Genetic Diseases, Immunology, Viruses, and Gene Therapy

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MY INTEREST IN GENE THERAPY STARTED AS an MD–PhD student in Bill Kelley’s laboratory at the University of Michigan. I was studying the molecular basis for the X-linked recessive disease Lesch-Nyhan syndrome (LNS), caused by a defect in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). This devastating childhood disease is associated with gout and nephrolithiasis caused by hyperuricemia and a bizarre constellation of neurologic and behavioral findings including spasticity and choreoathetoid movements, as seen in Huntington’s disease, and a compulsion to self-mutilate. I successfully sequenced the normal HGPRT enzyme (Wilson *et al.*, 1982) and identified a number of missense mutations that caused LNS (Wilson *et al.*, 1983). While I was excited by the technical progress we made in understanding the molecular basis of LNS, I was concerned that our research was unlikely to improve the health of those living with LNS. My perspective changed in 1980 when, during my graduate training, I read about the work of a Stanford graduate student in Paul Berg’s laboratory, Richard Mulligan, who corrected the metabolic abnormalities in cells from a patient with LNS by transferring the prokaryotic homolog of HGPRT into the cells (Mulligan and Berg, 1980). This remarkable but understated article suggested the potential of treating recessive diseases like LNS by gene therapy. At that moment I decided to dedicate my career to the development of gene therapy for inherited diseases like LNS. Little did I know it would take us more than 30 years to show that the concept of gene therapy actually works in human beings!

A consistent theme of my career is the impact that individual patients have had on my research. After completing my thesis research, I entered the clinical phase of medical school where I was confronted with several graphic examples of the tremendous burden that genetic diseases have in children. During my pediatric rotation, I met a boy with metachromatic leukodystrophy who had lost most of his neuromuscular and cognitive function, requiring around-the-clock nursing care. Soon thereafter I was assigned to take care of a young boy with a severe form of epidermolysis bullosa. A single gene defect affected the structural integrity of his skin, which would spontaneously peel away from his body leaving him with large areas of exposed tissue similar to what you would see in severe burns. In each case, there was nothing that we could do for these boys other than

provide them with supportive care. Again, I thought gene therapy may ultimately be the answer for children afflicted with these devastating diseases.

I moved to Boston in 1984 to do a residency in internal medicine at the Massachusetts General Hospital (MGH) with the hope of following up with a postdoctoral fellowship with Richard Mulligan, who had moved from Stanford to the Massachusetts Institute of Technology (MIT). During one evening, while on call at MGH, a group of residents got together to talk about interesting papers. We discussed a fascinating case study of a young girl with familial hypercholesterolemia (FH) caused by a deficiency in low-density lipoprotein (LDL) receptor (Starzl *et al.*, 1984). At the age of six she developed severe congestive heart failure (CHF) due to repeated myocardial infarctions. The article described an attempt to save her life by subjecting her to a combined heart–liver transplant; the heart transplant was necessary to treat her CHF, while the liver transplant provided her a source of hepatic LDL receptor. The results were dramatic. She survived the procedure and realized a virtually complete normalization of LDL cholesterol. This case directed my thoughts about gene therapy to liver as a target. I felt liver would be more tractable than bone marrow, which was the focus of the majority of the work in gene therapy at the time, or the brain, which would be the target for LNS. Furthermore, the outcome of gene therapy for FH is easily monitored through LDL cholesterol measurements in much the same way that the efficacy of liver transplant was assessed in this young girl. I was anxious to get back into the laboratory!

Following completion of my residency in 1986, I moved to MIT to work with Richard Mulligan who was developing retroviruses as gene transfer vehicles to target hematopoietic stem cells for the treatment of patients with inherited severe combined immune deficiency disorders. Richard allowed me to explore the potential of liver as a target for gene therapy using a rabbit model of FH. The approach was to engineer autologous hepatocytes *ex vivo* with retroviral vectors and to transplant the engineered cells back into the individual from which they were derived (Wilson *et al.*, 1988).

I moved to the University of Michigan in 1989 to begin my independent academic career and to further my research in gene therapy. Our studies of *ex vivo* gene therapy in the FH rabbit model looked promising and formed the basis for the first liver gene therapy experiment in humans

(Chowdhury *et al.*, 1991). This phase 1 clinical trial demonstrated modest evidence of gene transfer in some FH patients (Grossman *et al.*, 1995). It became clear to me that the most effective way to treat liver metabolic diseases such as FH would be to directly target hepatocytes *in vivo* with gene delivery vehicles that could safely and efficiently transduce hepatocytes following direct injection; however, vectors were not up to this task.

While at Michigan, I enjoyed many collaborations including work on gene therapy for the disease cystic fibrosis (CF) with Francis Collins, with whom I shared research space. We used retroviral vectors to correct the CF defect in cultured CF cells, although retroviruses (Drumm *et al.*, 1990) were not capable of transducing airway cells when injected *in vivo*, the same limitation that we observed with liver. Ron Crystal and colleagues brought forward a new vector system based on human adenoviruses, which was able to transduce airway cells *in vivo* (Rosenfeld *et al.*, 1991). We established the adenoviral vector system and showed equally promising results in targeting conducting airway cells in various animal models. Three clinical trials of *in vivo* gene therapy with adenovirus vectors were conducted in patients with CF, including Ron Crystal, who at the time was at NIH (Crystal *et al.*, 1994), Mike Welsh from Iowa (Zabner *et al.*, 1993), and my group (Zuckerman *et al.*, 1999).

I moved to the University of Pennsylvania in 1993, where we established large multidisciplinary research programs in gene therapy. At the time of this move, my research focused on adenovirus vectors, which showed promise for applications of *in vivo* gene therapy in targets other than the lung, such as the liver. The transduction efficiencies in the liver were remarkable, although our enthusiasm was tempered because transgene expression was transient and associated with mononuclear cell inflammation, and attempts to readminister the vector were unsuccessful. A young graduate student in my laboratory, Yiping Yang, was assigned to study these problems and to design ways to overcome them. He performed a very simple but elegant experiment on the hunch that some of the problems we were seeing with adenoviral vectors were caused by immune responses. Yiping simply injected vector into mice that were void of T cells and showed that expression was stable over time with little concomitant inflammation; in addition, vector could be readministered (Yang *et al.*, 1994). This represented one of those rare eureka moments and suggested to me that successful *in vivo* gene therapy would require a much greater understanding of potential host immune responses and the development of strategies to manage them. This epiphany essentially dictated the direction of our research for almost a decade.

A number of students from my lab, including Yiping, conducted studies that delineated the detailed cellular and molecular pathways of the adaptive immune response to adenoviral vectors with a focus on the generation of destructive cytotoxic T lymphocyte (CTL) responses (Gao *et al.*, 1996). Based on this information, we created second- and third-generation adenoviral vectors that were associated with substantially blunted CTL responses. These improved adenovirus vectors were evaluated in the clinic in several diseases including a liver metabolic disease caused by a deficiency of the enzyme ornithine transcarbamylase (OTC)

(Raper *et al.*, 2002). There were no apparent problems associated with the generation of CTLs in these trials, although one subject in the OTC deficiency trial developed a lethal episode of systemic inflammation that upon further evaluation was found to be caused by the activation of innate immunity by the vector capsid (Raper *et al.*, 2003). This adverse event highlighted the role of innate immunity in acute toxicity and its proximal role in initiating adoptive immune responses. There was no easy way to engineer around these problems if the capsid was essentially the trigger. We redirected our studies of adenoviral vectors to their use as genetic vaccines, where their inherent immunogenicity would be an asset. Soumitra Roy, also from my lab, focused our vaccine work on the creation of better adenovirus vaccine vectors created from novel adenoviruses isolated from nonhuman primates (Roy *et al.*, 2009, 2011).

My fascination with viruses grew as our lab continued its work on vector technology. One type of virus that caught my attention was a family of primate parvoviruses called adeno-associated virus (AAV). Six different AAVs (*i.e.*, AAV1–6) were discovered as contaminants in laboratory preparations of primate adenoviruses in the 1960s to 1970s and serologic studies suggested that AAVs may circulate as infectious agents in humans. Soon after I arrived at Michigan, I visited the laboratory of Jude Samulski about work he had done with AAV as a postdoctoral fellow in Tom Shenk's laboratory (Samulski *et al.*, 1987). Jude helped us set up the AAV2 system, although progress was slow because of difficulties in producing vectors. *In vivo* studies were eventually performed by a number of laboratories, including ours, with fascinating results. Expression of highly immunogenic transgenes was stable without evidence of destructive cellular immune responses, suggesting that AAV2 somehow evades the problems that plagued adenoviral vectors (Kessler *et al.*, 1996; Xiao *et al.*, 1996; Fisher *et al.*, 1997). Unfortunately, transduction efficiencies with AAV2 vectors in tissues/organs relevant to our work were too low to be of use in human therapeutic applications.

Our first attempt to improve the performance of AAV vectors was conducted by a postdoctoral fellow, Weidong Xiao. Weidong and I decided to develop a vector based on AAV1, which was isolated in the 1960s but never characterized. Sequence analysis showed substantial differences between the AAV1 and AAV2 capsids. Our logic in pursuing this project was that capsid structure should drive vector performance and that natural isolates of AAV would differ from one another with respect to capsid structure because of variation that allows for escape from the host's immune response. Our hope was that the capsid differences in AAV1 would translate to improved vector performance—although it was also possible that these differences would have no effect or even diminish vector performance. Weidong showed that AAV1 was indeed more efficient in transducing muscle and that the prevalence of neutralizing antibodies in human populations to AAV1 was diminished relative to AAV2 (Xiao *et al.*, 1999). AAV1 was ultimately licensed to a number of biopharmaceutical companies, including Celladon, which is in phase 2a clinical trials with an AAV1 product for the treatment of CHF, (Zsebo *et al.*, 2014) and UniQure, who recently received marketing approval for Glybera, an AAV1-based product which is administered intramuscularly to treat a rare genetic form of

hypertriglyceridemia (Bryant *et al.*, 2013). While AAV1 did afford significant advantages for targeting skeletal and cardiac muscle, it was no better in transducing other tissues/organs such as liver.

At the turn of the century, the field of gene therapy saw a significant erosion of support for a variety of reasons (Wilson, 2009). Despite extensive clinical trial activity, the field had no clear demonstration of clinical success. My assessment was that available vector technology was not suitable for most of the clinical applications that were contemplated. I approached Tachi Yamada, who at the time was chief scientific officer of SmithKlineBeecham (SB), for advice about the current state of the field and what I could do to move it forward. Tachi was the chair of my department at Michigan and a very important mentor. He encouraged me to “go back to the bench” and focus on creating better vectors and offered to have SB help support this effort through a sizable sponsored research agreement with Penn. We had the financial support—we now needed a plan!

My thought was to leverage the favorable immune biology of AAV but to substantially improve its performance beginning with better *in vivo* gene transfer across a wide scope of tissue/organ targets. We considered three strategies. Rational engineering of existing capsids by specific mutagenesis to direct cell interactions was considered but dismissed because of a paucity of structural information available at the time. We also considered the so-called “*in vitro* evolution” approach in which improved vectors could be selected from large libraries of capsid variants generated through *in vitro* mutagenesis. This was not pursued because of concerns about authenticity of the selection systems, which were likely to be conducted *in vitro*. We decided to expand on the concept used for AAV1, which was to use capsid variation generated through natural infections to find improved vectors. Success would hinge on isolating a large and diverse array of natural AAV variants. I viewed this exercise like a game of soccer—that *is*, each novel vector is like a shot on goal; the more shots you have on goal, the more likely it is that you will score. Previous work did not inform a strategy for isolating AAVs from natural sources since infection with AAV was not associated with any illness and all previous isolates were recovered as contaminants of adenovirus preparations. Based on *in vitro* work done with AAV2, which demonstrated the existence of latent AAV2 genomes, we hypothesized that silenced AAV genomes persist in the host following the resolution of natural AAV infections. We tested this hypothesis by probing DNA recovered from healthy human and nonhuman primates using PCR with oligonucleotides to conserved regions of the six known AAVs (see the cover for a description of the strategy and data). The results were absolutely amazing and quite frankly shocking. We detected endogenous AAV sequences across a wide array of tissues from human and nonhuman primates (Gao *et al.*, 2002, 2003, 2004). Strikingly, we discovered that over 40% of human livers harbor endogenous AAV sequences. What was even more remarkable was the diversity of capsid sequences represented in this family of endogenous viruses. We had over 120 novel AAV capsids representing quite a few shots on goal, but would any of them score?

Vectors made with the initial two capsids isolated from primate tissues, subsequently named AAV7 and AAV8,

were injected into mice intravenously and livers were analyzed for transgene expression. I was stunned when the chief scientist on the project, Guangping Gao, presented the data: transduction efficiency in liver with AAV8 was almost 100-fold better than what we had achieved with the existing AAV capsids (Gao *et al.*, 2002). I knew immediately that this was an important result that had the chance to materially change the clinical development of AAV gene therapy. We went on to screen a substantial number of the other novel capsids for transduction to liver, heart, muscle, and retina-finding capsids with equally impressive improvements in transduction. Anticipating significant interest in this new AAV technology, we scaled up our vector production capacity to serve outside investigators in what is called “Penn Vector”. The response was muted at the beginning, although interest very quickly picked up. Recipients of materials from Penn Vector progressed applications into novel targets such as the central nervous system (CNS) (Sondhi *et al.*, 2008; Foust *et al.*, 2010) and into some clinical applications, the most celebrated being the use of AAV8 in patients with hemophilia B (Nathwani, *et al.*, 2011).

Conclusions

We are entering a remarkable era of gene therapy research that will accelerate its development and lead to a number of commercial products across a spectrum of diseases. Several forces have contributed to this period of unprecedented growth. After 30 years of science, we have the technology and know-how to safely and efficiently transfer genes into human cells. Careful selection of target diseases has yielded unambiguous proof-of-concept of the efficacy of these technologies in clinical trials. The first market authorization of a gene therapy in Europe was a key milestone that demonstrated both technical feasibility and regulatory receptivity for a commercial gene therapy product. The most tractable diseases for demonstrating clinical success of gene therapy are orphan diseases, which the biopharmaceutical industry has recently embraced as attractive targets for investment and development of novel products. Finally, a stronger economy has contributed to a surge in biotechnology investments and an almost unprecedented environment for successful initial public offerings (IPOs). This is bringing much-needed capital to gene therapy product development.

Despite these exciting developments, we need to recognize that the commercialization of gene therapy, which is the critical path to providing access to patients, is still in its infancy. The technologies have not been completely derisked in all applications. There will be setbacks as the community expands the clinical evaluation of gene therapy into new tissue/organ targets and a wider array of diseases. The regulatory science around the development of commercial products is nascent and likely to evolve. This is particularly relevant to the production of clinical grade vector, where few reference standards exist and analytical characterizations of the products need improvement.

It has been 34 years since I read Richard Mulligan’s article on transfection of LNS cells and decided to focus my career on gene therapy. It has been my privilege to be a member of a core group of colleagues who have doggedly pursued the dream of gene therapy over the decades.

Recently I read with pride several articles describing encouraging clinical data from gene therapy trials in children with the diseases metachromatic leukodystrophy (Biffi *et al.*, 2013) and epidermolysis bullosa (De Rosa *et al.*, 2014), both of which had such an influence on me as a medical student. Our research group plans to leverage the successful experiences of liver-directed gene therapy with AAV8 for hemophilia B into clinical trials of AAV8 gene therapy for patients with FH and OTC deficiency. My ultimate goal is to exploit the neurotrophic properties of some of the vectors we discovered toward the treatment of genetic diseases with disabling and degenerative CNS manifestations. We believe the most tractable approach is intrathecal gene therapy in patients with neuropathic manifestations of lysosomal diseases, which hopefully will pave the way to gene therapy of the LNS and will bring me full circle.

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J.M.W. is a consultant to ReGenX Holdings, and is a founder of, holds equity in, and receives a grant from affiliates of ReGenX Holdings; in addition, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings.

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