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# Basic Biology of Adeno-Associated Virus (AAV) Vectors Used in Gene Therapy

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**Abstract:** Adeno-associated virus (AAV) based vectors have emerged as important tools for gene therapy in humans. The recent successes seen in Phase I/II clinical trials have also highlighted the issues related to the host and vector-related immune response that preclude the universal application of this promising vector system. A fundamental insight into the biological mechanisms by which AAV infects the host cell and a thorough understanding of the immediate and long-lived cellular responses to AAV infection is likely to offer clues and help design better intervention strategies to improve the therapeutic efficiency of AAV vectors. This article reviews the biology of AAV-host cellular interactions and outlines their application in the development of novel and improved AAV vector systems.

**Keywords:** Adeno-associated virus, gene therapy, integration, serotype, viral vectors.

## INTRODUCTION

Adeno-associated virus (AAV) is one of the smallest viruses (~22nm) known and belongs to the family *Parvoviridae* and genus *Dependovirus*. It is a non-enveloped virus and is dependent on other helper viruses to complete its life cycle/replication. AAV was first described by Atchinson *et al.,* in 1965 as a contaminant of adenoviral stocks. A high concentration of small uniform virus-like particles was observed when cultures of monkey kidney cells infected with simian adenovirus type 15 (SV15) were analysed by negative staining with phosphotungstic acid under a transmission electron microscope. These virions were found to be replication-defective and their escape from the host cell required super-infection with adenovirus and hence aptly named as AAV. The new virus was also immunologically distinct from Adenovirus [1]. Hoggan *et al.,* in 1966 confirmed these findings and studies carried out later that decade demonstrated the non-pathogenic nature of AAV [2]. The viral genome which was speculated to be double stranded was later identified as a single-stranded DNA [3]. Further extensive studies between 1965-1983 on the properties of AAV led to its development as a gene transfer vehicle (Fig. **1**) [4]. In a seminal study by Hermonat *et al.,* in 1984, the utility of AAV as a mammalian gene transfer vector was first demonstrated. The wild-type AAV capsid coding region was replaced with the neomycin resistance (*neo*) gene under the control of an SV40 promoter and recombinant AAV stocks comprising *neo* transgene were generated by providing *cap* gene in *trans*. The recombinant AAV-*neo* vectors were then shown to successfully transduce mammalian cells. Since then, numerous pre-clinical and clinical studies using AAV vectors have been carried out with the first approved human gene therapy product, Glybera (AAV1 vector encoding lipoprotein lipase) licensed recently in Europe. Despite the many successes achieved with AAV vectors, issues related to its limited packaging capacity, immune response directed against the vector capsid or the transgene product and cross-neutralization by antibodies generated by the host continue to remain [5-8]. Thus a thorough understanding of the biology of AAV- host cellular interactions is necessary to further optimize and expand the utility of this promising vector system.

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## LIFE-CYCLE

Two distinct phases have been recognized in the life cycle of AAV: (1) Lysogenic or latent phase and (2) the lytic phase (Fig. **2**). In the absence of helper viruses such as adenovirus or herpes simplex virus, AAV establishes latency in the host by integration into a specific site in chromosome [(Chr) 19 (q13.4)] termed as the Adeno- associated virus integration site 1 (AAVS1). The earliest evidence of this phenomena was shown in human bone marrow derived fibroblast-like *Detroit 6* cells, which upon infection with AAV2 demonstrated the persistence of the viral DNA for ~47 passages, with a genome copy number ranging between 3-5 per diploid cellular genome [9]. Further characterization has revealed a tandem head-to-tail repeat integration through the viral inverted terminal repeat (ITR) elements, thus allowing the virus to be quiescent in the host cell [10]. Several reports suggest that this insertion disrupts the myosin binding subunit 85 (MBS85), a protein involved in actin-myosin fiber assembly [11-14]. However, the consequence of this gene disruption is not clear/understood. A majority of the integration sites are within the first exon/intron region in MBS85 gene, that has only one intact and functional allele for MBS85 protein generation [12, 15]. The survey of integration sites for wild type AAV2 in human genome has revealed other integration sites such as chr. 5p13.3 (AAVS2) and chr. 3p24.3 (AAVS3) which constitute the conserved rep-binding elements within the host genome [16]. The fre-

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quency of integration in the AAVS1 site in human and rhesus macaque tissues has been estimated to be 0.5% [17-19].

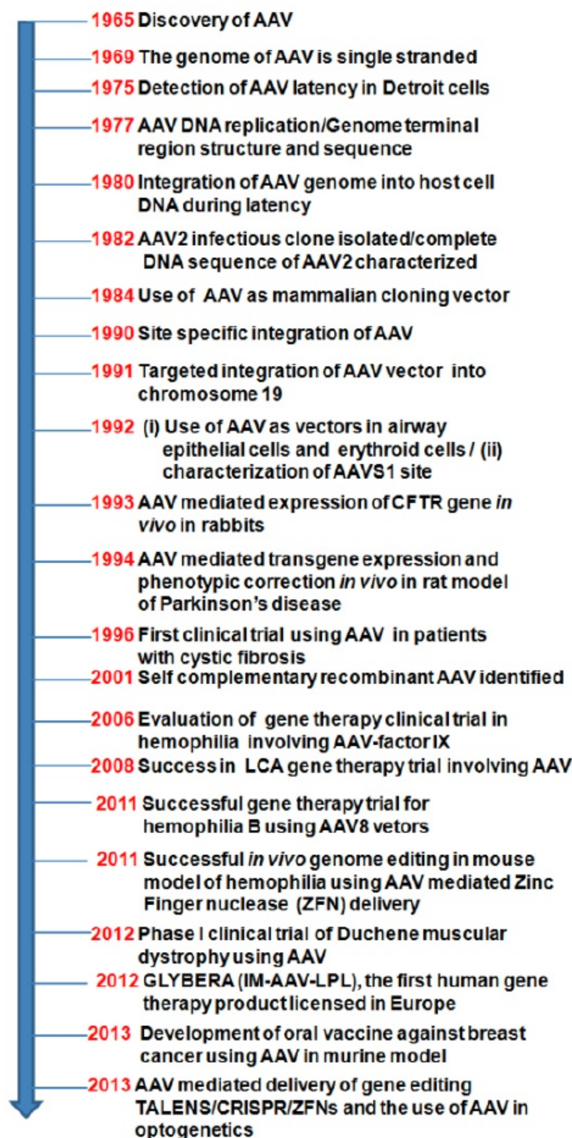



**Fig. (1).** Milestones in understanding of AAV vector biology

The lytic phase is initiated when cells containing AAV are infected with a helper virus. This phase is characterized by Rep-mediated excision of the provirus from the host genome, its replication, viral gene expression and the packaging of the viral genome. The newly assembled AAV is then released by helper virus induced cell lysis. Various helper virus gene products in this phase that favour virion generation are listed in Table **1**.

## GENOME

AAV genome consists of a linear single stranded DNA which is ~4.7kb in length (Fig. **3A**). The genome consists of two open reading frames (ORF) flanked by an inverted terminal repeat (ITR) sequence that is 145bp in length. The ITR consists of a 125 base palindromic sequence that forms T-shaped hairpin structure by complementary base pairing. The remaining 20 bases remain unpaired and are designated as the D-sequence. The ITR also has a rep-protein binding site (RBS) and a terminal resolution site (TRS) both of which function as the origin of replication. The secondary structure of the ITR has a free 3' hydroxyl group which initiates DNA replication through self priming and second strand synthesis. The double stranded form of the genome serves as the replication intermediate. Strand displacement mechanism ensures the production of single stranded AAV genome for packaging and the double stranded intermediate for viral gene expression. During the packaging of viral genome into the capsid, both the sense and anti-sense strands are packaged with comparable frequency and efficiency.

### Rep Gene

The left ORF encodes proteins namely Rep78, Rep68, Rep52 and Rep40 (designated based on molecular weight) which are required for replication of the virus (Fig. **3B**). There are two internal promoters within the left ORF. The P5 promoter facilitates Rep78 and Rep68 transcription and the P19 promoter facilitates Rep52 and Rep40 transcription. These promoters are activated by co-infecting helper virus. Rep78 and Rep68 are involved in various stages of AAV life cycle such as DNA replication, site-specific integration, excision of the genome from the host and regulation of gene expression. Overexpression of Rep78 is known to have a significant anti-proliferative effect on the host cells as shown in NIH3T3, mouse embryonic fibroblasts (MEFs) and human lung fibroblasts [31]. The Rep78 protein has also been shown to nick the cellular chromatin at rep binding sites which occur at a frequency of ~$2x10^5$ sites in the human genome [32]. Thus by inducing DNA damage, the Rep78 protein is known to completely arrest the host cell in S phase and facilitate the latent infection by the virus [33]. Rep68 is a site-specific endonuclease that can unwind DNA. It is generated as a splice variant under the control of the P5 promoter. This protein shares a modified version of AAA+ domain belonging to SF3 family of helicases and an amino terminal DNA binding domain with Rep78. This protein thus exhibits flexible oligomeric behaviour necessary for its multiple functions such as initiating DNA replication, regulating AAV DNA transcription and site-specific integration [34-36]. Rep52 and Rep40 proteins are DNA helicases with 3'-to-5' polarity essential for the accumulation and packaging of the genome into the capsid. These Rep proteins also play an important role in gene regulation by repressing P5 transcription in the absence of helper virus infection [37, 38].

### Cap Gene

The right ORF encodes three viral proteins VP1, VP2 and VP3 that constitute the icosahedral capsid (Fig. **3A**, Fig. **4**). The P40 promoter initiates the transcription of the cap gene and generates two transcripts by alternative splicing. While the unspliced transcript forms the 87KDa VP1 protein, the splice variants encode the 72KDa VP2 protein from an alternate start codon, ACG and the 61KDa VP3 from the conventional start codon, ATG (Fig. **3**). Approximately 60 copies of VP1, VP2 and VP3 are assembled into an icosahedral particle of 260A$^o$ diameter in a molar ratio of 1:1:10 [39-41]. The capsid is the metastable entity protecting the infectious genome and its prominent biological function

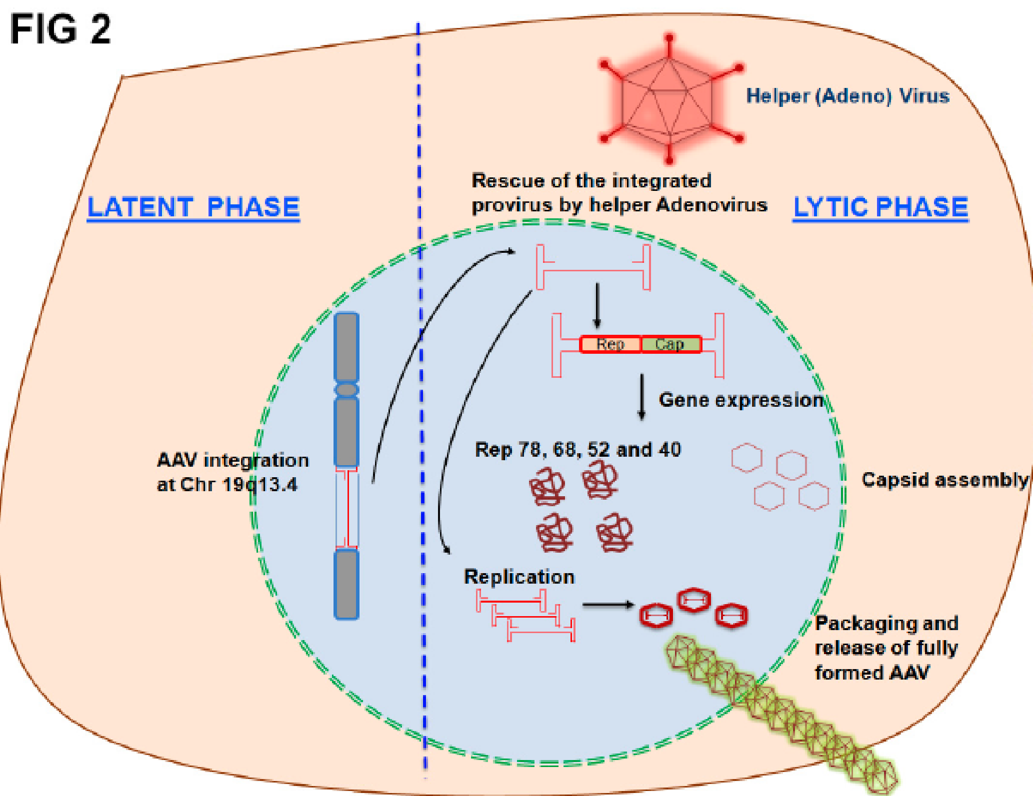
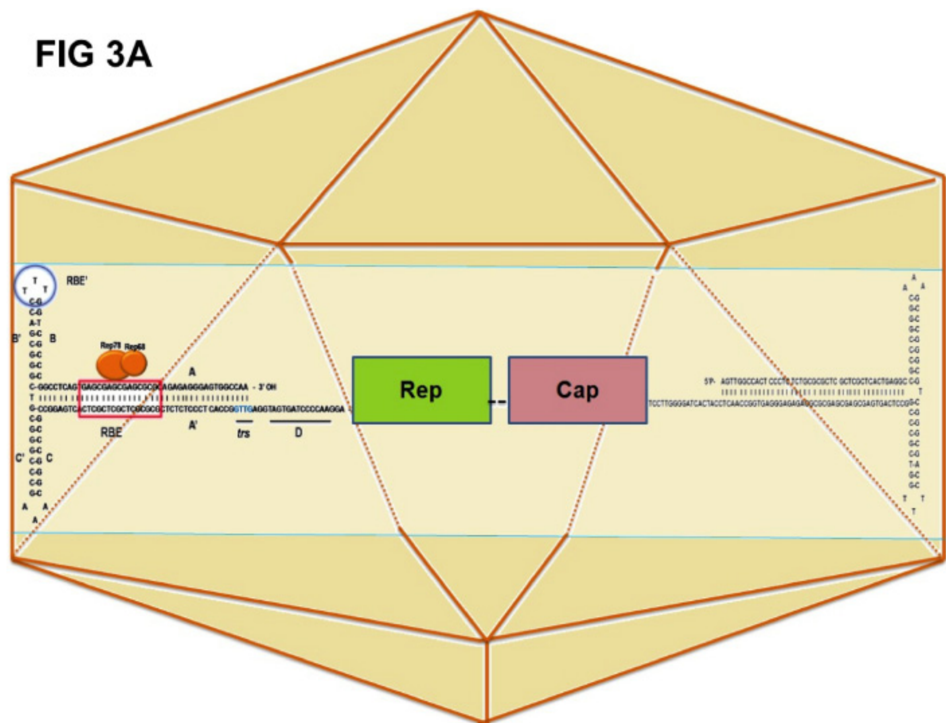



**Fig. (2). AAV life cycle.** AAV has two distinct phases in its life cycle. (1) latent phase and the (2) lytic phase. In the absence of super infection with a helper virus (adenovirus or herpes simplex virus), AAV establishes latency in the host by integration into AAVS1 site in chromosome 19 (q13.4) [9]. The lytic phase is initiated when cells containing integrated AAV vectors are super-infected with a helper virus. This phase is characterized by a series of events like Rep-mediated rescue (excision) of the provirus from the host genome, replication, viral gene expression and virion production involving packaging of the viral genome. The helper viruses activate promoters P5 and P40 to initiate transcription of the Rep proteins (Rep 78, 68, 52 and 40) and the Cap proteins. Rep52 and Rep40 mediates accumulation and packaging of the single stranded genome into the capsid [70]. The newly assembled AAV is then released by helper virus induced cell lysis.

**Table 1. List of helper virus gene products that facilitate AAV replication.**

| Helper Virus Genes | Function | Reference |
|---|---|---|
| Adenoviral early 1a gene (E1a) | Relieves repression of AAV P5 promoter | Chang *et al.*, 1989 [20]<br>Shi *et al.*, 1991 [21] |
| Adenoviral early 2a gene (E2a) | Single strand DNA binding protein found in AAV replication center | Weitzman *et al.*, 1996 [22]<br>Ward *et al.*, 1998 [23] |
| Adenoviral early genes (E1b55k and E4) | Promotes AAV replication and second strand synthesis | Samulski *et al.*, 1988 [24]<br>Fisher *et al.*, 1996 [25] |
| Adenoviral Viral associated (VA) RNA | Stimulates AAV protein expression | Nayak *et al.*, 2007 [26] |
| Herpes simplex virus-1 helicase/primase proteins UL5, UL8 and UL52, ICP8 | AAV replication | Weindler *et al.*, 1991 [27]<br>Stracker *et al.*, 2004 [28]<br>Alex *et al.*, 2012 [29] |
| Herpes simplex virus-1 infected cell proteins (ICP20, ICP4 and ICP22) | Activates AAV rep gene expression | Geoffroy *et al.*, 2004 [30] |

involves cell surface receptor binding as well as facilitating intracellular trafficking of the virus. The capsid protein and symmetry is also known to determine the tissue tropism of AAV. The N-terminal region of VP1 has a unique phospholipase A2 (PLA2) motif and a putative nuclear localization signal (NLS). Targeted disruption of the PLA2 motif at select histidine and aspartic acid catalytic amino acids to alanine and asparagine is known to decrease viral infection by a magnitude of three orders [42]. This has been attributed to the prevention of virion escape from the endosomes, thus suggesting a major role for the phospholipase domain in this process [43-45]. Johnson *et al.*, demonstrated that a BR3+K



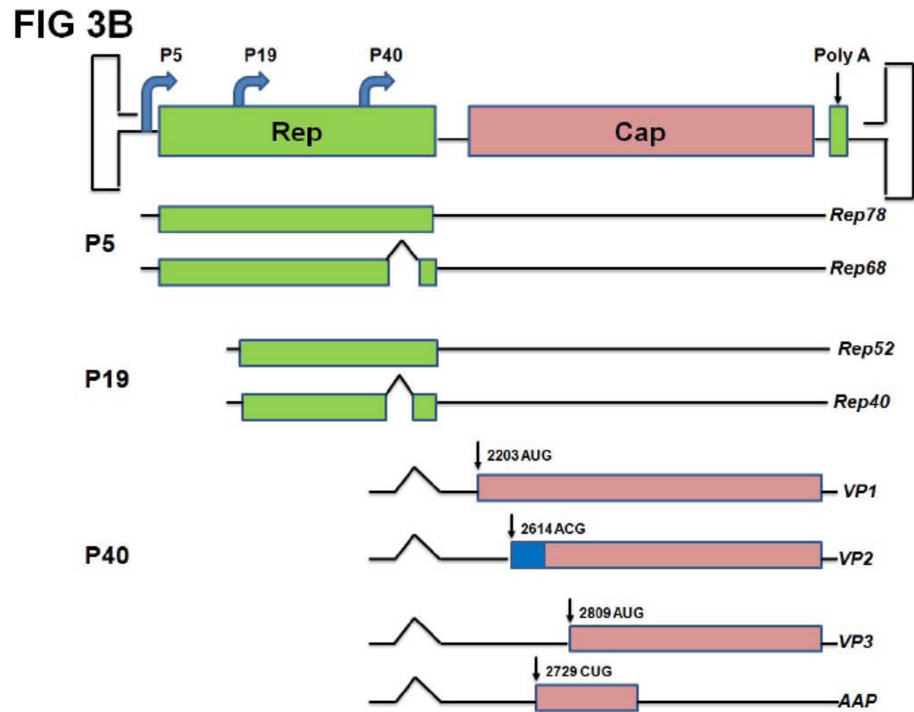




**Fig. (3). AAV genome. A.** AAV genome is a linear single stranded DNA of ~4.7kb in length. The genome consists of two open reading frames (ORF) flanked by a 145bp inverted terminal repeat (ITR) sequence. One ORF codes for the Rep proteins and the other for the capsid proteins. The ITR consists of a 125bp palindromic sequence that forms T-shaped hairpin structure by complementary base pairing. The remaining 20 bases remain unpaired and are designated as the D-sequence. The ITR also has a rep-protein binding site (RBS) and a terminal resolution site (*trs*) which together function as origin of replication. **B.** The left ORF of AAV encodes four non-structural proteins namely Rep78, Rep68, Rep52, and Rep40 which are required for viral replication. The P5 internal promoter facilitates Rep78 and Rep68 transcription, while the P19 promoter facilitates Rep52 and Rep40 transcription. The right ORF encodes three capsid viral proteins-VP1, VP2 and VP3 that constitute the icosahedral capsid. The cap gene is transcribed from the internal P40 promoter. Alternative splicing generates two transcripts; (i) unspliced transcript forms the entire capsid (VP1) (ii) splice variant encodes the 72kDa VP2 protein from an alternate start codon (ACG) and the 61kDa VP3 from the conventional start codon (ATG). An alternate ORF that encoding a protein called assembly activating protein (AAP~22kDa) is present upstream of VP3 coding sequence, whose expression is through an unconventional translational initiation codon, CTG [44].

mutant (alanine to lysine mutation in VP1) resulted in 10 to 100 fold less infection due to impaired trafficking. These studies confirm the role of VP1 in cellular intracellular trafficking [46]. More recently an alternate ORF that encodes a protein, assembly activating protein (AAP, ~22KDa) has been described upstream of VP3 coding sequence whose expression is known to be driven by an unconventional translational initiation codon, CTG. This protein has been shown to be involved in the transportation of the native VP proteins into the nucleolar region for capsid assembly [47].

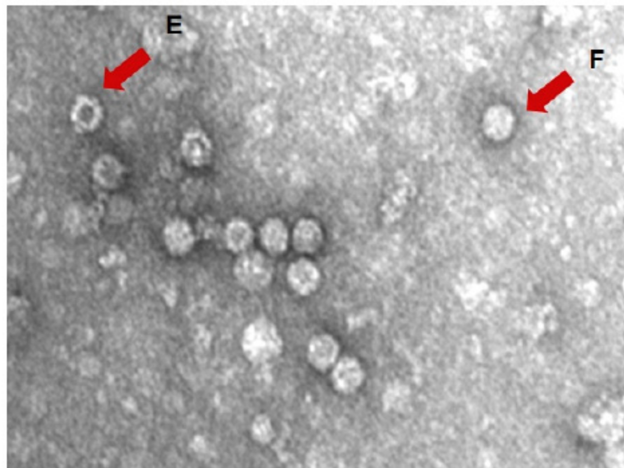



**Fig. (4). Electron micrograph of AAV vectors.** AAV vector preparations were negatively stained by phosphotungstic acid and loaded on to a foamvar copper coated grid. The images were captured using a transmission electron microscope at a magnification of 60000X. The figure shows the presence of both empty capsids (E) and fully formed capsids (F).

## SEROTYPES

So far, 12 different AAV serotypes and 108 new isolates (serovars) have been classified based on phylogenetic analyses [48, 49]. AAV serotypes 1, 3, 4, 7, 8, 10 and 11 were originally isolated from non-human primates while serotypes 2, 5, 6 and 9 are of human origin (Table **2**). These serotypes exhibit diverse tissue tropism by virtue of their use of different cellular receptors and co-receptors (Table **2**). High resolution structural analysis of the capsids by cryo-electron microscopy or by X-ray crystallography has comprehensively characterized the most abundant VP3 capsid protein. Each VP has a core region containing an eight-stranded β-barrel motif (βB to βI) and an alpha helix (αA). The surface loops connect the β-strands to produce structurally variable regions (VR). These VRs cluster together and contribute to local variations in the capsid structure. Such conformational variations in these VRs of different serotypes are believed to determine their host cellular tropism and transduction efficiency. It is based on this phenomenon that human trials have attempted to use specific AAV serotype vectors for various disease states and for efficient tissue targeting (Fig. **5**).

## TROPISM

Unlike wildtype AAV vectors, pseudotyping of recombinant AAV has been useful to generate vectors with substantial diversity in tissue tropism [60, 61]. Pseudotyping refers to cross packaging of the AAV genome between various serotypes. This process is generally carried out with the AAV2 genome and packaged with different capsid proteins [62]. Even manipulations to the packaging DNA sequence (ITR) of these viruses to their parent serotypes (*i.e.* AAV6 pseudotype having AAV2 ITR and AAV6 capsid) does not alter their tissue tropism confirming that the capsid proteins alone are responsible for tissue specificity [63]. Based on *in vivo* studies, vectors specific to retina (AAV2, AAV4 and AAV5) [64-68], brain (AAV2, AAV5, AAV8, AAV9 and AAVrh10) [69-78], cardiac tissue (AAV1, AAV2, AAV6, AAV8 and AAV9) [79-83], liver (AAV1, AAV2, AAV5, AAV6, AAV7, AAV8, AAV9 and AAV10) [49, 84-93] and lungs (AAV1, AAV2, AAV5 and AAV9) [86-89, 92-98] have been identified. It has been shown that intra venous (i.v.) injection of AAV vectors facilitates their rapid distribution to the vasculature of different organs [98]. Studies have also sought to alter the receptor foot print of AAV vectors [99]. These studies underscore the power of molecular tools to generate viral vectors with innumerable receptor footprints.

## INFECTION

AAV infection is a dynamic multi-step process. It includes the attachment of the virus to the cell surface receptor, its internalization, endosomal trafficking, nuclear import and gene expression/replication (Fig. **6**). The first step is the attachment of the viral capsid to its primary receptor on the host cell surface. As listed in Table **2**, various AAV serotypes use different cell surface receptors. However, proteoglycan conjugates generally act as their primary receptor while O- or N- linked sugars and proteins act as co-receptors. AAV2 is the prototype vector whose primary receptor, the heparin sulphate proteoglycan (HSPG) was first described in 1998 [100]. It must be noted that the natural AAV2 variants found in humans do not use HSPG as the receptor, suggesting that the HSPG usage in cell lines could be an *in vitro* adaptation [101]. Nevertheless, competition assays *in vitro* and subsequent structural analysis has led to the discovery of multitude of primary and co-receptors for other AAV serotypes. The receptor binding site on AAV capsid is believed to be on the 3-fold proximal spike. In case of AAV2, the binding site is centered at $Arg_{585}$ and $Arg_{588}$ on the sides of the 3-fold proximal spike [102, 103]. The binding of the capsid to the receptor also induces a change on capsid structure which is speculated to further enhance the intracellular trafficking of the virus [104, 105].

### Endocytosis

Cellular endocytosis is a process through which cells uptake macromolecules from the surrounding environment. Available evidence suggests that internalization of AAV is due to the receptor-mediated endocytosis. Upon binding of the viral particle, the plasma membrane invaginates to form a vesicle around the virions. Several mechanisms have been proposed for this process, including dynamin dependent endocytosis [106, 107], clathrin coated vesicle formation [106, 107] or a dynamin/clathrin independent mechanism such as macropinocytosis [108, 109]. Initial experiments with HeLa cells demonstrated that AAV2 is internalized by

**Table 2. Characteristics of AAV serotypes 1-12.**

| Serotype | Origin | Source | Primary receptor | Secondary receptor/co-receptor | Capsid homology to AAV2 | Crystal structure-PDB ID |
|---|---|---|---|---|---|---|
| AAV1 | Non human primate | As a contaminant in simian Adenovirus type 15 stock (SV15) [2] | α2–3/ a 2–6 N linked sialic acid | | ~83% | 3NG9 |
| AAV2 | Human | As a contaminant in Adenovirus type 12 stock [2] | Heparin sulphate proteoglycan (HSPG) | FGFR1, HGFR, integrins, 37/67 kDa LamR | 100% | 1LP3 [50] |
| AAV3 | Non human primate | As a contaminant in Adenovirus type 7 stock [2] | HSPG | 37/67 kDa LamR, HGFR | 88% | |
| AAV4 | Non human primate | African green monkeys infected with SV15 [51] | α2–3 O linked sialic acid | | 60% | 2G8G [52] |
| AAV5 | Human | Human penile condylomatous wart [53] | α2–3 N linked SA, | PDGFR | 57% | 3NTT |
| AAV6 | Human | Contaminant in lab adenoviral stock [54] | HSPG, α2–3/ α 2–6 N linked Sialic acid | EGFR | 83% | 3OAH [55] |
| AAV7 | Non human primate | Rhesus monkeys[49] | | | 82% | |
| AAV8 | Non human primate | Rhesus monkeys[49] | | 37/67 kDa LamR | 83% | 2QA0 [56] |
| AAV9 | Human | [48] | N-linked galactose | 37/67 kDa LamR | 82% | 3UX1 [57] |
| AAV10 | Non human primate | cynomolgus monkeys[58] | | | | |
| AAV11 | Non human primate | cynomolgus monkeys[58] | | | | |
| AAV12 | Non human primate | Simian Adenovirus type18 stock [59] | | | | |

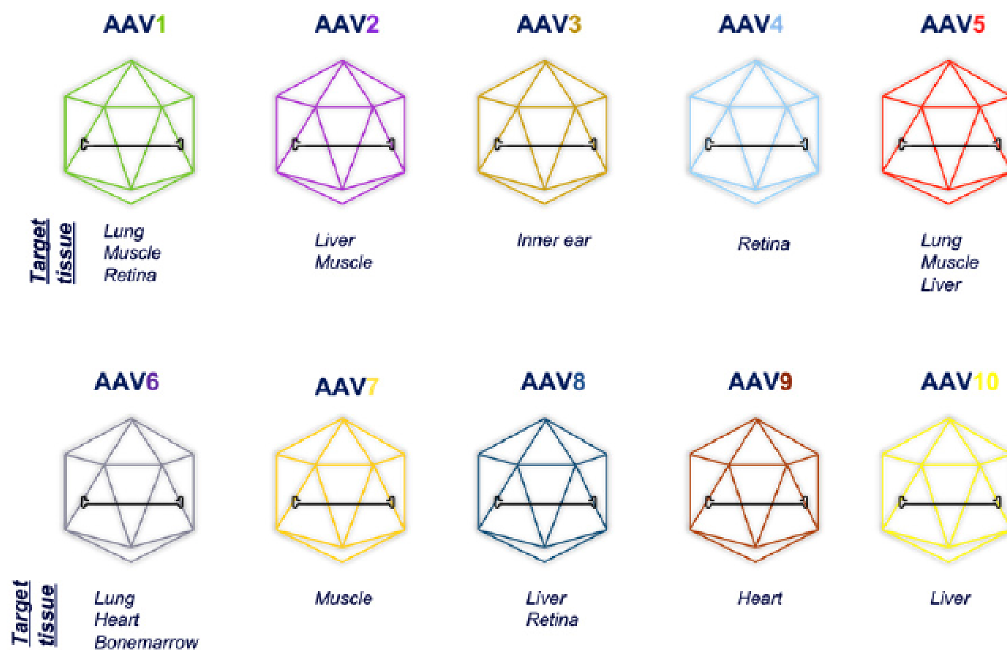



**Fig. (5).** Ten common serotypes of AAV vectors and their tissue tropism.

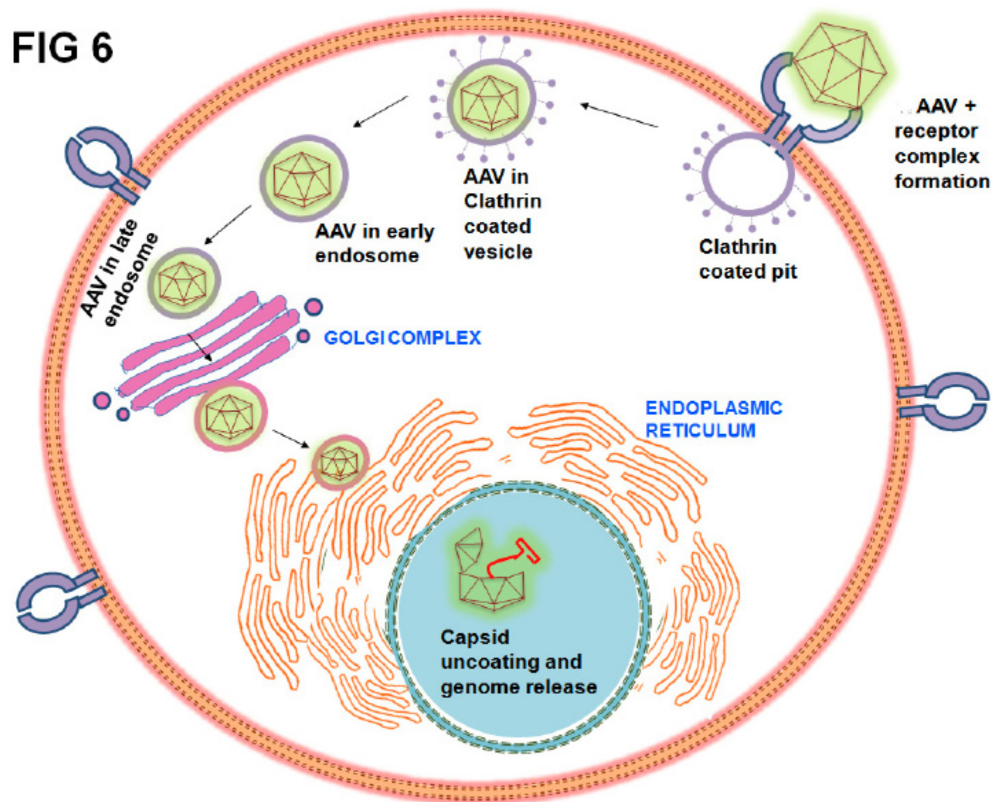



**Fig. (6). AAV infection.** The first step of infection is the binding of the AAV particles with the cell surface receptor (e.g. HSPG) to form an AAV-receptor complex [97, 98]. These receptors are concentrated on specialized area called clathrin-coated pits. This complex is then internalized *via* endocytosis [106, 107]. Intra-cellular trafficking is then initiated with the formation of endosomes. A typically low pH of 6 in early endosome and in late endosome (pH 5) induces a conformational change in the AAV capsid to expose the N-terminal phospholipase A2 domain and nuclear localization signal [42, 46, 116, 117]. This facilitates their endosomal escape and the AAV particles traverse through golgi complex and endoplasmic reticulum *via* the retrograde transport mechanism. Upon translocation into the nucleus, the vector undergoes uncoating of its capsid and the genome is released [109, 123]. The genome might then integrate into the host genome at the AAVS1 site. An elegant model for such an integration process has been discussed in detail elsewhere [14, 189]

clathrin-mediated endocytosis. The host cell surface receptors are concentrated in a specialized area called clathrin-coated pits [106, 110]. HeLa cells expressing a mutant form of dynamin (K44A), a protein involved in cleaving clathrin coated pits, when transduced with AAV2 demonstrated a marked reduction in gene expression despite rapid internalization (<10 mins) of the clathrin coated vesicles [106]. However, the inhibition of dynamin by overexpression of a dominant negative mutant K44A did not completely inhibit this process suggesting that AAV2 can also use alternative routes of cellular entry [107]. More recently studies using chlorpromazine a drug that inhibits the formation of clathrin-coated vesicles has confirmed that AAV2 transduction is independent of this process and that clathrin-independent carriers (CLIC) and GPI-anchored-protein-enriched endosomal compartment (GEEC) pathways may be involved [111]. Similar data has also been obtained with AAV2 intracellular tracing studies [112].

## Retrograde Transport

The intra-cellular trafficking is a major rate-limiting step in AAV transduction [113]. For e.g., experiments performed with AAV tagged with a fluorescent marker (Cy3) has shown that only a small fraction of the virus enters into the nucleus while the rest accumulates in the perinuclear region [106]. It is now well recognized that AAV particles traverse through early endosome, late endosomes and the recycling endosomes before their entry into the nucleus [114, 115]. Evidence for this has been generated with quantum dot tagged AAV where co-localization was observed with early endosome antigen 1 (EEA1), cation-independent mannose 6-phosphate receptor (CI-MPR, late endosomal marker) and Rab11 (recycling endosome marker) at 15 to 30 mins post infection [112]. The low pH (pH 6.0 to 5.5) of the endosomal compartment imparts conformational change to the capsid that facilitates its trafficking [42]. The acidification of the capsid, triggers the exposure of N-terminal phospholipase A2 domain and nuclear localization signal of VP1 protein, facilitating their endosomal escape and nuclear entry by traversing the cellular organelles such as golgi and endoplasmic reticulum [42, 46, 116, 117]. Agents like bafilomycin or ammonium chloride which can buffer the endosomal pH, have been shown to inhibit AAV infection by ~10 fold, confirming that this is a crucial step in intracellular trafficking. The involvement of golgi apparatus in this process was deduced from the increased localization of mutant AAV capsids within the golgi apparatus and golgi-associated regions [46] when dilysine residues within the N-terminal region of VP1 protein were mutated (BR1⁻ [120QAKKR/QANNR]).

Salganik *et al.*, 2012 demonstrated that the acidic pH (5.5) in the late endosomal compartment can induce the autolytic protease activity of capsids of AAV serotypes 1, 2, 5 and 8 and result in structural changes in the capsid [118]. In classical retrograde trafficking [119], the cytoplasmically exposed dilysine residues (KKXX) in the trafficked molecule is presented by the golgi transmembrane proteins to the cytoplasm where the coatomer- protein-complex transfers these vesicular targets to the endoplasmic reticulum. The role of endoplasmic reticulum in AAV trafficking has been demonstrated by the activation of an endoplasmic reticulum stress response pathway, the unfolded protein response (UPR) [120]. In this study, AAV vectors were shown to activate specific pathways of the cellular unfolded protein response (UPR) (PERK and IRE1α) and which seem to target the AAV capsids for degradation. Pharmacological inhibition of this process modestly increased transgene expression by about 3 fold [120]. Data from cells treated with pharmacological agents (e.g. nocodozole) and live cell imaging has also shown that the cellular microtubule network plays an important role in virus trafficking. The disruption of the cellular microtubules by nocodozole, allows only 3% of AAV2 particles into the nucleus while ~10-15% of virus gain nuclear entry in case of untreated cells. The association of AAV2 virions and the microtubules has been further confirmed by their co-localization using confocal microscopy[121].

## Nuclear Translocation and Uncoating

The translocation of AAV across the nuclear membrane is poorly understood. Several studies have tried to address if AAV enters the nuclear compartment as intact virions or if only the genome is transported after the uncoating of the capsid. Studies have shown the presence of a majority of capsid encoded genome accumulated in the perinuclear region by *fluorescent in situ hybridization* suggesting that uncoating happens either just before or during nuclear entry [122]. On the contrary, immunofluorescence microscopy and microinjection of anti-AAV antibodies that bind specifically to intact particles have presented structural and functional evidence that AAV uncoats in the nucleus [42, 109, 123]. It is also known that the virions accumulate in the nucleolus after infection, but empty capsids are excluded from further nuclear entry. Isolation of these virions from the nucleolus by sub-cellular fractionation and their secondary infection has demonstrated their infectivity as well [124]. In line with these observations, proteasome inhibitors (MG132) are known to increase nucleolar accumulation while hydroxyurea mobilizes capsids to the nucleoplasm [124]. Since the two functional elements on the N-terminal domain of VP1/2 (i) phospholipase A2 domain and (ii) putative NLS play an important role in sub-cellular trafficking, several studies have attempted to disrupt this process [42, 116]. In particular, a BR3+K mutant (alanine to lysine mutation in VP1 to disrupt the NLS) was 10- to 100-fold-less infectious than wild type AAV2. It was found that this mutant trafficked identically to the wildtype but seem to accumulate preferentially in the nucleolar region. This study presents strong evidence that AAV uncoating happens in the nucleus [46, 124].

## Gene Expression and Replication

Once the genome of AAV is released into the nucleus, the single stranded DNA is converted into double stranded DNA initiating its replication and gene expression. A general model of AAV DNA replication that is accepted is the unidirectional strand displacement mechanism. The AAV ITR, due to its self complementary nature, anneals together to form a secondary hairpin structure and has a free 3' hydroxyl group. The self annealed structure of the ITR thus serves as the replication primer. This leads to unidirectional synthesis of the secondary strand which is believed to occur using host cellular replication machinery. The ITR replication is completed by the Rep78 and 68 proteins. The Rep proteins bind to the RBS within the ITR leading to the regeneration of the 3'OH group by site specific nicking at the terminal resolution site (TRS) in the ITR [125, 126]. This leads to replication of the ITR sequence. The large Rep 78/68 proteins possess endonuclease, helicase and ATPase enzymatic activities. The N-terminal domain of these proteins recognise GAGC sequence motif that is repeated imperfectly four times within the ITR [127, 128]. The Rep helicase unwinds the ITR and the rep endonuclease activity nicks the DNA. This whole process generates either the double stranded intermediate AAV genome or the single stranded full length genome. Apart from the ITR and Rep proteins, *in vitro* AAV replication assays demonstrate the requirement of replication factor C (RFC), proliferating cell nuclear antigen (PCNA) and polymerase δ as critical factors required for viral replication. RNA interference studies both *in vitro* and *in vivo* have also suggested that a cellular helicase complex- minichromosome maintenance complex (MCM) along with RCF, PCNA and polymerase δ forms a minimal protein complex required for this replication [129]. A large scale proteomic analysis of HEK 293 cells infected with AAV and affinity tagged *rep* protein has identified 188 cellular proteins as binding partners of rep proteins. As proposed by Nash *et al.*, these proteins normally function as transcription factors, translation factors, potential splicing factors and are generally involved in DNA replication or repair. Further co-immunoprecipitation and co-localization studies has revealed additional rep associated cellular proteins, such as reticulocalbin-1 (RCN1) (membrane transport), structural maintenance of chromosomes protein 2 (SMC2) (chromatin dynamics), EDD1 (ubiquitin ligase), insulin receptor substrate 4 (IRS4) (signal transduction) and FUS (splicing). However their exact role in virus replication is still not clear [130].

As outlined in Table **1**, co-infection with the helper virus can activate the viral promoter and initiates the transcription of the Rep and Cap genes. The Rep proteins can activate the gene expression only in the presence of a helper virus [131, 132]. The P5 and the P19 promoters are the first to be activated by the helper virus. This primarily drives the expression of Rep proteins as well as cellular factors such as YY1, a human GLI-Kruppel-related protein [21, 133]. Both P5 and P19 promoters act as a switch element involved in transcription, replication, and site-specific integration [134, 135]. The activation of these promoters leads to the generation of Rep78, Rep68, Rep 52 and Rep 40. The activation of P40 leads to the synthesis of VP1, VP2 and VP3 proteins which are responsible for assembling the AAV capsids. Upon capsid formation, the single stranded genome is packaged into the capsids, a process mediated by Rep52 and Rep40 proteins (42).

### AAV and Mitochondria – The Missing Link?

Even though a vast majority of the studies have tried to dissect out the steps in intracellular trafficking of AAV, it is still not clear if AAV trafficks through the cellular mitochondria. It is generally believed that mitchondrial infection requires retargeting of AAV. In a mouse model of Leber's hereditary optic neuropathy (LHON), a degenerative vision disorder caused due to mutations in mitochondrial NADH dehydrogenase subunit 4 (ND4) gene, a retargeted AAV vector comprising a mitochondrial targeting sequence (MTS) fused to VP2 capsid protein was used to successfully deliver the wild type ND4 gene [136]. However, conflicting data has been obtained in a recent study where five lipoprotein lipase deficient patients were adminstered witth AAV1-LPLS447X vectors. Analysis of samples from these patients by LAM-PCR has revealed a number of hotspots or common integration sites (CIS) in the mitochondria. Mitochondrial DNA enrichment and direct sequencing further validated this concept of AAV integration in mitochondria in these subjects [137]. Further detailed studies are necessary to better understand the link between AAV and mitochondria and the factors that favour AAV integration into this organalle.

### Integration

As described above, in the absence of helper function the virion tends to establish latency by integrating into the AAVS1 loci in the human genome [92]. The region around AAVS1 site in chromosome 19 (q13.4) contains characteristic CpG islands and tandem GCTC repeat elements. These serve as binding sites for the Rep78/68 proteins. The AAVS1 site also contains a Rep specific nicking site called *trs* [138]. Initial biochemical assays demonstrated that Rep78/68 stably binds to the GCTC repeat in the AAVS1 site and mediates the complex formation between AAVS1 site and the ITR. Interestingly, the integration frequency was enhanced (5% to 47%) when the rep gene function was provided in *trans*. This provided robust evidence that integration of AAV is mediated by the large Rep proteins [139]. Similar functional assays with the use of episomal AAVS1 fragments revealed that the RBS and *trs* at the AAVS1 site are alone sufficient for the site-specific integration [140]. A general model accepted for site specific integration involves the rep78/68 mediated replication from the chr19 origin of replication (*ori*). This is followed by tethering of the strands between the chromosomal and proximal viral DNA template and the subsequent incorporation of many copies of AAV genome.

The fact that AAV Rep proteins can mediate site specific integration has been exploited to enhance gene targeting using AAV vectors. By co-transfecting the AAV Rep78 expressing plasmids along with the AAV vector comprising the transgene, targeted gene integration has been demonstrated at the AAVS1 site [139, 141, 142]. However the episomal maintenance of the Rep78 plasmid is known to be toxic to the host cells and may lead to chromosomal instability as well. Nevertheless this can be potentially circumvented by direct introduction of the Rep protein [143] or by conditional activation of the Rep protein [144]. The Rep68/78 has also been transiently expressed as mRNA to facilitate targeted gene integration [145]. However, Rep mediated cytotoxicity still remains a major concern. The concept of Rep mediated site specific integration has also been reviewed in detail elsewhere [146].

The mechanism of integration is postulated to be through non homologous end joining (NHEJ) as limited homology exists between AAV and the chromosomal AAVS1 site[147]. Numerous DNA repair proteins interacting with Rep protein have been identified by pull down assays. They are Ku70, Ku80, replication protein A, Rad50, poly (ADP) polymerase I, DNA-PK and proliferating cell nuclear antigen [130]. Later it was demonstrated that a NHEJ protein, DNA ligase IV also plays an important role in this process [147, 148]. But the exact mechanism of how these NHEJ proteins facilitate AAV integration is unclear. Nonetheless, the high level of targeted integration has spawned the use of AAVS1 loci for targeted integration using various recent technologies, such as Zinc finger nucleases and TALENS [149-152].

## RECOMBINANT AAV VECTORS

There are several informative reviews on this subject [153-155]. Briefly, AAV has been used as a gene therapy vector in numerous preclinical and clinical studies due to its non pathogenic nature and in their ability to infect dividing and quiescent cells both *in vitro* and *in vivo* [156-160]. The design of AAV as a vector for gene therapy is relatively simple. The Rep and the cap genes are replaced by the transgene and their function is provided in *trans* by plasmid constructs encoding them. Thus, the overall method for production of recombinant AAV involves transient transfection of modified HEK293 cells with three plasmids; (i) plasmid encoding transgene flanked by ITRs; (ii) plasmid encoding the rep and the cap genes and (iii) helper plasmids providing adenoviral protein function. Several other scale–up packaging protocols such as the use of SF9 insect cells and baculovirus expression systems have also been described [161, 162]. Stable cell lines containing AAV rep-cap genes or systems containing herpesvirus or Adenovirus-AAV hybrid systems have been also used for recombinant AAV production. These strategies are attractive as they are amenable to bulk vector production [163, 164] [165]. Recombinant AAV was first used as a gene transfer vector for use in humans for cystic fibrosis [166]. Further research on AAV vector biology had led to an explosion of knowledge on the cellular receptors, mechanisms of intracellular trafficking and factors affecting gene expression. The following is a brief overview of how this knowledge has been applied in improving AAV as a gene therapy vector.

### Retargeting Vector Entry

The availability of a variety of naturally occurring serotypes and variants of AAV and their broad tissue tropism is promising as they could potentially be used to target many tissues. These vectors are generally administered by i.v injections. It is known that his mode of adminstration allows the vector to pass through the vasculature efficiently and facilitate its homing into different organs and peripheral tissues. However, Zincarelli *et al.*, have demonstrated that all naturally occurring AAV serotypes invariably accumulate in the liver upon systemic administration[98]. Such accumulated viral particles are also known to be cleared by hepatic sequestration [167, 168]. Apart from this, the cross neutrali-

zation of AAV vectors by neutralizing antibodies (NAbs) is known to be another major impediment in AAV gene therapy. Thus, strategies to rationally engineer AAV capsids in order to redirect the AAV vectors from liver to other tissues have emerged. AAV2.5 was generated by mutagenesis and swapping of 5 surface residues at the two-fold (VP3 dimer) interface of AAV2 capsid with AAV1 [169]. These vectors demonstrated enhanced transduction of the muscle tissue and a concomitant immune evasion potential in a phase I trial for Duchenne muscular dystrophy [169]. Using a similar approach, the generation of a hybrid AAV2i8 vector was shown to traverse vasculature and transduce skeletal muscle tissues with high efficiency. This hybrid vector comprising AAV2 /AAV8 was generated by substituting [585-RGNRQA-590 with QQNTAP] the heparin sulfate receptor binding site that retargets them from the liver to the muscle tissue. These chimeric capsids are also only modestly neutralized by anti-AAV2 serum, suggesting that AAV2i8 vectors have an altered antigenic profile [99]. Such meticulously engineered vectors are likely to expand the repertoire of AAV vectors available for human applications [170]. Contrary to the rational approach, directed evolution has also been employed to generate vectors with improved immune evasion properties. These AAV capsids are generated by a PCR-based random mutagenesis protocol and subsequently screened for their infectivity in the presence of neutralizing antibodies [171]. Such approaches have the power of generating a repertoire of vectors with varying transduction and targeting abilities.

### Improvement in Intra-Cellular Trafficking

It is well recognized that intra-cellular trafficking is a rate limiting step in AAV mediated gene transfer. In particular, the degradation of the capsid protein by the cellular ubiquitin-proteasomal system is a major impediment in this process [172]. A variety of pharmacological agents that inhibit the cellular kinases or the proteasomal machinery have been shown to improve transgene expression from AAV vectors [173-175]. Alternatively, AAV capsids are also being bioengineered to overcome this limitation. AAV capsids that have single or multiple amino acid substitution at specific serine, threonine, lysine and tyrosine residues have been shown to evade cellular ubiquitin mediated degradation. Mutagenesis of the surface exposed tyrosine to phenylalanine amino acids on the AAV2 capsid have been shown to improve their transduction by ~ 10 fold [176, 177], while modifications at specific serine, threonine or lysine residues improved the transgene expression of various serotypes (AAV1, AAV2, AAV5 and AAV8) by several-fold [178] [179, 180].

### Improved Gene Expression

The single stranded nature of AAV genome hinders its efficient second strand synthesis and leads to sub-optimal gene expression [181, 182]. The design of the self complementary (sc) AAV to bypass this crucial rate-limiting step is very significant. These self complementary vectors are generated as transcriptionally active genome as they reanneal spontaneously by virtue of the mutated *trs* in the right ITR. This leads to a consistently 10 fold higher transgene expression from these vectors in comparison to single-stranded AAV[89, 183, 184]. However, the size of the transgene in scAAV is halved to ~2.4Kb which limits its use for delivering large genes [185, 186]. Nevertheless, scAAV of many different serotypes have been successfully used in clinical trials for treating diseases like hemophilia, Leber's congenital amaurosis (LCA), and lipoprotein lipase deficiency. For delivering large genomes, a *trans*-splicing strategy, where the transgene is split between two segments and appropriate splicing sites are incorporated can be used. Such vectors when administered will form concatamers, leading to the transcription and optimal splicing of the transcribed mRNA. These vectors are, however, known to be less efficient than scAAV vectors [187, 188].

## CONCLUSIONS

Forty eight years since its discovery, AAV vectors have been transformed from a mere contaminant in culture to one of the most promising vector systems for gene therapy in humans. Nonetheless, challenges related to the universal application of vectors remain. The identification of novel serotypes from various sources and the repertoire of engineered vectors are likely to further enhance and expand the scope of application of AAV vectors. In addition, careful vector designing with the knowledge on the key biological aspects of AAV-host cellular interactions is likely to overcome most of the barriers to successful transduction and facilitate wide-spread use of this vector system.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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## PATIENT CONSENT

Declared none.

## ABBREVIATIONS

| | | |
|---|---|---|
| AAP | = | Assembly Activating Protein |
| AAV | = | Adeno-Associated Virus |
| CI-MPR | = | Cation-independent mannose 6-phosphate receptor |
| CLIC | = | Clathrin-independent carriers |
| EEA1 | = | Early endosome antigen 1 |
| GEEC | = | GPI-anchored-protein-enriched endosomal compartment |

| | | |
|---|---|---|
| HSPG | = | Heparan sulphate proteoglycan |
| IRE1α | = | Inositol-requiring enzyme 1 |
| IRS4 | = | Insulin receptor substrate 4 |
| ITR | = | Inverted Terminal Repeat |
| LCA | = | Leber's Congenital amaurosis |
| MBS85 | = | Myosin Binding Subunit 85 |
| MCM | = | Minichromosome maintenance complex |
| MEF | = | Mouse Embryonic Fibroblast |
| Nabs | = | Neutralizing antibodies |
| NHEJ | = | Non homologous end joining |
| NLS | = | Nuclear Localization Signal |
| ORF | = | Open Reading Frame |
| PCNA | = | Proliferating cell nuclear antigen |
| PERK | = | PKR-like ER-resident eIF2α kinase |
| PLA2 | = | Phospholipase A2 |
| RBS | = | Rep-protein binding site |
| RCN1 | = | Reticulocalbin-1 |
| RFC | = | Replication factor C |
| SMC2 | = | Structural maintenance of chromosomes protein 2 |
| SV15 | = | Simian Adenovirus type 15 |
| TRS | = | Terminal Resolution Site |
| UPR | = | Unfolded protein response |
| VR | = | Variable Region |

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