Gene therapy for hemophilia
Katherine P. Ponder

Purpose of review
This review will highlight the progress achieved in the past 2 years on using gene therapy to treat hemophilia in animals and humans.

Recent findings
There has been substantial progress in using gene therapy to treat animals with hemophilia. Novel approaches for hemophilia A in mice include expression of Factor VIII in blood cells or platelets derived from ex-vivo transduced hematopoietic stem cells, or in-vivo transfer of transposons expressing Factor VIII into endothelial cells or hepatocytes. Advances in large-animal models include the demonstration that neonatal administration of a retroviral vector expressing canine Factor VIII completely corrected hemophilia A in dogs, and that double-stranded adeno-associated virus vectors resulted in expression of Factor IX that is 28-fold that obtained using single-stranded adeno-associated virus vectors. In humans, one hemophilia B patient achieved 10% of normal activity after liver-directed gene therapy with a single-stranded adeno-associated virus vector expressing human Factor IX. Expression fell at 1 month, however, which was likely due to an immune response to the modified cells.

Summary
Gene therapy has been successful in a patient with hemophilia B, but expression was unstable due to an immune response. Abrogating immune responses is the next major hurdle for achieving long-lasting gene therapy.

Keywords
adeno-associated virus vector (AAV vector), adeno viral vector, gene therapy, hemophilia, retroviral vector

Introduction
This review will summarize the major advances made over the past 2 years in the use of gene therapy to treat hemophilia. A brief introduction of hemophilia, gene therapy vectors, and target organs will be given. New approaches that have been evaluated in mouse models for the treatment of hemophilia A or B will follow. Since success in mouse models does not necessarily translate into large animals, a summary of progress in the use of gene transfer in large animal models will be provided. The status of the liver-directed adeno-associated virus (AAV) vector-mediated gene therapy trial in humans with hemophilia B will be presented. Finally, concerns about the risks of gene therapy will be discussed.

Overview of hemophilia and gene therapy
Hemophilia is due to a deficiency in a coagulation factor that results in the inability of the blood to clot efficiently. Hemophilia A is due to Factor VIII (FVIII) deficiency and occurs in 1 in 5000 males, while hemophilia B is due to Factor IX (FIX) deficiency and occurs in 1 in 30,000 males [1]. Hemophilia can also be due to very rare autosomal recessive deficiencies of other coagulation factors. Patients with hemophilia experience spontaneous bleeding into joints, soft tissues, and other sites.

Hemophilia is treated with intravenous infusion of the appropriate coagulation factor. Purified preparations of FVIII and FIX are readily available in developed countries, although access is limited in developing countries. Purified factors are not available for the treatment of the rare hemophilies, and so prothrombin complex concentrates or plasma, which have a higher risk of viral transmission or other adverse effects, have to be used to treat bleeding episodes. Inhibitors are antibodies that block the function of a coagulation factor and make bleeding episodes difficult to treat [2,3].

For hemophilia, gene therapy usually involves transfer of a wild-type or minimally modified gene into cells in the body, which results in secretion of a functional protein into the blood [4–8]. Viral vectors contain viral proteins that bind to receptors on the outside of cells and facilitate efficient delivery, and are modified to contain the therapeutic gene. Commonly used viral vectors include retroviral (both gamma retroviral and lentiviral), AAV, and adenoviral vectors. Retroviral vectors integrate into the chromosome, which ensures stable maintenance of the DNA, but can result in insertional mutagenesis. Although AAV and adenoviral vectors do not usually integrate, they
are generally maintained in non-dividing cells. All of these vectors can be engineered to lack viral coding sequences and to be replication-incompetent. Plasmid DNA vectors are often perceived to be safer than viral vectors, although those that integrate non-specifically can cause insertional mutagenesis. Plasmid vectors do not enter cells efficiently.

The most effective target cells for gene therapy for hemophilia have been liver and muscle. The liver is transduced efficiently after parenteral injection, which can result in long-lasting expression even with non-integrating vectors, as adult hepatocytes have a low rate of turnover. The muscle has been another popular target for gene therapy for hemophilia, due to its accessibility via an intramuscular injection. Limitations are difficulties in distributing the vector throughout the muscle and the diffusion barriers imposed for secretion into the blood. Although successful muscle-directed gene therapy has been achieved for the 55 kDa FIX protein, it has not been reported for the ~200 kDa FVIII protein.

Progress in gene therapy for hemophilia in mouse models

Mouse models are generally evaluated first, due to their small size and low costs. A variety of vectors and approaches have resulted in therapeutic expression of FIX in mice. Since expression of therapeutic levels of FVIII without inhibitor formation has been more difficult, this review will focus on progress in gene therapy for hemophilia A.

Hematopoietic stem cells (HSC) can be re-infused into patients after ex-vivo transduction. In addition, tolerance can be achieved by expressing a variety of genes in blood cells, which may involve central tolerance due to expression in the thymus. Previous attempts to perform gene therapy in HSC did not achieve therapeutic expression [9,10]. More recently, murine HSC were modified ex vivo with retroviral vectors expressing human [11*] or porcine [12*] FVIII, as summarized in Table 1. Transduced cells were infused into mice with hemophilia A whose bone marrow was partially ablated with irradiation or busulfan. Recipients of retroviral vectors expressing human or porcine FVIII achieved 25% and 100% of normal FVIII activity respectively, without inhibitor formation, and the former group was tolerant to human FVIII protein challenge. The porcine FVIII has a higher specific activity than human FVIII, and might be used in humans that have already developed inhibitors to human FVIII.

A very novel approach to achieving hemostasis despite the presence of inhibitors is to express FVIII in α-granules of platelets. Since platelet granules are released at the site of injury, expression of FVIII in platelets of transgenic mice can prevent bleeding in mice with hemophilia A [13,14]. A recent study [15] extended this result by showing that transfer of platelets from transgenic to hemophilia A mice resulted in achievement of hemostasis in animals with very high inhibitor titers. In addition, ex-vivo HSC transduction with a lentiviral vector expressing human FVIII from a platelet-specific promoter could prevent bleeding in mice with hemophilia A (R.R. Montgomery, Medical College of Wisconsin and Blood Research Institute, Milwaukee, WI, USA, personal communication). This could serve as an effective treatment in patients who have already developed inhibitors.

Another approach to treating hemophilia A is to transpose a plasmid into the host chromosome. Endothelial cells express von Willebrand factor, and can secrete functional FVIII into the medium in culture. Endothelial cells from adult mice resulted in achievement of hemostasis in animals with very high inhibitor titers. In addition, ex-vivo HSC transduction with a lentiviral vector expressing human FVIII from a platelet-specific promoter could prevent bleeding in mice with hemophilia A (R.R. Montgomery, Medical College of Wisconsin and Blood Research Institute, Milwaukee, WI, USA, personal communication). This could serve as an effective treatment in patients who have already developed inhibitors.

Table 1 Summary of advances in gene therapy for hemophilia A in mouse models

<table>
<thead>
<tr>
<th>Blood cells derived from HSC [11*,12*]</th>
<th>Adult</th>
<th>Retroviral</th>
<th>Human FVIII</th>
<th>25% of normal activity without inhibitors</th>
<th>Partial bone marrow ablation; integration</th>
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<tbody>
<tr>
<td>Platelets derived from HSC [15**]</td>
<td>Adult</td>
<td>Transgenic mice</td>
<td>Human FVIII</td>
<td>Achieve hemostasis even with high inhibitors</td>
<td>Bone marrow ablation; integration</td>
</tr>
<tr>
<td>Endothelial cells [16**]</td>
<td>Newborn</td>
<td>Plasmid with transposase</td>
<td>Human FVIII</td>
<td>10% of normal activity</td>
<td>Toxicity of polyethylenimine; integration</td>
</tr>
<tr>
<td>Hepatocytes [17*]</td>
<td>Adult</td>
<td>Plasmid with transposase</td>
<td>Human FVIII</td>
<td>10–100% of normal, but need to tolerate with neonatal FVIII protein</td>
<td>Hydrodynamic injection; integration</td>
</tr>
<tr>
<td>Hepatocytes [18**]</td>
<td>Newborn</td>
<td>Retroviral</td>
<td>Canine FVIII</td>
<td>139% of normal activity without inhibitors</td>
<td>Integration</td>
</tr>
<tr>
<td>B cells [19*]</td>
<td>Adult</td>
<td>Retroviral</td>
<td>A2 and C2 domains of human FVIII</td>
<td>Reduces inhibitor formation</td>
<td>Duration of response unclear; integration</td>
</tr>
</tbody>
</table>

FVIII, Factor VIII; HSC, hematopoietic stem cells.
promoter, and was flanked by the transposition sequence. This resulted in endothelial-specific expression and FVIII activity that was 10% of normal, without inhibitor formation. Another study used the Sleeping Beauty transposition system delivered by hydrodynamic injection to adults to express human FVIII from a ubiquitous promoter in the liver of mice with hemophilia A [17*]. This resulted in 10–100% of normal FVIII activity and prevention of bleeding. Since human FVIII protein is antigenic in adult mice, animals were tolerized with a neonatal injection of human FVIII protein.

Another study took advantage of the immaturity of the newborn immune system to achieve tolerance to canine FVIII in mice [18**]. Newborn mice with hemophilia A were injected intravenously with a retroviral vector expressing canine FVIII. They achieved 139 ± 22% of normal activity without inhibitor formation and did not bleed.

A third approach for inducing tolerance to FVIII involved transfer of a retroviral vector expressing a fusion protein between domains of human FVIII and IgG into B-cell blasts [19*], an approach that has been shown to induce tolerance to other proteins. B-cell blasts were transduced \textit{ex vivo} with retroviral vectors expressing the C2 or the A2 domain of human FVIII in-frame with an IgG heavy-chain backbone, and injected into hemophilia A mice. Animals were then challenged with human FVIII protein starting 1 week later. Prior injection of transduced B cells reduced inhibitor titers to ~1% of the value in mice that were immunized in a similar fashion, but did not receive transduced B cells. The tolerized mice still produced substantial levels of anti-C2 domain antibodies, however, as determined by immunoassay, demonstrating that tolerance was not complete.

There have also been advances in gene therapy for hemophilia B. AAV vectors expressing human FIX proteins that were modified to have low affinity for the extracellular matrix, or to have a higher specific activity, resulted in FIX activity that was 2–5-fold higher after intramuscular injection than was observed with a similar dose of a vector expressing wild-type FIX [20*]. Mucosal administration of the immunodominant peptide from human FIX to mice with hemophilia B reduced inhibitor formation on subsequent challenge with an AAV2 vector expressing human FIX [21*], although some animals still produced inhibitors; this approach may be difficult to apply in humans with marked heterogeneity in their major histocompatibility complexes.

**Progress in gene transfer in large-animal models**

Gene therapy approaches that are effective in inbred mice often fail in humans. This may relate to difficulties in scaling up to larger animals, or to the biology of animals with a longer life span. In addition, immune responses are more potent in outbred large animals than in inbred mice, making it likely that responses in large animals will be more predictive for humans. For these reasons, many investigators have evaluated gene transfer in large animals, primarily dogs and non-human primates, as summarized in Table 2.

Although AAV vectors with alternative capsid proteins are more effective than AAV2 vectors in mice, these have been disappointing in large animals. The capsid proteins on the outside of AAV particles bind to cell surfaces, which facilitates entry into the cell. The first vectors that were developed used AAV2 capsid proteins. Pseudotyped vectors with capsid proteins from other AAV serotypes are more efficient at transducing liver in mice than are AAV2 vectors [22,23*,24,25]. In contrast, neither AAV6 nor AAV8 vectors were more effective than AAV2 at expressing canine FVIII in dogs with hemophilia A in one study [26]. In another study [23*], expression of canine FIX from an AAV8 vector was ~2-fold that from an AAV2 vector in dogs with hemophilia B, while expression from AAV5 was lower than that from AAV2. Similarly, AAV5 or AAV8 vectors expressing human FIX were not more effective than AAV2 vectors at transducing hepatocytes in Rhesus macaque monkeys [27*,28*]. Although alternative capsid proteins do not improve transduction of hepatocytes in large animals, they can allow transduction to be achieved when anti-serotype neutralizing antibodies are present [23*,26*,27*], which is a common problem for AAV2 [29].

Utilization of double-stranded, or so-called self-complementary, AAV vectors has markedly increased the expression of FIX over that observed with single-stranded AAV vectors in mice and non-human primates [28*,30]. AAV has a single-stranded DNA genome that is packaged as either a negative or a positive strand, with inverted repeats at the ends that form a hairpin. Upon entering a cell, expression requires that the DNA be copied into double-stranded DNA, or that negative and positive strands from different particles anneal. A recent advance was the development of AAV vectors with DNA that is double-stranded at the time of gene transfer, which results in more rapid and higher levels of expression than vectors with single-stranded DNA. Use of a double-stranded AAV8 vector markedly increased expression in Rhesus macaques as compared with a single-stranded AAV8 vector, resulting in 21% of normal human FIX levels with a relatively low dose [28*].

Therapeutic expression of FVIII in large-animal models has been very difficult to achieve. In a recent study [18**] a retroviral vector expressing canine FVIII was injected into newborn dogs with hemophilia A. This resulted in
<table>
<thead>
<tr>
<th>Target organ</th>
<th>Age</th>
<th>Species</th>
<th>Promoter and dose</th>
<th>Gene</th>
<th>Result (% of normal)</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>AAV</td>
<td>Liver</td>
<td>Adult Hemophilia A dogs</td>
<td>Single-stranded AAV with trnasfibrin promoter [(6–27) × 10^{12} vg/kg] [26*]</td>
<td>Canine FVIII</td>
<td>AAV2 = 2.1%</td>
<td>AAV8 and AAV6 were not better than AAV2</td>
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<td></td>
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<td>Two separate single-stranded AAV vectors with synthetic liver promoter expressing heavy and light chains [(6–30) × 10^{12} vg/kg] [31*]</td>
<td>Canine FVIII</td>
<td>AAV8 = 3%</td>
<td>No direct comparison with AAV2</td>
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<td>AAV9 = 3%</td>
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<td>Rhesus macaque</td>
<td>Single-stranded AAV5 with CAGG promoter or AAV8 with hAAT-HCR promoter (4 × 10^{12} vg/kg) [27*]</td>
<td>Human FIX</td>
<td>AAV5 = 3%</td>
<td>AAV8 not better than AAV2; some inhibitors</td>
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<tr>
<td></td>
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<td></td>
<td>Double-stranded AAV8 with liver promoter (1 × 10^{12} vg/kg) [28**]</td>
<td>Human FIX</td>
<td>AAV8 = 21%</td>
<td>Double-stranded AAV8 28-fold better than single-stranded AAV8 Integration</td>
</tr>
<tr>
<td>Retro-viral</td>
<td>Liver</td>
<td>Newborn Hemophilia A dogs</td>
<td>Retroviral with hAAT promoter (10^{10} TUKg) [16**]</td>
<td>Canine FVIII</td>
<td>116%</td>
<td></td>
</tr>
<tr>
<td>Adeno-viral</td>
<td>Liver</td>
<td>Adult Hemophilia B dogs</td>
<td>Highly deleted adenoviral vector (3 × 10^{12} vg/kg) [35*]</td>
<td>Canine FIX</td>
<td>4%</td>
<td>Inflammatory responses</td>
</tr>
<tr>
<td>AAV</td>
<td>Muscle</td>
<td>Adult Hemophilia B dogs</td>
<td>AAV2 vector with CMV promoter and isolated arterial injection (3 × 10^{12} vg/kg) [37*]</td>
<td>Canine FIX</td>
<td>8%</td>
<td>Somewhat invasive; transient hypotension; some inhibitors</td>
</tr>
<tr>
<td>AAV</td>
<td>Liver</td>
<td>Adult Humans with hemophilia B</td>
<td>Single-stranded AAV2 vector with hAAT-HCR promoter (highest dose 2 × 10^{12} vg/kg) [43**]</td>
<td>Human FIX</td>
<td>10% for 1 month in one patient</td>
<td>Expression fell in conjunction with increase in liver enzymes; likely due to cytotoxic T-lymphocyte response</td>
</tr>
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</table>

AAV, adeno-associated virus; CAGG, CMV-enhancer β-actin promoter; CMV, cytomegalovirus; FVIII, Factor VIII; FIX, Factor IX; hAAT, human α₁-antitrypsin; HCR, hepatic control region locus; LSP, liver-specific promoter; TU, transducing units; vg, vector genomes.
FVIII activity that was 116 ± 5% of that in normal dogs, and no bleeding episodes. In contrast, the size-constrained AAV vectors have only achieved ~5% of normal FVIII activity using a very small promoter [26*] or with two vectors expressing the heavy and light chains separately [31*].

Although highly deleted adenoviral vectors have generally resulted in only transient expression in large animals [32–34], a recent study [35?] demonstrated that stable and therapeutic expression of canine FIX could be achieved with this vector. In this study, a very high dose (3 × 10^{12} vector particles/kg) was injected into adult dogs with hemophilia B, resulting in FIX activity of 2–5% of normal. Problems with this approach include the induction of an inflammatory response, and the likelihood that expression will decline further over time.

Improved methods for dissemination of an AAV vector throughout muscle have been described. In the human muscle-directed AAV2 gene therapy trial, up to 100 separate injections were required for a high dose of AAV2 [36]. Regional delivery of AAV to muscle was achieved by cannulating the femoral artery and vein to create a closed system, and injecting an AAV2 vector and agents that increase permeability [37*]. More recently, AAV vectors were injected intravenously into a leg where the blood flow was occluded with an external tourniquet. This resulted in efficient transduction of muscle without administration of a permeability agent [38], and achieved 15% of normal canine FIX levels at a dose of 3 × 10^{12} vector genomes of an AAV2 vector/kg (V. Arruda, Children’s Hospital of Philadelphia, Philadelphia, PA, USA, personal communication).

Gene therapy in humans

There have been five gene therapy trials for treatment of hemophilia in humans. Ex-vivo transduction of fibroblasts [39] or intravenous injection of a retroviral vector [40] for hemophilia A had at most a marginal effect on FVIII levels, and these approaches are no longer being pursued. Intravenous administration of a helper-dependent adenoviral vector expressing FIX induced the acute-phase response and was abandoned due to safety concerns [41]. The muscle-directed AAV vector-mediated gene therapy trial for hemophilia B did not provide convincing evidence of expression [36], and enrolment has been stopped.

The liver-directed AAV-vector-mediated gene therapy trial was considered very promising, as this approach resulted in ~10% of normal FIX levels in dogs with hemophilia B [42]. Indeed, one of the patients who received the highest dose (2 × 10^{12} vector genomes/kg) achieved ~10% of normal activity during the first month, and had reduced factor needs [43**]. The FIX activity fell at 1 month after transduction, however, in conjunction with an increase in liver enzymes, which was believed to be due to a cytotoxic T-lymphocyte response directed against AAV capsid proteins. This trial will be modified to include immunosuppression at the time of gene transfer (K.A. High, Children’s Hospital of Philadelphia, Philadelphia, PA, USA, personal communication).

Risks of gene therapy

Inhibitor formation is a very important concern for gene therapy. Although Chapel Hill dogs with a missense mutation in their FIX gene that received liver-directed gene therapy have generally not developed inhibitors, some dogs that were treated with muscle-directed gene therapy have done so [37*,44], suggesting that a muscle approach may be more immunogenic than a liver approach. On the other hand, some Rhesus macaques have developed inhibitors to human FIX despite the use of a liver-restricted promoter [28**], and there are only 11 amino acid differences between the human and the Rhesus macaque FIX sequences [45]. Neonatal administration of protein [17*] or gene [16**,18**] therapy induced tolerance to human or canine FVIII in mice; however, one out of five dogs that received neonatal gene therapy with a human FVIII cDNA developed high-titer inhibitors (K.P. Ponder, unpublished observation). Thus, results in mice may not predict results in large animals and humans. Patients have not developed inhibitors after gene therapy, although these adults had been treated extensively with factor without inhibitor formation, and were likely to be at low risk. Clearly, the problem of inhibitor development needs to be evaluated further.

A major concern for integrating vectors is the risk of cancer from insertional mutagenesis. Although the common gamma chain used for gene therapy in patients with X-linked severe combined immunodeficiency may have contributed to the leukemias that developed in ~20% of these patients, integration of the retroviral vector near an oncogene also played a role [46,47]. In addition, integration within the Evi1 locus or other sites may have promoted clonal expansion of hematopoietic cells in non-human primates [48] or in humans with chronic granulomatous disease [49] after ex-vivo transduction of HSC. Thus insertional mutagenesis continues to be a very serious concern for HSC transduction.

Most studies have not reported cancers in small or large animals that received gene therapy with a viral or plasmid vector to the liver or muscle. One exception is the report that neonatal intravenous injection of an AAV2 vector resulted in liver tumors in mice with mucopolysaccharidosis VII [50]. Another exception is the demonstration that fetal or neonatal transfer of some lentiviral vectors resulted in liver tumors in adults, although administration of other integrating vectors at
the same ages was not carcinogenic. This suggests that there was a specific oncogenic element present in the vectors that caused cancer [51]. It will be important to obtain long-term data in animals with therapeutic levels of expression to assess this risk further.

There are other potential adverse effects of gene therapy. Some studies have used HSC transduction, which requires at least partial bone marrow ablation to achieve engraftment, and has substantial morbidity and mortality. The use of polyethyleneimine or hydrodynamic injection to enhance delivery of plasmids, or injection of highly-deleted adenoviral vectors, can be toxic.

Conclusion
Gene therapy continues to hold promise for the permanent correction of hemophilia. A variety of approaches have been effective in animal models, and an AAV2 vector resulted in transient expression in one patient. Future studies need to address immunological and safety issues.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 386).


12. Gangadharan B, Parker ET, Idle LM, Spencer HT, Doering CB. High-level expression of porous factor VIII from genetically modified bone marrow-derived stem cells. Blood 2006; 107:3859–3864. This paper demonstrates that HSC-directed gene therapy with partial bone marrow ablation and a human FVIII gene can result in therapeutic levels of FVIII and prevention of bleeding in mice with hemophilia A.


19. Lee TC, Scott DW. Induction of tolerance to factor VIII inhibitors by gene therapy with immunodominant A2 and C2 domains presented by B cells as Ig fusion proteins. Blood 2005; 105:4865–4870. This paper demonstrates that expression of fusion proteins of domains of FVIII with IgG in B cells can result in partial tolerance to FVIII in mice.


21. Cao O, Armstrong E, Schlichtermeier A, et al. Immune deviation by mucosal antigen administration suppresses gene transfer-induced inhibitor formation to Factor IX. Blood 2006 Mar 16; [Epub ahead of print]. This paper demonstrates that mucosal administration of immunodominant peptides from human FIX can tolerate mice to human FIX.


24. This paper demonstrates that transposition of a plasmid into the liver can result in correction of hemophilia A. This paper demonstrates that neonatal injection of a retroviral vector expressing canine FVIII can completely correct hemophilia A in mice and dogs.


26. This paper demonstrates that an AAV8 vector is 2-fold more efficient per copy at expressing canine FVIII in dogs with hemophilia B than an AAV2 vector.

27. This paper demonstrates that AAV8 and AAV6 vectors are not more efficient at expressing canine FVIII in hepatocytes of dogs with hemophilia A than an AAV2 vector.

28. This paper demonstrates that single-stranded AAV5 and AAV6 vectors are not more efficient than an AAV8 vector.

29. This paper demonstrates that use of genes that express modified human FIX proteins can improve the efficacy of AAV2-vector-mediated muscle-directed gene therapy over that seen when using wild-type human FIX.

30. This paper demonstrates that use of genes that express modified human FIX proteins can improve the efficacy of AAV2-vector-mediated muscle-directed gene therapy over that seen when using wild-type human FIX.

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51. This paper demonstrates that use of genes that express modified human FIX proteins can improve the efficacy of AAV2-vector-mediated muscle-directed gene therapy over that seen when using wild-type human FIX.
This paper demonstrates that improved dissemination of an AAV2 vector through-