Molecular Therapy
Review

Gene Therapy for Hemophilia

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The X-linked bleeding disorder hemophilia causes frequent and exaggerated bleeding that can be life-threatening if untreated. Conventional therapy requires frequent intravenous infusions of the missing coagulation protein (factor VIII [FVIII] for hemophilia A and factor IX [FIX] for hemophilia B). However, a lasting cure through gene therapy has long been sought. After a series of successes in small and large animal models, this goal has finally been achieved in humans by in vivo gene transfer to the liver using adeno-associated viral (AAV) vectors. In fact, multiple recent clinical trials have shown therapeutic, and in some cases curative, expression. At the same time, cellular immune responses against the virus have emerged as an obstacle in humans, potentially resulting in loss of expression. Transient immune suppression protocols have been developed to blunt these responses. Here, we provide an overview of the clinical development of AAV gene transfer for hemophilia, as well as an outlook on future directions.

Hemophilia is an X-linked bleeding disorder that occurs approximately in 1 of 5,000 male births worldwide. It is caused by mutations in blood clothing factor VIII (FVIII, hemophilia A) or factor IX (FIX, hemophilia B).[1,2] Both proteins are normally produced in the liver (FIX [factor IX] by hepatocytes and FVIII by endothelial cells) and secreted into the blood circulation. FIX is a vitamin K-dependent serine protease that plays a vital role in the coagulation cascade. Its cofactor, FVIII, is critical for FIX enzymatic activity. Therefore, mutations in either protein may lead to lack of coagulation activity, resulting in frequent spontaneous bleeds in patients with activities of <1% of normal. If left untreated, bleeding may become fatal.[3] Both hemophilia A and B can be treated with recombinant factor replacement with significant improvement in morbidity and mortality.[3] However, such treatment is extremely costly and is often still marred by clinical complications, including bleeding, particularly bleeding into the joints despite factor replacement. The need for frequent administration of recombinant factor concentrates infused two to three times per week in order to maintain minimal therapeutic levels is demanding and highly invasive. Some improvement in outcome has been achieved in highly developed countries, but the majority of subjects throughout the world lack resources for optimal treatment.[1–4] These considerations prompted efforts to develop novel approaches for treatment of hemophilia using gene therapy, which has the potential for lasting treatment and even curing of the disease.

Our early gene therapy studies focused on the treatment of hemophilia B as work had progressed most rapidly for the disorder. Ongoing efforts have also accelerated with respect to the development of vectors suitable for the treatment of hemophilia A, as will be discussed in detail in this work. The work in my laboratory dedicated to the development of gene therapy for hemophilia, based on gene transfer with recombinant adeno-associated virus (rAAV), began after Dr. Amit C. Nathwani arrived from the University College London to work as a post-doctoral fellow in my lab (July 1997). Dr. Andrew Davidoff, a fully trained pediatric surgeon, was recruited to the project because of the belief that a portal-vein injection in mice was needed to ensure liver transduction. This turned out not to be true, as we have subsequently found equivalent transduction following portal versus peripheral vein infusion. They directed all of the preclinical studies and our clinical trial.

rAAV vectors have been derived from the many serotypes of AAV that exist in nature.[5,6] Serotype rAAV2 was used most extensively in early studies because had been the first serotype which was characterized. AAV viruses are thought to be nonpathogenic. The AAV genome is single stranded DNA and 7 kb in length. It includes inverted terminal repeats (ITRs) of approximately 145 base pairs. The ITRs are the only elements that must be retained in the vector genome to allow its packaging. AAV viruses depend on a helper virus for replication, usually adenovirus. Production of viral vector occurs in packaging cells that have been transfected with a multi-plasmid system in which a number of adeno-viral proteins are expressed as well as the AAV rep and capsid proteins.[7,8] Alternatively, the vector is produced using herpes virus helper or by infection of insect cells with baculovirus.[7,9]

Initial studies attempting to exploit rAAV vectors for treatment of hemophilia B relied on the use of these single-stranded rAAV2 vectors. Two clinical trials have been completed, with limited results. The initial trial focused on intramuscular injection of rAAV encoding FIX in a classic dose-escalation study.[10–12] Minimal expression was observed in only one of eight participants in this trial and FIX expression was at a very low level. The next trial focused on liver-targeted

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delivery.[13] As liver is the natural site for FIX synthesis, this seemed like an appropriate target. Seven patients with severe hemophilia B received an rAAV2 vector via hepatic artery injection. Initial expression was observed in those trial participants who received the highest dose, but expression was lost as cytotoxic T lymphocytes appeared in the blood. They were judged to have destroyed the FIX-expressing hepatocytes.[13–15]

Many studies had resulted in the identification of multiple AAV serotypes.[16,17] Serotype AAV8 was particularly intriguing, in that it does not cross-react with antibodies directed against other AAV serotypes. Because AAV8 rarely infects humans, the incidence of neutralizing antibodies is relatively low, with only approximately 20% of individuals having neutralizing antibodies. Recent studies, however, suggest a significantly higher incidence of anti-AAV8 antibodies in humans.[18] Furthermore, relatively low titers of the antibodies abrogate liver transduction. Two studies have shown that transient immunosuppression may facilitate transduction of the liver in vivo in animal models.[19,20]

We choose serotype 8 for our studies because of its efficacy in transducing hepatocytes from peripheral vein.[5] Another favorable factor for the AAV8 serotype is that the genome is rapidly uncoated, allowing for prompt expression.[21] A technical advance that we incorporated into our vector design was the use of a self-complementary vector.[22,23] Self-complementary AAV vectors (scAAV) have each strand of the FIX-coding genome in an inverted orientation, between which there is an ITR which has been mutated to prevent its digestion. Self-complementary genomes quickly self-anneal when cells are transduced, enhancing the rate of gene expression as well as the level of gene expression achieved.[22] We designed our vector to have a FIX expression cassette that was small enough to accommodate the self-complementary genome by modifying transcriptional control elements (enhancer, promoter, and polyadenylation site) to minimize size and therefore to allow efficient packaging of the self-complementary genome.[24] The FIX coding sequences were codon optimized by synthesis with the most frequently used codons in natural cellular transcripts. We observed a 20-fold improvement in FIX expression in mice compared to ssAAV vectors. Expression of FIX was very high despite the use of much lower vector doses than had been applied in earlier studies with ssAAV vectors.

Extensive preclinical studies were performed, initially in mice.[25] Vector was given by tail vein administration, as early studies had indicated equivalent liver uptake by peripheral injection compared to portal vein injection.[26] scAAV2/8 vector particles were found to be much more efficient at transducing hepatocytes than ssrAAV2/8. A dose-dependent increase in FIX was observed in mice, with animals receiving the highest dose achieving 100% of the FIX levels that are found in normal human plasma. Preclinical studies were extended to evaluate transduction of non-human primate hepatocytes.[25] This was achieved very successfully with a peripheral vein infusion. The vector genome was found predominantly in the liver, with lesser amounts in the spleen. Expression of the vector-encoded transcript was found exclusively in the liver as predicted based on the construction of the liver-specific enhancer promoter combination that was used. Noteworthy is the fact that no toxicity was observed in any of the animals that received vector as part of the preclinical studies. Despite the superiority of AAV8 in mice, the two serotypes seemed more equivalent in non-human primates and in the clinical trials to date.

The initial clinical trial was designed as a phase I/II dose-escalation study with the classic design.[27,28] The initial dose was 2×10^{11} vector genomes (vg)/kg, with an intermediate dose of 6×10^{11} vg/kg and a higher dose of 2×10^{12} vg/kg. Overall, 12 subjects have participated in this trial. This report provides detailed results on the first 10, each of which have been followed 3 years or more after the single vector infusion. All 10 participants have had measurable levels of FIX. The two patients treated most recently also have stable production of hFIX. Among the initial six patients who received the highest dose, the average FIX level was 5.1% ± 1.7%, with each having significant production of $\geq$ 2%. FIX expression resulted in a dramatic reduction in requirements for FIX infusions. Several of the patients had a mild elevation in transaminase levels. After observing this phenomenon in the first high-dose patient, we resolved to begin prednisolone as soon as there was a 50% or greater increment in transaminases above the baseline values even if the values remained within the normal range. With this treatment approach, the transaminase elevations resolved over a period of a week or two and steroids could be withdrawn after 4 weeks with no recurrence of the transaminitis. Fortunately, the prompt treatment permitted maintenance of the FIX levels in the individual participants.

Several other pharmaceutical companies have recently used the AAV vector pseudotyped with a variety of capsid proteins to deliver either wild-type F9 cDNA or one containing the gain-of-function mutation known as Padua (R338L).[29–32] UniQure and Dimension have ongoing studies with the wild-type factor sequence, whereas Baxalta and Spark have studies entail the use F9 cDNA that incorporates the Padua mutation, which leads to an 8-fold enhancement of expression of FIX. The results of these trials, which involve over 30 patients, remain largely unpublished, but they support our initial observations.[33] The majority of these studies confirm the efficiency and durability of AAV-mediated gene transfer following systemic administration of the vector. There have been no reports of serious adverse events in any of the trials. An asymptomatic rise in liver enzymes is often associated with a decline in FIX levels has been observed in most of these studies usually between 4 and 10 weeks after gene transfer.[34] Treatment with corticosteroid appears to be effective at limiting the hepatocellular toxicity as well as preserving expression of transgenic FIX, especially when commenced early in some of the affected patients. The best emerging data so far is from a study sponsored by Spark Therapeutic using a novel engineered AAV capsid and a codon-optimized, and a gain-of-function Padua variant. Data presented thus far show that the low dose (5×10^{11} vg/kg) of their vector resulted in sustained FIX activity levels between 12% and 63% of normal following a single administration of SPK-9001 at the initial dose level studied in the trial.[29,31]

Hemophilia A is a far more common disorder than hemophilia B. Accordingly, we had great interest in developing an effective rAAV vector suitable for treating hemophilia A. Development of such a vector presented several challenges.[35] The human factor VIII (FVIII) coding sequence is 7 kb in length, which is too large to be packaged into an AAV capsid with its limiting packaging capacity. FVIII has three functional domains. The B domain can be deleted, and in the construction of our vector, a 226-amino-acid spacer was included in place of the B domain in the construction of our vector. Amino acid triplets which function as glycosylation sites that are normally part of the B domain were included within this spacer. The resulting 5.2-kb vector was efficiently packaged after its coding sequences were codon optimized. Super physiological levels of FVIII were achieved in mice and non-human primates.[35] An immune response mediated by antibodies targeted to FVIII was eliminated with transient immunosuppression.

Encouraging results from our clinical trials have stimulated a great deal of interest in the application of gene therapy for the treatment of hemophilia. Using an AAV5 containing the SQ linker codon-optimized FVIII expression cassette described above, in a study sponsored by Biomarin nine subjects with severe hemophilia A have been treated at doses ranging from 6×10^{12} to 6×10^{13} vg/kg. FVIII levels in the seven patients treated at a dose of 6×10^{13} vg/kg have consistently been within the normal range of 40%–150%[36,37] beyond 12 weeks after gene transfer, a feat which was thought impossible to achieve just a few years ago. These data were presented at the 35th Annual J. P. Morgan HealthCare Conference and show a 91% drop in the mean annualized bleeding rate and a 98% drop in prophylactic infusions. Thus, BMN 270 opens up an exciting potentially curative treatment opportunity severe hemophilia A patients. Several review articles have also been published which outline in detail the anticipated future progress.[30,33,38–50]

The future of gene therapy for hemophilia looks bright. Several ongoing studies are focused on evaluating various vectors for gene delivery, strategies to enhance transduction efficiency in human hepatocytes, definition of the immune and stress responses to vector administration, and the potential application of genome editing for the treatment of these disorders.[51–66] The possibility of using hepatic AAV gene transfer as an immune modulatory therapy to induce tolerance to FVIII and FIX is being explored.[67–69] A number of critical questions remain to be answered, however. What is the best way to avoid or overcome the CD8+ T-cell response to AAV capsid? Is it routine transient immunosuppression? Or lower, but presumably still therapeutic, doses of vector? Or manipulation of the capsid protein? What is the best AAV serotype to use, both in terms of tropism for and efficiency of infection of human hepatocytes and prevalence of pre-existing neutralizing antibodies resulting from prior wild-type AAV infection? At what point do the data suggest that this approach is safe for testing in pediatric patients, the population that would benefit most from early intervention before extensive joint damage has occurred? And will transgene expression mediated by AAV, a largely non-integrating virus, be maintained for the lifetime of a child? Will repeat administration of AAV be required, which would necessitate either the use of an alternative, non-cross-reactive serotype or immunosuppression either at initial vector administration and/or subsequent dosing(s)? Or would it be better to use an integrating virus such as lentivirus or genome editing approaches for long-term expression of normal clotting factor? Finally, can the chronic synthesis and delivery of clotting factors, as is achieved with liver-targeted gene transfer approaches, be used to induce immune tolerance in patients in whom neutralizing antibodies to FVIII or FIX have developed, a particularly difficult clinical problem?

CONFLICTS OF INTEREST

A.W.N. has no conflicts of interest. A.M.D. receives patent income from Uniqure for the FIX vector and a patent has been granted for the FVIII vector. A.C.N. receives income from Uniqure for the FIX vector and income from Biomarin for FVIII. He has recently licensed a new AAV-FIX expression cassette and capsid to Freeline Therapeutics and was a founder of this company, in which he holds equity.

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REFERENCES

1. Nathwani, A.C., Nienhuis, A.W., and Davidoff, A.M. (2014). Our journey to successful gene therapy for hemophilia B. Hum. Gene Ther. *25*, 923–926.
2. Nienhuis, A.W., Nathwani, A.C., and Davidoff, A.M. (2016). Gene therapy for hemophilia. Hum. Gene Ther. *27*, 305–308.
3. Darby, S.C., Kan, S.W., Spooner, R.J., Giangrande, P.L., Hill, F.G., Hay, C.R., Lee, C.A., Ludlam, C.A., and Williams, M. (2007). Mortality rates, life expectancy, and causes of death in people with hemophilia A or B in the United Kingdom who were not infected with HIV. Blood *110*, 815–825.
4. Lheriteau, E., Davidoff, A.M., and Nathwani, A.C. (2015). Haemophilia gene therapy: Progress and challenges. Blood Rev. *29*, 321–328.
5. Gao, G.P., Alvira, M.R., Wang, L., Calcedo, R., Johnston, J., and Wilson, J.M. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc. Natl. Acad. Sci. USA *99*, 11854–11859.
6. Samulski, R.J., Srivastava, A., Berns, K.I., and Muzyczka, N. (1983). Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. Cell *33*, 135–143.
7. Clement, N., and Grieger, J.C. (2016). Manufacturing of recombinant adeno-associated viral vectors for clinical trials. Mol. Ther. Methods Clin. Dev. *3*, 16002.
8. Grieger, J.C., Soltys, S.M., and Samulski, R.J. (2016). Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector. Mol. Ther. *24*, 287–297.
9. Mietzsch, M., Grasse, S., Zurawski, C., Weger, S., Bennett, A., Agbandje-McKenna, M., Muzyczka, N., Zolotukhin, S., and Heilbronn, R. (2014). OneBac: platform for scalable and high-titer production of adeno-associated virus serotype 1-12 vectors for gene therapy. Hum. Gene Ther. *25*, 212–222.
10. Buchlis, G., Podsakoff, G.M., Radu, A., Hawk, S.M., Flake, A.W., Mingozzi, F., and High, K.A. (2012). Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. Blood *119*, 3038–3041.
11. Kay, M.A., Manno, C.S., Ragni, M.V., Larson, P.J., Couto, L.B., McClelland, A., Glader, B., Chew, A.J., Tai, S.J., Herzog, R.W., et al. (2000). Evidence for gene transfer

and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat. Genet. *24*, 257–261.

12. Manno, C.S., Chew, A.J., Hutchison, S., Larson, P.J., Herzog, R.W., Arruda, V.R., Tai, S.J., Ragni, M.V., Thompson, A., Ozelo, M., et al. (2003). AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood *101*, 2963–2972.
13. Manno, C.S., Pierce, G.F., Arruda, V.R., Glader, B., Ragni, M., Rasko, J.J., Ozelo, M.C., Hoots, K., Blatt, P., Konkle, B., et al. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat. Med. *12*, 342–347.
14. Mingozzi, F., Maus, M.V., Hui, D.J., Sabatino, D.E., Murphy, S.L., Rasko, J.E., Ragni, M.V., Manno, C.S., Sommer, J., Jiang, H., et al. (2007). CD8(+) T-cell responses to adeno-associated virus capsid in humans. Nat. Med. *13*, 419–422.
15. Pien, G.C., Basner-Tschakarjan, E., Hui, D.J., Mentlik, A.N., Finn, J.D., Hasbrouck, N.C., Zhou, S., Murphy, S.L., Maus, M.V., Mingozzi, F., et al. (2009). Capsid antigen presentation flags human hepatocytes for destruction after transduction by adeno-associated viral vectors. J. Clin. Invest. *119*, 1688–1695.
16. Asokan, A., Schaffer, D.V., and Samulski, R.J. (2012). The AAV vector toolkit: poised at the clinical crossroads. Mol. Ther. *20*, 699–708.
17. Muzyczka, N., and Berns, K.I. (2015). AAV's golden jubilee. Mol. Ther. *23*, 807–808.
18. Hurlbut, G.D., Ziegler, R.J., Nietupski, J.B., Foley, J.W., Woodworth, L.A., Meyers, E., Bercury, S.D., Pande, N.N., Souza, D.W., Bree, M.P., et al. (2010). Preexisting immunity and low expression in primates highlight translational challenges for liver-directed AAV8-mediated gene therapy. Mol. Ther. *18*, 1983–1994.
19. Jiang, H., Couto, L.B., Patarroyo-White, S., Liu, T., Nagy, D., Vargas, J.A., Zhou, S., Scallan, C.D., Sommer, J., Vijay, S., et al. (2006). Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. Blood *108*, 3321–3328.
20. McIntosh, J.H., Cochrane, M., Cobbold, S., Waldmann, H., Nathwani, S.A., Davidoff, A.M., and Nathwani, A.Cl. (2012). Successful attenuation of humoral immunity to viral capsid and transgenic protein following AAV-mediated gene transfer with a non-depleting CD4 antibody and cyclosporine. Gene Ther. *19*, 78–85.
21. Thomas, C.E., Storm, T.A., Huang, Z., and Kay, M.A. (2004). Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. J. Virol. *78*, 3110–3122.
22. McCarty, D.M., Fu, H., Monahan, P.E., Toulson, C.E., Naik, P., and Samulski, R.J. (2003). Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. Gene Ther. *10*, 2112–2118.
23. Wang, Z., Ma, H.I., Li, J., Sun, L., Zhang, J., and Xiao, X. (2003). Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors in vitro and in vivo. Gene Ther. *10*, 2105–2111.
24. Nathwani, A.C., Gray, J.T., Ng, C.Y., Zhou, J., Spence, Y., Waddington, S.N., Tuddenham, E.G., Kemball-Cook, G., McIntosh, J., Boon-Spijker, M., et al. (2006). Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. Blood *107*, 2653–2661.
25. Nathwani, A.C., Gray, J.T., McIntosh, J., Ng, C.Y., Zhou, J., Spence, Y., Cochrane, M., Gray, E., Tuddenham, E.G., and Davidoff, A.M. (2007). Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. Blood *109*, 1414–1421.
26. Davidoff, A.M., Gray, J.T., Ng, C.Y., Zhang, Y., Zhou, J., Spence, Y., Bakar, Y., and Nathwani, A.C. (2005). Comparison of the ability of adeno-associated viral vectors pseudotyped with serotype 2, 5, and 8 capsid proteins to mediate efficient transduction of the liver in murine and nonhuman primate models. Mol. Ther. *11*, 875–888.
27. Nathwani, A.C., Reiss, U.M., Tuddenham, E.G., Rosales, C., Chowdary, P., McIntosh, J., Della Peruta, M., Lheriteau, E., Patel, N., Raj, D., et al. (2014). Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N. Engl. J. Med. *371*, 1994–2004.
28. Nathwani, A.C., Tuddenham, E.G., Rangaraian, S., Rosales, C., McIntosh, J., Linch, D.C., et al. (2011). Adeno-associated virus vector-mediated gene transfer in hemophilia B. N Eng. J. Med. *365*, 2357–2365.
29. Dolgin, E. (2016). Early clinical data raise the bar for hemophilia gene therapies. Nat. Biotechnol. *34*, 999–1001.
30. Hartmann, J., and Croteau, S.E. (2016). 2017 clinical trials update: innovations in hemophilia therapy. Am. J. Hematol. *91*, 1252–1260.
31. Herzog, R.W. (2016). A cure for hemophilia: the promise becomes a reality. Mol. Ther. *24*, 1503–1504.
32. Kumar, S.R., Markusic, D.M., Biswas, M., High, K.A., and Herzog, R.W. (2016). Clinical development of gene therapy: results and lessons from recent successes. Mol. Ther. Methods Clin. Dev. *3*, 16034.
33. Kattenhorn, L.M., Tipper, C.H., Stoica, L., Geraghty, D.S., Wright, T.L., Clark, K.R., and Wadsworth, S.C. (2016). Adeno-associated virus gene therapy for liver disease. Hum. Gene Ther. *27*, 947–961.
34. Herzog, R.W. (2015). Hemophilia gene therapy: caught between a cure and an immune response. Mol. Ther. *23*, 1411–1412.
35. McIntosh, J., Lenting, P.J., Rosales, C., Lee, D., Rabbanian, S., Raj, D., Patel, N., Tuddenham, E.G., Christophe, O.D., McVey, J.H., et al. (2013). Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. Blood *121*, 3335–3344.
36. Hoffman, B.E., and Herzog, R.W. (2013). Covert warfare against the immune system: decoy capsids, stealth genomes, and suppressors. Mol. Ther. *21*, 1648–1650.
37. Mingozzi, F., Anguela, X.M., Pavani, G., Chen, Y., Davidson, R.J., Hui, D.J., et al. (2013). Overcoming preexisting humoral immunity to AAV using capsid decoys. Sci. Transl. Med. *5*, 194ra192.
38. Arruda, V.R., and Samelson-Jones, B.J. (2015). Obstacles and future of gene therapy for hemophilia. Expert Opin. Orphan Drugs *3*, 997–1010.
39. Brimble, M.A., Reiss, U.M., Nathwani, A.C., and Davidoff, A.M. (2016). New and improved AAVenues: current status of hemophilia B gene therapy. Expert Opin. Biol. Ther. *16*, 79–92.
40. George, L.A., and Fogarty, P.F. (2016). Gene therapy for hemophilia: past, present and future. Semin. Hematol. *53*, 46–54.
41. Lowdell, M.W., and Thomas, A. (2017). The expanding role of the clinical haematologist in the new world of advanced therapy medicinal products. Br. J. Haematol. *176*, 9–15.
42. Monahan, P.E. (2015). Emerging genetic and pharmacologic therapies for controlling hemostasis: beyond recombinant clotting factors. Hematology Am. Soc. Hematol. Educ. Program *2015*, 33–40.
43. Nichols, T.C., Whitford, M.H., Arruda, V.R., Stedman, H.H., Kay, M.A., and High, K.A. (2015). Translational data from adeno-associated virus-mediated gene therapy of hemophilia B in dogs. Hum. Gene Ther. Clin. Dev. *26*, 5–14.
44. Sharma, A., Easow Matthew, M., Sriganesh, V., and Reiss, U.M. (2016). Gene therapy for haemophilia. Cochrane Database Syst. Rev. *12*, CD10822.
45. Spencer, H.T., Riley, B.E., and Doering, C.B. (2016). State of the art: gene therapy of haemophilia. Haemophilia *22* (*Suppl 5*), 66–71.
46. Swystun, L.L., and Lillicrap, D. (2016). Gene therapy for coagulation disorders. Circ. Res. *118*, 1443–1452.
47. VandenDriessche, T., and Chuah, M.K. (2015). Moving forward toward a cure for hemophilia B. Mol. Ther. *23*, 809–811.
48. Ward, P., and Walsh, C.E. (2016). Current and future prospects for hemophilia gene therapy. Expert Rev. Hematol. *9*, 649–659.
49. Zhang, R., Wang, Q., Zhang, L., and Chen, S. (2015). Optimized human factor IX expression cassettes for hepatic-directed gene therapy of hemophilia B. Front. Med. *9*, 90–99.
50. Rogers, G.L., and Herzog, R.W. (2015). Gene therapy for hemophilia. Front Biosci. (Landmark Ed) *20*, 556–603.
51. Barzel, A., Paulk, N.K., Shi, Y., Huang, Y., Chu, K., Zhang, F., Valdmanis, P.N., Spector, L.P., Porteus, M.H., Gaensler, K.M., et al. (2015). Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. Nature *517*, 360–364.
52. Guan, Y., Ma, Y., Li, Q., Sun, Z., Ma, L., Wu, L., Wang, L., Zeng, L., Shao, Y., Chen, Y., et al. (2016). CRISPR/Cas9-mediated somatic correction of a novel coagulator factor IX gene mutation ameliorates hemophilia in mouse. EMBO Mol. Med. *8*, 477–488.

53. Lange, A.M., Altynova, E.S., Nguyen, G.N., and Sabatino, D.E. (2016). Overexpression of factor VIII after AAV delivery is transiently associated with cellular stress in hemophilia A mice. Mol. Ther. Methods Clin. Dev. *3*, 16064.

54. Li, H., Haurigot, V., Doyon, Y., Li, T., Wong, S.Y., Bhagwat, A.S., Malani, Nirav, Anguela, X.M., Sharma, R., Ivanciu, L., et al. (2011). In vivo genome editing restores haemostasis in a mouse model of haemophilia. Nature *475*, 217–221.

55. Martino, A.T., Basner-Tschakarjan, E., Markusic, D.M., Finn, J.D., Hinderer, C., Zhou, S., Ostrov, D.A., Srivastava, A., Ertl, H.C., Terhorst, C., et al. (2013). Engineered AAV vector minimizes in vivo targeting of transduced hepatocytes by capsid-specific CD8+ T cells. Blood *121*, 2224–2233.

56. Pang, J., Wu, Y., Li, Z., Hu, Z., Wang, X., Hu, X., Wang, X., Liu, X., Zhou, M., Liu, B., et al. (2016). Targeting of the human F8 at the multicopy rDNA locus in hemophilia A patient-derived iPSCs using TALENickases. Biochem. Biophys. Res. Commun. *472*, 144–149.

57. Park, C.Y., Lee, D.R., Sung, J.J., and Kim, D.W. (2016). Genome-editing technologies for gene correction of hemophilia. Hum. Genet. *135*, 977–981.

58. Perrin, G.Q., Zolotukhin, I., Sherman, A., Biswas, M., de Jong, Y.P., Terhorst, C., Davidoff, A.M., and Herzog, R.W. (2016). Dynamics of antigen presentation to transgene product-specific CD4+ T cells and of Treg induction upon hepatic AAV gene transfer. Mol. Ther. Methods Clin. Dev. *3*, 16083.

59. Prakash, V., Moore, M., and Yáñez-Muñoz, R.J. (2016). Current progress in therapeutic gene editing for monogenic diseases. Mol. Ther. *24*, 465–474.

60. Sack, B.K., Merchant, S., Markusic, D.M., Nathwani, A.C., Davidoff, A.M., Byrne, B.J., and Herzog, R.W. (2012). Transient B cell depletion or improved transgene expression by codon optimization promote tolerance to factor VIII in gene therapy. PLoS One *7*, e37671.

61. Yen, C.-T., Fan, M.-N., Yang, Y.-L., Chou, S.-C., Yu, I.-S., and Lin, S.-W. (2016). Current animal models of hemophilia: the state of the art. Thromb. J. *14* (*Suppl 1*), 22.

62. Zolotukhin, I., Markusic, D.M., Palaschak, B., Hoffman, B.E., Srikanthan, M.A., and Herzog, R.W. (2016). Potential for cellular stress response to hepatic factor VIII expression from AAV vector. Mol. Ther. Methods Clin. Dev. *3*, 16063.

63. Li, S., Ling, C., Zhong, L., Li, M., Su, Q., He, R., Tang, Q., Greiner, D.L., Schultz, L.D., Brehm, M.A., et al. (2015). Efficient and targeted transduction of nonhuman primate liver with systemically delivered optimized AAV3B vectors. Mol. Ther. *23*, 1867–1876.

64. Lisowski, L., Dane, A.P., Chu, K., Zhang, Y., Cunningham, S.C., Wilson, E.M., Nygaard, S., Grompe, M., Alexander, I.E., and Kay, M.A. (2014). Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. Nature *506*, 382–386.

65. Vercauteren, K., Hoffman, B.E., Zolotukhin, I., Keeler, G.D., Xiao, J.W., Basner-Tschakarjan, E., High, K.A., Ertl, H.C., Rice, C.M., Srivastava, A., et al. (2016). Superior In vivo Transduction of Human Hepatocytes Using Engineered AAV3 Capsid. Mol. Ther. *24*, 1042–1049.

66. Wang, L., Bell, P., Somanathan, S., Wang, Q., He, Z., Yu, H., McMenamin, D., Goode, T., Calcedo, R., and Wilson, J.M. (2015). Comparative Study of Liver Gene Transfer With AAV Vectors Based on Natural and Engineered AAV Capsids. Mol. Ther. *23*, 1877–1887.

67. Arruda, V.R., and Samelson-Jones, B.J. (2016). Gene therapy for immune tolerance induction in hemophilia with inhibitors. J. Thromb. Haemost. *14*, 1121–1134.

68. Sack, B.K., Herzog, R.W., Terhorst, C., and Markusic, D.M. (2014). Development of Gene Transfer for Induction of Antigen-specific Tolerance. Mol. Ther. Methods Clin Dev *1*, 14013.

69. Wang, X., Terhorst, C., and Herzog, R.W. (2016). In vivo induction of regulatory T cells for immune tolerance in hemophilia. Cell Immunol. *301*, 18–29.