU/dl, 6.2 IU/dl at 10 weeks post-dosing, 19.6 IU/dl at 12 weeks post-dosing, 13 IU/dl at 16 weeks post-dosing and 13 IU/dl

at 19 weeks post-dosing, well above the concentration of Factor VIII required for satisfactory blood coagulation in humans and decrease in bleeding time during a bleeding event in the patient.

[00305] Patient Six was treated therapeutically with corticosteroid at concentrations ranging from 5 mg/day to 60 mg/day over the continued period of observation. Therapeutic corticosteroid treatment maintained hepatotoxicity-related ALT concentrations at acceptable levels without concomitant decrease in Factor VIII levels or any associated serious adverse events.

Results - Patient Seven

[00306] Patient Seven was dosed by single intravenous perfusion of 6E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight as described above. At the time of dosing, Patient Seven had a circulating blood Factor VIII level of <1.0 UL/dl. Twenty-one days after dosing, Patient Seven's circulating blood Factor VIII level had increased to 10.4 IU/dl, 56.4 IU/dl at 10 weeks post-dosing, 58 IU/dl at 12 weeks post-dosing, 93.1 IU/dl at 16 weeks post-dosing and 135.8 IU/dl at 18 weeks post-dosing, well above the concentration of Factor VIII required for satisfactory blood coagulation in humans and decrease in bleeding time during a bleeding event in the patient.

[00307] Patient Seven was treated prophylactically with corticosteroid at concentrations ranging from 5 mg/day to 40 mg/day over the continued period of observation. Prophylactic corticosteroid treatment maintained hepatotoxicity-related ALT concentrations at acceptable levels without concomitant decrease in Factor VIII levels or any associated serious adverse events.

Results - Patient Eight

[00308] Patient Eight was dosed by single intravenous perfusion of 6E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight as described above. At the time of dosing, Patient Eight had a circulating blood Factor VIII level of <1.0 UL/dl. Twenty-one days after dosing, Patient Eight's circulating blood Factor VIII level had increased to 5.1 IU/dl, 35.2 IU/dl at 10 weeks post-dosing, 42.7 IU/dl at 12 weeks post-dosing, 49.7 IU/dl at 16 weeks post-dosing and 68.8 IU/dl at 17 weeks post-dosing, well above the concentration of Factor VIII required for

satisfactory blood coagulation in humans and decrease in bleeding time during a bleeding event in the patient.

[00309] Patient Eight was treated prophylactically with corticosteroid at concentrations ranging from 10 mg/day to 40 mg/day over the continued period of observation. Prophylactic corticosteroid treatment maintained hepatotoxicity-related ALT concentrations at acceptable levels without concomitant decrease in Factor VIII levels or any associated serious adverse events.

Results - Patient Nine

[00310] Patient Nine was dosed by single intravenous perfusion of 6E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight as described above. At the time of dosing, Patient Nine had a circulating blood Factor VIII level of <1.0 UL/dl. Twelve weeks after dosing, Patient Nine's circulating blood Factor VIII level had increased to 78.7 IU/dl, well above the concentration of Factor VIII required for satisfactory blood coagulation in humans and decrease in bleeding time during a bleeding event in the patient.

[00311] Patient Nine was treated therapeutically with corticosteroid at concentrations ranging from 10 mg/day to 40 mg/day over the continued period of observation. Therapeutic corticosteroid treatment maintained hepatotoxicity-related ALT concentrations at acceptable levels without concomitant decrease in Factor VIII levels or any associated serious adverse events.

Summary

[00312] The results presented in this Example 8 demonstrate that successful therapy of hemophilia A in human patients can be achieved using the compositions and methods of the present invention. More specifically, demonstrated herein is that treatment of humans suffering from hemophilia A with at least 2E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight results in stable FVIII activity of ≥2 IU/dl over at least 26 weeks post-dosing and that treatment of humans suffering from hemophilia A with at least 6E13 vector genomes [vg] of AAV5-FVIII-SQ per kilogram of body weight results in high, sustained FVIII activity of >10 IU/dl in all patients treated. Moreover, the data provided herein demonstrates that treatment with AAV5-FVIII-SQ is well-tolerated and results in no clinically-relevant sustained rises in ALT levels or other markers of hepatotoxicity. Prophylactic and/or therapeutic corticosteroid

treatment of patients is capable of maintaining hepatotoxicity-related ALT concentrations at acceptable levels without concomitant decrease in Factor VIII levels or any associated serious adverse events. Finally, initial data demonstrates that patients treated either prophylactically or therapeutically with corticosteroids can be successfully tapered off steroid treatment with no adverse impact on FVIII expression or ALT concentration levels.

CLAIMS

1. Use of a recombinant AAV5-FVIII-SQ virus in the manufacture of a medicament for treating a subject suffering from hemophilia A, wherein the medicament comprises sodium phosphate dibasic at a concentration of from 0.1 mg/ml to 3 mg/ml, sodium phosphate monobasic at a concentration of from 0.1 mg/ml to 3 mg/ml, sodium chloride at a concentration of from 1 mg/ml to 20 mg/ml, mannitol at a concentration of from 5 mg/ml to 40 mg/ml, and poloxamer 188 at a concentration of from 0.1 mg/ml to 4 mg/ml,

wherein AAV5-FVIII-SQ comprises an AAV2 5′ inverted terminal repeat (ITR), a liver specific transcriptional regulatory region, a functionally active FVIII coding region, a polyadenylation sequence, an AAV2 3′ ITR, and optionally one or more introns, wherein the functionally active FVIII coding region comprises nucleotides 403-4776 of SEQ ID NO: 1

and wherein the medicament is to be administered intravenously.

2. The use of Claim 1, wherein from 1E12 vg/kg to 1E14 vg/kg recombinant AAV5-FVIII-SQ virus is to be administered to said subject.

3. The use of Claim 1, wherein from 6E12 vg/kg to 6E13 vg/kg recombinant AAV5-FVIII-SQ virus is to be administered to said subject.

4. The use of any one of Claims 1-3 which results in expression of at least 5 IU/dl of functional Factor VIII protein in said subject.

5. The use of any one of Claims 1-4, wherein said subject expresses at least 5 IU/dl of functional Factor VIII protein 3 weeks or more after said administration.

6. The use of any one of Claims 1-4 which results in an increase in functional FVIII activity of at least 1 IU/dl in said subject.

7. The use of any one of Claims 1-6, wherein the medicament is stable during storage at ≤ 65°C for at least 2 weeks.

8. The use of any one of Claims 1-7, wherein the medicament is liquid.

9. The use of any one of Claims 1-8, wherein the medicament comprises said AAV5-FVIII-SQ virus at a concentration of from 1E12 vg/ml to 2E14 vg/ml.

10. The use of claim 9, wherein the medicament comprises said AAV5-FVIII-SQ virus at a concentration of 2E13 vg/ml.

11. The use of any one of Claims 1-10, wherein the medicament is useful for IV administration to a patient suffering from hemophilia A.

12. The use of any one of Claims 1-11, wherein said subject is to be treated prophylactically with a corticosteroid at a concentration ranging from 5 mg/day to 60 mg/day.

13. The use of any one of Claims 1-12 wherein said subject is to be treated prophylactically with a corticosteroid at a concentration ranging from 5 mg/day to 60 mg/day over a continuous period of at least 3, 4, 5, 6, 7, 8, 9 or 10 weeks or greater.

14. The use of any one of Claims 1-13 wherein said subject is to be treated therapeutically with a corticosteroid at a concentration ranging from 5 mg/day to 60 mg/day.

15. The use of any one of Claims 1-14 wherein said subject is to be treated therapeutically with a corticosteroid at a concentration ranging from 5 mg/day to 60 mg/day over a continuous period of at least 3, 4, 5, 6, 7, 8, 9 or 10 weeks or greater.

16. The use of any one of Claims 1-15 wherein the absence or presence of anti-AAV capsid antibodies in the serum of said subject is to be determined before administration of said therapeutically effective amount of said recombinant AAV5-FVIII-SQ virus.

17. The use of any one of Claim 1-15 wherein the absence or presence of hepatotoxicity in the subject is to be determined after administration of said therapeutically effective amount of said recombinant AAV5-FVIII-SQ virus.

18. A pharmaceutical formulation comprising a recombinant AAV5-FVIII-SQ virus, a buffering agent, an isotonicity agent, a bulking agent and a surfactant, wherein the formulation comprises sodium phosphate dibasic at a concentration of from 0.1 mg/ml to 3 mg/ml, sodium phosphate monobasic at a concentration of from 0.1 mg/ml to 3 mg/ml, sodium chloride at a

concentration of from 1 mg/ml to 20 mg/ml, mannitol at a concentration of from 5 mg/ml to 40 mg/ml, and poloxamer 188 at a concentration of from 0.1 mg/ml to 4 mg/ml,

wherein AAV5-FVIII-SQ comprises an AAV2 5′ inverted terminal repeat (ITR), a liver specific transcriptional regulatory region, a functionally active FVIII coding region, a polyadenylation sequence, an AAV2 3′ ITR, and optionally one or more introns, wherein the functionally active FVIII coding region comprises nucleotides 403-4776 of SEQ ID NO: 1

and wherein the formulation is to be administered intravenously.

19. The pharmaceutical formulation of Claim 18 which is liquid.

20. The pharmaceutical formulation of Claim 18 which comprises said AAV5-FVIII-SQ virus at a concentration of from 1E12 vg/ml to 2E14 vg/ml.

21. The pharmaceutical formulation of Claim 20 which comprises said AAV5-FVIISQ virus at a concentration of 2E13 vg/ml.