Chapter 2
The Role of the Adeno-Associated Virus Capsid in Gene Transfer

Kim M. Van Vliet, Veronique Blouin, Nicole Brument, Mavis Agbandje-McKenna, and Richard O. Snyder

Abstract Adeno-associated virus (AAV) is one of the most promising viral gene transfer vectors that has been shown to effect long-term gene expression and disease correction with low toxicity in animal models, and is well tolerated in human clinical trials. The surface of the AAV capsid is an essential component that is involved in cell binding, internalization, and trafficking within the targeted cell. Prior to developing a gene therapy strategy that utilizes AAV, the serotype should be carefully considered since each capsid exhibits a unique tissue tropism and transduction efficiency. Several approaches have been undertaken in an effort to target AAV vectors to specific cell types, including utilizing natural serotypes that target a desired cellular receptor, producing pseudotyped vectors, and engineering chimeric and mosaic AAV capsids. These capsid modifications are being incorporated into vector production and purification methods that provide for the ability to scale-up the manufacturing process to support human clinical trials. Protocols for small-scale and large-scale production of AAV, as well as assays to characterize the final vector product, are presented here.

The structures of AAV2, AAV4, and AAV5 have been solved by X-ray crystallography or cryo-electron microscopy (cryo-EM), and provide a basis for rational vector design in developing customized capsids for specific targeting of AAV vectors. The capsid of AAV has been shown to be remarkably stable, which is a desirable characteristic for a gene therapy vector; however, recently it has been shown that the AAV serotypes exhibit differential susceptibility to proteases. The capsid fragmentation pattern when exposed to various proteases, as well as the susceptibility of the serotypes to a series of proteases, provides a unique fingerprint for each serotype that can be used for capsid identity validation. In addition to serotype identification, protease susceptibility can also be utilized to study dynamic structural changes that must occur for the AAV capsid to perform its various functions during the virus life cycle. The use of proteases for structural studies in solution complements the crystal structural studies of the virus. A generic protocol based on proteolysis for AAV serotype identification is provided here.

Keywords AAV; capsid; Proteolysis; capsid structure; Serotype; Vector production; Column chromatography; Gene therapy

1 Introduction

Adeno-associated virus (AAV) is a single-stranded DNA virus that is currently being utilized for gene therapy applications. AAV is a member of the family Parvoviridae, genus *Dependovirus*. Several features of AAVs that make them promising candidates as gene transfer vectors are as follows: (1) Good safety profile: the members of this genus are not associated with disease in humans. The virus is replication-deficient, and, except under special circumstances, it will not replicate or spread [1], and AAV has been well tolerated in human clinical trials. (2) Stable long-term expression of the transgene: AAV has successfully been used to express transgenes in several tissues, including brain, muscle, liver, lung, vascular endothelium, and hematopoietic cells [2–9]. (3) Ability to transduce dividing and nondividing cells [10]. (4) Episomal maintenance: wild-type AAV exhibits site-specific integration of its genome into chromosome 19; however, the majority of vector genomes appear to be maintained episomally [11–13]. Therefore, the risk of integration is minimal, compared with retroviral vectors that require integration into the host genome and have the potential to activate proto-oncogenes. (5) Low immunogenicity: unlike adenoviral vectors, a robust T cell response to the vector is not generated by AAV [14], although humoral immune responses are generated, which may result in viral neutralization [15]. The use of other AAV serotypes may circumvent this response and allow for repeated treatments [16]. Alternatively, immunosuppressive therapy may be used during treatment with AAV to avoid the immune response [17]. (6) Physiochemical stability: AAV virions are highly stable over a wide range of pH and temperature, a feature that is important for production and purification methods for clinical-grade AAV vectors, as well as for stability of the final vector product [18].

The genome of AAV is ~4,700 bases of linear, single-stranded DNA. There are two genes, *rep* and *cap*, which are flanked by the inverted terminal repeats (ITRs). The ITRs consist of 145 nucleotides, which form a characteristic T-shaped hairpin. These are the only sequences required in *cis* for viral DNA replication and packaging. The *rep* gene encodes four nonstructural proteins, Rep78, Rep68, Rep52, and Rep40, which play a role in viral genome replication and transcription, as well as packaging. Rep78 and Rep68 are translated from mRNAs transcribed from the p5 promoter, while Rep52 and Rep 40 are derived from mRNAs transcribed from the p19 promoter. Alternative splicing replaces a 92 amino acid C-terminal element in Rep78 and Rep52 with a 9 amino acid element in Rep68 and Rep40 [19]. The *cap* gene encodes the three structural proteins of the AAV capsid, VP1 (87 kDa), VP2 (72 kDa), and VP3 (63 kDa), translated from mRNA transcribed from the p40 promoter. Differential splicing yields major and minor spliced products. VP1 is translated from the minor spliced mRNA, yielding less VP1 protein. VP2 and VP3 are both translated from the more abundant major spliced mRNA; however, VP2 is translated less efficiently because it initiates at an ACG codon, while VP3 is translated very efficiently because of a favorable Kozak context [20]. As a result, the AAV capsid proteins, which differ only in their N-terminal region, are present in the mature virion in a ratio of 1:1:10 (VP1:VP2:VP3). The single-stranded DNA genome of AAV and its products are depicted in Fig. 2.1.
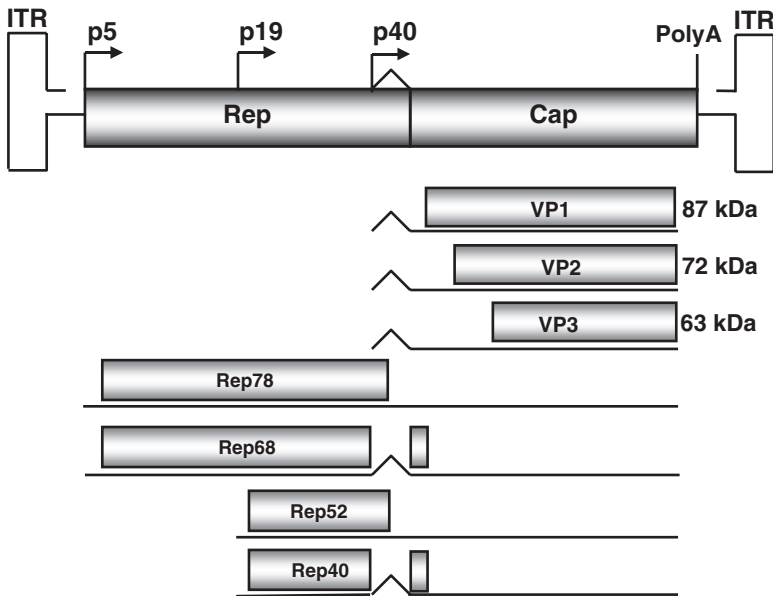
Fig. 2.1 The single-stranded DNA genome of AAV. The inverted terminal repeats (ITRs) flank the two open reading frames *rep* and *cap*. The *rep* gene encodes four nonstructural proteins – Rep78, Rep68, Rep52, and Rep40. The *cap* gene encodes three structural proteins – VP1, VP2, and VP3. The location of the promoters, p5, p19, and p40 are depicted by arrows

2 The AAV Capsid

2.1 Capsid Assembly and Packaging

In 1980, the work of Myers and Carter provided evidence that the structural proteins for AAV assemble into empty capsids, and then the genome is packaged into these preformed capsids [21]. Pulse-chase experiments showed that empty particles rapidly accumulate (10–20 min), but that mature "full" virions accumulated more slowly (4–8 h). They also showed that the number of empty viral particles decreases at the same rate as the number of DNA-containing mature virions increases during the course of infection. Additionally, de la Maza and Carter showed that DI particles, particles with deletions in the AAV genome, are packaged into apparently normal capsids, indicating that full-length viral genomic DNA is not required for the assembly or structural integrity of the AAV capsid [22]. In the absence of capsid assembly, ssDNA does not accumulate, further suggesting that empty capsids form first. Interactions between the preformed AAV capsid and Rep52 provides a mechanism where the nonstructural protein's helicase activity inserts the viral DNA [23]. Within the cell, capsid assembly occurs at centers within the nucleus where there is colocalization of Rep proteins, capsid proteins, and DNA [24]. Empty AAV capsids

can be produced by expressing the AAV *cap* gene in insect cells using a baculovirus expression system [25] or in mammalian cells utilizing a recombinant adenovirus expressing the AAV capsid proteins. These systems have advanced the structural studies of the AAV capsid as a result of the large amount of empty capsids or virus-like particles (VLPs) that can be produced. Studies of AAV assembly have demonstrated that VP3 alone is sufficient to form VLPs [26], but VP1 is required for infectivity [27]. Subsequently, it was shown that the unique N-terminus of VP1 has phospholipase A2 activity and contains a nuclear localization signal (NLS) [28]. The N-terminus of VP2 also has a NLS and may play a role in transporting VP3 into the nucleus; however, it has been shown that the N-terminus of VP2 is nonessential and that infectious virus can be produced that lack VP2 entirely [29]. Additionally, the N-terminus of VP2 has been replaced with green fluorescent protein and these capsids still assemble and maintain infectivity. This demonstrates that VP2 can tolerate peptide insertions and may be useful for incorporating peptides into the capsid for cell-specific targeting of AAV.

2.2 Capsid Structure

The adeno-associated virus capsid is ~25 nm in diameter and is composed of 60 subunits arranged in T = 1 icosahedral symmetry. Because AAV has a number of features that make it attractive as a gene transfer vector, many studies have focused on the basic biology of the virus, including studies that address the structural characteristics. Cryo-EM or crystal structures for AAV2, AAV4, and AAV5 have been determined [30–34], and the crystal structure for AAV8 is currently in progress [35]. Dependoviruses share the same subunit fold as the other members of the family Parvoviridae, including the insect densoviruses, and the autonomously replicating parvoviruses such as canine parvovirus (CPV) and minute virus of mice (MVM), even though AAV shares low capsid primary sequence identity (7–22%) [36–38]. The monomeric subunit of AAV has a conserved β-barrel core that is common in viral capsid proteins. Figure 2.2 depicts the structure of a monomeric subunit of AAV2 as determined by Xie et al. [31].

The motif is an eight-stranded antiparallel β-barrel motif (jelly-roll β-barrel), with the β strands labeled B-I [36]. The β-strand labeled A is present in some parvoviruses, including AAV. Not surprisingly, genetic capsid mutants of AAV2 in the conserved core β-barrel, such as mut19 (βA mutation), mut20 (βB mutation), mut25 (βD mutation), and mut46 (βI mutation), are unable to assemble into capsids [40]. AAV has long loop insertions between the strands of the core β-barrel that are labeled according to the β strands that they flank. These long interstrand loops contain β ribbons and elements of secondary structure that form much of the outer surface features of the AAV capsid. The GH loop is the longest interstrand loop, and three VPs interact extensively at each three-fold axis of symmetry, forming a prominent spike. Five DE loops each form an antiparallel β ribbon at the five-fold axis of symmetry that results in a cylindrical structure surrounding a canyon-like
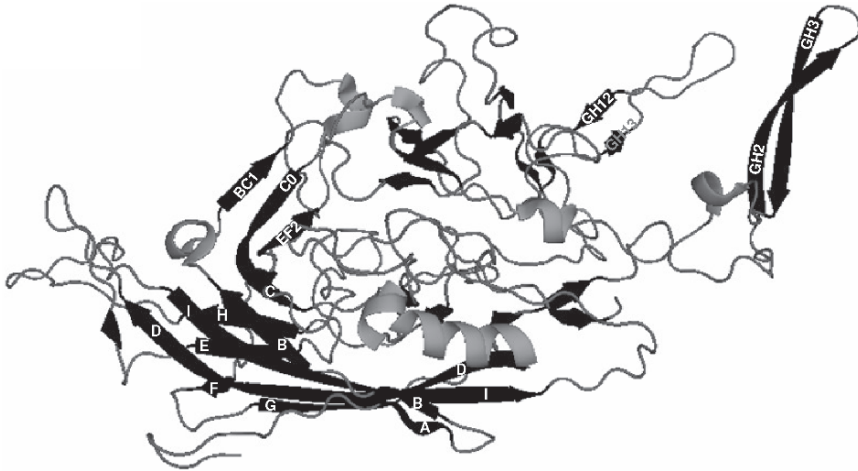
Fig. 2.2 The structure of a monomeric subunit of AAV2 as determined by Xie et al. [31]. This image was produced using the AAV2 coordinates from the Protein Databank, (PDB Accession no. 1lp3), with the molecular modeling software PyMOL (www.pymol.org) provided by DeLano Scientific, Palo Alto, CA [39]

depression. At the two-fold axis of symmetry there is a small depression, often referred to as the two-fold dimple [41, 42]. Analysis of newly discovered AAV genotypes identified a total of 12 hypervariable regions on the AAV capsid [43]. Overlaying these regions onto the X-ray crystallographic model of AAV2 showed that these regions are exposed on the capsid surface. Most of the variability is located between the G and H β strands, which are implicated in the formation of the valley and the peaks of the protruding three-fold axis of symmetry. These surface features of the virus are responsible for the interactions of the capsid with cellular receptors, as well as antibodies.

2.3 Cellular Receptors

Motifs on the capsid surface are critical for attachment to the host cell, which is the first step required for infection. Different serotypes of AAV utilize unique cellular receptors. The primary receptor for AAV2 is heparin sulfate proteoglycan (HSPG). After binding the cell surface, AAV2 can utilize secondary receptors, such as αVβ5 integrin, human fibroblast growth factor receptor-1 (FGFR-1), or hepatocyte growth factor (c-met), which mediate entry [44–47]. Recently, it was demonstrated that AAV2 utilizes α5β1 as an alternative co-receptor in HEK293 cells which lack αVβ5 integrin. The integrin recognition sequence, NGR, is at amino acid 511–513 within VP3, which, in assembled capsids, is located at the three-fold axis of symmetry,

adjacent to R585 and R588 of AAV2, which have previously been implicated in heparin binding [48]. The NGR motif is conserved among AAV serotypes 1–11, with the exception of AAV4, AAV5, and AAV11. Similar to AAV2, AAV3H has also been reported to bind heparin, heparan sulfate, as well as FGFR-1 [49]. The receptor for AAV5 has been shown to be PDGFR [50], and AAV5 binds to α-2-3-N-linked sialic acids [51], while AAV4 binds to α2-3-O-linked sialic acids [52]. AAV1 and AAV6 utilize α-2-3-N-linked sialic acids, as well as α-2-6-N-linked sialic acids; however, unlike AAV4, they are unable to utilize O-linked sialic acids [53]. Domains on the surface of the AAV capsid interact with the cellular receptors found on specific cells, resulting in the range of tissue tropism seen for the various AAV serotypes.

2.4   Serotypes

There are 11 known serotypes of AAV with different cellular targets and antigenic properties. Recently, about 100 genomic variants of these primary AAV serotypes have been discovered [54–56]. These new variants may provide expanded tropism and unique cellular targets for AAV-mediated gene delivery. Utilizing the natural differences in tropism of the AAV serotypes provides one strategy to efficiently deliver AAV vectors to specific target tissues, and selecting the appropriate capsid serotype for the target tissue is an important consideration. The rAAV genome can be packaged into capsids of its own serotype, "isotype," or alternatively the rAAV genome can be "cross-packaged" into capsids derived from another serotype, a process called pseudotyping [57–59]. For pseudotyped vectors, the capsid gene of each serotype can be cloned with AAV2 *rep,* and the transgene is flanked by ITRs of AAV2. The resulting vector has a capsid of some serotype other than AAV2 and the packaged transgene flanked by the ITRs of AAV2. This provides a method to compare the transduction efficiency of various serotypes and the ability to choose the most efficient one for the specific application [58]. Table 2.1 lists several target tissues and the comparative transduction efficiency of selected AAV serotypes studied in animal models. Efficient transduction could involve cell entry associated with receptor binding and internalization, or post-entry events such as cellular processing pathways, intracellular transport, nuclear entry, or processing of virions and vector genomes. Thomas et al. showed that AAV6 and AAV8 vectors uncoat faster and, as a result, transgene expression is seen sooner than AAV2 [90, 91].

Repeated vector delivery might be needed in order to increase transgene expression or to deliver two genes in a coordinated fashion. For these applications, the same transgene can be packaged into capsids from different AAV serotypes for readministration and to circumvent an antibody neutralization response [92]. Differences in the amino acid composition of the viral capsid present unique epitopes to the host's immune system. Studies with rAAV2 and rAAV8 vectors

Table 2.1 Transduction efficiencies of AAV serotypes in different tissues

Tissue type	Serotype	Reference
Muscle	AAV1 > AAV2	[60]
	AAV1-AAV6-AAV7 > AAV5 > AAV3 > AAV2 > AAV4	[58]
	AAV6 > AAV5 or AAV2	[61]
	AAV5 > AAV2	[62]
	AAV7	[63]
	AAV8 highest systemic; AAV1 and AAV6 highest local injection	[64]
	Transducing remote sites when injected locally:	
	AAV7 = AAV8 > AAV1, AAV5 and AAV6. AAV8 > AAV2; AAV1 > AAV2 and AAV8	
Liver (hepatocytes)	AAV8 > AAV7 > AAV5 > AAV2; AAV1 > AAV5 > AAV3 > AAV2 > AAV4	[58]
	AAV5 > AAV2	[65]
	AAV1 > AAV8 > AAV6 > AAV2	[66]
	AAV8	[63]
	AAV9 = AAV8	[67]
	AAV8-AAV9 > AAV2	[54]
Pancreas	AAV8 > AAV2	[91]
	AAV1 > AAV2	[68]
Kidney	AAV1 = AAV2 = AAV5	[69]
	AAV2 transduces tubular epithelium but not glomerular, blood vessel or interstitial cells	
Lung	AAV5 and AAV6	[70]
	AAV9 > AAV5	[71]
Retina	AAV5 > AAV4 > AAV1 = AAV2 = AAV3	[58]
Photoreceptor cells	AAV5 > AAV2	[72]
Hematopoietic stem cells	AAV1 > AAV2-AAV5	[73]
Dendritic cells	AAV6	[74]

(continued)

Table 2.1 (continued)

Tissue type	Serotype	Reference
Cochlear inner ear cells	AAV1 and AAV2 > AAV5	[75]
Solid tumors and melanoma	AAV2 > AAV1 and AAV3	[76]
Glioblastoma	AAV8 = AAV7 > AAV6 > AAV2 > AAV5	[77]
Glioma cells	AAV2 > AAV4 and AAV5	[78]
Brain	AAV5 > AAV1 > AAV2	[58]
	AAV7 > AAV8 > AAV5 > AAV2 = AAV6	[77]
	AAV1 and AAV5 > AAV2; AAV1 and AAV5 transduced pars reticulate; AAV1 transduced entire midbrain; AAV1 and AAV5 transduced pyramidal cell layers; AAV2 transduced the dentate gyrus	[79]
	AAV5 > AAV4 > AAV2	[80]
	AAV1 > AAV2	[81, 82]
	AAV8 > AAV1 or AAV2	[83]
	AAV5 > AAV2	[84]
	AAV2 transduces neurons	[85]
	AAV5 and AAV1 transduce neurons and glial cells	[85]
	AAV4 transduces ependymal cells	[85]
	AAV1 transduces glial cells and ependymal cells	[82]
Cardiac tissue	AAV8 > AAV2	[64]
	AAV1 > AAV2 > AAV5 > AAV4 > AAV3	[86]
	AAV9 > AAV8	[67]
	AAV9 > AAV8 > AAV1	[87]
	AAV1 > AAV2	[88]
	AAV6 > AAV2	[89]

expressing human factor IX indicate that the AAV2 capsid (amino acids 373–381) and the AAV8 capsid (amino acids 50–58) can elicit a cytotoxic T-lymphocyte (CTL) response [93]. In mice, the AAV8 capsid amino acids 126–140 elicited a Th1 response, whereas a Th2 response was elicited by AAV2 amino acids 475–489. The magnitude of the immune response depends on several factors, including vector dose, as well as the route and site of administration. Antibodies formed after transduction with one AAV serotype are likely to show only weak interaction with other serotypes, or may not cross-react. In animals that are transduced with AAV6 and then transduced with AAV2, delivery of the transgene was not hampered; however, animals transduced with AAV2 and then retransduced with AAV2 developed a neutralizing antibody response [92]. The region of the AAV2 capsid that has been shown to be responsible for generating a CD8$^+$ T cell response is the RXXR motif in VP1, VP2, and VP3, which is involved in heparin binding for AAV2. This region was identified as being involved in uptake into dendritic cells, as well as the activation of capsid-specific T cells [94].

Alternative serotypes may also provide for treating cells that have heretofore been refractory to AAV infection, such as stem cells. It has been demonstrated that AAV2 is not efficient at transducing hematopoietic stem cells because of suboptimal levels of expression of the cell surface receptor for the viral vector (HSPG), impaired cellular trafficking of the vector, inefficient vector uncoating, and the lack of viral second-strand DNA synthesis [7]. However, AAV1 is able to transduce hemotopoietic stem/progenitor cells when evaluated in short-term colony assays, as well as in long-term bone marrow transplantation assays *in vivo* [73]. There is also a low transduction efficiency for AAV2 in the airway epithelium of the lung due to a low abundance of HSPG on the apical surface; however, sialic acid is an abundant sugar on the apical surface of airway epithelium and pseudotyped AAV5 and AAV6 vectors have been shown to be efficient at gene transfer to murine airway epithelia *in vivo* [95, 96]. Encapsidated AAV6 vectors achieve transduction rates that should be sufficient for treating lung diseases, such as cystic fibrosis (CF). Recently, it was determined that the transduction efficiency of rAAV2 for the *in vivo* treatment of pancreatic and colon carcinoma is insufficient; however, other AAV serotypes, which have not yet been tested, may result in better transduction of this target tissue [97].

AAV2 and AAV1 bind to different cellular receptors, resulting in better transduction of muscle tissue for AAV1, and since the vectors in these studies contained the same *cis* elements, the AAV capsid is responsible for the differences in transduction efficiency between serotypes [98, 99]. Hauck and Xiao produced a series of capsid mutants to investigate the major regions of the AAV1 capsid responsible for the increased transduction efficiency of AAV1 in muscle tissue [100]; the major tissue tropism determinants were located in the surface region of VP1 (amino acid 350–423). Similar functional studies of the AAV capsid will provide a better understanding of the surface features and the specific requirements for engineering AAV capsids to efficiently target specific cells.

3 Methods

3.1 Retargeting

A receptor, like HSPG, which is present on a variety of cell types poses difficulties for targeting AAV2 vectors to a specific tissue. One approach to limiting gene expression in the target tissue is to utilize a viral vector that harbors a tissue-specific promoter [4, 72, 101]. Genetic modifications of the AAV capsid also provide an alternative approach for retargeting AAV2. Regions of the capsid have been identified that will accept insertion of heterologous peptides for retargeting. Girod et al., first demonstrated that the insertion of a 14 amino acid integrin binding peptide at amino acid 587 allowed for retargeting of AAV2 [102]. To obtain a genetic map of the AAV2 capsid, Wu et al. constructed alanine scanning mutants at 59 different positions in the AAV capsid gene by site-directed mutagenesis [103]. Studies of these mutants showed that the capsid can tolerate some modifications and still maintain infectivity. A 34 amino acid IgG binding domain was inserted into rAAV2 capsids at amino acid 587, and this, coupled with antibodies against B1 integrin, CXCR4, or the c-kit receptor, mediated the targeting of these rAAV vectors to specific cell surface receptors [104]. Endothelial cell binding peptides were identified using phage display and inserted into the AAV2 capsid at amino acid 587 to transduce venous endothelial cells and significantly reduce hepatocyte transduction [105]. Recently, it was demonstrated that AAV2 could be retargeted to the heart utilizing a capsid with mutations at amino acids 484 and 585 to eliminate heparin binding, which is required for AAV2 to infect the liver [106]. Perabo et al. also demonstrated that insertion at amino acid 587 with peptides that confer a net negative charge will ablate binding to HSPG, and this correlated with liver and spleen detargeting for AAV2. Insertion of peptides between 585 and 588 can either cause spatial separation or sterically block heparin binding of AAV2 (using bulky amino acids), and the insertion of positively charged peptides reconstitutes the ability to bind HSPG [107]. This provides a strategy to improve AAV targeting in other tissues. For retargeting AAV to tumor cells, an RGD-4C motif can be inserted at amino acids 520 and 584, or 588 to confer a novel tropism and eliminate heparin binding. The RGD motif binds the cellular integrin receptor, which is expressed on tumor cells, including ovarian cancer cells, but these cells express only low levels of HSPG and are nonpermissive for AAV2 transduction [108, 109].

3.2 Capsid Mosaics

Introduction of a peptide into the AAV2 capsid is a viable strategy for retargeting AAV2 to additional cell types; however, modifications in the AAV2 capsid often result in a reduction in vector yields, especially if the insertion is large. The unique N-terminal region of VP2 allows for peptide insertions without a loss of titer, when

VP1 and VP3 are supplied in *trans*. AAV2 mosaics that have ligand insertions in a subset of VP1, VP2, and VP3 molecules result in increased vector yields and transducing titers, compared with viruses that carry the insertion in all 60 capsid protein subunits [110]. Insertion of an HA epitope (YPVDVPDYA) at amino acid 522 or 533 of VP1 results in noninfectious particles [103]; however, the inclusion of wild-type capsid proteins can restore viral infectivity. Ried et al. [104] inserted an immunoglobulin-binding fragment of protein A (Z34C) that resulted in at least a tenfold reduction in particle titers, most likely due to its large size (34 amino acids). Compared with wild-type virus, infectious titers were 4 orders of magnitude lower. Gigout et al. generated mosaic viruses that contained between 25 and 75% of Z34C capsid proteins, with the rest of the capsid being composed of wild-type subunits to produce AAV2 mosaics that were infectious. Compared with wild-type virus, the Z34C mosaics showed up to a tenfold increase in titer, and those containing 25% Z34C capsid protein (an average of 15 subunits per capsid) were 4–5 orders of magnitude more infectious than all mutant viruses. By mutating R585 and R588 to alanine, they were able to eliminate HSPG binding and then the insertion of Z34C resulted in retargeting of AAV2 [110]. This work demonstrated for the first time that a combined approach of generating AAV2 mosaics to alter tropism and mutating two residues to reduce HSPG binding could be used to retarget AAV2 to specific cell types. For AAV1, incorporating an RGD4C motif in VP1 at amino acid 590 enables targeting to integrin receptors which are present on vascular endothelial cells [111]. It is also possible to incorporate a small biotin acceptor peptide (BAP) in this position for the purpose of metabolically biotinylating the AAV1 capsid for purification using a commercially available avidin affinity column. Mosaics with both of these modifications have been generated, which enable retargeting of the AAV1 capsid, and simplify purification.

3.3 Capsid Chimeras

Another strategy to broaden tissue tropism is to generate AAV with mixed or chimeric capsids [112]. This is accomplished by supplying the capsid gene from two distinct AAV serotypes during production. By varying the ratio of the two capsid genes, the resulting mixed virus may exhibit altered tropism. Rabinowitz et al. showed that mixed capsids of AAV2/5 at a ratio of 3:1 resulted in a loss of heparin binding. The ability of these virions to transduce HeLa cells, which have high levels of heparin sulfate, decreased, while the ability to transduce the heparin-sulfate-deficient cell line CHO pgsD increased from 2% to > 30% as the composition of the virion changed from AAV2-like to AAV5-like. Chimeras also may show combined tropism of both serotypes, broadening the tropism for these virions. Kohlbrenner et al. utilized chimeric capsids to improve infectivity when expressing AAV5 and AAV8 in insect cells [113]. It was determined that AAV5 and AAV8 vectors generated using the baculovirus system had low infectivity, which was due to insufficient phospholipase A2 activity. Substituting the entire VP1 protein from AAV2 in chimeric

AAV5 or AAV8 capsids resulted in increased phospholipase activity levels and enhanced transduction. Generating chimeras also provides a method to functionally define structural relatedness for newly discovered serotypes. The ability to generate stable chimeric capsids suggests that the subunits from these different serotypes are structurally compatible. Conversely, AAV2 when mixed with AAV4 inefficiently packages genomes. The inability to generate stable virions may reflect a failure of essential structural subunit interactions at one or more axes of symmetry. Alternatively, the nonstructural Rep protein interactions that occur on the surface of the capsid for packaging may not be compatible in the AAV2/AAV4 chimeras, as suggested by the structures for AAV2 and AAV4 [30, 31].

3.4   Vector Utility

The AAV capsid provides a potent gene delivery vehicle and has shown great promise in animal models, as well as in human clinical trials conducted to date. AAV vectors have been developed for a multitude of diseases, including disorders of the central nervous system for which other vectors and methods of treatment have been inadequate. Examples include Parkinson's disease and Alzheimer's disease. Gene therapy approaches utilizing AAV have also addressed classic genetic disorders, such as lysosomal storage disorders, hemophilia, and cystic fibrosis. Many diseases that are amenable to AAV gene therapy approaches are listed in Table 2.2. AAV vectors to treat these diseases have shown significant and persistent gene transfer without toxicity [3–5, 117–124, 138–141, 159–160] in human clinical trials. Vectors are administered to effect intracellular expression of proteins such as dystrophin or expression of secreted therapeutic proteins that result in cross correction of cells. AAV may also be utilized as a vaccine to deliver specific antigens; for example, AAV5 targets dendritic cells, allowing for antigen presentation, and AAV vectors have been produced that express components of papillomavirus, the causative agent of cervical cancer, as well as the receptor for the spike protein of SARS coronavirus [181, 182]. AAV-based gene transfer has the therapeutic potential to arrest or reverse the course of these inherited and acquired diseases.

3.5   Vector Production

An important consideration for the development of a suitable gene delivery vector is the ability to produce the quantities of vector required to treat human patients. Historically, production methods have been a limitation in the development of AAV vectors. Improvements in production and purification methods have resulted in better yields, increased purity, and higher titers through the development of scalable systems [113, 183–187]. Recombinant AAV vectors can be manufactured using a two-plasmid system, as shown in Fig. 2.3. One plasmid harbors the therapeutic

Table 2.2 Disease targets

| Disorder	Target tissue	Species	Serotype	Reference
Cystic fibrosis	Nasal and lung epithelium	Mice	AAV2/5, AAV2/9	[71]
	Lung	Mice	AAV1, AAV2, AAV5	[114]
	Airway epithelium	Cell culture	AAV5	[115]
	Lung	Mice	AAV2, AAV5	[116]
	Lung	Mice	AAV2, AAV6	[96]
	Lungs – airway epithelium and maxillary sinus	Human	AAV2	[117–120, 121–124]
Hemophilia B	Liver	Mice	AAV8, AAV9	[67]
	Liver	Mice	AAV1, AAV2, AAV6	[125]
	Liver	Mice	AAV5	[65]
	Liver	Mice, Monkey	AAV2, AAV5, AAV2/8	[126]
	Liver	Mice	AAV2	[127, 128]
	Liver	Dog	AAV2/8	[129]
	Liver	Dog	AAV2	[130, 131]
	Liver	Human	AAV2	[4]
	Muscle	Mice	AAV1-AAV2 hybrid	[60]
	Muscle	Mice	AAV1	[132, 133]
	Muscle	Mice	AAV1, AAV2, AAV3, AAV4, AAV5	[98]
	Muscle	Mice; Dog	AAV1	[134]
	Muscle	Dog	AAV2	[135–137]
	Muscle	Human	AAV2	[138–141]
Anemia	Muscle	Mice	AAV2	[142, 143]
Parkinson's disease	Brain	Rat	AAV2	[144]
	Brain	Monkey	AAV2	[145–149]
	Brain	Human	AAV2	[5]
Lysosomal storage disease	Muscle	Mice	AAV2	[150]
	Muscle	Mice	AAV1 and AAV2	[151]
	Muscle	Mice	AAV2/6	[152]

(continued)

Table 2.2 (continued)

Disorder	Target tissue	Species	Serotype	Reference
	Brain	Mice	AAV2	[153]
	Brain	Mice	AAV2, AAV5	[154]
	Brain	Human	AAV2	[3]
	i.v. – systemic delivery	Mice	AAV8	[155]
	i.v. – systemic delivery	Mice	AAV2/8	[156]
Canavan disease	Brain	Mice	AAV2	[157]
	Brain	Rodents, monkey, human	AAV2	[158]
	Brain	Human	AAV2	[159, 160]
Type I diabetes	Pancreas – acinar cells, beta cells	Mice	AAV6, AAV8	[161]
	Islets	Mice	dsAAV2, dsAAV6, dsAAV8	[162]
	Islet cells	Mice	AAV1, AAV2	[68]
		Mice		[68]
Alzheimer's disease	Brain – Abeta vaccine	Mice	AAV2	[163–165]
	Brain	Rat	AAV2/5	[84]
	Brain	Rat	AAV1, AAV2, AAV4, AAV5	[85]
Cardiovascular	Cardiac tissue	Rodent	AAV2	[166]
	Cardiac tissue	Mice	AAV2/1, AAV2/8, AAV2/9	[87]
	Cardiac muscle	Mice	AAV2	[151, 167]
	Cardiac tissue	Rat	AAV6, AAV2	[89]
Cancer	Glioma	Tumor cell lines	AAV2, AAV4, AAV5	[78]
	Ovarian carcinoma	Ovarian carcinoma cell line	RGD modified AAV2	[168]
		Mice	AAV2	[169]
		Mice	AAV2	[170]
	Breast carcinoma	Mice	AAV2	[171]
	Glioblastoma	Mice	Pseudotyped AAV7 and AAV8	[77]
		Rat	AAV2	[172]

|				
	Prostate cancer	Mice	AAV2	[173]
	Liver cancer	Mice	AAV2	[174, 175]
	Lung adenocarcinoma	Mice	Hybrid AAV2/5	[176]
Vaccine development	Bone-marrow-derived dendritic cells	Mouse	AAV6	[74]
	Papillomavirus antigen HPV16 L1 protein delivery	Mice – intranasal delivery	AAV5	[182]
	Dendritic cells or muscle, HIV gp160 protein delivery	Mice	AAV1, AAV5, AAV7 and AAV8	[181]
	Dendritic cells, siRNA delivery for dengue virus vaccine	Human cells	AAV2	[177]
Obesity	Vector expressing leptin	Mouse	AAV2	[178]
	Vetor expressing leptin receptor	Rat	AAV2	[179]
	Vector expressing adiponectin	Rat	AAV1, AAV2, AAV3, AAV4, AAV5	[180]
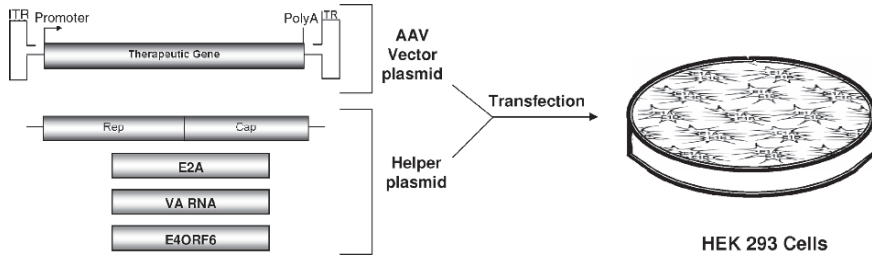
Fig. 2.3 Viral vector production. The rAAV Vector plasmid contains the therapeutic gene flanked by the ITRs, usually of AAV2. The helper plasmid contains the *rep* and *cap* genes, as well as the adenoviral genes needed for replication. Both plasmids are transiently transfected into HEK293 cells that express the adenovirus E1A and E1B gene products

gene flanked by the ITRs and the other one contains the AAV *rep* and *cap* genes, as well as the helper functions for AAV2 replication. The adenovirus helper functions are provided by expression of E2A, E4ORF6, and VA RNAs in the helper plasmid, while the adenovirus E1A and E1B gene products are expressed in the HEK293 cells utilized for production [188]. The two plasmids are co-transfected into 293 cells, and this results in production of an AAV virion that contains the therapeutic gene flanked by the ITRs [189]. The ITRs are the only viral sequences remaining after purification of rAAV vectors.

Protocol 1 (see Appendix) describes the small-scale production and purification of rAAV vectors as described by Zolotukhin et al. [190]. Generally, HEK293 cells are expanded, transfected, and harvested at 72h post-transfection. The cells are then lysed, and loaded onto either a cesium chloride or an iodixanol gradient to separate infectious virions from empty capsids and other cellular proteins. Virus is then purified using either heparin affinity chromatography or ion-exchange chromatography. The chromatography method used is dependent on the natural receptors for AAV, as well as the charge characteristics of the viral particle, as shown in Fig. 2.4. Following column chromatography, the virus is concentrated and characterized. These protocols yield vector stocks that are relatively pure; however, owing to the density gradient requirement, these methods are not easily scalable.

Protocols 2 and 3 describe the large-scale production and purification of rAAV vectors as described by Snyder et al. [18]. Protocol 3 provides a method suitable for large-scale production of rAAV2 vectors that is scalable for the production of vectors under cGMP conditions for clinical trials [191]. Cell factories of HEK293 cells are transfected, incubated for 72h, and then harvested. The cell pellet is resuspended in lysis buffer containing 0.5% deoxycholate to reduce viral particle aggregation and 50U Benzonase/ml to degrade cellular, plasmid, and nonpackaged nucleic acid. A microfluidizer is utilized to lyse the cells in order to form a fine suspension that can be loaded directly onto a Streamline Heparin column. The column is washed and the virus is eluted from the column with phosphate-buffered saline (PBS) containing 0.2M NaCl (for a total ionic strength of 350mM). The peak fraction from the Streamline Heparin column is brought to 1M NaCl and loaded on a Phenyl
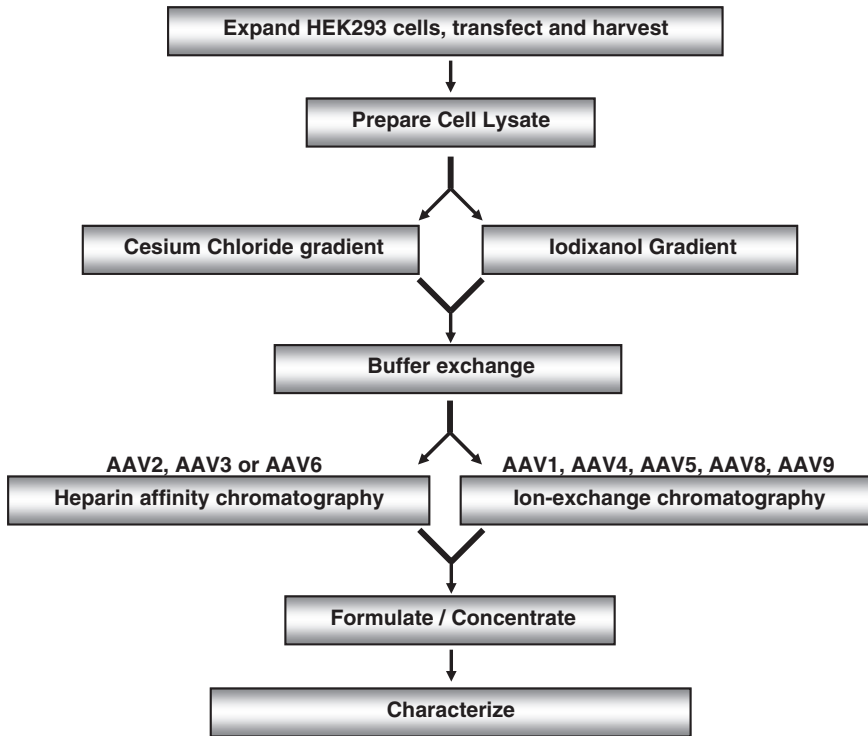
Fig. 2.4 Flowchart of the steps for rAAV production as described in Protocol 1. HEK293 cells are expanded, transfected, and harvested at 60h posttransfection. The cells are lysed, and loaded onto either a cesium chloride gradient or an iodixanol gradient to separate infectious virions from empty capsids. Virus is purified using either heparin affinity chromatography or ion-exchange chromatography. Virus preparations are formulated and concentrated, and characterized

Sepharose column. This column is a hydrophobic interaction column (HIC) and the virus will remain in the flow through. The flow through from the Phenyl Sepharose column is diluted using sterile water to bring the salt concentration to 0.150 M NaCl. This is loaded onto a SP Sepharose column, washed in column buffer, and eluted in PBS with 0.135 M NaCl (for a total ionic strength of 0.285 M).

3.6 Vector Characterization

Several assays to characterize the final product with respect to titer, purity, identity, potency, particle to infectious ratio, and stability are listed in Table 2.3. In addition, vector product safety testing should be conducted prior to animal or human studies. Assays used for product characterization and safety testing are included in Table 2.3.

Table 2.3 Assays used for vector characterization and safety testing of rAAV vectors produced for clinical trials under cGMP conditions

Assay type	Method and reference	Purpose
Titer		
Infectious	Infectious Center Assay (ICA) [192, 193]	Determine titer of infectious particles produced
	Serial Dilution Replication Assay (dRA) [194]	
Vector genome	Dot-blot hybridization	Determine genome-containing vector concentration
	PCR [195]	
Capsid	ELISA [24]	Determine total capsid protein concentration – enables a determination of empty particles in the prep
	Bradford	
	Western	
	Electron microscopy [196]	
	Optical density (OD) [197]	
Purity		
Protein	SDS-PAGE	Determine the presence or absence of contaminating proteins
Cellular DNA	DNA hybridization or PCR	Determine the presence or absence of cellular DNA
Identity		
Transgene cassette	DNA sequencing or restriction enzyme digestion	Verification of the transgene
Capsid	SDS-PAGE with silver and Coomassie stain	Expected AAV banding pattern
	Limited proteolysis	Serotype identification
Potency		
Transgene expression	Transduction assay in cells or animals	Ensure that the active transgene product is expressed
Safety		
Adventitious agents	qPCR-based assays to detect infectious adventitious viral agents	Detect contaminating infectious viral agents of human or animal origin (serum, trypsin)
Mycoplasma	Growth assays on cells in antibiotic-free conditions, followed by dye or PCR to detect mycoplasma	Determine the presence or absence of mycoplasma
	Growth assay in appropriate agar media	
Endotoxin	Rabbit pyrogen assays	Determine the presence or absence of endotoxin

(continued)

Table 2.3 (continued)

Assay type	Method and reference	Purpose
Sterility	Bacteriostasis/fungistasis	Determine the presence or absence of microbial contaminants
Stability		
Physiochemical	SDS-PAGE	Demonstrate that the product is not degrading over time
Infectious	ICA	Demonstrate that infectivity is maintained over time
Sterility	Bacteriostasis/fungistasis	Demonstrate the integrity of final product container over time

3.7 Proteolytic Structural Mapping

In the near future, customized AAV gene therapy vectors may consist of modified capsids that allow for specific targeting to treat patients with various diseases. The 3D structures of the AAV capsids will provide a basis for rational vector design; however, the 3D structures available for autonomous parvoviruses and dependoviruses provide only a "snapshot" of the capsid topology in a low energy conformation. Our knowledge about the AAV viral capsid structure in solution is limited; however, this structure must be dynamic to carry out the various functions required for viral attachment and entry, as well as trafficking within the cell. Cryo-EM studies have shown that the unique N-terminus of VP1 is internal to the capsid based on additional density at the two-fold axis of symmetry [198]. *In vitro*, upon heat treatment of AAV capsids, it has been shown that this region can be externalized. Mutagenesis experiments have shown that this externalization occurs through the pore at the five-fold axis of symmetry [199]. Previously, antibodies have been produced that detect various regions of the capsid proteins [200]. The B1 antibody epitope is on the C-terminal end of the capsid protein, and is primarily internal at the two-fold axis of symmetry in assembled capsids. This antibody is useful in detecting denatured AAV proteins and the epitope is highly conserved among the AAV serotypes. A1 antibody recognizes the unique N-terminal region of VP1, while the A69 epitope is in the N-terminal region of VP2 for AAV2. Polyclonal antibodies have been produced to AAV2 capsids, as well as to other serotypes. In addition, antibodies have been produced that recognize conformational epitopes that are present only on assembled capsids for AAV2, as well as AAV1. Capsid antibodies can be utilized for serotype identity testing of the final vector product; however, many of these antibodies cross-react with multiple serotypes. Historically,

AAV has been shown to be remarkably stable and is generally resistant to proteases. However, we have demonstrated that when exposed to proteases, specific regions of the capsid are susceptible to proteolysis [201]. Owing to differences in the primary sequence of the capsid proteins of different serotypes, as well as the resulting differences in capsid structure, proteolysis can be utilized for AAV serotype determination. Different serotypes provide unique fragmentation patterns when cleaved with protease, with some serotypes such as AAV5 being relatively resistant, while others such as AAV2 are more susceptible to trypsin and chymotrypsin. Figure 2.5 depicts protease mapping of the AAV capsid for capsid serotype determination. A generic assay is described in Protocol 4 where proteolysis is used together with specific antibodies to provide a powerful mapping technique and a method to identify and confirm the serotype of the AAV capsid.
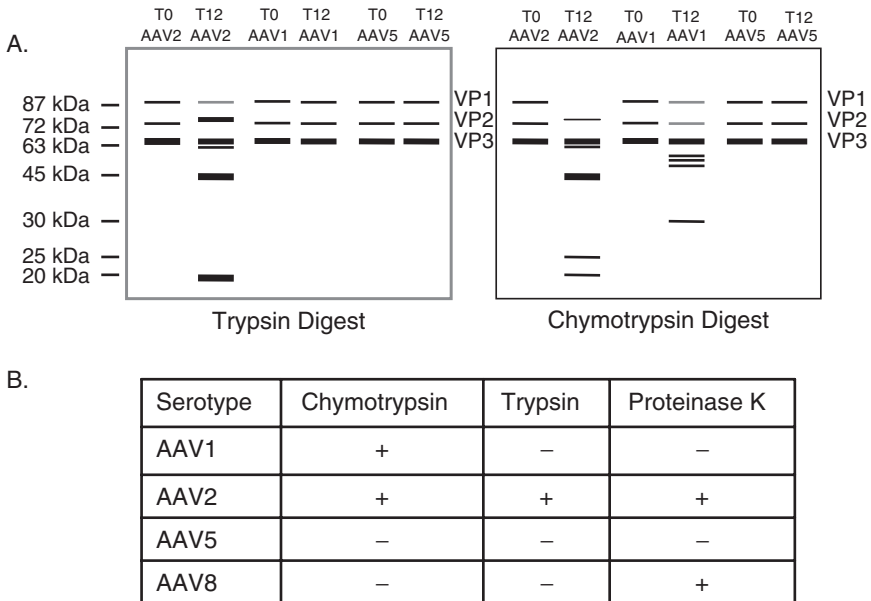
Fig. 2.5 Protease mapping of the AAV capsid for capsid serotype determination. **A** Samples are digested with a protease, in this case trypsin for one set of samples and chymotrypsin for the other set. A Western blot is performed using polyclonal antisera to AAV capsids, and based on the fragmentation pattern, a serotype determination can be made. Undigested sample (T_0) and samples digested for 12 h (T_{12}) for AAV2, AAV1, and AAV5 are shown. AAV5 is resistant to these proteases, while AAV1 and AAV2 exhibit differences in their fragmentation patterns. T_0 samples represent the undigested capsid proteins VP1, VP2, and VP3. **B** Different AAV serotypes demonstrate different susceptibilities to proteases due to the differences in their primary amino acid sequences. This differential susceptibility provides a unique signature for each serotype and allows for capsid serotype identification

3.8 Concluding Remarks on AAV Capsid

In summary, the AAV capsid is the major determinant in gene transfer efficiency and targeting. Several approaches are available to target AAV viral vectors to specific cell types, including utilizing natural serotypes that target a desired cellular receptor, producing pseudotyped vectors, as well as engineering chimeric and mosaic capsids. Genetic mutations of the AAV capsid have identified regions that will accommodate peptide insertions and modifications for specific targeting. The use of modified capsids may enhance efficiency and target specificity to make a more potent and safe vector. Several genetic diseases are amenable to treatment with AAV vectors, making AAV a valuable gene delivery vehicle, especially for disorders for which current therapies are inadequate or non-existent. Current production methods allow for the generation of quantities of virus needed for clinical trials, and purification methods have been developed for several serotypes. In addition, assays have been developed for final product characterization to ensure patient safety.

Appendix

Protocol 1: AAV Small-Scale Production (~3 × 10⁸ cells/prep)

Transfection Protocol

1. Seed [20] 15-cm plates with 293 cells so that they will be 75–80% confluent the following day (12–16 h prior to transfection). 293 cells are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin.
2. Check plates prior to transfection to ensure optimal confluency, and change media to ensure cells are in a volume of 20 ml. Be careful not to disrupt the monolayer.
3. For transfection, prepare the DNA mixture at room temperature in a 50-ml conical tube as follows: Mix 900 µg of helper plasmid with 300 µg rAAV vector plasmid. This is 60 µg DNA per plate in a 1:1 molar ratio. Bring the final volume of the DNA mixture to 22.5 ml with sterile dH$_2$O. Add 2.5 ml of 2.5 M CaCl$_2$ to the DNA for a CaCl$_2$ concentration of 0.250 M.
4. Prepare ten 15-ml conical tubes each with 2.5 ml 2× HBS to transfect the 20 plates (25 ml 2× HBS total). Transfect two plates at a time by adding 2.5 ml of DNA mixture to a tube of 2.5 ml 2× HBS, mix, and incubate for 1 min at room temperature to allow precipitate to form. Add 5 ml of media to the tube and mix. (Total volume in the tube is 10 ml.) Pipette 5 ml onto each of the two plates. Repeat for the remainder of the plates.
5. Incubate at 37°C for 72 h.

6. At 72 h, harvest cells. Use media to dislodge the cells. Collect media and cells and centrifuge at $1,000 \times g$, for 10 min at 4°C to pellet the cells. Discard the media. Resuspend pellet in PBS to wash the pellet. Centrifuge at $1,000 \times g$, for 10 min at 4°C. Discard PBS and freeze pellet. Frozen pellets can be stored at −20°C for processing at a later time.

Transfection Reagents and Materials

– 15-cm tissue culture plates.
– 293 cells.
– Culture media: DMEM + 5% FBS + 1% penn/strep.
– Trypsin for splitting cells.
– PBS without magnesium and calcium.
– Vector plasmid, 300 μg.
– Helper plasmid, 900 μg.
– Sterile dH$_2$O.
– 2.5 M CaCl$_2$ – (stock 147.02 g/mol calcium chloride dihydrate) – for 1 l add 367.55 g CaCl$_2$·2H$_2$O. Prepare 10-ml aliquots and store at −20°C.
– 2× HBS (HEPES-buffered saline; prepare 1 l):

   0.274 M NaCl (stock 58.44 g/mol) – 16.0 g.
   0.010 M KCl (stock 74.56 g/mol) – 0.75 g.
   0.002 M Na$_2$HPO$_4$ anhydrous (stock 141.96 g/mol) – 0.28 g.
   0.011 M dextrose (stock 180.16 g/mol) – 2.0 g.
   0.042 M HEPES (stock 238.3 g/mol) – 10.0 g.

   Adjust the pH to 7.05 with 0.5 N NaOH, bring to a final volume of 1 l with dH$_2$O, and sterile filter. Store in 50-ml aliquots at −20°C. (Note: the optimal pH range for 2× HBS is 7.05–7.12. The pH of the 2× HBS is a key factor that influences precipitate formation.)

Cell Lysate and Cesium Chloride Gradient Protocol

7. Resuspend pellet in 30 ml lysis buffer. Lyse cells by three freeze/thaw cycles. Thaw pellet in 37°C water bath and freeze cells in EtOH/dry ice bath; repeat twice.
8. Transfer the lysate to a 40-ml Dounce homogenizer. Homogenize the lysate with 20 strokes to shear cellular DNA.
9. For each 10 ml of lysate, add 5 g CsCl and homogenize until the CsCl is dissolved completely.
10. Fill six 12.5 ml ultra clear ultracentrifuge tubes (Beckman) with 10 ml of lysate and underlay with 0.5 ml each of CsCl (1.5 g/ml). Balance the tubes using CsCl (1.37 g/ml) prior to ultracentrifugation.

11. Centrifuge for 24 h at 265,000 × *g* (40,000 rpm in SW 41 rotor), 21°C.
12. Collect AAV from the gradient by dripping 1-ml fractions, and verify the density of the fractions by refractometry. The density of infectious AAV virions is 1.40–1.42 g/cm^3. Empty AAV particles and DI particles have a density of 1.32–1.35 g/cm^3.
13. Dialyze AAV-containing fractions into the buffer that will be used for column chromatography (1× TD buffer for heparin column or 15 mM NaCl, 20 mM Tris (pH 8.5) for Q Sepharose purification).
14. Purify using either heparin affinity chromatography (for AAV2, AAV3, or AAV6) or ion-exchange Q Sepharose chromatography (for AAV1, AAV4, AAV5, AAV8, or AAV9). Use a 1-ml column for 20–40 plates, as described in steps 1–7 under "AAV Purification by Heparin Chromatography" or "AAV Purification by Q Sepharose Chromatography." The purification method is as described by Zolotukhin et al. [190].

Cell Lysate and Cesium Chloride Gradient Reagents

Lysis buffer (prepare 1l):

–   150 mM NaCl (stock 58.44 g/mol) – 8.766 g.
–   50 mM Tris (stock 121.14 g/mol) – 6.055 g.
–   Adjust pH to 8.5 with HCl, bring to a final volume of 1l with dH$_2$O, and filter sterilize. Store at room temperature.

1.37 g/ml CsCl in PBS – Dissolve 50 g CsCl in PBS and adjust the final volume to 100 ml. Weigh 1 ml to verify the density is 1.37 g/ml. Filter sterilize. Store at room temperature.

1.5 g/ml CsCl in PBS – Dissolve 67.5 g CsCl in PBS and adjust the final volume to 100 ml. Weigh 1 ml to verify the density is 1.5 g/ml. Filter sterilize. Store at room temperature.

5× TD buffer (prepare 1l):

–   5× PBS −500 ml of 10× PBS (Invitrogen).
–   5 mM MgCl$_2$·6H$_2$O (stock 203.3 g/mol) – 1.0165 g.
–   12.5 mM KCl (stock 74.56 g/mol) – 0.932 g.

1× TD buffer – Prepare from 5× TD buffer stock: 200 ml 5× TD stock, 800 ml dH$_2$O.

Q Sepharose low salt column buffer:

–   15 mM NaCl (stock 58.44 g/mol) – 0.877 g.
–   20 mM Tris (stock 121.14 g/mol) – 2.423 g.
–   Adjust pH to 8.5 with HCl and bring to a final volume of 1l with dH$_2$O.

Alternative Cell Lysate and Iodixanol Gradient Protocol

7a. Resuspend pellet in 30 ml lysis buffer. Lyse cells by three freeze/thaw cycles. Thaw pellet in 37°C water bath and freeze cells in EtOH/dry ice bath; repeat twice.

8a. To the cell suspension, add 3 μl of 4.82 M $MgCl_2$ and vortex. Add 1 μl of Benzonase (250 U/μl) and vortex. Incubate at 37°C for 30 min. Centrifuge for 20 min at $1,000 \times g$.

9a. Pipette the supernatant into two quick seal tubes for a 70 Ti rotor. Underlay lysate with iodixanol gradient:

   – 7.5 ml of 15% iodixanol
   – 5.0 ml of 25% iodixanol
   – 7.5 ml of 40% iodixanol
   – 5.0 ml of 60% iodixanol

10a. Weigh tubes, cap, and seal with heat sealer.

11a. Centrifuge at $350,333 \times g$, 18°C, for 1 h (69,000 rpm in a 70 Ti rotor for 1 h).

12a. Collect the 40% iodixanol band and the interface between the 60% and the 40% bands. This is done by setting up a ring stand and placing the tube in a clamp. Swab the top and bottom of the tube with an alcohol swab. Vent the top of the tube with a needle and collect fractions from bottom of the tube.

13a. Dialyze virus-containing fractions into the buffer that will be used for column chromatography (1× TD buffer for heparin column or 15 mM NaCl, 20 mM Tris (pH 8.5) for Q Sepharose purification).

14a. Purify using either heparin affinity chromatography (for AAV2, AAV3, or AAV6) or ion-exchange Q Sepharose chromatography (for AAV1, AAV4, AAV5, AAV8, or AAV9). Use a 1-ml column for 20–40 plates, as described in steps 1–7 under "AAV Purification by Heparin Chromatography" or "AAV Purification by Q Sepharose Chromatography." The purification method is as described by Zolotukhin et al. [190].

Cell Lysate and Iodixanol Gradient Reagents

Lysis buffer (*150 mM NaCl, 50 mM Tris, pH 8.5*) (prepare 1l):

– 150 mM NaCl (stock 58.44 g/mol) – 8.766 g.
– 50 mM Tris (stock 121.14 g/mol) – 6.055 g.
– Adjust pH to 8.5 with HCl, bring the final volume to dH$_2$O with 1l, and filter sterilize. Store at room temperature.

5 M NaCl (*stock 58.44 g/mol*) – 292.2 g and dH$_2$O to 1l.
   For iodixanol gradient, mix the following:

Step	Optiprep (ml)	5 M NaCl (ml)	5× TD (ml)	dH$_2$O (ml)	Phenol red (µl)	Total volume (ml)
15%	45	36	36	63	–	180
25%	50	–	24	46	300	120
40%	68	–	20	12	–	100
60%	100	–	–	–	250	100

AAV Purification by Heparin Chromatography

1. Equilibrate a 1-ml HiTrap heparin HP column (GE Healthcare) by washing with ten column volumes of 1× TD. The flow rate for all chromatography steps is 1 ml/min.
2. Activate the column by washing with five column volumes of 1× TD/1 M NaCl.
3. Re-equilibrate the column by washing with ten column volumes of 1× TD.
4. Apply the dialyzed virus from either the cesium gradient or iodixanol gradient to the column.
5. Wash the column with ten volumes of 1× TD.
6. Elute with five column volumes of elution buffer using either a continuous or step gradient. AAV2 can be eluted in 1× TD/0.5 M NaCl [190] and collect [5] 1-ml fractions.
7. Dialyze the virus preparation into storage buffer or suitable formulation and freeze at −20°C.

Heparin Column Chromatography Reagents

1× TD buffer – prepare from 5× TD buffer stock: 200 ml 5× TD stock, 800 ml dH$_2$O.
1× TD/1 M NaCl buffer: 200 ml 5× TD buffer, 58.44 g NaCl; bring to 1 l with dH$_2$O.

Storage buffer:

– 100 mM NaCl (stock 58.44 g/mol) – 5.844 g.
– 50 mM Tris (stock 121.14 g/mol) – 6.055 g.
– Adjust pH to 8.0 and bring to 1 l with dH$_2$O.

AAV Purification by Q Sepharose Chromatography

1. Equilibrate a 1 ml HiTrap Q HP column (GE Healthcare) by washing with ten column volumes of low salt Q column buffer (0.020 M Tris, 0.015 M NaCl, pH 8.5). The flow rate for all chromatography steps is 1 ml/min.

2. Activate the column by washing with five column volumes of high salt Q column buffer (0.020 M Tris, 1.0 M NaCl, pH 8.5).
3. Re-equilibrate the column by washing in ten column volumes of low salt Q column buffer.
4. Apply the dialyzed virus from either the cesium or iodixanol gradient to the column. (Note: After recovering the virus from the cesium or iodixanol gradient, virus must be dialyzed into low salt buffer or a buffer exchange into low salt buffer must have been performed in order to bind to the Q column.)
5. Wash the column with ten volumes of low salt Q column buffer.
6. Elute with five column volumes of elution buffer (20 mM Tris, 0.5 M NaCl, pH 8.5) [190] and collect [5] 1-ml fractions.
7. Dialyze the virus preparation into storage buffer (50 mM Tris, 100 mM NaCl, pH 8.0) or suitable formulation aliquot, and freeze at −20°C.

Q Sepharose Chromatography Reagents

Q Sepharose low salt column buffer:

– 15 mM NaCl (stock 58.44 g/mol) − 0.877 g.
– 20 mM Tris (stock 121.14 g/mol) − 2.423 g.
– Adjust pH to 8.5 and bring the final volume to 1 l with dH$_2$O.

Q Sepharose high salt column buffer:

– 1 M NaCl (stock 58.44 g/mol) − 58.44 g.
– 20 mM Tris (stock 121.14 g/mol) − 2.423 g.
– Adjust pH to 8.5 and bring the final volume to 1 l with dH$_2$O.

Q Sepharose elution buffer:

– 0.5 M NaCl (stock 58.44 g/mol) − 29.22 g.
– 20 mM Tris (stock 121.14 g/mol) − 2.423 g.
– Adjust pH to 8.5 and bring the final volume to 1 l with dH$_2$O.

Storage buffer:

– 100 mM NaCl (stock 58.44 g/mol) − 5.844 g.
– 50 mM Tris (stock 121.14 g/mol) − 6.055 g.
– Adjust pH to 8.0 with HCl and bring to 1 l with dH$_2$O.

Protocol 2: AAV Large-Scale Production (10 Cell Factories ~1 × 10¹⁰ cells)

Transfection Protocol

1. Seed [10] ten-layer cell factories (Nunc) each with 5×10^8 293 cells so that each will be 75–80% confluent the next day. (Incubate 12–16 h prior to transfection.) 293 cells are cultured in DMEM supplemented with 5% fetal bovine serum, 1% penicillin, and 1% streptomycin.
2. Check each cell factory microscopically prior to transfection to ensure optimal confluency.
3. For transfection, prepare the DNA mixture in a 250-ml conical tube: Each cell factory requires 2,400 μg total DNA: 1,800 μg helper plasmid and 600 μg rAAV vector plasmid. (This is a 1:1 molar ratio.) Calculate the volume of total input DNA required. Bring the final volume of the DNA mixture to 46.8 ml with sterile dH_2O. Add 5.2 ml 2.5 M $CaCl_2$ to the DNA for a final $CaCl_2$ concentration of 0.25 M. The total volume of the DNA/$CaCl_2$/dH_2O is 52 ml.
4. Add the DNA to 52 ml of 2× HBS, mix well, and incubate for 1 min at room temperature to allow precipitate to form. Add the transfection mix to 1,000 ml of prewarmed DMEM-Complete Media. Discard media from cell factory. Pour media with transfection mix into the cell factory. Repeat for all cell factories.
5. Incubate at 37°C for 72 h.
6. At 72 h, harvest cells. Collect media and cells and centrifuge at $1,000 \times g$, for 10 min at 4°C to pellet the cells. Discard the media. Resuspend pellet in 300 ml PBS to wash the pellet. Centrifuge at $1,000 \times g$, for 10 min at 4°C. Discard PBS and freeze pellet. Frozen pellets can be stored at −20°C for processing at a later time.

Protocol 3: Large-Scale Purification Protocol for AAV2

1. Resuspend the cell pellet from one cell factory in 60 ml large-scale lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 0.5% deoxycholate, and Benzonase (50 U/ml)). Deoxycholate is used in the lysis buffer to reduce aggregation.
2. For ten cell factories, lyse the cells in a single-pass by using a microfluidizer (Microfluidics M-110S). This will form a fine suspension that can be loaded onto the column.
3. Load the lysate onto a 150-ml Streamline Heparin column (Pharmacia) at a flow rate of 20 ml/min using an AKTA-FPLC (Pharmacia).
4. Wash the column with four column volumes of lysis buffer, followed by five column volumes of PBS.
5. Elute the virus with PBS containing 0.2 M NaCl (0.350 M total ionic strength) and monitor the absorbance at 280 λ. The peak fraction will be ~90 ml.

6. Bring the NaCl concentration of the peak fraction to 1 M NaCl and load this onto a Phenyl Sepharose column (5 ml column, Pharmacia). This is a hydrophobic interaction column and the flow-through that contains the virus is collected.
7. The flow-through is diluted to 150 mM NaCl with sterile water.
8. The virus is loaded onto a 5 ml SP Sepharose column (Pharmacia) at a flow rate of 5 ml/min.
9. Wash the column with ten column volumes of PBS.
10. Elute the virus with PBS containing 0.135 M NaCl (285 mM total ionic strength) and monitor the absorbance at 280 λ. Aliquot and store at −20°C.
11. Dialyze the virus prep into storage buffer (50 mM Tris-Cl, 100 mM NaCl, pH 8.0), aliquot, and freeze at −20°C.

Protocol 4: Proteolytic Digestion for AAV Capsid Serotype Validation [201]

1. Dialyze AAV vector into protease digestion reaction buffer, 50 mM Tris-Cl, 100 mM NaCl, pH 8.0, if needed.
2. Digest 0.8 µg (~1.2×10^{11}) capsids with either 5 µg (0.02% final concentration) of trypsin, 1 µg of proteinase K, or 80 µg of α-chymotrypsin in a 25 µl reaction at 37°C for up to 24 h. For each serotype, an undigested sample should be included as a control. These proteases are commercially available from Sigma.
3. Add an equal volume of Laemmli sample buffer containing 1% sodium dodecyl sulfate (SDS) and 655 mM β-mercaptoethanol and boil the samples at 100°C for 5 min.
4. Separate the proteolytic fragments on a 10% SDS-PAGE gel for 90 min at 125 V (constant voltage) until the dye front reaches the bottom of the gel.
5. Transfer the proteins to nitrocellulose (Western blot) in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, and 20% methanol) for 2 h at 0.5 A (constant amperes).
6. Probe the membrane with rabbit polyclonal antisera to AAV capsids. Duplicate samples can be run to probe with other AAV antibodies to determine the origin of the fragments that are produced. For example, a signal with monoclonal B1 antibody demonstrates that the fragment is from the common C-terminal end of VP1, VP2, and VP3. Alternatively, by probing with monoclonal A1 antibody, a signal on the Western blot would demonstrate that the origin of the fragment is the unique N-terminus of VP1. The B1 epitope is conserved among most AAV serotypes and polyclonal antibodies have been developed for a few AAV serotypes. Anti-AAV capsid antibodies are available from Progen or American Research Products, Inc.

References

1. Berns KI, Bohenzky RA. Adeno-associated viruses: an update. Adv Virus Res 1987;32:243–306.
2. Bankiewicz KS, Forsayeth J, Eberling JL, Sanchez-Pernaute R, Pivirotto P, Bringas J, Herscovitch P, Carson RE, Eckelman W, Reutter B, Cunningham J. Long-term clinical improvement in MPTP-lesioned primates after gene therapy with AAV-hAADC. Mol Ther 2006;14:564–570.
3. Crystal RG, Sondhi D, Hackett NR, Kaminsky SM, Worgall S, Stieg P, Souweidane M, Hosain S, Heier L, Ballon D, Dinner M, Wisniewski K, Kaplitt M, Greenwald BM, Howell JD, Strybing K, Dyke J, Voss H. Clinical protocol. Administration of a replication-deficient adeno-associated virus gene transfer vector expressing the human CLN2 cDNA to the brain of children with late infantile neuronal ceroid lipofuscinosis. Hum Gene Ther 2004;15:1131–1154.
4. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, Ozelo MC, Hoots K, Blatt P, Konkle B, Dake M, Kaye R, Razavi M, Zajko A, Zehnder J, Rustagi PK, Nakai H, Chew A, Leonard D, Wright JF, Lessard RR, Sommer JM, Tigges M, Sabatino D, Luk A, Jiang H, Mingozzi F, Couto L, Ertl HC, High KA, Kay MA. Successful transduction of liver in hemophilia by AAV-factor IX and limitations imposed by the host immune response. Nat Med 2006;12:342–347.
5. During MJ, Kaplitt MG, Stern MB, Eidelberg D. Subthalamic GAD gene transfer in Parkinson disease patients who are candidates for deep brain stimulation. Hum Gene Ther 2001;12:1589–1591.
6. Stedman H, Wilson JM, Finke R, Kleckner AL, Mendell J. Phase I clinical trial utilizing gene therapy for limb girdle muscular dystrophy: alpha-, beta-, gamma-, or delta-sarcoglycan gene delivered with intramuscular instillations of adeno-associated vectors. Hum Gene Ther 2000;11:777–790.
7. Srivastava A. Hematopoietic stem cell transduction by recombinant adeno-associated virus vectors: problems and solutions. Hum Gene Ther 2005;16:792–798.
8. Work LM, Buning H, Hunt E, Nicklin SA, Denby L, Britton N, Leike K, Odenthal M, Drebber U, Hallek M, Baker AH. Vascular bed-targeted in vivo gene delivery using tropism-modified adeno-associated viruses. Mol Ther 2006;13:683–693.
9. De BP, Heguy A, Hackett NR, Ferris B, Leopold PL, Lee J, Pierre L, Gao G, Wilson JM, Crystal RG. High levels of persistent expression of alpha1-antitrypsin mediated by the nonhuman primate serotype rh.10 adeno-associated virus despite preexisting immunity to common human adeno-associated viruses. Mol Ther 2006;13:67–76.
10. Kauffman SL. Cell proliferation in the mammalian lung. Int Rev Exp Pathol 1980; 22:131–191.
11. Duan D, Sharma P, Yang J, Yue Y, Dudus L, Zhang Y, Fisher KJ, Engelhardt JF. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. J Virol 1998;72:8568–8577.
12. McCarty DM, Young SM, Jr, Samulski RJ. Integration of adeno-associated virus (AAV) and recombinant AAV vectors. Annu Rev Genet 2004;38:819–845.
13. Schnepp BC, Jensen RL, Chen CL, Johnson PR, Clark KR. Characterization of adeno-associated virus genomes isolated from human tissues. J Virol 2005;79:14793–14803.

14. Jooss K, Yang Y, Fisher KJ, Wilson JM. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. J Virol 1998; 72:4212–4223.
15. Zaiss AK, Muruve DA. Immune responses to adeno-associated virus vectors. Curr Gene Ther 2005;5:323–331.
16. Peden CS, Burger C, Muzyczka N, Mandel RJ. Circulating anti-wild-type adeno-associated virus type 2 (AAV2) antibodies inhibit recombinant AAV2 (rAAV2)-mediated, but not rAAV5-mediated, gene transfer in the brain. J Virol 2004;78:6344–6359.
17. Jiang H, Couto LB, Patarroyo-White S, Liu T, Nagy D, Vargas JA, Zhou S, Scallan CD, Sommer J, Vijay S, Mingozzi F, High KA, Pierce GF. Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. Blood 2006;108:3321–3328.
18. Snyder RO, Francis J. Adeno-associated viral vectors for clinical gene transfer studies. Curr Gene Ther 2005;5:311–321.
19. Timpe J, Bevington J, Casper J, Dignam JD, Trempe JP. Mechanisms of adeno-associated virus genome encapsidation. Curr Gene Ther 2005;5:273–284.
20. Becerra SP, Koczot F, Fabisch P, Rose JA. Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiations from a single transcript. J Virol 1988;62:2745–2754.
21. Myers MW, Carter BJ. Assembly of adeno-associated virus. Virology 1980;102:71–82.
22. de la Maza LM, Carter BJ. Molecular structure of adeno-associated virus variant DNA. J Biol Chem 1980;255:3194–3203.
23. King JA, Dubielzig R, Grimm D, Kleinschmidt JA. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. EMBO J 2001; 20:3282–3291.
24. Wistuba A, Kern A, Weger S, Grimm D, Kleinschmidt JA. Subcellular compartmentalization of adeno-associated virus type 2 assembly. J Virol 1997;71:1341–1352.
25. Ruffing M, Zentgraf H, Kleinschmidt JA. Assembly of viruslike particles by recombinant structural proteins of adeno-associated virus type 2 in insect cells. J Virol 1992;66:6922–6930.
26. Steinbach S, Wistuba A, Bock T, Kleinschmidt JA. Assembly of adeno-associated virus type 2 capsids in vitro. J Gen Virol 1997;78 (Pt 6):1453–1462.
27. Hermonat PL, Labow MA, Wright R, Berns KI, Muzyczka N. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J Virol 1984;51:329–339.
28. Girod A, Wobus CE, Zadori Z, Ried M, Leike K, Tijssen P, Kleinschmidt JA, Hallek M. The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. J Gen Virol 2002;83:973–978.
29. Warrington KH, Jr, Gorbatyuk OS, Harrison JK, Opie SR, Zolotukhin S, Muzyczka N. Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminus. J Virol 2004;78:6595–6609.
30. Govindasamy L, Padron E, McKenna R, Muzyczka N, Kaludov N, Chiorini JA, Agbandje-McKenna M. Structurally mapping the diverse phenotype of adeno-associated virus serotype 4. J Virol 2006;80:11556–11570.
31. Xie Q, Bu W, Bhatia S, Hare J, Somasundaram T, Azzi A, Chapman MS. The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. Proc Natl Acad Sci USA 2002;99:10405–10410.
32. DiMattia M, Govindasamy L, Levy HC, Gurda-Whitaker B, Kalina A, Kohlbrenner E, Chiorini JA, McKenna R, Muzyczka N, Zolotukhin S, Agbandje-McKenna M. Production, purification, crystallization and preliminary X-ray structural studies of adeno-associated virus serotype 5. Acta Crystallogr Sect F Struct Biol Cryst Commun 2005;61:917–921.
33. Padron E, Bowman V, Kaludov N, Govindasamy L, Levy H, Nick P, McKenna R, Muzyczka N, Chiorini JA, Baker TS, Agbandje-McKenna M. Structure of adeno-associated virus type 4. J Virol 2005;79:5047–5058.

34. Walters RW, Agbandje-McKenna M, Bowman VD, Moninger TO, Olson NH, Seiler M, Chiorini JA, Baker TS, Zabner J. Structure of adeno-associated virus serotype 5. J Virol 2004;78:3361–3371.
35. Lane MD, Nam H-J, Padron, E, Gurda-Whitaker B, Kohlbrenner E, Aslanidi G, Byrne B, McKenna R, Muzyczka N, Zolotukhin S, Agbandje-McKenna M. Production, purification, crystallization and preliminary X-ray analysis of adeno-associated virus serotype 8. Acta Crystallogr Sect F 2005;61:558–561.
36. Chapman MS, Rossmann MG. Structure, sequence, and function correlations among parvoviruses. Virology 1993;194:491–508.
37. Xie Q, Chapman MS. Canine parvovirus capsid structure, analyzed at 2.9 A resolution. J Mol Biol 1996;264:497–520.
38. Llamas-Saiz AL, Agbandje-McKenna M, Wikoff WR, Bratton J, Tattersall P, Rossmann MG. Structure determination of minute virus of mice. Acta Crystallogr D Biol Crystallogr 1997;53:93–102.
39 DeLano WL. The PyMOL Molecular Graphics System (2002) on the World Wide Web http://www.pymol.org.
40. Wu P, Xiao W, Conlon T, Hughes J, Agbandje-McKenna M, Ferkol T, Flotte T, Muzyczka N. Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. J Virol 2000;74:8635–8647.
41. Agbandje-McKenna M, Chapman MS. Correlating structure with function in the viral capsid. In: Kerr JR, Cotmore SF, Bloom ME, Linden RM, Parrish CR (eds) Parvoviruses. Edward Arnold, Ltd., New York, 2006:125–139.
42. Chapman MS, Agbandje-McKenna M. Atomic structure of viral particles. In: Kerr JR, Cotmore SF, Bloom ME, Linden RM, Parrish CR (eds) Parvoviruses. Edward Arnold, Ltd., New York, 2006:107–123.
43. Gao G, Alvira MR, Somanathan S, Lu Y, Vandenberghe LH, Rux JJ, Calcedo R, Sanmiguel J, Abbas Z, Wilson JM. Adeno-associated viruses undergo substantial evolution in primates during natural infections. Proc Natl Acad Sci USA 2003;100: 6081–6086.
44. Qing K, Mah C, Hansen J, Zhou S, Dwarki V, Srivastava A. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. Nat Med 1999; 5:71–77.
45. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J Virol 1998;72:1438–1445.
46. Summerford C, Bartlett JS, Samulski RJ. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. Nat Med 1999;5:78–82.
47. Kashiwakura Y, Tamayose K, Iwabuchi K, Hirai Y, Shimada T, Matsumoto K, Nakamura T, Watanabe M, Oshimi K, Daida H. Hepatocyte growth factor receptor is a coreceptor for adeno-associated virus type 2 infection. J Virol 2005;79:609–614.
48. Asokan A, Hamra JB, Govindasamy L, Agbandje-McKenna M, Samulski RJ. Adeno-associated virus type 2 contains an integrin alpha5beta1 binding domain essential for viral cell entry. J Virol 2006;80:8961–8969.
49. Blackburn SD, Steadman RA, Johnson FB. Attachment of adeno-associated virus type 3H to fibroblast growth factor receptor 1. Arch Virol 2006;151:617–623.
50. Di Pasquale G, Davidson BL, Stein CS, Martins I, Scudiero D, Monks A, Chiorini JA. Identification of PDGFR as a receptor for AAV-5 transduction. Nat Med 2003;9: 1306–1312.
51. Walters RW, Yi SM, Keshavjee S, Brown KE, Welsh MJ, Chiorini JA, Zabner J. Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. J Biol Chem 2001;276:20610–20616.
52. Kaludov N, Brown KE, Walters RW, Zabner J, Chiorini JA. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. J Virol 2001;75: 6884–6893.

53. Wu Z, Miller E, Agbandje-McKenna M, Samulski RJ. Alpha2,3 and alpha2,6 *N*-linked sialic acids facilitate efficient binding and transduction by adeno-associated virus types 1 and 6. J Virol 2006;80:9093–9103.

54. Gao G, Vandenberghe LH, Wilson JM. New recombinant serotypes of AAV vectors. Curr Gene Ther 2005;5:285–297.

55. Chen CL, Jensen RL, Schnepp BC, Connell MJ, Shell R, Sferra TJ, Bartlett JS, Clark KR, Johnson PR. Molecular characterization of adeno-associated viruses infecting children. J Virol 2005;79:14781–14792.

56. Schmidt M, Grot E, Cervenka P, Wainer S, Buck C, Chiorini JA. Identification and characterization of novel adeno-associated virus isolates in ATCC virus stocks. J Virol 2006;80:5082–5085.

57. Grimm D. Production methods for gene transfer vectors based on adeno-associated virus serotypes. Methods 2002;28:146–157.

58. Rabinowitz JE, Rolling F, Li C, Conrath H, Xiao W, Xiao X, Samulski RJ. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J Virol 2002;76:791–801.

59. Grimm D, Kay MA, Kleinschmidt JA. Helper virus-free, optically controllable, and two-plasmid-based production of adeno-associated virus vectors of serotypes 1 to 6. Mol Ther 2003;7:839–850.

60. Hauck B, Xu RR, Xie J, Wu W, Ding Q, Sipler M, Wang H, Chen L, Wright JF, Xiao W. Efficient AAV1-AAV2 hybrid vector for gene therapy of hemophilia. Hum Gene Ther 2006;17:46–54.

61. Ghosh A, Yue Y, Duan D. Viral serotype and the transgene sequence influence overlapping adeno-associated viral (AAV) vector-mediated gene transfer in skeletal muscle. J Gene Med 2006;8:298–305.

62. Duan D, Yan Z, Yue Y, Ding W, Engelhardt JF. Enhancement of muscle gene delivery with pseudotyped adeno-associated virus type 5 correlates with myoblast differentiation. J Virol 2001;75:7662–7671.

63. Denby L, Nicklin SA, Baker AH. Adeno-associated virus (AAV)-7 and -8 poorly transduce vascular endothelial cells and are sensitive to proteasomal degradation. Gene Ther 2005;12:1534–1538.

64. Wang Z, Zhu T, Qiao C, Zhou L, Wang B, Zhang J, Chen C, Li J, Xiao X. Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. Nat Biotechnol 2005;23:321–328.

65. Mingozzi F, Schuttrumpf J, Arruda VR, Liu Y, Liu YL, High KA, Xiao W, Herzog RW. Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. J Virol 2002;76:10497–10502.

66. Seppen J, Bakker C, de Jong B, Kunne C, van den Oever K, Vandenberghe K, de Waart R, Twisk J, Bosma P. Adeno-associated virus vector serotypes mediate sustained correction of bilirubin UDP glucuronosyltransferase deficiency in rats. Mol Ther 2006;13:1085–1092.

67. Vandendriessche T, Thorrez L, Acosta-Sanchez A, Petrus I, Wang L, Ma L, De Waele L, Iwasaki Y, Gillijns V, Wilson JM, Collen D, Chuah MK. Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 versus lentiviral vectors for hemophilia B gene therapy. J Thromb Haemost 2007;5:16–24.

68. Loiler SA, Conlon TJ, Song S, Tang Q, Warrington KH, Agarwal A, Kapturczak M, Li C, Ricordi C, Atkinson MA, Muzyczka N, Flotte TR. Targeting recombinant adeno-associated virus vectors to enhance gene transfer to pancreatic islets and liver. Gene Ther 2003;10:1551–1558.

69. Takeda S, Takahashi M, Mizukami H, Kobayashi E, Takeuchi K, Hakamata Y, Kaneko T, Yamamoto H, Ito C, Ozawa K, Ishibashi K, Matsuzaki T, Takata K, Asano Y, Kusano E. Successful gene transfer using adeno-associated virus vectors into the kidney: comparison among adeno-associated virus serotype 1–5 vectors in vitro and in vivo. Nephron Exp Nephrol 2004;96:e119–e126.

70. Seiler MP, Miller AD, Zabner J, Halbert CL. Adeno-associated virus types 5 and 6 use distinct receptors for cell entry. Hum Gene Ther 2006;17:10–19.
71. Limberis MP, Wilson JM. Adeno-associated virus serotype 9 vectors transduce murinealveolar and nasal epithelia and can be readministered. Proc Natl Acad Sci USA 2006; 103:12993–12998.
72. Glushakova LG, Timmers AM, Pang J, Teusner JT, Hauswirth WW. Human blue-opsin promoter preferentially targets reporter gene expression to rat s-cone photoreceptors. Invest Ophthalmol Vis Sci 2006;47:3505–3513.
73. Zhong L, Li W, Li Y, Zhao W, Wu J, Li B, Maina N, Bischof D, Qing K, Weigel-Kelley KA, Zolotukhin I, Warrington KH, Jr, Li X, Slayton WB, Yoder MC, Srivastava A. Evaluation of primitive murine hematopoietic stem and progenitor cell transduction in vitro and in vivo by recombinant adeno-associated virus vector serotypes 1 through 5. Hum Gene Ther 2006;17:321–333.
74. Aldrich WA, Ren C, White AF, Zhou SZ, Kumar S, Jenkins CB, Shaw DR, Strong TV, Triozzi PL, Ponnazhagan S. Enhanced transduction of mouse bone marrow-derived dendritic cells by repetitive infection with self-complementary adeno-associated virus 6 combined with immunostimulatory ligands. Gene Ther 2006;13:29–39.
75. Stone IM, Lurie DI, Kelley MW, Poulsen DJ. Adeno-associated virus-mediated gene transfer to hair cells and support cells of the murine cochlea. Mol Ther 2005;11:843–848.
76. Hacker UT, Wingenfeld L, Kofler DM, Schuhmann NK, Lutz S, Herold T, King SB, Gerner FM, Perabo L, Rabinowitz J, McCarty DM, Samulski RJ, Hallek M, Buning H. Adeno-associated virus serotypes 1 to 5 mediated tumor cell directed gene transfer and improvement of transduction efficiency. J Gene Med 2005;7:1429–1438.
77. Harding TC, Dickinson PJ, Roberts BN, Yendluri S, Gonzalez-Edick M, Lecouteur RA, Jooss KU. Enhanced gene transfer efficiency in the murine striatum and an orthotopic glioblastoma tumor model, using AAV-7- and AAV-8-pseudotyped vectors. Hum Gene Ther 2006;17: 807–820.
78. Thorsen F, Afione S, Huszthy PC, Tysnes BB, Svendsen A, Bjerkvig R, Kotin RM, Lonning PE, Hoover F. Adeno-associated virus (AAV) serotypes 2, 4 and 5 display similar transduction profiles and penetrate solid tumor tissue in models of human glioma. J Gene Med 2006;8:1131–1140.
79. Burger C, Gorbatyuk OS, Velardo MJ, Peden CS, Williams P, Zolotukhin S, Reier PJ, Mandel RJ, Muzyczka N. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. Mol Ther 2004;10:302–317.
80. Davidson BL, Stein CS, Heth JA, Martins I, Kotin RM, Derksen TA, Zabner J, Ghodsi A, Chiorini JA. Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. Proc Natl Acad Sci USA 2000;97:3428–3432.
81. Shen F, Su H, Liu W, Kan YW, Young WL, Yang GY. Recombinant adeno-associated viral vector encoding human VEGF165 induces neomicrovessel formation in the adult mouse brain. Front Biosci 2006;11:3190–3198.
82. Wang C, Wang CM, Clark KR, Sferra TJ. Recombinant AAV serotype 1 transduction efficiency and tropism in the murine brain. Gene Ther 2003;10:1528–1534.
83. Broekman ML, Comer LA, Hyman BT, Sena-Esteves M. Adeno-associated virus vectors serotyped with AAV8 capsid are more efficient than AAV-1 or -2 serotypes for widespread gene delivery to the neonatal mouse brain. Neuroscience 2006;138:501–510.
84. Wu K, Meyer EM, Bennett JA, Meyers CA, Hughes JA, King MA. AAV2/5-mediated NGF gene delivery protects septal cholinergic neurons following axotomy. Brain Res 2005;1061:107–113.
85. Tenenbaum L, Chtarto A, Lehtonen E, Velu T, Brotchi J, Levivier M. Recombinant AAV-mediated gene delivery to the central nervous system. J Gene Med 2004;6 (Suppl 1): S212–S222.

86. Du L, Kido M, Lee DV, Rabinowitz JE, Samulski RJ, Jamieson SW, Weitzman MD, Thistlethwaite PA. Differential myocardial gene delivery by recombinant serotype-specific adeno-associated viral vectors. Mol Ther 2004;10:604–608.

87. Pacak CA, Mah CS, Thattaliyath BD, Conlon TJ, Lewis MA, Cloutier DE, Zolotukhin I, Tarantal AF, Byrne BJ. Recombinant adeno-associated virus serotype 9 leads to preferential cardiac transduction in vivo. Circ Res 2006;99:e3–e9.

88. Su H, Huang Y, Takagawa J, Barcena A, Arakawa-Hoyt J, Ye J, Grossman W, Kan YW. AAV Serotype-1 mediates early onset of gene expression in mouse hearts and results in better therapeutic effect. Gene Ther 2006;13:1495–1502.

89. Kawamoto S, Shi Q, Nitta Y, Miyazaki J, Allen MD. Widespread and early myocardial gene expression by adeno-associated virus vector type 6 with a beta-actin hybrid promoter. Mol Ther 2005;11:980–985.

90. Thomas CE, Storm TA, Huang Z, Kay MA. Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. J Virol 2004; 78:3110–3122.

91. Wang AY, Peng PD, Ehrhardt A, Storm TA, Kay MA. Comparison of adenoviral and adeno-associated viral vectors for pancreatic gene delivery in vivo. Hum Gene Ther 2004; 15:405–413.

92. Halbert CL, Rutledge EA, Allen JM, Russell DW, Miller AD. Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. J Virol 2000;74:1524–1532.

93. Chen J, Wu Q, Yang P, Hsu HC, Mountz JD. Determination of specific CD4 and CD8 T cell epitopes after AAV2- and AAV8-hF.IX gene therapy. Mol Ther 2006;13:260–269.

94. Vandenberghe LH, Wang L, Somanathan S, Zhi Y, Figueredo J, Calcedo R, Sanmiguel J, Desai RA, Chen CS, Johnston J, Grant RL, Gao G, Wilson JM. Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. Nat Med 2006; 12:967–971.

95. Zabner J, Seiler M, Walters R, Kotin RM, Fulgeras W, Davidson BL, Chiorini JA. Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. J Virol 2000;74:3852–3858.

96. Halbert CL, Allen JM, Miller AD. Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. J Virol 2001;75:6615–6624.

97. Teschendorf C, Warrington KH, Jr, Shi W, Siemann DW, Muzyczka N. Recombinant adeno-associated and adenoviral vectors for the transduction of pancreatic and colon carcinoma. Anticancer Res 2006;26:311–317.

98. Chao H, Liu Y, Rabinowitz J, Li C, Samulski RJ, Walsh CE. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. Mol Ther 2000;2:619–623.

99. Grimm D, Pandey K, Nakai H, Storm TA, Kay MA. Liver transduction with recombinant adeno-associated virus is primarily restricted by capsid serotype not vector genotype. J Virol 2006;80:426–439.

100. Hauck B, Xiao W. Characterization of tissue tropism determinants of adeno-associated virus type 1. J Virol 2003;77:2768–2774.

101. Aikawa R, Huggins GS, Snyder RO. Cardiomyocyte-specific gene expression following recombinant adeno-associated viral vector transduction. J Biol Chem 2002;277: 18979–18985.

102. Girod A, Ried M, Wobus C, Lahm H, Leike K, Kleinschmidt J, Deleage G, Hallek M. Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. Nat Med 1999;5:1052–1056.

103. Wu P, Xiao W, Conlon T, Hughes J, Agbandje-McKenna M, Ferkol T, Flotte T, Muzyczka N. Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. J Virol 2000;74:8635–8647.

104. Ried MU, Girod A, Leike K, Buning H, Hallek M. Adeno-associated virus capsids displaying immunoglobulin-binding domains permit antibody-mediated vector retargeting to specific cell surface receptors. J Virol 2002;76:4559–4566.
105. White SJ, Nicklin SA, Buning H, Brosnan MJ, Leike K, Papadakis ED, Hallek M, Baker AH. Targeted gene delivery to vascular tissue in vivo by tropism-modified adeno-associated virus vectors. Circulation 2004;109:513–519.
106. Muller OJ, Leuchs B, Pleger ST, Grimm D, Franz WM, Katus HA, Kleinschmidt JA. Improved cardiac gene transfer by transcriptional and transductional targeting of adeno-associated viral vectors. Cardiovasc Res 2006;70:70–78.
107. Perabo L, Goldnau D, White K, Endell J, Boucas J, Humme S, Work LM, Janicki H, Hallek M, Baker AH, Buning H. Heparan sulfate proteoglycan binding properties of adeno-associated virus retargeting mutants and consequences for their in vivo tropism. J Virol 2006;80:7265–7269.
108. Shi X, Fang G, Shi W, Bartlett JS. Insertional mutagenesis at positions 520 and 584 of adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors with eliminated heparin-binding ability and introduced novel tropism. Hum Gene Ther 2006;17:353–361.
109. Ruffing M, Heid H, Kleinschmidt JA. Mutations in the carboxy terminus of adeno-associated virus 2 capsid proteins affect viral infectivity: lack of an RGD integrin-binding motif. J Gen Virol 1994;75 (Pt 12):3385–3392.
110. Gigout L, Rebollo P, Clement N, Warrington KH, Jr, Muzyczka N, Linden RM, Weber T. Altering AAV tropism with mosaic viral capsids. Mol Ther 2005;11:856–865.
111. Stachler MD, Bartlett JS. Mosaic vectors comprised of modified AAV1 capsid proteins for efficient vector purification and targeting to vascular endothelial cells. Gene Ther 2006;13:926–931.
112. Rabinowitz JE, Bowles DE, Faust SM, Ledford JG, Cunningham SE, Samulski RJ. Cross-dressing the virion: the transcapsidation of adeno-associated virus serotypes functionally defines subgroups. J Virol 2004;78:4421–4432.
113. Kohlbrenner E, Aslanidi G, Nash K, Shklyaev S, Campbell-Thompson M, Byrne BJ, Snyder RO, Muzyczka N, Warrington KH, Jr, Zolotukhin S. Successful production of pseudotyped rAAV vectors using a modified baculovirus expression system. Mol Ther 2005;12:1217–1225.
114. Virella-Lowell I, Zusman B, Foust K, Loiler S, Conlon T, Song S, Chesnut KA, Ferkol T, Flotte TR. Enhancing rAAV vector expression in the lung. J Gene Med 2005;7:842–850.
115. Ostedgaard LS, Rokhlina T, Karp PH, Lashmit P, Afione S, Schmidt M, Zabner J, Stinski MF, Chiorini JA, Welsh MJ. A shortened adeno-associated virus expression cassette for CFTR gene transfer to cystic fibrosis airway epithelia. Proc Natl Acad Sci USA 2005;102:2952–2957.
116. Sirninger J, Muller C, Braag S, Tang Q, Yue H, Detrisac C, Ferkol T, Guggino WB, Flotte TR. Functional characterization of a recombinant adeno-associated virus 5-pseudotyped cystic fibrosis transmembrane conductance regulator vector. Hum Gene Ther 2004;15:832–841.
117. Wagner JA, Messner AH, Moran ML, Daifuku R, Kouyama K, Desch JK, Manley S, Norbash AM, Conrad CK, Friborg S, Reynolds T, Guggino WB, Moss RB, Carter BJ, Wine JJ, Flotte TR, Gardner P. Safety and biological efficacy of an adeno-associated virus vector-cystic fibrosis transmembrane regulator (AAV-CFTR) in the cystic fibrosis maxillary sinus. Laryngoscope 1999;109:266–274.
118. Wagner JA, Nepomuceno IB, Messner AH, Moran ML, Batson EP, Dimiceli S, Brown BW, Desch JK, Norbash AM, Conrad CK, Guggino WB, Flotte TR, Wine JJ, Carter BJ, Reynolds TC, Moss RB, Gardner P. A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. Hum Gene Ther 2002;13:1349–1359.
119. Flotte TR, Zeitlin PL, Reynolds TC, Heald AE, Pedersen P, Beck S, Conrad CK, Brass-Ernst L, Humphries M, Sullivan K, Wetzel R, Taylor G, Carter BJ, Guggino WB. Phase I trial of

intranasal and endobronchial administration of a recombinant adeno-associated virus sero-
type 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: a two-part clinical study. Hum
Gene Ther 2003;14:1079–1088.

120. Aitken ML, Moss RB, Waltz DA, Dovey ME, Tonelli MR, McNamara SC, Gibson RL,
Ramsey BW, Carter BJ, Reynolds TC. A phase I study of aerosolized administration of
tgAAVCF to cystic fibrosis subjects with mild lung disease. Hum Gene Ther 2001;
12:1907–1916.

121. Wagner JA, Moran ML, Messner AH, Daifuku R, Conrad CK, Reynolds T, Guggino WB,
Moss RB, Carter BJ, Wine JJ, Flotte TR, Gardner P. A phase I/II study of tgAAV-CF for the
treatment of chronic sinusitis in patients with cystic fibrosis. Hum Gene Ther 1998;
9:889–909.

122. Moss RB, Rodman D, Spencer LT, Aitken ML, Zeitlin PL, Waltz D, Milla C, Brody AS,
Clancy JP, Ramsey B, Hamblett N, Heald AE. Repeated adeno-associated virus serotype 2
aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of
patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. Chest
2004;125:509–521.

123. Wagner JA, Nepomuceno IB, Shah N, Messner AH, Moran ML, Norbash AM, Moss RB,
Wine JJ, Gardner P. Maxillary sinusitis as a surrogate model for CF gene therapy clinical tri-
als in patients with antrostomies. J Gene Med 1999;1:13–21.

124. Flotte T, Carter B, Conrad C, Guggino W, Reynolds T, Rosenstein B, Taylor G, Walden S,
Wetzel R. A phase I study of an adeno-associated virus-CFTR gene vector in adult CF
patients with mild lung disease. Hum Gene Ther 1996;7:1145–1159.

125. Grimm D, Zhou S, Nakai H, Thomas CE, Storm TA, Fuess S, Matsushita T, Allen J, Surosky
R, Lochrie M, Meuse L, McClelland A, Colosi P, Kay MA. Preclinical in vivo evaluation of
pseudotyped adeno-associated virus vectors for liver gene therapy. Blood 2003;102:
2412–2419.

126. Davidoff AM, Gray JT, Ng CY, Zhang Y, Zhou J, Spence Y, Bakar Y, Nathwani AC.
Comparison of the ability of adeno-associated viral vectors pseudotyped with serotype 2, 5,
and 8 capsid proteins to mediate efficient transduction of the liver in murine and nonhuman
primate models. Mol Ther 2005;11:875–888.

127. Wang L, Takabe K, Bidlingmaier SM, Ill CR, Verma IM. Sustained correction of bleeding
disorder in hemophilia B mice by gene therapy. Proc Natl Acad Sci USA 1999;96:
3906–3910.

128. Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, Nagy D, Gown AM, Winther B,
Meuse L, Cohen LK, Thompson AR, Kay MA. Persistent and therapeutic concentrations of
human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat Genet
1997;16:270–276.

129. Wang L, Calcedo R, Nichols TC, Bellinger DA, Dillow A, Verma IM, Wilson JM. Sustained
correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8-mediated,
liver-directed gene therapy. Blood 2005;105:3079–3086.

130. Wang L, Nichols TC, Read MS, Bellinger DA, Verma IM. Sustained expression of therapeu-
tic level of factor IX in hemophilia B dogs by AAV-mediated gene therapy in liver. Mol Ther
2000;1:154–158.

131. Mount JD, Herzog RW, Tillson DM, Goodman SA, Robinson N, McCleland ML, Bellinger
D, Nichols TC, Arruda VR, Lothrop CD, Jr, High KA. Sustained phenotypic correction of
hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. Blood
2002;99:2670–2676.

132. Liu YL, Mingozzi F, Rodriguez-Colon SM, Joseph S, Dobrzynski E, Suzuki T, High KA,
Herzog RW. Therapeutic levels of factor IX expression using a muscle-specific promoter and
adeno-associated virus serotype 1 vector. Hum Gene Ther 2004;15:783–792.

133. Chao H, Monahan PE, Liu Y, Samulski RJ, Walsh CE. Sustained and complete phenotype
correction of hemophilia B mice following intramuscular injection of AAV1 serotype vec-
tors. Mol Ther 2001;4:217–222.

134. Arruda VR, Schuettrumpf J, Herzog RW, Nichols TC, Robinson N, Lotfi Y, Mingozzi F, Xiao W, Couto LB, High KA. Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1. Blood 2004;103:85–92.

135. Herzog RW, Mount JD, Arruda VR, High KA, Lothrop CD, Jr. Muscle-directed gene transfer and transient immune suppression result in sustained partial correction of canine hemophilia B caused by a null mutation. Mol Ther 2001;4:192–200.

136. Monahan PE, Samulski RJ, Tazelaar J, Xiao X, Nichols TC, Bellinger DA, Read MS, Walsh CE. Direct intramuscular injection with recombinant AAV vectors results in sustained expression in a dog model of hemophilia. Gene Ther 1998;5:40–49.

137. Chao H, Samulski R, Bellinger D, Monahan P, Nichols T, Walsh C. Persistent expression of canine factor IX in hemophilia B canines. Gene Ther 1999;6:1695–1704.

138. Kay MA, Manno CS, Ragni MV, Larson PJ, Couto LB, McClelland A, Glader B, Chew AJ, Tai SJ, Herzog RW, Arruda V, Johnson F, Scallan C, Skarsgard E, Flake AW, High KA. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet 2000;24:257–261.

139. Larson PJ, High KA. Gene therapy for hemophilia B: AAV-mediated transfer of the gene for coagulation factor IX to human muscle. Adv Exp Med Biol 2001;489:45–57.

140. Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, Tai SJ, Ragni MV, Thompson A, Ozelo M, Couto LB, Leonard DG, Johnson FA, McClelland A, Scallan C, Skarsgard E, Flake AW, Kay MA, High KA, Glader B. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood 2003;101:2963–2972.

141. Jiang H, Pierce GF, Ozelo MC, de Paula EV, Vargas JA, Smith P, Sommer J, Luk A, Manno CS, High KA, Arruda VR. Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. Mol Ther 2006;14:452–455.

142. Tan M, Qing K, Zhou S, Yoder MC, Srivastava A. Adeno-associated virus 2-mediated transduction and erythroid lineage-restricted long-term expression of the human beta-globin gene in hematopoietic cells from homozygous beta-thalassemic mice. Mol Ther 2001;3:940–946.

143. Johnston J, Tazelaar J, Rivera VM, Clackson T, Gao GP, Wilson JM. Regulated expression of erythropoietin from an AAV vector safely improves the anemia of beta-thalassemia in a mouse model. Mol Ther 2003;7:493–497.

144. Hayashita-Kinoh H, Yamada M, Yokota T, Mizuno Y, Mochizuki H. Down-regulation of alpha-synuclein expression can rescue dopaminergic cells from cell death in the substantia nigra of Parkinson's disease rat model. Biochem Biophys Res Commun 2006;341: 1088–1095.

145. Hadaczek P, Kohutnicka M, Krauze MT, Bringas J, Pivirotto P, Cunningham J, Bankiewicz K. Convection-enhanced delivery of adeno-associated virus type 2 (AAV2) into the striatum and transport of AAV2 within monkey brain. Hum Gene Ther 2006;17:291–302.

146. Forsayeth JR, Eberling JL, Sanftner LM, Zhen Z, Pivirotto P, Bringas J, Cunningham J, Bankiewicz KS. A dose-ranging study of AAV-hAADC therapy in Parkinsonian monkeys. Mol Ther 2006;14:571–577.

147. Sanftner LM, Sommer JM, Suzuki BM, Smith PH, Vijay S, Vargas JA, Forsayeth JR, Cunningham J, Bankiewicz KS, Kao H, Bernal J, Pierce GF, Johnson KW. AAV2-mediated gene delivery to monkey putamen: evaluation of an infusion device and delivery parameters. Exp Neurol 2005;194:476–483.

148. During MJ, Samulski RJ, Elsworth JD, Kaplitt MG, Leone P, Xiao X, Li J, Freese A, Taylor JR, Roth RH, Sladek JR, Jr, O'Malley KL, Redmond DE, Jr. In vivo expression of therapeutic human genes for dopamine production in the caudates of MPTP-treated monkeys using an AAV vector. Gene Ther 1998;5:820–827.

149. Bankiewicz KS, Leff SE, Nagy D, Jungles S, Rokovich J, Spratt K, Cohen L, Libonati M, Snyder RO, Mandel RJ. Practical aspects of the development of ex vivo and in vivo gene therapy for Parkinson's disease. Exp Neurol 1997;144:147–156.

150. Daly TM, Okuyama T, Vogler C, Haskins ME, Muzyczka N, Sands MS. Neonatal intramuscular injection with recombinant adeno-associated virus results in prolonged beta-glucuronidase expression in situ and correction of liver pathology in mucopolysaccharidosis type VII mice. Hum Gene Ther 1999;10:85–94.

151. Fraites TJ, Jr, Schleissing MR, Shanely RA, Walter GA, Cloutier DA, Zolotukhin I, Pauly DF, Raben N, Plotz PH, Powers SK, Kessler PD, Byrne BJ. Correction of the enzymatic and functional deficits in a model of Pompe disease using adeno-associated virus vectors. Mol Ther 2002;5:571–578.

152. Sun B, Zhang H, Franco LM, Brown T, Bird A, Schneider A, Koeberl DD. Correction of glycogen storage disease type II by an adeno-associated virus vector containing a muscle-specific promoter. Mol Ther 2005;11:889–898.

153. Watson G, Bastacky J, Belichenko P, Buddhikot M, Jungles S, Vellard M, Mobley WC, Kakkis E. Intrathecal administration of AAV vectors for the treatment of lysosomal storage in the brains of MPS I mice. Gene Ther 2006;13:917–925.

154. Passini MA, Dodge JC, Bu J, Yang W, Zhao Q, Sondhi D, Hackett NR, Kaminsky SM, Mao Q, Shihabuddin LS, Cheng SH, Sleat DE, Stewart GR, Davidson BL, Lobel P, Crystal RG. Intracranial delivery of CLN2 reduces brain pathology in a mouse model of classical late infantile neuronal ceroid lipofuscinosis. J Neurosci 2006;26:1334–1342.

155. McEachern KA, Nietupski JB, Chuang WL, Armentano D, Johnson J, Hutto E, Grabowski GA, Cheng SH, Marshall J. AAV8-mediated expression of glucocerebrosidase ameliorates the storage pathology in the visceral organs of a mouse model of Gaucher disease. J Gene Med 2006;8:719–729.

156. Cardone M, Polito VA, Pepe S, Mann L, D'Azzo A, Auricchio A, Ballabio A, Cosma MP. Correction of Hunter syndrome in the MPSII mouse model by AAV2/8-mediated gene delivery. Hum Mol Genet 2006;15:1225–1236.

157. Matalon R, Surendran S, Rady PL, Quast MJ, Campbell GA, Matalon KM, Tyring SK, Wei J, Peden CS, Ezell EL, Muzyczka N, Mandel RJ. Adeno-associated virus-mediated aspartoacylase gene transfer to the brain of knockout mouse for Canavan disease. Mol Ther 2003;7:580–587.

158. Leone P, Janson CG, Bilaniuk L, Wang Z, Sorgi F, Huang L, Matalon R, Kaul R, Zeng Z, Freese A, McPhee SW, Mee E, During MJ. Aspartoacylase gene transfer to the mammalian central nervous system with therapeutic implications for Canavan disease. Ann Neurol 2000;48:27–38.

159. McPhee SW, Janson CG, Li C, Samulski RJ, Camp AS, Francis J, Shera D, Lioutermann L, Feely M, Freese A, Leone P. Immune responses to AAV in a phase I study for Canavan disease. J Gene Med 2006;8:577–588.

160. Janson C, McPhee S, Bilaniuk L, Haselgrove J, Testaiuti M, Freese A, Wang DJ, Shera D, Hurh P, Rupin J, Saslow E, Goldfarb O, Goldberg M, Larijani G, Sharrar W, Liouterman L, Camp A, Kolodny E, Samulski J, Leone P. Clinical protocol. Gene therapy of Canavan disease: AAV-2 vector for neurosurgical delivery of aspartoacylase gene (ASPA) to the human brain. Hum Gene Ther 2002;13:1391–1412.

161. Wang Z, Zhu T, Rehman KK, Bertera S, Zhang J, Chen C, Papworth G, Watkins S, Trucco M, Robbins PD, Li J, Xiao X. Widespread and stable pancreatic gene transfer by adeno-associated virus vectors via different routes. Diabetes 2006;55:875–884.

162. Rehman KK, Wang Z, Bottino R, Balamurugan AN, Trucco M, Li J, Xiao X, Robbins PD. Efficient gene delivery to human and rodent islets with double-stranded (ds) AAV-based vectors. Gene Ther 2005;12:1313–1323.

163. Fukuchi K, Tahara K, Kim HD, Maxwell JA, Lewis TL, Accavitti-Loper MA, Kim H, Ponnazhagan S, Lalonde R. Anti-Abeta single-chain antibody delivery via adeno-associated virus for treatment of Alzheimer's disease. Neurobiol Dis 2006;23:502–511.

164. Hara H, Monsonego A, Yuasa K, Adachi K, Xiao X, Takeda S, Takahashi K, Weiner HL, Tabira T. Development of a safe oral Abeta vaccine using recombinant adeno-associated virus vector for Alzheimer's disease. J Alzheimer Dis 2004;6:483–488.

165. Zhang J, Wu X, Qin C, Qi J, Ma S, Zhang H, Kong Q, Chen D, Ba D, He W. A novel recombinant adeno-associated virus vaccine reduces behavioral impairment and beta-amyloid plaques in a mouse model of Alzheimer's disease. Neurobiol Dis 2003;14:365–379.

166. Pachori AS, Melo LG, Zhang L, Solomon SD, Dzau VJ. Chronic recurrent myocardial ischemic injury is significantly attenuated by pre-emptive adeno-associated virus heme oxygenase-1 gene delivery. J Am Coll Cardiol 2006;47:635–643.

167. Woo YJ, Zhang JC, Taylor MD, Cohen JE, Hsu VM, Sweeney HL. One year transgene expression with adeno-associated virus cardiac gene transfer. Int J Cardiol 2005;100: 421–426.

168. Shi W, Hemminki A, Bartlett JS. Capsid modifications overcome low heterogeneous expression of heparan sulfate proteoglycan that limits AAV2-mediated gene transfer and therapeutic efficacy in human ovarian carcinoma. Gynecol Oncol 2006: 103:1054–1062.

169. Koppold B, Sauer G, Buning H, Hallek M, Kreienberg R, Deissler H, Kurzeder C. Efficient gene transfer of CD40 ligand into ovarian carcinoma cells with a recombinant adeno-associated virus vector. Int J Oncol 2005;26:95–101.

170. Subramanian IV, Ghebre R, Ramakrishnan S. Adeno-associated virus-mediated delivery of a mutant endostatin suppresses ovarian carcinoma growth in mice. Gene Ther 2005; 12:30–38.

171. Li ZB, Zeng ZJ, Chen Q, Luo SQ, Hu WX. Recombinant AAV-mediated HSVtk gene transfer with direct intratumoral injections and Tet-On regulation for implanted human breast cancer. BMC Cancer 2006;6:66.

172. Yanamandra N, Kondraganti S, Gondi CS, Gujrati M, Olivero WC, Dinh DH, Rao JS. Recombinant adeno-associated virus (rAAV) expressing TFPI-2 inhibits invasion, angiogenesis and tumor growth in a human glioblastoma cell line. Int J Cancer 2005;115: 998–1005.

173. Watanabe M, Nasu Y, Kashiwakura Y, Kusumi N, Tamayose K, Nagai A, Sasano T, Shimada T, Daida H, Kumon H. Adeno-associated virus 2-mediated intratumoral prostate cancer gene therapy: long-term maspin expression efficiently suppresses tumor growth. Hum Gene Ther 2005;16:699–710.

174. Sun X, Krissansen GW, Fung PW, Xu S, Shi J, Man K, Fan ST, Xu R. Anti-angiogenic therapy subsequent to adeno-associated-virus-mediated immunotherapy eradicates lymphomas that disseminate to the liver. Int J Cancer 2005;113:670–677.

175. Ma H, Liu Y, Liu S, Xu R, Zheng D. Oral adeno-associated virus-sTRAIL gene therapy suppresses human hepatocellular carcinoma growth in mice. Hepatology 2005;42: 1355–1363.

176. Shi J, Zheng D, Liu Y, Sham MH, Tam P, Farzaneh F, Xu R. Overexpression of soluble TRAIL induces apoptosis in human lung adenocarcinoma and inhibits growth of tumor xenografts in nude mice. Cancer Res 2005;65:1687–1692.

177. Zhang W, Singam R, Hellermann G, Kong X, Juan HS, Lockey RF, Wu SJ, Porter K, Mohapatra SS. Attenuation of dengue virus infection by adeno-associated virus-mediated siRNA delivery. Genet Vaccines Ther 2004;2:8.

178. Murphy JE, Zhou S, Giese K, Williams LT, Escobedo JA, Dwarki VJ. Long-term correction of obesity and diabetes in genetically obese mice by a single intramuscular injection of recombinant adeno-associated virus encoding mouse leptin. Proc Natl Acad Sci USA 1997;94:13921–13926.

179. Keen-Rhinehart E, Kalra SP, Kalra PS. AAV-mediated leptin receptor installation improves energy balance and the reproductive status of obese female Koletsky rats. Peptides 2005;26:2567–2578.

180. Shklyaev S, Aslanidi G, Tennant M, Prima V, Kohlbrenner E, Kroutov V, Campbell-Thompson M, Crawford J, Shek EW, Scarpace PJ, Zolotukhin S. Sustained peripheral expression of transgene adiponectin offsets the development of diet-induced obesity in rats. Proc Natl Acad Sci USA 2003;100:14217–14222.

181. Xin KQ, Mizukami H, Urabe M, Toda Y, Shinoda K, Yoshida A, Oomura K, Kojima Y, Ichino M, Klinman D, Ozawa K, Okuda K. Induction of robust immune responses against HIV is supported by the inherent tropism of AAV5 for DC. J Virol 2006;80: 11899–11910.

182. Kuck D, Lau T, Leuchs B, Kern A, Muller M, Gissmann L, Kleinschmidt JA. Intranasal vaccination with recombinant adeno-associated virus type 5 against human papillomavirus type 16 L1. J Virol 2006;80:2621–2630.

183. Blouin V, Brument N, Toublanc E, Raimbaud I, Moullier P, Salvetti A. Improving rAAV production and purification: towards the definition of a scaleable process. J Gene Med 2004;6 (Suppl 1):S223–S228.

184. Booth MJ, Mistry A, Li X, Thrasher A, Coffin RS. Transfection-free and scalable recombinant AAV vector production using HSV/AAV hybrids. Gene Ther 2004;11:829–837.

185. Conway JE, Rhys CM, Zolotukhin I, Zolotukhin S, Muzyczka N, Hayward GS, Byrne BJ. High-titer recombinant adeno-associated virus production utilizing a recombinant herpes simplex virus type I vector expressing AAV-2 Rep and Cap. Gene Ther 1999;6:986–993.

186. Farson D, Harding TC, Tao L, Liu J, Powell S, Vimal V, Yendluri S, Koprivnikar K, Ho K, Twitty C, Husak P, Lin A, Snyder RO, Donahue BA. Development and characterization of a cell line for large-scale, serum-free production of recombinant adeno-associated viral vectors. J Gene Med 2004;6:1369–1381.

187. Urabe M, Ding C, Kotin RM. Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum Gene Ther 2002;13:1935–1943.

188. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1997;36:59–74.

189. Matsushita T, Elliger S, Elliger C, Podsakoff G, Villarreal L, Kurtzman GJ, Iwaki Y, Colosi P. Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther 1998;5:938–945.

190. Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Fraites TJ, Jr, Chiodo VA, Phillipsberg T, Muzyczka N, Hauswirth WW, Flotte TR, Byrne BJ, Snyder RO. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 2002;28:158–167.

191. Francis JD, Snyder RO. Production of research and clinical grade recombinant adeno-associated viral vectors. In: Berns KI, Flotte TR (eds) Laboratory techniques in biochemistry and molecular biology. Elsevier, Amsterdam, 2005:19–56.

192. Zen Z, Espinoza Y, Bleu T, Sommer JM, Wright JF. Infectious titer assay for adeno-associated virus vectors with sensitivity sufficient to detect single infectious events. Hum Gene Ther 2004;15:709–715.

193. Clark KR, Liu X, McGrath JP, Johnson PR. Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. Hum Gene Ther 1999;10:1031–1039.

194. Drittanti L, Rivet C, Manceau P, Danos O, Vega M. High throughput production, screening and analysis of adeno-associated viral vectors. Gene Ther 2000;7:924–929.

195. Veldwijk MR, Topaly J, Laufs S, Hengge UR, Wenz F, Zeller WJ, Fruehauf S. Development and optimization of a real-time quantitative PCR-based method for the titration of AAV-2 vector stocks. Mol Ther 2002;6:272–278.

196. Gao G, Qu G, Burnham MS, Huang J, Chirmule N, Joshi B, Yu QC, Marsh JA, Conceicao CM, Wilson JM. Purification of recombinant adeno-associated virus vectors by column chromatography and its performance in vivo. Hum Gene Ther 2000;11:2079–2091.

197. Sommer JM, Smith PH, Parthasarathy S, Isaacs J, Vijay S, Kieran J, Powell SK, McClelland A, Wright JF. Quantification of adeno-associated virus particles and empty capsids by optical density measurement. Mol Ther 2003;7:122–128.

198. Kronenberg S, Kleinschmidt JA, Bottcher B. Electron cryo-microscopy and image reconstruction of adeno-associated virus type 2 empty capsids. EMBO Rep 2001;2:997–1002.

199. Bleker S, Pawlita M, Kleinschmidt JA. Impact of capsid conformation and Rep-capsid interactions on adeno-associated virus type 2 genome packaging. J Virol 2006;80:810–820.

200. Wobus CE, Hugle-Dorr B, Girod A, Petersen G, Hallek M, Kleinschmidt JA. Monoclonal antibodies against the adeno-associated virus type 2 (AAV-2) capsid: epitope mapping and identification of capsid domains involved in AAV-2-cell interaction and neutralization of AAV-2 infection. J Virol 2000;74:9281–9293.
201. Van Vliet K, Blouin V, Agbandje-McKenna M, Snyder RO. Proteolytic mapping of the adeno-associated virus capsid. Mol Ther 2006;14:809–821.