REVIEW ARTICLE

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Adeno-associated Virus Serotypes: Vector Toolkit for Human Gene Therapy

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Recombinant adeno-associated viral (AAV) vectors have rapidly advanced to the forefront of gene therapy in the past decade. The exponential progress of AAV-based vectors has been made possible by the isolation of several naturally occurring AAV serotypes and over 100 AAV variants from different animal species. These isolates are ideally suited to development into human gene therapy vectors due to their diverse tissue tropisms and potential to evade preexisting neutralizing antibodies against the common human AAV serotype 2. Despite their prolific application in several animal models of disease, the mechanisms underlying selective tropisms of AAV serotypes remain largely unknown. Efforts to understand cell surface receptor usage and intracellular trafficking pathways exploited by AAV continue to provide significant insight into the biology of AAV vectors. Such unique traits are thought to arise from differences in surface topology of the capsids of AAV serotypes and variants. In addition to the aforementioned naturally evolved AAV isolates, several strategies to engineer hybrid AAV serotype vectors have been formulated in recent years. The generation of mosaic or chimeric vectors through the transcapsidation or marker-rescue/domain-swapping approach, respectively, is notable in this regard. More recently, combinatorial strategies for engineering AAV vectors using error-prone PCR, DNA shuffling, and other molecular cloning techniques have been established. The latter library-based approaches can serve as powerful tools in the generation of low-immunogenic and cell/tissue type-specific AAV vectors for gene delivery. This review is focused on recent developments in the isolation of novel AAV serotypes and isolates, their production and purification, diverse tissue tropisms, mechanisms of cellular entry/trafficking, and capsid structure. Strategies for engineering hybrid AAV vectors derived from AAV serotypes and potential implications of the rapidly expanding AAV vector toolkit are discussed.

Key Words: adeno-associated virus, serotype, tropism, vector, hybrid

Contents

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INTRODUCTION

Adeno-associated viruses (AAV) are members of the *Parvoviridae* family. The viruses belong to the genus Dependovirus, the members of which require a helper virus, such as adenovirus or herpes simplex virus, to facilitate productive infection and replication. In the absence of helper virus, AAVs establish a latent infection within the cell, either by site-specific integration into the host genome or by persisting in episomal forms. The virion shell is approximately 25 nm in diameter and encapsidates a single-stranded DNA genome of 4.7 kb that consists of two large open reading frames (ORFs) flanked by inverted terminal repeats (ITR). The ITRs are the only *cis*-acting elements required for genome replication and packaging. The left ORF encodes four replication proteins responsible for site-specific integration, nicking, and helicase activity, as well as regulation of promoters within the AAV genome. The right ORF encodes the viral structural proteins, VP1, VP2, and VP3, that assemble into icosahedral virion shells comprising 60 subunits each.

Following the establishment of the first infectious clone of AAV serotype 2 (AAV2) in 1982 [1], AAV2 vectors have rapidly gained popularity in gene therapy applications, due to their lack of pathogenicity, wide range of infectivity, and ability to establish long-term transgene expression. Recombinant AAV2 vectors have been tested in preclinical studies for a variety of diseases such as hemophilia, α1 anti-trypsin deficiency, cystic fibrosis, Duchenne muscular dystrophy, and rheumatoid arthritis. At least 20 clinical trials have been completed or initiated with 15 different AAV2-based vectors being administered in several hundred patients thus far [reviewed in 2]. Despite the well-established safety and efficacy of AAV2 vectors for *in vivo* gene transfer, several studies suggest that the transduction efficiency of AAV2 vectors falls short of requirements for adequate and organ-specific transgene expression. As a result, ongoing research efforts in the field are focused on modifying both vector genomes and capsid proteins to improve the transduction efficiency and/or specificity of AAV2-based vectors. For example, self-complementary AAV2 vectors [3–5], which were developed to bypass rate-limiting second-strand DNA synthesis, display enhanced transduction in comparison with conventional AAV vectors in liver [3–5], muscle [4], brain [6], retina [7], and cancer cells [8]. Other efforts have focused on manipulating the AAV2 capsid shell using site-directed and insertional mutagenesis, peptide display libraries, and chemical conjugation. These studies have been reviewed elsewhere [9,10].

To date, a number of AAV serotypes and over 100 AAV variants have been isolated from adenovirus stocks or from human/nonhuman primate tissues [11–19]. Utilization of alternative AAV serotypes can not only lower the vector load due to their potentially higher transduction efficiency, but also help evade preexisting neutralizing antibodies generated as a result of humoral immune response to natural infection or prior treatment with AAV-based vectors. In addition, AAV serotypes and variants can serve as templates for design of tissue-targeted capsid constructs that will serve to expand and complement the current range of AAV vectors. The current review is focused on naturally occurring AAV serotypes and engineering hybrid AAV vectors that can achieve high levels of transduction in specific tissue types. The isolation, serology, classification, and gene delivery applications of AAV serotypes have been reviewed previously [20,21]. The following report will highlight recent developments in the isolation and production of AAV serotypes and provide a detailed perspective on the potential mechanisms underlying diverse tissue tropisms of AAV serotypes, including receptor usage, intracellular processing, and capsid structure. Strategies for generation of hybrid AAV vectors derived from different serotypes are also discussed.

AAV SEROTYPES

Prior to discussing AAV serotypes in detail, it is noteworthy to mention that a new serotype, by definition, is a newly isolated virus that does not efficiently cross-react with neutralizing sera specific for all other existing and characterized serotypes. Based on such, only AAV1–5 and AAV7–9 can be defined as true serotypes. Variants AAV6, 10, and 11 do not appear to fit into this definition, since the serology of AAV6 is almost identical to that of AAV1 [16,17,22], and serological profiles of AAV10 and AAV11 are not well characterized [18]. Nevertheless, for the sake of discussion, the numbering previously assigned to each AAV isolate has been utilized throughout this article. Since the serology of the over 100 new AAV isolates is not currently available, these isolates are called AAV variants. Several reports have shown that *in vivo* transduction efficiency of one serotype AAV vector is often unaffected by the presence of preexisting neutralizing antibodies to another AAV serotype [23–25]. However, this is not always the case, since the extent of cross-reactivity between some AAV serotypes appears to be species specific or dependent on tissue type and route of administration. For example, initial treatment with AAV2 vectors diminished AAV1-mediated transduction by about 20-fold in murine liver [23]. Interestingly, this effect was much weaker during muscle transduction.

Isolation

AAV serotypes 1 to 6 were isolated as contaminants in laboratory adenovirus stocks, with the exemption of AAV5, which was isolated from a human penile condylomatous wart [14]. Among these, AAV2, 3, and 5 are thought to be of human origin based on the prevalence of

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neutralizing antibodies in the human population [26–29]. In contrast, AAV4 appears to have originated potentially in monkeys since antibodies against AAV4 are common in nonhuman primates [27]. Whether AAV1 originated from human or nonhuman primates remains inconclusive. While antibodies to AAV1 were found in monkey sera [27], AAV1 viral genomes have been isolated from human tissues [17]. Interestingly, AAV6 is thought to be a hybrid recombinant between AAV1 and AAV2, since the left ITR and p5 promoter regions are virtually identical to those of AAV2, while the rest of the genome is nearly identical to that of AAV1 [15,23]. However, whether recombination occurred *in vivo* or in cell culture remains unclear. Recently, AAVs found in 10 simian adenovirus isolates showed greater than 96% homology to AAV1 and AAV6 [19].

In the past 4 years, several novel AAV serotypes, including AAV7, AAV8, and AAV9, and over 100 AAV variants have been found in human or nonhuman primate tissues ([16–19]; for review, see [21]). Different from AAV1 to AAV6, the new AAV serotypes and variants were not isolated as live virus forms; instead, they were isolated as DNA sequences using a novel PCR-based strategy. Briefly, a "signature PCR" spanning a short variable region of capsid gene was adopted to screen for new AAV isolates. Additional rounds of PCR were then carried out to isolate full-length Rep or Cap sequences. The results show that diverse AAV genomes were widely disseminated throughout multiple tissue types in a variety of human or nonhuman primate species. These newly isolated AAV serotypes and variants, along with the other existing AAV serotypes, have been subdivided into six "clades" or several "clones" based on their genetic relatedness [17,21]. Some of these isolates show enhanced transduction in comparison with previously identified AAV serotypes in several tissue types. For instance, AAV8 displays a propensity for liver transduction, while AAV7 transduces muscle with efficiency similar to that of AAV1, the most efficient serotype for muscle transduction identified so far [16]. In addition, AAV9 appears to be a vector that can outperform other AAV serotypes in most tissues [17].

Using a similar strategy, Mori *et al.* [18] recently isolated AAV10 and AAV11 from cynomolgus monkeys. Phylogenetic analysis showed that AAV10 and AAV11 resembled AAV8 and AAV4, respectively. The lack of cross-reactivity between mouse antisera against AAV2, AAV10, and AAV11 capsids suggests that AAV10 and 11 can potentially be utilized for gene transfer in individuals with high antibody titers against AAV2. However, as mentioned earlier, the cross-reactivity of AAV10 and 11 with antisera against existing serotypes other than AAV2 has not been characterized. In addition to primates, AAV genomes have also been isolated from other species such as horse [30], cow [31], chicken [32], snake [33], lizard [34], and goat [35,36]. Among these, bovine, avian, and caprine AAV have been developed into vectors in gene transfer studies [31,32,36].

Vector Production and Purification

As mentioned previously, AAV2 was the first AAV isolate to be developed into recombinant vectors for transgene delivery. Initially, adenoviral helper functions to produce AAV2 were provided directly using adenovirus, which raised concerns pertaining to contamination of AAV2 stocks with adenoviral capsids. Significant improvements have been made regarding AAV2 production in the past decade. Plasmid-based protocols, which include a plasmid containing the Ad genes instead of using adenovirus, are primarily used for AAV production [37,38]. Currently, the production of AAV2 vectors requires transfection of the following components into host 293 cells: (1) vector genome containing the transgene expression cassette flanked by two ITRs, (2) expression of Rep and Cap proteins provided by a helper plasmid *in trans*, and (3) adenovirus genes encoding E1, E2A, E4, and virus-associated RNA. Since the transfection method is often considered unsuitable for large-scale production, the infection of cell lines stably expressing Rep and Cap with adenovirus carrying a vector genome has afforded a choice for scale-up [39–41]. Alternatively, infection of proviral cell lines with adenovirus or herpes simplex virus vector carrying a Rep and Cap expression cassette has served as a viable alternative [42,43]. However, these methods still require the complete elimination of adenovirus (or herpesvirus) during the production process.

More recently, the development of baculovirus expression vector systems for rAAV2 vector production in insect SF9 cells [44] has shown promise for large-scale production. In this system, components of AAV production, including Rep and Cap proteins, as well as vector genomes are provided by separate recombinant baculoviruses. The Ad helper functions needed in mammalian cells appear to be unnecessary in insect cells or provided by baculovirus. To maintain proper stoichiometry of AAV proteins as well as to increase AAV yield, a noncanonical VP1 start codon and an attenuated baculovirus promoter driving Rep78 expression were used. As shown by Urabe *et al.,* the resulting AAV vectors displayed infectivity similar to that of vectors generated in mammalian cells both *in vitro* and *in vivo*. Thus, the baculovirus–SF9 suspension culture system holds promise to afford a high yield of AAV (5×10^4 vector genomes/cell) and provides a convenient way for large-scale production of AAV vectors [44,45]. However, as demonstrated by Kohlbrenner *et al.,* all of the baculovirus helpers were prone to passage-dependent loss-of-function deletions resulting in considerable decrease in rAAV titers [46]. Splitting the palindromic orientation of the Rep genes into two separate helpers increased the passaging stability of Rep-helper. Nevertheless, using baculovirus-helper components at a

passage number of 2 or less is recommended for large-scale AAV production.

In theory, all of the above production methods can be applied to the production of non-AAV2 serotype vectors. Most studies to date have adopted a "cross-packaging" strategy to generate pseudotyped AAV vectors based on the discovery that expression of AAV2 Rep proteins together with capsid proteins of a different serotype results in formation of viral particles that package AAV2 vector genomes [47,48]. To achieve such, AAV2 ITRs and Rep sequences are left unchanged within the vector genome and helper plasmids, respectively, irrespective of the serotypes to be produced. Serotype-specific Cap expression cassettes are then placed downstream of AAV2 Rep to generate capsids packaging recombinant genomes. The ability to package identical genomes within different serotype virion shells (hybrid viruses) utilizing this strategy enables unbiased comparison of transduction efficiencies of different serotypes without influence of ITR on transgene expression. In summary, the transfection-based production of non-AAV2 serotype vectors has been commonly utilized, despite efforts to exploit herpesvirus or baculovirus systems for production of AAV5 [49,50] and AAV8 [46] vectors.

Conventional methods for purification of AAV vectors are based on cesium chloride density gradient ultracentrifugation. Zolotukhin *et al.* [51] described purification strategies that involve the use of nonionic iodixanol gradients followed by ion-exchange or heparin-affinity column chromatography for the purification of AAV2. With the rapid identification of new AAV serotypes, several purification protocols based on ion-exchange chromatography have emerged for AAV1, 2, 4, 5, and 8 [52–57]. In addition, AAV4 and AAV5 can be purified using mucin columns, based on the ability of these serotypes to bind sialic acid residues in mucin [58]. Due to its ability to bind heparan sulfate (albeit at lower affinity than AAV2), AAV6 can be purified using heparin-affinity columns [59,60]. It is important to note that such methods are not generic, in that the conditions for purification of each serotype often require selective optimization. In this regard, a universal purification scheme for AAV serotype vectors would prove extremely valuable for the generation of large-scale clinical-grade vectors. The development of such protocols, notably based on histidine tags and endogenous biotinylation sequences, has been attempted by several labs [61,62]. However, these methods often require insertional or site-directed mutagenesis of the capsid sequence, which in turn might affect transduction efficiency or tissue tropism.

Tissue Tropism

Several researchers have exploited the cross-packaging strategy to compare the transduction efficiencies of serotypes of AAV vectors in different tissues. While results pertaining to AAV serotype tissue tropism are generally difficult to interpret due to interstudy variations in vector titers and doses, promoters, and transgenes, a general hierarchy of transduction efficiency in major tissues has been established (Table 1). For example, AAV2 is known to transduce a wide range of tissue types, including liver, muscle, lung, and central nervous system, with moderate efficiency. AAV9 exhibits a similar profile with widely disseminated transduction, albeit with much higher efficiency than AAV2 [17]. In skeletal muscle, AAV1 and AAV7 are known to perform well with rapid onset and high levels of transduction [16,17,63]. AAV6, which differs from AAV1 capsid by only six amino acid residues, has also shown a propensity for transduction of skeletal muscle [60]. However, a direct comparison of these two closely related serotypes has not been reported to date. An illustrative example of the hierarchy in transduction of mammalian retina and skeletal muscle tissue by AAV serotypes 1–5 determined in our lab is shown in Fig. 1. Comprehensive studies to elucidate the transduction efficiency and tissue tropisms of other serotypes are currently in progress.

TABLE 1: Hierarchy of transduction efficiency in major tissues of AAV serotype vectors

Tissue	Optimal serotype(s)	References
Liver	AAV8, AAV9	[16,17,68,71]
Skeletal muscle	AAV1, AAV7, AAV6, AAV8, AAV9	[16,17,23,60,63]
CNS	AAV5, AAV1, AAV4	[64–66,123]
Eye		[67]
RPE	AAV5, AAV4	
Photoreceptor cells	AAV5	
Lung	AAV9	[17]
Heart	AAV8	[69]
Pancreas	AAV8	[125,126]
Kidney	AAV2	[124]

Several AAV serotypes have revealed distinct patterns of transduction within the nervous system. In general, AAV1 and 5 exhibit higher transduction frequencies than AAV2 in all regions injected within the CNS [64,65]. While AAV2 shows widespread transduction throughout the entire midbrain, AAV4 appears to transduce specific cell types [66], such as the ependyma and astrocytes in the subventricular zone. AAV4 has also been shown to transduce the retinal pigmented epithelium, although at levels lower than those achieved with AAV5 ([67] and Fig. 1). In liver, AAV8 has been shown to be a robust vector for achieving high levels of transgene expression [16,17]. In addition, AAV6 appears to transduce the liver with efficiency higher than AAV2 and AAV1 [22]. It is noteworthy to mention that AAV8 not only transduces liver efficiently, but also other organs with higher efficiency than most other serotypes. Nakai *et al.* [68] demonstrated that a high dose of AAV8 transduced skeletal muscle

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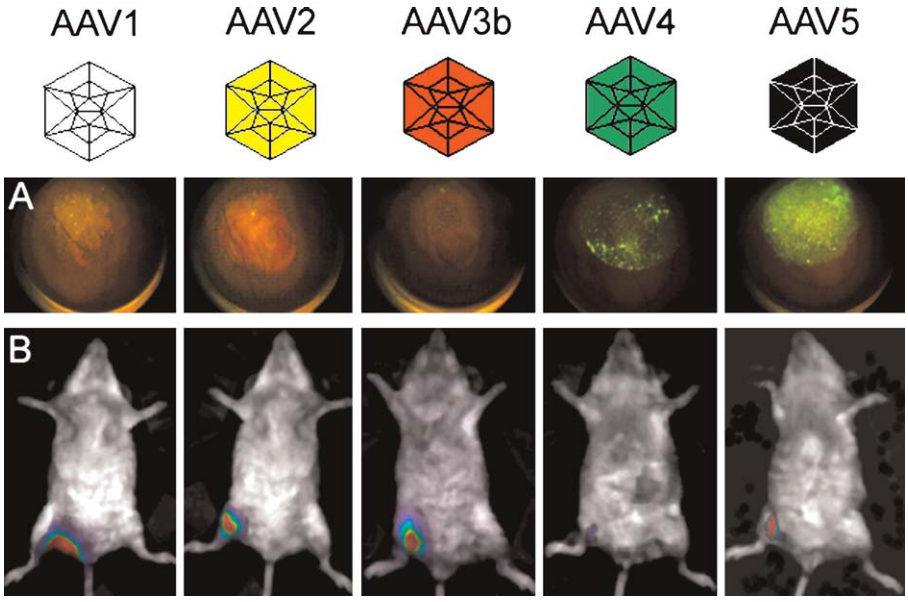

FIG. 1. Illustrative example of the hierarchy of transduction of AAV serotypes 1–5 cross-packaged with AAV type 2 vector genomes in different tissues. (A) GFP expression in the retina of Wistar rats after 1 month subsequent to subretinal injection with AAV serotypes 1 through 5. (B) Luciferase expression in skeletal muscle of Balb/C mice 2 months following intramuscular injection of AAV serotypes 1 through 5.

throughout the body, including the diaphragm; the entire cardiac muscle, and, at substantial levels, the pancreas, smooth muscle, and brain. A recent study by Wang *et al.* [69] demonstrated efficient transduction of skeletal muscle and heart after systemic injection of AAV8. On the other hand, AAV6, which transduces skeletal muscle with high efficiency, may require coadministration of VEGF to traverse the blood vessel barrier and achieve whole-body transgene expression [70]. The brain and skeletal muscle are, in general, difficult to transduce upon systemic administration of AAV due to blood–brain and blood vessel barriers, respectively. However, the aforementioned studies suggest that AAV8 might be able to traverse the endothelial cell lining of blood vessels to transduce such tissues. Thus, it is important to understand that the expanded tissue tropism of AAV serotypes, while useful for certain gene delivery applications, can result in transduction of nontarget tissues, and their successful application in human clinical trials will require control and manipulation of their endogenous tissue tropisms.

It is important to note that the final transduction efficiency as well as the kinetics of transgene expression varies significantly among different serotypes. A particularly well-suited example to illustrate this phenomenon is the efficiency of liver transduction by AAV2, 6, and 8 vectors. Transgene expression by AAV2 vectors increases at a relatively slower rate after portal vein injection and usually takes 6 to 8 weeks to attain maximum levels. In contrast, AAV6 and AAV8 have a rapid onset of transgene expression and achieve maximum levels within 4 weeks after administration ([71], Z. Wu and R. J. Samulski, unpublished). Although the mechanism(s) underlying such distinct transduction profiles is currently unclear, it is likely that differences between serotypes arise due to differences in cellular uptake and intracellular trafficking mechanisms of AAV serotype vectors in each tissue type. These diverse infectious pathways are in turn thought to arise from structural differences between AAV serotypes at the capsid level.

Cell Surface Receptors

The cellular entry of nonenveloped viruses is often initiated by interaction of the capsid with cell surface glycosaminoglycan receptors. Subsequent secondary interactions of the viral capsid with coreceptors appear to dictate the intracellular trafficking pathway and biological fate of the capsid. It is this stage of the infectious pathway (and eventually transduction efficiency) that can be most significantly influenced by the choice of AAV serotype or hybrid vectors. For AAV2, an interaction with heparan sulfate proteoglycans is important for cell binding and transduction [72]. Subsequent interactions with human fibroblast growth factor receptor 1 (FGFR1) [73], hepatocyte growth factor receptor [74], and integrins $\alpha_V\beta_5/\alpha_5\beta_1$ ([75], A. Asokan and R. J. Samulski, unpublished) have also been reported. Mutagenesis of AAV2 has identified a clustering of basic residues, particularly R585 and R588, contributed by icosahedral threefold axis symmetry-related VP3 molecules that is involved in mediating heparin binding [76,77]. The structural determinants of AAV2 binding to secondary receptors (coreceptors) have not been mapped thus far.

AAV1, which lacks the critical heparin binding amino acid residues R585 and R588, does not bind heparin [47]. However, AAV6, which differs from AAV1 by only six amino acid residues and shares ~85% homology with the AAV2 capsid sequence, binds heparan sulfate and can be

purified using heparin-affinity chromatography [59,60]. Unlike AAV2, the transduction of cells by AAV6 is not inhibited upon co-incubation with soluble heparin. It is interesting to note that the AAV6 capsid does not possess the R585 and R588 residues that are primarily responsible for heparan sulfate binding by AAV2, but contains an additional lysine residue at position 531, which could contribute to heparin binding (Z. Wu and R. J. Samulski, unpublished). Similarly, the ability to bind heparin is conserved in AAV3 [47,78], which is ~87% identical to AAV2, albeit with weaker affinity. Again, AAV3 lacks R585 and R588, and the residues that contribute to heparin binding remain undetermined. The AAV serotypes 4 and 5, which display different tropism with respect to AAV2, utilize sialic acid with different linkage specificities for cell surface binding and transduction; AAV4 uses α-2,3-O-linked while AAV5 uses α-2,3-N-linked sialic acid for infection [79,80]. In addition, the platelet-derived growth factor receptor has been identified as a coreceptor for AAV5 [81]. Recently, several groups have independently established the role of sialic acid for AAV1- or AAV6-mediated transduction on certain cell types ([19,82,83], Z. Wu and R. J. Samulski, submitted for publication). We have recently determined that both α-2,3- and α-2,6-N-linked sialic acids facilitate efficient binding and transduction by AAV1 and AAV6 (Z. Wu *et al.,* submitted for publication). The nature of cell surface carbohydrates and receptors utilized for cell binding by other AAV serotypes and variants remains to be determined.

Intracellular Processing

Intuitively, the availability of specific cell surface receptors and coreceptors is often thought to dictate the tropism of a specific AAV serotype. However, this is patently not always the case. For example, AAV2 binds and enters NIH3T3 cells efficiently, but fails to transduce this cell type efficiently, despite moderate expression levels of heparan sulfate and FGFR. Further analysis revealed impaired trafficking to the nucleus as a major limiting step for AAV2 transduction in NIH3T3 cells [84,85]. Nevertheless, the ever-increasing number of new AAV serotypes and variants warrants a clear understanding of similarities and differences in receptor usage and intracellular processing of AAV serotypes in various cell types. Currently, the intracellular trafficking of AAV2 remains most well characterized among AAV serotypes. This could partly be due to the fact that most non-AAV2 serotypes display reduced transduction efficiencies *in vitro*. In this regard, identification of cell lines that are permissive to enhanced infection by other serotypes is critical for further studies on the intracellular trafficking of alternative AAV serotypes [e.g., 81].

Following receptor binding, AAV2 particles are endocytosed into the cell via clathrin-coated pits [86]. This event requires dynamin, a 100-kDa cytosolic GTPase that regulates clathrin-mediated endocytosis [87]. Although the role of $\alpha_V\beta_5$ integrin has been demonstrated in cellular uptake of AAV2 [75], the exact mechanism(s) remains undetermined. The intracellular trafficking pathway of AAV5 in HeLa cells has been studied [88]. Endocytosis of AAV5 was predominantly through clathrin-coated vesicles, although particles were also detected in non-coated pits. Surprisingly, AAV5 appears to be routed mainly to Golgi, within cisternae of the *trans*-Golgi network and within vesicles associated with cisternae and dictyosomal stacks of the Golgi apparatus. These studies suggest that AAV5 utilizes an atypical endocytic route that has not been described as a pathway for viral entry. A recent study by Ding *et al.* [89] suggests that AAV2 traffics through both late and recycling endosomes in a dose-dependent fashion. Interestingly, the trafficking of AAV2 through recycling endosomes appeared to be favorable for efficient transgene expression. Current understanding of the infectious pathway of AAV from a cellular perspective has been reviewed elsewhere [90]. Another alternative trafficking pathway exploited by AAV is transcytosis across barrier epithelial and endothelial cells [91]. The process appears to be serotype and cell-type specific, with particles isolated subsequent to apical-to-basolateral transport across polarized cells being able to transduce permissive cell types *in vitro*. Such unique trafficking mechanisms can be exploited in the development of AAV vectors capable of systemic dissemination for wide-spread gene expression.

Subsequent to endocytosis, processing of AAV virions within the endosomal compartment appears to be closely linked to transduction. For example, the acidic pH of the endosomal lumen is likely to induce conformational changes of key capsid subunits necessary for priming the virus for successful endosomal release or other downstream events [92]. One possible event that could occur during endosomal processing is the exposure of the phospholipase A2 (PLA2) domain located at the N-terminus of VP1. This PLA2 domain is conserved in parvoviruses and has been shown to play an important role in viral infectivity. While PLA2-inactivating point mutations do not affect capsid assembly, packaging, or cellular uptake of AAV2, they result in delayed onset and low levels of transgene expression [93]. Subsequent to endosomal escape, perinuclear accumulation of AAV2 virions has often been observed. A particularly interesting observation is the ability of proteasome inhibitors to enhance transduction by AAV serotypes. Studies from several groups [94–98] have shown that proteasome inhibitors increase transduction by AAV2, AAV5, AAV7, and AAV8 vectors. However, this effect appears to be cell/tissue-type selective. For example, proteasome inhibitors enhanced AAV2 transduction of mouse lung and liver, but did not affect transduction efficiency in skeletal or cardiac muscle [94]. A more recent study showed that proteasome inhibitors can increase AAV7 and AAV8 transduction of vascular endothelial cells, but have no effect on smooth muscle cells [98]. In similar *in vitro* studies, proteasome

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inhibitors increased transduction by AAV2 and AAV5 incubated on the apical surface of human polarized airway epithelia. However, transduction by these vectors incubated on the basolateral side remained unaffected [97]. The mechanism of enhanced transduction in the presence of proteasome inhibitors is unclear [90]. As suggested by Yan *et al.* [96], the increase in AAV capsid ubiquitination caused by proteasome inhibitors might facilitate efficient completion of the AAV latent life cycle. However, it is important to note that such chemical inhibitors can potentially impact AAV transduction at several levels of intracellular processing, including trafficking, as well as at the level of transgene expression.

The intracellular events underlying disassembly (uncoating) and nuclear translocation of AAV virions remain largely unknown. AAV appears to enter the nucleus through a mechanism independent of the nuclear pore complex, since agents that block the nuclear pore do not affect AAV nuclear entry [99]. Whether AAV uncoating happens before or after nuclear entry is not totally clear. Controversial results have been reported by different groups [86,100–103], probably due to interstudy variations in the use of cell lines/detection methods. It is likely that capsid disassembly might occur upon interaction with nuclear membrane components or other nuclear proteins. Such a scenario is supported by the ability of AAV2 virions to uncoat when infecting nuclei that are isolated or when the virions are incubated with nuclear extract [71,99]. Although generic, it is likely that capsid uncoating and nuclear entry mechanisms might differ between AAV serotypes. For example, Thomas *et al.* [71] have suggested that rapid uncoating could contribute to the faster onset and higher levels of transgene expression in the liver by AAV8 in comparison with AAV2.

Capsid Structure

Tissue tropisms of AAV vectors likely arise due to the cumulative effects of viral binding to multiple cell surface receptors, cellular uptake, intracellular processing, nuclear delivery of vector genomes, uncoating, and second-strand DNA conversion. Such events are often modulated by specific interactions between capsid proteins and cellular components. An understanding of the structural and functional correlates of AAV serotype capsids is therefore critical for elucidating the mechanism(s) underlying their diverse tissue tropisms as well as the design of hybrid vectors. To date, the crystal structure of AAV2 has been determined at a resolution of 3 Å using X-ray crystallography [104], while the structures of AAV4 and 5 have been solved using cryoelectron microscopy (cryo-EM) and image reconstruction at a resolution of 13 and 16 Å, respectively [105,106]. Preliminary X-ray crystallography analysis of AAV5 and AAV8 has also been carried out recently [107,108]. Crystal structures of other key AAV serotype capsids, notably AAV1 and AAV9, are currently in progress (Mavis Agbandje-McKenna, personal communication). Whether the viral genome ITRs of different serotypes also contribute to tissue tropism remains inconclusive. Recent work by Grimm *et al.* showed that ITRs from AAV1 to AAV6 do not affect the liver transduction when they are packaged into AAV8 capsids [109]. However, Zhou *et al.* [110] have recently shown that the combination of AAV8 ITRs with AAV8 capsids resulted in at least fivefold higher transgene expression than AAV8 capsids pseudotyped with AAV2 ITRs.

As a general rule, AAV virions have a $T = 1$ icosahedral capsid consisting of 60 copies of three related proteins, VP1, VP2, and VP3, at a ratio of 1:1:18. The three proteins share a common C-terminal region, but have different N-termini resulting from alternative start codon usage. As a result, the entire sequence of VP3 is present in VP2, which in turn is entirely contained within VP1. In solving the structures of AAV2, 4, and 5, only the common C-terminal region, namely the VP3 subunit, was observed. The core of the protein comprises a conserved eight-stranded antiparallel β-barrel motif. The majority of the variable surface structure consists of large loops inserted between strands of the β-barrel. Structural features on the capsid surfaces of these viruses include projections at or surrounding the icosahedral threefold axis and the depressions at the twofold axis and around the fivefold axis of symmetry. A conserved cylindrical channel is present at the icosahedral fivefold axis formed by symmetry-related β-ribbons. Differences between the serotypes can primarily be mapped to their surface topology, which may account for their binding to different receptors.

To understand the observed surface loop variability at the icosahedral two- and threefold axes in AAV serotypes, Padron *et al.* [105] aligned structural models generated for the VP3 amino acid sequences of AAV1 through AAV9. The loops equivalent to those making up the threefold protrusions were found to be the most variable among serotypes. This variable region spans the center of the primary sequence (residues ~440 to 600, AAV2 VP1 numbering), while residues located at the N- and C-termini are conserved. Other regions that show the most variability among AAV serotypes are near residues 260 and 380 (AAV2 VP1 numbering). AAV4 and AAV5 are the most antigenically distinct AAV serotypes, with AAV4 being unable to cross-react with antibodies generated to linear AAV2 epitopes, such as the AAV2 antibody-binding B1 site, which cross-reacts with AAV1, AAV3, and AAV5. Variations within the loop regions are also thought to eliminate antibody recognition of serotypes AAV1 and AAV3–9 by the conformation-specific antibody A20, which binds to AAV2. These observations highlight the diverse antigenic nature of AAV serotype capsids and their ability to utilize different capsid surface regions for the recognition of cell surface receptors during cell recognition and infection. Current efforts to understand

the structural attributes of AAV capsid interactions with cell surface receptors are focused on cocrystallization or cryo-EM studies of AAV virion shells bound to the soluble form of their primary and/or secondary receptors (Mavis Agbandje-McKenna, personal communication).

Engineering Hybrid Vectors From AAV Serotypes

The purpose of developing new AAV vectors is to enable the transduction of tissues that are refractory to transduction by naturally occurring AAV vectors or limit AAV tropism to specific tissues. Strategies for engineering such custom-designed AAV capsids by insertion of peptide ligands, conjugate-based targeting, and presentation of large protein ligands on the AAV capsid have been described in detail elsewhere [9]. The following section is focused on strategies to engineer hybrid AAV vectors derived from different AAV serotypes that serve as starting templates.

Mosaic AAV Vectors

A mosaic virion can be defined as a capsid structure composed of a mixture of capsid subunits from different serotypes. Such particles can be generated by using a mixture of helper constructs that encode capsid proteins from different serotypes or wild-type and mutant capsid proteins of the same serotype or from two different mutant capsid subunits of the same serotype. In theory, the ratio of capsid subunits from different sources in the mosaic virion must reflect the input ratio of different helper constructs, although this has not been proved experimentally. The unique advantage of this technique is the ability to combine selective features from different sources that synergistically enhance transgene expression. Using a mixture of AAV1 and 2 helper constructs, Hauck *et al.* [111] generated mosaic viruses that combine the transduction characteristics of AAV1 in muscle and AAV2 in liver. The resulting mosaic particles inherited the heparin-binding property from AAV2, which can be utilized for affinity column purification. Rabinowitz *et al.* [112] generated a more comprehensive panel of mosaic vectors by mixing pair-wise combinations of serotypes 1 through 5 at several input ratios. Mosaic particles with dual binding characteristics of parent serotypes were generated in this study, with relative binding preferences determined by the serotype present at the higher concentration. Interestingly, new properties different from either parental virus were also seen in some mosaics. For example, even though AAV1 and AAV2 do not transduce C2C12 muscle cells efficiently in culture, mosaic AAV1/2 virions, produced by transfection of AAV1 and 2 helper constructs at a ratio of 1:19, exhibited dramatically increased transduction in C2C12 cells. Such could occur due to altered intracellular trafficking of mosaic AAV1/2 virions in C2C12 cells. Other reports [113,114] have also shown that mosaic AAV generated from two noninfectious AAV2 mutants can regain infectivity when prepared using optimal plasmid ratios.

The mosaic strategy also provides insight into the biology of AAV serotypes, such as capsid assembly, receptor binding, and intracellular trafficking [112]. For example, the generation of mosaics using combinations of AAV1, 2, and/or 3 all resulted in high-titer virions, whereas mixing of AAV5 capsid subunits with other serotypes resulted in moderate titers. Mosaics generated with AAV4 provided the lowest yields. These results highlight the importance of capsid subunit compatibility in the assembly of mosaic virions. Another interesting observation was the ability of AAV1/5, 2/5, or 3/5 mosaics to bind mucin like parental AAV5 at capsid subunit levels as low as 10%. In contrast, the AAV2/5 mosaic does not bind heparin even with 75% AAV2 subunits. These results suggest that AAV5 could bind mucin through fewer subunits than AAV2 requires for heparin binding. Application of this transcapsidation approach can provide important insight into the biology of other important serotypes such as AAV6–9 and aid in the development of hybrid vectors with altered tropism.

Chimeric AAV Vectors

"Chimeric virions" refers to the vectors containing capsid proteins that have been modified by domain or amino acid swapping between different serotypes. Strategies for the generation of chimeric virions primarily involve the marker rescue approach or mutagenesis of AAV virions to swap surface domains ranging from single to multiple amino acid residues. The marker rescue strategy, developed by Bowles *et al.* [115], exploits the sequence homology between AAV serotypes to serve as crossover points for recombination initiated by cellular proteins. The recombination of sequences can result in the "rescue" of infectious or targeted phenotypes in mutant virions through directed selection of functional capsid subunits that assemble into viable virions. For example, three AAV2 capsid mutant sequences previously characterized as noninfectious and unable to bind heparin were rescued after cotransfection with AAV3 capsid DNA sequences. Such "forward engineering" could serve as a powerful tool for generation of chimeric virions tailored with unique properties governed by the criteria set in the screening process. *In vitro* versions of marker rescue are typically derived from DNA shuffling or error-prone PCR techniques (see below).

Domain swapping involves the transfer of specific capsid domains such as surface loops or specific residues from one serotype to another. Such techniques complement the transcapsidation strategy, in that they can provide vital information pertaining to compatibility of serotype capsid subunit domains as well as help identify regions that are determinants for tissue tropism. For

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example, Hauck *et al.* [116] utilized the domain-swapping strategy to identify that the 350–430 region (AAV1 VP1 numbering) is critical for AAV1 muscle tropism. However, it is important to note that simply swapping domains may not always transfer specific tropisms to the newly generated chimeric vector. For example, substitution of the heparin-binding residues from AAV2 onto a similar region of AAV5 produced vector particles at good yields and conferred heparan sulfate binding to this chimeric virion. However, the chimeric virus was noninfectious in cells normally transduced by AAV2 [76]. Such phenomena have been observed in other AAV serotypes as well (J. E. Rabinowitz and J. R. Samulski, unpublished data). In contrast, recent studies in our lab have shown that the muscle-tropic characteristics of AAV1 can indeed be transferred to AAV2 by swapping as little as five amino acid residues between serotypes (D. E. Bowles and J. R. Samulski, unpublished data). It is noteworthy that this vector, dubbed AAV2.5, is currently being utilized in a Phase I clinical trial for the treatment of Duchenne muscular dystrophy (J. R. Samulski *et al.,* unpublished). Substituting similar amino acid residues on AAV3 also appears to produce a phenotype that enables efficient transduction of the mouse heart at higher efficiency than parental AAV3 (D. E. Bowles and J. R. Samulski, personal communication). Such strategies not only hold tremendous potential for the generation of chimeric vectors, but can also be exploited along with crystal structure data to establish structure–function correlates of AAV serotype capsids.

Combinatorial AAV Vector Libraries

DNA shuffling and error-prone PCR are powerful library-based approaches for directed evolution, which generate diversity by recombination and combining useful mutations from individual genes. Single and multigene traits that require many mutations for improved phenotypes can be evolved rapidly. Libraries of hybrid genes can be generated by random fragmentation of a pool of related genes, followed by reassembly of the fragments in a self-priming polymerase reaction. Template switching causes crossovers in areas of sequence homology [117]. DNA shuffling technology has been significantly enhanced in the past year, extending its range of applications to pharmaceutical proteins, antibodies, enzymes, vaccines, gene therapy vectors, and transgenes [for review, see 118]. To achieve accelerated evolution of novel phenotypes, Powell *et al.* and Soong *et al.* performed breeding of six ecotropic murine leukemia virus strains by DNA shuffling [119,120]. The envelope regions were shuffled to generate a recombinant library of 5×10^6 replication-competent retroviruses. Several viral clones with greatly improved stabilities and completely new tropisms were isolated. The envelopes of these novel variants differed in DNA and protein sequence, and in all cases complex chimeras derived from multiple parental strains.

More recently, Maheshri *et al.* and Perabo *et al.* have extended the error-prone PCR and DNA-shuffling strategy into the realm of AAV vectors by using a single AAV2 capsid gene and random mutations as a source of diversity to select for variants that can escape neutralizing antibodies [121,122]. The generation of AAV libraries with increased diversity can be achieved by shuffling the capsid genomes of several AAV serotypes. The lack of bias associated with such directed evolution strategies can not only be exploited for molecular breeding of novel cell/tissue-specific AAV variants, but will also enable mapping the structural attributes of the diverse tissue tropisms of AAV serotypes. Studies focused on the biological characterization of AAV serotypes and resolution of their crystal structures have dramatically increased in recent years. Although primarily AAV2-based vectors have entered clinical trials thus far, alternative AAV serotypes and hybrid vectors will soon be available as part of a versatile toolkit for human gene therapy applications in the near future. A thorough understanding of the mechanisms and molecular determinants underlying the infectious pathway of such vectors and establishment of their safety profile is critical for the successful development and application of AAV vectors tailored to fit individual disease and/or patient profiles.

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