Title: Biosafety of recombinant adeno-associated virus vectors.
Authors: Dismuke DJ, Tenenbaum L, Samulski RJ
Journal: Current gene therapy
Year: 2013 Dec
Volume: 13
Issue: 6
Pages: 434-52

Biosafety of Recombinant Adeno-associated Virus Vectors

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Summary

It is hoped that the use of gene transfer technology to treat both monogenetic and acquired diseases may soon become a common therapy option in medicine. For gene therapy to achieve this objective, any gene delivery method will have to meet several criteria, including ease of manufacturing, efficient gene transfer to target tissue, long-term gene expression to alleviate the disease, and most importantly safety in patients. Viral vectors are an attractive choice for use in gene therapy protocols due to their relative efficiency in gene delivery. Since there is inherent risk in using viruses, investigators in the gene therapy community have devoted extensive efforts toward reengineering viral vectors for enhance safety. Here we review the approaches and technologies that are being evaluated for the use of recombinant vectors based upon adeno-associated virus (AAV) in the treatment of variety of human diseases. AAV is currently the only known human DNA virus that is non-pathogenic and AAV-based vectors are classified as Risk Group 1 agents for all laboratory and animal studies carried out in the US. Although its apparent safety in natural infection and animals appears well documented, we examine the accumulated knowledge on the biology and vectorology of AAV, lessons learned from gene therapy clinical trials, and how this information is impacting current vector design and manufacturing with an overall emphasis on biosafety.

Keywords:

Adeno-Associated Virus

Biosafety

Capsid modification

Clinical trials

Gene delivery

Immune response

Insertional mutagenesis

Targeting

Introduction

Gene transfer technology has gone through multiple stages in the last 45 years. The initial concepts were conceived in the late 1960s with the first clinical trial occurring in the early 1970s (1). While the idea of "gene surgery" was optimistic in the beginning, public concern heightened over the next three decades due to clinical trials that were hampered by inefficient technologies and safety concerns associated with viral vectors (2-4). Recent advances in vector technologies have enabled investigators to increase the safety and efficacy of gene delivery and these improvements have led to renewed interest in gene therapy, successful clinical trials, and the first approved gene delivery product in Europe (5). One of the most commonly used vectors is based on adeno-associated virus (AAV). Recombinant AAV (rAAV) vectors have several advantages, including long-term gene expression, relatively low immunogenicity, minimal toxicity, and no association of AAV with any disease (6). In addition, rAAV vectors have a demonstrated safety record in research laboratories, preclinical animal studies, and many early-stage clinical trials. While this biosafety profile is encouraging, there are still risks that investigators and patients should consider.

AAV Biology

AAV is a member of the parvovirus family and has been classified as a dependovirus due to the requirement for co-infection with helper viruses such as Adenovirus (Ad) or Herpes Simplex virus (HSV) for productive infection (7). AAV is one of the smallest known viruses and

contains a linear, single-stranded DNA genome of about 4.7 kilobases (Figure 1). The wild-type (wt) AAV genome is made up of three genes flanked by two 145-bp inverted terminal repeats (ITRs) that form T-shaped hairpin structures. The ITRs function in viral genome packaging, serve as the origin of replication, and as integration elements (8,9). The first gene, *rep*, encodes four replication proteins. The larger replication proteins, Rep 78 and 68, are splice variants originating from the p5 promoter. Rep 68/78 play a role in many aspects of the lifecycle of AAV; the large Rep proteins are necessary for transcription, nicking and unwinding of the ITRs, viral DNA replication, and site-specific integration into human chromosome 19 (10). The small replication proteins, Rep 40 and 52, have been shown to be important for packaging of the viral DNA into viral capsid within the nucleolus of producer cells. The second gene, *cap*, encodes three capsid proteins and viral assembly factor. The capsid proteins are produced from the same open reading frame (ORF) but utilize different translational start sites. The assembly-activating protein (AAP) is encoded from a nested, alternative ORF and targets the capsid to the nucleolus for virion assembly.

AAV particles are non-enveloped, icosahedral structures of approximately 25 nm in diameter and have a triangulation number of 1. The virion is made up of a total of 60 of the three capsid proteins Vp1, Vp2 and Vp3 at a 1:1:10 ratio, respectively. All three capsids share the common VP3 region, while VP1 and VP2 have additional amino-terminal structures. The exposed surface residues in the VP3 region are used for binding to cellular matrices and recognition of cellular receptors defining the tropism of AAV. The VP1 capsid subunit is required for infectivity and contains a phospholipase domain which is thought to function in endosomal escape (11,12). It has been shown that AAV particles that lack VP2 are fully functional, suggesting that this protein is dispensable for infectivity (13). The capsid structures for a number of the different AAV serotypes have been solved, including AAV1, AAV2, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8, and AAV9 (14-25). These studies have revealed that the

different serotypes share a very common structure. These include depressions at the 2-fold axis and around both the 3-fold axis and the 5-fold axis, variable loops at the 3-fold axes protrusions, and a pore at the 5-fold axis of symmetry. The loops and base at the 3-fold axis are thought to be critical for binding to target cells through glycan residues in the extracellular matrix (26,27), while the 5-fold channel is involved in viral genome packaging and possibly genome release (28). The 2-fold axis of symmetry has been implicated in uncoating (29,30), but the role of the other capsid regions in viral disassembly is not completely understood.

The AAV virus infectious pathway can take two forms, latent and productive, depending on whether the cells are co-infected with a helper virus such as Ad. In both pathways the transduction of target cells begins by AAV first binding to cell surface glycans on target cells such as heparan sulfate for AAV2 and AAV6, sialic acid for AAV1, AAV4, and AAV5, or galactose for AAV9 (Figure 2) (31,32,32-35). After attachment the virus utilizes coreceptors for internalization. Integrins (such as αVβ5 and αVβ1), fibroblast growth factor (36), platelet-derived growth factor receptor (37), and the laminin receptor (38) have been shown to function in AAV receptor mediated endocytosis in a clathrin-dynamin-mediated manner. Following cellular uptake the virus is thought to undergo conformational changes during acidification of the endosome, resulting in exposure of a phospholipase region in the VP1 subunit and subsequent endosomal escape (12). Basic regions in VP1 and VP2 have been shown to act as nuclear localization signals (39-42). The microtubule network has been shown to be important for the virus to intracellular transport to the perinuclear region (43). AAV is thought to translocate into the nucleus through active import (44). Once in the nucleus, the viral genome is released from the capsid and must undergo synthesis of the complementary strand that is initiated from the 3' ITR. AAV can undergo productive infection in the presence of a helper virus and the viral genome can integrate in the host chromosome in a Rep-dependent fashion (45). Following transcription and translation, nascent viral particles are formed in the nucleus of producer cells.

A newly identified AAV viral protein, AAP, has been shown to promote the shuttling of capsid proteins to the nucleolus for particle assembly (46,47). The single-stranded AAV genomes are packaged into capsids presumably by spooling the DNA through the 5-fold pore; the 3' end first through the action of Rep78/52 (28,48). Fully assembled particles are then released from the cells during helper virus mediated cellular lysis or potentially through exocytosis (49).

AAV has traditionally been thought of as a safer platform for a viral vector due to the natural requirement of a helper virus for productive infection. Ad, HSV, Vaccinia virus, and Human Papillomavirus have been shown to be able to rescue AAV infection (50-53). It has been shown that genotoxic agents that induce cell stress, such as hydroxyurea or UV light can also promote AAV replication in some cell lines (54-56). In addition, AAV has also been shown to productively infect cultures of differentiating keratinocytes (57). Therefore, it is possible that AAV can autonomously replicate under some conditions. Interestingly, AAV has been shown to inhibit the replication of a number of viruses (52,58-61), suggesting that in some cases AAV infection may be protective against many of the same viruses it utilizes for helper functions.

While the functions of the necessary components for AAV replication have been investigated for a variety of helper viruses, the function of a subset of the Ad genes have been the most extensively studied. Four Ad proteins have been shown to be necessary to provide the Ad helper functions; these are the early region E1A, E1B, E4orf6, and E2A. In addition, the transcription of the Ad virus-associated (VA) RNAs is essential. The E1A protein is an oncogene that has pleiotropic effects on cellular transcription and growth. It also transcriptionally activates the Ad genes and AAV p5 promoter (62). The E4orf6 and E1B proteins interact as a heterodimer and promote viral gene expression by aiding in the transport of AAV mRNA to the cytoplasm as well as arresting the cell cycle in G2 or S phase (63-65). E4orf6 has also been shown to enhance the conversion of the ssDNA viral genomes into the

dsDNA form (66). E2A is a ssDNA binding protein that aids in activating transcription from AAV promoters and may be involved in AAV DNA replication (67,68). The small, non-coding Ad VA RNAs are highly synthesized and enhance the translation of AAV capsid mRNAs by increasing the steady-state levels of the transcripts, improving the translation of the proteins, and inhibiting the serine-threonine kinase protein kinase R -induced inhibition of capsid subunits (69,70).

Importantly, AAV has not been reported to cause any disease. AAV was originally revealed in electron microscopic studies of preparations of human and simian adenovirus (71). This early work showed that the particles were in fact not adenovirus subunits, but a contaminating virus that required a helper virus for replication. Due to the association of AAV with adenovirus, early assumptions pointed to a possible participation of AAV in the disease response to acute adenovirus infection. However, epidemiology studies in the late 1960s established that both children and adults infected with AAV showed no signs of disease and that AAV did not appear to alter the clinical expression of acute adenovirus infection (72-76). Furthermore, serological surveillance collected from a variety of sources has shown that up to 80% of the human population has been infected with the AAV2 serotype and there has been no correlation with any disease (77-81).

The integration profile of AAV is also advantageous. AAV can establish a latent infection by integrating into a 4-kb unique site on human chromosome 19 that is mediated in part by the ITRs and Rep68/Rep78 (82-85). This locus, known as the AAVS1 site, exists in an open chromatin conformation and contains an insulator element (86). The AAVS1 site is located in the MBS85 gene, so genetic mis-regulation has been a concern (87). Further work by the Linden laboratory has determined that there were no discernable gene expression differences due to this rearrangement, despite the partial duplication of the MBS85 gene (88). Since integration into the AAVS1 site has not been shown to cause tumor formation or to be

cytopathic in cells, it has been deemed a “safe site” for integration. Due to this lack of toxicity, others have targeted this site for integration using a number of vectors in an effort to avoid insertional mutagenesis associated with random integration (89-91). However, most of the work associated with integration into the AAVS1 site, has been conducted *in vitro*. Further studies in animals will be required to definitively establish the AAVS1 region as a safe-site for integration.

It has also been shown that wild-type AAV exists as extra-chromosomal concatemers in normal human tissue or integrates randomly into open chromatin (92,93). Rep expression in target cells is usually dependent on acute co-infection of the cells with a helper virus such as Ad or HSV. In the absence of Rep expression, the viral DNA cannot integrate into the AAVS1 site, so the transgene usually exists in episomal form (Figure 2). In tissues that are largely composed of non-dividing cells such as muscle or the brain, the viral DNA can remain for lifespan of the infected animal or individual. In rapidly dividing cells, the viral episomes are quickly diluted from the population. From a safety standpoint, the low level of integration implies a relatively slim chance of tumorigenesis, due to the small probability of disruption of tumor suppressor genes or the activation of oncogenes. This reasoning matches the lack of epidemiological evidence for exposure to AAV as a risk factor for cancer. A study that investigated the infection of rhesus macaques with wild-type AAV by intravenous, intramuscular, and intranasal routes showed no evidence of increased risk of tumorigenesis in the animals (94). Since AAV integrates preferentially in mammalian cells that have faulty DNA repair and that are lacking expression of the tumor suppressor p53, it suggests that cancerous cells are more prone to AAV integration at the AAVS1 site (95). It has also been demonstrated that Rep can interact with p53 (96). Therefore, it is possible that in normal, non-cancerous cells that are infected with wild-type AAV without helper virus, the little Rep68/78 that is expressed is bound by p53. This scenario could lead to inhibition of AAVS1 integration, and subsequent

concatemerization or non-specific integration of AAV genomes observed in normal human tissue.

Parvoviruses are known to cause cell cycle arrest and cell death. For AAV, this is largely due to the expression of Rep. The expression of Rep78 in cells has shown to cause an S phase arrest of cells (97). Further studies showed that Rep induces a DNA damage response (DDR) that is caused by two separate activities (98). First, Rep78/68 activity is responsible for nicking of the host cell chromatin at purported Rep-binding sites; an estimated 2×10^5 such recognition sequences are thought to exist in the human genome (45). Secondly, the zinc-finger domain of Rep functions to bind and inhibit the activity of Cdc25A. This action interferes with the Cdc25A-dependent activation of Cdk1 and Cdk2, which contributes to the lack of cell cycle progression. Rep has also shown to be cytotoxic through the induction of apoptosis. Rep78 is capable of activating the apoptosis pathway in a caspase-3-dependent manner (99). Further studies have also demonstrated that caspase-independent pathways can be triggered by AAV (100), which appear to be due to Rep78 expression. In addition to the actions of Rep78, genomic elements of the virus have similarly shown the capacity to stimulate a DNA damage response. The ITRs have been suggested to mimic stalled replication forks and that this structure can mediate the death of p53-deficient cells (101,102). However, later work failed to show a DDR invoked by rAAV vectors and instead pointed to cis-acting AAV DNA sequences, such as p5 promoter (103) or DNA-dependent protein kinase catalytic subunit (DNA-PKcs) -mediated DDR caused by viral replication itself (104).

Vectorology of rAAV

AAV has become one of the most common viral vector platforms in gene delivery due to a number of desirable features. These include their simple design, ease of manufacturing, wide

range of infectivity, long-term expression of delivered genes, and excellent safety record in clinical trials. Furthermore, reengineering of the viral genome and capsid has given rise to recombinant AAV (rAAV) vectors that have enhanced tropism, expression, and safety. While numerous clinical trials have been conducted using rAAV vectors with no serious adverse events, it has become clear that there are some outstanding issues that have yet to be tackled for rAAV to be successful for various indications.

In conventional rAAV vectors, the *rep* and *cap* genes are removed and are replaced by an exogenous promoter, gene of interest, and polyadenylation signal. The ITRs which flank the transgene are the only cis-acting elements that are retained in the vector genome (Figure 1). The AAV *rep* and *cap* genes are provided in trans during vector production. This separation is necessary due to the 4.7 kb size constrain of the rAAV vector and it also serves to limit the production of wild-type AAV particles. To avoid the possible contamination of rAAV preparations with Ad or HSV helper viruses, most production systems utilize a two or three plasmid transfection method (105,106). Recently, the NIH has classified all serotypes of rAAV vectors made in the absence of helper viruses as Risk Group 1 agents, if the transgene does not encode for a toxic or potentially tumorigenic gene. A practical application of this guideline is that many rAAV vectors can be used in most laboratories and animal facilities without additional biosafety concerns. Therefore, it is important to consider the source of the vector and how it was generated and purified to ensure that the NIH classification in applicable. In addition, the manufacturing of rAAV vectors should be performed BSL2 facilities for production platforms that utilize HEK293 cells, HeLa cells, Ad, or HSV (Risk Group 2).

Early in the development of AAV as a recombinant vector system most laboratories utilized the AAV2 capsid and ITRs. As other serotypes were discovered it was noted that some had distinct bio-distribution profiles (107-110). To develop these novel serotypes as vectors, most vector particles are constructed to contain the ITR from AAV2 and the capsid from the

different serotype, since transgenes containing the AAV2 ITRs can be packaged into capsids from almost any serotype (107,111-113). By transencapsidating reporter genes into various capsids it was observed that many had important clinical applications (Figure 3). For example, rAAV5 vectors have enhanced transduction of neurons (114), rAAV8 vectors display an increased tropism for the liver, while rAAV7 and rAAV9 vectors allow for a more systematic and enhanced expression of transgenes (107,113).

Second-strand synthesis is a major rate-limiting step for the transduction of cells with rAAV vectors and this is especially evident in cells that are post-mitotic with limited DNA replication machinery. Self-complementary (sc) vectors were developed to bypass this step (115,116). During production, the sc-transgene plasmid contains one normal ITR and another that has a deletion of the terminal resolution site. This mutation prevents the mutant ITR from being nicked by Rep during replication in producer cells and results in double-stranded form of the transgene being incorporated into nascent particles. It has been well demonstrated that recombinant scAAV vectors exhibit an enhanced expression *in vitro* and *in vivo* animal studies (117,118). One downside of this strategy is that the packaging capacity of recombinant scAAV vectors is approximately 2.5 kb. This further limits many of the genes that can be packaged into AAV-based vectors and often requires smaller promoters and poly A signals to retain expression. Another challenge with these vectors is that they may be more easily recognized by innate intracellular pathways. Several studies have indicated that Toll-like receptor 9 is activated by scAAV vectors (119,120), although vectors depleted of CpG sequences may avoid this activation (121).

Another option that is being explored to overcome the natural packaging size of AAV is to produce vectors that allow the production of larger genetic material during concatemerization in target cells (122,123). This process is call trans-splicing and relies on the propensity for the ITRs from two separate transgenes to recombine and the RNA message to be correctly spliced. This technique has shown the ability to deliver genes up to 10-kb in size, which drastically

improves the applications that could use rAAV (124). The drawback to this method is the dependence on multiple vectors transducing the same cell to accumulate sufficient mRNA and produce a therapeutic protein. Target cells are usually transduced by one particle or less in most clinical applications. Therefore, it is presently unclear what doses might be required for efficient delivery of donor and acceptor vectors and whether these doses would be acceptable for patient safety.

Risks Associated with rAAV Gene Therapy

Risk of Dissemination

A biosafety concern for rAAV is the risk of secondary spread of the vector transgene by shedding, mobilization, or germ line transmission. Shedding is the dissemination of the vector from treated animals or patients in urine, feces, tears, or other bodily fluids (125). For rAAV vectors, shedding is primarily due to the release of vectors that did not infect target cells at the time of administration, but were cleared or released from the patient or animal. Most studies of rAAV vector shedding have been performed in the context of clinical trials (125-131) and have concluded that the risk of shedding is low and dependent on the route of administration. Preclinical evaluations of rAAV shedding have also shown that shedding is transient and that exposure to the vector is limited to relatively low numbers of particles (132,133). In addition, a wealth of preclinical and clinical data has shown that successful transduction requires relatively larger quantities of vector than the amount of the vector that is shed, and that the vector must be administered through systemic or localized delivery. Despite this low safety hazard, it is advisable for laboratory, vivarium, and hospital workers who may come in contact with bodily secretions or excreta to utilize proper protective equipment such as surgical masks, lab coats, protective eyewear, and disposable gloves (134).

For rAAV vectors, mobilization of the vector differs from fundamentally from shedding. Mobilization of rAAV vectors requires that target cells be infected with the vector in order to deposit the vector transgene. Vector mobilization also necessitates that the same cells are also infected with a suitable helper virus and wild-type AAV in order to mobilize copies of the transgene out of the cell or host and into nascent vector particles. Nearly all rAAV vectors utilize the ITR structures from AAV2. Due to the prevalence of AAV2 in the human population there is the potential for cells transduced with a rAAV vector to also be infected with wild-type AAV2 and a helper virus. This could allow the rAAV vector genome to be replicated and mobilized into new cells or individuals (135). Data from a number of laboratories has shown that this is theoretically possible (82,135,136). To overcome this risk, it will be important to develop rAAV vector transgenes with novel ITRs that cannot be recognized by wild-type Rep or packaged into the capsids of wild-type virus.

A serious concern for vertical and horizontal transmission of rAAV vectors is through seminal fluid. While the gene transfer risk is low for the sexual partners of the male, the possibility of germ line transmission increases the chance of genetic transmission to offspring of rAAV recipients. This is theoretically possible, since it has been shown that rAAV vectors can be used to generate transgenic animals by the transduction of male germ line stem cells (GSCs) with rAAV (137,138). Even in these studies that bypassed the natural barriers and replaced the endogenous GSCs, the rates of transgenic animals were relatively low, suggesting the likelihood of vertical transmission is minimal. In a Phase I open label dose-escalation safety trial evaluating the safety of rAAV-FIX administered via injection of the hepatic artery vector transgenes were found in the semen of a 63-year-old research participant, raising the concern for transmission of genetic material in humans (139). While semen samples tested positive for AAV-FIX transgenes until week 12 post-administration, no copies of the vector genomes were observed in the patient's motile sperm. Earlier data from this same group also showed that intramuscular injection of mice and intrahepatic injection of rats with rAAV-FIX showed a dose-

dependent level of vector transgene in the animal's gonads (140). The authors demonstrated that intramuscular injection of rAAV-FIX into rabbits revealed that the vector could be localized to the basement membrane and vessel walls of the testicular tissue, but was not detected in semen samples. Additionally, semen samples from injected dogs did not show the presence of vector transgenes. In work that analyzed the bio-distribution of rAAV in nonhuman primates vector genomes were undetectable in male germ line cells. A study of the safety of rAAV in rhesus macaques found that the gonads were consistently negative for transgene sequences following intramuscular injection (141) and the presence of vector genomes was not detected in the gonads of baboons following intramuscular administration of rAAV-AAT (142). However, other work has shown that vector genomes can be found in almost all gonads of macaques following intramuscular injection and regional intravenous administration of rAAV1 and rAAV8 vectors (143). In addition, evaluations of AAV1-AAT in mice and rabbits found vector in rabbit semen. Risk is still considered low since rAAV apparently cannot infect sperm cells and infectious virus cannot be recovered from semen (140,144,145). In addition, rAAV bio-distribution to the semen appears to clear by 12 weeks for serotypes 2, 5, 6, and 8 (132,145), so any risk would be transient and simply addressed by barrier contraception.

Risks Associated with rAAV Production

Contamination with helper virus

Early rAAV production strategies relied on the use of adenoviral vectors to provide the helper virus functions required for AAV replication. These platforms depend on the heat stability of AAV and temperature sensitivity of adenovirus. By heating the partially purified viruses at 56°C for 30 minutes, the majority of adenovirus is inactivated, while the AAV vector is stable. Intact adenovirus is then further purified from the AAV vector by ultracentrifugation or chromatography, although adenoviral proteins are often still present and difficult to remove. Adenovirus is not required in current production platforms, since the genes required for helper

virus functions have been identified and can be provided in trans (105). For systems that still employ adenovirus or herpes virus for helper functions, it is imperative to demonstrate that the final preparations of rAAV vector are devoid of contaminants from the helper viruses.

Risk related to the creation of replication-competent AAV by recombination

Although wild-type AAV has not been associated with any disease, it is considered an unwanted impurity in vector preparations. The possibility exists that the presence of wild-type AAV could heighten non-specific integration, increase the risk of vector mobilization, or cause toxicity in target cells due the presence of Rep (146). Although most production methods utilize separate plasmids for the *rep-cap* AAV helper and the vector transgene, it appears that recombination between these plasmids can give rise to replication competent AAV (RC-AAV) particles that resemble the wild-type virus (147,148). The RC-AAV particles can contribute to 10% of the total vector preparation if not properly controlled (148,149). Due to this propensity for recombination, several strategies have been employed to reduce the chance of RC-AAV contamination. One approach is to place the *rep* and *cap* genes in opposite orientations, on separate plasmids, or by inserting introns within the *rep* and *cap* genes. This method has been shown to decrease the presence of RC-AAV to undetectable levels during large-scale production (149-151). Another innovative method was developed by the laboratory of Weidong Xiao; this system uses vaccinia virus to deliver and restrict the *cap* and *rep* genes to the cytoplasm, thereby avoiding the potential for recombination and RC-AAV production (152). It is also important to develop validated assays for the detection of RC-AAV particles, especially if the vector being produced is intended for preclinical or clinical use. These assays generally employ three or more rounds of infection cycles on a permissive cell line in the presence of adenovirus followed by detection by end-point PCR and agarose gel electrophoresis.

Risk Related to Integration

Serious health risks have been seen in clinical trials with retroviral vectors due to insertional mutagenesis and the subsequent induction of oncogenesis (4,153,154). While rAAV vectors display a much lower likelihood of integration into the target cell genome, the hazard of insertional mutagenesis is worrying and numerous investigators have explored this risk. Unlike wild-type AAV, most rAAV vectors lack the *rep* gene and are therefore deficient for targeted integration into the host chromosome at the AAVS1 site. Instead rAAV vectors predominantly circularize to form episomal structures. While this limits the effectiveness of AAV-based vectors to non-dividing cells, concatameric DNA assemblies of the transgene are generally considered to be safe. Most studies that have assessed the non-specific integration capability of rAAV vectors have concluded that this background integration is relatively low (155-157). In contrast, several reports have suggested that the risk of insertional mutagenesis is potentially much higher, especially in the liver (158-162). During a study evaluating the treatment of neonatal mucopolysaccharidosis type VII (MPS VII) mice with a rAAV2 carrying a β-glucuronidase gene driven by the chicken β-actin promoter, a significant incidence of tumorigenesis was observed in the animals (160). In agreement with this troubling finding, subsequent work found that rAAV integration events were potentially deleterious and tumorigenic (158,159,163,164). The findings included evidence for integration hot-spots near regulatory sequences, observations that rAAV vector integration sites were associated with a substantial percentage of deletions, and signs that integration predominantly occurred in active genes or fragile sites in the chromosomes (158,163,164). Recent work (161,162), and in the 2007 study by Donsante et al. (159), proviral insertions were found in rAAV transformed cells that mapped to a locus on chromosome 12 that contains small nucleolar RNAs and microRNAs that could affect cell cycle regulation. Two of the integration sites were found within the mir-341 microRNA transcript, suggesting that mir-341 may be a preferential target for rAAV vector integration. In more recent work, significantly higher rates of tumor formation in hepatocellular carcinoma-prone mice following administration

with scAAV vectors has been observed, with integration patterns that likely led to read-through transcription, enhancer activation, and tumor suppressor deregulation (162). However, in these reports the authors tended to use tumor-prone or neonatal mouse models to investigate the likelihood of AAV-mediated tumorigenesis. Furthermore, the vector transgenes often incorporated bacterial origins of replication, evaluated a limited number of integration events, and utilized vector preparations that were not of clinical-grade quality.

In a recent high-throughput analysis that investigated the potential for insertional mutagenesis, a high dose of cGMP-grade rAAV2 human Factor IX vector was administered in an adult mouse model and linker-mediated polymerase chain reaction (LM-PCR) and 454 pyro sequencing was performed from tissue approximately 18 months post-injection (165). Although numerous integration events were identified, no evidence for increased genotoxicity was seen. This data is in agreement with investigations in non-human primates and clinical trial patients that utilized linear amplification-mediated (LAM)-PCR and deep sequencing and found random and low frequency of integration (10^{-5} to 10^{-4}) without tumorigenesis (157,166,167). These findings are supported by studies that have not found any increase of tumorigenesis in rAAV treated animals (168-170). Furthermore, no evidence for insertional mutagenesis has been reported for any individual treated with a rAAV vector, suggesting that the risk to patients or laboratory workers is low.

One solution to the risk of non-specific integration is to develop rAAV vectors that can exclusively target the AAVS1 site. This strategy has been explored in a number of studies that have suggested incorporating the Rep protein or using hybrid vectors the rAAV vectors may be capable of more specific chromosomal targeting (171,172). However, the problem with illegitimate integration by rAAV vectors may be difficult to avoid. Recent work that used high-throughput sequencing identified that wild-type AAV integration at the AAVS1 site only accounts for about 45% of the total integration events (161). This suggests that even with strategies that use the natural integration pattern of AAV, there might still be considerable off-target integration.

Off-target Effects

Like any drug product, all gene therapy vectors exhibit some degree of off-targeting. For gene delivery, these effects are seen when the gene of interest is expressed in non-intended tissues. Although the use different serotypes has been shown enhanced expression in certain tissues, off-targeting remains a serious concern for the treatment of some indications with rAAV vectors. To build upon the unique properties of different capsids, several groups have advanced strategies to improve upon the natural occurring serotypes. These approaches included making simple amino acid changes to a serotype, the mixing of capsid genes to create vectors pseudotyped with chimeric capsids, random mutagenesis of the *cap* gene, or introducing targeting ligands (26,173).

While the discovery of novel serotypes and isolates, as well as the capsid engineering efforts have vastly increased the set of useful capsids for clinical objectives, the goal of specific targeting vectors has yet to be realized. In complementary work, several approaches have been taken to control gene expression in the event of off target transduction. One scheme is the use of inducible or tissue specific promoters. For example, promoters have been identified that help restrict expression to the heart (174) or human rhodopsin kinase gene to specifically drive transgene expression in rods and cones of the eye (175). An alternative means to control expression is through incorporating microRNA targeting sequences into the transgene (176). This approach has been demonstrated to restrict expression with rAAV5 vectors in pig eyes and rAAV9 vectors outside of the CNS (177-179). Taken together these different approaches offer the potential to tightly control transgene expression to specific organs or cell types.

Control of transgene expression

With the exception of suicide gene delivery in cancer gene therapy (180), the administration of recombinant viral vectors is an irreversible process. So far, in clinical trials, the

level of transgene expression cannot be controlled after vector administration, raising the question of the long-term safety of continuous unregulated delivery of therapeutic transgene products. Several regulated systems have been successfully used in animal research but so far, none of them meets the requirements for clinical applications.

Promising switches for *in vivo* regulation of transgene expression are those regulated at the transcriptional level such as the tetracycline-inducible (181) and repressible systems (182), the rapamycin- (183,184), and mifepristone-inducible systems (185,186), or at the post-transcriptional level, using a destabilizing domain regulated by trimethoprim fused to the therapeutic protein (187) that has been optimized for *in vivo* applications (188).

The limitations of these systems include the inducers toxicity (189,190) or off-target effects to the patients (191,192), raising of antibiotic resistance (193), and deleterious effects on the environment (194).

Interestingly, the tetracycline-repressible system was shown to drive biologically-active GDNF brain levels upon treatment with doxycycline doses which are below those recommended for patients undergoing antimicrobial or anti-inflammatory treatments (195-197).

In contrast, the more clinically-relevant tetracycline-inducible system requires a one order of magnitude higher doxycycline dose to induce biological effects (198). Efforts to reduce inducer's dose (199) or use alternative inducers combined with rationally-designed (200) or evolution-selected (201) tetracycline repressors might ultimately lead to a clinically useful tetracycline-inducible genetic switch.

A second important limitation of the tetracycline-inducible/repressible system is the immunogenicity of the transactivator, which contains bacterial (tetracycline repressor) and viral (a deleted form of the HSV1 VP16 activation domain) components (202). Indeed, after intramuscular delivery via AAV recombinant virus into non-human primates, a rapid loss of transgene expression correlating with a cellular immune response has been described (203).

Nevertheless, no immune response has been reported so far in immune-privilege sites such as retina (204) and brain (205).

A fact that further complicated the use of the tet system in clinical applications is that a large proportion of the human population (60%) has been exposed to herpes simplex virus (206). Replacing the viral VP16-derived activator domain by human activation domains, could help reducing the immunogenicity of the Tet transactivator (207).

In addition, most of these systems do not allow expression to reach the same level of transgene expression as constitutive promoters especially in the brain in which the bioavailability of the inducers is reduced due to the blood-brain barrier (183,188,208).

Risk Management Measures

Although wild-type AAV has not been associated with any disease and rAAV vectors have shown an excellent safety profile in a large number of animal studies and many clinical trials, it is advisable to use good laboratory and clinical practices to avoid any inadvertent exposure. The virus is very stable across a wide range of pH and temperatures; work has shown that temperatures of greater than 60°C are required for denaturation of the vector capsid (209). AAV-based vectors have also shown to be stable for a month or longer following desiccation or lyophilization (210,211). Therefore, work areas should be disinfected with the use of chemical denaturants such as 0.5% sodium hypochlorite, ionic detergents, or alkaline solutions (pH > 9.5). Personal protective equipment should include protective eyewear, laboratory coats, and disposable gloves. If contact with contaminated aerosolized material is possible (e.g. soiled animal bedding), then adsorption masks should be worn. When possible, materials that have come in contact with AAV or rAAV vectors should be autoclaved (134). While preexisting immunity to AAV limits the ability of the vector to effectively transduce cells *in vivo*, care should be taken to avoid accidental exposure to open cuts or wounds. Used needles containing AAV or rAAV vectors should be disposed of after use to prevent inadvertent needle

sticks and possible infection. In the event of an accidental needle stick the recommendations of the Center for Disease Control and Prevention should be followed (212).

Clinical Trials with rAAV

The rapidly increasing use of AAV vectors in diverse fields of fundamental and translational research (from 8 articles in 1990 to 374 in 2010 and 469 in 2012 indexed in PubMed- key words: “adeno-associated virus” and “vector”) necessitates a thorough evaluation of potential risks related to their dissemination into the environment. Abundant studies using rAAV have been conducted in mice, rats, pigs, dogs, horses, non-human primates, and other animal models. However, these preclinical evaluations have not always been predictive of administration during clinical trials with AAV-based vectors. While rAAV vectors have advantageous properties for *in vivo* gene delivery and the vector has been used safely for gene delivery in over fifty clinical trials, efficacy has only been reported in a handful of cases. A number of notable clinical trials are summarized below with some important lessons learned from each. A summary of the more striking data illuminating the some important biosafety aspects such as shedding, integration of vector DNA, and risk of insertional mutagenesis, as well as immune responses is provided in Table 1.

Lung Diseases - Cystic Fibrosis and Alpha-antitrypsin Deficiency

Cystic Fibrosis (CF) is the most common autosomal recessive genetic disease in the Caucasian population, occurring in 1 of every 3000 live births (213). The disease results from mutations in the CF transmembrane conductance regulator (CFTR) gene which leads to mis-regulation of the ion channel in organs, especially the upper and lower respiratory tract, and typically death due to chronic lung infections. Despite the problems associated with packaging

4.4 kb CFTR transgene, preclinical studies using AAV2 to deliver CFTR to the lungs of rabbits and nonhuman primates demonstrated long-term expression of CFTR and no safety concerns (135,214). Importantly, experiments that were performed to address the possibility of vector shedding, which the European Medicines Agency and International Conference on Harmonization has defined as the "dissemination of the virus/vector through secretions and/or excreta of the patient" (215). These evaluations showed that rAAV2 exhibited no appreciable shedding or mobilization when co-infected with adenovirus. The lack of toxicity and vector shedding seen in these studies was also observed in patients treated with rAAV2-CFTR. Several Phase I clinical trials showed that rAAV could be safely administered at various doses and by different routes, which led to Phase II trials that continued this safety profile (126,216,217). While these studies demonstrated that rAAV2-CFTR was safe, the therapy itself was limited. The weakness was likely due to the intrinsic low transduction capacity of AAV2 for airway epithelia. Further studies have shown that AAV5-derived vectors can improve the ability to target the lung (218).

Another monogenetic lung disease is alpha-antitrypsin (AAT) deficiency. Severe loss of the AAT glycoprotein causes destructive diseases of the lung, liver and pancreas and can lead to pulmonary emphysema and the need for liver transplantation. The current standard of care for affected individuals is life long treatment with weekly infusions of AAT derived from human plasma. This protein replacement therapy is costly and has some associated risks. Several clinical trials are addressing the ability of AAV-mediated delivery of AAT through intramuscular injection (129,219,220). In a Phase II dose-escalation study led by Terry Flotte a rAAV1 vector carrying the human gene was able to achieve peak levels of AAT between 412 and 694nM after administration of the highest dose (6×10^{12} vector genomes/kilogram of body mass (VG/kg)), and the concentration of AAT corresponded to vector dose. Unfortunately, the serum levels of AAT were substantially lower than therapeutic concentrations (220). While these studies

confirmed the safety of rAAV vectors even at doses as high as 4.3×10^{14} VG, they also demonstrated the induction of an immune response against AAV that correlated with loss of transgene expression. It will be necessary to enhance the vector or delivery method to overcome this hurdle and attain a functional therapy using rAAV.

Neurological Diseases

rAAV vectors are being developed for the treatment of several diseases of the central nervous system (CNS), including Parkinson's disease (PD) (221), Alzheimer's (222), amyotrophic lateral sclerosis (ALS) (223), Batten's disease (224), epilepsy, and Canavan disease. The brain is generally considered an excellent target organ for therapy with rAAV; vectors serotyped with either AAV2 or AAV5 capsids have been shown to effectively transduce neurons and the brain is immune-privileged. The route of administration, however, is usually by direct injection into the brain due to inability of most rAAV vectors to cross the blood-brain barrier (BBB) and these infusions carry potential risks, such as hemorrhaging or infection. In addition, there is limited spread of rAAV within the brain, which necessitates multiple injection sites for the treatment of some indications. It has been shown recently that self-complementary rAAV vectors pseudotyped with the capsid of AAV9 can infect cells in the CNS after intravenous injection (225,226). While rAAV9 vectors can be used to circumvent the BBB, their expression must be tightly controlled since rAAV9 vectors can transduce multiple organs, such as the heart, liver, and kidneys, increasing risk of off-target transduction.

The first clinical trial that used rAAV to target the CNS was for the treatment of Canavan disease, a childhood form of leukodystrophy caused by mutations in the gene coding the enzyme aspartoacylase (ASPA) (227). This study used a rAAV2-ASPA vector that was delivered by intracranial injection into 10 patients, the youngest of which was 3 months old. The safety data collected for this trial showed that administration of rAAV was well tolerated. The

immune response to the vector and transgene appeared to be low and no serious adverse events related to the vector were reported. A 10-year follow up of the patients showed an excellent long-term safety profile and some clear signs of efficacy for the therapy (228). The authors reported no serious adverse events related to the vector itself and evidence that the treatment lowered N-acetyl-aspartate levels, slowed brain atrophy, and improved seizure frequency.

This work was followed up by trials that aimed to treat PD, a degenerative disorder of the CNS caused by a loss of dopaminergic neurons in the substantia nigra (SN) region of the midbrain. Thanks to the discovery of the important role of dopamine depletion in PD, a breakthrough in the pharmacological treatment of PD was made in the 1960s, in the form of the oral administration of L-dopa, the precursor to dopamine (229). Exogenous L-dopa is taken up into remaining DA neurons where it is converted into dopamine by aromatic acid decarboxylase (AADC). With the ongoing loss of DA neurons, though, the uptake of extracellular L-dopa declines and increasing doses of L-dopa need to be administered leading to important fluctuations (e.g. peak-dose dyskinesias). Interestingly, three different therapeutically paradigms were developed. In the first, it was hypothesized that continuous delivery of L-dopa directly to the striatum via gene therapy could be a significant improvement because L-dopa would reach only the clinically relevant target area (230). A rAAV2 vector expressing AADC has been injected in PD patients' putamen in an attempt to reduce and stabilize the L-dopa dose necessary to alleviate the symptoms (231). Patients received bilateral intra-putaminal infusions of rAAV2-AADC. Data based on PET imaging (221) demonstrated stable transgene expression over 4 years confirming the preclinical data on the longevity of rAAV-mediated transgene expression (232). This clinical trial further confirmed the safety and tolerability of rAAV intracerebral administration. The clinical data showed dose-dependent improvements but even in the high-dose group in which all patients improved in the first 12 months, a slow deterioration was observed in subsequent years that is thought to be related to continued degeneration of the

remaining DA neurons. The second paradigm consisted in reducing the overactivity of the subthalamic nucleus (STN), an excessively active output nuclei of the motor loop in PD, in order to restore a normalized output to the cortex. A rAAV2 vector expressing glutamic acid decarboxylase, the enzyme that converts glutamate, an excitatory neurotransmitter into the inhibitory neurotransmitter GABA was injected in the STN. A Phase I clinical trial demonstrated the safety and tolerability of rAAV2 injections into the STN. Furthermore, 10 of 12 patients treated showed clinical improvements at 12 months (233,234).

These therapeutic strategies are however only compensatory and are not expected to interfere with DA neuron cell death. Providing neurotrophic support might constitute the first disease-interfering approach for PD. A rAAV2 vector expressing Neurturin (NTN) has been evaluated in a Phase II clinical trial (235). The virus was injected in the putamen. At 12 months post-surgery, no statistical improvement in the main clinical parameters could be established. However, in a subset of patients reaching 18 months post-surgery, several parameters were significantly improved. For 2 patients who died for unrelated causes, post-mortem analysis revealed that NTN covered approximately 15% of the putamen (236). Contrary to data obtained with similar amounts of virus in monkey, though, very few NTN-positive cells were detected in the SN, suggesting a poor retrograde transport of NTN in patients. A second clinical trial has been launched using both putamen and SN as delivery area. However, several preclinical studies (237-239) and one clinical using GDNF (a NTN analog) recombinant protein (240) have described adverse effects related to uncontrolled dosage and off-target delivery of GDNF. In order to address these safety issues, e.g. in the case of neuroprotective strategies, it will be necessary to regulate transgene expression in order to adapt the dose of the transgene product to the patients needs or even interrupt the treatment. The on-going development of rAAV vectors with enhanced targeting and better genetic regulation also could provide a opportunity to deliver and express GDNF in a more controlled manner.

Correction of lysosomal enzyme deficiencies has also generated promising preclinical data. This has been demonstrated in rAAV-mediated gene transfer for mucopolysaccharidosis type III (MPSIII) a disorder caused by the absence of one of the lysosomal enzymes required for the degradation of heparan sulfate (HS) resulting in the accumulation of heparan sulfate oligosaccharides (HSOs). In particular, in MPSIIIB, alpha-N-acetylglucosaminidase (NaGlu) deficiency is responsible for progressive mental neurodegeneration. Intracerebral stereotactic injections of AAV5 vectors coding for the missing enzyme reversed alterations of HS degradation, corrected pathology in neuronal cells and prevented neuroinflammation at the organ level in animal models (241). Recently, a clinical trial using a rAAVrh10 vector has been launched for MPSIIIA, a deficiency in heparan-N-sulfamidase (242).

Muscular Diseases

Some of the earliest studies in animal models established the potential of rAAV vectors for long-term muscle-directed gene transfer (243). This discovery led to intense efforts to use rAAV to treat devastating illnesses such as limb girdle muscular dystrophy 2D (LGMD2D), Duchenne muscular dystrophy (DMD), Pompe disease, as well as cardiovascular diseases. Two clear obstacles exist for using rAAV vectors to treat LGMD2D and DMD: the genes far exceed the packaging size 4.7kb limit of rAAV; and the doses required for systemic administration of skeletal muscle are incredibly high. To overcome transgene restriction mini and micro-forms of the transgene and exon-skipping vectors have been engineered. Due to the impracticality and safety of infusing patients with very large doses of vector, the clinical trials have usually focused on critical muscle areas in hopes of improving lifespan or quality of life. In 2006 a Phase I clinical trial was initiated for the treatment of DMD at the Nationwide Children's Hospital in Columbus, Ohio (244). Six boys with frame-shift mutations were treated with rAAV2.5 vectors carrying a functional mini-dystrophin transgene. Three of the patients received a dose of 2.0×10^{10} vector genomes per kilogram body weight (VG/kg) and the three others were

administered 1.0×10^{11} VG/kg. There were some mild adverse events described that included nausea or upset stomach, macular rash, fungal rash, and sore throat, but no moderate or severe adverse events were reported. A dystrophin-specific T-cell response was observed and two of the patients had detectable levels of spontaneously primed T cells before treatment, indicating that these patients had developed self-reactive T cells during the course of their disease. The authors of this study suggest that cellular immune responses should be examined during gene therapy trials for DMD. One possible means to circumvent the immunotoxicity is by isolated limb perfusion. It has been shown that the selective regional administration of rAAV vectors to limbs of mice, dogs, and non-human primates allows for substantial transduction with decreased immune activation (245-247).

Diseases of the Eye

One of the most promising target tissues for gene delivery using rAAV vectors is the eye. Due the lower doses required and the fact that the eye is relatively immune privileged, some notable trials have demonstrated success. In particular the treatment of Leber's congenital amaurosis (LCA) and the wet form of macular degeneration have provided evident safety profiles and exciting clinical results. All of these trials have used subretinal injection and vectors based on the AAV2 serotype to deliver the therapeutic genes, due to the propensity for this platform to efficiently target the retina (248,249).

LCA is the most common and severe form of inherited childhood blindness and is caused by severe retinal degeneration of the photoreceptor cells or the retinal pigment epithelium (250). A number of different genes have been shown to be involved in this dystrophy, with mutations in the retinal pigment epithelium-specific 65-kDa protein (RPE65) gene causing disease in approximately 6% of the patients (251). The RPE65 gene codes for an enzyme that is responsible for the isomerohydrolase activity in the retinal pigment epithelium. Studies in animal models showed that rAAV2-RPE65 gene delivery could safely replace

functional protein for more than 7 years, establishing a clear rationale for human therapy (252). In 2008, three reports were published from independent early-phase clinical trials that explored the gene delivery of RPE65 in young LCA patients (128,130,253). A total of nine subjects between the ages of 17 and 26 were treated in these early studies, with vector administration in one eye and the contralateral eye as a control. While retinal complications did occur in two of the patients, these were likely related to the surgical procedure and no serious adverse events were reported. Furthermore, there was no evidence for systemic toxicity or immunogenicity to the vector. Despite the differences in transgene construction, dose delivered, and retinal degeneration in the patients, there was evidence for visual improvement in each trial. A substantial deficiency in low-light vision is a hallmark deficiency in LCA; seven of the nine patients reported improved vision in dim light environments. Subsequent reports, which included vector re-administration in 3 patients, have continued to demonstrate the long-term efficacy of rAAV2-RPE65 gene delivery, without safety difficulties or the induction of cytotoxic T-cells against the vector or transgene (254-256).

AAV is also being evaluated in two separate clinical trials that are delivering the soluble vascular endothelial growth factor (VEGF) Flt-1 (sFlt-1) gene to block neo-vascularization of the eye in the “wet” form of age-related macular degeneration (AMD), a leading cause of vision loss in the elderly (257). Preclinical data performed in mice, rats, and monkeys in support of these studies has demonstrated that the treatment is capable of long-term expression and well tolerated (258,259). Early reports from the trial led by Rakoczy et al. indicate that no signs of inflammation or increase in T-cell activation, even though patients with preexisting neutralizing antibodies to AAV2 were not excluded from the study (260). While transgene sequences were detected in the tear samples from two of the subjects the day after dosing, this vector shedding was transient. In addition, evidence of efficacy was also reported, since the most of the patients had improved vision and were able to discontinue the standard injections of the monoclonal antibody fragment, ranibizumab.

Together these findings suggest that AAV-mediated gene delivery is a safe and effective means to treat ocular diseases and has created renewed enthusiasm in gene therapy. The current route of delivery, however, has been through injection into the subretinal space, a procedure that may not be well tolerated in other disorders like retinitis pigmentosa in which the retina is weak. Newer delivery methods and enhanced vectors are currently being developed that offer the capability to transduce numerous cell types in the retina though less invasive means such as intravitreal injections (261).

Hemophilia B

Hemophilia B is an X-linked bleeding disorder that is caused by the lack of blood coagulation Factor IX (FIX) in affected patients. The current standard of care is prophylactic injections of recombinant or plasma-derived FIX on a weekly or twice-weekly basis to maintain low bleeding rates and on demand treatment to control a bleeding episode. While this enables management of the disease, inhibitor development, the burden of regular injections, and the very high cost for the clotting factor infusions are problematic. The treatment of hemophilia B using gene delivery has been considered promising for many years. This is due to the fact that FIX is in the circulation and therefore can be expressed from multiple tissue types. Another reason is that only a small portion of normal protein expression is needed for a therapeutic effect; hemophilia B can be treated with as little as 1% of normal circulating levels of Factor IX and levels above 5% are considered mild.

The first trial was a dose-escalation study that used a rAAV2 vector to deliver FIX to skeletal muscle (262). Importantly, the inclusion criteria included that the subjects have missense mutations and no history of FIX inhibitor development, and did not exclude patients with neutralizing AAV2 antibodies. The dose range was 2×10^{11} to 1.8×10^{12} VG/kg and safety results were encouraging for all the doses: there was no indication of vector-related toxicity, increased humoral response, or dissemination to patient semen. In addition, the persistence of

transgene sequence and modest FIX expression suggested that the treatment offered a low level of gene delivery, though circulating levels of FIX did not reach therapeutic levels (263). The second trial also used an AAV2-based vector, but in this study the administration was through the hepatic artery (264). No acute or systemic toxicity was reported, but a short-lived rise in liver transaminases was observed. The authors described a transient level of therapeutic levels of FIX with the highest vector dose and it was proposed that CD8-positive T-cell responses to the AAV2 capsid were the cause of vector clearance (265,266).

In a more recent trial, a rAAV8 vector was used to deliver a codon optimized self-complementary FIX transgene driven by a tissue-specific promoter to the liver of hemophilia B patients by intravenous injection, thereby utilizing the natural tropism of AAV8 for the liver (267). Vectors based on AAV8 are also thought to be less likely to induce an immune response, since the prevalence of neutralizing antibodies to AAV8 in the human population is relatively low and AAV8 does not utilize heparin sulfate for cellular attachment and therefore may not be presented on dendritic cells as readily (81,268). No neutralizing antibodies or T-cell response to FIX were detected in any of the patients. Humoral responses to AAV8 capsid were observed in all the subjects and varied AAV8-capsid specific T-cell responses that correlated with vector dose. A significant rise in liver enzymes was seen in one of the six patients and correlated with a decrease in FIX expression, suggesting that a cytotoxic T-cell against transduced hepatocytes was the cause. This immune response was treated with prednisolone without further complications. Notably, the results of this trial indicated that gene delivery was very successful in treating the disease. All patients developed circulating FIX levels of between 1% and 6% for more than 2 years and exhibited less dependence on FIX protein replacement treatments. Four of the patients were able to stop taking prophylactic injections, while the two other subjects were able to reduce their number of infusions. This trial has been seen as a critical milestone in rAAV-mediated gene delivery due to its clear efficacy and safety endpoints.

Storage Diseases

Another area where treatment with rAAV vectors has demonstrated success is in the treatment of storage diseases. Lipoprotein lipase (LPL) deficiency is a rare autosomal recessive disease mainly characterized by recurrent and potentially fatal pancreatitis (269). LPL is the main enzyme in the catabolism of triglyceride (TG)-rich lipoproteins called chylomicrons. In LPL-deficient patients, impaired chylomicron clearance from the circulation leads to extremely elevated levels of serum triglycerides. A natural gain-of-function variant, LPLS447X (270), has been used to generate alipogene tiparvovec (or Glybera), a rAAV1-LPLS447X vector that restored normal levels of chylomicrons in patients 12 weeks after intramuscular injection. However, at long-term, the plasma triglyceride levels, which were chosen as primary efficacy endpoint, returned to baseline (271). It was first hypothesized that T-cells responses directed against AAV1 could be responsible for this apparent drop of vector activity (272), but it was afterwards realized that the triglyceride level was not an adequate primary endpoint to assess treatment efficacy. Other characteristics of the TG-rich lipoproteins rather than plasma triglycerides content were better reflecting pancreatitis risk and were shown to be stably corrected. In addition, despite the presence of anti-capsid antibodies, vector DNA and transgene expression persisted at least until 26 weeks and a strong tendency for reduced pancreatitis frequency during a 2-years follow-up in an open-label trial (273). Apart from muscle pain due to the high number of muscle injections, no adverse effect was reported. Interestingly, this clinical trial offered the possibility to study AAV integration profile in a large number of events in 5 LPL-deficient patients (444,433 reads from 25 muscle biopsies) (167). The study, using LAM-PCR and deep sequencing revealed a largely random integration profile. In contrast to other AAV integration studies, no preferential integration within genes has been observed. Only very few integration hotspots were found in the nuclear as well as (even more frequently) in mitochondrial DNA. The integration frequency (10^{-4} to 10^{-5}) is approximately one order of magnitude higher than in other studies but it is still much lower than that of retroviral vectors.

This is in striking contrast to the data obtained in very specific mice models involving partial hepatectomy in a cancer-prone model which raised the potential of AAV-mediated oncogene activation via insertional mutagenesis in particular in one locus, called AAV-HCC l (162). In the AAV1-LPLS447X, no integration in the AAV-HCC locus was discovered. Interestingly, no accumulation in the AAVS1 locus, the wild-type AAV preferential integration site was observed.

This clinical trial was also the first to establish the safety of clinical-grade AAV vectors manufactured with the bacullovirus technology that offers to possibility to produce larger scale batches than previously (273).

Due to the restricted number of patients worldwide and to the initial difficulty to find a reliable output measure of efficacy, it has been difficult to statistically establish the benefit of the therapy. Therefore, despite the very extensive and convincing data regarding safety (minimal shedding, limited and transient elevation of anti-capsid antibodies and no cellular immune response; see table1), the EMA repeatedly rejected the application for market authorization due to an estimated low benefit in balance with the expected discomfort of the high number of injections and risks related to the transient immune-suppression in the protocol. However, when postprandial chylomicrons clearance and triglyceride content of the chylomicron lipoprotein fraction was taken as the efficacy endpoint, a prolonged effect of the gene therapy with levels in treated patients similar to those of normal subjects has been demonstrated (273). Glybera has finally been accepted as the first commercialized gene therapy product in the Western world in November 2012 (274,275).

Importantly, as required by Directives 2001/83/EC and 726/2004 from the European Parliament and the Council of the European Union, a thorough environmental risk assessment was performed for Glybera (281). This biosafety review evaluated the risks associated with vector dissemination through shedding, germ line transfer, and mobilization, wild-type AAV contamination, non-specific integration, and other biosafety possibilities. The study concluded

that the vector poses only a negligible risk to the environment, including healthcare workers, friends and family, pets, or others that come in contact with Glybera treated individuals.

Future

AAV-based vectors have an impressive safety record and this has advanced their use to more laboratories and clinical trials. The approval of Glybera illustrates the potential for rAAV vectors and will possibly be the first of many. For the widespread use of rAAV vectors to become a reality, it will be necessary to overcome the current challenges of efficacy and safety. These include decreasing the immunogenicity of AAV-based therapies, and limiting the non-specific integration, off-target effects of transgene expression.

While not directly related to safety of the vector, it is now becoming clear that immune activation by rAAV vectors is the major challenge that will need to be overcome to fulfill the promise of AAV-mediated gene therapy (276-278). Unlike Ad vectors, rAAV vectors have not been associated with a high risk of tissue and organ inflammation, but both innate and adaptive immune responses are known to limit rAAV vector transduction and eliminate transduced cells. In the future it will be important to identify the best means to decrease the immune activation to the vector and the transgene product. This path will likely involve developing vectors with better immune evasion, the limited use of immunosuppression, and controlling vector production to create higher purity drug products.

Concluding Remarks

Every form of new and important therapy has come with inherent risks. Blood transfusion, for example, was once a risky and controversial procedure (279). In the past 100 years the scientific community has worked diligently to overcome the problems of immune rejection and infections that have complicated the procedure. Likewise, gene therapy will need

to address the pitfalls that could limit the effectiveness of treatment or endanger patient health. For rAAV vectors, many of the hurdles have been identified and work is currently underway to enhance further its safety and efficacy which now has accumulated from this platform vector that has now been tested in brain, eye, heart, lung, liver and muscle with minimum risk. As these improvements are made there will be benefits for basic scientists as well as for patients.

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Figure Legends

Figure 1. Organizational structure of AAV genome. The genome of AAV is divided into two main gene segments, *rep* and *cap*. The two larger *rep* transcripts (*rep78* and *rep68*) are produced from the p5 promoter. The full-length transcript is *rep78*, while alternative splicing is used to generate *rep68*. The two smaller *rep* sequences are coded from the p19 promoter, and produce *rep52* and an alternatively spliced transcript, *rep40*. The three capsid subunits are coded by the *cap* gene from the p40 promoter, using a combination of alternative splicing and the unique ACG start codon for VP2. A second open reading frame that is nested within *cap* is used for translation of AAP. The Inverted Terminal Repeats (ITRs) that flank the viral genome are the only components conserved in rAAV vector transgenes.

Figure 2. AAV transduction pathway. AAV or rAAV vectors can effectively transduce target cells in the absence of helper virus through the following steps: Receptor binding, Receptor-mediated endocytosis, subcellular trafficking with endosomal compartments, activation of viral phospholipase and escape from the endosome, entry into the nucleus, capsid uncoating and genome release, second-strand synthesis, and persistence of the transgene as episomal DNA concatamers.

Figure 3. Tropism of natural AAV serotypes. Preclinical and clinical data has confirmed that numerous AAV serotypes can effectively transduce a number of tissues including the brain, eye, lung, liver, skeletal muscle, and heart.

Figure 1.

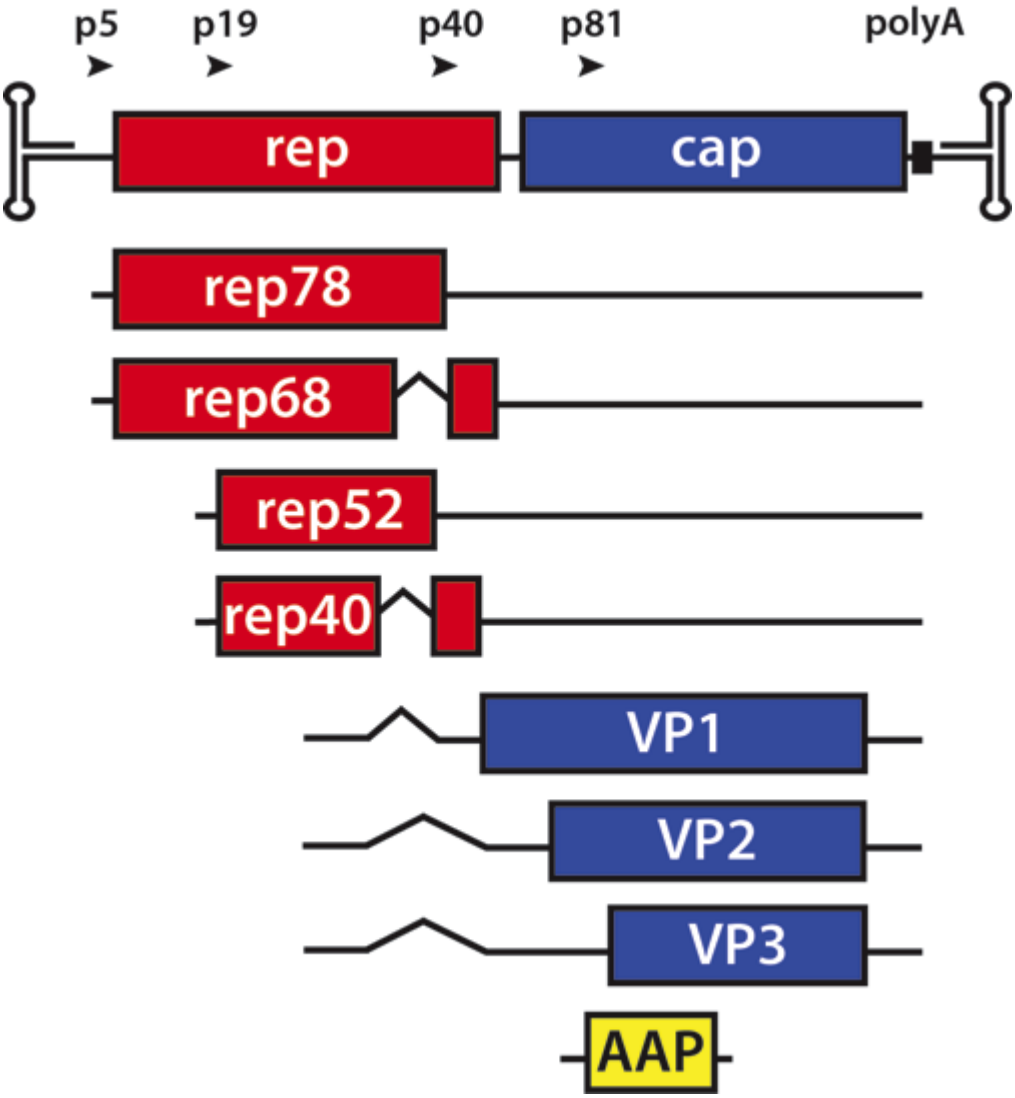
p5
p19
p40
p81
polyA
rep
cap
rep78
rep68
rep52
rep40
VP1
VP2
VP3
AAP

Figure 2.

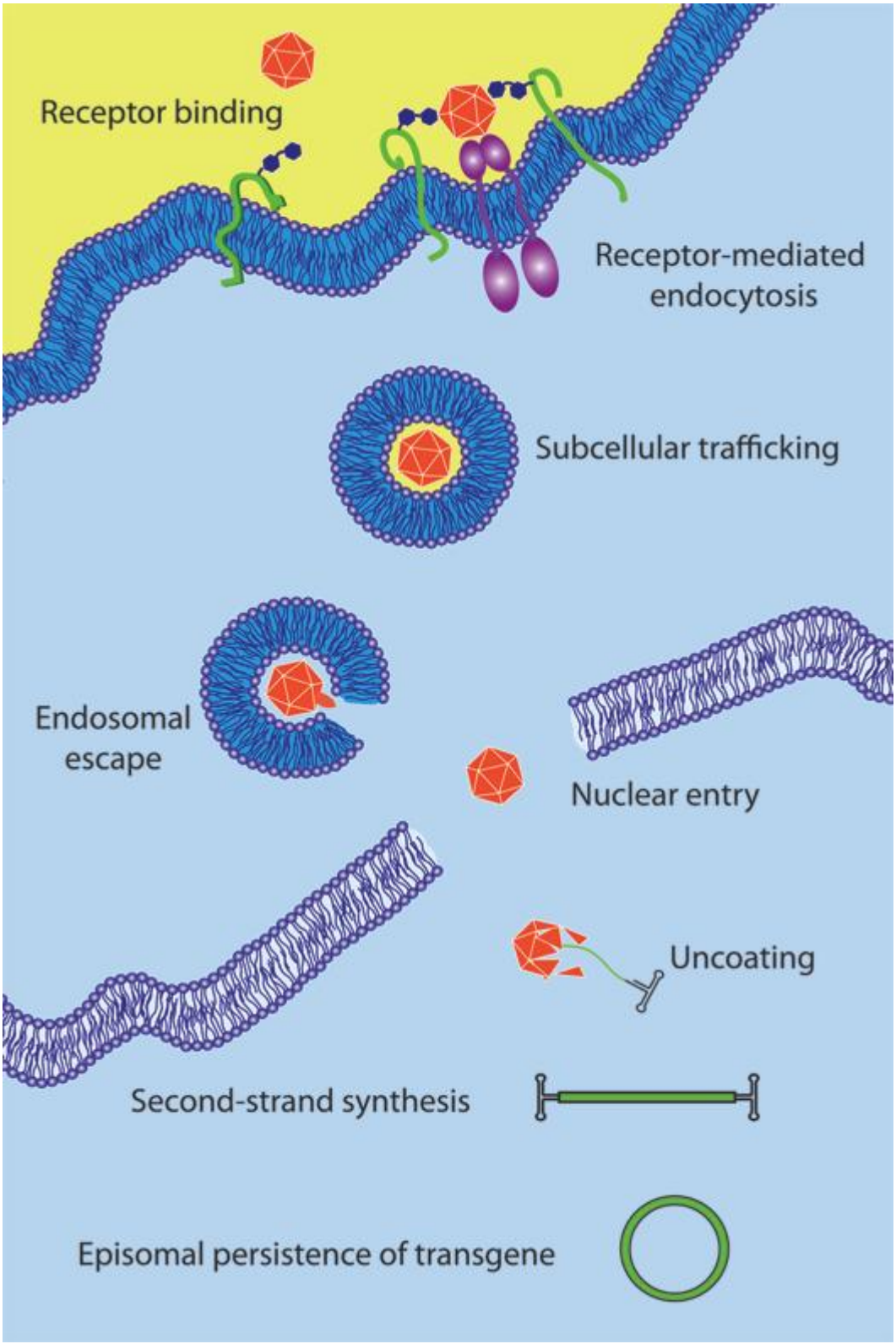
Receptor binding
Receptor-mediated endocytosis
Subcellular trafficking
Endosomal escape
Nuclear entry
Uncoating
Second-strand synthesis
Episomal persistence of transgene

Figure 3.

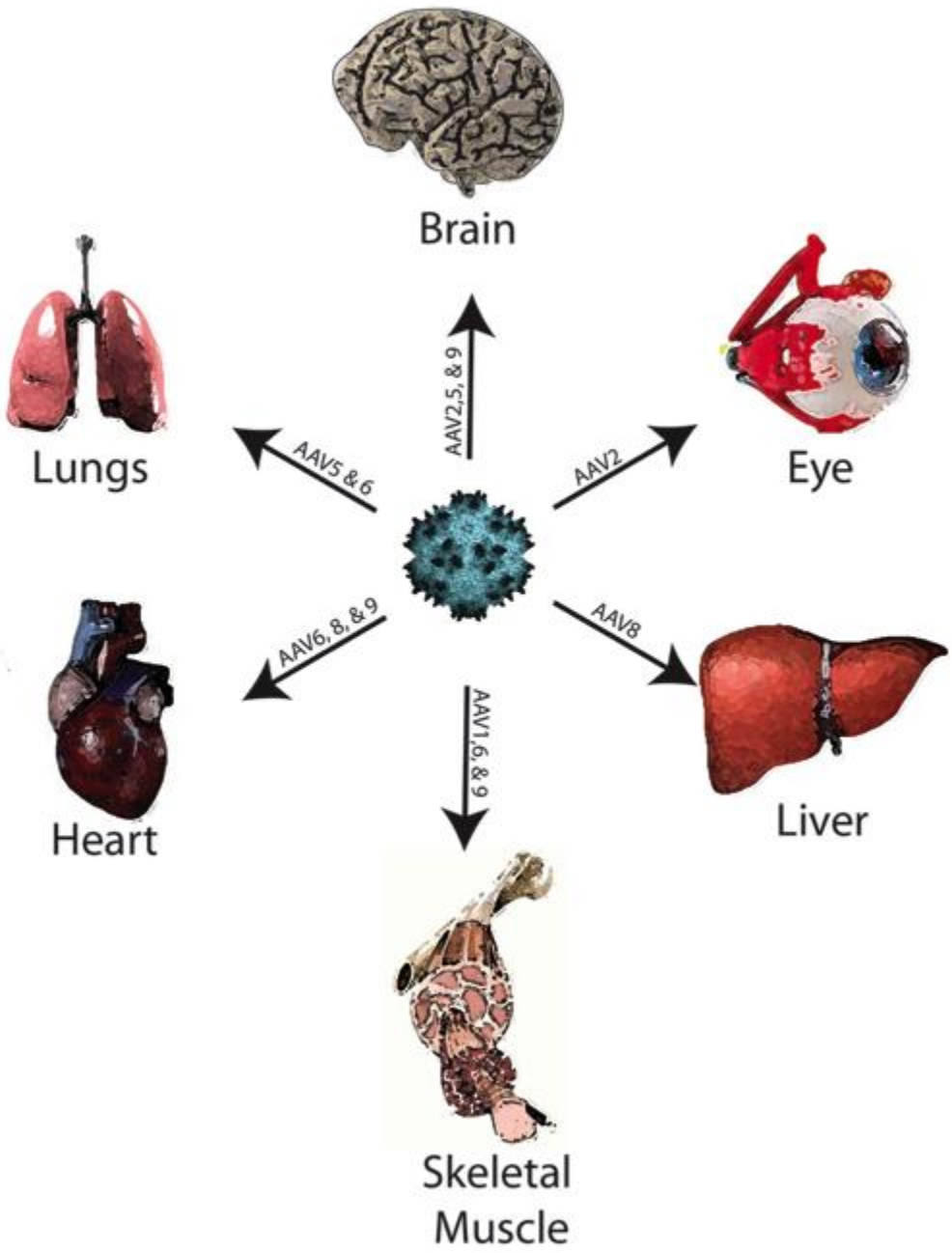
Brain
Lungs
Eye
Heart
Liver
Skeletal Muscle
AAV2,5, & 9
AAV5 & 6
AAV2
AAV6, 8, & 9
AAV8
AAV1,6, & 9

Table 1.

Table 1: Summary of the lessons from clinical trials regarding the biosafety of AAV vectors

	Mode of administration	**Disease/vector**	**Conclusion**	**References**
Shedding and biodistribution				
	Intra-muscular quadriceps (40-60 injections)	LPL deficiency/AAV1-LPL^{S447X}	Shedding (i.e. the dissemination in any form into the environment via excreta (urine, feces, sweat, saliva, nasopharyngeal fluids)) at 24H. serum, saliva, urine. undetectable after 4-6 weeks except in one subject. Presence of viral sequences in semen at low levels at one week, clearance between 6-10 weeks.	(273)
	subretinal	Leber congenital amaurosis type 2 (LCA2)/AAV2.hRPE65	Transient positivity in serum and tears	(280)
Integration				
	Intra-muscular quadriceps (40-60 injections)	LPL deficiency/AAV1-LPL^{S447X}	Largely random	(167)
Immune response				
	Intra-muscular quadriceps (40-60 injections)	LPL deficiency/AAV1-LPL^{S447X}	Anti-capsid antibodies not associated with a drop of transgene expression. No anti-LPL^{S447X} antibodies	(273) (272)
	Intra-hepatic artery	Hemophilia B/AAV2-FIX Hemophilia B/scAAV8-FIX Liver specific promoter/codon optimized-FIX	CD8-positive T-cell responses to the AAV2 capsid/vector clearance in the high-dose cohort. Therapeutically-relevant FIX levels reached in the high-dose cohort. Transient rise in liver transaminases accompanied by a raise in anti-capsids T cells treated by transient glucocorticoïd treatment. Maintenance of transgene expression.	(265) (267)
	subretinal	RPE/AAV2.hRPE65	No neutralizing antibodies, except transiently in one subject. No anti-transgene antibodies. No cellular immune response. No rejection of transduced cells after re-administration in contralateral eye in 3 patients.	(256) (280)

Note: This table reviews only data from clinical trials. The numerous preclinical data available are not thoroughly described in the present review. An extensive review covering pre-clinical and clinical data on the shedding of AAV2 depending on the route of administration until 2010 has been provided by Brandon and colleagues (133).