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Our Journey to Successful Gene Therapy for Hemophilia B

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IN 1997, I (A.C.N.) SECURED a 3-year Wellcome Trust Advanced Training Fellowship in the United Kingdom to pursue a gene therapy approach for sickle cell disease, using adeno-associated viruses (AAVs) under the supervision of Dr. Arthur Nienhuis at St. Jude Children's Research Hospital, Memphis, Tennessee. Within 6 months, we realized that AAV vectors, which have the best safety profile among vectors of viral origin, may not be ideally suited for gene therapy of sickle cell disease because of their inability to maintain stable transgene expression following gene transfer into human hematopoietic stem cells (HSCs). The AAV genome is maintained predominantly in an episomal format following gene transfer and is rapidly jettisoned as HSCs undergo division (Nathwani *et al*., 2000). Searching for inspiration, I recalled how my Ph.D. examiner, Prof. George Brownlee, who had isolated the gene for factor IX, suggested that I work on gene therapy for hemophilia B. With Dr. Nienhuis's support I, therefore, switched tack, though by then other groups had already stolen a march. In particular, Dr. Katharine High, a pioneer in this field, was at this time preparing to conduct a clinical trial in hemophilia B patients entailing intramuscular administration of AAV vectors encoding human FIX.

Our initial efforts were focused on a head-to-head comparison of the safety and efficacy of the intramuscular, intravenous, and liver (the site of factor IX production)-targeted modes of AAV delivery in murine models. I sought help from Dr. Davidoff, a newly recruited faculty member in the Department of Surgery at St. Jude, for the challenging task of AAV administration into the portal vein of mice. This was the start of a long collaboration and friendship. In the course of conducting these experiments, Dr. Davidoff and I started bouncing ideas off each other, and before he fully realized, he had become integrally involved in the hemophilia gene therapy project.

We discovered that, for the same dose of vector, expression was significantly higher following systemic or portal vein delivery of AAV when compared with intramuscular injections. More concerning was the fact that muscle delivery of AAV was more likely to trigger factor IX antibody production, which in the context of a clinical trial would be disastrous because of its potential to render gene therapy as well as FIX protein replacement therapy ineffective (Nathwani *et al*., 2001). As it happens, intramuscular delivery of AAV in the first trial of this vector in severe hemophilia B patients did not result in any toxicity. However, sustained increase in plasma FIX levels of $>1\%$ was not observed in any of the seven subjects recruited, despite promising long-term efficacy data in the murine and canine models of hemophilia B (Herzog *et al*., 1997, 1999; Hagstrom *et al*., 2000; Kay *et al*., 2000; Manno *et al*., 2003).

Further exploration of liver-targeted delivery of AAV2 vector was needed as concerns were being raised about the inability of the murine and canine models of hemophilia to be reliable models for predicting outcomes in humans. We, therefore, turned to rhesus macaques because like humans they are natural hosts for AAV and had been shown to be better predictors of outcomes in humans in relation to retroviral gene transfer of HSCs (Donahue and Dunbar, 2001). However, difficulties in distinguishing native NHP FIX from the highly homologous human cognate had impeded the use of NHP for preclinical evaluation of AAV vector-mediated expression of FIX. Working with Dr. Jay Lozier at the NIH, we developed an assay to detect human FIX in rhesus plasma using serum from a macaque (RQ1305) in which high titers of antihuman FIX antibodies developed after adenovirus-mediated gene transfer of a vector encoding the human *FIX* gene, but without significant perturbation of rhesus blood coagulation (Lozier *et al*., 1999). This antiserum enabled us to reliably detect human FIX expression in rhesus plasma down to 1% of physiologic values (Nathwani *et al*., 2002). With this assay we were able to demonstrate stable (>5 years) therapeutic expression of human FIX without toxicity following a single administration of AAV2 vector encoding FIX into the portal circulation of NHP (Nathwani *et al*., 2002).

At around this time, unexpected results were emerging out of the second gene therapy trial, which entailed a bolus

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infusion of AAV2 vector containing a strong liver-specific FIX expression cassette into the hepatic artery of patients with severe hemophilia B. Most concerning was the occurrence of a rise in liver enzymes and elimination of transgene expression in first subject to be treated at the high dose. The lower doses evaluated in this study were safe but not efficacious. These events were not observed in animal models even after administration of a log higher dose than administered in humans. Further immunological studies led to the hypothesis that the decline in FIX expression and the transaminitis were likely because of a memory AAV2 capsid-specific cytotoxic T cells response directed against the transduced hepatocytes, which was thought to be a legacy of prior exposure to wild-type AAV2 (Manno *et al*., 2006; Mingozzi *et al*., 2007). This was highly concerning as sero-epidemiological studies showed that >60% of adults had preexisting immunity to AAV2 because of prior exposure to wild-type AAV2, which could preclude them from future gene therapy trials (Blacklow *et al*., 1968; Parks *et al*., 1970).

We, therefore, turned our attention to alternative serotypes, and in particular AAV8, a new serotype that had been isolated from NHP tissues by the Wilson group (Gao *et al*., 2002). Early studies showed that AAV8 could mediate between 10- and 100-fold higher transduction of murine liver then observed with equivalent numbers of AAV2 particles (Gao *et al*., 2002; Grimm *et al*., 2003; Sarkar *et al*., 2004; Davidoff *et al*., 2005), which raised the possibility of therapeutic gene transfer using lower potentially safer doses, thus reducing the risk of transaminitis, while easing the burden on vector production. Critically, <20% of humans had antibodies to AAV8 and, in addition, it seemed that AAV8 could mediate efficient gene transfer in animals with neutralizing anti-AAV2 antibodies (Gao *et al*., 2002; Davidoff *et al*., 2005). But a major limitation to the clinical use of AAV8 vectors was the lack of an efficient method for generating clinical-grade vector particles. Dr. Jenny McIntosh in our group diligently explored an array of chromatography materials to define a scalable ion exchange chromatographic method for purification of AAV8 vectors (Davidoff *et al*., 2004). This method was later used to generate clinical-grade vector in the GMP manufacturing facility at St. Jude Children's Research Hospital for use in our pivotal trial (Allay *et al*., 2011).

To improve liver transduction beyond that achieved with AAV8 vectors, we capitalized on the observations by Dr. David Russell and Dr. Jude Samulski, who had independently shown that AAV expression cassettes that were half the size of the wild-type AAV genome were naturally packaged as two complementary strands within a single AAV particle. This enhanced the efficiency with which transcriptionally active double-stranded proviral DNA was formed, resulting in an increase in the transduction efficiency of hepatocytes, muscle, and retina by 10- to 100-fold (Hirata and Russell 2000; Yang *et al*., 2002; McCarty *et al*., 2003; Wang *et al*., 2003). However, their smaller packaging capacity ($\sim$2.5 kb) had limited the clinical application of these self-complementary vectors (scAAV). A new recruit to St. Jude at that time, Dr. John Gray, helped us to create a more compact (2.1 kb) human FIX expression cassette (scAAV-LP1-hFIXco) that met the packaging requirements of scAAV, while maintaining liver-restricted expression through the creation of a new small synthetic liver-specific promoter. Another key aspect of this cassette was the alteration of the coding sequence of human FIX by using a subset of codons most frequently found in highly expressed eukaryotic genes (''codon optimization'') (Nathwani *et al*., 2006). The scAAV-LP1-hFIXco vector when pseudotyped with serotype 5 or 8 capsid improved transduction of the murine liver by 20-fold and mediated therapeutic levels of human FIX in NHP following liver-targeted administration of significantly lower doses of vector than required with single-stranded AAV.

The next important advance in developing our gene therapy strategy for the clinic was the demonstration that the remarkable tropism of AAV8 for the liver could enable safe and highly effective transduction of the liver following a simple bolus infusion of AAV8 vectors into the peripheral venous circulation of NHP. This validated similar observation in murine models by our group and others (Nathwani *et al*., 2001, 2007; Thomas *et al*., 2004; Davidoff *et al*., 2005). The peripheral vein route of vector delivery appeared better suited for patients with a bleeding diathesis such as hemophilia B as it was simple and dispensed with the need for invasive procedures such as selective catheterization of the hepatic artery.

Having developed a body of safety and efficacy data for our distinct approach for hemophilia B, we embarked on a path to gain regulatory approval both in the United Kingdom and the United States. Our encounters with the regulators were helpful and instructive. We did, however, encounter some resistance from physicians during ethics review by the Gene Therapy Advisory Committee in the United Kingdom. These physicians had experienced firsthand the catastrophe of contaminated blood products in the hemophilia community. They argued against further experimental therapies for hemophilia patients on the grounds that the current standard of care with recombinant factor concentrate was safe and highly effective, resulting in an overall increase in life expectancy to near-normal levels (Darby *et al*., 2007). In contrast, our gene therapy approach for hemophilia B included several features that had not been tested in humans before, including AAV serotype 8, self-complementary genomic format, and systemic administration of vector, and carried the potential risk of integration oncogenesis and T cell-mediated liver injury, among others. Patients, in contrast, argued that there was a need to develop alternative treatment options that reduced the need for lifelong injection of factor concentrates.

Despite the reservation among physicians, the first six patients recruited to our trial were from the United Kingdom. They were divided into 3 cohorts of 2 participants each and received scAAV8-LP1-hFIXco vector by peripheral vein at vector doses of 2×10^{11}, 6×10^{11}, and 2×10^{12} vg/kg, respectively. All patients had to undergo an elaborate three-stage consent process to ensure that they fully understood the experimental nature of gene therapy and the potential risks associated with this procedure. The low-dose subjects who consented did so in the full knowledge that the dose they would receive was not likely to give them any benefit based on our dose-finding studies in animals (Nathwani *et al*., 2011a). Furthermore, they realized that they would not be able to have a further dose of the same vector as their immune system would then prevent subsequent successful gene transfer with AAV8. Nevertheless, two of our patients, who were motivated purely by altruistic desire to help the progress of treatment for their condition, volunteered for this dose level. They are, therefore, the real heroes of this story.

To our great surprise, we observed stable expression of FIX between 1% and 2% in both the low-dose subjects, who, as with all patients in the study, had ''severe'' hemophilia B with well-documented baseline FIX activity at <1% of normal. For the first subject, this level of expression has been ''life-changing'' as he has been able to stop twice weekly prophylaxis and remains free of spontaneous bleeds. The second subject, like the third subject after him, who received the intermediate dose of vector, had significant hemophilic arthropathy characterized by a frail inflamed synovial lining, which increased the risk of joint bleeds. Neither subject has been able to stop prophylaxis altogether after gene transfer despite evidence of endogenous wild-type FIX expression at 1–3%, but both have been able to increase the period between prophylaxis from twice weekly to once every 10–14 days.

Of the first six patients, four were able to stop prophylaxis completely within 6 weeks after gene transfer. The highest level of transgene expression of between 8% and 12% of normal was observed in the two subjects treated at the high dose level and remained stable up to 6 weeks after gene transfer. This level of expression was enough to convert their bleeding phenotype from severe to mild. Patients with mild hemophilia typically have plasma FIX levels of between 5% and 40% of normal and have very few or no spontaneous bleeding episodes though they are still at risk of excessive hemorrhage after trauma or surgery. Unexpectedly, at around 7 weeks after gene transfer, the first of the high-dose patients had a 10-fold increase in liver enzymes associated with a drop in human FIX expression to 2% of normal, which was thought to be because of a cellular immune response to the transduced hepatocytes. We commenced him on prednisolone and this was followed by resolution of the transaminitis but without complete loss of FIX expression.

The other high-dose patient also developed a slight elevation of liver enzyme levels over his baseline at around 9 weeks after gene transfer. Although the liver enzyme levels remained within the normal range, he was commenced on prednisolone. His liver enzyme levels promptly returned to baseline values and FIX expression is maintained at 4% of normal levels. He has not required any treatment with FIX concentrates despite living a very active life. He was on thrice-weekly FIX prophylaxis prior to gene transfer (Nathwani *et al*., 2011b). The prednisolone regimen used in our study was based on that used in the treatment of autoimmune hepatitis and proved to be an important intervention for minimizing liver inflammation while preserving transgene expression. This study continues to enroll participants with several unanswered questions remaining to be addressed, including cause of and risk factors for transaminitis, detrimental or beneficial effects of empty capsid particles that do not contain vector genomes in the vector preparations, and the optimal serotype for the most efficient transduction of human hepatocytes.

Encouraged by this success, we have begun to look at hemophilia A—a more common bleeding diathesis but technically more challenging because the factor VIII protein is inefficiently synthesized in humans. In addition, the factor VIII cDNA at 7 kb is too larger (7 kb) to be packaged into AAV, which have a maximum packaging capacity of 5 kb. Nevertheless, we have addressed both these obstacles to develop an AAV-based gene transfer approach that we hope to take to clinic in the next 18 months. Expression of human FVIII was improved 10-fold by codon optimization of the wild-type cDNA of human FVIII (Nathwani *et al*., 2006; Radcliffe *et al*., 2008; Ward *et al*., 2011; McIntosh *et al*., 2013). Expression from the B domain-deleted codon-optimized FVIII molecule was further enhanced by the inclusion of a 17 amino acid peptide that contains the six N-linked glycosylation signals from the B domain required for efficient cellular processing. These changes have resulted in a novel 5.2 kb AAV expression cassette (AAV-HLP-codop-hFVIII-V3) that is efficiently packaged into recombinant AAV vectors and capable of mediating supraphysiological level of FVIII expression in animal models over the same dose range of AAV8 that proved to be efficacious in subjects with hemophilia B.

Progress at times can seem agonizingly slow in the field of gene therapy. However, the fact that our initial cohort of hemophilia B patients is still benefiting from the one-off gene transfer treatment delivered in 2010 represents an important and encouraging advance in this field. As well as clinical improvement, the study has also generated economic benefits, with a saving of £300K in just the first year of the trial from reduction in FIX concentrate usage. Our gene therapy success was achieved by an international wide-reaching collaborative effort that brought together many of the key players in the field of gene therapy who were all fully committed to the success of our strategy. Further improvements in this technology are clearly needed, but it is our hope that one day gene therapy will become the standard of care for patients with hemophilia as well as other disorders affecting the liver with greater unmet treatment needs.

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