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AAV Hybrid Serotypes: Improved Vectors for Gene Delivery

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Abstract

In recent years, significant efforts have been made on studying and engineering adeno-associated virus (AAV) capsid, in order to increase efficiency in targeting specific cell types that are non-permissive to wild type (wt) viruses and to improve efficacy in infecting only the cell type of interest. With our previous knowledge of the viral properties of the naturally occurring serotypes and the elucidation of their capsid structures, we can now generate capsid mutants, or hybrid serotypes, by various methods and strategies. In this review, we summarize the studies performed on AAV retargeting, and categorize the available hybrid serotypes to date, based on the type of modification: 1) transcapsidation, 2) adsorption of bi-specific antibody to capsid surface, 3) mosaic capsid, and 4) chimeric capsid. Not only these hybrid serotypes could achieve high efficiency of gene delivery to a specific targeted cell type, which can be better-tailored for a particular clinical application, but also serve as a tool for studying AAV biology such as receptor binding, trafficking and genome delivery into the nucleus.

Keywords

Adeno associated virus; serotypes; capsids; tissue tropism

ABBREVIATIONS

AAV = Adeno-associated virus; Ad = Adeno Virus; $\alpha_v\beta_5$ = Integrin alpha-V-beta-5; Cap = Capsid protein; FGFR1 = Fibroblast growth factor receptor - 1; GAG = Glycosaminoglycan; GFP = Green fluorescent protein; HGFR = Hepatocyte growth factor receptor; HSPG = Heparan sulfate proteoglycans; HSV = Herpes simplex virus; ITR = Inverted terminal repeat; RBS = Rep binding site; Rep = Replication protein; scAAV = Self-complementary adeno-associated virus; SV = Simian adeno virus; TRS = Terminal resolution site; VP = Viral proteins; WT = Wild-type

INTRODUCTION

Adeno-Associated Virus (AAV) is a non-pathogenic single-stranded DNA parvovirus, with a capsid diameter of 26nm. Each end of the single-stranded DNA genome contains an inverted terminal repeat (ITR), which is the only cis-acting element required for genome replication and packaging. The genome carries two viral genes: *rep* and *cap*. The virus utilizes two promoters and alternative splicing to generate four proteins necessary for replication (Rep78,

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Rep 68, Rep 52 and Rep 40), while a third promoter generates the transcript for three structural viral capsid proteins, 1, 2 and 3 (VP1, VP2 and VP3), through a combination of alternate splicing and alternate translation start condons [Berns, KI and Linden, RM, 1995]. The three capsid proteins share the same C-terminal 533 amino acids, while VP2 and VP1 contain additional N-terminal sequences of 65 and 202 amino acids, respectively. The AAV virion contains a total of 60 copies of VP1, VP2, and VP3 at a 1:1:20 ratio, arranged in a T=1 icosahedral symmetry [Rose, JA *et al.*, 1971; Muzyczka, N. 1992].

As a dependovirus, AAV requires Adenovirus (Ad) or Herpes Simplex Virus (HSV) as a helper virus to complete its lytic life-cycle [Atchison, RW *et al.*, 1965; Hoggan, MD *et al.*, 1966; Conway, JE *et al.*, 1997]. In the absence of the helper virus, wt AAV establishes latency by integration with the assistance of Rep proteins through the interaction of the ITR with the chromosome [Berns, KI and Linden, RM, 1995]. Recombinant AAV (rAAV) gene delivery vectors can be produced by removing the two viral genes (*rep* and *cap*) and inserting a transgene expression cassette between the two ITRs, with Rep and Cap provided *in trans*. Because there are no viral genes in the rAAV, toxicity associated with this vector is minimal. Therefore, rAAV vectors are a desirable tool used in delivering a vast range of transgenes applicable to many disease models.

For a gene delivery vector, a successful therapeutic outcome can only be achieved by efficient infection of target tissues and establishment of long term gene expression. It has been extensively demonstrated that rAAV can successfully infect and transduce a broad variety of cell and tissue types, such as brain, liver, muscle, etc [During, MJ *et al.*, 1998; Song, S *et al.*, 1998; Ye, X *et al.*, 1999; Acland, GM *et al.*, 2001; Flotte, TR, 2001] and has the ability to infect both dividing and quiescent cells [Lewis, PF and Emerman, M. 1994; Alexander, IE *et al.*, 1996]. Not only can rAAV infect a broad spectrum of cell types, long term gene expression greater than 1.5 years has also been demonstrated in animal models including canine, murine and hamster [McCown, TJ *et al.*, 1996; Xiao, X *et al.*, 1996; Monahan, PE *et al.*, 1998; Herzog, RW *et al.*, 1999; Snyder, RO *et al.*, 1999; Cottard, V *et al.*, 2000; Song, S *et al.*, 2001; Asfour, B *et al.*, 2002; Li, J *et al.*, 2003].

At the cellular level, AAV undergoes 5 major steps prior to achieving gene expression: 1) binding or attachment to cellular surface receptors, 2) endocytosis, 3) trafficking to the nucleus, 4) uncoating of the virus to release the genome and 5) conversion of the genome from single-stranded to double-stranded DNA as a template for transcription in the nucleus. The cumulative efficiency with which rAAV can successfully execute each individual step, determines the overall transduction efficiency. Rate limiting steps in rAAV transduction include the absence or low abundance of required cellular surface receptors for viral attachment and internalization, inefficient endosomal escape leading to lysosomal degradation, and slow conversion of single-stranded to double-stranded DNA template. Therefore, with a better understanding of AAV biology, vectors with modifications to the genome and/or the capsids can be designed to facilitate more efficient or more specific transduction or cells or tissues for gene therapy.

In this review, we will focus our discussion on the ongoing efforts made in 1) understanding the naturally existing AAV serotypes (1–11); 2) the importance of AAV crystal structure and its applications; and 3) methods and strategies for generating hybrid serotypes with capsid protein modifications that increase the efficiency and efficacy of viral infection in tissues of interest, including i) transcapsidation, ii) adsorption of antibody to capsid surface, iii) mosaic capsid, and iv) chimeric capsid. Also, we will discuss the implications of these AAV hybrid serotypes for gene therapy as well as AAV biology, including signal transduction, trafficking, and virion assembly. Important intricacies of using these hybrid serotypes will also be discussed.

1. AAV SEROTYPES

Definition of Serotypes

Before discussing the development of novel rAAV hybrid vectors, it is important to define AAV serology, because there has been controversy in the field regarding what constitutes a new serotype. Serology is defined as the inability of an antibody that is reactive to the viral capsid proteins of one serotype in neutralizing those of another serotype. As more and more naturally occurring isolates of AAV are discovered and AAV capsid mutants generated, the true distinctions between serotypes can become blurred. Theoretically, a new serotype can only be named when the newly isolated virus of interest has been tested for neutralization against serum specific for all existing and characterized serotypes. If there is no serological difference with any of the currently existing serotypes, then the new virus is a subgroup or variant of the corresponding serotype. Therefore, naturally occurring serotypes discussed in this review are under the working definition of serotype as discussed in published studies and mutant viruses with modifications are considered as hybrid serotypes. In most cases, serology testing has yet to be performed on mutant viruses with capsid sequence modifications.

1.1. Isolation of AAV Serotypes from Animal Tissues: AAV1 - 11

AAV serotype 2 (AAV2) was the first AAV serotype to be cloned into bacterial plasmids [Samulski, RJ *et al.*, 1982] after its discovery as a contaminant in an Adenovirus type 12 stock [Hoggan, MD *et al.*, 1966]. A tremendous amount of work has been performed on understanding the biology of AAV2, and therefore it is the best characterized among all naturally discovered serotypes. As a consequence, early AAV clinical trials have been heavily focused on the use of AAV2. Since the discovery of AAV2, 10 other serotypes have been discovered and cloned for development into gene therapy vectors.

AAV1 and AAV3 were found to be contaminants of a simian Adenovirus type 15 (SV15) stock [Atchison, RW *et al.*, 1965] and Adenovirus type 7 [Hoggan, MD *et al.*, 1966], respectively. AAV3 has an overall sequence similarity of 82% with AAV2 [Muramatsu, S *et al.*, 1996]. A genetic variant, AAV3B, with differences in only 16 nucleotides was also identified in the stock from which AAV3 was isolated. AAV4 particles, and their corresponding antibodies, were isolated only in African green monkeys infected with SV15 stock [Parks, WP *et al.*, 1967] and were subsequently cloned and used as a vector by Chiorini and co-workers [Chiorini, JA *et al.*, 1997]. Interestingly, its genome has been demonstrated to integrate preferentially into simian chromosomes in CV-1 cells [Amiss, TJ *et al.*, 2003]. AAV5, originally identified from a human clinical sample [Bantel-Schaal, U and zur Hausen, H, 1984; Rutledge, EA *et al.*, 1998], contains ITRs that are structurally similar to AAV2, but are only 58% identical at the sequence level [Chiorini, JA *et al.*, 1999]. The rep-binding site (RBS) in the AAV5 ITR is conserved between AAV5 and AAV2, but the terminal resolution site (*trs*) for Rep mediated DNA endonuclease activity is different. The AAV5 RBS is larger and the spacing between the *trs* and the RBS is greater than AAV2. The nucleotide sequence of AAV5 *rep* is 67% identical to AAV2 Rep, while the sequence of AAV5 *cap* is only 56% identical to AAV2 *cap*. AAV5 is one of the most divergent of the AAV serotypes [Chiorini, JA *et al.*, 1999]. AAV6 was isolated as a contaminant of an adenovirus type 5 stock and is believed to be either a variant of AAV1, with only 6 amino acids difference between their capsids [Rutledge, EA *et al.*, 1998], or a natural recombinant between AAV1 and AAV2 [Xiao, W *et al.*, 1999]. AAV7 and AAV8 were isolated from rhesus monkey tissues [Gao, GP *et al.*, 2002] by PCR, utilizing primers to highly conserved regions of the capsid sequence, AAV9, which was isolated from human tissue, demonstrated high transduction efficiency in the lung, muscle and liver [Gao, G *et al.*, 2004]. AAV10 and AAV11 were isolated from cynomolgus monkey tissue using a similar PCR strategy, and demonstrated the ability to infect both human and monkey cells [Mori, S *et al.*, 2004]. In addition to human and primates, AAV has also been isolated from

different species including cow [Schmidt, M *et al.*, 2004], chicken [Bossis, I and Chiorini, JA, 2003], sheep [Clarke, JK *et al.*, 1979], snake [Farkas, SL *et al.*, 2004], lizard [Jacobson, ER *et al.*, 1996], and goat [Olson, EJ *et al.*, 2004]. The isolation of new AAV serotypes from animal tissues and the development of these new variants into gene therapy vectors are discussed in a separate review in this issue [Gao *et al.*].

1.2. The Best Characterized AAV Serotype: AAV2

Among all naturally occurring serotypes isolated to date, AAV2 is the best characterized and extensively studied and has served as the archetype for AAV biology. The first step of AAV infection is to utilize cell surface receptor as the initial step in docking and interacting with cell surface moieties. Different AAV serotypes are infectious in different cell types, depending on the availability of suitable high-affinity receptor molecules. Heparan sulfate proteoglycan (HSPG), a widely-expressed cell surface receptor, is the only type of glycosaminoglycan (GAG) that AAV2 uses as a primary receptor for cell attachment, and the efficiency of AAV infection is directly related to the presence and concentration of HSPG receptors [Summerford, C and Samulski, RJ, 1998]. AAV2 also utilizes co-receptors to assist its internalization, including the fibroblast growth factor receptor-1 (FGFR1) [Qing, K *et al.*, 1999], integrin α -V- β 5 ($\alpha_v\beta_5$) [Summerford, C *et al.*, 1999], and hepatocyte growth factor receptor (HGFR) [Kashiwakura, Y *et al.*, 2005]. Qing and co-workers suggested that AAV infection is more efficient when HSPG and FGFR are co-expressed on the cell surface, as HSPG serves as the initial docking receptor for AAV2 and FGFR assists viral internalization into the cell. [Qing, K *et al.*, 1998]. While the cellular receptors for some AAV serotypes are still unknown, significant progress has been made in our understanding of the interaction between cellular surface receptors and other AAV serotypes. It has been demonstrated that AAV4 binds to O-linked α 2–3 linked sialic acid, while AAV5 binds to N-linked α 2–3 or 2–6 sialic acid [Kaludov, N *et al.*, 2001; Walters, RW *et al.*, 2001]. A co-receptor, platelet-derived growth factor receptor (PDGFR- α), has also been identified for AAV5 [Di Pasquale, G *et al.*, 2003].

Following receptor binding, rAAV2 particles enter the cell via a dynamin-dependent clathrin-coated pit endocytosis pathway. The virus is then trafficked from early endosome to late endosome, and is believed to escape from the endosome and accumulate in a perinuclear pattern before the genome is delivered to the nucleus [Weitzman, MD *et al.*, 1996; Duan, D *et al.*, 1999; Bartlett, JS *et al.*, 2000; Hansen, J *et al.*, 2000; Sanlioglu, S *et al.*, 2000; Xiao, W *et al.*, 2002]. With the assistance of host cell DNA polymerase, the genome is converted from single-stranded to double-stranded DNA, which serves as a transcription template. While the full scope of differences between the known wt serotypes, and their relationship to the establishment of successful transduction is yet to be fully investigated, it is already recognized that these differences can be exploited and expanded upon to achieve the desired target cell specificity for gene therapy applications. These efforts have been greatly aided, in terms of effective modification of the features that give rise to the different serotypes, through a detailed knowledge of the AAV capsid structure and the spatial distribution of these features.

1.3. Capsid Crystal Structure: So Much to Learn

The viral capsid protein is the first element that cellular receptor encounters during a viral infection. In order to rationally design vectors for a particular target, it is important for us to know the structure of the viral capsid and its surface topology and understand the interactions between each AAV serotype and its corresponding cellular receptor. X-ray crystallography is a powerful tool to study the structure of proteins, and advances in this technology have dramatically increased the number of viral capsid proteins that have been characterized in recent years. Not only can the existing conformation of capsid proteins be analyzed by this technique, but also the computational methods used to visualize these structures can facilitate *in silico* prediction of the structures of various mutant capsids.

The first few parvovirus capsid structures to be solved were the canine parvovirus [Tsao, J *et al.*, 1991], followed by feline panleukopenia virus [Agbandje, M *et al.*, 1993], B19 [Chipman, PR *et al.*, 1996; Kaufmann, B *et al.*, 2004], the minute virus of mice (MVM) [Llamas-Saiz, AL, 1997] and Galleria mellonella densovirus [Simpson, AA *et al.*, 1998]. The structural details from these parvovirus capsids have been used as a foundation for many AAV studies because of their structural similarity in several domains. A hypothetical AAV surface model was developed by comparing the canine parvovirus VP2 to the AAV VP3 proteins [Girod, A *et al.*, 1999], which served as a guide for designing capsid modifications which could alter cell tropism. However, because canine parvovirus binds to different types of cell surface molecules, knowledge of the exact AAV2 capsid topology is critical in fully understanding how the virus interacts with its cellular surface receptor. The structure of AAV2 was eventually determined at a resolution of 3-Å by X-ray crystallography in 2002 [Xie, Q *et al.*, 2002]. The crystal structure resolved the VP3 capsid subunit, except for the 14 amino acids near its N-terminus, and the unique residues of the N-terminus of VP1 and VP2 (amino acids 1-202). The β -barrel core structure of the AAV capsid is a common feature among other parvoviruses, but the long loops between the β -strands contribute to the formation of the 3-fold proximal peaks, giving a unique surface topology to AAV2. Structural analysis of AAV2 and its primary cellular receptor HSPG revealed that the 3-fold peaks, which are composed of loops contributed from three different capsid subunits, are responsible for its binding. In light of this information, capsid modifications in previous studies [Bartlett, JS *et al.*, 1999; Girod, A *et al.*, 1999; Rabinowitz, JE *et al.*, 1999; Wu, P *et al.*, 2000] can now be re-examined in terms of the interplay between their overall structure and the corresponding functionality.

Structural analysis of other AAV serotypes suggests that their capsid surface topology differs, which may account for their unique tropism. For example, Walters *et al.* determined the structure of AAV5 at a resolution of 16-Å, and demonstrated that the spikes on the three-fold axis of AAV2 are larger and more pointed than the mounds on AAV5 on the same axis [Walters, RW *et al.*, 2004]. Surprisingly, no specific domain on the AAV5 capsid has been identified to be responsible for sialic acid binding. Because the preliminary crystal structure of AAV4 has been determined at a resolution of 3.2-Å [Kaludov, N *et al.*, 2003], it is possible that comparisons of the AAV4 and AAV5 structures could provide useful hints to determine the domain responsible for cellular receptor specificity. With the availability of the AAV crystal structure, the design of the mutant virus is much more systematic, highlighting the importance of visualization of the surface topology in creating better binding to target receptors.

The structural data for AAV could only be achieved through the generation of pure crystals, which required improvement in AAV purification methods [Xie, Q *et al.*, 2004]. The large scale AAV2 production was accomplished through adaptation of producer cells to suspension culture and re-optimization of the times of infection and transfection, yielding highly concentrated AAV (10^{15} particles/ml) at 99% purity for crystallization. While affinity column purification is feasible for AAV2, using a heparin column [Summerford, C and Samulski, RJ, 1999], and AAV5 using a mucin column [Auricchio, A *et al.*, 2001], other serotypes could not be purified in the same fashion, and rely on CsCl density fractionation. Recent developments in AAV purification mostly utilize ion exchange column purification. Kaludov *et al.* (2002) described a two step purification utilizing an anion-exchange column and high molecular weight retention filters [Kaludov, N *et al.*, 2002]. This method is suitable for all serotypes without using affinity chromatography, which requires knowledge of the cellular receptor, and serves as an alternative protocol to CsCl density gradients. These advancements in purification can potentially accelerate the process of purifying high quality crystals for structural analysis of other AAV serotypes.

The following sections describe AAV capsid studies conducted both before and after the crystal structure of AAV2 were available. With the structure known, the exact position of an epitope

to be tagged or a mutation to be made can be studied *in silico* before performing the biological studies, thus providing a more rational design of capsid modification.

2. AAV HYBRID SEROTYPES

Since the development of naturally occurring AAV serotypes into gene therapy vectors, researchers have spent much effort in understanding the tropism of each serotype so that further modification to the virus could be performed to enhance the efficiency of gene transfer. These efforts also encompassed the idea of swapping domains from one serotype capsid to another, to create hybrid vectors with desirable qualities from each parent. As the viral capsid is responsible for cellular receptor binding, the understanding of viral capsid domain(s) critical for binding is important. Mutation studies on the viral capsid (mainly on AAV2) performed before the availability of the crystal structure were mostly based on capsid surface functionalization by adsorption of exogenous moieties, insertion of peptide at a random position, or comprehensive mutagenesis at the amino acid level. In this review, we would like to discuss the properties of various types of AAV hybrid serotypes according to the following categories:

2.1 Transcapsidation - Packaging the genome containing ITRs from one serotype in the capsid of a different serotype;

2.2 Adsorption modification - Carries foreign peptides adsorbed on the capsid surface;

2.3 Mosaic Capsid - Packaging a mixture of unmodified capsid proteins from two different serotypes;

2.4 Chimeric Capsid - Packaging capsid proteins with foreign peptide sequence fused at either the N- or C-terminus of viral protein or, as an insertion into the VP sequence.

2.1. Transcapsidation – ITR from One Serotype Cross-Packaged into Capsid of a Different Serotype

AAV transcapsidation is to package an AAV genome containing an ITR from one serotype into the capsid of another serotype. Because AAV2 is the most studied serotype, and served as the archetype for AAV replication research, its genetic and biochemical properties, and the host cell response to the AAV2 ITR, is the best understood. In addition, most of the vectors used in previous studies were built with the AAV2 ITR, therefore it is logical to choose the AAV2 ITR to be cross-packaged into different serotype capsids, in order to expand the use of the vector for better targeting of cell types that do not express the receptors that AAV2 utilizes.

Several groups first cross-packaged AAV2 ITR into other serotype capsids by complementing with the homologous Rep proteins for either the ITR or the capsid. Davidson *et al* (2000) cross-packaged AAV2 ITR into AAV4 capsid and compared the transduction efficiency of this virus in the central nervous system to AAV5 vector containing a genome with AAV5 ITR [Davidson, BL *et al.*, 2000]. However, the transduction efficiency of the above two vectors cannot be directly compared because the genome was flanked by ITRs from two different serotypes, and these may influence gene expression independently of the behavior of the capsid. A better strategy is to package the same serotype ITR into the capsids of all serotypes to be studied. In two other studies, an AAV2 ITR genome was cross-packaged into AAV 1, 2 and 5, respectively, for investigating muscle [Hildinger, M *et al.*, 2001] and retina [Auricchio, A *et al.*, 2001] tropism. In these studies, the transduction efficiency from different serotype capsids can be evaluated in the absence of potential effects from the expression or stability of the genome. However, all of the cross packaging viruses were prepared by providing cells with Rep from AAV2 (Rep2) only. A drawback of this strategy is that the transencapsidated AAV2 ITR into

AAV5 capsid vector suffered from low titer yield, because the *trs* of AAV5 is significantly different from the *trs* of AAV1, 2, 3, 4 and 6, and Rep2 cannot efficiently nick or process the AAV5 ITR *trs* [Chiorini, JA *et al.*, 1999]. A further complication is that while the N-terminus of Rep mediates specific ITR binding, the C-terminus appears to interact specifically with the capsid [Dubielzig, R *et al.*, 1999]. Therefore, an alternative strategy is required for generating higher titer cross-packaged virus. A more comprehensive study was performed to cross-package AAV2 ITR-containing genomes into the capsids of AAV1, 2, 3, 4 and 5 by engineering chimeric rep fusion proteins from AAV2 and the Rep proteins specific for each serotype [Rabinowitz, JE *et al.*, 2002]. The efficiency of interaction between the hybrid Reps and the serotype-specific capsids is increased, producing titers of cross-packaged vectors similar to that of AAV2.

2.2. Adsorption of Receptor Ligands to AAV Capsid Surface

Even though there are 11 (to date) identified AAV serotypes that can transduce different tissues efficiently, there are still specific cell types that are non-permissive to any of these serotypes and therefore, AAV transduction cannot improve the transduction of those cell types. In order to take advantage of the attractive properties of AAV vectors for therapeutic purposes, including long-term gene expression with minimal toxicity, capsid modification studies on AAV were performed with the aim of retargeting the virus to infect new cell types of interest. One of the many approaches was to use an AAV-specific antibody that is chemically linked to another antibody that binds specifically to a cellular receptor known to be highly expressed on the targeted cell type. This is one of the first approaches in altering AAV capsid tropism without extensive knowledge of capsid structures. AAV2 was modified with a bi-specific F (ab' gamma)₂ antibody, using monoclonal A20 antibody recognizing AAV2 capsid [Wistuba, A *et al.*, 1995], and chemically crosslinked to Fab' arms of the $\alpha_{11}\beta_3$ integrin binding monoclonal AP-2 antibody [Bartlett, JS *et al.*, 1999]. This vector was shown to display altered tropism to target $\alpha_{11}\beta_3$ integrin expressing DAMI and MO7e megakaryocyte cells [Ponnazhagan, S *et al.*, 1996] instead of HSPG expressing cells. This study is considered to be one of the first milestones in the effort to retarget AAV to non-permissive cell types. Another study that utilized ligand-adsorption on the viral surface for re-targeting was performed by Ponnazhagan and co-workers (2002). The purified virus was biotinylated and allowed to react with a bispecific protein, a fusion between the FGF (or EGF) and avidin. The modified virus was able to alter the tropism of AAV to target EGF and FGFR expressing cells and successfully lead to transduction [Ponnazhagan, S *et al.*, 2002].

2.3. Mosaic Capsid – Capsid subunits from Different Serotypes are Assembled into a Single Virion

A mosaic capsid AAV is a virion that is composed of a mixture of viral capsid proteins from different serotypes. The capsid proteins are provided by complementation with separate plasmids, which were mixed at a various ratios. During viral assembly, the different serotypes capsid proteins are theoretically mixed in each virion, at subunit ratios stoichiometrically reflecting the ratios of the complementing plasmids. Two studies were performed using this approach by Xiao *et al.* and Rabinowitz *et al.* In an effort to combine the muscle tropism of AAV1 [Xiao, W *et al.*, 1999] and the HSPG binding ability of AAV2 [Summerford, C and Samulski, RJ, 1998], mosaic virus was generated out of a mixture of AAV1 and 2 capsid components [Hauck, B *et al.*, 2003]. The mosaic virus, composed of, theoretically, 50% of viral capsids from each parental serotype, can bind to heparin for purification, and has superb transducing efficiency in both muscle and liver cells *in vivo*. Even though the actual ratio of viral capsid contributed from each serotype cannot be directly determined, the result strongly suggested that the mosaic virus is a promising approach to generate more sophisticated viruses with properties of both parental serotypes.

Apart from combining the advantages of parental serotypes, the unexpected gain of function of mosaic virus vectors can also provide counter-intuitive insights in understanding both functionality and biological significance of various capsids. A more comprehensive study was performed to produce mosaic viruses from 5 parental serotypes (AAV1 to 5) with 5 different ratios (1:19, 1:3, 1:1, 3:1, 19:1) of input plasmids encoding different serotype capsid sequences [Rabinowitz, JE *et al.*, 2004]. The results demonstrated that the AAV1 to 3 serotypes are more compatible to each other in terms of co-assembly into capsids, while AAV5 capsid components are less compatible with these three serotypes. Likewise, a transfection containing only 5% plasmids expressing AAV4 capsid, in a mixture of plasmids expressing AAV1, 2, or 3, capsids resulted in inhibition of viral assembly. The mixture of AAV3 and AAV5 at a 3:1 ratio produced a mosaic virus that exhibited both heparin and mucin binding ability, demonstrating that the virus carries properties from both parental serotypes. In addition, even though AAV1 and AAV2 alone do not transduce C2C12 cells efficiently, mosaic virus AAV1/2, with only 5% of input expressing AAV1 capsid mixed with that expressing AAV2 capsids, displays dramatically increased transduction efficiency on C2C12 cells when compared to the two parental wt serotypes. Further studies suggested that AAV1 could be trafficked differently than AAV2, which suggested that the AAV1 and 2 mosaic virus described above could be trafficked in a different route than AAV2, and more efficiently deliver the genome to the nucleus for transduction. The series of mosaic virus generated in this study are important tools for us to further understand the biology of different AAV serotype capsids and warrant revisiting in an *in vivo* setting.

2.4. Chimeric Capsids

Chimeric capsid AAV can be defined as an insertion of a foreign protein sequence, either from another wt AAV sequence or an unrelated protein, into the open reading frame of the capsid gene. Among all the methods and strategies used in generating chimeric capsid sequences, most studies performed can be categorized into four groups:

- 2.4.1. Using naturally existing serotypes as templates;
- 2.4.2. Using an epitope coding sequence fused to the N- or C-terminus of the capsid coding sequence;
- 2.4.3. Using an epitope sequence inserted into a specific position in the capsid coding sequence;
- 2.4.4. Using an epitope identified from a peptide library to inserted into a specific position in the capsid coding sequence.

2.4.1. Chimeric Capsid 1 – Chimeric Capsid Proteins Generated by Recombination between Two (or more) Naturally Occurring AAV Serotypes Capsid Sequences—A marker rescue approach was applied to generate chimeric capsid sequences. Three AAV2 capsid mutant sequences that had been previously characterized as non-infectious and unable to bind to heparin were rescued after co-transfection of the coding sequences with wt AAV3 capsid DNA sequence [Bowles, DE *et al.*, 2003]. Microhomology between the mutant AAV2 and wt AAV3 capsid coding sequences served as crossover points for homologous recombination initiated by cellular proteins. The recombined sequences encoded functional chimeric proteins due to directed selection, leading to the production of infectious vectors that could bind to heparin sulfate. This method could serve as a tool to generate a large number of chimeric viruses with unique properties depending on the criteria set in the screening process.

2.4.2. Chimeric Capsid 2 – Epitope Tagged at N- or C-terminus of Capsid Proteins—The structure of the viral capsid and the viral genes carried within has evolved for

millions of years to naturally select for stable and efficient variants for survival. Alignment of the viral capsid gene among all naturally occurring serotypes demonstrated that some conserved domains exist for structural stability of the AAV capsid. To alter the natural tropism of the virus for cell-type retargeting is challenging, especially if a foreign epitope is to be inserted into the capsid sequence.

The important parameters for epitope tagging onto AAV capsids are as follows:

A. The Site of Insertion of the Epitope Must be Such That

- i. assembly of the viral capsid, internalization, trafficking and nuclear import are not affected; and
- ii. peptides must be exposed on the surface of the virus;

B. The Choice of Retargeting Peptide Requires

- i. high affinity binding of the targeting receptor/binding site of interest; and
- ii. high specificity to increase efficacy, especially if the virus is to be administered via a systemic route for clinical studies.

Several groups performed insertional or mutational studies to generate infectious virus that carries an epitope tag to alter tissue tropism. In order to generate a new AAV that infects hematopoietic stem/progenitor cells with higher efficiency, Yang and co-workers (1998) utilized the single-chain variable region (sFv) of an anti-CD34 antibody and fused it to the N-terminus of each of the three AAV2 capsid genes [Yang, Q *et al.*, 1998]. While the N-terminal tag on each of the three capsid proteins did not yield infectious virus, a mosaic virus assembled from a mixture of the three wt capsid proteins and the N-terminal tagged sFv-VP2 capsid, can generate virus that gives improved infection efficiency of CD34 expressing cell line, KG-1. This was the first study that claimed to report successful generation of infectious and functional virus with a foreign peptide fused to the N-terminus of VP2 of AAV2. However, these results have not been reproduced, and it is clear that the ability to form viral like particles that are not assembled properly is a common phenomena. The lack of physical characterization of these proposed chimeric capsid proteins and the virion structure left the field wanting to believe the possibility, but lacking the rigor and proof to confirm that chimeric AAV capsid proteins would be a ready substrate for molecular tinkering. However, these were early days and many of the techniques commonly used were not developed and lack of ability to reproduce the report left this primary example lacking for supporters.

In a separate study, the influenza virus hemagglutinin (HA) tag (YPYDVPDYA) was inserted at the N-terminus of each of the three capsid genes and at the C-terminus of the capsid open reading frame, which is shared by all three proteins [Wu, P *et al.*, 2000]. The results showed that peptide insertion could be tolerated at the N-terminus of VP1 (amino acid 1 in the cap *ORF*), the N-terminus of VP2, which is also the amino acid 138 of VP1 (amino acid 138 in the cap *ORF*) but not at the N-terminus of VP3 or the shared C-terminus. In addition, AAV with serpin receptor ligand peptide (KFNKPFVFLI) fused to the N-terminus of VP1 or VP2 can increase infection efficiency in IB3 cells. Similarly, insertion of a ligand derived from apolipoprotein E (ApoE) at position 138, generated infectious vector with a higher transduction efficiency than wt AAV2 for low-density lipoprotein receptor (LDL-R) expressing human islets cells [Loiler, SA *et al.*, 2003]. Furthermore, Warrington *et al.* (2004) determined the best position for terminal epitope tagging to generate near wt titer and infectivity to be the N-terminus of VP2 in the presence of unaltered sequences of VP1 and VP3 [Warrington, K.H, Jr. *et al.*, 2004]. The infectivity of the epitope tagged viruses expressing an 8 kDa chemokine binding domain of rat fractalkine (FKN) or 18 kDa human hormone leptin (LEP) at this position showed no significant difference in viral properties when compared to wt virus, except the gain

of ability for the tagged virus to target the corresponding cellular receptors. It has also been shown that the addition of a 30 kDa GFP tag at the N-terminus of VP2 (with wt sequences of VP1 and VP3) can yield a virus only 3 fold less infectious than wt, further suggesting that VP2 is non-essential for AAV infectivity. In a different study, Zhang et al (2002) described a chimeric AAV, composed of wt AAV VP1 and 2 proteins, and a C-terminal 6-His-tagged VP3, which yielded infectious and wt-like virus that can be readily purified by using a Ni-nitrilotriacetic acid column [Zhang, HG *et al.*, 2002]. Nevertheless, the VP2 N-terminus is by far the best terminal position for epitope tagging for AAV capsid protein.

2.4.3. Chimeric Capsid 3 – Epitope Tagged in the ORF of the Capsid Proteins—

The position chosen for epitope insertion is very critical for maintaining the efficiency of viral infection, in terms of assembly, DNA packaging, and trafficking, with an increased efficacy in transducing the desired target cells. The VP2 N-terminal epitope tag strategy described in the previous section is useful for generating infectious virus with additional tropism. However, the disadvantage of this approach is that the HSPG binding ability (in the case of AAV2) was not completely abolished. While this property can be useful for vector purification, it also mediates infection of a very broad range of cell types. This may lead to transgene expression in cell-types where it could be deleterious, or simply to dilution of the vector dose in tissues where it is not useful. It would therefore be advantageous in some therapeutic applications to mutate the HSPG binding amino acids to abrogate binding and deter the natural cell tropism. Before the availability of the x-ray crystallographic structure, many mutational and epitope tagging studies contributed significantly to identifying positions that are on the surface of the viral capsid. These studies also pointed to regions of the capsid which might be involved in HSPG binding and are summarized as follows.

One of the first studies on AAV capsid mutagenesis was performed using small peptide insertional mutagenesis randomly in the AAV2 capsid gene and screening for positions that can tolerate insertions without the loss of function [Rabinowitz, JE *et al.*, 1999]. Eighteen mutants were generated, ranging from the phenotype completely incapable of assembling virions, to those which generate vector titers similar to wt virus. In particular, one mutant that is only composed of VP3 (H2634) can assemble into an intact virus that binds to HSPG but fails to transduce cells. This suggests that VP1 and/or VP2, despite being minor contributors to the viral capsid composition, are responsible for viral infectivity, and VP3 is responsible for HSPG binding and structural support of the virus.

In another study, a 14-amino-acid peptide L14 (QAGTFALRGDNPQG), containing an RGD sequence, was inserted into specific positions in the AAV2 capsid gene for retargeting AAV2 to infect cells displaying $\alpha_v\beta_5$ integrin, but are otherwise non-permissive to wt AAV2 [Girod, A *et al.*, 1999]. Taking advantage of the crystal structure solved for Canine parvovirus [Tsao, J *et al.*, 1991], Girod *et al.* built a hypothetical model by sequence alignment between the AAV2 VP3 and canine parvovirus VP2 proteins. Based on this alignment, six positions on the AAV2 VP3 capsid protein were proposed for epitope insertion that potentially would not disturb the viral life cycle (1–261, 1–381, 1–447, 1–534, 1–573 and 1–587). In particular, insertion at 1–587 altered the viral tropism to gain specificity in binding to integrin and significantly dampen the wt AAV2 HSPG binding ability. A few years after this study, residues R-585 and R-588, which were neighboring sites of 1–587, were demonstrated to be responsible for HSPG binding [Opie, SR *et al.*, 2003].

As mentioned in the previous section, Wu *et al.* (2000) performed a comprehensive mutagenesis study on the AAV2 capsid before the availability of the crystal structure. Other than placing insertions at the terminus of the capsid proteins, the authors tested a total of 93 mutants at 59 different positions for the acceptance of foreign peptide insertion. Among all tested sites, only insertion at residue 138 in the VP1 sequence, which is also the N-terminus

of VP2, yielded a virus with reduced infectivity, but still acceptable for practical use. In other studies, similar capsid positions were tested for cell-type retargeting using an epitope that is a ligand of human luteinizing hormone receptor (LH-R) [Shi, W *et al.*, 2001]. These modifications at residues 139 (N-terminus) and 161 in VP2 sequence and residues 459, 584 and 587 in VP3 sequence, are able to retarget AAV2 to LH-R expressing OVCAR-3 cells. Even with 15 amino-acid peptide insertion, the viruses were able to display the epitope on their surfaces and yield titers similar to wt. Further experiments demonstrated that the insertion at residue R588 can inhibit AAV2 from binding to HSPG [Shi, W and Bartlett. JS, 2003]. The strategy of VP2 N-terminus insertion was further confirmed by Warrington *et al.*'s study (2004), in which the viral production and infectivity could be rescued if the insertion was exclusively at the N-terminus of VP2 (as discussed above). Also, other studies have shown that an insertion at residue 587 can disrupt the interaction of AAV2 to primary receptor HSPG, and allow retargeting of the virus to bind to the receptor of interest [Nicklin, SA *et al.*, 2001].

2.4.4. Chimeric Capsid 4 – Using a Library Approach in to Screen for Useful Ligand Specific Epitopes—

The previous sections on chimeric capsids describe an insertion of a known peptide sequence as a ligand to a receptor expressed on the target cell type. The search for a position that can tolerate peptide insertion for receptor retargeting and yet be able to retain most of the biological properties of AAV was difficult and tedious. After all, even a slight alteration in viral capsid structure could affect receptor binding and potentially downstream events, thus reducing transduction efficiency. Three recent studies used a library, or a directed evolution, approach to generate new chimeric capsid AAV for a more versatile retargeting vector. Muller *et al.* (2003) employed a random 7-residue peptide library inserted next to the R588 position of AAV2 capsid sequence to ensure that the peptide is displayed on the virus surface and, at the same time, to ablate the HSPG binding ability. The AAV library was then screened for the ability to infect human coronary artery endothelial cells, and the sequences from infectious virus were cloned for analysis. A chimeric capsid AAV carrying the peptide sequence NDVRAVS was shown to efficiently infect coronary cells *in vitro*, while one with peptide sequence NSSRDLG was efficient for infection of the mouse heart after tail vein injection *in vivo* [Muller, OJ *et al.*, 2003]. The same approach of 7-amino-acid random peptide insertion into the same capsid domain was performed by Perabo *et al.* [Perabo, L *et al.*, 2003]. More recently, a study demonstrated the feasibility of a “staggered extensive process”, analogous to DNA shuffling [Maheshri, N and Schaffer, DV, 2003], to develop a method to generate interesting viral capsid mutants (Maheshri N. *et al.*, personal communication). Maheshri *et al.* used this DNA shuffling approach to generate rAAV2-based capsids that evade neutralizing antibodies and also have reduced heparin binding ability. These studies could potentially be extended to select chimeric capsid AAV for hybrid AAV for *in vivo* systemic delivery with high efficiency and efficacy.

In conclusion, the development of chimeric hybrid serotypes, with epitope tags up to 30kDa at the N-terminus of VP2, is the ideal position for insertion if the HSPG binding ability is required to be maintained, while amino acid position 1–587 is the best position for epitope insertion if an alteration of tropism is mandatory for the hybrid virus.

3. IMPLICATIONS FOR USE OF AAV HYBRID SEROTYPES

Long term gene expression and varied tissue tropism from different serotypes of AAV had been demonstrated previously. However, more specific modification on the viral capsids is required to further expand the use of AAV as a gene delivery vector. For instance, transcapsidation of the AAV2 genome into other serotype capsids allows the genome from a well characterized serotype to gain the ability to infect cells that are more susceptible to other serotypes [Rabinowitz, JE *et al.*, 2002]. Other than transcapsidation, capsid modification with an epitope tag can greatly expand the use of AAV in many different target cell types or disease

models, A small suicide protein can be fused to the N-terminus of VP2, with an enzymatic cleavage sequence that could be processed by a common host cell enzyme and used for delivering the toxic peptide to cancer cells. In addition, the attachment of a receptor-specific ligand to the viral surface can further increase the specificity of the virus in infecting cells/tissues of interest. Multiple epitope labeling of AAV can potentially facilitate binding to specific primary and secondary receptors, causing the virus to be efficiently bound and internalized for transduction.

Other than for therapeutic uses, AAV hybrid serotypes greatly enhance our understanding of the virus biology. In the course of generating hybrid serotypes by a variety of methods, large scale mutagenesis studies were performed. Many of these mutants, ranging from single residue mutation to up to 30 kDa peptide insertions as an epitope tag, shed light on the residue or the region(s) of the capsid that is/are responsible for virion packaging, genome import, attachment of cellular surface receptor, internalization, trafficking, endosomal escape and nuclear localization. These results would provide enormous valuable knowledge for AAV basic virology.

3.1. Receptor Targeting to Specific Cell Types and Viral Assembly

Mosaic viruses produced by mixing the plasmids that express AAV3 and 5 capsids generated hybrid serotypes that gain properties from both parental serotypes in binding to HSPG and mucin [Rabinowitz, JE *et al.*, 2004]. Using the same strategy, mosaic virus can be used to screen for efficient binding to primary and secondary receptor of the target cells to improve the infection efficiency of wt virus. In addition, this study described a new method to classify AAV serotypes into subgroups according to the compatibility of the subunits of one serotype to the other serotypes. The results indicated that AAV 1–3 capsids (subgroup A, as classified by Rabinowitz *et al.*) are more compatible to each other, with moderate compatibility to mix with AAV5 capsids (subgroup B). However, AAV4 capsids (subgroup C) do not mix well with AAV1–3 capsids. Similar studies to be performed on other available AAV serotypes would provide us with a better understanding of the different properties of these serotypes, so that hybrid vectors with multiple desired properties from each parental serotype can be generated.

Viral assembly can also be studied with hybrid serotypes. Mutants generated in capsid mutation studies can be used to identify important domains of the capsid that are critical for viral assembly. For example, Wu *et al.* (2000) demonstrated that alanine substitution at specific capsid positions carrying charged amino acids generated mutant viruses that were not able to assemble (amino acids 228–232, 235–239, 285–289, 291–295, 307–311, 607–611, 681–683, 689–693). These mutants could be further analyzed *in silico* and *in vitro* to determine the amino acids that contribute to the interactions among viral capsid subunits and assembly kinetics of the capsid. In addition, a mutation at R-432 to A-432 alone can generate empty capsid. Further analyses of this mutant would explain the DNA packaging deficiencies that lead to formation of empty capsid, and potentially provide details in the mechanism of genome packaging. Moreover, the function of individual viral proteins (VP1, 2 and 3) of different serotypes (AAV1-11 and/or hybrid serotypes capsids) can be studied by mixing plasmids that express specific VP proteins (e.g. VP1) from one serotype and other VP proteins (e.g. VP2 and 3) from a different serotype. The regions of the proteins that contribute to the specific properties of the wt and/or modified virus can allow us to understand the various functions of each capsid protein.

3.2. Signal Transduction

A microarray study of gene expression profiles following infection with AAV2 or an empty AAV capsid showed that cell proliferation proteins were down-regulated and p21 was up-regulated in infected cells. The authors further demonstrated that the AAV2 capsid is

responsible for initiating the G₂/M delay [Stilwell, JL and Samulski, RJ, 2004]. It is possible that the capsid binding to its receptor can trigger such a response through a signal transduction cascade from the receptor to the nucleus during the initial step of viral infection. The swap of a domain of AAV2 capsid with that of AAV3 capsid, which does not cause the G₂/M delay, reduced the delay observed with wt AAV2 capsid, highlighting the important role of HSPG binding in triggering signaling events that caused cell cycle delay (Stilwell JL, unpublished data). In another study, Rac1 and PI3 kinase cascade activation was demonstrated to be a result of AAV2 endocytosis mediated by the $\alpha_v\beta_5$ integrin internalization pathway [Sanlioglu, S *et al.*, 2000]. The domain on the viral capsid responsible for assisting the interaction between the virus and integrin is unknown, but can be mapped out using similar strategies used in identifying the capsid binding domain for HSPG.

3.3. AAV Trafficking

Hybrid serotypes can also be used to study AAV trafficking. Investigations on AAV receptor binding, which is the first step of AAV infection, allow us to identify a variety of cell surface receptors that are utilized by AAV (HSPG, $\alpha_v\beta_5$ integrin, FGFR-1 and HGF). The subsequent step in the infection process involves clathrin-dependent and independent internalization. It has been shown that AAV must be transported from early to late endosomal compartments, followed by an escape from the endosome to reach the nucleus [Douar, AM *et al.*, 2001]. Researchers have demonstrated that the lack of AAV transduction in certain cell types (eg. differentiated human airway epithelial cells) was due to inefficient endosomal processing and nuclear trafficking [Duan, D *et al.*, 2000]. In addition, a separate study using NIH3T3 cells showed that the endosomal pathway of this cell line is functional, but the endosomal processing from early to late endosome is impaired such that AAV is not trafficked to the nucleus [Hansen, J *et al.*, 2001]. Proteasome inhibitors such as MG-132 and LLnL can increase the number of virus transported to the nucleus and eventually leading to transduction [Duan, D *et al.*, 2000; Douar, AM *et al.*, 2001]. Therefore, mutation studies on the capsid that lead to altered endosomal trafficking and/or processing can help us to identify the essential regions of the capsid that mediate this process, and help to identify the host components that they interact with. Moreover, AAV virion and capsid proteins are ubiquitinated after endocytosis [Yan, Z *et al.*, 2002]. However, the target residue(s) and/or domain(s) on the capsid to be ubiquitinated are still unclear. Capsid mutational studies and hybrid viruses can direct us to identify the target sites and allow us to understand the role of AAV capsid ubiquitination in improving AAV transduction.

In addition, hybrid AAV vectors can be used to study AAV trafficking in a real time manner. Confocal microscopy was previously used to visualize AAV trafficking through the cytoplasm using a Cy3-labeled AAV virus [Sanlioglu, S *et al.*, 2000]. Even though the labeled virus is a useful tool in studying trafficking, a minor drawback of the study is that the conjugated label can fall off from the virus, leading to the tracking of the dye instead of the virus. Warrington *et al.* (2004) described a VP2-N-terminal GFP-tagged virus to perform real-time imaging to study viral trafficking [Sanlioglu, S *et al.*, 2000; Warrington, KH, Jr. *et al.*, 2004]. This is an improved tool for trafficking study because the fluorochrome is covalently attached to the viral capsid sequence.

4. IMPORTANT CONSIDERATIONS WHEN USING HYBRID SEROTYPES

Despite the fact that hybrid serotypes have enormous potential in therapeutic applications and in solving a wide spectrum of questions in AAV biology, researchers and clinicians should be cautious when using hybrid serotype for their studies. First, there is no universal cell line in testing the transduction efficiency of all serotypes. For example, AAV1 (a naturally isolated serotype) transduces muscle tissue very well [Xiao, W *et al.*, 1999] but is not very efficient in transduction *in vitro*, including C2C12 cells (a muscle cell line) [Rabinowitz, JE *et al.*, 2004].

Therefore, screening new mutants using cell lines alone may lead to false negatives, because mutants that work extremely well *in vivo* could be neglected. However, animal experiments are often expensive and time consuming for screening purposes. Second, normalizing the infectious doses of vectors among different serotypes in comparative studies, based on their ability to infect a standard cell type such as HeLa, can be misleading. Because each serotype has different transduction efficiency for a particular cell-type, infectious titers derived in this way are not directly comparable. In this case, quantification of genome containing particles by dot blots and/or quantitative PCR (QPCR) becomes more important. A standardized protocol for virus quantification and a widely available viral stock to serve as a reference should be used across the field to provide a better comparison among results obtained in different laboratories. However, this does not allow the investigator to evaluate the viability of new vector stocks. Third, the screening process for identifying a desired hybrid serotype should meet stringent criteria in efficiency, efficacy, purity and safety. For serotypes to be tested in a clinical setting, the viruses should allow efficient delivery to the target cells and specifically to the tissue of interest. Since post modification (e.g. antibody adsorption on the surface of the virus) or mixing of reagents (e.g. plasmids expressing different AAV serotype capsids in mosaic virus) are used in generating the virus, potential unforeseen interactions and/or properties can result. Quality control of the output virus needs to be carefully and vigorously tested and validated.

5. FUTURE DIRECTIONS: WHERE DO WE GO FROM HERE?

This review has focused on the use of hybrid serotypes as one of the strategies for improving viral genome delivery. However, to further improve AAV as a gene delivery vector, modifications of the viral genome is as important as capsid modifications, in order to achieve an exceptional level of transduction. Second-strand synthesis has been identified as a rate-limiting step in rAAV vector transduction in many tissue cell types [McCarty, DM *et al.*, 2001]. Self-complementary AAV (scAAV) has been one of the most important advances in AAV vector development. By deleting the *trs* from one of the ITRs, the Rep protein continues to replicate along the genome using the coding sequence as a template, thus generating a single-stranded genome that contains both the coding and the complementary sequence packaged into the viral capsid [McCarty, DM *et al.*, 2003]. After the delivery of the genome to the nucleus, the genome folds from the TR at the mutated end. Since the complementary strand of the genome is already provided to the host cells, gene expression immediately occurs after the genome gets into the nucleus. This vector has been demonstrated to be an improved transduction vector with a fast onset of gene expression. In spite of the reduction of the packaging size, scAAV vectors can deliver smaller transgene expression cassette (up to 2.3kb). Recently, scAAV has been exploited for delivering small-interference RNA (siRNA) expressing cassette [Tomar, RS *et al.*, 2003; Han, W *et al.*, 2004; Pinkenburg, O *et al.*, 2004; Xia, H *et al.*, 2004; Zhang, W *et al.*, 2004]. These studies demonstrated that scAAV could efficiently deliver the siRNA expression cassette and lead to an optimum level of siRNA silencing. The scAAV genomes can be packaged into the hybrid serotypes and can potentially lead to an even better and broader expression in the target tissues when compared to the conventional single-stranded genomes. With this strategy in mind, development of AAV with mutant capsids that can assemble the virus to package a bigger genome can further expand the forefront of the use of this vector to carry a larger transgene expression cassette.

Hybrid serotypes of AAV have been shown to have tremendous potential for efficient gene delivery and for understanding AAV biology in the past. In the future, we can foresee that more hybrid serotypes will be designed and generated depending on the specific criteria for each application, thus further broadening the potential use of AAV as a gene delivery vector. With better knowledge of how to overcome other limiting factors of AAV transduction (e.g. internalization, endosomal trafficking, nuclear import, etc), researchers can re-examine cell types or organs that could not be targeted before, or disease models that could not be treated

using AAV vectors. Further advances in understanding AAV biology can allow researchers to generate more efficient gene delivery vectors to the benefit of the patients that are in need of gene therapy.

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