Grand Rounds

AAV Vectors for Hemophilia B Gene Therapy

HENGJUN CHAO, M.D., AND CHRISTOPHER E. WALSH, M.D.

Abstract

Adeno-associated viral (AAV) vector is attracting significant interest for use in gene therapy for genetic diseases, because of its unique and advantageous characteristics, compared to other currently available viral vectors. Eight natural serotypes of AAV have been identified, of which AAV serotype 2 is the one best characterized and most widely used in current gene delivery studies. The application of AAV serotype 2 in hemophilia B gene therapy is a promising development in gene therapy for genetic diseases such as hemophilia. Preliminary studies have demonstrated relation and distinction of host, genome sequences, replication, tropism, packaging of recombinant virions and cross-reactivity of neutralizing antibodies among different serotypes of AAV. This review summarizes the progress of studies in AAV serotypes and pertinent applications in hemophilia B gene therapy. The latest progress in gene delivery of coagulant factor IX (for hemophilia B) using AAV serotype vectors is described in detail.
Key Words: Hemophilia B, gene therapy, adeno-associated virus, serotype.

Hemophilia B Gene Therapy

HEMOPHILIA B is a sex-linked hemorrhagic disease resulting from deficiency in coagulant factor IX (FIX) (1). It occurs in one of 30,000 live male births in all populations (1, 2). The main clinical manifestation of the disease is unpredictable, recurrent, spontaneous bleeding in soft tissues and/or major joints. Recurrent bleeding in large joints usually leads to crippling arthropathies in a majority of severely affected patients (3, 4). The clinical severity of hemophilia B corresponds to the level of circulating FIX. Severe hemophilia occurs in less than 1% of FIX activity. With moderate hemophilia, 1–5% of FIX activity, there is infrequent, spontaneous bleeding. The presence of at least 5% of FIX seems to protect those with mild hemophilia against spontaneous bleeding (1, 4). Currently, the standard treatment for hemophilia B is infusion of FIX concentrates in response to a bleeding event (4). Prophylactic infusions of factor concentrates in children significantly reduce the frequency of bleeding episodes and subsequent joint disease, thus improving the quality of life of these patients (5). However, the high cost of purified factor products makes lifelong prophylactic infusion impractical. Administration of recombinant factors would enable the patients to be free of the potential pathogenic contamination associated with plasma-derived FIX concentrates (6). However, many patients in undeveloped countries, and even in developed countries, lack adequate medical insurance coverage; they are still at risk for infection from contaminant viral pathogens while receiving plasma-derived FIX concentrates (1, 3, 4). In addition, prevailing responsive replacement treatment cannot prevent the occurrence of progressive joint damage, which is a result of recurrent bleeding episodes occurring in those patients with the most severe cases (3, 4). Somatic gene therapy could provide sustained expression of functional FIX safely and conveniently via the introduction of the correct FIX gene into patients, circumventing the above-mentioned problems in hemophilia B treatments.

From the Division of Hematology/Oncology, Department of Medicine, Mount Sinai School of Medicine, New York, NY.

Address all correspondence to Christopher E. Walsh, M.D., Box 1097, Mount Sinai School of Medicine, New York, NY 10029; christopher-e.walsh@msnyuhealth.org

Adapted from a Grand Rounds presentation to the Department of Medicine, Mount Sinai School of Medicine, New York, NY, on April 29, 2003 and updated as of March 2004.

Hemophilia B has been one of several targets in an effort to achieve the first definitive gene correction of a genetic disease (7). Factors contributing to this effort include: the relatively small size of the FIX coding sequence (~1.5 kb), which makes it easy to be packaged and delivered by most available vectors; the relatively long half-life of the FIX protein (~20 hours); the fact that no precise regulation or specific tissue/organ is required for functional FIX expression; the well-established *in vitro* and *in vivo* assays necessary to evaluate FIX antigen and activity; the well-characterized hemophilia B animal models (FIX knock-out mouse (8) and naturally occurring hemophilia B canine (9)); and most important, the fact that only a slight increase of the circulating FIX level (5% of physiological level) results in significant clinical benefit for patients (4).

Generally, the FIX gene can be delivered via either non-viral or viral mediation. Currently, viral vectors are being generated mainly from murine Moloney leukemia retrovirus, herpes simplex virus, adenovirus, adeno-associated virus (AAV) and lentivirus. They are being used as a gene therapy modality in hemophilia B and other genetic diseases (7). In recent years, dramatic progress has been made using viral vectors in gene therapy for hemophilia B (10–22). *Ex vivo* transduction of fibroblasts with murine Moloney retroviral vectors has expressed high levels of FIX *in vitro* but has failed to produce the similar FIX yield *in vivo*, for several reasons (7). Although a clinical trial has shown that subcutaneous injections of *ex-vivo* transduced fibroblasts with murine Moloney retrovirus-based vectors have expressed functional FIX and reduced hemorrhagic frequency in two hemophilia B children (19), reproducible outcomes from more patients are needed to verify the efficacy of this procedure. One group has also demonstrated long-term expression and partial hemophilia phenotype correction in the Chapel Hill strain of hemophilia B canines, following intraportal injections of retroviral vectors and partial hepatectomy (14). Low transduction efficiency to post-mitotic cells and aggressive procedures (partial hepatectomy) required to promote cell cycling restrict the practical application of retroviral vectors (7). Recombinant adenoviral vectors express transient high levels of functional FIX, but the severe vector-related immune response and toxicity significantly limit the application of adenovirus vectors (23), especially in patients with chronic genetic diseases such as hemophilia. The advent of gutless adenoviral vectors, with the dramatic reduction of immune response and toxicity associated with adenoviral elements, may extend the application of adenoviral vectors in hemophilia gene therapy (24). The novel lentiviral vector has demonstrated promising preliminary results in hemophilia B gene therapy (25, 26). However, compared to other vectors, the relatively low FIX yield associated with the potential incapability of infecting quiescent cells vector-related toxicity remain optimized before this vector system comes to practical application in human patients (25–27). Compared to retrovirus, adenovirus and lentivirus, which are the only non-pathogenic viral vectors, recombinant adeno-associated virus (rAAV) is considered one of the best vectors for FIX gene transfer. This is based on its unique and advantageous characteristics including vector-related toxicity and safety, target tissue/organ tropism, and persistence of transgene expression (22, 28–30).

Gene Therapy for Hemophilia B Using AAV Serotype 2 Vectors

In the past decade, AAV has attracted significant interest as a promising gene delivery vector, due to its unique characteristics (22, 28–30). AAV is a group of nonpathogenic, replication-defective small DNA viruses. Out of the six natural serotypes of AAV, AAV serotype 2 (AAV2) is the best characterized and most widely used (30). Current understanding of AAV biology, the expertise in manipulating recombinant AAV, and the production and purification of rAAV have resulted from studies on AAV2 (30). A majority of applications of AAV as gene transfer vectors were also performed on the basis of AAV2 (28–30).

AAV2 is a small (~20 nm of diameter) DNA virus containing 4675 bp of linear, single-stranded genome. The nonstructure rep genes are located within the left part of the genome and encode four proteins, designated Rep78, Rep68, Rep52 and Rep40. The structure cap genes encode the three capsid proteins—VP1, VP2 and VP3—of which protein VP3 is the most abundant and accounts for 90% of the total virion capsid. The only cis components required to generate AAV2 vectors are the two 145 nucleotide-inverted terminal repeats (30). These terminal repeats are required for the replication and packaging of the recombinant genome into newly formed AAV virions (30). Advances in the production and purification of

rAAV type 2 vectors, including the elimination of helper virus (ad) contamination (31), and purification by column chromatography (32), have helped in the advancement of AAV2 use in gene therapy applications. Like nonpathogenic wild type AAV type 2 (wtAAV2), rAAV infects both dividing and nondividing cells and establishes latency for the life of the cell. Although the natural route of infection for wtAAV is upper respiratory, rAAV has demonstrated efficient infection and long-term expression of transgenes in the brain, liver, muscle, retina, and vasculature of experimental animals (30).

Hemophilia B gene therapy using rAAV2 has made great progress in recent years (33, 34). Intraportal vein injection of rAAV2 has expressed sustained therapeutic levels of FIX in hemophilia B mouse and dog models, and partially corrected the abnormal hemostasis parameters of the animals (18, 21, 35, 36). With a canine FIX (cFIX) expression cassette carrying a liver-specific promoter, a group reported expression of supra-physiological levels of functional cFIX (up to 20 μg/mL) and complete hemophilia phenotype correction in hemophilia B mice following intraportal delivery of the rAAV2/FIX vectors (20). This vector was optimized by adding a post-transcriptional element (37) and administered to the liver of the Chapel Hill strain of hemophilia B dogs. A subsequent report from the same group demonstrated that this optimized cFIX expression cassette expressed therapeutic levels of FIX (~240 ng/mL, ~5% of physiological level) and achieved the complete correction of the abnormal whole blood clotting time (WBCT) in one of the two dogs (21). Both dogs received the high dose of rAAV2 vectors (2.8×10^{12} and 4.6×10^{12} rAAV2 particles/kg). This was the first report to demonstrate expression of therapeutic levels of FIX (5% of physiologic level) from AAV2 vectors in large animal models, though only one dog showed this therapeutic level of FIX and complete correction of WBCT, and a dramatic reduction in the frequency of spontaneous bleeding, after receiving a high dose of rAAV2 vectors (21). Another group recently reported expression of therapeutic levels of FIX in hemophilia B dogs after hepatic delivery of rAAV2 vectors carrying a strong liver promoter (36). However, due to frequent exposure to blood products, a high prevalence of hepatitis virus infections and subsequent liver diseases was observed in the hemophilia population (1, 3, 4), which may restrict the application of AAV targeting liver for gene therapy in these patients. In fact, a patient enrolled in a recent phase I clinical trial using hepatic delivery of AAV2 vector for hemophilia B gene therapy attained therapeutic levels but transient expression of FIX. The peak-and-through plasma levels of FIX coincided with an increase and decrease of the patient's serum transaminase (38). The real cause(s) responsible for diminished FIX expression was unclear and may be complicated. However, the potential contribution from cryptic liver damage could not be completely excluded.

Skeletal muscle is a good targeting tissue for somatic gene therapy (39, 40). The large bulk of this muscle allows for multiple injections of a large volume of the viral vectors. Direct intramuscular injection is in general well-established medical practice and is applicable to vector delivery. Skeletal muscle has shown the capability of secreting a range of functional proteins, especially those capable of secreting FIX and carrying out post-translational modification of FIX (γ-carboxylation) (41–45). The pilot study demonstrated that direct intramuscular injections of rAAV2 vectors are capable of facilitating long-term (over 1.5 years) transgene (LacZ) expression without detectable cellular immune response against AAV vector and the transgene (46). An rAAV2 vector carrying human FIX canine DNA (cDNA) was then produced and administrated via direct intramuscular injections to the Chapel Hill strain hemophilia B dogs. Formation of anti-human FIX inhibitory antibodies disturbed assays on circulating FIX antigen and activity in the dogs, even though evidence demonstrated expression of rAAV-derived human FIX in the injected skeletal muscle in the dogs (17). To bypass interference of anti-human FIX inhibitor, two rAAV2 vectors expressing cFIX were constructed and high-titer rAAV2 vectors (~10^{13}) were produced (10). After direct intramuscular injection of rAAV2/cFIX into two Chapel Hill strain hemophilia B canines, expression of about 1% of normal level of canine FIX was detected by enzyme-linked immunosorbent assay (ELISA) assay (10). The activity of the cFIX resulted in reduction of WBCT from more than 60 minutes before injections, down to less than 20 minutes after AAV2 injections in two canines. The cFIX level and reduction of WBCT persisted for three years in one dog and for nine months in the second dog, at which time it was sacrificed (10). The cFIX expressed was not sufficient to completely correct the bleeding phenotype of the dogs. Both canines still suffered from sponta-

neous bleeding, which required intermittent infusion of normal dog plasma (10). Our results were consistent with a report using a similar strategy in the same strain of hemophilia B canines. In that study, where as much as 1×10^{13} rAAV2/cFIX virion particles per kilogram of the canine body weight was administered, up to 1.2% of normal level of cFIX was expressed into the circulation of the Chapel Hill strain hemophilia B dogs (13). The dogs showed reduction of WBCT from over 60 minutes before AAV injection, down to 15–20 minutes after injections, while still suffering from spontaneous bleeding (13). Further investigation determined that formation and persistence of neutralizing antibodies (NAB) against AAV2 in both dogs was detected after virus injection (10). The NAB significantly inhibited rAAV2 vectors from transducing target cells *in vitro* (10).

Investigation of biodistribution of rAAV2 vectors after intramuscular injections revealed that a majority of the AAV2 vectors were trapped around the injection site, which acts as the resource to express functional FIX. Heparan sulfate proteoglycan (HSP) acts as receptor for AAV2 (47). It was also found that AAV2 tropism is limited to slow-twitch muscle fibers rather than fast-twitch fibers, associated with an overexpression of HSP on slow muscle fibers (48). The limited tropism of AAV2 to certain myofibers may partially account for the relatively low FIX yield from injected skeletal muscle.

These studies demonstrate the promising outlook for rAAV2 as a safe and effective approach for hemophilia B gene therapy. Compared to the aggressive intraportal injection, and taking into account the high prevalence of hepatic viral infections in adult hemophilia patients, the safe and convenient intramuscular injection of AAV has its own advantages in hemophilia B gene therapy. However, FIX levels in hemophilia B canines were subtherapeutic (less than 2% of normal level, failing to completely correct hemophilia phenotype) despite high doses of rAAV2 (up to 1×10^{13} AAV particles per kilogram of animal body weight) (10, 13). When scaling up from a 10 kg dog to a 70 kg adult patient, it is difficult to produce enough rAAV2 vectors for expressing minimal therapeutic FIX levels in patients, based on current AAV production protocol (31). In fact, a gene therapy clinical trial based on intramuscular delivery of rAAV2 exhibited undetectable FIX in the circulation of the recipients despite FIX expression detected at the injection site (15). Alternate strategies to re-administer AAV2 vectors may be impeded due to the formation and persistence of high titer of NAB against AAV2 in the animal after primary injections of AAV2, which probably makes re-administration of rAAV2 ineffective (10, 49–52). In addition, high prevalence of NAB against AAV2 in the general human population (53–55) may also restrict application of rAAV2 in human gene therapy. Due to the frequent exposure to blood products, and thus more susceptibility to viral infection (3, 4, 56), the hemophilia population may have a higher prevalence of AAV2 NAB than does the general population. Strategies are required to bypass AAV NAB and/or increase rAAV transduction efficiency in target tissue to reach a therapeutic level of FIX.

AAV Serotypes as Gene Transfer Vectors

Eight natural serotypes of AAV have been cloned and sequenced (30, 49, 57–59). They were termed serotypes 1–8 in accordance with their unique serological (immunological) characteristics, and the sequences of isolation and characterization (60–65). AAV serotype 1 was first isolated from the rhesus monkey, based on seroepidemiological evidence, but frequently infects human population; however, it has not yet been isolated from the human species (49, 53, 60, 62, 64, 65). AAV serotype 2, 3 and 5 are human viruses and each demonstrates a high prevalence of infection in the general human population, based on seroepidemiological investigation results (53, 60, 62, 63, 65, 66). AAV serotype 4 is of African green monkey origin and rarely infects humans, based on seroepidemiological studies, although it can infect human cells *in vitro* (58, 61, 62). Serotypes 1 and 6 share >99% amino acid homology in their capsid proteins, and sequence analysis supports a recombination event between serotypes 1 and 2, resulting in the genesis of AAV6 (49).

Comparison of the serotype capsid amino acid sequences suggests that heterogeneity exists among AAV serotype capsids (49, 57–59, 67), a finding consistent with serological study results (53, 62, 63, 65, 66). Preliminary studies on AAV serotypes as gene transfer vectors also suggest different tropism and transduction efficiency on different cell types (when compared to AAV2 or with each other) (11, 49, 51, 57, 68–70). Xiao and colleagues reported that AAV1 demonstrated higher transduction efficiency and transgene expression than AAV2 in skeletal muscle in C57BL/6 mice, while using α anti-trypsin as the reporter gene; however, it is

the reverse when targeting the liver of the mice (49). Although AAV3 shows high homology with AAV2 in genome sequence, a study demonstrated capacity of AAV3-based vector to infect erythroid hematopoietic cells in which AAV2-based vector failed (70), indicating that differential tropism exists between the two genetically close vectors. In addition, AAV3 vector was reported to be capable of evading anti-AAV2 NAB and successfully infecting rabbit airway epithelial cells (51, 52) despite high homology and cross-reactivity between AAV2 and AAV3. However, AAV6, which is more constant from AAV2 than from AAV3, demonstrated a higher efficiency in evading pre-existing AAV2 NAB and infecting the airway epithelial cells (51, 52). It was also reported that AAV serotypes 4 and 5 have different host cell tropisms in the brain when compared to each other and to AAV2 (69). A vector based on AAV serotype 5 but not on serotype 2 demonstrated the capacity to infect the apical surface of airway epithelium and facilitate gene transfer (68). AAV serotype 6-derived vectors showed 15–74-fold higher transduction efficiency than AAV2 vectors in airway epithelia (71). A very recent report shows superhepatic transduction of the newly identified AAV serotype 7 and 8 vectors in rodents (72). In summary, the above reports demonstrated higher tropism of AAV serotypes vectors, with the exception of AAV2, indicating the feasibility of increasing AAV-mediated gene transfer efficiency in different cell types by using non-AAV-serotype-2 vectors. They also show the feasibility of evading pre-existing anti-AAV2 NAB by using other AAV serotype vectors.

We tested the transduction efficiency of rAAV serotype 1–5 vectors in the skeletal muscle of the mouse. Our results demonstrated super-intramuscular transduction efficiency of rAAV1 and rAAV5 over rAAV2 vectors (11). A subsequent investigation of rAAV serotype vectors targeting rat retina and mouse liver found that rAAV1 vector was superior for efficient transduction of liver, followed in order by rAAV serotypes 5, 3, 2, and 4 (73, 74), which is in accord with that in skeletal muscle (11, 75, 76). Surprisingly, this order changed when the vector was introduced into rat retina. Types 5 and 4 were most efficient, followed by type 1 (73).

AAV-Serotype-Mediated Gene Therapy for Hemophilia B

Direct intramuscular injection of AAV2 vector has proven to be a safe and convenient procedure to provide sustained expression of the transgene (FIX, etc.) in gene therapy for hemophilia B and other genetic diseases (34). However, only subtherapeutic expression of FIX was seen in large animal models and human patients after intramuscular injection of even high doses of AAV2 vectors, which is attributed to the limited muscular transduction efficiency of AAV2. In our attempt to pursue more efficient intramuscular transduction of AAV vectors and higher FIX expression, we found higher skeletal muscle transduction efficiency using serotypes of AAV vectors, especially AAV1 vector, than of AAV2 (11, 77).

We then tested the five serotype AAV/cFIX vectors for FIX expression in skeletal muscle. A dose of 2.5×10^{11} vector genomes of AAV/cFIX serotypes 1, 2, 3, 4 and 5 was injected into the hind limbs of nonobese diabetic/severe combined immune deficient (NOD/SCID) mice (n=5, for each experimental group). Plasma was tested at monthly intervals for cFIX using an established ELISA assay specific for cFIX. We detected cFIX in all experimental animals. No cFIX antigen was detected in plasma samples taken from control animals receiving equivalent doses of serotyped AAV/GFP. As expected and consistent with the green fluorescent protein (GFP) studies, cFIX antigen levels in mice receiving AAV types 1, 3, 4 and 5 were higher than those in the AAV2/cFIX mice. The amount of cFIX in the plasma of mice receiving types 1, 3 and 5 was unexpected. Levels higher than 100 μg/mL and 10 μg/mL of cFIX were detected in the mice receiving AAV types 1 and 5, respectively. This represents a 10^3 and 10^2 increase of cFIX, respectively, when compared to mice receiving AAV2/cFIX. Western blot results verified the ELISA data showing that supraphysiological levels of cFIX were expressed in the plasma of AAV1/cFIX-injected mice. We determined the AAV1/cFIX protein to have a molecular weight of approximately 64 Kd. FIX levels detected in the AAV2-injected mice in our studies were lower than those in a previous report using AAV2 to express human FIX (hFIX) in muscle of Rag1 knock-out mice (~100 ng/mL vs. 300 ng/mL) (78). Differences in the transgene (cFIX vs. hFIX), transgene cassette (including different polyA signal sequence and position of intron), AAV production and titration, and mouse strains (NOD/SCID vs. Rag1) between the two studies may be responsible for the different FIX expression outcomes.

To extend the results observed in SCID mice, we tested AAV1 and AAV2 serotype vec-

tors for FIX expression and hemophilia B phenotype correction in FIX knock-out mice (77). These mice develop spontaneous bleeding consistent with the hemophilic phenotype and have no detectable FIX mRNA, due to deletion of the 5′ untranslated region through exon 3 of the mouse FIX gene (8). In addition to spontaneous bleeding, these animals show prolonged *in vitro* clotting activity (APTT) and cannot survive tail-clip-induced trauma, due to sustained bleeding (8). A dose of 1×10^{11} AAV1/cFIX vector genomes was injected into the hind limbs of FIX knock-out mice (n=10).

Compared to AAV1/GFP controls, high levels of cFIX antigen were measured only in plasma of rAAV1/cFIX injected animals. Peak antigen expression was observed 3–5 weeks post-injection, consistent with all previous studies using AAV2 vectors. Western blot quantitation verified the presence of ~64 Kd protein at levels approximately 2–5 times that of normal plasma. Accordingly, the abnormal APTT of the mice was completely corrected in the AAV1/cFIX-treated animals and all the AAV1/cFIX treated mice survived the tail clip challenge.

In comparison, cohorts of FIX knock-out mice injected with 1×10^{11} AAV2/hFIX vector genomes generated anti-FIX antibody which was detected as early as one week after AAV injection. When we gave cyclophosphamide (CTX) to the AAV2-injected mice, a peak cFIX level of 100 ng/mL (2% of normal) was measured. APTT results in these animals were consistently elevated above the normal range, but were not manifested by normal clotting activity. The differences between serotype specific transduction were further distinguished by tail clip challenge resulting in survival of all AAV1-treated mice compared to 50% of AAV2-treated mice.

Based on our studies, AAV type 1 vectors appeared to be most efficient in muscle and liver transduction, followed by types 5, 3, 4, and finally 2 (11). A previous report comparing AAV1 and AAV2 vectors in muscle using different reporter genes in mouse strain also described increased vector transduction for type 1, albeit at lower levels (49). However, a very recent report verified our observation of rAAV1 transduction superiority in muscle while delivering FIX gene (79).

Discussion

The accumulated evidence suggests that current protocols of gene therapy for hemophilia B using rAAV2 targeting skeletal muscle are unable to provide the therapeutic levels of FIX necessary to prevent spontaneous hemorrhages in large animal models and/or human patients (10, 13, 15), although they seemed promising for small rodent models (78). Our preliminary studies demonstrate that rAAV1 vectors may bring about a dramatic increase in FIX expression after transducing skeletal muscle in large animal models, thus leading to successful gene therapy for hemophilia B; however, it remains to be proven. Our preliminary results in the hemophilia B mice show complete phenotype correction of hemophilia after intramuscular injection of rAAV1 vectors. To our knowledge, this is the first time hemophilia phenotype correction has been achieved by intramuscular injection of AAV vector. However, we realized, from our own experience (10) and the literature (12, 18, 20, 21), that there is a lower FIX yield in canine models than in rodent models, when both receive the same dose of AAV vectors. While our preliminary studies demonstrate that rAAV1 led to particularly robust transduction in mouse skeletal muscle, it is conceivable that human skeletal muscle may not demonstrate the same improved tropism. However, experience with rAAV2 infection across many mammalian species suggests that this is unlikely (10, 12, 17, 80–82).

Our preliminary studies also demonstrated that a simple change of the rAAV/cFIX capsid without modifying the cFIX expression cassette would dramatically increase the cFIX yield (11). rAAV tropism difference, based on the unique capsid structure, may account for promotion of cFIX expression. Optimization of the FIX expression cassette by the use of stronger (tissue-specific) enhancers/promoters (83), and post-transcriptional elements will be performed to increase FIX expression (21, 37).

Because the AAV serotype capsid is the only difference among our serotype AAV/cFIX vectors, it is reasonable to hypothesize that virus binding and entry into the target cells (47, 84, 85), intracellular trafficking (86, 87), and nuclear transportation, all of which are associated with the unique capsid of the AAV serotypes, may be the major machinery accounting for significant differential transgene (cFIX) expression efficiency of AAV serotype vectors. This seems true in the case of AAV serotypes targeting skeletal muscle for cFIX expression (11), and hence should be universal in all gene transfer. However, in some cases, the robust transgene expression from non-AAV-

serotype 2 may be impeded due to the silence of the promoter in certain cells and/or the limited capacity of certain cells to process the transgene (like post-translational modification), even though the cells were efficiently infected or the transgene mRNA was transcribed abundantly. We need further testing of targeting more than skeletal muscle and delivering more than canine FIX gene in more than mouse animal models.

References

1. Roberts H, Hoffman M. Hemophilia and related conditions—inherited deficiencies of prothrombin (factor II), factor V, and factor VII to XII. In: Beutler E, Lichtman M, Coller B, Kipps T. Williams hematology. 5th ed. New York: McGraw-Hill, Inc.; 1995. pp. 1415–1416.
2. Soucie JM, Evatt B, Jackson D. Occurrence of hemophilia in the United States. The Hemophilia Surveillance System Project Investigators. Am J Hematol 1998; 59(4):288–294.
3. Center for Diseases Control and Prevention. Report on the Universal Data Collection Program (UDC), Includes data collected from May 1998 through February 2000. Atlanta (GA); US Department of Health and Human Services. 2000. Vol. 2. No. 2.
4. Roberts H, Lozier J. Clinical aspects and therapy for hemophilia B. In: Hoffman R, Benz E, Shattil S, et al., editors. Hematology, basic principles and practice. New York; Churchill-Livingstone; 1991. pp. 1325–1331.
5. Lofqvist T, Nilsson I, Berntorp E, Pettersson H. Haemophilia prophylaxis in young patients—a long-term follow-up. J Intern Med 1997; 241(5):395–400.
6. Lee C. Recombinant clotting factors in the treatment of hemophilia. Thromb Haemost 1999; 82(2):516–524.
7. Verma IM, Somia N. Gene therapy—promises, problems and prospects. Nature 1997; 389(6648):239–242.
8. Lin H, Maeda N, Smithies O, et al. A coagulation factor IX-deficient mouse model for human hemophilia B. Blood 1997; 90(10):3962–3966.
9. Evans JD, Brinkhous KM, Brayer GD, et al. Canine hemophilia B resulting from a point mutation with unusual consequences. Proc Natl Acad Sci U S A 1989; 86(24):10095–10099.
10. Chao H, Samulski R, Bellinger D, et al. Persistent expression of canine factor IX in hemophilia B canines. Gene Ther 1999; 6:1695–1704.
11. Chao H, Liu Y, Rabinowitz J, et al. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. Mol Ther 2000; 2(6):619–623.
12. Herzog RW, High KA. Adeno-associated virus-mediated gene transfer of factor IX for treatment of hemophilia B by gene therapy. Throm Haemost 1999; 82(2):540–546.
13. Herzog RW, Yang EY, Couto LB, et al. Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. Nat Med 1999; 5(1):56–63.
14. Kay MA, Rothenberg S, Landen CN, et al. In vivo gene therapy of hemophilia B: sustained partial correction in factor IX-deficient dogs. Science 1993; 262(5130):117–119.
15. Kay MA, Manno CS, Ragni MV, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet 2000; 24(3):257–261.
16. Koeberl DD, Alexander IE, Halbert CL, et al. Persistent expression of human clotting factor IX from mouse liver after intravenous injection of adeno-associated virus vectors. Proc Natl Acad Sci U S A 1997; 94(4):1426–1431.
17. Monahan PE, Samulski RJ, Tazelaar J, et al. Direct intramuscular injection with recombinant AAV vectors results in sustained expression in a dog model of hemophilia. Gene Ther 1998; 5(1):40–49.
18. Snyder RO, Miao C, Meuse L, et al. Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. Nat Med 1999; 5(1):64–70.
19. Wang J, Han F, Yang J, et al. Clinical study on hemophilia B gene therapy. Chinese J Hematl 1994; 15(6):282–285.
20. Wang L, Takabe K, Bidlingmaier SM, et al. Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. Proc Natl Acad Sci U S A 1999; 96(7):3906–3910.
21. Wang L, Nichols TC, Read MS, et al. Sustained expression of therapeutic level of factor IX in hemophilia B dogs by AAV-mediated gene therapy in liver. Mol Ther 2000; 1(2):154–158.
22. Kaufman RJ. Advances toward gene therapy for hemophilia at the millennium. Hum Gene Ther 1999; 10(13):2091–2107.
23. Kung SH, Hagstrom JN, Cass D, et al. Human factor IX corrects the bleeding diathesis of mice with hemophilia B. Blood 1998; 91(3):784–790.
24. Hammerschmidt DE. Development of a gutless vector. J Lab Clin Med 1999; 134(3):C3.
25. Buchschacher GL Jr, Wong-Staal F. Development of lentiviral vectors for gene therapy for human diseases. Blood 2000; 95(8):2499–2504.
26. Park F, Ohashi K, Kay MA. Therapeutic levels of human factor VIII and IX using HIV-1-based lentiviral vectors in mouse liver. Blood 2000; 96(3):1173–1176.
27. Park F, Ohashi K, Chiu W, et al. Efficient lentiviral transduction of liver requires cell cycling in vivo. Nat Genet 2000; 24(1):4952.
28. Monahan PE, Samulski RJ. AAV vectors: is clinical success on the horizon? Gene Ther 2000; 7(1):24–30.
29. Rabinowitz JE, Samulski J. Adeno-associated virus expression system for gene transfer. Curr Opin Biotechnol 1998; 9(5):470–475.
30. Samulski RJ, Sally M, Muzyczka N. Adeno-associated viral vectors. In: Friedman T. The development of human gene therapy. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1999. pp. 131–172.
31. Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J Virol 1998; 72(3):2224–2232.
32. Zolotukhin S, Byrne BJ, Mason E, et al. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 1999; 6(6):973–985.
33. VandenDriessche T, Collen D, Chuah MK. Gene therapy for the hemophilias. J Thromb Haemost 2003; 1(7):1550–1558.
34. Walsh CE. Gene therapy progress and prospects: gene therapy for the hemophilias. Gene Ther 2003; 10(12):999–1003.
35. Snyder RO, Miao CH, Patijn GA, et al. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat Genet 1997; 16(3):270–276.
36. Mount JD, Herzog RW, Tillson DM, et al. Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. Blood 2002; 99(8):2670–2676.
37. Loeb JE, Cordier WS, Harris ME, et al. Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy. Hum Gene Ther 1999; 10(14):2295–2305.

38. Glader B, Kay MA, Hutchinson S, et al. A phase I safety study in patients with severe hemophilia B (factor IX deficiency) using adeno-associated viral vector to delivery the gene for human factor IX into the liver. The National Hemophilia Foundation's Fifth Workshop on Gene Therapies for Hemophilia. Apr 30, 2002. La Jolla, CA. 2003. Abstract.
39. Breithaupt H. AAV muscles ahead. Nat Biotechnol 1999; 17(9):838.
40. Blau H, Springer M. Muscle-mediated gene therapy. N Engl J Med 1995; 333(23):1554–1556.
41. Arruda V, Hagstrom J, Deitch J, et al. Posttranslational modifications of recombinant myotube-synthesized human factor IX. Blood 2001; 97(1):130–138.
42. Kurachi S, Pantazatos DP, Kurachi K. The carboxyl-terminal region of factor IX is essential for its secretion. Biochemistry 1997; 36(14):4337–4344.
43. Wu SM, Cheung WF, Frazier D, Stafford DW. Cloning and expression of the cDNA for human gamma-glutamyl carboxylase. Science 1991; 254(5038):1634–1636.
44. Yao SN, Kurachi K. Expression of human factor IX in mice after injection of genetically modified myoblasts. Proc Natl Acad Sci U S A 1992; 89(8):3357–3361.
45. Yao SN, Smith KJ, Kurachi K. Primary myoblast-mediated gene transfer: persistent expression of human factor IX in mice. Gene Ther 1994; 1(2):99–107.
46. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J Virol 1996; 70(11):8098–8108.
47. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J Virol 1998; 72(2):1438–1445.
48. Pruchnic R, Cao B, Peterson ZQ, et al. The use of adeno-associated virus to circumvent the maturation-dependent viral transduction of muscle fibers. Hum Gene Ther 2000; 11(4):521–536.
49. Xiao W, Chirmule N, Berta SC, et al. Gene therapy vectors based on adeno-associated virus type 1. J Virol 1999; 73(5):3994–4003.
50. Manning WC, Zhou S, Bland MP, et al. Transient immunosuppression allows transgene expression following readministration of adeno-associated viral vectors. Hum Gene Ther 1998; 9(4):477–485.
51. Halbert C, Rutledge E, Allen J, et al. Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. J Virol 2000; 74(3):1524–1532.
52. Beck S, Jones L, Chesnut K, et al. Repeated delivery of adeno-associated virus vectors to the rabbit airway. J Virol 1999; 73(11):9446–9455.
53. Blacklow N, Hoggan M, Rowe W. Serologic evidence for human infection with adenovirus-associated viruses. J Natl Cancer I 1968; 40(2):319–327.
54. Chirmule N, Propert K, Magosin S, et al. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther 1999; 6(9):1574–1583.
55. Erles K, Sebokova P, Schlehofer J. Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). J Med Virol 1999; 59(3):406–411.
56. Grossman Z, Mendelson E, Brok-Simoni F, et al. Detection of adeno-associated virus type 2 in human peripheral blood cells. J Gen Virol 1992; 73:961–966.
57. Rutledge E, Halbert C, Russell D. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. J Virol 1998; 72(1):309–319.
58. Chiorini J, Yang L, Liu Y, et al. Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. J Virol 1997; 71:6823–6833.
59. Chiorini J, Kim F, Yang L, Kotin, R. Cloning and characterization of adeno-associated virus type 5. J Virol 1999; 73((1309–1319.
60. Athchison R, Casto, B. Adenovirus-associated defective virus particles. Science 1965; 149:754–756.
61. Parks W, Melnick J, Rongey R, Mayor H. Physical assay and growth cycle studies of a defective adeno-satellite virus. J Virol 1967; 1(1):171–180.
62. Parks W, Boucher D, Melnick J, et al. Seroepidemiological and ecological studies of the adenovirus-associated satellite viruses. Infect Immun 1970; 2(6):716–722.
63. Bantel-Schaal U, Hausen H. Characterization of the DNA of a defective human parvovirus isolated from a genital site. Virology 1984; 134:52–63.
64. Hoggan M, Blacklow N, Rowe W. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. Proc Natl Acad Sci U S A 1966; 55:1467–1474.
65. Blacklow NR, Hoggan MD, Kapikian AZ, et al. Epidemiology of adenovirus-associated virus infection in a nursery population. Am J Epidemiol 1968; 88(3):368–378.
66. Georg-Fries B, Biederlack S, Wolf J, Hausen H. Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. Virology 1984; 134:64–71.
67. Muramatsu S, Mizukami H, Young N, Brown, K. Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. Virology 1996; 221:208–217.
68. Zabner J, Seiler M, Walters R, et al. Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. J Virol 2000; 74(8):3852–3858.
69. Davidson B, Stein C, Heth J, et al. Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. Proc Natl Acad Sci U S A 2000; 97:3428–3432.
70. Handa A, Muramatsu S, Qiu J, et al. Adeno-associated virus (AAV)-3-based vectors transduce haematopoietic cells not susceptible to transduction with AAV-2-based vectors. J Gen Virol 2000; 81:2077–2084.
71. Halbert C, Allen J, Miller A. AAV6 vectors for airway gene delivery. 2000 Annual Meeting of Cystic Fibrosis Foundation. Baltimore (MD). 2000.
72. Gao GP, Alvira MR, Wang L, et al. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci U S A 2002; 99(18):11854–11859.
73. Rabinowitz J, Rolling F, Li C, et al. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J Virol 2002; 76(2):791–801.
74. Grimm D, Zhou S, Nakai H, et al. Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. Blood 2003; 102(7):2412–2419.
75. Duan D, Yan Z, Yue Y, et al. Enhancement of muscle gene delivery with pseudotyped adeno-associated virus type 5 correlates with myoblast differentiation. J Virol 2001; 75(16):7662–7671.
76. Hildinger M, Auricchio A, Gao G, et al. Hybrid vectors based on adeno-associated virus serotypes 2 and 5 for muscle-directed gene transfer. J Virol 2001; 75(13):6199–6203.
77. Chao H, Monahan P, Liu Y, et al. Sustained and complete phenotype correction of hemophilia B mice following intramuscular injection of AAV1 serotype vectors. Mol Ther 2001; 4(3):217–222.
78. Herzog RW, Hagstrom JN, Kung SH, et al. Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. Proc Natl Acad Sci U S A 1997; 94(11):5804–5809.

79. Arruda VR, Schuettrumpf J, Herzog RW, et al. Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1. Blood 2004; 103(1):85–92.
80. Alexander I, Russell D, Spence A, Miller A. Effects of gamma irradiation on the transduction of dividing and nondividing cells in brain and muscle of rats by adeno-associated virus vectors. Hum Gene Ther 1996; 7(7):841–850.
81. Chao H, Mao L, Bruce A, Walsh C. Sustained expression of human factor VIII in mice using a parvovirus-based vector. Blood 2000; 95(5):1594–1599.
82. Rudich S, Zhou S, Srivastava R, et al. Dose response to a single intramuscular injection of recombinant adeno-associated virus-erythropoietin in monkeys. J Surg Res 2000; 90(2):102–108.
83. Hagstrom J, Couto L, Scallan C, et al. Improved muscle-derived expression of human coagulation factor IX from a skeletal actin/CMV hybrid enhancer/promoter. Blood 2000; 95:2536–2542.
84. Bartlett JS, Wilcher R, Samulski RJ. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. J Virol 2000; 74(6):2777–2785.
85. Kaludov N, Brown KE, Walters RW, et al. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. J Virol 2001; 75(15):6884–6893.
86. Bantel-Schaal U, Hub B, Kartenbeck J. Endocytosis of adeno-associated virus type 5 leads to accumulation of virus particles in the Golgi compartment. J Virol 2002; 76(5):2340–2349.
87. Yan Z, Zak R, Luxton GW, et al. Ubiquitination of both adeno-associated virus type 2 and 5 capsid proteins affects the transduction efficiency of recombinant vectors. J Virol 2002; 76(5):2043–2053.