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My Pathway to Adeno-Associated Virus and Adeno-Associated Virus Gene Therapy: A Personal Perspective

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When i began my research career, I had no idea where it would ultimately lead me. Indeed, my whole career in science never had any sort of master plan but rather has been one of following my nose and taking advantage of some opportunities that luckily appeared in front of me along the way.

DOCTORAL RESEARCH IN NEW ZEALAND

I began in the biochemistry department at the University of Otago in my birthplace in Dunedin, New Zealand, where I did my PhD from 1966 to 1969 with Mervyn Smith, who had just returned from Al Hershey's lab at Cold Spring Harbor. He had analyzed lambda phage DNA replication in Hershey's lab, and I continued those studies and became steeped in phage molecular biology. After that, I wanted to transition to mammalian cells, and I thought a good way to do this would be via study of mammalian viruses.

Merv Smith on his family sheep station in New Zealand.

POSTDOCTORAL SOJOURN IN LONDON

In the fall of 1969, due to Mervyn Smith's connections, I joined Lionel Crawford's lab at the Imperial Cancer Research Fund in London to work on polyoma virus DNA replication. However, several serendipitous events sparked my interest in adeno-associated virus (AAV), which had been discovered just four years previously. In 1969, Jim Rose and Ken Berns at the National Institutes of Health (NIH) reported that AAV packages plus and minus single-strand DNA genomes into separate particles, and they demonstrated how to separate these complementary strands.[1] This suggested that the 5 kb AAV genome was a useful and tractable model to understand gene expression in mammalian cells. Also, mutants of the AAV helper virus, adenovirus, were becoming available, suggesting that this system would allow biochemistry and genetics to be combined. In another stroke of serendipity, as a result of a letter Jim Rose wrote to Lionel Crawford in 1970, I arrived in late October of that year at the NIH, Bethesda, to work on AAV in Jim's lab, which was part of the

Jim Rose (*right*) with Ken Berns (*center*), Nic Muzyczka (*left*), c. 1986.

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Laboratory of Biology of Viruses at the National Institute of Allergy and Infectious Diseases (NIAID).

TWO DECADES AT THE NIH

My time at the NIH was an extraordinary opportunity, and when I first arrived there, I did not realize that I would stay for 22 years. I had planned to return to the biochemistry department in Dunedin, but several considerations led me to change this plan. The overall environment of the whole NIH intramural research community offered an enormous opportunity for interaction and collaboration on broad fronts in basic and clinical research. The clinical focus at the NIH ultimately helped guide me toward clinical development of gene therapy. Also, NIH intramural scientists did not have to apply for research funds. So, it was an unfettered environment to focus on research. For a young scientist starting out, these were halcyon days.

After 3 years in the Rose lab, I was able to set up my own lab at the National Institute for Diabetes and Digestive and Kidney Diseases (NIDDK). AAV had little apparent connection to any of these diseases, and AAV was not a disease-causing pathogen, but Ed Rall, the enlightened Scientific Director of NIDDK, agreed that AAV was an interesting experimental system and fully supported my work and my attainment of tenure. In the 1970s, the focus was on the molecular biology of AAV, and this continued into the 1980s. Beginning in 1980, we were able to begin work that led to the development of AAV vectors, and by the end of the decade, we were working on a specific AAV vector for cystic fibrosis (CF). I have described the development of AAV and AAV vectors elsewhere.[2] So, I will summarize that work briefly here.

In the Rose lab, I began basic studies on the AAV genome and adenovirus helper functions. However, the most fascinating work there was the initial characterization of the unusual properties of the AAV2 DNA terminal sequences that eventually lead to their characterization as inverted terminal repeats (ITRs). With George Khoury, we observed unusual behavior of purified single strands of AAV DNA on hydroxylapatite columns. We thought this might be annealing of terminal repeats, which was confirmed by electron microscopy. At NIDDK, with David Denhardt at McGill University, we observed that these termini could form nonlinear structures. Meanwhile, at Johns Hopkins, Ken Berns and Tom Kelly did enzymatic digestion experiments that suggested the AAV termini were both direct and inverted repeats. That the terminal repeats appeared to be palindromic sequences was confirmed by direct sequencing.[3] As subsequent studies have shown, the only AAV DNA sequences required in AAV vectors are the ITRs because they represent the replication origins and the packaging sequences, and also mediate formation of circular episomes.

Ed Rall (*center*) in 1992.

At NIDDK, our studies on AAV were advanced by many highly talented individuals. Cathy Laughlin established the basic transcription map showing the three transcription promoters (labeled p5, p19, and p40) and overlapping sets of messenger RNAs. Later, Jim Trempe provided definitive evidence for the alternate splicing of the AAV intron to provide access to separate initiation codons in order to generate the three AAV capsid proteins. Luis de La Maza analyzed the variant genomes that accumulate with undiluted passage of wild-type AAV, which led to several important insights. These molecules are internally deleted and enriched for the ITRs, which helped to establish the importance of the ITR as the replication origin. Second, because these genomes were less than half-size, they could be encapsidated as dimeric molecules that annealed (or snapped back) to form duplex molecules, and subsequently this was the basis of the conception of self-complementary AAV vectors.[4] Finally, the AAV variants required wild-type AAV, in addition to adenovirus, to replicate. So, we could predict that AAV must code for a replication (or Rep) function in addition to the capsid proteins.

The full sequencing of the AAV2 genome[5] by Arun Srivastava in the Berns lab showed a large open reading frame in the left half of the genome. Ella Mendelson and Jim Trempe identified the four proteins coded by the region, which we labeled Rep78, Rep68, Rep52, and Rep40 to reflect their approximate molecular weights.

Maureen Myers analyzed product and precursor relationship of AAV DNA replication and the generation of AAV particles. This led to our model that AAV DNA single strands were packaged into preformed capsids, which subsequently became relevant for understanding the importance of self-complementary vectors and oversized vectors, as is required for hemophilia A.

Gene therapy was discussed in the early 1970s,[6] but we could not entertain serious thoughts about AAV and gene therapy. Under the early NIH DNA guidelines, cloning of an entire human viral genome required a P4 (BL4) containment. However, in 1980, this was reduced to BL2 containment. This allowed us to clone intact genomes of

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AAV2 in bacterial plasmids and to observe that transfection of the plasmid into adenovirus-infected cells generates infectious AAV. This facilitated both the analysis of AAV genetics and biochemistry and the development of AAV vectors led in my lab by Jon-Duri Tratschin. Thus, in 1984, we[7] and the Muzyczka lab[8] described the first AAV vectors that could express foreign genes and the initial genetic analysis of AAV from both labs.[9,10] Subsequently, Nor Chejanovsky, Roland Owens, and Matt Weitzman analyzed aspects of the genetics and biochemistry of the Rep proteins and showed, for instance, that Rep52 and Rep40 are required for the accumulation of AAV single strands but not for AAV DNA replication *per se*.

In early 1989, several events stimulated me to focus on a specific disease: CF. Terry Flotte, a pulmonologist at John Hopkins Medical School, came to work in the lab with funds provided by the Cystic Fibrosis Foundation (CFF). The CFF was very enthusiastic about gene therapy, as it was expected that the CF gene would be sequenced by late that year, and the pulmonary tract seemed easily accessible for gene delivery. I was also afforded additional funds with a research grant from the CFF.

Because AAV is not a pathogen and is easy to produce in suspension culture, it was a potentially a safe starting point for the generation of a gene delivery system. The main downside appeared to be the payload size, since the AAV2 genome is only 4,681 nucleotides long. This became a challenge when we learned that the coding sequence for the CFTR protein is about 4,400 nucleotides, and it was not known then if any portions of the protein were dispensable for function. Consequently, I began my first experience of developing AAV vectors with a gene that needed an oversized vector. Nevertheless, we managed to generate an AAV-CFTR vector that did express CFTR and could correct the defect in CF airway cells. This began a long collaboration with Terry Flotte and Bill Guggino, also at Hopkins.

CLINICAL DEVELOPMENT OF AAV VECTORS AT TARGETED GENETICS IN SEATTLE

By the early 1990s the establishment of the first gene-therapy start-up companies began. In this context, I was recruited to join Targeted Genetics Corporation (TGC) in Seattle as the Chief Scientific Officer. When I arrived at TGC, with Terry Flotte, we had results from the first animal experiments with an AAV vector in the lungs of rabbits, and we decided to move ahead with clinical development of the AAV2-CFTR vector. This CF program became one of the first substantial collaborations between industry, academia, and a nonprofit patient-oriented organization, the CFF. In addition to Guggino at Hopkins, and Flotte at Hopkins and Florida, we added Phyllis Gardner and Rick Moss at Stanford as clinical collaborators. The clinical trials were supported by NIH clinical center funding at all three centers, and as we progressed to Phase II trials, we also had support from the CFF Center for Therapeutic Development organized by Bonnie Ramsey in Seattle.

CF as a gene therapy target now is seen as much more difficult and probably not the optimal choice, and gene therapy for CF has not succeeded with any delivery vector. However, the support and effort of the CFF to involve a large cohort of investigators with multiple types of gene delivery systems was extremely important in understanding and developing the multidisciplinary approach that would be required for the clinical development of any gene therapy and in instructing us all in appreciating the complexity of such a task.

AAV-CFTR was the first AAV gene therapy program to enter the clinic, beginning in 1995, and thus it initiated the regulatory and clinical pathway of AAV vector development. The history of this program has been described in detail elsewhere.[11] In preclinical studies, we observed that the vector existed in rhesus macaque lungs as an unintegrated species, which was one of the earliest indications that *rep* AAV vectors persist primarily as episomes.[12] The clinical trials showed general safety of dosing up to 10^{13} AAV particles and of repeated delivery, but also showed generation of anti-capsid humoral response. In the course of the CF program, TGC developed Good Manufacturing Practice (GMP) production of AAV vectors in the adenovirus-induced producer line that was originated in Phil Johnson's lab at Columbus, Ohio. This was much more efficient than the DNA transfection procedures we previously used and could be readily scaled in bioreactors.

A second program at TGC that began in 1997 also was a collaboration with industry, academia (Phil Johnson at Columbus, Ohio), and a nonprofit organization, the International AIDS Vaccine Initiative (IAVI) that provided substantial funding, and again eventually involved additional substantial funding from the NIAID at the NIH. The objective of this program was to develop an acquired immune deficiency syndrome vaccine via intramuscular injection of an AAV vector expressing human immunodeficiency virus (HIV) antigens. After a series of clinical trials in Europe and Africa, this program did not continue because of general uncertainty about the appropriate HIV antigen. However, this program also helped the development of the AAV platform. Although AAV persisted mainly as an episome, the Food and Drug Administration pressed us to provide some measure or upper limit of the possible integration frequency of the vector genomes. A combination of very extensive biodistribution studies in mice and rabbits, together with a DNA polymerase chain reaction assay designed in the Johnson lab to detect vector–cell DNA junctions, provided the first estimates of the low frequency of integration.[13]

The most successful program at TGC was a collaboration with Robin Ali in London who was developing an

AAV2 vector for the rare disease Leber congenital amaurosis (LCA) caused by a mutation in the RPE65 protein that leads to retinal degeneration and loss of vision. At TGC, we manufactured the GMP AAV2-RPE65 vector and helped with the preclinical program. Subretinal injection of the vector showed improved retinal sensitivity and visual mobility in low light. This result,[14] published in 2008 along with similar results from two other groups in that year, was the first AAV program to a give clear indication of a clinical benefit. Ultimately, an AAV2 vector, Luxturna®, was approved as a treatment for LCA in 2017.

The enthusiasm generated by the LCA results in 2008 was temporarily muted by the worldwide liquidity crisis later that year, which immediately caused many companies to take somewhat drastic steps. As a result of one such step, I became a consultant, and TGC eventually dissolved as a gene therapy entity.

Investment in gene therapy was dramatically accelerated in 2011 by a watershed achievement of AAV gene therapy for hemophilia B. Amit Nathwani and Andrew Davidoff and their respective groups reported that in six subjects with hemophilia B intravenous, administration of an AAV8 human Factor IX (FIX) vector provided sustained circulating amounts of the coagulation factor at 5–7% of normal levels accompanied by dramatic decreases in the annual rate of bleeding and need for prophylactic administration of the protein factor. This hemophilia work, following the LCA results, and the parallel clinical successes with gene-modified T-cell therapies further fueled the fires. Additionally, in 2012, the first AAV gene therapy, Glybera®, for the treatment of dyslipidemia by intramuscular injection was approved in Europe. Although Glybera® was not a commercial success, it checked a final box that had worried investors for two decades: namely, whether a gene therapy could ever obtain regulatory approval.

GENE THERAPY FOR HEMOPHILIA AT BIOMARIN PHARMACEUTICAL

In the midst of the renewed excitement for gene therapy, I began consulting for BioMarin Pharmaceutical at the behest of their CSO Len Post, and by the end of 2012, the company licensed an AAV gene therapy program for hemophilia A, which again had been developed by the Nathwani–Davidoff team. A large part of the FVIII protein, the B-domain, is not needed for its coagulation function, and removal of this region from the cDNA facilitates generation of an AAV vector genome that is <5 kb.

BioMarin was already a successful company with several approved biologic products for rare diseases and extensive experience in rapid clinical development and manufacturing of these protein products. However, the company had no prior institutional experience with gene therapy, and I was prevailed upon to join the company as an employee at the beginning of 2013 to help get gene therapy at BioMarin off the ground. When I retired after 6 years at the end of 2018, the program was poised to complete pivotal clinical trials.

BioMarin Vector Biology Group 2018. Peter Colosi seated *front row left.*

It was a new experience for me to develop a gene therapy program in an environment where the full infrastructure for clinical trials, commercial manufacturing, and worldwide marketing already existed. By a remarkable coincidence, Gordon Vehar in BioMarin's R&D group had been a lead player at Genentech when the FVIII gene was cloned and published in 1984 at the same time that we described our first AAV vector. Also, we immediately recruited a group of gene therapists, led by Peter Colosi, many of whom had been at Avigen, another early AAV gene therapy company in the Bay area.

Development of the AAV-FVIII program proceeded rapidly, and with initial preclinical studies completed,[15] a Phase I trial began in the fall of 2014. The outcome of this trial[16] was highly encouraging, and pivotal trials were initiated in 2017. Also, the manufacturing group at BioMarin built an in-house commercial facility to manufacture AAV vectors at 2,000 L scale. The pivotal trials[17] have now led to recent filings, in both Europe and the United States, for approval of the AAV hemophilia A product that is now known as valoctocogene roxaparvovec.

SOME FINAL THOUGHTS

I began and ended my involvement in developing AAV gene therapy with genes, *CFTR* and *FVIII*, respectively, which each exceeded the packaging capacity of AAV. The success of the FVIII and not CF reflects important developments in AAV gene therapy. The judicious choice of target cells matters; the slowly dividing hepatocytes are better suited to the episomal persistence of AAV vectors than the rapidly turning over CF airway cells. Availability of clear clinical trial readouts is highly important; clinical

endpoints in hemophilia are direct measures and more predictive of clinical impact than are CF clinical trial endpoints. Repeat delivery of AAV vectors, however, is still severely limited by preexisting or induced humoral immune responses to AAV capsids.

There is now a large body evidence from well in excess of 100 clinical trials regarding the safety profile of AAV vectors. Manufacturing of AAV vectors has advanced enormously in terms of both scale and purity of the product and the analytics required. It is now possible to deliver much higher individual patient doses that are greater than we could manufacture in a single run two decades ago. Some caution is still required, as the higher doses and intravenous delivery have highlighted additional host responses that need to be understood and managed.

I look expectantly for the outcome of the regulatory discussions this year. To have been able to contribute significant improvement in the lives of patients afflicted with debilitating disease is something I never imagined remotely when I started my research career on bacteriophage DNA replication. Approval of valoctocogene roxaparvovec would be a satisfying outcome of my long pathway for AAV and AAV vectors.

ACKNOWLEDGMENTS

I have mentioned a few individuals in the above summary, but I would be remiss not to acknowledge the very much larger number of others who have been involved along the way. I have been mostly a very fortunate observer of, and participant in, successively larger teams that have progressively advanced the field as a whole. I only regret that it is not feasible to acknowledge here the many hundreds of people involved.

AUTHOR DISCLOSURE

Author is an external consultant to BioMarin and holds BioMarin stock and stock options.

REFERENCES

1. Rose JA, Berns KI, Hoggan MD, et al. Evidence for a single-stranded adenovirus-associated virus genome: formation of a density hybrid upon release of viral DNA. Proc Natl Acad Sci U S A 1969;64:863–869.
2. Carter BJ. Adeno-associated virus and the development of adeno-associated virus vectors: a historical perspective. Mol Ther 2004;10:981–989.
3. Lusby E, Fife KH, Berns KI. Nucleotide sequence of the inverted terminal repetition of adeno-associated virus DNA. J Virol 1980;32:402–409.
4. Carter BJ. Metabolically activated recombinant viral vectors and methods for their preparation and use. US Patent 6,596,535, issued July 22, 2003.
5. Srivastava A, Lusby EW, Berns KI. Nucleotide sequence and organization of the adeno-associated virus 2 genome. J Virol 1983;45:555–564.
6. Friedmann T, Roblin R. Gene therapy for human genetic disease? Science 1972;175:949–955.
7. Tratschin JD, West MHP, Sandbank T, et al. A human parvovirus, adeno-associated virus as a eukaryotic vector: transient expression and encapsidation of the prokaryotic gene for chloramphenicol acetyl transferase. Mol Cell Biol 1984;4:2072–2081.
8. Hermonat PL, Muzyczka N. Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells. Proc Natl Acad Sci U S A 1984;81:6466–6470.
9. Hermonat PL, Labow MA, Wright R, et al. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J Virol 1984;51:329–339.
10. Tratschin JD, Miller IL, Carter BJ. Genetic analysis of adeno-associated virus. Properties of deletion mutants constructed *in vitro* and evidence for an adeno-associated virus replication function. J Virol 1984;51:611–619.
11. Loring HS, ElMallah MK, Flotte TR. Development of RAAV2-CFTR: history of the first RAAV vector product to be used in humans. Hum Gene Ther 2016;27:49–58.
12. Afione SA, Conrad CK, Adams R, et al. An *in vivo* model of AAV vector rescue. J Virol 1996;70:3235–3241.
13. Schnepp BC, Clark KR, Klemanski DL, et al. Genetic fate of recombinant adeno-associated virus vector genomes in muscle. J Virol 2003;77:3495–3504.
14. Bainbridge JW, Smith AJ, Barker S, et al. Effect of gene therapy on visual function in adults with Leber's congenital amaurosis. New Engl J Med 2008;358:2231–2239.
15. Bunting SB, Zhang L, Xie L, et al. Therapeutic levels of human AAV FVIII in mice and primates and normalization of bleeding in hemophilic mice following gene therapy with BMN 270. Mol Ther 2018;26:496–509.
16. Ranjarajan S, Walsh L, Lester W, et al. AAV5-factor VIII gene transfer in severe hemophilia A. N Engl J Med 2017;377:2519–2530.
17. Pasi KJ, Rangarajan S, Mitchell N, et al. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. N Engl J Med 2020;382:29–40.