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Recombinant Adeno-Associated Virus Gene Therapy in Light of Luxturna (and Zolgensma and Glybera): Where Are We, and How Did We Get Here?

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Abstract

The recent market approvals of recombinant adeno-associated virus (rAAV) gene therapies in Europe and the United States are landmark achievements in the history of modern science. These approvals are also anticipated to herald the emergence of a new class of therapies for monogenic disorders, which had hitherto been considered untreatable. These events can be viewed as stemming from the convergence of several important historical trends: the study of basic virology, the development of genomic technologies, the imperative for translational impact of National Institutes of Health–funded research, and the development of economic models for commercialization of rare disease therapies. In this review, these historical trends are described and the key developments that have enabled clinical rAAV gene therapies are discussed, along with an overview of the current state of the field and future directions.

Keywords

adeno-associated virus; AAV; gene therapy; genetic disease; genome editing; clinical trial

1. KEY MILESTONES IN THE HISTORY OF rAAV GENE THERAPY

1.1. The Discovery of AAV and AAV Latency

Adeno-associated virus (AAV) was discovered by Atchison et al. (1) by electron microscopy–based screening of adenovirus (Ad) preparations in the mid-1960s. Shortly thereafter, AAV was found in human isolates as a coinfecting agent during outbreaks of Ad-induced diarrhea, conjunctivitis, and other illnesses (220135).

The Berns laboratory (6) initially worked out the hairpin self-priming mechanism now established as the mechanism of replication of both wild-type and recombinant AAV (rAAV) (Figure 1). Subsequently, the Berns laboratory (7) discovered that AAV infection in the absence of Ad would establish a persistent or latent infection. With regard to latency, great

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interest developed around the fact that wild-type AAV2 demonstrated integration, with a strong preference for integration into a specific site on human chromosome 19 (the AAVS1 site) (8–10).

1.2. The Development of Recombinant Gene Transfer Vectors Based on AAV and Early Preclinical Studies

Initially, there was some technical difficulty with cloning the AAV2 genome into bacterial plasmids, most likely due to the secondary structure of the AAV inverted terminal repeats (ITRs), which are palindromes capable of hairpin formation. This technical hurdle was overcome by the Muzyczka laboratory at the University of Florida and the Carter laboratory at the National Institutes of Health. Shortly thereafter, Arun Srivastava and Ken Berns (11) published the sequence of the AAV2 genome. Then the Muzyczka and Carter labs (12–14) both succeeded in adapting recombinant AAV2 as a eukaryotic gene transfer vector.

A major breakthrough in the use of rAAV as a gene transfer vector came when Samulski, Chang, and Shenk (15, 16) developed a two-plasmid system in which AAV2-ITR-flanked payload was supplied on one plasmid and the AAV2 *rep* and *cap* on a second, ITR-deleted plasmid (Figure 2). Cotransfection of these two plasmids into Ad-infected HeLa cells enabled packaging of rAAV preparations that were relatively free of wild-type AAV (16). The system has been shown to be highly recombinogenic, and further modifications were required to eliminate all replication-competent AAV. However, the Samulski plasmids, pSub201(+) and pAAV/Ad, allowed for widespread use of rAAV for gene transfer experiments.

The first concerted effort to develop rAAV-based therapy for a single-gene disorder was the effort by the Carter/Flotte laboratories and subsequently the Targeted Genetics Corporation to develop rAAV2-CFTR (cystic fibrosis transmembrane conductance regulator) vectors for in vivo use in the respiratory tract to treat patients with cystic fibrosis (CF). Initial efforts centered on the problem of packaging the CFTR coding sequence, which measures 4.4 kb, into rAAV with a suitable promoter and polyadenylation signal. These efforts resulted in the discovery of cryptic promoter elements within and near the AAV2-ITR (17). Packaging of rAAV2-CFTR enabled complementation of the CFTR chloride channel defect in CF bronchial epithelial cells in culture (18).

rAAV2-CFTR delivery to the airway in vivo was modeled using a fiberoptic bronchoscope to deliver rAAV2-CFTR to the airway epithelium of rabbits (19). These studies showed that the CFTR messenger RNA and protein could be expressed in vivo from a dose of up to 1×10^{10} physical particles of rAAV2-CFTR delivered to a single bronchopulmonary segment through the bronchoscope. This expression persisted at least 6 months, providing the pivotal proof of principle to justify moving forward to a clinical trial of rAAV2-CFTR to the nose and single bronchopulmonary segment of the lung in adult CF patients (20, 21).

A number of other early-stage in vivo rAAV2 gene therapy vector studies were published in the 1990s. Specific examples include studies using the *lacZ* reporter gene in skeletal muscle (22) and the central nervous system (CNS) (23, 24). Additional studies showed that rAAV2 delivery to muscle could mediate robust and sometimes stable expression of a secreted

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protein. This was initially tested with erythropoietin (25–27). More therapeutic examples of rAAV2 muscle injection for ectopic stable expression of secreted serum proteins included rAAV2-Factor IX (rAAV2-FIX) (28, 29) and rAAV2-alpha-1 antitrypsin (rAAV2-AAT) (30, 31).

The concept of using direct rAAV2 injection to transduce specific target cells and replace deficient gene products was also developed. This approach was used to create potential therapies to a number of disorders of the CNS and muscle. Specific examples included rAAV2-tyrosine hydroxylase vectors designed to increase dopamine formation in the basal ganglia in patients with Parkinson's disease (PD) (32–34). Another example was direct intraparenchymal injections of rAAV2 vector encoding *APSA* (AAV2-ASPA) for Canavan disease (35, 36). The rAAV2-delta sarcoglycan vector was similarly developed for direct muscle injection in patients with limb girdle muscular dystrophy (37).

1.3. Description of AAV Capsid Variants and Tropisms

As more in vivo and potential clinical applications of rAAV emerged, it became clear that it could be advantageous to find AAV capsid variants that had higher efficiency for gene transfer into various target cell and tissue types or that were immunologically distinct. The logic was that capsid variants with a higher affinity for a specific cell type could be adapted to become therapeutic vectors with higher potency when delivered to those cell types.

While there were a few specific instances of using different serotype capsids prior (38), Xiao and colleagues (39) worked out a simple system in which the AAV2-ITRs would be used for all vector cargo and the AAV2 *rep* gene would be used as a complementing gene for all variants. However, these AAV2-ITR-flanked genomes would be cross-packaged into any of a variety of other AAV capsids by complementing the vector with the *cap* gene corresponding to that serotype or variant. Simply using the first five known AAV serotypes, Xiao and colleagues (40) demonstrated important differences in vector potency, specifically the very high potency of rAAV1 when injected into muscle.

1.3.1. Biodiversity of AAV.—The real breakthrough in vector potency came with Gao and Wilson and their colleagues discovering the tremendous biodiversity of AAV in nature (41, 42), with over 2,000 genetically distinct clades and hundreds of genomic variants (genomovars) found in human and nonhuman primate tissues. Subsequent studies have demonstrated that thousands of such variants exist naturally.

Early on in this work, it became clear that certain of the newer rAAV serotypes had dramatically improved efficacy in certain sites. rAAV8 was found to be very efficient for liver and muscle delivery (40). Meanwhile, rAAV9 and other members of its clade, AAVrh10 and AAVrh8, were found to be efficient for CNS and cardiac muscle gene transfer (43–45). The ability of rAAV9 vectors, when delivered systemically, to cross the blood-brain barrier was of particular interest (46).

1.3.2. Targeted capsid modifications.—While the identification of novel, natural AAV capsid variants was successful, the conceptual proof that changing the capsid could lead to great improvements in vector potency led others to conclude that designed

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modifications of the capsid could potentially be even more effective and confer a degree of specificity not generally observed with naturally occurring genomovars. The Muzyczka laboratory (47) performed alanine-scanning mutagenesis of the AAV2 capsid and identified a number of loci at which amino acid substitutions or insertions could be tolerated. Initially, only short polypeptide stretches were inserted, such as the short version of the serpin-enzyme complex receptor (47, 48). Subsequent work from Muzyczka and colleagues (49) has shown, however, that much larger domain insertions are tolerated when genetically engineered into the N terminus of VP2.

1.3.3. Synthetic capsid libraries and directed evolution and ancestral reconstruction.—A third approach to the generation of new capsids in which to deliver rAAV has explored a more empiric approach. Combinatorial AAV capsid libraries were generated with a number of methods, including both randomly generated variations in the variable *cap* regions (by synthesis or error-prone polymerase chain reaction) and so-called capsid shuffling where portions of the *cap* gene from multiple different serotypes are used to reconstitute intact chimeric capsids (50, 51). These combinatorial libraries of capsids were then passaged over cultured cells and/or in vivo, selecting for and thereby enriching for those capsids that enable efficient infection. The error-prone nature of AAV replication may enable a modest degree of additional genetic evolution during serial passage and selection. Serial passage under these selective conditions was used in several different applications, in many cases focusing on a specific cell or tissue type or animal host species (52–56). Almost opposite of directed evolution, reconstruction of ancestral AAVs has led to some interesting candidate capsids with unique properties and tropisms (57, 58).

1.3.4. Overview of tropisms.—With this new palette of AAV capsids, translational investigators have utilized multiple different serotypes for multiple different applications in order to optimize the serotype, dose, and delivery method that could potentially achieve a therapeutic goal. The tropism of each capsid variant is likely largely attributed to expression of viral receptors on target tissue cell types, which can vary greatly from species to species. As an interesting example, AAV3b shows almost no expression in mouse liver but robust expression in human xenograft models and nonhuman primates (59), presumably because AAV3 uses huHGFR as a coreceptor (60). Therefore, it is important to consider, and sometimes it is overlooked, that the tropisms that have been best described are those for mice, while newer data from larger animals and humans show some important distinctions in potency of different AAV capsid variants across species. With that caveat considered, the current pattern of preference of use by investigators, largely based on in vivo efficacy, is shown in Table 1.

1.4. Improvements in rAAV Manufacturing Technology

Two main technologies exist for manufacturing rAAV, transfection and helper virus systems (Table 2).

1.4.1. Transfection technology.—Initially, it was assumed that the maximum possible expression of all the Rep proteins in the rAAV producer cells would increase the efficiency packaging and the yields of vector. This was subsequently disproven. The Samulski

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laboratory (61) was the first to show that highly regulated *rep* gene expression (particularly moderating the expression of Rep78/69) could increase the efficiency of transfection-mediated rAAV manufacturing. This was initially done in the context of transfecting modified *rep*/*cap* plasmid helpers into Ad-infected cells. The approach was further improved by eliminating productive Ad infection from the producer cells and substituting transient *transfection* of three of the adenoviral helper genes (E2a, E4, and VA RNA) into cells that constitutively express adenoviral E1a and E1b genes, such as HEK-293 cells (62). The triple transfection of the ITR-flanked vector plasmid, the Rep/cap plasmid, and the adenoviral gene helper plasmid was thus established as the most commonly used packaging platform. This process has been further streamlined by combining the Ad-gene and *rep/cap* gene plasmids into a single helper plasmid, pDG (63, 64).

1.4.2. Helper virus-based technologies.—Several attempts to enhance the efficiency and scalability of manufacturing of rAAV have aimed to eliminate transient transfection from the upstream process. Ideally, this could be accomplished by creating stable producer cell lines, thus mimicking the most common upstream process for recombinant protein production. However, the toxic and cytostatic effects of the AAV-Rep proteins present a fundamental limitation to that approach. A more feasible alternative that has been successfully pursued is the use of infectious recombinant helper viruses. The three primary approaches of this type have been the use of replication-competent Ad (rcAd) in HeLa cells with inducible AAV-Rep, the use of infectious baculovirus recombinants in the Sf9 insect cell line, and the use of recombinant herpes simplex virus (HSV) constructs supplying helper virus function, Rep/cap complementation, and the ITR-flanked proviral rAAV construct.

1.4.2.1. Replication-competent adenovirus in Rep-HeLa cells.: As described above, the earliest transient transfection approaches to rAAV manufacturing all relied on infecting the transfected producer cells with rcAd, initially with wild-type Ad5 or Ad2. Early studies suggested that cell lines may be able to tolerate an integrated AAV2-*rep* gene, most likely because the AAV2-p5 promoter was active in HeLa cells only when lytic phase rcAd was supplied externally (65). Clark et al. (66) developed such cell lines as a sustainable method for manufacturing of rAAV vectors. In fact, this technology was used for later-phase studies of the first rAAV2-CFTR vectors (the initial phase 1 was performed with transfection-based material). The rcAd method has proven to be scalable and capable of generating high titers of rAAV, as well as being adaptable to multiple different serotypes.

1.4.2.2. Baculovirus-Sf9.: The baculovirus-Sf9 rAAV manufacturing system relies on the basics of the baculovirus expression system, namely the ability to use the baculovirus polyhedron protein promoter to mediate very high-level expression of proteins when induced. Interestingly, it was discovered that insect cells undergoing lytic infection with baculovirus were able to support helper virus functions as well as carry both the Rep/cap construct and the rAAV proviral plasmid (67). As with the rcAd system, the baculovirus system has proven to be very scalable and adaptable into suspension culture, resulting in net production of very high titer rAAV preparations (68).

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1.4.2.3. Herpes simplex virus packaging.: Initial efforts combined HSV-1 amplicons capable of expression of AAV-*rep* and *cap* genes with replication-defective ICP27-deleted HSV-1 (69). Further optimization used a single rHSV1, capable of providing both helper virus functions and AAV-*rep* and *cap* gene functions (70), while separate rHSV-1 vectors can be used to provide the rAAV-proviral gene cassette. The rHSV-1 system has been used successfully to package rAAV vectors for both preclinical and clinical trials. Because all elements of the system are infectious, this system is also scalable into suspension cell bioreactor systems (71).

2. CLINICAL TRIALS AND CLINICAL PRODUCT DEVELOPMENT WITH rAAV

2.1. Early Cystic Fibrosis Transmembrane Conductance Regulator Work

As mentioned above, the rAAV2-CFTR program was the first clinical development program with rAAV vector. Following the rabbit proof-of-principle studies mentioned above, nonhuman primate studies for evaluating safety were performed (72, 73). Interestingly, the latter studies showed a clear demonstration that rAAV persistence in vivo was mediated by episomal persistence rather than integration, confirming a phenomenon that had been observed by the Flotte lab (74) two years prior. This episomal nature of rAAV persistence turned out to have major implications for the utility of rAAV, specifically that long-term persistence of rAAV was observed only in nondividing cells.

A number of human trials were then performed with the rAAV2-CFTR vector (20, 21, 75–80). In the case of the administration of rAAV2-CFTR both to the nasal and bronchial epithelium (21, 79) and in the maxillary sinus (75), rAAV2-CFTR delivery was associated with dose-related DNA transfer and expression of CFTR at low levels. Expression did not persist, as might have been predicted given the relatively rapid turnover of the airway epithelium in CF patients. However, these CF studies provided a strong set of evidence of the general safety of rAAV administration in vivo, setting the stage for later studies.

2.2. Hemophilia Trials

In a series of preclinical and clinical trials, a number of laboratories developed rAAV2-FIX vectors, initially delivered by intramuscular (IM) injection, for the continuous secretion of FIX in patients with hemophilia B (81–83). Subsequently, rAAV2-FIX, when delivered by intrahepatic artery, was shown to be capable of transient expression of FIX (84–87), which was the first trial that showed evidence of an effector T cell response to vector capsid being responsible for elimination of transduced cells. Nathwani et al. (88, 89) subsequently showed that rAAV8-FIX gene therapy was capable of sustained expression of a FIX from a self-complementary AAV construct. These studies also enabled the further elucidation of a common immune response profile with systemic rAAV vectors, the development of an effector T cell response against AAV capsid epitopes, occurring approximately 6 to 10 weeks after vector administration, apparently leading to a rise in serum transaminases and a suppression of transgene expression. These studies also demonstrated that this immune response could be controlled with oral anti-inflammatory corticosteroids. Similar studies have now been published by several other groups. More recently, a number of hemophilia B

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trials with rAAV8-FIX vectors have been developed by industry sponsors, including one with a gain-of-function variant, known as the Padua variant (R338L) (90).

Hemophilia A [Factor VIII deficiency (FVIII deficiency)] presented a more difficult challenge in the context of rAAV gene therapy, since the FVIII cDNA is substantially larger, exceeding the packaging capacity of most rAAV vector designs. The BioMarin group and Nathwani and colleagues have overcome this limitation with a B-domain deleted FVIII construct packaged in rAAV5 capsids (91).

2.3. Genetic Retinal Diseases

Another breakthrough in demonstrating the clinical efficacy of rAAV gene therapy came with a series of parallel programs aimed at using rAAV2-RPE65 vectors by subretinal injection as a means to treat inherited retinal dystrophy due to Leber congenital amaurosis RPE-65 deficiency (IRD-RPE65). In 2008, three similar trials of the first use of rAAV2-RPE65 were published (92–95). In light of these studies, the Spark Therapeutics program moved forward with carefully designing a multiluminance mobility test to measure the effect of the RPE-65 isomerase gene on retinoid recycling, specifically the restoration of dim light vision due to improvement in rod photoreceptor function (96). This multiluminance mobility test represents an important example of how gene therapies can diverge in design and endpoints based on their molecular mechanism of action. Because they often do not function in a manner equivalent to a small molecule drug, it is only logical that the most informative outcomes may also differ. These studies formed the basis for the market approval for the first Food and Drug Administration (FDA)-approved rAAV gene therapy product, voretigene neparvovec (Luxturna), in December 2017 and subsequent European Commission approval in November 2018, which has greatly impacted the field of AAV-based gene therapies moving forward. A number of other eye diseases are under investigation including retinitis pigmentosa, Leber hereditary optic neuropathy, choroideremia, and age-related macular degeneration.

2.4. Muscular Dystrophies

Several late-stage preclinical and clinical studies of rAAV delivery for treatment of muscular dystrophies have been performed (NCT01519349, NNCT03375164, NCT03368742, NCT02354781, NCT00428935, NCT03362502) by direct IM injection, isolated limb perfusion, or systemic IV injection. Diseases that have been addressed in this manner include limb girdle muscular dystrophy and Duchenne muscular dystrophy. In general, the systemic delivery approach that has attracted the most attention has been the use of rAAV9 or rAAV9-like vectors, which are efficient for generalized gene transfer to skeletal and cardiac muscle after IV administration. As with hemophilia gene therapy, there has been some limitation on these studies because of immune responses. Surprisingly, the very high doses of rAAV9 required for these studies have also resulted in an early-phase toxicity occurring within the first week after administration, which had not previously been reported (97, 98).

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2.5. Lipoprotein Lipase Deficiency and Alpha-1 Antitrypsin Deficiency

rAAV vectors have also been directed to muscle for the ectopic expression of proteins in single-gene disorders that are not associated with primary muscle disease. This has particularly been developed for AAT deficiency and lipoprotein lipase (LPL) deficiency. In the case of LPL deficiency, the deficiency state results in a form of hyperlipidemia that causes recurrent pancreatitis and premature atherosclerotic cardiovascular disease, including coronary artery disease. Clinical studies of rAAV1-LPL delivery to muscle resulted in actual clinical efficacy in patients with LPL deficiency (99–102). These results led to the European Medicines Agency (EMA) approval of Glybera for patients with LPL deficiency in 2012. The EMA approval of this therapy was, in fact, a milestone in rAAV therapeutic development, even though Glybera was subsequently withdrawn from the market in 2017 due to economic considerations.

In the case of AAT deficiency, the deficiency of the serum antiprotease AAT can result in emphysema due to degradation of interstitial elastin within the lungs. The use of muscle-based expression of AAT has been studied extensively in mice, rabbits, baboons, and rhesus macaques and more recently in clinical trials of both rAAV2-hAAT (NCT00377416) and rAAV1-hAAT (NCT00430768) delivery to the muscle of AAT-deficient patients. The use of rAAV2-hAAT for ectopic secretion of AAT was demonstrated in mice and baboons prior to the first human use (30, 31, 103, 104). Subsequently, the use of rAAV1 in muscle was shown to mediate a higher potency of gene transfer, and this platform was used to express wild-type (M) AAT from muscle in two human trials (105, 106). The more recent of these has been followed for five years with partial correction of biomarkers at a 2.5–3% level of serum protein reconstitution (107). In these studies, it was shown that rAAV1 delivery to muscle resulted in the generation of regulatory T cell responses to AAV1 capsid epitopes (108).

2.6. Spinal Cord Disease

A major therapeutic milestone was recorded by Mendell et al. (109), as a single IV injection of rAAV9-smn1 resulted in a dramatic restoration of lower motor neuron function in infants with spinal muscular atrophy 1 (NCT02122952), a disorder that previously was uniformly fatal without mechanical ventilation. In May 2019, Zolegensma, a one-time treatment for spinal muscular atrophy in pediatric patients less than 2 years of age, became the second FDA approved AAV gene therapy. The dramatic benefit observed in this study has served as an important proof of concept for other gene therapies of lower motor neurons. Phase 1 studies of rAAV-rh10-mediated delivery of therapeutic anti-superoxide dismutase 1 microRNAs have demonstrated efficacy and safety in nonhuman primate studies for treatment of amyotrophic lateral sclerosis (110) and in early expanded access human studies (Brown, personal communication).

2.7. Monogenic Brain Disorders Including Lysosomal Storage Disorders

The use of rAAV in single-gene disorders affecting the brain has a more ambitious therapeutic target than for disorders affecting the spinal cord. Most clinical trials originally focused on using AAV2 for treatment of monogenic brain disorders such as Canavan disease (36) and late infantile neuronal ceriod lipofuscinosis (LINCF) (NCT00151216) (111). New capsid variants, such as rAAVrh10 vectors, have been tested in patients with Batten disease

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(NCT0116576), metachromatic leukodystrophy (NCT01801709), LINCF (NCT01414985), and mucopolysaccharidosis IIIA (NCT01474343) (112). rAAV9 vectors have been used in both late-state preclinical studies and patients with Batten disease (NCT02725580), AADC deficiency (113, 114), and Canavan disease (115, 116). Meanwhile, late-stage preclinical studies with rAAVrh8 have been published in both GM1 gangliosidosis (117) and GM2 gangliosidosis, Tay-Sachs disease, and Sandhoff disease (118–122). Additionally, studies in patients with giant axonal neuropathy have added to the overall clinical experience with single-gene disorders affecting the brain (NCT02362438).

2.8. Other Brain Disorders

The use of AADC as a therapeutic transgene has been undertaken for patients with acquired PD to restore l-dopa responsiveness. Muramatsu et al. (123) used rAAV2-hAADC in a phase I trial in which vector was injected directly into the putamen, resulting in measurable improvements in motor score and by positron emission tomography–computed tomography. Similar results were obtained by Christine et al. (124), with rAAV2-hAADC vector administered by intraputaminal injection in PD patients. As recently reviewed by Cartier and colleagues (125), gene therapy for Alzheimer disease is under development in a number of laboratories. Gene therapy for CNS diseases has been extensively reviewed elsewhere (126).

2.9. Inborn Errors of Metabolism, Glycogen Storage Disorders, and Other Liver Disease Trials

A number of other genetic disorders affecting the liver, including inborn errors of metabolism such as phenylketonuria and ornithine transcarbamylase deficiency (NCT02991144), have been undertaken. Several clinical trials for Pompe disease (NCT00976352, NCT02240407, NCT03533673) have been conducted, including a study looking at readministration of AAV; in the study, one leg is injected with immune modulation to ablate B cells followed by redosing of a contralateral leg four months later. Clinical trials have been initiated for other glycogen storage diseases such as addition glycogen storage disease type 1a (NCT03517085).

3. LIMITATIONS AND RISKS OF rAAV

3.1. Packaging Capacity

The wild-type AAV2 genome, which is the backbone for most rAAV vectors, has a genome length of approximately 4,675 nucleotides, although it seems likely that there is natural variation of the wild-type genome length. One of the first systematic studies of the limitation on packaging capacity was published by Dong et al. (127), who showed a sharp decline in packaging efficiency between 4.8 and 5.0 kb. Subsequent studies indicated that certain oversized constructs might be packageable, especially in the context of certain serotypes or with elimination of VP2 expression (128, 129). Recently, it appears that the interpretation of many of these results was complicated by packaging of overlapping partial rAAV genomes, which were capable in some cases of recombining to regenerate full-length genomes in the target cell.

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Given this, several strategies have been undertaken to bypass the packaging capacity limitation. The most consistently productive strategy has been to optimize the smallest possible designs for large transgenes. In the case of CFTR this has been done by using highly compact promoter, enhancer, and polyadenylation elements (130). In the case of larger coding sequences, such as dystrophin, the optimization of minigenes capable of restoring partial function has been an effective strategy (131, 132). Alternative approaches have included the use of overlapping constructs that may recombine by homologous recombination within the target cell or *trans*-splicing constructs. The typical *trans*-splicing approach includes a split intron, with the 5′-end of the transgene and the splice donor in one rAAV cassette and the 3′-end of the gene and the splice acceptor in a second cassette. The process of head-to-tail vector genome concatemerization allows such constructs to yield a functional expression unit within the target cell (133, 134).

3.2. Immune Response

Although AAV is generally considered a nonpathogenic virus, immune responses are still observed to both wild-type and recombinant viruses. Both capsid-specific and transgene-specific immune responses can hinder AAV gene therapy.

3.2.1. Humoral immune responses.—Preexisting immunity due to natural wild-type AAV infection continues to be a limitation for clinical rAAV applications. Neutralizing antibodies (NAbs) against different AAV capsid variants are readily found in the human population and are capable of blocking rAAV administration (84, 85). Several strategies are being tested to overcome NAb responses, but currently most clinical trials have seropositivity as an exclusion criterion.

It has been uniformly observed that the administration of rAAV vectors to any site outside the retina results in the generation of serum NAbs against the AAV capsid that was administered (135). Interestingly, cross NAbs against other AAV capsid variants have also been observed, at least in the case of rAAV1-IM administration (106). The prevalence of NAbs against different AAV capsid variants within healthy populations is a concern for gene therapies (136). Immune modulation of the blocking of the humoral immune responses may allow for readministration (137), and a number of clinical trials are now using immunosuppressive drugs that ablate B cells (138).

3.2.2. T cell–mediated responses.—The characterization of the immune responses observed in the early AAV2 hemophilia B trials has formed the basis for understanding of the primary T cell responses creating limitations to gene transfer in human applications. Similar findings have been demonstrated in subsequent trials with rAAV8-FIX (139), where investigators demonstrated that cytotoxic T cell responses were elicited against specific AAV capsid epitopes and lead to clearance of transduced cells (87). Many groups have begun using oral anti-inflammatory corticosteroids to control such responses, particularly with IV delivery. However, in two trials utilizing AAV1 by IM injection, a T-regulatory response to AAV capsid was associated with long-lived transgene expression (101, 108). T cell–mediated responses to AAV capsid are not easily modeled in animal models, making them difficult to study.

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3.2.3. Innate immunity.—Initially, rAAV vectors were not commonly thought to activate early-phase innate immune response. However, some studies suggest that AAVs are sensed by Toll-like receptor 2 (TLR2) and TLR9 innate immune receptors in mice and humans (140–143). In addition, further questions about the innate immune response against AAV have been called into question with very high doses (2×10^{14} vg/kg) of rAAV9-like vectors (rAAVhu68 and rAAV-PHP.B) when given intravenously. In studies from the Wilson laboratory (97, 98), neonatal piglets and rhesus macaques were noted to have an early onset of liver toxicity with thrombocytopenia and toxicity of the dorsal root ganglia. Currently, it is unclear whether this represents a dose-limiting toxicity for rAAV generally or whether it is specific to certain variants.

3.2.4. Immune responses to transgene.—Transgenes can also elicit both B and T cell immune responses against transgenes, which is more frequently observed in patients who have null mutations. However, a T cell response against single amino acid common polymorphism was observed in a clinical trial for AAT deficiency (144). Additionally, delivery of therapeutic antibodies has been greatly limited by anti-antibody responses.

3.3. Risk of Tumorigenesis

There has been a long-standing controversy over whether wild-type AAV2 is occasionally associated with generation of tumors or whether in contrast it may actually be protective against tumorigenesis (145–147). While there is evidence on both sides of this question, it appears that the risk of carcinogenesis with this system is quite low, if it exists.

4. CURRENT TRENDS AND FUTURE DIRECTIONS OF rAAV GENE THERAPY

4.1. Further Technological Improvements in Capsids and Manufacturing

The widespread use of rAAV for gene therapy continues to be limited by the scalability of manufacturing methods. It is somewhat remarkable that there has not been a major change in the platforms in the past decade (148). It seems likely that the clinical development of rAAV will drive further investment in such innovations.

4.2. Use of rAAV to Deliver RNA Interference and Gene Editing Machinery

The ability of rAAV to deliver the molecular components required to silence genes by RNA inhibition and to engineer cells via gene editing is in its early stages of development (149). The Cas9 recombinase component of CRISPR/Cas9 systems will ideally be delivered transiently, which could be accomplished with rAAV in rapidly dividing cells, such as hematopoietic stem cells and epithelial surface cells. Meanwhile, ss-AAV genomes seem to be a particularly favorable template for homology directed repair–mediated editing. New Cas9s have considerable promise for in vivo genome editing using rAAVs with increased efficiencies, reduced size, and dinucleotide protospacer-adjacent motif sequences (150).

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4.3. Future Clinical Applications

The future of rAAV gene therapy appears to be overwhelmingly positive, as clinical programs are advancing for a wide range of genetic diseases, including those affecting the retina, CNS, muscle, and liver. The most studied applications include hemophilia, Duchenne muscular dystrophy, and other retinal diseases, while the most remarkable improvements have been seen in patients with spinal muscular atrophy. These studies show promise of getting FDA approval, following in the footsteps of Luxturna and Zolgensma.

4.4. Economic Models of Rare Disease Treatment and Pricing of Approved Drugs

A considerable amount of resurgence in the field has come from biotechnology and pharmaceutical investment in gene therapy product development due to successes from Glybera and Luxturna but also economic models suggesting profitability from treatment of rare diseases. However, it is important to point out that of the two currently approved AAV-based therapies, Glybera has been removed from the market due to economic concerns. Glybera treatment costs were announced to be €1.11 million for an average adult (100, 151). However, the seemingly high price tag is not completely unreasonable when compared to yearly costs of enzyme replacement therapy, small molecule drugs, or monoclonal antibodies over a period of time, when AAV-based therapeutics would ideally be one-time treatments (152). Luxturna has also received criticism for a high price tag of $850,000 ($425,000 per eye), but it is difficult to put a price on regaining vision. Zolgensma treatment costs were announced at $2.1 million, or an annualized cost of $425,000 per year for 5 years, making it the world's most expensive drug on the current market. However, when compared to Spinraza, the only other approved therapy for spinal muscular atrophy, which requires regular spinal infusions versus a one-time treatment, the 5-year costs for Zolgensma are lower considering Spinraza costs $750,000 the first year and $375,000 annually thereafter for life.

5. CONCLUSION

Our view is that rAAV will achieve its rightful place in the armamentarium of molecular therapies as the most safe and efficient means to correct single-gene defects in nondividing cells, such as neurons, retinal photoreceptors, and uninjured muscle and liver cells. It seems likely that the characteristics of AAV as a virus that exists in long-term symbiosis with its human host will suit it well as the vehicle for long-term gene transfer in humans. The limitations of this system related to immune responses seem likely to be overcome with specific immunosuppression, while those intrinsic to the packaging capacity of the virus will likely continue to exist. Overall, rAAV represents one of the most straightforward vehicles for direct translation of genomic revolution in medicine. In that context, rAAV-mediated gene therapy validates public investments in molecular genetics over the past half-century. The most valued outcome of such therapy will be to offer children and families with genetic diseases the opportunity to live longer and healthier lives.

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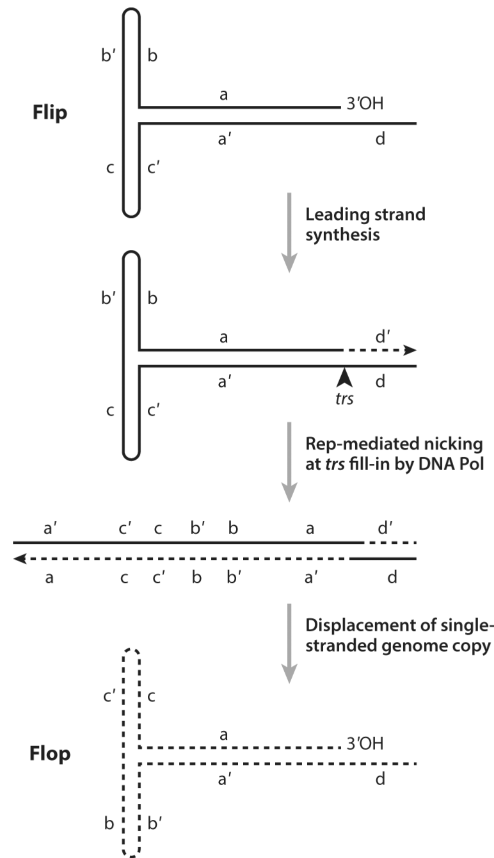

Figure 1.

Adeno-associated virus (AAV) replication scheme. The steps in AAV replication and terminal resolution result in flip and flop configurations of inverted terminal repeats (ITR). The 3′ end of the hairpin configuration of the ITR acts as template for leading strand synthesis. Rep-mediated nicking at the *trs* allows for terminal resolution. Other abbreviations: trs, terminal resolution site, DNA Pol-DNA Polymerase. Figure adapted from Reference 148.

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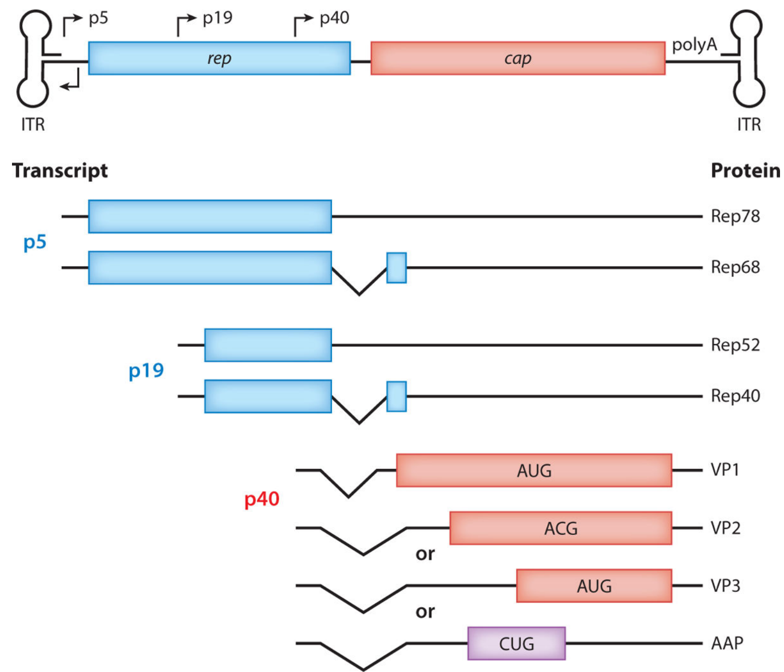

Figure 2.

The wild-type AAV genome contains three parts: noncoding ITRs and two genes, *rep* and *cap*. From both alternative splicing and non-AUG start sites, eight different proteins are produced. Four Rep proteins are produced, two from the p5 promoter (Rep78 and Rep68) and two from the p19 promoter (Rep52 and Rep40). Three capsid and AAPs are produced from the p40 promoter. VP2, VP3, and AAP are produced from the same transcript but from different initiation start sites. Abbreviations: AAP, assembly-activating protein; AAV, adeno-associated virus; ITR, inverted terminal repeat.

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Table 1

Examples of adeno-associated virus (AAV) capsid specificities currently utilized for preclinical and clinical applications

Organ	Mouse	Other large animal	Nonhuman primate	Human
Central nervous system	AAV9, AAVrh10, AAVrh8, AAV2, AAV5, ANC80L65, AAV-PHP.B	AAV9, AAVrh10, AAVrh8, AAV5	AAV9, AAVrh10, AAVrh8, AAV5	AAV2, AAV9, AAVrh10, AAVrh8
Liver	AAV8, AAV9	AAV8, AAV5	AAV8, AAV9, AAV3B	AAV2, AAV5, AAV8, AAV9, AAV3B
Lung	AAV5, AAV6, AAV8, AAV1, AAV6.2, AAV6.2FF, AAVrh10, AAV2-ESGHGYF	AAV5, AAV2/HBoV1	AAV1, AAV5, AAVrh10	AAV2
Muscle	AAV1, AAV8, AAVrh74, AAV9, AAV-B1	AAV1, AAV8, AAVrh74, AAV9	AAV1, AAV8, AAVrh74, AAV9	AAV1, AAVrh74, AAV8, AAV9, AAV2.5
Retina	AAV2, AAV5, AAV7, AAV8, AAV9, ANC80L65	AAV2, AAV5	AAV2, AAV5, AAV9, ANC80L65	AAV2, AAV4, AAV5

Different AAV capsid variants have differing tropisms both for tissue targets and across different species. Many variants are initially tested preclinically in mouse models. However, the most promising capsids are tested in larger animal models and nonhuman primates often prior to initiating clinical trials in human patients.

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Table 2

AAV packaging methods

Packaging method	Host cell(s)	Source(s) of helper genes	Source of Rep and cap	Source of ITR construct	Reference(s)
Triple transfection	HEK-293	Helper plasmid and host cell	Second helper plasmid	Proviral plasmid	27
Double transfection	HEK-293	Dual helper plasmid and host cell	Dual helper plasmid	Proviral plasmid	28, 29
Baculovirus	Sf-9	rBEV-Rep/cap	rBEV-Rep/cap	rBEV-rAAV	18, 30
HSV1 helper	Vero-27, BHK, HEK-293	rHSV1	rHSV1-Rep/cap	rHSV1-rAAV	31–34
Rescuable HeLa lines	HeLa	rcAd5 or rcAd2	Integrated in HeLa	Integrated in HeLa	35

Abbreviations: AAV, adeno-associated virus; HSV1, herpes simplex virus 1; ITR, inverted terminal repeat; rcAd, replication-competent Ad. Table adapted from Reference 153.