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Directed Evolution of Novel Adeno-Associated Viruses for Therapeutic Gene Delivery

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Directed Evolution of Novel Adeno-Associated Viruses
for Therapeutic Gene Delivery

By
Melissa Ann Kotterman

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requirements for the degree of
Doctor of Philosophy
in
Chemical and Biomolecular Engineering
in the
Graduate Division
of the
University of California, Berkeley

Committee in charge:
Professor David V. Schaffer, Chair
Professor Danielle Tullman-Ercek
Professor Seung-Wuk Lee

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Abstract

Directed Evolution of Novel Adeno-Associated Virus for Gene Therapy Applications

by

Melissa Kotterman

Doctor of Philosophy in Chemical and Biomolecular Engineering

University of California, Berkeley

Professor David V. Schaffer, Chair

Gene delivery vehicles, or vectors, based on adeno-associated viruses (AAV) have demonstrated success in both preclinical disease models and recently in human clinical trials for several disease targets, including muscular dystrophy, hemophilia, Parkinson’s disease, Leber’s congenital amaurosis, and macular degeneration. AAV gene therapy vectors have become increasingly utilized because the parent virus is nonpathogenic in humans, they can transduce both dividing and non-dividing cells, and they are efficient for some important cell and tissue types. AAV’s simple genome contains two open reading frames, which encode the nonstructural proteins needed for viral replication and virus assembly (rep) and the three structural proteins that assemble to form a 60-mer viral capsid (cap). To create a gene therapy vector, a therapeutic gene of interest is inserted in place of the viral open reading frames to be packaged during vector production. Despite its considerable promise and emerging clinical success, several challenges impede the broader implementation of AAV gene therapy, including the prevalence of anti-AAV neutralizing antibodies in the human population due to natural exposure to the parent virus, low transduction of a number of therapeutically relevant cell types, and an inability to overcome physical transport barriers in the body. These challenges arise since the demands we place on AAV vectors are often different from or even at odds with the properties nature bestowed on their parent viruses. Viral directed evolution – the iterative generation of large, diverse libraries of viral mutants and selection for variants with specific properties of interest – offers a promising means to address these problems.

Directed evolution is a high-throughput, molecular engineering approach that our group has adapted and implemented to create AAV variants with novel properties, such as altered receptor binding, altered cell transduction, and altered tissue transduction in the body. In general, the method emulates the process of natural evolution, in which repeated genetic diversification and selection enable the accumulation of key mutations or genetic modifications that progressively improve a molecule's function, even without knowledge of the underlying mechanistic basis for the problem. For AAV, this process has involved mutating wild-type AAV cap genes to create large genetic libraries, which can be packaged to generate libraries of viral particles, each of which is composed of a variant capsid surrounding a viral genome encoding that capsid. A selective pressure – such as high-affinity antibodies against the AAV capsid, the ability to infect adult neural stem cells, or the ability to infect human pluripotent stem cells – is then applied to promote the emergence of variants able to surmount these barriers. After each such selection step, the successful variants can be recovered and used as the starting material for
the next selection step to further enrich for improved variants. After several such selection steps, the resulting \textit{cap} gene pool is subjected to additional mutagenesis and selection. After several rounds of mutagenesis and selection, the resulting variants can be analyzed individually for the desired property.

Using directed evolution, I have engineered several novel AAV variants capable of enhanced gene delivery in several applications. First, variants selected in the presence of pooled human antibodies were 2- to 35-fold less susceptible to neutralization by anti-AAV antibodies compared to parental AAV \textit{in vitro}. The antibody neutralization properties also translated to enhanced transduction in an \textit{in vivo} mouse model in the presence of neutralizing antibodies. The isolation of such novel variants resistant to anti-AAV antibodies may enable the future treatment of patients with pre-existing immunity that are currently ineligible for AAV gene therapy. Second, a novel variant selected for the ability to infect adult neural stem cells was capable of more efficient gene delivery to rat, mouse, and human neural stem cells \textit{in vitro}. Furthermore, the variant demonstrated more efficient and specific transduction of rat and mouse neural stem cells \textit{in vivo} compared to natural AAV serotypes. Gene delivery to neural stem cells using this variant could be used as a gene therapy option to better harness these cells for tissue regeneration. Finally, a novel variant selected for the ability to infect human pluripotent stem cells was able to transduce several human embryonic stem cell and induced pluripotent stem cell lines 3-fold more efficiently than natural AAV serotypes, which enabled a 10-fold increase in the efficiency of a genome-editing technique termed gene targeting. These results demonstrate that engineered and evolved AAV vectors are highly promising for a range of applications from the lab to the clinic.
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CHAPTER 1

DIRECTED EVOLUTION OF NOVEL ADENO-ASSOCIATED VIRUSES FOR THERAPEUTIC GENE DELIVERY

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Abstract

Gene therapy vectors based on adeno-associated virus (AAV) are currently in clinical trials for numerous disease targets, such as muscular dystrophy, hemophilia, Parkinson’s disease, Leber’s congenital amaurosis, and macular degeneration. Despite its considerable promise and emerging clinical success, several challenges impede the broader implementation of AAV gene therapy, including the prevalence of neutralizing antibodies in the human population, low transduction of a number of therapeutically relevant cell and tissue types, an inability to overcome physical and cellular barriers in vivo, and a relatively limited carrying capacity. These challenges arise since the demands we place on AAV vectors are often different from or even at odds with the properties nature bestowed on their parent viruses. Viral directed evolution – the iterative generation of large, diverse libraries of viral mutants and selection for variants with specific properties of interest – offers an approach to address these problems. Here, we outline progress in creating novel classes of AAV variant libraries and highlight the successful isolation of variants with novel and advantageous in vitro and in vivo gene delivery properties.

Introduction

AAV Biology

Adeno-associated virus (AAV) is a nonpathogenic parvovirus composed of a 4.7 kb single-stranded DNA genome within a non-enveloped, icosahedral capsid.¹ The genome contains three open reading frames (ORF) flanked by inverted terminal repeats (ITR) that function as the viral origin of replication and packaging signal.¹ The rep ORF encodes four nonstructural proteins that play roles in viral replication, transcriptional regulation, genomic integration, and virion assembly.¹ The cap ORF encodes three structural proteins (VP1-3) that assemble to form a 60-mer viral capsid.¹ Finally, an ORF present as an alternate reading frame within the cap gene produces the assembly-activating...
protein (AAP), a viral protein that localizes AAV capsid proteins to the nucleolus and functions in the capsid assembly process.[2]

The virus’s capsid governs its ability to transduce cells, from initial cell surface receptor binding to gaining entry into the nucleus. Briefly, AAV2 – the variant most broadly studied to date – is internalized via receptor-mediated endocytosis, with evidence supporting a role for both the clathrin-coated pit pathway[4,5] and the clathrin-independent carriers/GPI-anchored-protein-enriched endosomal compartment pathway.[6] Following cellular entry, the virion escapes from early endosomes and traffics to the perinuclear area. There is evidence supporting both AAV trafficking into the nucleus prior to uncoating[7-9] and AAV uncoating prior to viral DNA entry into the nucleus.[10] Upon nuclear entry, second-strand synthesis – i.e. conversion of its single-stranded genome into double-stranded, transcriptionally available DNA – must occur for viral gene expression.[11] Finally, in the absence of helper virus co-infection, AAV enters a latent life-cycle in which viral genomes can integrate selectively into the AAV1S locus on human chromosome 19 (for replication competent, wild-type AAV) or persist as extrachromosomal episomes (for both wild-type and recombinant AAV).[12-14]

In recombinant versions of AAV, a gene of interest is inserted between the ITRs in place of rep and cap, and the latter are provided in trans, along with helper viral genes, during vector production.[15] The resulting vector can transduce both dividing and non-dividing cells with stable transgene expression in the absence of helper virus for years in post-mitotic tissue. There are eleven naturally occurring serotypes and over 100 variants of AAV, each of which differs in amino acid sequence, particularly within the hypervariable regions of the capsid proteins, and thus in their gene delivery properties.[16,17] Importantly, no AAV has been associated with any human disease, making recombinant AAV attractive for clinical translation.[1]

AAV has yielded promising results in an increasing number of clinical trials. As a prominent example, during Phase I clinical trials for Leber’s congenital amaurosis (LCA), numerous patients who received a subretinal injection of AAV2 encoding a protein required for the isomerohydrolase activity of retinal pigment epithelium showed sustained improvement in both subjective and objective measurements of vision.[18-20] Furthermore, there were no significant adverse events during either the pre-trial efficacy studies or the trial.[18-20] As a second recent example, AAV8-mediated delivery of cDNA encoding factor IX to the liver of hemophilia B patients resulted in sufficient levels of secreted protein to alleviate the patients’ bleeding phenotype.[21] AAV vectors are also being clinically explored for muscular dystrophy, Parkinson’s disease, and Alzheimer’s disease.[22] AAV thus has considerable promise. Nevertheless, there are impediments that may limit its utility, such as anti-capsid immune responses, low transduction of certain tissues, an inability for targeted delivery to specific cell types, and a relatively low carrying capacity. Rational design has made progress in creating AAV variants with enhanced properties.[23,24] In many situations, however, there is insufficient mechanistic knowledge to effectively empower rational design with the capacity to improve AAV. As an alternative, directed evolution has been emerging as a strategy to create novel AAV variants that meet specific biomedical needs.
**Directed Evolution**

Directed evolution is a high-throughput molecular engineering approach that has been successfully utilized to generate protein pharmaceuticals with enhanced biological activities, antibodies with enhanced binding affinity, and enzymes with new specificities.\(^\text{[25]}\) The method emulates the process of natural evolution, in which repeated genetic diversification and selection enable the accumulation of key mutations or genetic modifications that progressively improve a molecule’s function, even without knowledge of the underlying mechanistic basis for the problem. For AAV (Figure 1a), this process has involved mutating wild-type AAV *cap* genes to create large genetic libraries (described below), which can be packaged to generate libraries of viral particles, each of which is composed of a variant capsid surrounding a viral genome encoding that capsid. A selective pressure is then applied – such as high-affinity antibodies against the AAV capsid (Figure 1b), the need to bind new cell surface receptors or circumvent intracellular barriers (Figure 1c), or tissue structures that bar the virus from accessing target cells in vivo (Figure 1d) – to promote the emergence of variants able to surmount these barriers. After each such selection step, the successful variants can be recovered (e.g. by superinfection with a helper virus or PCR amplification) and used as the starting material for the next cycle of selection (Figure 1a, step 6) to further enrich for improved variants. If the process is halted after a single library diversification and selection step, it is referred to as library selection. However, after several such selection steps, directed evolution can be conducted by subjecting the resulting *cap* gene pool (Figure 1a, step 7) to additional mutagenesis and selection (Figure 1a, step 8). After library selection or directed evolution, the resulting variants can be analyzed clonally for the desired property.
Figure 1. Directed Evolution of AAV. (a) In a schematic of the process, 1) a viral library is created by mutating the cap gene. 2) Viruses are packaged (typically in HEK293T cells using plasmid transfection), such that each particle is composed of a mutant capsid surrounding the cap gene encoding that protein capsid. 3) Viruses are harvested and purified. 4) The viral library is placed under selective pressure. 5) Successful viruses are amplified and recovered. 6) Successful clones are enriched through repeated selection steps. 7) Isolated viral DNA reveals selected cap genes. 8) Selected cap genes are again mutated to serve as a new starting point for further selection steps to iteratively increase viral fitness. (b) – (d) Examples of selective pressure for directed evolution. (b) Evasion of antibody neutralization. (c) Altered receptor binding. (d) Cell specificity within complex tissue structures.

Successful library selection or directed evolution begins with the creation of high quality, high diversity libraries. In the last few years, a variety of library types have been created using several in vitro and in vivo techniques for viral DNA mutagenesis. The resulting libraries can be used individually or in combination to isolate novel variants. In addition, the techniques used to create the libraries can be used singly or in tandem for additional genetic diversification midway through the evolution process.

Library Classes

As the most straightforward library, sub-optimal polymerase chain reaction (PCR), i.e. error-prone PCR, can be used to amplify and introduce random point mutations into the AAV cap ORF at a defined and tunable rate (Figure 2a). This approach has been used to introduce mutations into either single or multiple AAV serotypes for subsequent selection.

In addition to point mutations, genetic recombination has been used to generate chimeric capsids. The earliest report of random chimeras of AAV cap genes was an in vivo viral rescue method, in which cellular co-transfection of a defective AAV2 genome with PCR fragments of the cap gene of another serotype (AAV3) led to rescued viral chimeras capable of replication. DNA shuffling, an in vitro PCR-based method, has subsequently been implemented to create large chimeric cap gene libraries composed of multiple serotypes (Figure 2b).

Furthermore, random peptide sequences have been inserted into defined sites of the viral capsid, such as in the heparin binding domain of the AAV2 capsid (at capsid residue R588), via ligation of degenerate oligonucleotides into the cap ORF (Figure 2c). This technique has recently been extended to AAV9. Defined peptide-encoding sequences can also be inserted into random locations of the AAV2 cap ORF via transposon mutagenesis. This approach was used to incorporate hexahistadine tags randomly throughout the AAV2 capsid to explore AAV clones capable of immobilized metal affinity chromatography purification.

Finally, diversity can be focused on several hypervariable regions of the AAV capsid, which lie on surface-exposed loops. One of the first studies to conduct DNA shuffling of the AAV capsid noted that many of the functionally selected variants were composed primarily of a single serotype (AAV1 and AAV6) with surface loops exchanged with other serotypes. This motivated the development of a “loop swap” library in which four loops of AAV2 were replaced with a library of peptide sequences designed based on the level of conservation of each amino acid position among natural AAV serotypes and
variants.\textsuperscript{[38]}

\textbf{Figure 2.} Schematic of Library AAV Capsid Protein Engineering Strategies. Methods for generation of highly diverse viral libraries include (a) Random point mutagenesis (error-prone PCR), (b) \textit{in vitro} recombination (e.g. DNA shuffling), and (c) Insertion of random peptides. Directed evolution strategies use these approaches as part of an iterative strategy to increase AAV’s fitness for various applications.

\textbf{In Vitro Selection and Evolution}

\textit{Alternate Receptor Targeting}

AAV mutant libraries are being utilized in an increasing number of selection strategies to isolate novel variants, both \textit{in vitro} and \textit{in vivo}. For example, there is a strong biomedical motivation to generate AAV variants capable of transducing previously non-permissive cell types and/or to target gene delivery to specific, therapeutically relevant cell types. The important finding that insertion of small (seven amino acid) peptides into the heparin binding domain of the AAV2 capsid could alter viral tropism without disrupting capsid stability lay the groundwork for initial library selection approaches.\textsuperscript{[34,39]} In particular, Perabo \textit{et al.} inserted random peptide sequences into AAV2’s heparin binding domain and selected the resulting library for the capacity to infect a human megakaryocytic cell line and a B-cell lymphocytic leukemia cell line.\textsuperscript{[39]} Variants isolated from the human megakaryocytic cell selections shared an RGD motif and were capable of up to a 100-fold increase in transduction versus AAV2.\textsuperscript{[39]} Müller \textit{et al.} also generated a random peptide insertion library, selected for the capacity to infect primary human coronary artery endothelial cells, and thereby created variants capable of 4- to 40-fold increased gene transfer to endothelial cells \textit{in vitro} compared to AAV2. These mutants also showed increased accumulation in heart tissue and decreased localization to liver \textit{in vivo}, demonstrating that \textit{in vitro} selections can produce variants with improved \textit{in vivo} transduction properties.\textsuperscript{[34]}
AAV2 random peptide library selections have subsequently been applied to improve transduction of primary human venous endothelial cells, lung carcinoma cells, prostate carcinoma cells, acute myeloid leukemia cells (and other hematopoietic cancer cell lines), and primary human hematopoietic progenitor cells. Recent selection of an AAV9 random peptide insertion library for the capacity to transduce human coronary artery endothelial cells yielded a variant with a 200-fold improved infection efficiency compared to AAV9 on postnatal human umbilical vein endothelial cells.

In addition to AAV peptide display, other forms of mutagenesis can alter receptor binding. As a proof-of-concept, Maheshri et al. showed that directed evolution can be applied to modulate the affinity of AAV for its primary receptor. Specifically, they evolved AAV2 variants with both increased and decreased affinity for heparan sulfate proteoglycans by repeatedly selecting an error prone AAV2 library for elution from a heparin affinity chromatography column at sodium concentrations either higher or lower than those needed to elute wild-type AAV2. In addition, shuffling the cap genes of several AAV serotypes can enable a shift in viral tropism, potentially modulating affinity for existing viral receptors, pairing different primary and secondary receptors, or creating binding domains for new receptors. Li et al. used a shuffled library of AAV1-9 (excluding AAV7) to isolate a variant capable of melanoma cell transduction. A chimeric variant of AAV1, 2, 8, and 9 was more efficient at infecting hamster, mouse, and human melanoma cell lines in vitro, as well as hamster melanoma cell-derived tumors in vivo following direct injection. Mutational studies to map the melanoma specific tropism identified residues 705 to 735 contributed by AAV9 as playing a critical but not sufficient role in the new tropism, demonstrating that these variants can contribute to knowledge of the structure-function relationships of capsid regions.

Adult and Pluripotent Stem Cells

AAV can potentially be applied as an in vitro tool to improve the biomedical utility of stem cells. It is now well-recognized that AAV vector genomes carrying gene targeting constructs can mediate homologous recombination with target loci in a cellular genome at efficiencies $10^3$-$10^4$-fold higher than corresponding plasmid constructs. AAV-mediated gene targeting could thus aid in creating stem cell lines harboring mutations involved in human disease for basic investigation of disease mechanisms or high throughput in vitro small molecule drug discovery and toxicity studies. Gene targeting can also mediate the safe harbor integration of transgenes that guide differentiation into specific lineages, mediate secretion of therapeutic products, or enable cells to better resist the toxic effects of a diseased tissue. Finally, in the long term, gene correction of disease-causing mutations may enhance the therapeutic potential of individualized cell replacement therapies, such as ones based on pluripotent stem cells. However, while AAV-mediated gene targeting has been successfully applied to cells that AAV can effectively transduce, naturally occurring AAV variants are typically highly inefficient at infecting a number of stem cell types.

Directed evolution was first applied to stem cells by Jang et al. to isolate a variant capable of efficient transduction of neural stem cells (NSCs). Selections using an error-prone AAV2 library, an AAV2 with random peptide insert library, and an AAV2 pairwise shuffled library on NSCs (from the adult hippocampus) yielded an AAV2 variant containing a peptide insertion that mediated 50-fold increased transduction of rat...
NSCs, as well as increased transduction of murine NSCs, human fetal NSCs, and human embryonic stem cell (hESC) derived neural progenitor cells. Presumably as a result of the increased transduction, the variant also exhibited a 5-fold increased rate of targeted gene correction compared to natural serotypes in NSCs.

The most successful report of gene targeting to human pluripotent stem cells using wild-type AAV demonstrated correct targeting of 1.3% of all colony forming units, which corresponds to an overall gene targeting frequency for the originally infected hESCs of approximately 0.03%. To build upon this result, Asuri et al. applied directed evolution to create an AAV variant capable of enhanced gene delivery and gene targeting in human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). Selecting an AAV2 and AAV6 error-prone library, an AAV2 loop-swap library, a shuffled library (containing AAV 1, 2, 4-6, 8, and 9), and a random peptide insert library for enhanced infection of hESCs yielded a simple AAV2 variant harboring a single R459G mutation. This mutant, which exhibited higher heparin affinity, was capable of increased transduction of the hESCs used in the selection, as well as other hESC and human iPSC lines. In addition to increased transduction, the variant exhibited a 0.1% gene targeting efficiency, already substantially higher than plasmid-mediated gene targeting, which increased to over 1% in the presence of zinc-finger nuclease-induced double-stranded breaks at the target locus. This increase in gene targeting efficiency was most likely due to an increase in both the total number of cells transduced by the variant, as well as an increase in the number of viral copies that infected each cell.

**Antibody Evasion**

AAV has been successful in clinical studies involving delivery to immune privileged regions, and immunosuppression is a promising approach for reducing antiviral cellular immune responses in general. However, humoral immunity – for example anti-AAV antibodies resulting from childhood exposure to one or more serotypes, or from prior administration of an AAV vector – poses a significant challenge, particularly for intrahepatic and intravascular administration. Recent analysis indicated that the prevalence of anti-AAV IgG antibodies in humans was highest for AAV2 (72%) and AAV1 (67%), but AAV9 (47%), AAV6 (46%), AAV5 (40%), and AAV8 (38%) antibodies were also present in a large portion of the population studied. Other studies have shown a lower but significant prevalence of anti-AAV antibodies in the population against multiple AAV serotypes.

Directed evolution can create AAV variants that evade such neutralizing antibodies. Using potent serum from rabbits immunized with AAV2 vector, Maheshri et al. evolved an antibody-resistant AAV2 variant using error-prone PCR and staggered extension process (StEP) mutagenesis over two rounds of evolution (i.e. two mutagenesis and six selection steps). An AAV2-based variant containing E12A, K258N, T567S, N5871, and T716A point mutations emerged from the selections. Compared to vector with wild-type AAV2 capsid, a 96-fold higher neutralizing antibody concentration was required to neutralize the variant in vitro, and 100-1000 fold higher levels of antiserum were required in vivo.

Perabo et al. selected an error-prone AAV2 library for variants capable of transduction in the presence of human serum. Mutations to amino acids 459 and 551 were
dominant among the analyzed clones, and a variant with R459K and N551D substitutions withstood 5.5-fold higher neutralizing antibody levels compared to wild-type AAV2 in vitro.\cite{26} Grimm et al. selected a library of shuffled cap genes from wild-type AAV serotypes of primate (AAV2, AAV4, AAV5, AAV8, AAV9) and nonprimate (caprine, bovine, avian) origin for variants that could more efficiently transduce HepG2 liver cells in the presence of intravenous immunoglobulin (IVIG), the pooled IgG fraction from over 1000 human donors.\cite{30} A single clone with sequence contributions from AAV2, AAV8, and AAV9 emerged from the selection and showed higher transduction efficiency compared to wild-type AAV1, 2, 3, 4, 5, 6, 8, and 9 in liver, kidney, fibroblast, and lung cell lines.\cite{30} In addition, the variant exhibited antibody resistance levels comparable to AAV8 and AAV9, but much higher than AAV2 in vivo.\cite{30}

Library selection can be guided by some aspects of rational design to analyze the effects of comprehensive amino acid substitutions at certain sites of interest. For example, Maersch et al. performed saturation mutagenesis at amino acid positions 449, 458, 459, 493, and 551 of the AAV2 capsid,\cite{54} positions previously implicated in key antibody epitopes.\cite{26,27} Though the resulting variants did not reach the antibody resistance levels of some natural serotypes, the mutants isolated from selections in the presence of human serum importantly reduced the antibody susceptibility of AAV2 while conserving tropism.\cite{54}

In addition to selections designed specifically to reduce antibody neutralization, modulating other AAV properties (such as tissue or cell tropism) can serendipitously yield variants that also exhibit anti-AAV antibody resistance. For example, Koerber et al. selected functional AAV chimeras from a DNA shuffled library of cap genes from AAV serotypes 1, 2, 4-6, 8, and 9.\cite{31} Though the selections did not involve antibodies, four out of the seven clones analyzed withstood higher neutralizing antibody concentrations than their parent serotypes, potentially through the loss of key epitopes at the junctures of capsid regions from different serotypes.\cite{31} As another example, a chimeric variant isolated by Li et al. during selections on hamster melanoma cells had no cross-reactivity to antisera of mice immunized with AAV1, AAV8, or AAV9, and low cross-reactivity with AAV2.\cite{32} In addition, a chimeric variant isolated by Yang et al. from an in vivo selection to identify a muscle-targeting variant (described in more detail below) exhibited a similar level of in vitro resistance to IVIG as compared to AAV8 and a higher level of resistance compared to AAV2.\cite{33} Finally, Varadi et al. demonstrated that random peptide insertions can alter AAV immunoreactivity, as AAV9-SLRSPPS and AAV9-RDVRAVS vectors exhibited enhanced in vitro transduction in the presence of IVIG compared to wild-type AAV2 and AAV9.\cite{35} Furthermore, the anti-AAV9 antibody ADK9 did not neutralize the variants.\cite{35} These results indicate that it may be possible to simultaneously select for two independent properties: antibody resistance and maintenance of existing or engineering of novel tropism.

In Vitro Models of Human Disease

Similar to the selections to alter the receptor binding of AAV, in vitro selections can be performed to increase transduction of specific cell types used in culture models of human disease. Excoffon et al. selected several libraries for efficient transduction of human airway epithelial cell cultures from the apical surface, a property that is critical for cystic fibrosis (CF) gene therapy and that natural AAV variants lack.\cite{55} A chimera of the
VP1-unique domain of AAV2 with the remainder of the AAV5 capsid, along with a key A581T point mutation in the sialic acid binding domain, emerged from two rounds of evolution. This evolved variant, AAV2.5T, bound to the apical surface of human airway epithelial cells at 100-fold higher levels than AAV5, transduced human airway nearly 100-fold more efficiently than AAV2 and AAV5, and as a result was able to correct the chloride ion transport defect of human cystic fibrosis airway epithelia upon delivery of the CFTR gene.\[^{55}\] Li \textit{et al.} created a library of shuffled \textit{cap} genes from AAV1-6, 8, and 9 and selected for transduction of primary differentiated ciliated airway epithelium. The selections yielded two chimeras composed of AAV1, 6, and/or 9.\[^{56}\] These variants were able to increase CFTR mRNA 25-fold over endogenous CF levels and rescue up to 31\% of the normal CFTR response to forskolin in cystic fibrosis airway epithelia.\[^{56}\]

Neurodegenerative diseases, in both the central and peripheral nervous systems, are also clinical targets that could significantly benefit from improved vectors. As one example, AAV typically has strong tropism for neurons; however, for a number of reasons it would be beneficial to develop variants that transduce glia. Astrocytes outnumber neurons in some regions of the nervous system, often play natural neuroprotective roles that can be further enhanced, and have even been shown to contribute to disease pathology in Alzheimer’s Disease and Amyotrophic Lateral Sclerosis.\[^{57,58}\] Using an AAV2 error-prone library, an AAV2 loop-swap library, a shuffled library containing AAV 1, 2, and 4-9, and AAV2 with random peptide insertions, Koerber \textit{et al.} evolved variants for the ability to infect primary human astrocytes.\[^{38}\] In addition to enhanced transduction of astrocytes \textit{in vitro}, two AAV2-based variants from the shuffled and loop-swap libraries transduced 5.5- and 3.3-fold more astrocytes than AAV2 within the striatum following intracranial injection in rats.\[^{38}\] As described below, one of these variants had highly advantageous properties in another neural tissue, the retina. Finally, Maguire \textit{et al.} created a library of shuffled \textit{cap} genes from AAV1, 2, 5, 8, 9, and 10 and selected for infection of glioblastoma multiforme cells.\[^{59}\] The isolated variant was capable of efficient transduction of these cells \textit{in vitro} and performed as well as or better than AAV2 on a panel of other glioma cells.\[^{59}\]

\textit{In Vivo} Selection and Evolution

\textit{Tissue-Specific Transduction}

A number of variants selected \textit{in vitro} have exhibited correspondingly promising properties \textit{in vivo}. However, in numerous situations cell culture models cannot adequately emulate the properties of some complex tissues, such as those with complex physical and cellular barriers that can impede viral gene delivery, delicate cells that cannot be cultured, or situations in which targeted delivery to one cell type within a heterogeneous tissue is desired. In one of the first examples of \textit{in vivo} selection, Michelfelder \textit{et al.} isolated variants from an AAV peptide insertion library for the potential to transduce either the lung or a tumor cell graft.\[^{60}\] The resulting clones mediated much higher gene expression in the target tissue; however, isolated vectors also transduced heart tissue (both tumor and lung variants) and other tissues (lung variants),\[^{60}\] indicating that targeted \textit{in vivo} delivery can be challenging.

Similarly, Yang \textit{et al.} isolated a chimeric variant composed of AAV1, 6, 7, and 8 through \textit{in vivo} biopanning for variants that infect muscle. Compared to AAV9 (the most efficient muscle-transducing serotype), the variant showed nearly equal cardiac
infectivity and significantly decreased localization to the liver.\textsuperscript{[33]} Interestingly, analysis of the variant’s \textit{in vitro} transduction of cardiomyocytes and \textit{in vivo} transduction after direct muscle injection showed that its increased infectivity of muscle tissue was due to more efficient crossing of tight endothelial barriers, an example where library selection provided both high infectivity of target cells and the ability to overcome a cellular barrier within the tissue.\textsuperscript{[33]}

\textbf{Crossing Physical and Cellular Barriers}

Cellular and extracellular matrix barriers significantly impede the transport of macromolecules to target sites within tissues,\textsuperscript{[61]} and AAV can be evolved for the ability to overcome these limitations. For example, the majority of retinal diseases afflict photoreceptors (PR) and retinal pigment epithelia (RPE), cells that lie deep within the retina. Numerous AAV serotypes can infect PR and RPE when administered subretinally (injected underneath the retina), which contributed to success in three recent clinical trials for Leber’s congenital amaurosis.\textsuperscript{[18-20]} However, unlike LCA, in the majority of retinal diseases the retina undergoes comparatively rapid cell death and tissue degeneration and can be further damaged by the retinal detachment that accompanies subretinal injection. This concern motivates the need for gene delivery approaches that can transduce or otherwise rescue PRs upon non-invasive administration to the vitreous humor of the eye.

One type of retinal cell, the Müller glia, spans the entire length of the retina and contacts all retinal neuronal cell types. Klimczak \textit{et al.} tested wither AAV variants previously selected for the ability to infect central nervous system (CNS) glia were capable of Müller cell transduction.\textsuperscript{[62]} \textit{In vivo} analysis revealed an AAV6-based mutant (ShH10) capable of highly specific (94\%) and efficient infection of Müller cells compared to AAV2 and AAV6.\textsuperscript{[62]} In subsequent work using a rat model of retinitis pigmentosa, infection of Müller glia with ShH10 encoding glial-derived neurotrophic factor (GDNF) slowed the progression of retinal degeneration and enhanced retinal electrophysiological responses for five months.\textsuperscript{[63]}

While using Müller cells to secrete factors within the retina is a useful strategy, in other situations – such as dominant disorders directly affecting the outer retina – it would be advantageous to directly transduce photoreceptors. Dalkara \textit{et al.} used a randomly mutagenized AAV2 \textit{cap} library, a library of chimeric AAV \textit{cap} genes from serotypes 1, 2, and 4-9, and a library with randomized peptides inserted near the three-fold axis of symmetry on the AAV2 capsid to evolve variants capable of transducing PRs from the vitreous humor of the eye.\textsuperscript{[64]} A resulting variant mediated strong, pan-retinal expression in the photoreceptors with both ubiquitous and photoreceptor-specific promoters,\textsuperscript{[64]} work with implications for the treatment of a range of retinal degenerative diseases.

Recent promising clinical studies have investigated CNS gene therapy to treat Canavan’s disease\textsuperscript{[46,65]} and Parkinson’s Disease.\textsuperscript{[66]} However, in some situations the target cells are surgically inaccessible, delicate, or span large regions of a tissue that would require multiple direct vector injections. Gray \textit{et al.} selected a library of shuffled \textit{cap} genes from wild-type AAV serotypes 1-6, 8, 9, and AAV8 with an E531K mutation for the ability to gain access to regions of the brain in which seizure had compromised the blood-brain barrier.\textsuperscript{[67]} Two clones composed primarily of AAV1, 8, and 9 could transduce either the piriform cortex or both the piriform cortex and ventral hippocampus upon tail vein administration of the vectors after induction of CNS seizure,\textsuperscript{[67]} and no
transduction occurred in brain areas where the blood-brain barrier was not compromised. Within the targeted brain areas, the variants efficiently transduced oligodendrocytes and neurons, but not astrocytes or microglia, demonstrating a tropism consistent with that of AAV8. This approach highlights the potential for targeting specific regions of the CNS upon systemic administration.

**Future Directions**

Directed evolution is a powerful approach that enables relatively rapid selection and isolation of AAV variants with novel and therapeutically valuable properties. Numerous reports have now demonstrated the utility of this methodology to create AAV mutants capable of utilizing alternate cell-surface receptors for transduction, transducing specific cells and tissues in vitro and in vivo, and evading neutralizing antibodies. There are considerable additional opportunities to further improve the AAV vector repertoire and in turn, through investigation of the structure-function relationships of the resulting variants, to progressively enhance our understanding of AAV biology. For example, evolution could be employed as a forward genetic screen to identify and investigate functionally important regions of the AAV capsid. In addition, the discovery of an alternative reading frame within the cap gene encoding the assembly activating protein (AAP) potentially complicates the analysis of variants isolated from directed evolution. Mutations that are silent in the cap ORF could constitute non-synonymous, advantageous mutations in the AAP that affect viral assembly. In addition, while in vitro selections have generated vectors with properties that are useful in vivo, it is likely that increasingly complex challenges for AAV vector engineering will require in vivo selection.

Finally, as novel AAV variants enter into the therapeutic pipeline, they will likely progress closer to the clinic. Phase I and Phase I/II clinical trials involving wild-type AAV1, 2, 5, 6, and 8 serotypes have been approved by the U.S. Food and Drug Administration, and they are increasingly yielding promising results. Recently, the first clinical trial involving an engineered AAV variant established that this rationally designed AAV1/2 chimera was safe and well-tolerated for treatment of Duchenne muscular dystrophy, laying the foundation for future trials involving additional AAV variants designed to suit a given clinical objective. Engineered and evolved AAV vectors are therefore highly promising for a range of applications from the lab to the clinic.

**References**


CHAPTER 2

ENHANCING THE CLINICAL POTENTIAL OF AAV VECTORS BY CAPSID ENGINEERING TO EVADE PRE-EXISTING IMMUNITY

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Abstract

Vectors based on adeno-associated viruses have shown considerable promise in both preclinical models and increasingly in clinical trials. However, one formidable challenge is pre-existing immunity due to widespread exposure to numerous AAV variants and serotypes within the human population, which affect efficacy of clinical trials due to the accompanying high levels of anti-capsid neutralizing antibodies. Transient immunosuppression has promise in mitigating cellular and humoral responses induced by vector application in naïve hosts, but cannot overcome the problem that pre-existing neutralizing antibodies pose towards the goal of safe and efficient gene delivery. Shielding of AAV from antibodies, however, may be possible by covalent attachment of polymers to the viral capsid or by encapsulation of vectors inside biomaterials. In addition, there has been considerable progress in using rational mutagenesis, combinatorial libraries, and directed evolution approaches to engineer capsid variants that are not recognized by anti-AAV antibodies generally present in the human population. While additional progress must be made, such strategies, alone or in combination with immunosuppression to avoid de novo induction of antibodies, have strong potential to significantly enhance the clinical efficacy of AAV vectors.

Introduction

AAV Biology

Adeno-associated virus (AAV) is a nonpathogenic parvovirus composed of a 4.7 kb single-stranded DNA genome packaged into a nonenveloped, icosahedral capsid.[1] The viral genome contains three open reading frames (ORF) between two inverted terminal repeats (ITR), which function as the origin of replication and as the packaging signal.[1] The rep ORF encodes four nonstructural proteins that function in viral replication, transcriptional regulation, site-specific integration, and virion assembly.[1] The cap ORF encodes three structural proteins that assemble to form a 60-mer viral capsid.[1]
alternative ORF located in the same region of the genome produces the assembly-activating protein (AAP), a viral protein that localizes AAV capsid proteins to the nucleolus and functions in the capsid assembly reaction.

The capsid proteins determine the virus’ ability to interact with and infect cells, from their initial binding to various cell surface receptors, to their trafficking inside the cell, to gaining access to the nucleus. Specifically, AAV is internalized rapidly via receptor-mediated endocytosis from clathrin-coated pits. Following cellular internalization, the virion escapes from early endosomes and traffics to the perinuclear area. Evidence exists for AAV uncoating prior to viral DNA entry into the nucleus, as well as viral trafficking into the nucleus prior to uncoating. In either case, once AAV localizes to the nucleus, second-strand synthesis – i.e. conversion of its single-stranded genome into double-stranded, transcriptionally active DNA – must occur for viral gene expression.

In the absence of helper virus co-infection, AAV enters a latent life-cycle with viral genomes integrated in human chromosome 19 or non-integrated as extrachromosomal episomes.

Recombinant versions of AAV can be created in which a gene of interest is inserted between the ITRs, and the ORFs for structural and non-structural proteins are supplied separately. This system allows the gene of interest to be packaged inside the viral capsid and delivered to the cell. Dividing as well as non-dividing cells are transduced, and gene expression – which occurs in the absence of helper virus function – is stable for years in post-mitotic tissue. There are several naturally occurring variants and serotypes of AAV, each of which differs in amino acid sequence, in particular in the hypervariable regions of the capsid proteins, and thus in their gene delivery properties. Importantly, no AAV has been associated with any human disease, making it a desirable gene delivery vector for clinical applications.

Clinical Trials Involving AAV

AAV has been employed with promising results in a number of clinical trials. During a Phase I dose-escalation trial for gene therapy of Leber’s congenital amaurosis (LCA), for example, all 12 patients who received a subretinal injection of AAV2 encoding a protein required for the isomerohydrolase activity of retinal pigment epithelium showed sustained improvement in both subjective and objective measures of vision. Furthermore, there were no significant adverse events during either the pre-trial efficacy studies or the Phase I trials. As another example, in a Phase I study for gene therapy of Canavan disease, ten patients received intracranial infusions of AAV2 encoding human aspartoacylase. Of importance with respect to vector reapplication, seven out of ten patients in this trial had low or undetectable levels of neutralizing antibodies to AAV2 following administration of the gene therapy vector.

In contrast to these clinical studies, which targeted immune privileged sites, anti-AAV host responses most likely mediated by cytotoxic T cells limited the therapeutic efficacy of rAAV vectors following intrahepatic and intramuscular administration. Briefly, a Phase I/II dose-escalation study in which patients received a hepatic artery infusion of AAV2 encoding human Factor IX initially achieved therapeutic levels of Factor IX expression. However, the therapeutic levels were only present for two months, and follow-up experiments concluded that T-cell-mediated immunity to the AAV capsid antigens induced the destruction of AAV2-transduced hepatocytes.
Furthermore, all subjects had a several log increase in neutralizing antibody titer following vector administration.\textsuperscript{13} Similarly, in a gene therapy study on lipoprotein lipase (LPL) deficiency, all eight patients achieved decreased median triglyceride levels and increased local LPL protein levels and activity twelve weeks post-administration of the AAV1 vector encoding LPI\textsuperscript{S447X}.\textsuperscript{14} But, also in this trial, triglyceride levels returned to baseline levels eighteen to thirty-one months post-administration.\textsuperscript{14}

**Immune Responses Towards AAV**

Viruses are, in general, recognized by the innate immune system and induce the production of inflammatory cytokines, chemokines and/or interferons that foster adaptive immune responses. The latter mainly comprises induction of antibodies produced by B lymphocytes following presentation of viral antigens by antigen-presenting cells, as well as direct cell killing mediated by cytotoxic T cells.\textsuperscript{1}

Knowledge on the mechanisms that lead to recognition and elimination of AAV in particular is still limited. Only recently, Zhu and colleagues provided evidence that AAV’s genome is recognized by the innate immune system through the Toll-like receptor 9-MyD88 pathway.\textsuperscript{15} In this study, plasmacytoid dendritic cells (pDCs) were identified as sentinel cells, and activation of the Toll-like receptor 9-MyD88 pathway was shown to elicit humoral and cytotoxic T-lymphocyte immune responses in mice.

Humans become naturally infected by numerous AAV serotypes, and in particular AAV2, during childhood, as indicated by sero-conversion. As a result of this exposure, memory B and eventually memory T cells are induced. The latter are assumed to be the cause for the lack of long-term gene expression and loss of therapeutic efficacy in the clinical trials on hemophilia and on LPL mentioned above. Currently, it is not clear how this response is launched, and consequently strategies for its avoidance are lacking. One hypothesis is that memory T cells were re-activated upon vector administration through presentation of capsid fragments following intracellular degradation of incoming capsids. Alternatively, contamination of the gene therapy vector preparations with cap DNA impurities could result in persistent expression of capsid antigens and thus the destruction of transduced cells, though a detailed analysis was unable to find cap DNA sequences in the vector preparation used for the hemophilia trial.\textsuperscript{16} Should this prove to be a problem, microRNA approaches or an oversized cap ORF could be employed to avoid de novo capsid production and consequently MHC loading following AAV vector application.\textsuperscript{17,18} Employing these strategies may help to reduce the cytotoxic immune response to successfully transduced cells. Furthermore, immunosuppression is exploited as a strategy to avoid induction of T as well as of B cell responses.\textsuperscript{19} An as of yet even greater challenge is the high prevalence of pre-existing neutralizing antibodies that significantly hamper the efficacy of de novo cell transduction.

Natural exposure to AAV serotypes 1, 2, 5, 6, 8, and 9 results mainly in production of IgG1, but low amounts of IgG2, IgG3, and IgG4 antibodies are also produced.\textsuperscript{20} With respect to the total anti-AAV IgG prevalence for each serotype, a recent, comprehensive analysis revealed that AAV2 (72%) and AAV1 (67%) antibodies were the most common, but AAV9 (47%), AAV6 (46%), AAV5 (40%), and AAV8 (38%) antibodies were also present in a large portion of the population studied.\textsuperscript{20} Furthermore, these anti-AAV antibodies may cross-react with other related AAV serotypes. Differences in antibody
prevalence in the human population are likely due to human genetic variation and/or frequency of exposure to the various serotypes.

Animal studies on the mechanisms of anti-AAV antibody induction using class I-deficient (unable to mount a cellular immune response) and class II-deficient (unable to mount a humoral response) mice found that the latter could readily express high levels of transgenes upon a second administration of the vector.\[^{21}\] In addition, using several types of immunodeficient mice (RAG1 knockout, CD40 ligand knock-out, and nude), Chirmule \textit{et al.} showed that the AAV neutralizing antibody response is T-cell dependent.\[^{22}\] Viral capsid antigens are presented to B cells in the lymph nodes, resulting in CD4\(^+\) T cell activation.\[^{23}\] The B cells then differentiate into plasma cells, which produce antibodies against the viral capsid proteins.\[^{23}\] The route of administration has been shown to also have an impact on the induction of immune responses. Rhesus macaques receiving wild-type AAV via intramuscular or intravenous injection developed a humoral immune response to the AAV capsid, even without helper virus co-infection, while animals that received vectors via the intranasal route did not develop an immune response unless co-infected with a helper virus.\[^{24}\] Analogous results on the role of route of administration have been found in mice. Ge \textit{et al.} found that intramuscular vector administration was more effective at inducing a humoral immune response than intraportal vein delivery.\[^{25}\] Nevertheless, independent of the initial administration route, re-administration of AAV vectors via the tail vein did not lead to transgene expression.\[^{25}\]

Recently, an AAV variant derived from rhesus macaques (AAVrh32.33) was found to elicit an immune response in mice similar to that against other AAV vectors seen in primate species.\[^{26}\] Specifically, this AAV generates a strong CD8\(^+\) T-cell response to the AAV capsid and to the delivered transgene in mice, and its use may thus provide a more accurate murine model of AAV immune activation, and help to better explain the loss of transgene expression in human gene therapy clinical trials. In parallel to developing a deeper mechanistic understanding of anti-vector immune responses, for AAV to effectively function in clinical gene therapy, strategies must be developed to create vectors that evade the body’s immune response.

\section*{Strategies to Avoid Neutralization by Humoral anti-AAV Immune Responses}

\subsection*{Transient Immunosuppression}

In the naïve host, humoral immune responses are elicited upon AAV vector application. In order to circumvent induction of this response, transient immunosuppression has been exploited. This strategy has been tested in preclinical animal models, using antibodies or small molecule inhibitors against T cell functionality. Manning \textit{et al.} reported that successful vector re-administration was achieved in 60\% of mice treated with anti-CD40 antibodies during the first vector administration.\[^{21}\] However, the mice developed neutralizing antibodies to AAV following the second administration of the vectors, which was conducted without immunosuppression.\[^{21}\] Another study by Halbert \textit{et al.} attempted transient immunosuppression using MR1 (a monoclonal anti-CD40 ligand antibody) and CTLA4Ig (a CTLA4-immunoglobulin fusion protein) alone or in combination.\[^{27}\] Mice that received only MR1 or CTLA4Ig were capable of expressing the delivered transgene, but at a lower level than the combination treatment in which the animals also developed low to undetectable levels of
neutralizing antibodies to AAV. Also in these animals, neutralizing antibodies were elicited upon re-administration.\cite{27}

In response to the above mentioned clinical trial that reported destruction of AAV2-FIX transduced hepatocytes,\cite{13} Jiang et al. tested transient immunosuppression as a means to prevent AAV capsid-directed T-cell response against liver cells expressing the FIX transgene. Immunosuppression of rhesus macaques was achieved using a combination of mycophenolate mofetil and tacrolimus (FK506). Neither transaminitis nor expansion of memory T cells was observed in any of the animals, and consequently only the impact of immunopression on humoral, but not on anti-capsid T cell responses, could be investigated. Of the three animals receiving immunosuppression during vector administration, one developed a strong anti-AAV antibody response. Furthermore, withdrawal of the immunosuppression therapy after 6 weeks lead to a 2-log increase in neutralizing antibody titer, revealing that at least in rhesus macaques long time immunosuppression is required.\cite{19}

In summary, immune suppression is an effective strategy to mitigate the body’s immune response long enough to allow the capsid proteins to clear from the cell surface and prevent the formation of neutralizing antibodies in order to facilitate readministration of the vector. However, transient immunosuppression is not a solution to preexisting neutralizing antibodies to the AAV capsid. Modifications, either chemical modifications that protect surface exposed parts of the protein capsid or genetic modifications that result in changes to the protein capsid, must be made to the vector to evade these neutralizing antibodies.

**Chemical Modification of AAV Capsid to Avoid Antibody Neutralization**

One approach to reduce antibody neutralization of vector particles is to graft chemical moieties onto the virion surface to shield neutralizing epitopes, as recently reviewed.\cite{28} For example, crosslinking synthetic polymers onto the vector can reduce neutralization by anti-virus antibodies as well as enable evasion of innate immune responses, thereby enhancing gene transfer in the presence of existing antibodies and facilitating repeated administration by reducing subsequent adaptive immune responses to the vector.\cite{29} Polymeric materials that have been explored in conjunction with adenoviral and adeno-associated viral vectors have included polyethylene glycol (PEG),\cite{30-39} poly-N-(2-hydroxypropyl) methacrylamide (poly-HPMA),\cite{40,41} polysaccharides,\cite{42} and others.\cite{43,44} They are typically covalently conjugated through the reaction of active groups on the polymer termini to nucleophilic amino acid side chains on the viral surface, such as lysines and cysteines.

PEG is a non-toxic material known for its capacity to resist protein binding – likely through steric hindrance and blocking of protein surface charges – and it has been extensively conjugated to proteins to extend their circulatory half-life and reduce immune responses.\cite{45-48} Likewise, PEGylation has been utilized to protect viral vectors from neutralizing antibodies,\cite{30-34,36,38,49} enable vector retargeting,\cite{37} and enhance vector stability.\cite{35} Several studies have PEGylated AAV, using several different crosslinking chemistries. In one report, high molecular weight PEG conjugated to AAV using a terminal N-hydroxysuccinimidyl ester to attach the polymer to viral surface lysines modestly protected AAV from neutralizing serum in culture (2.3-fold) at intermediate levels of PEG/virus. However, above a key stoichiometric ratio of PEG/AAV, viral
infectivity was lost for both high and low molecular weight PEG, presumably due to a loss of key lysine residues and/or steric hindrance of viral surface regions critical for viral infectivity. This loss of infectivity was accompanied by alterations of AAV particles that could be visualized by electron microscopy (i.e., virion shape and size). Another study, which used different PEGylation chemistries, found more positive results. Specifically, AAV reacted with PEG using succinimidyl succinate chemistry (SSPEG) was partially protected from antibodies. In contrast, AAV coated with PEG via tresyl chloride reactive groups (TMPEG) was more effectively protected from neutralizing antibodies both in vitro and in vivo. Over time, hydrolysis of the SSPEG linkages may progressively unmask antigen binding sites, rendering this conjugation less effective.

Other human viruses also face challenges with pre-existing immunity, and chemical conjugation has, for example, been more extensively explored to shield adenoviral vectors from serum, including both PEG and other polymers such as poly-HPMA and other polymers such as poly-HPMA. In one study, adenoviral vectors were coated with a random copolymer containing N-(2-hydroxypropyl)methacrylamide and methacryloyl-Gly-Gly-4-nitrophenoxy (pHPMA-ONp), via reaction with adenoviral surface amino groups. A binding assay involving ELISA measurement of free antibodies found that even at high excess, coated virus did not deplete anti-viral antibodies from solution, indicating the polymer shielded the adenovirus from the antibodies. Furthermore, since polymer coatings can block regions responsible for natural viral tropism, this modification afforded the further opportunity to retarget the virus via addition of Fibroblast Growth Factor (FGF)-2 or Vascular Endothelial Growth Factor (VEGF) to the polymer. Adenoviral vectors coated with the polymer plus FGF-2 were 10-fold more resistant to antibody neutralization in vitro. In addition, the vectors exhibited selective delivery to cell lines expressing receptors for FGF-2 or VEGF, depending on the ligand attached to the particle surface.

In addition to direct polymer grafting, an alternate strategy for protecting vectors from serum is to encapsulate them inside polymeric gels or particles designed to progressively degrade and release the virus. In early work, Beer et al. encapsulated adenovirus in poly-lactic glycolic acid using a double emulsion technique. They found that the encapsulated virus retained 27–50% and 62–65% infectivity in culture at 1:100 and 1:500 dilutions of rat serum, respectively, while non-modified vectors retained less than 1% of infectivity at both dilutions. In addition, they assessed the development of neutralizing antibodies in naïve mice upon administration of protected vector and found that animals receiving encapsulated adenovirus did not develop anti-adenovirus antibodies until after the third dose, and the resulting titers were 45-fold lower than mice receiving unmodified adenovirus. In another report, adenovirus was encapsulated in microspheres generated from alginate, a linear copolymer of anionic saccharides isolated from brown algae. Upon intranasal or intraperitoneal administration of virus-loaded microspheres, high level LacZ expression was observed in numerous organs in mice (including spleen, liver, lung, kidney, and lymph node) and was not significantly inhibited by the presence of neutralizing anti-adenovirus antibodies. By contrast, administration of un-encapsulated control vector was significantly reduced in animals harboring neutralizing antibodies.

In summary, direct chemical conjugation of protective polymers to AAV shows promise, though with somewhat mixed results, and future work may investigate the
application of alternate polymers and controlled release strategies that have shown promise with adenoviral and other vectors.

**Genetic Modification of AAV Capsid Proteins to Avoid Antibody Neutralization**

As suggested by the term “serotype”, AAV variants with unique serological characteristics have evolved in nature. Variations in the amino acid composition of the capsid proteins, in particular at protruding sites, can lead to low serum cross-reactivity between serotypes. For example, mice immunized with AAV2 show only a slight reduction in transduction efficacy if subsequently injected with AAV1 (which differs from AAV2 at 16.3% of the capsid amino acids), while anti-AAV2 antibodies impair re-application of AAV2. In principle, such findings raise the idea that one could harness natural AAV diversity to overcome problems of pre-existing immunity. That is, identification of one or more serotypes to which specific patients have not previously been exposed could allow recombinant vector administration or even re-administration. This idea suffers from several problems, however. First, such personalized therapy poses practical and regulatory concerns. In addition, capsid variability among serotypes affects not only antigenicity but also tropism, so that different serotypes could not be readily alternated. Furthermore, the patterns of pre-existing AAV immunity within the human population are complex. As already mentioned, sera are frequently found to neutralize more than one serotype, and for example in > 93% of sera positive for AAV2, neutralizing antibodies towards AAV1 are additionally present. Interestingly, antibodies against these two serotypes were also detectable in sera positive for AAV5, 6, 8, or 9. However, the observation that different serotypes exhibit different extents of cross-reactivity raises the idea that a given capsid can be engineered or mutated to retain the natural tropism of, yet evade pre-existing antibodies directed against, its parental serotype. Both rational design and directed evolution approaches have been pursued towards this goal.

**Peptide Scanning for Immunogenic Epitopes**

The rational design of AAV variants that evade pre-existing humoral immunity necessitates basic knowledge of immunogenic epitopes. In an early study, Moskalenko et al. utilized peptide scanning to map neutralizing epitopes for antibodies present in human serum samples and found a total of six linear epitopes that are targets of neutralizing antibodies (Table 1). Wobus and colleagues mapped additional linear and conformational immunogenic epitopes neutralized by mouse monoclonal antibodies. Linear epitopes in the VP1 unique region, in VP1/VP2, and at the C'-terminus of the VP3 region are recognized by antibodies A1, A69, and B1, respectively (Table 1), while conformational epitopes were bound by C24-B, C37-B, D3, and A20 (Table 2). In order to map the latter, Wobus and colleagues used peptide insertion mutants of AAV2 that displayed an integrin binding ligand, L14, at defined and surface exposed positions of the capsid.
additional sequences likely contribute to the immunogenic epitope\textsuperscript{[57]}

Table 1. Immunogenic Sites of the AAV2 Capsid Mapped by Peptide Scan

<table>
<thead>
<tr>
<th>Amino Acid Position</th>
<th>Localization</th>
<th>Sera</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E\textsubscript{17}GIRQWWKLKPG</td>
<td>VP1</td>
<td>polyclonal human</td>
<td>[56]</td>
</tr>
<tr>
<td>K\textsubscript{123}RVLEPLGL</td>
<td>VP1</td>
<td>A1</td>
<td>[57]</td>
</tr>
<tr>
<td>N\textsubscript{113}LGRAVFQAKKR</td>
<td>VP1</td>
<td>polyclonal human</td>
<td>[56]</td>
</tr>
<tr>
<td>L\textsubscript{171}NFGQTGADSV</td>
<td>VP1/VP2</td>
<td>A69</td>
<td>[57]</td>
</tr>
<tr>
<td>K\textsubscript{321}EVT\textsuperscript{#}</td>
<td>VP3 region</td>
<td>polyclonal human</td>
<td>[56]</td>
</tr>
<tr>
<td>T\textsubscript{337}STV</td>
<td>VP3 region</td>
<td>polyclonal human</td>
<td>[56]</td>
</tr>
<tr>
<td>V\textsubscript{369}FMVPQYGYL (main contribution)</td>
<td>VP3 region</td>
<td>A20</td>
<td>[57]</td>
</tr>
<tr>
<td>H\textsubscript{381}YFGYSTPWG (minor contribution)</td>
<td>VP3 region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R\textsubscript{S56}TTNPVAT573EQ (minor contribution)</td>
<td>VP3 region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q\textsubscript{477}SRNWLPGPCY</td>
<td>VP3 region</td>
<td>polyclonal human</td>
<td>[56]</td>
</tr>
<tr>
<td>S\textsubscript{479}RNWLPGPCY\textsuperscript{##}</td>
<td>VP3 region</td>
<td>D3</td>
<td>[57]</td>
</tr>
<tr>
<td>S\textsubscript{503}ADNNNSEYSWT (main contribution)</td>
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<td>C37-B</td>
<td>[57]</td>
</tr>
<tr>
<td>L\textsubscript{601}PGMVWQDRD (minor contribution)</td>
<td>VP3 region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I\textsubscript{726}GTRYLTR</td>
<td>VP3 region C’-terminus</td>
<td>B1</td>
<td>[57]</td>
</tr>
</tbody>
</table>

\textsuperscript{#} additional sequences likely contribute to the immunogenic epitope\textsuperscript{[57]}

\textsuperscript{##} described as part of a conformational epitope\textsuperscript{[56]}

The same mutants were subsequently screened for their immune escape phenotype by Huttner and colleagues using a panel of human polyclonal sera.\textsuperscript{[59]} 42\% of the sera showed a significant reduction (~31\%) in antibody binding affinity when the capsid position 534 or 573 were subjected to peptide insertion, while 21\% of the sera additionally showed a reduced binding affinity for the other six mutants (Table 2). These findings for polyclonal human sera agreed with the results of Wobus and colleagues obtained with mouse monoclonal antibodies and identified positions 534 and 573 as major antigenic determinants in humans.\textsuperscript{[57]} Peptide insertion not only impacts antibody binding affinity, but also the transduction ability. Further investigations by Huttner and colleagues revealed that the peptide insertion mutant I-587, which displayed the L14 peptide at position 587 as ligand for targeting B16F10 cells, transduced this cell line despite the presence of neutralizing antibodies. Exchanging the peptide for a 7\textsuperscript{mer} peptide targeting Mec1 cells\textsuperscript{[60]} yielded similar results, demonstrating that insertion of peptides at 587 can modulate both cell tropism and antibody neutralization.\textsuperscript{[59]}

25
<table>
<thead>
<tr>
<th>Amino Acid Position</th>
<th>Localization</th>
<th>Sera</th>
<th>Detection Method</th>
<th>Reference</th>
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<tr>
<td>E12</td>
<td>VP1 unique region</td>
<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
<tr>
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<td>VP1 unique region</td>
<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
<tr>
<td>A117</td>
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<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
<tr>
<td>A152</td>
<td>VP1/VP2</td>
<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
<tr>
<td>D180</td>
<td>VP1/VP2</td>
<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
<tr>
<td>K258</td>
<td>VP3 region</td>
<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
<tr>
<td>S261-S262</td>
<td>VP3 region</td>
<td>A20 C37-B D3 (*)</td>
<td>peptide insertion</td>
<td>[57], [59]</td>
</tr>
<tr>
<td>Q263</td>
<td>VP3 region</td>
<td>A20</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>S264</td>
<td>VP3 region</td>
<td>A20</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>G265</td>
<td>VP3 region</td>
<td>IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>D269</td>
<td>VP3 region</td>
<td>IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>N381-N382</td>
<td>VP3 region</td>
<td>A20 D3 (*)</td>
<td>peptide insertion</td>
<td>[57], [59]</td>
</tr>
<tr>
<td>S384</td>
<td>VP3 region</td>
<td>A20</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>Q385</td>
<td>VP3 region</td>
<td>A20</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>V418</td>
<td>VP3 region</td>
<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
<tr>
<td>R447-T448</td>
<td>VP3 region</td>
<td>C37-B</td>
<td>Peptide insertion</td>
<td>[59]</td>
</tr>
<tr>
<td>R459</td>
<td>VP3 region</td>
<td>polyclonal human</td>
<td>in vitro evolution</td>
<td>[62]</td>
</tr>
<tr>
<td>R471</td>
<td>VP3 region</td>
<td>polyclonal human</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>T491</td>
<td>VP3 region</td>
<td>IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>A493</td>
<td>VP3 region</td>
<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
<tr>
<td>N497</td>
<td>VP3 region</td>
<td>polyclonal human</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>S498</td>
<td>VP3 region</td>
<td>polyclonal human</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>W502</td>
<td>VP3 region</td>
<td>IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>K527</td>
<td>VP3 region</td>
<td>IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>E531</td>
<td>VP3 region</td>
<td>polyclonal human</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>K532</td>
<td>VP3 region</td>
<td>IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>F534-F535</td>
<td>VP3 region</td>
<td>A20 C24-B C37-B D3 (*)</td>
<td>peptide insertion</td>
<td>[57], [59]</td>
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</table>
Table 2. Immunogenic Sites at the AAV2 Capsid

<table>
<thead>
<tr>
<th>Patient</th>
<th>Region</th>
<th>Other</th>
<th>Method</th>
<th>References</th>
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<tbody>
<tr>
<td>K544</td>
<td>VP3 region</td>
<td>IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>E548</td>
<td>VP3 region spike region</td>
<td>A20 polyclonal human</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>T550</td>
<td>VP3 region</td>
<td>polyclonal human IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>N551</td>
<td>VP3 region three fold symmetry axis</td>
<td>polyclonal human</td>
<td>in vitro evolution</td>
<td>[62]</td>
</tr>
<tr>
<td>T567</td>
<td>VP3 region</td>
<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
<tr>
<td>T573-E574</td>
<td>VP3 region</td>
<td>A20 C24-B C37-B D3 (*) polyclonal human</td>
<td>peptide insertion</td>
<td>[57], [59]</td>
</tr>
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<td>E574</td>
<td>VP3 region</td>
<td>IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>G586</td>
<td>VP3 region</td>
<td>polyclonal human IVIG</td>
<td>rational design</td>
<td>[54]</td>
</tr>
<tr>
<td>N587</td>
<td>VP3 region three fold symmetry axis heparin binding motif</td>
<td>polyclonal rabbit polyclonal human</td>
<td>in vitro insertion rational design</td>
<td>[61], [54]</td>
</tr>
<tr>
<td>N587-R588</td>
<td>VP3 region</td>
<td>C24-B C37-B polyclonal human</td>
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<tr>
<td>N705</td>
<td>VP3 region</td>
<td>polyclonal human IVIG</td>
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<td>VP3 region</td>
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<td>[54]</td>
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<tr>
<td>V708</td>
<td>VP3 region edge of plateau</td>
<td>A20 polyclonal human IVIG</td>
<td>rational design</td>
<td>[54]</td>
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<tr>
<td>T713</td>
<td>VP3 region</td>
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<td>[61]</td>
</tr>
<tr>
<td>T716</td>
<td>VP3 region surface of the twofold dimple</td>
<td>polyclonal rabbit</td>
<td>in vitro evolution</td>
<td>[61]</td>
</tr>
</tbody>
</table>

"viral vectors, i.e. intact capsid assayed

(*) reduced binding affinity maybe due to a conformational change in the epitope caused by the peptide insertion[57]

A20 = mouse monoclonal antibody,[63] conformational epitope

C37-B = mouse monoclonal antibody,[57] conformational epitope

human sera = serum obtained from single donors

IVIG = purified human IgG prepared from thousands of blood donors

Rational Design of AAV Variants via Peptide Insertion or Site Directed Mutagenesis

Lochrie and colleagues utilized extensive site-directed mutagenesis of the AAV2 capsid to develop variants with immune escaping properties.[54] In silico structural analysis of potential docking for a murine IgG2a antibody with the AAV2 surface yielded a number of sterically accessible, candidate positions that were then subjected to point
mutagenesis. The resulting collection of mutants were assessed for antibody binding using A20 (mouse anti-AAV2 capsid antibody), human sera (three different donors), and IVIG (pooled human IgG isolated from thousands of blood donors). As may be anticipated for polyclonal antibody mixtures, in contrast to work with A20 that identified a set of variants with mutations at a specific epitope, analysis with human sera (single donors) and IVIG did not map a single epitope. At any rate, point mutations at distinct sites spread across the capsid reduced immune neutralization.

**Directed Evolution of AAV Variants via Random Mutagenesis**

In contrast to rational design based approaches, directed evolution strategies can be exploited in the absence of knowledge on the capsid biology or on immunogenic sites. This strategy is based on the generation and high-throughput selection of diverse genetic libraries to create variants with enhanced biological functions. In 2006, Maheshri et al. and Perabo et al. applied this technology to AAV to overcome the obstacle of pre-existing immunity. Both approaches used error prone mutagenesis to randomize the AAV2 capsid. Random point mutations to the AAV cap ORF are introduced using a “sloppy” polymerase chain reaction (PCR) to amplify and mutate the cap DNA sequence at a defined rate. The PCR conditions can be adjusted to tune the average number of mutations in each cap gene sequence (Figure 1a). Viral capsid mutants are then packaged by standard production protocols and screened for infectivity despite the presence of neutralizing antibodies. Progeny production is induced by helper virus co-infection and followed by a new round of selection.

**Figure 1.** Schematic of Library AAV Capsid Protein Engineering Strategies for Evasion of Antibody Neutralization. Methods for generation of highly diverse viral libraries include a) random point mutagenesis (error-prone PCR) and (b) in vitro recombination (DNA shuffling). Directed evolution strategies use these approaches as part of an iterative strategy to increase AAV’s ability to avoid antibody neutralization.

Perabo and colleagues selected in presence of human sera on a highly permissive cell line, HeLa. In contrast to selections in the presence of non-neutralizing sera, selections in
the presence of neutralizing antibodies yielded viral mutants in which 73% of point
mutations clustered in the same, surface exposed region. The most frequent selected
clones carried mutations at capsid positions 459 and 551. Introduction of selected amino
cacid substitution at position 459 and/or 551 of capsids of recombinant AAV vectors
conferred the vectors with an improved ability to evade neutralization. The highest
immune evasion was observed for the sera used to select the mutants, but with each of the
seven different sera assayed, a notable higher serum concentration was required to halve
the transduction efficacy of the mutants compared to AAV2 (up to 5.3-fold).

The viral library used by Perabo and colleagues possess on average 0.9 mutations per
clonen making it unlikely that multiple combinations of mutations are found on a single
clone. The latter, however, increases the likelihood of selecting potent immune escaping
variants. An efficient strategy to achieve this goal was exploited by Maheshri and
colleagues, who combined error prone PCR mutagenesis and DNA shuffling,[61] in their
approach to establish directed evolution strategies for AAV. Random DNA recombinatin (Figure 1b)
can be achieved using several different methods, including the staggered extension process recombination[66] (used by Maheshri et al.) and DNase I
digestion followed by fragment reassembly with DNA polymerase[67] (used by Grimm et al. and Koerber et al.). The staggered extension process (StEP) consists of repeated
cycles of denaturation, annealing, and short polymerase-catalyzed extension steps. The
abbreviated extension step results in short fragments of DNA that can then anneal to new
templates and further extend, creating genes that contain sequence information from
multiple templates. DNA shuffling uses DNase I digestion to create small fragments of
DNA similar to the fragments created in StEP. These fragments are then reassembled into
new genes through repeated annealing cycles in the presence of DNA polymerase. Both
methods can be used to create point mutations (when all cap gene templates are from the
same serotype) or to generate chimeric capsids (when cap gene templates are from
different serotypes).

The viral library produced by Maheshri and colleagues was selected for mutants that
productively infect HEK293 in the presence of a strongly neutralizing rabbit anti-AAV2
serum. Interestingly, each mutant selected in this screen contained a threonine to alanine
substitution at position 716, near the C-terminus of the common VP3 region. In order to
further optimize the selection procedure, the viral library was subjected to a further round
of mutagenesis. While transduction of AAV2 in the presence of rabbit sera was
neutralized at a 1:1,500 dilution, the most promising candidate of the second screen was
only mildly neutralized at a 1:2.35 serum dilution. More importantly, this mutant
mediated transgene expression in vivo following pre-incubation with anti-AAV serum at
levels 2-3 orders of magnitude higher than serum concentrations that neutralized
AAV2.[61]

To date, AAV libraries based on error prone PCR mutagenesis possess a diversity of
$10^7$ to $10^8$ clones.[68] However, in order to screen every possible amino acid substitution
for their immune escape properties, a diversity of $3.2 \times 10^{17}$ would be required.[68] A
possibility to overcome this technical limitation and identify the most effective amino
acid substitution for a given position, and hence improve the antibody evasion capacity of
immune escape mutants was proposed by Märsch and colleagues.[68] In this procedure,
only regions implicated as immunogenic sites are randomized, and these mutants are
subjected to high-throughput selections for viral infectivity in the presence of neutralizing
sera. In their proof-of-principle study, Märsch and colleagues focused on five immunogenic sites: positions 449, 558, 459, 551, and 493,[61,62,68] which were subjected to saturation mutagenesis. Due to this restriction, the library purportedly contained all possible amino acid substitutions for these positions (required diversity $2^5$, obtained diversity $6 \times 10^6$). From the mutants selected in the presence of human sera on HEK293 cells, six mutants were analyzed in comparison to AAV1, AAV2, and AAV2 R459K/N551D, a double mutant selected in the previous study of Perabo and colleagues.[62] Two of these mutants were significantly less neutralized by human sera from single donors and by IVIG compared to AAV2 and AAV2 R459K/N551D. In agreement with the initial hypothesis that the level of immune evasion can be improved if an optimal amino acid substitution is introduced to disrupt an immunogenic epitope, both mutants contained novel substitutions at positions 459, 493, and 551. In addition, the relatively minor sequence changes – compared for example to the 120 amino acid differences between AAV2 and AAV1 – likely did not change the mutant’s tropism relative to AAV2.

**Directed Evolution of AAV Variants via Serotype Shuffling**

Grimm and colleagues implemented an *in vitro* evolution strategy that utilized a library of chimeric capsids.[69] DNA shuffling through fragment reassembly was exploited to randomly combine *cap* sequences of eight AAV serotypes (AAV2, 4, 5, 8, and 9, and caprine (CAAV), avain (AAAV), and bovine AAV (BAAV)). The library was firstly selected for the ability to transduce human hepatoma cell lines, followed by further selection in the presence of IVIG. The result was a single mutant, AAV-DJ, a chimera of AAV2, 8, and 9 in which the majority of non-AAV2 amino acid sequences were found in immunogenic epitopes of the capsid. For in depth analysis of the immune profile of AAV-DJ, Grimm and colleagues passively immunized mice with IVIG followed by vector injection. At lower IVIG levels, AAV-DJ possessed an *in vivo* immune evasion capacity comparable to AAV8 and AAV9 as indicated by the comparable level of transgene expression, though both serotypes clearly outperformed AAV-DJ at a higher IVIG concentration. Interestingly, although re-administration of AAV-DJ was impaired, and although mice pre-immunized with AAV8 or 9 could not be transduced by AAV-DJ, AAV2, 8 and 9 were able to mediate transgene expression in AAV-DJ pre-immunized mice.

In another study involving DNA shuffling of AAV, Koerber and colleagues performed a detailed analysis of seven chimeric AAV vectors, which had been selected in the absence of antibodies in a single selection round for viability.[70] The library used for their selection contained DNA shuffling based virus chimeras of AAV1, 2, 4, 5, 6, 8, and 9, i.e. they included additional non-human primate serotypes but not more distantly related serotypes. Sequencing of candidate chimeras revealed a greater than 90% similarity to AAV2 for three of the mutants, and to AAV1 and 6 for the other four mutants. In addition, all clones contained a high proportion of sequences originating from non-AAV2 and non-AAV1/6 serotypes, respectively, in the surface exposed regions of the capsid. Of note, four of these seven mutants showed, in the absence of a selection pressure, a naturally greater resistance to neutralization by IVIG than the parental serotypes. The highest immune evasion capability was detected for a mutant with a greater than 90% similarity to AAV1/6. This mutant, cB4, was 400-fold more resistant to
neutralization by IVIG than AAV2 and is as of yet one of the most potent immune escaping AAV vectors. This work by Koerber et al. and Grimm and colleagues clearly reveal the great potential of chimeric virions. The immune evasion phenotype is, however, due to a new combination of cap sequences of different serotypes and thus requires the selection of chimeric mutants which in addition to immune evasion possess a desired tropism in order to fully exploit this strategy to improve AAV’s in vivo application.

Conclusion

Adeno-associated virus possesses several characteristics that have contributed to its growing popularity as a gene delivery vector for clinical gene therapy applications. Unfortunately, pre-existing neutralizing antibodies against the AAV capsid and humoral immune responses following vector administration have prevented AAV from reaching its full potential as a gene therapy vector. Chemical modifications that protect exposed immunogenic epitopes of the capsid and genetic modifications that mutate these epitopes have demonstrated that AAV vectors can be altered to decrease antibody neutralization, while still effectively delivering genetic material, both in vitro and in vivo. The modification methods described above can be used to isolate new AAV variants with more improved immune evasion characteristics and thus possibly stronger clinical potential.

References
CHAPTER 3

EFFECTS OF SYSTEMIC ANTIBODIES TOWARDS INTRAVITREAL GENE DELIVERY VECTORS IN MACAQUES

Abstract
Gene delivery vectors based on adeno-associated viruses (AAV) have demonstrated promise in both preclinical disease models and more recently in human clinical trials for several disease targets. Pre-existing immunity, in addition to subsequent development of immunity due to vector administration, can reduce therapeutic transgene expression from AAV gene delivery vectors. Although anti-AAV antibodies are known to be a potential obstacle to successful gene therapy in tissues accessed by intravenous delivery, antibodies are also a challenge for delivery to tissues that are often thought of as immune privileged, such as the eye. For example, intravitreal administration, the injection of AAV vectors into the vitreous of the eye, allows for broad ocular delivery, but is more susceptible to interactions with the immune system than subretinal administration. To help understand the immune response to ocular AAV gene therapy vectors, as well as the effects of systemic anti-AAV antibody levels on transgene expression in the eye, we quantified the anti-AAV antibodies present in the sera collected from non-human primates before and after intravitreal injections with various AAV capsids. Studying anti-AAV antibody development will aid in understanding the interactions between gene therapy vectors and the immune system during ocular administration and can form a basis for future clinical studies applying intravitreal gene delivery.

Introduction
Adeno-associated virus (AAV) is a nonpathogenic parvovirus composed of a 4.7 kb single-stranded DNA genome within a non-enveloped, icosahedral capsid. The genome contains three open reading frames (ORF) flanked by inverted terminal repeats (ITR) that function as the viral origin of replication and packaging signal. The rep ORF encodes four nonstructural proteins that play roles in viral replication, transcriptional regulation, site-specific integration, and virion assembly. The cap ORF encodes three structural proteins (VP1-3) that assemble to form a 60-mer viral capsid. Finally, an ORF present as an alternate reading frame within the cap gene produces the assembly-activating protein (AAP), a viral protein that localizes AAV capsid proteins to the nucleolus and functions in the capsid assembly process. In recombinant versions of AAV, a gene of interest is inserted between the ITRs in place of rep and cap, and the latter are provided in trans along with helper viral genes during vector production. The resulting vector can transduce both dividing and non-dividing cells, resulting in stable transgene expression in the absence of helper virus for years in post-mitotic tissue. There are eleven naturally occurring serotypes and over 100 variants of AAV, each of which differs in amino acid sequence, particularly within the hypervariable regions of the capsid proteins, and thus in their gene delivery properties.
As of 2013, there were over 90 completed or ongoing clinical trials that used AAV as the gene delivery vehicle. AAV has not been associated with any human disease, which makes recombinant AAV attractive for clinical applications. During Phase I clinical trials for Leber’s congenital amaurosis (LCA), over 30 patients who received a subretinal injection of AAV2 encoding a protein required for the isomerohydrolase activity of retinal pigment epithelium showed sustained improvement in both subjective and objective measurements of vision. Furthermore, there were no significant adverse events during either the pre-trial efficacy studies or the trial. These trials demonstrate the significant promise AAV possesses as a therapeutic ocular gene delivery vector for the treatment of several inherited retinal degenerative diseases, including glaucoma, macular degeneration, and retinitis pigmentosa.

One potential hurdle to the broad application of AAV ocular therapy is the current administration technique. Subretinal injection of AAV vectors allows for efficient gene expression in several retinal cell types, including photoreceptors and retinal pigment epithelial cells. During subretinal injection, AAV is delivered via a needle puncture through the neurosensory retina, and a portion of the photoreceptor layer is mechanical detached from the supporting epithelial layer. As a result, this method of injection only delivers the therapeutic vector to a specific region of the eye. This is not an optimal solution, since degeneration occurs throughout the retina in most diseases. Furthermore, the subretinal administration causes retinal detachment in the injection area, which can lead to a variety of ocular damage, including reactive gliosis, retinal disorganization, photoreceptor degeneration, and functional losses in vision. More importantly, retinas already weakened by degenerative diseases are more susceptible to the damage caused by subretinal injection. Intravitreal administration, the injection of AAV vectors into the vitreous of the eye, allows for broad delivery without the risk of retinal detachment. Recent work has developed AAV variants that are capable of specifically infecting Muller glia and photoreceptors within the retina following intravitreal administration, making intravitreal injections a viable alternative.

To date, AAV has been most successful in clinical studies involving delivery to immune privileged regions. However, evidence exists that even gene delivery to these regions may be susceptible to neutralization by antibodies. For example, the presence of extremely high titers of neutralizing antibodies in the serum can decrease transduction of AAV vectors in the rat brain or in the mouse eye following intravitreal injection. Furthermore, increases in neutralizing antibody titer were seen in the serum following subretinal injections of AAV vectors in dogs and nonhuman primates as well as some intracranial injections in several clinical trials. In addition to antibodies in serum, antibodies are also present in other bodily fluids, such as synovial fluid and vitreal fluid, and have the potential to inhibit vector transduction. These antibodies present a challenge for intravitreal administration and make this area not as immune privileged as the subretinal space. Immunosuppression is a promising approach for reducing cellular immune responses, but humoral immunity – anti-AAV antibodies resulting from childhood exposure to one or more serotypes, or from prior administration of AAV vectors – poses a significant challenge to AAV gene therapy. Recent analysis indicated that the prevalence of anti-AAV IgG antibodies in humans was highest for AAV2 (72%) and AAV1 (67%), but AAV9 (47%), AAV6 (46%), AAV5 (40%), and AAV8 (38%) antibodies were also present in a large portion of the population.
studied.[32] During the LCA clinical trial, patients were excluded “if immunological studies show presence of neutralizing antibodies to AAV2 above 1:1000.”[9] Recent clinical trials for hemophilia have used even more stringent criteria for patient exclusion, removing patients whose neutralizing antibodies were above 1:5.[33]

As part of an effort to study the role of optogenetic proteins in the non-human primate retina, serum samples were collected from non-human primates before and after injections with various AAV capsids. As a result, we have been able to use these serum samples to explore the immune response (through the presence of anti-AAV antibodies) to AAV vectors, as well as the effects of systemic anti-AAV antibody levels on transgene expression following intravitreal administration. Because the initial goal of the study was not to investigate immune responses to the gene therapy, the monkeys were not injected in the most optimal way for this study. In an effort to provide as much information as possible, AAV injection history for each monkey studied is included in the presentation of the results. Although the data generated lack the ability to display statistical significance in the findings, the trends elucidated through this study are extremely valuable in helping to understand the interactions between gene therapy vectors and the immune system during ocular administration, and it can provide insight for future clinical studies applying intravitreal gene delivery.

Materials and Methods

Cell Lines and Adeno-Associated Virus Production

HEK293T cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C and 5% CO2.

Recombinant AAV vectors were packaged using HEK293T cells using the calcium phosphate transfection method, and the viruses were purified by iodixonal gradient centrifugation.[34,35] AAV vectors for use during in vivo injections were further purified by Amicon filtration. DNase-resistant genomic titers were determined via quantitative PCR.[34,35]

Intravitreal Injections

Monkeys were anesthetized with Ketamine, and the cornea was locally anesthetized with Proparacaine drops. The palpebral fissure (eye and lids) was flushed with 50% strength betadine/saline to disinfect the injection site and then flushed out with copious amounts of sterile saline. An injection of 2-200 ul of the AAV vector was administered through a 30 gauge needle into the posterior chamber of the eye. Finally, an ophthalmic steroid ointment and atropine ointment was applied to the cornea post-injection to minimize inflammation. This procedure is conducted according to the ARVO Statement for the Use of Animals and the guidelines of the Office of Laboratory Animal Care at the University of Rochester.

Serum and Vitreal Fluid Extraction

Blood was collected at various time points pre- and post-AAV injection from a peripheral vein. Once collected, the sample was centrifuged and the serum was stored at -20°C.
In Vitro Transduction Analysis of Antibody Neutralization

HEK293T cells were plated at a density of 1.5x10^4 cells per well 24 hours prior to infection. Recombinant AAV serotypes 2, 5, 8, and 9 and AAV2 tyrosine mutant and AAV2 7mer insertion mutant variants expressing GFP under the control of the CMV promoter were incubated at 37°C for 1 hour with monkey serum, and cells were then infected at a genomic MOI of 2000. The percentage of GFP positive cells was assessed 48 hours post-infection using an ImageXpress Micro Cellular Imaging and Analysis System (Molecular Devices, Sunnyvale, CA) and MetaXpress Image Analysis Software, version 3.1.0, Multi Wavelength Cell Scoring Application Module (Molecular Devices).

Results

Increase of Anti-AAV2 Antibodies Pre-Infection vs. Post-Infection

Monkeys involved in a study to explore the role of optogenetic proteins in the non-human primate retina were injected with one or more of the following AAV gene delivery vectors: wild-type AAV2, wild-type AAV5, wild-type AAV9, AAV2 7mer insertion virus (an AAV2-based capsid containing an additional 7 amino acids inserted at position 588 on the viral capsid), and AAV2 tyrosine mutant virus (an AAV2-based capsid containing tyrosine to phenylalanine mutations at amino acid positions 272, 444, 500, 704, and 730). The type of vectors administrated are provided below. The levels of anti-AAV antibodies present in monkey serum samples were tested using an in vitro neutralization assay in which the inhibition of vector transduction of HEK 293 cells is measured following incubation with a range of serum dilutions. The neutralizing antibody titers are reported as the reciprocal of the serum dilution at which 50% of transduction is inhibited in vitro (Figure 1a). As shown in the representative images, there is no change in the level of transduction (measured as GFP positive cells) even in the most concentrated serum dilution (1:10) for a serum sample taken prior to intravitreal administration of an AAV2 7mer insertion virus, indicating that the serum does not contain a detectable level of anti-AAV2 antibodies. However, there is a 50% decrease in the amount of transduction in the 1:500 serum dilution in a serum sample taken from the same monkey after intravitreal administration of an AAV2 7mer insertion virus, indicating the presence of anti-AAV2 antibodies. There is no transduction in the 1:10 dilution, further illustrating the neutralization capacity of the anti-AAV2 antibodies found in the serum sample post-viral administration.

Serum samples were obtained before and after intravitreal injections of various AAV capsids in one or both eyes of adult macaques. Anti-AAV2 antibody titers increase following injection regardless of the capsid serotype, transgene, amount of virus, or number of eyes injected. This finding is consistent with previous reports in rodents indicating that intravitreal AAV and adenovirus delivery isn’t as immune-privileged as subretinal delivery.[36,37] However, these variables do change the magnitude of the antibody response. For example, the serotype used for delivery affects the magnitude of increase between pre- and post-injection AAV2 neutralizing antibody titers (Figure 1b – wild-type AAV2: 10-250 fold increase post-injection vs. Figure 1e – wild-type AAV5/AAV9: 2.5-50 fold increase post-injection). As expected, monkeys injected with AAV2 build a larger immune response to AAV2 than monkeys injected with other serotypes. Monkey 108 (injected with AAV5 and AAV9) has a larger anti-AAV2
response than Monkey 012 (only injected with AAV5), which could be due to the larger percentage of sequence similarity between AAV2 and AAV9 capsids, the larger amount of virus injected (since both eyes were injected), or the sequential eye injections in Monkey 108 heightening the immune response to the second injection. Another interesting point is that although the serotype injected (i.e AAV2 vs. AAV5 or AAV9) appears to change the level of antibody response mounted, small mutations to a serotype (i.e. wild-type AAV2 vs. AAV2 7mer insertion) do not affect the antibody response (Figure 1b – wild-type AAV2: 10-250 fold increase post-injection vs. Figure 1c – AAV2 7mer insertion capsid: 20-500 fold increase post-injection vs. Figure 1d – double injection of wild-type AAV2 + AAV2 tyrosine mutant capsid or AAV2 7mer insertion capsid: 50-200 fold increase post-injection). This is most likely because these changes do not make the mutant capsid different enough from wild-type AAV2 to disrupt the antibody binding sites on the capsid, so antibodies against a mutant will react to the other mutants or the wild-type capsid in the same way (Figure 2). To further support this finding, wild-type AAV2, an AAV2 tyrosine mutant capsid, and an AAV2 7mer insertion capsid were tested against sera from monkeys that had been exposed to various serotypes and variants (Figure 2 a-c) and human intravenous immunoglobulin (Figure 2d). Although the AAV2 tyrosine mutant capsid was slightly more infectious than wild-type AAV2 at the 1:2500 and 1:5000 serum dilutions, it was still neutralized (less than 50% transduction) at the same dilution as the AAV2 7mer insertion capsid and wild-type AAV2.

Comparing Monkeys 002, 109, 110, 906 and 909 to Monkeys 014, 736, and 902, injecting into one eye or both eyes (as long as both eyes are injected at the same time or within a short period of time) resulted in similar magnitudes of antibody response (Figure 1f – single eye: 10-500 fold increase post-injection vs. Figure 1g – double eye: 50-250 fold increase post-injection). However, for the seven monkeys injected with wild-type AAV2 and/or an AAV2 mutant, the variable that seems to most affect the production of anti-AAV2 antibodies is the amount of virus delivered (Figure 1h – total virus > 2x10^{12} vg: 200-500 fold increase vs. Figure 1i – total virus < 2x10^{12} vg: 10-50 fold increase). For example, Monkey 902 (50-fold increase post-injection) had less total virus injected into two eyes than Monkey 109 had into only one eye (500-fold increase post-injection).
Figure 1: Increase of Anti-AAV2 Antibodies Pre-Transduction vs. Post-Transduction. (a) Representative sample of fluorescent images taken during in vitro neutralization assay and used to determine the amount of viral transduction in the presence of serum dilutions. Top row: conditions of no serum, 1:500 serum dilution, and 1:10 serum dilution from a monkey before injection of a high dose (> 2x10^{12} viral genomes) of AAV2 7mer insertion viruses expressing GFP. Bottom row: same conditions following the injection. (b)-(i) Neutralizing antibody titers in serum pre- and post-injection of monkeys administered (b) Wild-type AAV2 capsid, (c) AAV2 7mer insertion capsid, (d) Mutant (tyrosine mutation or 7mer insertion) AAV2 and wild-type AAV2 capsids, (e) Alternative wild-type AAV capsids, (f) Single eye injections of AAV2 7mer insertion or wild-type AAV2 capsids, (g) Double eye injections of mutant (tyrosine mutation or 7mer insertion) AAV2 or wild-type AAV2 capsids, (h) Injection of high viral titers (> 2x10^{12} viral genomes), or (i) Injection of low viral titers (< 2x10^{12} viral genomes). Error bars indicate standard deviation (n=3). y-axis: reciprocal of the serum dilution at which 50% of viral transduction is inhibited in vitro. vg = viral genomes
Figure 2: *In Vitro* Antibody Evasion of AAV2 Variants. Antibody neutralization curves for wild-type AAV2, AAV2 tyrosine mutant, and AAV2 7mer insertion viruses in the presence of sera from monkeys that had been exposed to (a) AAV2 and AAV9 tyrosine mutant capsids, (b) Wild-type AAV5 and AAV9 capsids, (c) Wild-type AAV2 and AAV2 tyrosine mutant capsids, or (d) Human IVIG. Error bars indicate standard deviation (n=3).

Comparison of AAV2 Neutralization by Serum vs. Vitreous Fluid

Although the ideal bodily fluid to study to determine the presence of antibodies that may neutralize intraocular administrations of AAV vectors is the vitreous fluid, obtaining samples of this fluid is challenging. First, the process of removing vitreous fluid in a clinical setting can lead to a number of complications, including retinal detachment, high intraocular pressure, intraocular hemorrhaging, or cataracts. These complications are even more undesirable in patients whose retinas are already weakened by the degenerative diseases that are being treated. Secondly, it is difficult to obtain a large volume of vitreous fluid that could be analyzed for antibodies. Serum can be more easily obtained with fewer complications, making it a more desirable bodily fluid to evaluate. In order to determine whether anti-AAV antibody titers in the serum are predictive of antibody titers in the vitreous fluid, nine monkeys with previous intravitreal AAV administrations had matching serum and vitreous fluid samples extracted at the time of euthanasia.

For all nine monkeys, the anti-AAV2 antibody titers of the vitreous fluid from the right and left eyes were equal (Figure 3). This occurred even though monkeys were
injected with various combinations of AAV serotypes and AAV2-based 7mer insertion or tyrosine mutant vectors. Furthermore, even when one eye received a much larger amount of virus (as is the case with Monkey 108 and Monkey 622), the neutralizing titers in both eyes at the later time were equal. For over half of the samples tested, vitreous fluid and serum samples had the same level of AAV2 neutralization (Figure 3). For the other samples, anti-AAV2 titer in the serum was within an order of magnitude of the anti-AAV2 titer in the vitreous fluid (Figure 3). In general, the level of anti-AAV antibodies in the serum is predictive of anti-AAV antibody levels in the vitreal fluid, making this bodily fluid a good representation of both the systemic and the local ocular antibody titer.

**Figure 3:** Presence of Anti-AAV2 Antibodies in Matched Serum and Vitreal Fluid Samples. Neutralizing antibody titers against AAV2 in serum and vitreous fluid samples taken from both the right and left eyes post-administration of mutant (tyrosine mutation or 7mer insertion) AAV2 and wild-type AAV2 capsids (014, 505, 739, and 902), wild-type AAV5 capsids (001 and 622), wild-type AAV2 and wild-type AAV5 capsids (003), mutant AAV8 and wild-type AAV5 capsids (003A), or wild-type AAV5 and wild-type AAV9 capsids (108). Error bars indicate standard deviation (n=3).

**Presence of Anti-AAV2 Antibodies During Long Term Monitoring**

For a few monkeys, several serum samples that allowed monitoring of anti-AAV2 antibodies over a period of at least 7 months were available (Figure 4a). All injected monkeys showed consistent antibody titers for several months post-injection, but most seemed to experience a slight (2-5 fold) decrease in neutralizing antibody titer as early as 3 months post-injection. Monkey 013 had several pre-injection samples available with consistently low (or undetectable) antibody titers for about one year. Serum samples that allowed monitoring of Monkey 708 for two years were available (Figure 4b). The data from Monkey 708 show the same trend as the other long-term data from Monkeys 002, 012, 013, 014, and 902 and demonstrate that antibodies can persist for very long periods after gene therapy administration.
Figure 4: Presence of Anti-AAV2 Antibodies During Long Term Monitoring. (a) Presence of anti-AAV2 antibodies in the serum of several monkeys at various time points over 16 months. (b) Presence of anti-AAV2 antibodies in the serum of Monkey 708 at various time points over 2 years. Arrows indicate AAV administration to one or both eyes.

Cross-Reactivity of Antibodies to Various AAV Serotypes Pre-Infection vs. Post-Infection

Several rodent models have reported successful second administrations of AAV gene delivery vectors when different serotypes are used for the first and second administrations,[39-41] due at least in part to a lack of cross-reactivity of antibodies raised in rodents against AAV serotypes. However, these data have not been expanded to primate models of vector administration. Serum samples were evaluated for cross-reactivity of anti-AAV antibodies developed following vector administration. Consistent with the observation that anti-AAV2 antibody titers increase following injection of other serotypes, anti-AAV5, anti-AAV8, and anti-AAV9 antibody titers also increase following injection, regardless of the serotype administered (Figure 5). Once again, administration of AAV2 tyrosine mutant capsids or AAV2 7mer insertion capsids does not change the levels of antibodies developed against AAV5, AAV8, or AAV9 compared to the levels of antibodies developed against those serotypes by administration of wild-type AAV2 alone (Figure 5a-d). It also appears that high initial neutralizing titers result in a smaller magnitude of change between pre- and post-injection titers (Figure 5d, f, g). This could
be due to a large amount of neutralization of the viral vectors before they infect any cells (so less of an antibody response is mounted against them), a limitation of the assay (the lowest dilution measured was 1:5000, but some samples may have been neutralized at an even lower dilutions), or a saturation of the immune response. In 9 of 11 monkeys tested, initial antibody titers against AAV8 and AAV9 were higher than antibody titers against AAV2, most likely due to the high prevalence of natural AAV8 and AAV9 infections in rhesus macaques.\textsuperscript{42,43} These capsids have the highest sequence similarity of any of the four capsids studied,\textsuperscript{44} and showed consistent magnitudes of antibody induction in most monkeys. Furthermore, AAV5 has the most distinct sequence of the capsids (i.e. 55% amino acid similarity to AAV2),\textsuperscript{45} and consequently had the least predictable antibody response in monkeys administered other serotypes. As expected from the observation that administration of high vector doses lead to larger anti-AAV2 antibody responses (Figure 1h, i), high doses of AAV2 viral capsids also induce greater anti-AAV5, anti-AAV8, and anti-AAV9 antibody responses (Figure 6b, c). For example, Monkeys 109 and 110 were injected with the same AAV2 7mer insertion capsid into one eye. Monkey 109 received an approximately 10-fold higher dose of viral genomes, and developed an antibody response approximately 1-2 orders of magnitude higher towards all serotypes tested compared to Monkey 110.
**Figure 5:** Cross-Reactivity of Antibodies to Various AAV Serotypes Pre-Infection vs. Post-Infection. Neutralizing antibody titers against AAV2, AAV5, AAV8, and AAV9 in serum pre- and post-injection of monkeys administered (a) Wild-type AAV2 capsid, (b)-(d) Mutant (tyrosine mutation or 7mer insertion) AAV2 and wild-type AAV2 capsids, (e) Wild-type AAV5 capsid, (f) Wild-type AAV2 and AAV5 capsids, (g) Wild-type AAV5 and AAV9 capsids, or (h) AAV2 and AAV9 tyrosine mutant capsids. Error bars indicate standard deviation (n=3).

**Increase of Anti-AAV Antibodies Throughout Sequential Administrations**

Due to changes in the injection protocol that required monkeys receiving injections in both eyes to recover vision in the first eye prior to injection in the second eye, serum samples were available at multiple time points for three monkeys that had received sequential intravitreal injections of AAV vectors. Monkey 002 had a 10-fold increase in anti-AAV2 antibody titer following injection of AAV2, then a subsequent 2.5-fold increase in anti-AAV2 antibody titer following injection of AAV5 (Figure 6a), once again demonstrating the difference in anti-AAV2 antibody response resulting from administration of AAV2 capsids compared to the administration of capsids from different serotypes. Conversely, a 2-fold increase in anti-AAV5 antibody titer was observed following injection of AAV2, and a 100-fold increase in anti-AAV5 antibody titer following injection of AAV5 (Figure 6a). The same trend is observed in Monkey 109, which was injected with an AAV2 7mer insertion capsid, then an AAV5 capsid (Figure 6b). Interestingly, Monkey 110, which was injected with an AAV2 7mer insertion capsid, then an AAV9 capsid, did not follow this trend. The anti-AAV8 antibody response was larger than the anti-AAV9 antibody response following the injection of an AAV9 capsid. This may be due to the lower anti-AAV8 antibody prevalence after injection of an AAV2 7mer insertion capsid in the first eye (Figure 6c).

![Figure 6](image)

**Figure 6:** Increase of Anti-AAV Antibodies Throughout Sequential Infections. Neutralizing antibody titers against AAV2, AAV5, AAV8, and AAV9 in serum pre-injection, post-injection of the first eye, and post-injection of the second eye of monkeys administered (a) Wild-type AAV2 and AAV5 capsids, (b) AAV2 7mer insertion and wild-type AAV5 capsids, or (c) AAV2 7mer insertion and wild-type AAV9 capsids. Error bars indicate standard deviation (n=3).

**Effect of Pre-Injection Anti-AAV2 Antibodies on Expression**

The observation that pre-injection antibodies inhibit transgene expression from AAV gene delivery vectors has been documented following nonhuman primate liver and mouse muscle administrations. In a study monitoring GFP expression in livers of macaques following intravenous administration of AAV8 vectors, neutralizing antibody titers higher than 1:10 substantially decreased GFP expression in hepatocytes. For monkeys undergoing intravitreal administrations of gene delivery vehicles composed of wild-type
AAV2, AAV2 7mer insertion, or AAV2 tyrosine mutant capsids, the presence of neutralizing antibody titers of 1:10 or greater in the serum resulted in weak or no expression of the transgene (Table 1). Neutralizing titers in the range of 1:25 to 1:100 occasionally resulted in weak transgene expression or transgene expression that degenerated after initial observation. These data suggest that systemic anti-AAV antibodies result in loss of vector transduction in other body fluid compartments such as the vitreous.

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<td>50% neutralization at 1:25</td>
<td>yes, not typical</td>
</tr>
<tr>
<td>014</td>
<td>RE</td>
<td>AAV2 tyrosine mutant</td>
<td>$10^{13}$</td>
<td>50% neutralization at 1:25</td>
<td>very faint</td>
</tr>
<tr>
<td>623</td>
<td>LE</td>
<td>AAV2</td>
<td>$10^{11}$</td>
<td>50% neutralization between 1:25 and 1:50</td>
<td>no</td>
</tr>
<tr>
<td>906</td>
<td>RE</td>
<td>AAV2</td>
<td>$10^{11}$</td>
<td>50% neutralization between 1:25 and 1:50</td>
<td>weak foveal label</td>
</tr>
<tr>
<td>322</td>
<td>RE</td>
<td>AAV2</td>
<td>not listed</td>
<td>50% neutralization between 1:50 and 1:100</td>
<td>bright rim</td>
</tr>
<tr>
<td>322</td>
<td>RE</td>
<td>AAV2</td>
<td>$10^{12}$</td>
<td>not available*</td>
<td>no</td>
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<td>012</td>
<td>RE</td>
<td>AAV2 7mer insert</td>
<td>$10^{13}$</td>
<td>50% neutralization at 1:250</td>
<td>early euth.</td>
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<tr>
<td>707</td>
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<td>AAV2</td>
<td>$10^{11}$</td>
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<td>no</td>
</tr>
<tr>
<td>707</td>
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<td>AAV2</td>
<td>$10^{12}$</td>
<td>not available*</td>
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**Table 1:** Effect of Pre-Injection Anti-AAV2 Antibodies on Expression. The presence of low neutralizing antibody titers in serum pre-intravitreal injection of monkeys administered wild-type AAV2 or AAV2 mutant capsids reduces transgene expression in the eye, while the presence of intermediate to high neutralizing antibody titers completely prevents transgene expression. * = no serum collected

**Discussion**

Adeno-associated virus has been successful in clinical gene therapy trials for Leber’s congenital amaurosis, hemophilia, and lipoprotein lipase deficiency.\[^{8-10,30,31,33}\] Despite these exciting findings, further preclinical and clinical studies have highlighted the difficulty caused by immunological memory and pre-existing antibodies to AAV.\[^{46-48}\] Although intravitreal administration of gene therapy vectors may be advantageous because of a more broad delivery area and safer surgery, vectors delivered into the vitreous are more susceptible to neutralizing antibodies than vectors delivered subretinally in a murine model.\[^{22}\] We have found that the presence of pre-existing neutralizing antibody titers in the serum of monkeys correlates strongly with weak, degenerating, or no transgene expression following intravitreal administration of AAV gene delivery vectors. Furthermore, intravitreal gene delivery resulted in an increase in anti-AAV antibodies in the serum, showing the interactions between body fluid compartments during an immune response.

Anti-AAV antibody production following intravitreal injection increased regardless of the capsid serotype, transgene, amount of virus, or number of eyes injected. As expected, the antibody response to the serotype administered is greater than the antibody response against other AAV serotypes, but due to the high sequence similarity between AAV2, AAV8, and AAV9, administration of one of these vectors led to increases in antibody titers against all. AAV5 has the least sequence similarity to the other serotypes studied and had the least predictable antibody response in monkeys administered other serotypes. Although the serotype administered changes the antibody response, small mutations to a serotype do not affect the antibody response. For example, an AAV2 capsid variant containing a 7mer peptide insert was neutralized at the same serum dilution as wild-type AAV2 capsid using both individual monkey sera and pooled human intravenous immunoglobulin. The anti-AAV antibodies persist in monkeys for at least several months following AAV administration and were detected two years post-infection in an available sample. Antibody titers also increase following a second AAV administration, in a manner similar to the increase following the first administration.

As the number of gene therapy clinical trials continues to increase, a better understanding of the interaction with anti-vector antibodies is needed. Weak and degenerating transgene expression following intravitreal administration of AAV vectors to monkeys harboring anti-AAV antibodies provides more evidence that pre-existing immunity can decrease transduction of AAV vectors in immune privileged regions, such as the eye and brain.\[^{21}\] In addition, increases in neutralizing antibody titer were seen in the serum of these monkeys following intravitreal administration of AAV vectors. This observation is consistent with previous reports that subretinal injections of AAV vectors in dogs and nonhuman primates\[^{23}\] as well as some intracranial injections in several clinical trials\[^{24}\] led to increased serum anti-AAV antibody titers. Further studies should help to elucidate the full extent to which antibody presence in serum and other bodily fluids, including synovial fluid and vitreal fluid,\[^{25,26}\] inhibit vector transduction to
different organs. One possible solution to the improve the efficacy of gene therapy vectors administered in the presence of neutralizing antibodies is to utilize directed evolution to create new AAV variants capable of evading neutralizing antibodies (see Chapter 4). [35, 49]

In conclusion, analysis of sera collected from non-human primates before and after intravitreal injections of various AAV capsids has shown an increase in the immune response (through the presence of anti-AAV antibodies) following administration of AAV gene therapy vectors. The levels of anti-AAV antibodies in the serum correspond well with the amount of anti-AAV antibodies in the vitreal fluid into which AAV vectors are administrated. Additionally, the presence of these systemic anti-AAV antibodies correlates to weak, degenerating, or no transgene expression in the eye. This study therefore provides information on the interactions between AAV gene therapy vectors and the immune system during ocular administration. Furthermore, it may help to provide insight for future clinical studies applying intravitreal gene delivery.

References


40. Peden, C.S., C. Burger, N. Muzyczka, et al., *Circulating Anti-Wild-Type Adeno-Associated Virus Type 2 (AAV2) Antibodies Inhibit Recombinant AAV2 (rAAV2)-


CHAPTER 4

DIRECTED EVOLUTION OF ADENO-ASSOCIATED VIRUS FOR ENHANCED EVASION OF HUMAN NEUTRALIZING ANTIBODIES

Abstract

Gene delivery vectors based on adeno-associated viruses (AAV) have demonstrated promise in both preclinical disease models and recently in human clinical trials for several disease targets. However, the high prevalence of anti-capsid neutralizing antibodies, due to widespread exposure to numerous AAV variants and serotypes within the human population, decrease the efficacy of AAV gene therapy. This pre-existing immunity, as well as the development of immunity following vector administration, can impede the broader implementation of AAV gene therapy and should be addressed to build upon successful AAV results in immune privileged sites. Directed evolution has proven to be a powerful approach to generate AAV vectors with novel capabilities, and we demonstrate that AAV can be evolved to substantially overcome neutralization by human anti-AAV antibodies, both in vitro and in vivo. In particular, novel AAV variants exhibited 2- to 35-fold higher resistance to highly polyclonal, human neutralizing antibodies (intravenous immunoglobulin, or IVIG) than wild-type AAV in vitro. The antibody neutralization properties also translated to enhanced transduction in vivo in the presence of neutralizing antibodies. The creation of novel clones resistant to anti-AAV antibodies may enable the future treatment of patients with high antibody titers that are currently ineligible for AAV gene therapy.

Introduction

Adeno-associated virus (AAV) is a nonpathogenic parvovirus composed of a 4.7 kb single-stranded DNA genome within a non-enveloped, icosahedral capsid.\textsuperscript{[1]} The genome contains three open reading frames (ORF) flanked by inverted terminal repeats (ITR) that function as the viral origin of replication and packaging signal.\textsuperscript{[1]} The rep ORF encodes four nonstructural proteins that play roles in viral replication, transcriptional regulation, site-specific integration, and virion assembly.\textsuperscript{[1]} In addition, the cap ORF encodes three structural proteins (VP1-3) that assemble to form a 60-mer viral capsid.\textsuperscript{[1]} Finally, an ORF present as an alternate reading frame within the cap gene produces the assembly-activating protein (AAP),\textsuperscript{[2,3]} a viral protein that localizes AAV capsid proteins to the nucleolus and functions in the capsid assembly process.\textsuperscript{[2]} In recombinant versions of AAV, a gene of interest is inserted between the ITRs in place of rep and cap, and the latter are provided in trans along with helper viral genes during vector production.\textsuperscript{[4]} The resulting vector can transduce both dividing and non-dividing cells, resulting in stable transgene expression in the absence of helper virus for years in post-mitotic tissue.\textsuperscript{[1]} There are eleven naturally occurring serotypes and over 100 variants of AAV, each of which differs in amino acid sequence, particularly within the hypervariable regions of the capsid proteins, and thus in their gene delivery properties.\textsuperscript{[5,6]}
Importantly, no AAV has been associated with any human disease, making recombinant AAV attractive for clinical applications.\textsuperscript{[1]} As of 2013, there were over 90 completed or ongoing clinical trials that used AAV as the gene delivery vehicle.\textsuperscript{[7]} Most current studies have focused on AAV serotype 2. As a prominent example, during Phase I clinical trials for Leber’s congenital amaurosis (LCA), numerous patients who received a subretinal injection of AAV2 encoding a protein required for the isomerohydrolase activity of retinal pigment epithelium showed sustained improvement in both subjective and objective measurements of vision.\textsuperscript{[8-10]} Furthermore, there were no significant adverse events during either the pre-trial efficacy studies or the trial.\textsuperscript{[8-10]} AAV vectors are also being clinically explored for hemophilia, muscular dystrophy, Parkinson’s disease, and Alzheimer’s disease,\textsuperscript{[11]} but to date, AAV has been most successful in clinical studies involving delivery to immune privileged regions,\textsuperscript{[8-10,12]} or when patients harboring neutralizing antibodies are excluded from the trials.\textsuperscript{[11,13,14]} Immunosuppression is a promising approach for reducing cellular immune responses,\textsuperscript{[15-17]} but humoral immunity – for example anti-AAV antibodies resulting from childhood exposure to one or more serotypes, or from prior administration of AAV vector – remains a significant challenge, particularly for intrahepatic, intravascular, and even intravitreal administration.\textsuperscript{[13,14,18]} Recent analysis indicated that the prevalence of anti-AAV IgG antibodies in humans was highest for AAV2 (72%) and AAV1 (67%), but AAV9 (47%), AAV6 (46%), AAV5 (40%), and AAV8 (38%) antibodies were also present in a large portion of the population studied.\textsuperscript{[19]} Several studies found that antibodies raised against the AAV capsid during gene therapy could be prevented by reducing the dosage of rAAV particles delivered,\textsuperscript{[20]} suggesting that effective secondary administration could be achieved by an initial administration of a low vector dose.\textsuperscript{[15]} Unfortunately, administration of low vector doses leads to low transduction and thus low therapeutic gene expression. Thus, the development of novel AAV variants that are resistant to anti-AAV antibodies may enable the future treatment of patients that are currently ineligible for AAV gene therapy.

Two types of modifications to the AAV capsid have been previously attempted: 1) chemical modifications that protect the exposed immunogenic epitopes, and 2) rational genetic modifications that result in changes to the amino acids of the protein capsid. Chemical modification of the AAV2 capsid by polyethylene glycol (PEG) conjugation modestly protected vectors from antibody neutralization, but higher degrees of virion shielding with the polymer substantially reduced infectivity.\textsuperscript{[21,22]} Rational genetic modifications to the AAV capsid include using alternate natural AAV capsids or site-directed mutagenesis of antigenic epitopes within a given capsid. Numerous studies suggest that neutralizing capsid antibodies against one serotype have only limited cross-reactivity to other serotypes, thereby enabling sequential transduction of alternative serotypes.\textsuperscript{[23-25]} However, the variable tropism of different AAV serotypes and the presence of antibodies against multiple serotypes in the human population make the implementation of this strategy difficult. The rational mutation of a single epitope resulted in improved evasion of antibodies,\textsuperscript{[26]} but polyclonal antibody mixtures (i.e., those found in sera) harbor antibodies against many regions of the capsid. Thus, point mutations would need to be introduced across the capsid, rendering it difficult to identify which positions to mutate to reduce antibody binding while maintaining viral packaging, receptor binding, or downstream steps in the viral infection.
Directed evolution is a powerful high-throughput approach that has been implemented to create “designer” AAV mutants with novel properties, such as altered receptor binding, shifted cell tropism in vitro, and modified tissue transduction in vivo.\textsuperscript{27-33} The method emulates the process of natural evolution, in which repeated genetic diversification and selection enable the accumulation of key mutations or genetic modifications that progressively improve a molecule’s function. This approach provides a way to rapidly develop AAV variants with desired gene delivery properties without the need to rationally design the mutations, as information about the structure-function relationship of the AAV protein capsid is insufficient to enable the design of new variants for each desired application. For example, using potent serum from rabbits immunized with AAV2 vector, Maheshri et al. evolved an antibody-resistant AAV2 variant containing five point mutations. Compared to vectors with wild-type AAV2 capsid, a 96-fold higher neutralizing antibody concentration was required to neutralize the variant in vitro, and 2-3 orders of magnitude higher antiserum levels were required in vivo.\textsuperscript{29} Perabo et al. isolated an AAV2 variant containing two point mutations capable of withstanding 5.5-fold higher human sera neutralizing antibody levels compared to wild-type AAV2 in vitro.\textsuperscript{34} However, the variant was only tested on the serum sample that was also used in selections,\textsuperscript{34} which raises the possibility that the improvement could be specific towards only this individual serum. Furthermore, following selection of functional AAV chimeras from AAV serotypes 1, 2, 4-6, 8, and 9, Koerber et al. found that four out of the seven clones analyzed withstood higher neutralizing antibody concentrations than their parent serotypes, even in the absence of selective pressure from anti-AAV antibodies.\textsuperscript{28} Similar enhancements to anti-AAV antibody evasion were observed for chimeras selected for enhanced tropism of hamster melanoma cells and muscle tissue.\textsuperscript{33,35} Through selections involving human intravenous immunoglobulin (IVIG), Grimm et al. isolated a chimeric clone consisting of AAV2, AAV8, and AAV9 exhibiting antibody resistance levels comparable to AAV8 and AAV9, but much higher than AAV2 in vivo.\textsuperscript{36}

The previous work provides a strong basis for the promise of directed evolution to address the problem of pre-existing anti-AAV antibodies. However, there have been limited demonstrations of the success of these variants in vivo. We have used these findings as a basis for the creation of libraries that will build upon the previous applications of directed evolution to AAV antibody evasion. Using these libraries, we have created novel AAV variants that are capable of enhanced anti-AAV antibody evasion, both in vitro and in vivo. Stringent selections were performed with progressively stronger neutralizing, polyclonal human antibodies – from low and high potency human sera pools, then human IVIG – enabled the evolution of AAV variants capable of evading antibody neutralization by individual human sera, human IVIG in vitro and in vivo, and mouse sera, the most broadly evasive variants reported to date.

**Materials and Methods**

**Cell Lines**

Cell lines were cultured at 37°C and 5% CO\textsubscript{2}, and unless otherwise mentioned, were obtained from the American Type Culture Collection (Manassas, VA). HEK293T, HeLa, and HT1080 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin...
(Invitrogen, Carlsbad, CA). CHO K1 and CHO pgsA cells were cultured in F-12K medium (ATCC) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen). Pro5 and Lec1 cells were cultured in MEM-alpha medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen).

**Human Sera Pools for Selection**

Eighteen individual human serum samples were obtained from Innovative Research, Inc. (Southfield, MI) and the neutralizing antibody titer for wild type AAV2 was determined for each sample (Appendix A Table A1). Since individual samples likely possess variations in both the affinities and epitope specificities of the antibodies, three potent sera pools ($\alpha = A + F + G$, $\beta = B + H + M$, and $\gamma = I + J + N$) were generated by mixing equal volumes of individual serum samples. Selection in the presence of these variations of antibodies should result in a general enhancement of resistance to virtually all pre-existing human antibodies. Later selections were performed in the presence of Gamimune N, 10% Human IVIG (Bayer, Elkhart IN).

**Library Generation and Viral Production**

To create the saturation mutagenesis library, an AAV2 cap library was generated by error-prone PCR followed by the staggered extension process described by Zhao et al. using 5'-GCGGAAGCTTTGATCAACTACGC-3' and 5'-GGGGCGGCCGCAATTACAGATTACGAGTCAGGTATCTGGTG-3' as forward and reverse primers, respectively.$^{[37]}$ Selections using pooled individual human sera revealed a variant containing four point mutations (described in the results section) that served as the basis for the saturation mutagenesis library. The cap gene for this variant was subjected to further mutagenesis by changing the amino acids at specific sites. Primer 5'-CATTNNKGAACCAGTCTAGGAACTGG-3' and the corresponding reverse compliment primer were used to mutagenize the R471 amino acid site. Primer 5'-GCCAACAGGACGATGAAATNKTNTTTTCCTCAGAGCGGTGTTCTCATCTTT GGGAAAGCAAGGCTCCTANQKAAAACAAGTGTGGACATTG-3' and the corresponding reverse compliment primer were used to mutagenize the K532 and E548 amino acid sites. Primer 5'-CCAACCTCCAGAGGCNNKAGACAAGCAGCTACC3' and the corresponding reverse compliment primer were used to mutagenize the N587 amino acid site. Primer 5'- CAAACTACAACAAGTCTNNKAAATGTTGGACTTTACTGAGCNRKAAATGGCTGTATT-3' and the corresponding reverse compliment primer were used to mutagenize the V708 and T716 amino acid sites. A library consisting of AAV2 containing randomized cap loop regions and a library containing shuffled DNA from the wild type AAV1, AAV2, AAV4, AAV5, AAV6, AAV8, AAV9 cap genes were packaged and pooled for initial selection steps.$^{[28,38]}$ For the second and third rounds of evolution, random mutagenesis libraries were generated by subjecting cap genes from the Loop-Swap/Shuffle library and the Saturation Mutagenesis library to error-prone PCR using 5'-CATGGGAAGTGGCCAGACG-3' and 5'-ACCATCGGAGCCCATACCTG-3' as forward and reverse primers, respectively, as previously described.$^{[29]}$ The replication competent AAV libraries and recombinant AAV vectors expressing GFP under the control of a CMV promoter were packaged using
HEK293T cells (ATCC) using the calcium phosphate transfection method, and the viruses were purified by iodixonal gradient centrifugation.[27,29] Recombinant AAV vectors expressing GFP or luciferase under the control of a CMV promoter for use in vivo were further purified by Amicon filtration. DNase-resistant genomic titers were determined via quantitative PCR.[27,29]

**Library Selection and Evolution**

One round of selection is defined as HEK293T cell infection using the AAV starting library (incubated for 30 minutes at room temperature for the pooled individual human sera or for 1 hour at 37°C with heat inactivated IVIG prior to infection), followed by adenovirus rescue and harvest of successful variants. Each round of evolution consists of mutagenesis of the cap gene to create the starting library and three rounds of selection. Three rounds of evolution were performed with each library, with clonal analysis performed between each round of evolution. The starting libraries for each round of evolution were generated as described above. Following the third round of selection, AAV cap genes were isolated from the pool of successful AAV variants and amplified via PCR. Cap genes were inserted into the pXX2 recombinant AAV packaging plasmid using NotI and HindIII.[29] Cap genes were then sequenced at the University of California, Berkeley DNA sequencing facility, and analyzed using Geneious software (Biomatters, Auckland, New Zealand). Three-dimensional models of the AAV2 capsid (Protein Databank accession number 1LP3) were rendered in Pymol (DeLano Scientific, San Carlos, CA).

**In Vitro Transduction Analysis of Antibody-Evading Variants**

HEK293T were plated at a density of 3x10^4 cells/well 24 hours prior to infection. Variants were incubated at 37°C for 1 hour with heat inactivated IVIG, individual human sera, or individual mouse sera prior to infection, and cells were then infected with rAAV-GFP at a genomic MOI of 2000. The percentage of GFP positive cells was assessed 48 hours post infection using an ImageXpress Micro Cellular Imaging and Analysis System (Molecular Devices, Sunnyvale, CA) and MetaXpress Image Analysis Software, version 3.1.0, Multi Wavelength Cell Scoring Application Module (Molecular Devices).

**In Vitro Transduction Analysis**

To determine the relative transduction efficiencies the selected mutants compared to parental wild-type AAV serotypes, HEK293T, CHO K1, CHO pgsA, Pro5, Lec1, HeLa, and HT1080 cells were plated at a density of 2.5 x 10^4 cells per well 24 hours prior to infection. Cells were infected with rAAV1-GFP, rAAV2-GFP, rAAV6-GFP, Shuffle 100.1-GFP, Shuffle 100.3-GFP, SM 10.2-GFP, or SM 100.7-GFP at a range of MOI of 100-1000.[30] The percentage of GFP positive cells was assessed 48 hours post infection using a Beckman-Coulter Cytomics FC500 flow cytometer (Beckman-Coulter, Brea, CA).

**In Vivo Analysis of Antibody-Evading Variants**

For analysis of gene expression in vivo, eight week old, female, Balb/c mice were primed with 4 mg IVIG per mouse or phosphate buffered saline (for control mice) via tail vein injection 24 hours prior to administration of recombinant Shuffle 100-3, SM 10-2, or
AAV2 vectors. Mice were infected with $10^{11}$ viral genomes of recombinant AAV vectors encoding luciferase under the control of a CMV promoter via tail vein injection. For bioluminescence imaging, mice were anesthetized with 2% isofluorane and oxygen. D-luciferin substrate (GOLD Biotechnology, St. Louis, MO) was injected intraperitoneally, at a dose of 500 µg/g of body weight. Images were generated using a VivoVision IVIS Lumina imager (Xenogen, Alameda, CA). For each mouse, ventral images were taken 7–10 minutes after the substrate injection, every week for four weeks. Five weeks post-infection, serum was collected via cardiac puncture and mice were then perfused with 0.9% saline solution. Heart, liver, lungs, kidney, spleen, brain, spinal cord, and hind limb muscle were harvested and frozen. Frozen tissue samples were homogenized and resuspended in reporter lysis buffer (Promega, Mannheim, Germany) for in vitro luciferase analysis. Lysate containing luciferase was clarified by centrifugation for 10 minutes at 10,000g. To assay the samples, 20 µL of the lysate was added to 100 µL of the luciferase assay buffer, mixed, incubated for 5 minutes, and placed in the luminometer. The signal was integrated for 30 seconds with a 2 second delay and was reported in Relative Light Units (RLU) detected by a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). The luciferase signal was normalized to the total protein content determined by a bicinchoninic acid assay (Pierce).

Results

AAV Library Generation and Selection Through Directed Evolution: AAV2-based Variants

Figure 1a shows a schematic of the directed evolution approach used to isolate novel AAV variants capable of evading human antibody neutralization. Libraries of viruses were created using several DNA mutagenesis techniques – including error-prone PCR, DNA shuffling, and short peptide insertions – as described in greater detail below (Figure 1a, steps 1 and 2). The libraries were divided into two separate libraries, an AAV2-based library and a library containing DNA from several wild-type AAV serotypes, in order to pursue two goals in parallel: 1) to generate an AAV2 variant that is resistant, given the strong clinical safety profile of AAV2, and 2) to create a maximally resistant mutant that may be unrelated to AAV2. During initial selections, pools of viral libraries developed from error-prone PCR mutations to AAV2 cap genes were incubated with various dilutions of the low potency pool of three human sera (termed the α pool) for 30 minutes at room temperature prior to infection of HEK293T cells (step 3). Following three rounds of selection against the α sera pool (Figure 1a, steps 4 and 5), several variants with enhanced resistance were obtained (Appendix A Figure A1a). Variant 1.45 contained two point mutations (N312K, N449D), which resulted in >10-fold more resistance to neutralization by the α pool compared to wild type AAV2.
Figure 1. Directed Evolution of AAV for Enhanced Antibody Evasion. (a) Schematic of Directed Evolution. 1) A viral library is created by genetically diversifying the cap gene using several complementary approaches. 2) Viruses are packaged in HEK293T cells using plasmid transfection, then harvested and purified. 3) The viral library is incubated with human IVIG at several concentrations and introduced to HEK293T cells in vitro. 4) Successful viruses are amplified and recovered via adenovirus superinfection. 5) Successful clones are enriched through repeated selections at lower MOIs. 6) Isolated viral DNA reveals successful cap genes. 7) Successful cap genes are mutated again to serve as a new starting point for selection. (b) Selection of Antibody Evading Mutants from Loop-Swap/Shuffled, and Saturation Mutagenesis libraries. HEK293T cells were infected with viral libraries for 24 hours. Viral particles that productively infected cells were amplified by adenovirus infection, and the rescued AAV was quantified by qPCR. A 1:10 dilution of IVIG corresponds to a concentration of 10 mg IVIG/mL. Error bars indicate the standard deviation (n = 3).

The cap gene from variant 1.45 was subjected to additional random mutagenesis, and the resulting library was selected for three additional rounds of selection against the β and γ pools, in parallel. As only minor additional improvements in antibody evasion were observed (data not shown), the recovered cap genes were pooled and subjected to additional diversification via DNA shuffling and EP PCR. Three more rounds of selection against increasing levels of sera from the β and γ pools resulted in substantial enrichment in the levels of recovered virus from the viral library compared to wild type AAV2 (Appendix A Figure A1b, c). Sequencing of the successful cap genes from both pools revealed a single dominant mutant, variant γ4.3, and several low frequency mutants. γ4.3 contained four point mutations (N312K, N449D, N551S, and I698V). Although the α, β and γ human sera pools represent a more realistic estimation of the polyclonal anti-AAV antibodies present in the human population than any individual sera could, the variants created from selections against these pools still may not have the ability to evade the antibody diversity present within the broader human population. To more effectively model this diversity, we began testing variants against purified human intravenous immunoglobulin (IVIG) derived from the pooled human plasma of ~100,000 individuals, which is thought to represent about 99% of all immune responses within the donor population. In the presence of human IVIG, variant 1.45 demonstrated a modest 1.2-fold enhanced resistance to neutralization, whereas γ4.3 demonstrated 3.1-fold
enhanced resistance to neutralization (Appendix A Figure A1d). This observation confirms the hypothesis that pools of individual human sera can be used to isolate AAV variants capable of enhanced evasion of antibodies present in the general human population. The resistance of variant γ4.3 to neutralization by anti-AAV antibodies prompted the development of a library based on the γ4.3 cap gene for further improvement. Amino acid sites R471, K532, E548, N587, V708, T716, previously determined to be immunogenic sites on the AAV2 capsid,[26,29] were subjected to saturation mutagenesis in the context of the AAV2 mutant γ4.3 in an attempt to find amino acid mutations that may improve upon this variant’s antibody resistance. This “saturation mutagenesis” library, along with a library containing DNA from several wild-type AAV serotypes (described below) were then subjected to additional rounds of selection in the presence of IVIG to generate variants that would be more broadly evasive of anti-AAV antibodies present in the human population as a whole.

**AAV Library Generation and Selection Through Directed Evolution: non-AAV2-based Variants**

A “shuffled” library composed of random cap chimeras of 7 parent AAV serotypes[28] and a “loop-swap” library composed of AAV2 cap with substituted loop regions,[38] were subjected to three additional rounds of directed evolution, in which the pools of viral libraries were incubated with various dilutions of human IVIG for one hour at 37°C prior to infection of HEK293T cells. Following infection with AAV libraries, and amplification of the infectious AAV variants through adenovirus superinfection, the number of viral genomes, or viral titer, from each library condition was quantified and compared to titers of wild-type AAV2 as a method for determining the success of the selection (Figure 1b). For each round of selection using the saturation mutagenesis and loop-swap/shuffled libraries, viral pools from the 1:10 and 1:100 IVIG dilution conditions that produced higher viral titers than wild-type AAV2 were used as the starting point for the subsequent round of selection. After three rounds of selection, the successful viral cap genes were isolated and tested individually to determine the virus with the most efficient gene delivery. In addition, the cap genes isolated from the third round of selection were subjected to additional rounds of error-prone PCR mutagenesis, and the process was repeated to iteratively increase the fitness of the virus.

**Increased Antibody Evasion of the Novel Evolved AAV Variants In Vitro**

Of the twelve clones selected and packaged for individual analysis from the saturation mutagenesis and loop-swap/shuffled libraries after nine rounds of selection against human IVIG, all twelve withstood higher neutralizing antibody levels than both wild-type AAV1 and AAV2 – the most commonly used serotype in clinical trials (Figure 2a and Table 1). Variant Shuffle 100-3, which required a 35-fold higher in vitro IVIG concentration for neutralization than wild-type AAV2, was still capable of transducing approximately 10% of cells in the presence of 1 mg/mL IVIG (Figure 2b). Additionally, variant SM 10-2 from the AAV2 saturation mutagenesis library required a 6-fold higher in vitro IVIG concentration for neutralization than wild-type AAV2. Recently, AAV8 has demonstrated success in a clinical trial for hemophilia B, although patients with pre-existing anti-AAV8 antibodies were excluded.[11] In addition to the significant
improvement over AAV2, SM 10-2 showed similar neutralization to wild-type AAV8, and Shuffle 100-3 required a 5.7-fold higher *in vitro* IVIG concentration for neutralization than AAV8.

As demonstrated through the samples comprising the α, β and γ human sera pools, individual serum samples have large variation in the levels of neutralizing antibodies, and these antibodies may differ from the ones found in IVIG. In order to determine whether the variants created using a very diverse antibody pool could also evade neutralization by individual sera, the variants were tested against sera samples from individual patients excluded from a hemophilia B clinical trial due to pre-existing anti-AAV antibodies (Figure 3). Variants Shuffle 100-3 and SM 10-2 showed enhanced transduction in the presence of these sera, compared to wild-type AAV.

![Figure 2](image_url). Clonal Analysis – In Vitro Neutralization by IVIG. Neutralization Profiles of Antibody Evading Mutants. The cap genes of antibody evading mutants isolated after nine rounds of selection were used to package recombinant AAV encoding GFP and were incubated with human IVIG before infection of HEK293T cells. The fraction of remaining infectious particles was determined using high throughput fluorescence imaging and normalized to the infectious titer in
the absence of IVIG. Two clones from each library with resistance to IVIG are shown. Data for the other clones analyzed are displayed in Table 1. (a) Neutralization curves. Error bars indicate the standard deviation (n = 3). (b) Representative fluorescence images from several IVIG dilutions show that mutants are capable of HEK293T transduction in the presence of high concentrations of neutralizing antibodies.

### Table 1. IVIG Neutralizing Antibody Titers of Library Clones and Parent Serotypes

<table>
<thead>
<tr>
<th>Clone</th>
<th>Neutralizing IVIG Concentration (mg/mL)</th>
<th>Fold Resistance Relative to AAV2</th>
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<tbody>
<tr>
<td>AAV1</td>
<td>0.026</td>
<td>1.757</td>
</tr>
<tr>
<td>AAV2</td>
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<td>1.000</td>
</tr>
<tr>
<td>AAV8</td>
<td>0.092</td>
<td>6.113</td>
</tr>
<tr>
<td>Shuffle 10-2</td>
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<td>2.443</td>
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Table 1. IVIG Neutralizing Antibody Titers of Library Clones and Parent Serotypes. Human IVIG was used to neutralize recombinant AAV-GFP vectors with capsids from wild-type AAV1, AAV2, AAV8, and variants recovered from the loop-swap/shuffled and saturation mutagenesis libraries. The IVIG concentration (mg/mL) required to reduce gene delivery efficiency to 50% of that in the absence of IVIG is shown, and compared to the concentration required to reduce delivery of AAV2. All variants analyzed required higher concentrations of IVIG than wild-type AAV1 and AAV2. The neutralizing antibody titer was determined by fitting the curves in Figure 2 to an exponential curve.

### Figure 3. Clonal Analysis – In Vitro Neutralization by Individual Human Sera

Neutralization Profiles of Antibody Evading Mutants. Human sera were acquired from individuals that were excluded from hemophilia B clinical trials due to the presence of high neutralizing antibody titers against AAV. Recombinant AAV encoding GFP was incubated with individual human sera...
samples before infection of HEK293T cells. The fraction of remaining infectious particles was determined using fluorescence microscopy and normalized to the infectious titer in the absence of human sera. Error bars indicate the standard deviation (n = 3).

Sequence analysis of the twelve clones revealed that the two variants with the highest neutralizing antibody titers, Shuffle 100-3 and Shuffle 100-1, are almost identical shuffled capsids containing fragments of AAV1-4, AAV6, and AAV9 (Figure 4). Differences in amino acids 469 (AAV6 residue to AAV7 residue) and 595-599 (AAV6 residues to AAV1 residues) between the two variants apparently translate to almost a 3-fold increase in neutralizing antibody titer for Shuffle 100-3 (Table 1). Variant Shuffle 100-7, which had the third highest neutralizing antibody titer (Table 1), is also a shuffled capsid containing fragments of AAV1, AAV6, and AAV8 (Figure 4), which agrees well with reported data showing that wild-type AAV1 and AAV8 are effective at evading anti-AAV2 antibodies for natural serotypes.[36,39] Interestingly, variant SM 10-2 retained the point mutations acquired by variant γ4.3 and also retained wild type residues at the saturation mutagenesis sites. Variant SM 10-2 did acquire additional point mutations at surface residue D472N and internal residue L735Q, reinforcing the observation that a small number of point mutations can improve the antibody evasion capability of an AAV capsid considerably.[29]

**Figure 4.** Amino Acid Sequences of Loop-Swap/Shuffle and Saturation Mutagenesis Clones. (a) Schematics of the capsid protein are shown for the clones from each library with the highest neutralizing IVIG concentrations. Each region is shaded according to the parent serotype from which it is derived. Black arrows denote (from left to right) the start codons of VP1, VP2, and
VP3 capsid proteins. Gray arrows denote (from left to right) surface loop regions I, II, III, IV, and V based on the AAV2 capsid. (b) Molecular models of the full AAV2 capsid, based on the solved structure, are shown for the clones from each library with the highest neutralizing IVIG concentrations. Each region is shaded according to the parent serotype from which it is derived. For variant Shuffle 100-3, black arrows indicate differences from variant Shuffle 100-1. For variant SM 10-2, mutations N449D, D472N, N551S, and I698V are surface mutations (black).

To better understand how the antibody evading mutations may affect the in vitro transduction properties of the variants, infectivity of the variants was evaluated on a panel of cell lines with different surface receptors. Comparing the transduction profile of the variants to their parental AAV serotypes can provide evidence of how a set of mutations changes the variant’s dependence on cell surface receptors to enter the cell. Variants Shuffle 100-3 and Shuffle 100-1 have transduction profiles that mimic the transduction profiles of parent serotypes AAV1 and AAV6 (Figure 5). However, the mutations in SM 10-2 appear to shift transduction from a heparin dependence (as seen in parent serotype AAV2) to a sialic acid dependence, similar AAV1 and AAV6, while the Shuffle 100-7 shuffled clone appears to shift transduction to a profile similar to AAV2 (Figure 5).

![Figure 5. In Vitro Tropism of Novel AAV Variants. Recombinant AAV vectors expressing green fluorescent protein were used to transduce a panel of cell lines: CHO, pgsA (lacking all surface glycosaminoglycans), Pro5, Lec1 (lacking sialic acid), HEK293T, HeLa, and HT1080 (human fibrosarcoma cell line) to profile the transduction properties of the new AAV variants. Error bars indicate the standard deviation (n = 3).](image)

Increased Antibody Evasion of the Novel Evolved AAV Variants In Vivo

Although the in vitro transduction assay helped to elucidate the surface receptors used by the variants, it is also important to understand which tissues will be effectively transduced by the variants in vivo. Understanding the in vivo distribution of the gene delivery vectors will help to determine what diseases could be potential therapeutic
targets. To determine the localization pattern of variants Shuffle 100-3 and SM 10-2, luciferase enzyme activity was examined in various tissues of naïve mice injected with AAV2, Shuffle 100-3, or SM 10-2 (Figure 6a). Variant SM 10-2 displayed similar in vivo tropism to AAV2, except for 7-fold higher transduction of the heart, 5-fold higher transduction of the lungs, and 4.5-fold lower transduction of the liver. The Shuffle 100-3 variant exhibited over 4-fold higher transduction of the brain, over 3-fold higher transduction of the lungs, and 27-fold higher transduction of muscle than AAV2.

Certain future gene therapy applications for these variants may require multiple administrations of a gene therapy vector in order to achieve therapeutic levels of gene expression. Therefore, it is important to understand the extent to which these variants may be neutralized by antibodies generated from a gene therapy administration. Analysis of the serum from these mice used in the in vivo distribution study showed that variant Shuffle 100-3 required equal or higher in vitro serum concentrations for neutralization than AAV1 and AAV8 for serum from mice given AAV1, AAV2, AAV8 or Shuffle 100-3 gene delivery vectors (Table 2). SM 10-2 required equal or higher in vitro serum concentrations for neutralization than AAV1 for serum from mice given AAV1, AAV2, AAV8, Shuffle 100-3, or SM 10-2 gene delivery vectors (Table 2). Furthermore, both variants were less neutralized by serum from mice administered with AAV2 vectors than all wild-type AAV serotypes tested. Interestingly, variant Shuffle 100-3 was also less neutralized by serum of mice immunized against it than any of the other serotypes or variants tested (Table 2). These data illustrate the possibility that these variants could be used in combination with wild-type AAV serotypes or the other variant in applications requiring multiple sequential vector administrations.

To determine the ability of variants SM 10-2 and Shuffle 100-3 to evade antibody neutralization in vivo, mice were passively immunized with human IVIG prior to AAV injection. Variant SM 10-2 had significantly higher heart, liver, and muscle transduction than AAV2, as measured by luciferase enzyme activity (Figure 6b). Variant Shuffle 100-3 had significantly higher heart and muscle transduction compared to AAV2 (Figure 6b). In the presence of IVIG, AAV2 transduction levels of heart, liver, and muscle tissue were approximately 0.8%, 2%, and 5% of the transduction levels of those tissues in naïve mice. SM 10-2 and Shuffle 100-3 had much higher levels of transduction in IVIG immunized mice compared to naïve mice in the heart (23% - SM 10-2, 10% - Shuffle 100-3), liver (100% - SM 10-2, 21% - Shuffle 100-3), and muscle (84% - SM 10-2, 5% - Shuffle 100-3).
Figure 6. In Vivo Localization and Neutralization of Novel AAV Variants. (a) Recombinant AAV vectors encoding luciferase were administered via tail vein injection to female BALB/c mice. After 5 weeks, levels of luciferase activity were determined and normalized to total protein for each sample analyzed. (b) Recombinant AAV vectors expressing luciferase were administered via tail vein injection to female BALB/c mice 24 hours after tail vein injection of 4 mg of human IVIG. After 5 weeks, levels of luciferase expression were normalized to total protein for each sample analyzed. Error bars indicate the standard deviation (n = 3), * = p < 0.05. RLU, relative luciferase units.

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Table 2. Neutralizing Antibody Titers of Library Clones and Parent Serotypes in Immunized Mouse Sera. Sera from mice administered library clones or wild-type AAV was used to neutralize recombinant AAV-GFP vectors with capsids from wild-type AAV1, AAV2, AAV8, and variants recovered from the loop-swap/shuffled and saturation mutagenesis libraries. The serum dilution required to reduce gene delivery efficiency to 50% of that in the absence of serum is shown.

Discussion

AAV gene therapy vectors have demonstrated considerable promise in numerous clinical trials to date. However, circulating anti-AAV antibodies, resulting from childhood exposure to natural AAV serotypes, have led to the exclusion of many potential patients from trials, and likely from future gene therapies based on serotypes with high prevalence of neutralizing antibodies in the human population. We have used directed evolution to isolate two novel AAV variants that are capable of reduced neutralization by anti-AAV antibodies derived from individual human patients, pooled human serum, and mouse serum, both in vitro and in vivo. Directed evolution has proven to be a powerful approach to generate AAV vectors with novel properties, and previous work has shown that this technique can be used to increase resistance to anti-AAV antibodies. Directed evolution was previously paired with rational design by Maersch et al., who performed saturation mutagenesis at amino acid positions 449, 458,
459, 493, and 551 of the AAV2 capsid.\textsuperscript{[41]} Though the resulting variants did not reach the antibody resistance levels of some natural serotypes, the mutants isolated from selections in the presence of human serum importantly reduced the antibody susceptibility of AAV2 while conserving tropism.\textsuperscript{[41]} Furthermore, none of the variants were tested \textit{in vivo}.\textsuperscript{[41]}

A study by Lochrie \textit{et al.} reported that the immunogenic residues recognized by human sera and IVIG are different, suggesting that different individuals can produce various neutralizing antibodies to different sets of epitopes on the AAV capsid, and complete escape from neutralization is not easy.\textsuperscript{[29]} Our work demonstrates that the stringent implementation of multiple rounds of directed evolution using several different serum pools containing various amounts and potencies of anti-AAV antibodies will result in the isolation of novel AAV variants that are capable of enhanced cellular transduction, both \textit{in vitro} and \textit{in vivo}, in the presence of multiple anti-AAV antibody pools.

Adaptive immune responses to AAV vector components in animals and humans often prevent readministration of AAV vectors of the same serotype, making gene delivery applications requiring multiple vector administrations difficult. \textit{In vitro} neutralization assays using the serum from the mice used in the biodistribution studies demonstrate that the variants are less neutralized by these sera than wild-type AAV (Table 2), which may be useful for gene therapy strategies in which vector readministration is necessary. For example, Shuffle 100-3 was not neutralized by serum from mice injected with AAV2, and AAV2 was not neutralized by serum from mice injected with Shuffle 100-3, suggesting this variant can be used in combination with wild-type AAV serotypes or in applications requiring multiple vector administrations.

Variant γ4.3, isolated from an AAV2-based error-prone library selected against a pool of individual human sera, contained four point mutations (N312K, N449D, N551S, and I698V). Interestingly, two of these positions (N449 and N551) were previously identified as immunogenic residues using other pools of human serum, demonstrating that antigenic epitopes involving these sites are targeted by many different neutralizing antibodies.\textsuperscript{[34,38]} Thus, these sites are interesting and valuable targets for mutation. Pairing directed evolution and rational design in the saturation mutagenesis library resulted in the isolation of variant SM 10-2, which was capable of higher antibody resistance than both AAV1 and AAV2 and equal antibody resistance to AAV8 \textit{in vitro}. Variant SM 10-2 incorporates two additional point mutations (D472N and L735Q) to those found on variant γ4.3. The D472N mutation may reduce antibody binding within the heparin binding domain on the SM 10-2 capsid without disrupting heparin affinity (Figure 5), since previous mutations to positions 449 and 551 were found to have no effect on the heparin binding capacity of AAV2.\textsuperscript{[41]} Additionally, a D472A mutation was previously shown to increase the level of capsid synthesis in HEK293 cells,\textsuperscript{[26]} suggesting that the D472N mutation may have also been positively selected during the directed evolution for its ability to enhance virus packaging. Similarly, the replacement of the positively charged lysine side chain at amino acid position 735 with the uncharged glutamine side chain may function to stabilize the capsid, as it is also present in variant Shuffle 100-7 despite being located within the interior of the assembled capsid (Figure 4). Though variant SM 10-2 has only 6 mutations compared to the wild type AAV2, variant SM 10-2 displayed similar \textit{in vitro} tropism to AAV1/AAV6, including a shift from heparan sulfate dependence to sialic acid dependence (Figure 5). Although none of the mutations were made to the amino acids previously identified as responsible for heparin binding,\textsuperscript{[42]} it is
possible that since amino acids 449, 472, and 551 lie in close proximity to the heparin binding domain, changes to the side chains of these amino acids may alter the structure of the binding domain and prevent heparan sulfate binding. However, its in vivo tropism more closely mimicked that of parental serotype AAV2, with the exception of an increase in heart transduction and a decrease in liver transduction (Figure 6a). This change in the tissue transduction profile may be due to the rapid blood clearance of AAV serotypes containing a heparin binding domain.\textsuperscript{[43]}

The creation of chimeric AAV capsids allows for the creation of viral variants that can merge desirable properties from multiple AAV serotypes. Previous work by Koerber et al. and Grimm et al. demonstrated that one of the gene delivery characteristics that could be enhanced by chimeric AAV capsids was antibody neutralization.\textsuperscript{[28,36]} In this work, the shuffled library produced variants whose capsid exteriors were composed primarily of AAV1 and AAV6, consistent with previous data showing that AAV1 and AAV6 were less neutralized by IVIG than AAV2.\textsuperscript{[28]} Although AAV8 and AAV9 have also been shown to be much more resistant to IVIG neutralization than AAV2,\textsuperscript{[36]} amino acids specific to these capsids were only present in small spans on the surface of the shuffled variants isolated during our selections (Figure 4). The variant displaying the more efficient evasion of antibody neutralization in vitro, Shuffle 100-3, displayed similar in vitro tropism to its parental serotypes AAV1 and AAV6, but at a higher rate of infectivity than either wild-type serotype. Differences in amino acids 469 and 595-599 between variants Shuffle 100-1 and Shuffle 100-3 translate to almost a 3-fold increase in neutralizing antibody titer for Shuffle 100-3, once again demonstrating that changing only a few amino acids can result in significant changes to the gene delivery characteristics of an AAV vector.

References


CHAPTER 5

DIRECTED EVOLUTION OF ADENO-ASSOCIATED VIRUS FOR ENHANCED GENE DELIVERY AND GENE TARGETING IN HUMAN PLURIPOTENT STEM CELLS

This chapter is a postprint of a paper submitted to and accepted for publication as


The copy of record is available at: http://www.nature.com/mt/journal/v20/n2/full/mt2011255a.html

Abstract

Efficient approaches for the precise genetic engineering of human pluripotent stem cells (hPSCs) can enhance both basic and applied stem cell research. Adeno-associated virus (AAV) vectors are of particular interest for their capacity to mediate efficient gene delivery to and gene targeting in various cells. However, natural AAV serotypes offer only modest transduction of human embryonic and induced pluripotent stem cells (hESCs and hiPSCs), which limits their utility for efficiently manipulating the hPSC genome. Directed evolution is a powerful means to generate viral vectors with novel capabilities, and we have applied this approach to create a novel AAV variant with high gene delivery efficiencies (~50%) to hPSCs, which are importantly accompanied by a considerable increase in gene targeting frequencies, up to 0.12%. While this level is likely sufficient for numerous applications, we also show that the gene-targeting efficiency mediated by an evolved AAV variant can be further enhanced (>1%) in the presence of targeted double-stranded breaks generated by the co-delivery of artificial zinc finger nucleases (ZFNs). Thus, this study demonstrates that under appropriate selective pressures, AAV vectors can be created to mediate efficient gene targeting in hPSCs, alone or in the presence of ZFN-mediated double stranded DNA breaks.

Introduction

The capacity to mediate high efficiency gene delivery to human pluripotent stem cells (hPSCs) has numerous applications, ranging from the study of specific genes in stem cell self-renewal and differentiation to the directed differentiation of stem cells into specific lineages for therapeutic application. Furthermore, precise manipulation of the human stem cell genome using gene targeting techniques that exploit the natural ability of cells to perform homologous recombination (HR) has broad applications and implications, including safe harbor integration of genes for basic or therapeutic
application, creating *in vitro* models for investigating human development and disease, and high-throughput drug discovery and toxicity studies.\(^1,2\) However, while gene delivery and gene targeting are well established for various mammalian somatic cells,\(^3\) a readily generalized approach for efficient gene expression and gene targeting in hPSCs requires further development.

Current methods to deliver genes to hPSCs range from viral vectors to plasmid-based transient gene expression. Lentiviral vectors – which are highly efficient and result in long term gene expression – have been extensively employed in numerous studies in human stem cells.\(^4\) However, transient expression is desirable in some cases, such as for the temporary overexpression of regulatory signals to manipulate stem cell fate decisions.\(^5,6\) Also, vector integration into the genome can risk insertional mutagenesis, a potential concern for downstream clinical application.\(^4\) As an alternative, electroporation can be used to achieve transient gene expression, though this method can suffer from low transfection efficiencies and moderate toxicity in human stem cells.\(^7\)

In addition to gene delivery, there is a need to develop efficient gene targeting methods that rely on homologous recombination to introduce permanent and sequence-specific genome modifications in hPSCs. Several impressive studies have demonstrated successful gene targeting in hPSCs, though the initial rates reported using conventional methodologies are low (10\(^-7\) to 10\(^-5\) correctly targeted cells for every original cell in the population), which necessitates the use of positive and negative selection to improve the overall efficiency and specificity of the process.\(^8,9\)

Recently, several approaches have been utilized to improve gene targeting in hPSCs, including the introduction of double stranded breaks into the cellular genome by engineered nucleases.\(^10-14\) Such breaks stimulate the cellular DNA repair machinery and thereby greatly enhance the rate of homologous recombination with a donor DNA.\(^15\) For example, Zou *et al.* found that cotransfection of plasmids containing the donor DNA and zinc finger nucleases (ZFNs) significantly increased gene targeting in hPSCs up to a frequency of 0.24% (~1 targeted cell in 415 original cells), as compared to less than 1 targeted cell per 10\(^6\) original cells in the absence of the ZFNs.\(^13\) More recently, a new approach has been described to engineer DNA-binding specificities based on transcription activator–like effector (TALE) proteins from *Xanthomonas* plant pathogens and artificial restriction enzymes generated by fusing TALEs to the catalytic domain of *FokI* was used to generate discrete edits or small deletions within endogenous human genes at efficiencies of up to 25%.\(^16\) However, while the utilization of ZFNs and TALENs to enhance gene-targeting efficiencies in hPSCs is highly promising, the approach entails custom engineering of a new nuclease for each new target locus, which requires labor, time, and resources.

Adeno-associated virus (AAV) is a nonpathogenic, non-enveloped virus containing a 4.7 kb single-stranded DNA genome that encodes the structural proteins of the viral capsid (encoded by the *cap* gene) and the nonstructural proteins necessary for viral replication and assembly (encoded by the *rep* gene), flanked by short inverted terminal repeats (ITRs).\(^17\) Recombinant versions of AAV can be created by inserting a sequence of interest in place of *rep* and *cap*, and the resulting recombinant vectors can efficiently deliver a transgene and safely mediate long-term gene expression in dividing and non-dividing cells of numerous tissues.\(^17\) Such AAV-based vectors have proven safe, efficient, and recently very effective for clinical application.\(^18,19\)
An interesting property of AAV is that, as demonstrated by Russell and colleagues, AAV vector genomes carrying gene targeting constructs can mediate HR with target loci in a cellular genome at efficiencies $10^3$-$10^4$ fold higher than plasmid constructs.\[^3\] The AAV genome’s ITRs, which apparently mediate its entry into the RAD51/RAD54 component of the cellular homologous recombination pathway, play a central role in this property.\[^20\] AAV-mediated gene targeting has been successfully applied to cells that AAV can effectively transduce, but naturally occurring AAV variants are typically inefficient at infecting a number of stem cell types, particularly hESCs.\[^21,22\]

We have implemented directed evolution – a rapid, high-throughput selection approach to create and isolate novel mutants from millions of genetic variants – to rapidly engineer AAV variants with desired gene delivery properties in the absence of the extensive mechanistic information typically required for rational biomolecular design. Recent work highlights the ability to apply directed evolution to create AAV mutants with altered receptor binding, neutralizing antibody-evasion properties, and altered cell tropism in vitro and in vivo.\[^23-29\] Likewise, we have recently demonstrated that directed evolution can yield novel AAV variants that enhance gene delivery and gene targeting in neural stem cells.\[^21\] Here we implement this approach to create new AAV mutants with the enhanced capacity to infect and, subsequently, increase the efficiency of AAV-mediated gene targeting in hPSCs, both in the presence and absence of ZFN-mediated double stranded DNA breaks.

**Materials and Methods**

**Cell Lines**

Cell lines were cultured at 37°C and 5% CO2 and, unless otherwise mentioned, were obtained from the American Type Culture Collection (Manassas, VA). HEK293T and NT2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). CHO K1 and CHO pgsA cells were cultured in F-12K medium (ATCC) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen). HSF-6 hESCs (UC San Francisco) were cultured on Matrigel-coated cell culture plates (BD, Franklin Lakes, NJ) in X-Vivo medium (Lonza, Norwalk, CT) supplemented with 80 ng/mL fibroblast growth factor (FGF)-2 (PeproTech, Rocky Hill, NJ) and 0.5 ng/mL TGF-β1 (R&D Systems, Minneapolis, MN). H1 hESCs (WiCell, Madison, WI) and dermal fibroblast- and mesenchymal stem cell (MSC)-derived iPS cells (a kind gift from George Q. Daley, Children's Hospital Boston, Boston, MA) were cultured on Matrigel-coated cell culture plates (BD) in mTeSR1 maintenance medium (Stem Cell Technologies, Seattle, WA).

**Library Generation and Viral Production**

Four replication-competent viral libraries were used as starting materials for selections on HSF-6 hESCs. A random mutagenesis library was generated by subjecting cap genes from AAV2 and AAV6 to error-prone PCR using 5’-CATGGAAGGTGCCAGACG-3’ and 5’-ACCATCGGGAGCCATACCTG-3’ as forward and reverse primers, respectively, as previously described (EP Library).\[^23\] Libraries consisting of AAV2 containing random 7mer peptide inserts\[^30\] and AAV2 containing randomized cap loop regions\[^25\] were also utilized. Finally, a library
containing shuffled DNA from the wild-type AAV1, AAV2, AAV4, AAV5, AAV6, AAV8, AAV9 cap genes was packaged. Libraries were pooled for the selection steps. Following evolution, to create recombinant versions of selected viruses, the cap genes were inserted into the pXX2 recombinant AAV packaging plasmid using Not I and Hind III. Both the replication competent AAV library and recombinant AAV vectors expressing GFP under the control of a CMV promoter were packaged using HEK293T cells using the calcium phosphate transfection method, and the viruses were purified by iodixonal gradient centrifugation and Amicon filtration. DNase-resistant genomic titers were determined via quantitative PCR.

Library Selection and Evolution

One selection step is defined as hESC infection using a starting library, rescue by addition of adenovirus serotype 5 (at levels sufficient to induce a cytopathic effect 48 hours post-adenovirus infection), and harvest of successful variants. For each selection step, cells were grown on Matrigel-coated plates to eliminate the use of feeder cells, which could become infected and bias the selection towards viral variants that transduce the feeder layer. In addition, stromal cells were removed from the culture using collagenase digestion prior to the viral harvest to prevent the selection of viral variants that infect stromal cells instead of hESCs. One round of evolution consists of genetic diversification of the cap gene followed by three selection steps. Two rounds of evolution were performed, with clonal analysis (cap gene sequencing and hESC gene delivery assay for each selected virus) performed between each round of evolution. Following the third selection step, AAV cap genes were isolated from the pool of successful AAV variants and amplified via PCR. Cap genes were then sequenced at the University of California, Berkeley DNA sequencing facility and analyzed using Geneious software (Biomatters, Auckland, New Zealand). Three-dimensional models of the AAV2 capsid (Protein Databank accession number 1LP3) were rendered in Pymol (DeLano Scientific, San Carlos, CA).

hPSC Transduction Analysis

The human pluripotent cell lines were plated at a density of 10^5 cells/well 24 hours prior to infection. Cells were infected with rAAV-GFP at an MOI 10^4. Stromal cells were removed from the hESC and the iPSC culture using collagenase digestion prior to flow cytometry. The percentage of GFP positive cells was assessed 48 hours post infection using a Beckman-Coulter Cytomics FC 500 flow cytometer (Beckman-Coulter, Brea, CA).

Immunofluorescence Staining

Immunostaining was performed to visualize the expression of pluripotency markers following AAV infection and gene targeting. HSF-6 hESCs were plated on a 24-well plate and infected as described for the transduction analysis. 48 hours post-infection, cells were fixed in 4% paraformaldehyde for 15 minutes, washed three times with phosphate buffered saline (PBS), and blocked with 1% BSA and 0.1% Triton X-100 in PBS for 30 minutes. Cells were incubated overnight at 4°C with a mouse anti-Oct-3/4 primary antibody (1:200 dilution, Santa Cruz Biotechnology) or anti-Nanog primary antibody (1:200 dilution, Santa Cruz Biotechnology). Cells were then washed three times with...
PBS and incubated with a secondary fluorescent-conjugated Alexa Fluor 647 goat anti-mouse antibody (1:250 dilution, Invitrogen) for two hours, followed by 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining (Invitrogen) for 15 minutes. Cells were imaged using a Zeiss Axio Observer.A1 inverted microscope.

**In Vitro Differentiation Assay of Pluripotency**

Colonies of HSF-6 hESCs were isolated from stromal cells by collagenase enzymatic treatment and partially dissociated by gentle pipetting. The cells were cultivated as small clusters (5-10 cells) in suspension culture in ultra low-attachment plates (Corning, Corning, NY) to generate embryoid bodies (EBs) in X-Vivo medium. After 6 days, the EBs were transferred to plates precoated with Matrigel and further differentiated for 24 days. Immunocytochemistry as described above was used to investigate whether the cells express markers of the three germ layers using the following primary antibodies: ectodermal: rabbit anti- β-III tubulin (1:500 dilution, Covance, Princeton, NJ); mesodermal: mouse anti- α-smooth muscle actin (1:500 dilution, Sigma); endodermal: rabbit anti- hepatocyte nuclear factor 3 β (1:500 dilution Millipore). Fluorescence images were acquired using a Nikon TE2000E2 epifluorescence microscope.

**In Vitro Transduction and Cell Binding Analysis**

To determine the heparan sulfate dependence of AAV2 and the selected mutant, CHO K1 and CHO pgsA cells were plated at a density of 2.5 x 10⁴ cells per well 24 hours prior to infection. Cells were infected with AAV2-GFP, AAV6-GFP, or AAV1.9-GFP at MOIs ranging from 100-2500. The percentage of GFP positive cells was assessed 48 hours post infection using a Beckman-Coulter Cytomics FC 500 flow cytometer.

To analyze cell surface binding of AAV variants, cells were plated as described for the hESC transduction analysis. Stromal cells were removed from the hESC culture via collagenase digestion, and cells were incubated at 4°C for 10 minutes. 10⁵ cells were incubated with rAAV-GFP (MOI 10⁴) at 4°C for 1 hour. Cells were washed twice with ice-cold PBS to remove unbound virus. DNase-resistant genomic titers were determined via quantitative PCR.

**Heparin Column Chromatography**

rAAV-GFP vectors were subjected to heparin column chromatography as previously described. Briefly, 10¹⁰ vector genome containing AAV particles were loaded into a 1 mL HiTrap heparin column (GE Healthcare) previously equilibrated with 0.15 M NaCl and 50 mM Tris at pH = 7.5. Washes were performed using 1 mL volumes of Tris buffer containing increasing increments of 50 mM NaCl, starting at 150 mM NaCl and ending at 750 mM NaCl, followed by a 1 M NaCl wash. Genomic titers from each fraction were determined via quantitative PCR.

**Generation of Mutant GFP hPSC Lines**

An internal ribosome entry site and puromycin resistance gene cassette (IRES-PuroR) were cloned into the Eco RI and Xho I sites of pFUGW (a kind gift from David Baltimore, California Institute of Technology, Pasadena, CA) to replace the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and yield pFUGIP. Next, a
mutant GFP sequence harboring a 35 bp insertion (GFPΔ35) (a kind gift from Matthew H. Porteus, University of Texas Southwestern Medical Center, Dallas, TX) digested with Bam HI/Eco RI was inserted in place of the GFP sequence of similarly digested pFUGIP to construct pFUGΔ35IP. The lentiviral vector carrying the mutated GFP was packaged by the calcium phosphate transient transfection method. Briefly, 10 µg of pFUGΔ35IP, 5 µg of pMDL g/p PRE, 3.5 µg of pcDNA 3 IVS VSV-G, and 1.5 µg of pRSV Rev were transfected into HEK 293T cell lines (> 70% confluency), which were cultured on 10 cm tissue culture plates under Iscove’s modified Dulbecco’s medium (IMDM) supplemented by 10% FBS and 1% penicillin/streptomycin. The lentiviral vector was harvested and concentrated by ultracentrifugation (L8-55M Ultracentrifuge, Beckman Coulter), followed by resuspension in 100 µL of PBS with 20% sucrose. Stable hPSC lines expressing the mutant GFP was generated by infection with the lentiviral vector, followed by puromycin selection (1 µg/mL) for one week. GFP fluorescence was not detected in the GFPΔ35 hPSCs, as confirmed by flow cytometry.

**Gene Targeting Assay**

The donor plasmid was generated by subcloning a truncated GFP gene missing the first 37 base pairs (t37GFP), internal ribosome entry site, and puromycin resistance gene cassette into an AAV vector plasmid (pAAV CMV GFP SN), such that the resulting targeting vector lacked a promoter. The ZFN expression constructs, driven by a CMV promoter, were generated by subcloning gene cassettes containing the engineered zinc fingers targeting GFP (also a gift from Matthew H. Porteus) into pAAV CMV GFP SN (detailed cloning steps for the donor and ZFN expressing constructs available upon request). The donor construct and the ZFN expressing constructs were then packaged into the AAV2, AAV6, and AAV1.9 capsids. All viral vectors were harvested and purified as described earlier.

For gene targeting experiments mediated by AAV, hPSCs were seeded onto 12-well tissue culture plates at a density of 10⁵ cells/well 24 hours prior to AAV infection. The cells were infected with the AAV targeting vectors at an MOI 10⁵. For targeting experiments in the presence of ZFNs, the ZFN expressing vectors were added at an MOI 10² to the cells in addition to the AAV targeting vectors. For gene targeting experiments mediated by naked plasmid constructs, Rho kinase inhibitor (ROCK inhibitor, Y-27632, CalBioChem, San Diego, CA) was added to hPSC media 24 h before electroporation. Cells were harvested using collagenase digestion, resuspended at 10⁶ cells/mL in 100 µL Bio-Rad buffer (GPEB 2) containing 1 µg each of the donor plasmid and ZFN expression plasmids, and electroporated using the Bio-Rad Gene Pulser MXcell System (250 V, 500 µF, 1000 ohms; Bio-Rad, Hercules, CA). The cells were replated on Matrigel-coated plates in hPSC culture medium supplemented with ROCK inhibitor. The medium was replaced after one day. The percentage of GFP positive cells was assessed 48 hours post infection using flow cytometry as described earlier.

**Cell Sorting and Sequencing**

Cells infected with AAV1.9 were cultured for 30 days and sorted at the UC Berkeley Cancer Center with a BD Influx Sorter to isolate GFP positive cells. For DNA sequencing analysis, cellular genomic DNA was extracted with the QIAamp DNA Micro Kit (Qiagen, Velenica, CA) and amplified using 5’-CCACCCTCGTGACCACCTG-3’
and 5'-CGGCCATGATAGACGTTGTGGC-3' primers. The resulting PCR products were cloned using the StrataClone™ PCR cloning kit (Stratagene, La Jolla, CA), and individual clones were sequenced to confirm the gene correction.

Results

Evaluation of Wild-type Serotypes

There are numerous naturally occurring AAV variants and serotypes, each of which has different protein capsids and thus somewhat different gene delivery properties for various cell types and tissues.[32] AAV serotypes 1-6 and 8 were tested to assess their potential for hESC gene delivery. Initially, HSF-6 hESCs were infected with AAV vectors carrying GFP cDNA at a multiplicity of infection (MOI) of 10,000, and 48 hours post-infection, flow cytometry was used to determine the percentage of cells that express GFP and were thus infected by AAV. The most efficient was AAV6 (Figure 1); however, its low delivery efficiency (14.75% ± 3.08% GFP positive cells) places significant limitations on the fraction of cells that express a transgene or could undergo gene targeting. These results are consistent with earlier reports showing that natural AAV serotypes are typically inefficient in gene delivery to human stem cells,[33,34] thus demonstrating the need to find, or evolve, an AAV variant capable of more efficient transduction.

Figure 1. Gene Expression in hESCs Mediated by Various AAV Serotypes. HSF-6 cells were infected with numerous natural AAV serotypes at a MOI of 10,000. Transduction efficiency was assessed as the percentage of GFP positive cells measured by flow cytometry 48 hours post-infection. Error bars indicate standard deviation (n=3). AAV, adeno-associated virus; GFP, green fluorescent protein; hESC, human embryonic stem cell; MOI, multiplicity of infection.

AAV Library Generation and Selection through Directed Evolution

The AAV capsid proteins, encoded by the cap gene, determine the virus’ ability to infect cells through their initial binding to various cell surface receptors, intracellular trafficking, and entry into the nucleus. Directed evolution is a high throughput approach that involves the creation and functional selection of libraries of genetic mutants to isolate novel variants with desirable properties. We implemented a directed evolution strategy to create AAV variants capable of mediating efficient gene delivery to hESCs (Figure 2a). Briefly, hESCs were infected with pools of virus variants created using several different techniques: a library in which AAV2 and AAV6 cap were subjected to
error-prone PCR, a “shuffled” library composed of random cap chimeras of 7 parent AAV serotypes,\textsuperscript{[24]} an AAV2 cap library with random 7-mer inserts,\textsuperscript{[30]} and AAV2 cap with substituted loop regions.\textsuperscript{[25]} Following infection with AAV libraries (Figure 2a, step 4) and amplification of the infectious AAV variants through adenovirus superinfection – as AAV requires a helper virus such as adenovirus to induce replication – (step 5), the resulting titer of AAV rescued from each library condition was quantified and compared to titers of recovered wild-type AAV2 as a metric for relative success of the selection. For each selection step, viral pools from the library produced higher viral titers than wild-type AAV2 at MOIs of both 10 and 100 (data not shown). These viral pools were then used as the starting point for the subsequent selection step (step 6). After three such selection steps, the successful viral cap genes were isolated (step 7) and tested individually to identify variants with the most efficient gene delivery. In addition, the cap genes isolated after the third selection step were subjected to an additional round of evolution, i.e. additional mutagenesis (step 8) and three selection steps, to further increase the fitness of the pool.

Increased Transduction Efficiency of the Novel Evolved AAV Variant in hESCs

Nine cap variants from each library isolated after the third selection step of the first round of evolution were fully sequenced, and the AAV capsid protein variations and the frequency with which each clone was detected in the error-prone PCR-mutagenized AAV2 and AAV6 library are shown in Table 1. The cap genes of these variants were also used to package recombinant AAV carrying the green fluorescent protein (GFP) gene, under the control of the cytomegalovirus (CMV) promoter. These recombinant AAV variants were then used to infect hESCs, and the gene delivery efficiency of each virus was measured via flow cytometry. Of the mutants isolated from the error-prone PCR-mutagenized AAV2 and AAV6 library, the variant AAV EP 1.9 (or AAV1.9), which carries a single-point mutation (R459G) (Figure 2b), showed the highest infection efficiency at 28.25% ± 1.68% GFP positive cells, representing an approximately 3-fold increase over AAV2 (its parental serotype) and a 2-fold increase over AAV6 (the best serotype) at an MOI of 10,000 (Figure 2c). Variants isolated from the shuffled, 7-mer insertion, and substituted loop region libraries were sequenced and analyzed as well. Variants from these libraries showed increased infection efficiency compared to AAV2, but not AAV6 (data not shown). At an MOI of 100,000, the variant AAV1.9 achieved a strong infection efficiency of 48.21% ± 12.92% GFP positive cells (Figure 2d). It should be noted that hESCs grow as colonies that are typically multi-layered, making accessing and infecting all of the cells in a colony difficult. However, AAV1.9 provides an increase in gene delivery efficiency, by an increase in the number of cells infected (Figure 2c), an increase in the relative fluorescence intensity of infected cells (approximately 3-fold higher than AAV6), and a significantly increased number of donor DNA molecules delivered to each cell (Appendix B Supplementary Methods and Figure B1). Interestingly, sequencing of cap variants isolated after an additional round of mutagenesis to the cap library and three additional selection steps all matched the sequence of AAV1.9 (data not shown).
<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Mutations</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP 1.1</td>
<td>S85G, R459G</td>
<td>3/9</td>
</tr>
<tr>
<td>EP 1.9</td>
<td>R459G</td>
<td>2/9</td>
</tr>
</tbody>
</table>

**Table 1.** Summary of Variants Isolated from Three Rounds of Selection against hESCs. Protein sequence features and the frequency of appearance during analysis are listed for each clone isolated from the EP AAV2/AAV6 library. AAV, adeno-associated virus; hESC, human embryonic stem cell.

AAV1.9 was isolated through multiple rounds of selection using the HSF-6 hESC line; however, the increase in gene delivery efficiency also extended to the broadly utilized H1 hESC line, as well as induced pluripotent cell lines including MSC-derived, and dermal fibroblast-derived hiPSC lines (*Figure 2e*). In addition, while AAV1.9 is capable of higher gene delivery efficiency to hESCs and hiPSCs, it is less infectious towards several cell types typically permissive to AAV infection (*Figure 2f*), indicating that in this case directed evolution yielded a variant that efficiently and somewhat selectively transduces an ordinarily non-permissive cell. Furthermore, the hESCs maintained pluripotency marker expression following infection, assessed via Nanog immunostaining (*Figure 2g*).
Figure 2. Directed Evolution of AAV and hPSC Transduction by AAV1.9  
(a) Schematic of directed evolution. 1) A viral library is created by mutating the cap gene. 2) Viruses are packaged in HEK293T cells using plasmid transfection, such that each particle is composed of mutant capsid surrounding the cap gene encoding that protein capsid. 3) Viruses are harvested from 293 cells and purified. 4) The viral library is introduced to the HSF-6 hESCs in vitro. 5) Successful viruses are amplified and recovered using adenovirus rescue. 6) Successful clones are enriched through repeated selections. 7) Isolated viral DNA reveals selected cap genes. 8) Selected cap genes are mutated again to serve as a new starting point for selection.  
(b) Molecular model of the full AAV2 capsid, based on the solved structure,[36] shows the location of the R459G mutation (blue) on the surface of the capsid (VP3 region), near the three-fold axis of symmetry and residues known to be important for heparin and FGF receptor binding (pink).  
(c) AAV mediated gene expression in hESCs. HSF-6 cells were infected with selected mutants at a MOI of 10,000. Transgene expression was assessed as the percentage of GFP positive cells measured by flow cytometry 48 hours post infection. Error bars indicate the standard deviation (n=3), * = p < 0.01.  
(d) Elevating the MOI increases transduction. HSF-6 cells were infected with AAV1.9 at MOIs of 1,000, 10,000, and 100,000. Transgene expression was assessed as the percentage of GFP positive cells measured by flow cytometry 48 hours post infection. Error bars indicate standard deviation (n=3), * = p < 0.01.  
(e) AAV1.9 mediated gene expression in hPSCs. HSF-6 hESCs, human dermal fibroblast-derived hiPSCs, human MSC-derived hiPSCs, and H1 hESCs were
infected at a MOI of 10,000. Transgene expression was assessed as the percentage of GFP positive cells measured by flow cytometry 48 hours post infection. Error bars indicate the standard deviation (n=3), * = p < 0.01. (f) In vitro analysis of AAV1.9 tropism. Variant AAV1.9 (white), selected on hESCs, is less infectious on AAV-permissive cell types (HEK293T, CHO) than recombinant AAV2 (black) and AAV6 (gray), but more infectious on HSF-6 hESCs. Error bars indicate standard deviation (n=3), * = p < 0.01. (g) HSF-6 cells infected by AAV1.9 maintain pluripotency marker expression. HSF-6 cells infected with AAV1.9 encoding GFP at an MOI of 100,000 were fixed and immunostained one week after infection for the presence of GFP (green) and the pluripotency marker Nanog (red). AAV, adeno-associated virus; FGF, fibroblast growth factor; GFP, green fluorescent protein; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; MOI, multiplicity of infection; MSC, mesenchymal stem cell.

The R459G mutation in AAV1.9 lies close to but not within the heparin or FGF receptor binding domains on the AAV capsid surface (Figure 2b), though it could conceivably modulate binding to the cell surface. To determine whether the amino acid 459 mutation alters the heparin binding properties of the variant, AAV2, AAV6, and AAV1.9 were analyzed using heparin column chromatography, which can provide a measure of the heparin binding affinity of AAV variants. Despite its mutation residing outside the reported heparin binding domain,[37] and despite the loss of a positively charged residue, AAV1.9 displays a higher affinity for heparin compared to the parental serotype AAV2, as indicated by the higher NaCl concentration needed to elute the majority of AAV1.9 from the heparin column. (Figure 3a). However, in vitro transduction of CHO and pgsA (a mutant CHO cell line lacking surface glycosaminoglycans) cells showed that AAV1.9 had less dependence on cell-surface heparan sulfate proteoglycans (AAV2’s primary receptor[38]) for cell transduction (Figure 3b). Furthermore, while it exhibited higher transduction levels, AAV1.9 bound to hESCs at levels similar to AAV2 (Figure 3c). Collectively, these data indicate that the R459G mutation may exert its effects after initial docking to the cell surface, though future studies will be needed to elucidate its mechanism of enhanced hESC infection.

![Figure 3](image-url)

**Figure 3.** Mechanistic Analysis of Transduction by Variant AAV. a) AAV1.9 has a higher affinity for heparin than AAV2 and AAV6. The heparin affinity column chromatogram of recombinant AAV2 (black), AAV6 (gray), and AAV1.9 (white) is shown, where virus was eluted from the column using increasing concentrations of NaCl. Virus was quantified using qPCR. (b) In vitro characterization of AAV1.9 HSPG dependence. CHO (black) and pgsA (white) cells were transduced to demonstrate the decrease in HSPG dependence of AAV1.9 compared to AAV2. Error bars indicate standard deviation (n=3), * = p < 0.01. (c) In vitro characterization of
AAV1.9 binding affinity. AAV1.9 and its parental serotype AAV2 bind hESCs to similar extents, yet AAV1.9 is capable of significantly higher transduction of hESCs. Error bars indicate standard deviation (n=3), * = p < 0.01.

**Enhanced Gene Targeting Efficiency of the Novel Evolved AAV Variant in hESCs**

We assessed the ability of the novel variant to mediate gene targeting in hPSCs. To do so, we employed a mutated GFP based reporter system, previously described by Zou et al.\(^1\) Briefly, GFP cDNA containing a 35 bp insertion harboring a stop codon (GFP\(\Delta\)35) was introduced into HSF-6 hESCs using a lentiviral vector (see Materials and Methods and Figure 4a). The donor construct (t37GFP) consisted of a promoter-less, non-functional GFP with a 37 bp 5’ truncation, as well as 290 nucleotides of homology upstream and 1619 nucleotides downstream of the 35 bp insertion in GFP\(\Delta\)35. The donor plasmid was packaged into recombinant AAV vectors (AAV2, AAV6, and AAV1.9), and a hESC line carrying the mutated GFP was infected with these gene targeting constructs to test their capacity to mediate gene correction and thereby restore GFP fluorescence. The levels of gene correction achieved using recombinant AAV2 and AAV6 were below the limits of detection of flow cytometry in this assay (Figure 4b), whereas the variant AAV1.9 was capable of targeted gene correction resulting in fluorescent hESCs (0.12%). Furthermore, consistent with previous reports,\(^3\) we also show that at similar efficiencies of gene delivery, AAV vectors can mediate targeted gene correction at rates much higher than plasmid constructs (Figure 4b). Moreover, as discussed earlier, AAV1.9 is also capable of higher gene delivery efficiency to H1 hESCs, and MSC- and dermal fibroblast-derived hiPSCs (Figure 2e), and we observe a corresponding increase in gene targeting efficiencies (0.06–0.12%) for these cell lines as well (Table 2).

**Introduction of Double Stranded Breaks via ZFNs Improves the Gene Targeting Efficiencies of AAV Vectors**

Directed evolution thus created a novel AAV variant that mediates high levels of gene targeting in hESCs and hiPSCs, and previous reports indicate that in addition to enhancing the ability of the donor DNA to mediate HR, complementary approaches such as inducing local double-stranded breaks at the target locus can further enhance gene targeting.\(^39\) Recent years have witnessed the development of engineered restriction enzymes generated by fusing a DNA cleavage domain either to a zinc finger or a TAL effector DNA binding domain as a general approach to generate site-specific double-stranded chromosomal breaks and thereby greatly stimulate homology-directed gene repair in a number of human cell lines.\(^10\) These findings provided rationale to explore whether stimulation of gene targeting by nuclease-mediated double-stranded breaks can be combined with an evolved AAV to further enhance gene targeting in hPSCs. We first generated AAV vectors that expressed the ZFNs from a CMV promoter to target a site 12 bp upstream of the 35 bp insertion in the GFP gene (Figure 4a).\(^40\) hPSCs harboring the mutant GFP were infected with the two AAV vectors encoding a GFP-specific ZFN, as well as the AAV donor vector (t37GFP), and the gene targeting efficiency was measured via flow cytometry to detect cells expressing the corrected GFP. In the presence of a local DSB mediated by ZFNs, we observe a 5- to 13-fold increase in AAV1.9-mediated gene targeting rates (Figure 4b and Table 2), from an already relatively high efficiency of 0.06-0.12% to 1.2-1.6% across multiple hPSC lines. Also, while not as high as with AAV1.9, using other AAV serotypes to deliver the donor
construct and ZFNs did yield measurable gene correction in all cases, indicating the generality of using AAV to deliver both donor and nuclease for enhanced HR (Figure 4b and Table 2). Finally, we observed similar increases in gene targeting efficiencies when we infected human pluripotent embryonic carcinoma cells (NT2) and human embryonic kidney (HEK293T) cells harboring the mutant GFP with AAV2 vectors encoding the GFP-specific ZFN and the donor t37GFP plasmids, indicating the general utility of combining an AAV donor with double stranded breaks at the target locus (Appendix B Figure B2).

<table>
<thead>
<tr>
<th>Human pluripotent cell line</th>
<th>% Gene targeting without ZFNs</th>
<th>% Gene targeting with ZFNs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAV2</td>
<td>AAV1.9</td>
</tr>
<tr>
<td>HSF-6 hESCs</td>
<td>ND</td>
<td>0.08±0.02 *</td>
</tr>
<tr>
<td>H1 hESCs</td>
<td>0.02±0.01</td>
<td>0.12±0.03 *</td>
</tr>
<tr>
<td>MSC-iPSCs</td>
<td>ND</td>
<td>0.06±0.01 *</td>
</tr>
<tr>
<td>hFib2-iPSCs</td>
<td>ND</td>
<td>0.06±0.02 *</td>
</tr>
</tbody>
</table>

Table 2. Summary of Gene Targeting Experiments and Rates in Various Human Pluripotent Lines in the Presence and Absence of ZFN-mediated DSBs. Values reported as mean ± standard deviation (n=3), * = p < 0.01. AAV, adeno-associated virus; hESC, human embryonic stem cell; iPSC, induced pluripotent stem cell; MSC, mesenchymal stem cell; ND, not detectable; ZFN, zinc finger nuclease.

To verify correction of the GFPΔ35 sequence, genomic DNA from "corrected" (GFP positive cells isolated using fluorescence activated cell sorting) and "uncorrected" HSF-6 hESCs was subjected to PCR to amplify the GFP sequence. The 35 bp insert of the mutated GFP cell line harbors a unique Xho I site that enabled the confirmation of GFP correction by restriction digest analysis (Figure 4c). Also, direct sequencing of the PCR fragments confirmed the correction of the mutated GFPΔ35 gene (Figure 4d). Furthermore, the GFP positive hESCs, purified via FACS, were expanded and monitored by flow cytometry for 30 days, and >95% of cells maintained GFP expression (Figure 4e). These cells also maintained a pluripotent state during long-term culture following targeted gene correction as indicated by the expression of the pluripotency marker Oct4 (Figure 4f) and their ability to differentiate into cells originating from the three germ layers via embryoid body formation in vitro (Figure 4g).
Figure 4. AAV1.9 Mediated Correction of a Non-functional GFP Expressed in hESCs. (a) Schematic overview depicting the targeting strategy for the GFP gene correction. The defective GFP gene – GFPΔ35, mutated by the insertion of a 35 bp fragment containing a translational stop codon[13]—was integrated into HSF-6 cells using a lentiviral vector. A targeting vector (t37GFP) containing a 5’ truncated GFP coding sequence was packaged into a recombinant AAV vector lacking a promoter. Homologous recombination between donor vector and integrated defective GFPΔ35 gene would correct the 35 bp mutation and result in fluorescent hESCs. (b) Gene targeting frequencies assessed as the percentage of GFP positive cells measured via flow cytometry 72 hours post infection. Error bars indicate standard deviation (n=3), * = p < 0.01. ND = not detectable by flow cytometry. (c), (d) Representative analyses of the targeted correction of GFPΔ35 gene. Genomic DNA from the “corrected” and “uncorrected” HSF-6 cells was amplified using PCR. The 35 bp mutation within the GFPΔ35 gene contains an Xho I site, and therefore only the PCR products from the corrected cells (Sample 1) can be digested using Xho I, whereas the PCR products from the uncorrected cells (Sample 2) show the restoration of the functional GFP gene without the Xho I site. DNA sequencing analysis also shows AAV1.9 mediated correction of the 35 bp mutation in HSF-6 cells originally expressing the mutated GFPΔ35 gene.
GFPΔ35 gene. (e) Time course of AAV-mediated GFPΔ35 correction in HSF-6 cells. Error bars indicate standard deviations (n=3). (f) Maintenance of an undifferentiated state of gene-corrected, GFP-expressing cells for 30 days post-infection. GFP positive HSF-6 cells, isolated via FACS, were cultured for 30 days, then fixed and probed for the presence of GFP (green), Oct4 (red), and DAPI (blue). (g) Pluripotency of the HSF-6 cells carrying the corrected GFP gene was further confirmed through embryoid body mediated in vitro differentiation to generate derivatives of all three germ layers, as indicated by the expression of the ectodermal marker β-III tubulin, the mesodermal marker α-smooth muscle actin (α-SMA), and the endodermal marker hepatocyte nuclear factor 3 β (HNF3β) after 24 days of differentiation (scale bar=100µm). AAV, adeno-associated virus; CMV, cytomegalovirus; DAPI, 4′,6-diamidino-2-phenylindole; FACS, fluorescent-activated cell sorting; GFP, green fluorescent protein; hESC, human embryonic stem cell; IRES, internal ribosome entry site; ITR, inverted terminal repeat; LTR, long terminal repeat.

Finally, in addition to measuring targeted gene correction via HR, we also assessed the frequency of random chromosomal integration of AAV1.9 vectors. HSF-6 hESCs harboring GFPΔ35 were infected with AAV1.9 donor constructs and cultured for 14 days. The genomic DNA from the infected cells was subjected to PCR to specifically amplify a 822 bp fragment that partially spanned the AAV viral genome following the puromycin resistance gene (Appendix B Figure B3a), a region that should not participate in homologous recombination (Figure 4a) and would thus represent residual AAV sequence. This assay was calibrated by conducting PCR in parallel on naïve hESC DNA samples spiked with different amounts of AAV1.9 donor plasmid, ranging from 1 copy of AAV plasmid per 400 cellular genome copies to 1 copy of AAV plasmid per 20 cellular genome copies. Based on the quantification of the samples and the standards, approximately 1 copy of AAV1.9 genome was present per 100 cells that were originally exposed to the vector (Appendix B Supplementary Methods and Figure B3b), consistent with earlier reports that demonstrate low risk of random genomic integration of AAV in human cells. To determine whether introduction of site-specific DSBs using ZFNs increases this low frequency of random integration, we co-infected hESCs carrying the mutant GFP with AAV1.9 vectors encoding the donor plasmid, as well as the GFP-specific ZFNs. We did not observe a significant increase in the levels of random integration in the presence of ZFNs (Appendix B Figure B3), which suggests that at least in this system the introduction of site-specific DSBs may not have a strong effect on non-homologous integration frequencies of AAV vectors.

Discussion

AAV has attracted increasing attention for its ability to safely and efficiently mediate gene targeting in a variety of cell types. However, prior studies have indicated that naturally occurring AAV serotypes are typically inefficient in transducing numerous classes of stem cells, which can limit also the application of AAV-mediated gene targeting to hPSCs. Recently, two studies have reported the natural serotype AAV3 as the most efficient serotype for human pluripotent lines. In the hESC and hiPSC cell lines we examined, we did not observe a higher efficiency for AAV3 relative to other serotypes (Figure 2C and data not shown). Moreover, the reported transduction efficiency for AAV3 in the earlier studies was lower than 25% at a MOI of 200,000, and likely as a result, the maximum reported gene targeting frequency was 1.3 x 10⁻⁵. Collectively, these results indicate that different natural serotypes may be suited for
different hPSC lines, but importantly that gene targeting efforts in general may benefit from increasing AAV’s delivery efficiency to hPSCs.

Directed evolution has proven to be a powerful approach to create AAV vectors with novel capabilities, and we successfully demonstrate that under appropriate selective pressures AAV can evolve for improved transduction efficiencies in human pluripotent stem cells. Specifically, we isolated a new AAV variant, AAV1.9, that exhibited an enhanced infection efficiency of approximately 48% at a MOI of 100,000. AAV1.9 harbors a single R459G mutation that lies in close proximity to both the heparin and FGF receptor binding domains of AAV2. This variant displayed a slightly higher affinity for heparin compared to AAV2 (Figure 3a), though we observed similar levels of AAV1.9 and AAV2 binding to hESCs (Figure 3c). In their investigation of AAV2’s heparin binding domain, Opie et al. found that a R459A AAV2 mutant was incapable of infecting HeLa cells, despite heparin binding levels essentially the same as AAV2.[43] The authors hypothesized that the R459A mutation may result in a defect in a later stage of viral infection.[43] This finding, in conjunction with our observations that similar percentages of AAV2 and AAV1.9 bind to hESCs, suggests that the R459G mutation may increase hESC gene delivery by modulating viral infection at a point following initial cell surface docking, for example AAV structural rearrangement during heparan sulfate binding,[44] subsequent binding to secondary receptors, or cellular entry. Further studies are needed to elucidate such mechanisms.

An AAV variant with enhanced gene delivery to human embryonic stem cells can serve as a valuable tool for numerous applications. For example, AAV could mediate the controlled overexpression of regulatory proteins, shRNAs, or microRNAs, which would enable basic investigations of the roles of key factors in stem cell self-renewal or lineage commitment, as well as therapeutic applications that rely on the efficient generation of specific cell lineages. AAV has the potential to remain episomal and thereby mediate transient expression of such factors, compared to typically constitutive expression from lentiviral vectors, which may in some cases be advantageous. Furthermore, increased gene delivery efficiencies afford the opportunity to deliver multiple cargos effectively. For example, a 2-fold increase in the delivery of one AAV vector would translate to an 8-fold increase in the number of cells that would be transduced with three vectors carrying three different transgenes (such as the gene targeting construct and two ZFN-encoding vectors utilized in this study). Another consequence of generating AAV variants with higher transduction efficiencies in stem cells is the corresponding increase in gene-targeting efficiencies. When AAV1.9 was used to mediate gene correction in HSF-6 hESCs, we observed gene-targeting frequencies of $8 \times 10^{-9}$, approximately 10-fold higher than the reported targeting frequencies mediated by naturally occurring AAV serotypes.[33,34] We have also observed that AAV1.9 allows for increased gene delivery and gene targeting efficiencies to the H1 hESC line, as well as dermal fibroblast- and MSC-derived iPSCs, thus establishing the generality of the method.

It was previously shown that AAV gene targeting could be enhanced with a double stranded DNA break introduced at the target locus by the nuclease I-SceI,[39,41] and our results build upon this important prior work to show for the first time that AAV can function effectively in conjunction with zinc finger nucleases, which can now be engineered for genomic site specificity.[10,12,13] In addition to ZFNs, transcription activator–like effector (TALE) truncation variants have recently been linked to the
catalytic domain of Fok I to yield a new class of nucleases that can generate discrete edits or small deletions within endogenous human genes and induce gene modification in human cells by both non-homologous end joining and HR. Alternatively, the DNA recognition properties of homing endonucleases can be reengineered to yield enzymes that recognize endogenous genes in human cells. Such custom nucleases can be beneficial, as prior studies indicate that gene targeting frequencies vary from locus to locus and are lower at non-expressed genes, and the targeting efficiencies in this study may have benefitted from the use of a lentiviral vector – which preferentially integrates into active transcription units – as the target locus. Furthermore, researchers have observed that gene correction, gene knock-in, and knock-out vectors exhibit varying targeting frequencies. However, depending on the application, it is important to note that an appropriate AAV has the potential to mediate gene targeting at a given locus at rates that are sufficiently high to obviate the time, labor, and resources entailed in generating a custom nuclease. Furthermore, as ZFNs and other nucleases have the potential to exhibit off-target cleavage, the use of effective donor DNA in the absence of DSBs offers certain advantages.

In conclusion, we have used directed evolution to create a novel AAV variant with a single-point mutation that exhibited enhanced gene delivery efficiency, and subsequently gene targeting, in hPSCs. In addition, this work demonstrates that AAV-mediated gene-targeting frequencies can be further enhanced in the presence of targeted double-stranded breaks generated by the AAV vector co-delivery of ZFNs. These findings suggest that AAV vectors may find strong utility in investigations of stem cell biology and therapy, ranging from the generation of reporter cell lines to therapeutic gene correction.

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References


CHAPTER 6

ADENO-ASSOCIATED VIRAL VARIANT R3.45
ENHANCES SELECTIVE GENE DELIVERY TO NEURAL
STEM CELLS IN VIVO

Abstract

Neural stem cells are defined by their ability to self-renew and to differentiate into mature neuronal and glial cell types. Because of these properties, neural stem cells present interesting targets for gene and cell replacement therapies for neurons lost to injury or disease. Unfortunately, most gene delivery vectors are not capable of either efficient or specific gene delivery to neural stem cells in vivo. Vectors based on adeno-associated virus (AAV) present a number of advantages, including safety and efficiency for several important cell types, and have thus been utilized in many clinical trials. However, natural AAV variants are inefficient in transducing NSCs. We previously applied directed evolution, a high-throughput molecular engineering approach, to create an AAV variant capable of efficient transduction of adult neural stem cells in vitro. To build upon the in vitro success of this variant, we examined the in vitro and in vivo specificity of this novel AAV variant for neural stem cells. Variant r3.45 was more specific for neural stem cells than mature neurons in a human embryonic stem cell-derived culture containing a mixture of cell types including neural stem cells and neurons. Furthermore, variant r3.45 was capable of more efficient and specific transduction of both rat and mouse neural stem cells in vivo compared to natural AAV serotypes following intracranial vector administration. Delivery of a constitutively-active β-catenin transgene demonstrated that variant r3.45 can be used to study mechanisms through which key regulatory genes are acting in NSCs and that r3.45 can be used to deliver transgenes that can induce changes in the NSC population in the hippocampus.

Introduction

Neural stem cells (NSCs) are a type of adult stem cell characterized by their ability to self-renew and differentiate into different neural cell types, including neurons, astrocytes, and oligodendrocytes. In the adult brain, active neural stem cell populations exist only in the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus of the hippocampus. Efficient and specific gene delivery may be a way to study the function of various genes in the differentiation of neural stem cells in their natural in vivo environment by tracking of stem cell fate or elucidate basic mechanisms that regulate the quiescence, proliferation, self-renewal, and differentiation of the NSCs. This knowledge would add to the significant progress has been made toward understanding the mechanisms regulating adult neurogenesis and may provide an alternative therapeutic option to cell replacement therapy. For example, a gene delivery vector can be used to stimulate endogenous pathways through overexpression or knock-down of genes to differentiate neural stem cells into a specific type of neuron damaged by disease. Since neurogenesis is an important process that continues throughout the life of a mammal, neural stem cells present an interesting target for gene therapy and a source of replacement cells for neurons lost to injury or disease. Gene delivery to neural stem cells
can be used as a gene therapy option to express genes and secrete neurotrophic factors to protect against neurodegenerative diseases.\[3\] As an example, restoration of fragile X mental retardation protein expression specifically in adult NSCs rescues mice from learning deficits in a murine model of fragile X syndrome.\[4\]

Several attempts have been made to deliver genes to neural stem cells in vivo. Falk et al. administered polyethyleneimine (PEI) complexes, containing plasmids driving reporter gene expression via enhancer elements from the second intron of the human nestin gene, to the lateral ventricle of mice to show specific targeting to neural stem cells in the SVZ.\[5\] However, the efficiency was limited. Another study by Lemkine et al. using PEI-DNA complexes showed low specificity towards mouse SVZ neural stem cells compared to globular cells following delivery to the lateral ventricle.\[6\] Van Hooijdonk et al. used a vesicular stomatitis virus G glycoprotein-pseudotyped lentivirus to target neural progenitor cells and immature neurons in the sub-granular zone of the dentate gyrus of the mouse hippocampus. Although the lentivirus preferentially targeted neuronal progenitor cells and immature neurons in the subgranular zone, only 11% of cells infected with the virus were nestin positive one week after administration.\[7\]

Adeno-associated virus (AAV) is a nonpathogenic parvovirus composed of a 4.7 kb single-stranded DNA genome within a non-enveloped, icosahedral capsid.\[8\] The genome contains three open reading frames (ORF) flanked by inverted terminal repeats (ITR) that function as the viral origin of replication and packaging signal.\[8\] The rep ORF encodes four nonstructural proteins that play roles in viral replication, transcriptional regulation, site-specific integration, and virion assembly.\[8\] Additionally, the cap ORF encodes three structural proteins that assemble to form a 60-mer viral capsid.\[8\] In recombinant versions of AAV, a gene of interest is inserted between the ITRs in place of rep and cap, and the latter are provided within a plasmid in trans along with helper viral genes during vector production.\[9\] The resulting vector can transduce both dividing and non-dividing cells, resulting in stable transgene expression in the absence of helper virus for years in post-mitotic tissue. There are eleven naturally occurring serotypes and over 100 variants of AAV, each of which differs in amino acid sequence, and thus in their gene delivery properties.\[10,11\] Importantly, no AAV has been associated with any human disease, making recombinant AAV attractive for clinical translation.\[8\] Directed evolution is a high-throughput molecular engineering approach that has been successfully utilized to generate AAV variants with altered receptor binding, neutralizing antibody-evasion properties, and altered cell tropism.\[12-15\] The approach emulates the process of natural evolution, in which repeated genetic diversification and selection enable the accumulation of key mutations or genetic modifications that progressively improve a molecule’s function, even without knowledge of the underlying mechanistic basis for the problem. Recently, directed evolution was applied by Jang et al. to isolate a variant capable of efficient transduction of adult neural stem cells in vitro.\[16\] Selections on NSCs isolated from the adult rat hippocampus yielded r3.45, an AAV2 variant containing a peptide insertion that mediated 50-fold increased transduction of rat NSCs, as well as increased transduction of murine NSCs, human fetal NSCs, and human embryonic stem cell (hESC) derived neural progenitor cells.\[16\] In addition, the variant exhibited a 5-fold increased rate of targeted gene correction compared to natural serotypes in NSCs.\[16\]

Although a large portion of currently reported AAV variants developed through directed evolution utilized in vitro selections, some of these variants have demonstrated
success when translated to an in vivo model. For example, two variants evolved by Koerber et al. for the ability to infect primary human astrocytes in culture also transduced 3.3- and 5.5-fold more astrocytes, relative to neurons, than AAV2 within the striatum following intracranial injection in rats.\textsuperscript{[17]} In vivo analysis of another variant from the astrocyte selection revealed that this variant was capable of highly specific and efficient infection of Müller cells (a retinal glial cell type) compared to AAV2 and AAV6.\textsuperscript{[18]} The success of AAV variants created through in vitro selection to evade neutralization by human antibodies has also translated to increased antibody evasion in a mouse model of immunity (see Chapter 4).

Based on these successes, we investigated the in vivo transduction properties of AAV variant r3.45 and demonstrated this variant’s utility as a gene therapy tool. r3.45 has demonstrated efficient and specific transduction of adult mouse, rat, and human neural stem cells, both in vitro and in vivo. In addition, utilizing r3.45 as a vector to deliver constitutively-active β-catenin to NSCs in the mouse hippocampus enabled the study of the mechanism of action through which activation of the β-catenin pathway increases neurogenesis.

Materials and Methods

\textit{Viral Production}

HEK293T cells, obtained from the American Type Culture Collection (Manassas, VA), were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C and 5% CO\textsubscript{2}. Recombinant AAV vectors expressing green fluorescent protein (GFP) or firefly luciferase under the control of a CMV promoter were packaged using HEK293T cells using the calcium phosphate transfection method, and the viruses were purified by iodixonal gradient centrifugation and Amicon filtration. DNase-resistant genomic titers were determined via quantitative PCR.\textsuperscript{[12,14]}

\textit{In Vitro Infection of Human Embryonic Stem Cell-derived Mixed Neuronal Cultures}

H1 human embryonic stem cells (WiCell, Madison, WI) were cultured on Matrigel-coated cell culture plates (BD Biosciences, Franklin Lakes, NJ) in mTeSR1 maintenance medium (Stem Cell Technologies, Seattle, WA) for growth and expansion. To initiate cortical differentiation of hESCs, cells were seeded in adherent conditions at a density of 5×10\textsuperscript{4} cells/cm\textsuperscript{2} in growth medium. At 50% confluence, the medium was gradually changed to neural basal medium (Invitrogen) containing N2 and B27 supplements (Invitrogen). SMAD signaling inhibitors LDN193189 (1 µM, Stemgent, Cambridge, MA) and SB432542 (10 µM, Tocris Biosciences, Bristol, UK) were added for the first week of neural induction. Cyclopamine (400 ng/ml, Calbiochem, San Diego, CA) and FGF-2 (10 ng/ml, Peprotech, Rocky Hill, NJ) were added on days 3-14 of differentiation. After 12-14 days, cells were mechanically passaged into poly-L-ornithine (Sigma Aldrich, St. Louis, MO) and laminin (20µg/ml, Invitrogen) coated plates and allowed to undergo maturation for 3-6 weeks. BDNF (10ng/ml, Peprotech) was added to cultures one week after initiation of neuronal maturation.

Cells were infected at an approximate MOI of 10,000 with recombinant AAV2, AAV6, or r3.45 vectors encoding GFP. 48 hours post-infection, cells were fixed in 4% paraformaldehyde for 15 minutes, washed three times with phosphate buffered saline,
and blocked with 1% BSA and 0.1% Triton X-100 in phosphate buffered saline for 30 minutes. Cells were incubated overnight at 4°C with a mouse anti-Nestin (1:500 dilution, Abcam) or mouse anti-MAP2 primary antibody (1:500 dilution, BD Biosciences). Cells were then washed three times with phosphate buffered saline and incubated with a secondary fluorescent-conjugated donkey anti-mouse antibody (1:250 dilution, Invitrogen) for two hours. Cells were imaged using an Axio Observer.A1 inverted microscope (Zeiss). Quantification of the infected cells was performed using the Cell Counter function in ImageJ.

**Stereotaxic Injections**

Recombinant AAV2, AAV4, AAV6, or r3.45 vectors encoding GFP were stereotaxically injected into the hippocampus (AP, -3.5; ML, +/- 2.0; V/D, -3.5) of 12 week old female Fischer 344 rats. The animals were anesthetized with ketamine (Butler Animal Health Supply, San Leandro, CA – 68 mg/kg animal) and xylazine (Lloyd Laboratories, Shenandoah, Iowa – 38 mg/kg animal) prior to injection, and 3 µL of 5x10⁸ vg/µL AAV vector per hippocampus was injected using a Hamilton syringe as described. Recombinant AAV2, AAV6, or r3.45 vectors encoding GFP were stereotaxically injected into the hippocampus (AP, -2.12; ML, +/- 1.5; V/D, -1.55) of 9 week old female Balb/c mice. The animals were anesthetized with ketamine (Butler Animal Health Supply, San Leandro, CA – 50 mg/kg animal) and xylazine (Lloyd Laboratories, Shenandoah, Iowa – 50 mg/kg animal) prior to injection, and 1 µL of 1.5x10⁹ vg/µL AAV vector per hippocampus was injected using a Hamilton syringe as described. Mice were injected with 50 mg/kg 5-Bromo-2′-deoxyuridine (BrdU) for three consecutive days pre-stereotaxic injection, then injected with 50 mg/kg BrdU every other day until perfusion. Three weeks post-injection, animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Animal protocols were approved by the UCB Animal Care and Use Committee and conducted in accordance with National Institutes of Health guidelines. Brains were post-fixed in 4% paraformaldehyde overnight at 4°C and stored in 30% sucrose for cryoprotection.

**Histological Processing and Immunohistochemistry of Brain Tissue**

Brains were mounted onto a Series 8000 sliding microtome (Bright, Huntington, UK) with Clear Frozen Section Compound (VWR, Radnor, PA) and frozen with dry ice. Coronal sections (40 µm) were cut, and sections containing the hippocampus were stored at -20°C prior to immunostaining. Brains were stained as floating sections in a 12-well dish. Tissue sections were washed three times for 15 minutes each in phosphate buffered saline, then blocked in a solution containing 3% donkey serum and 0.3% Triton X-100 for 2 hours at room temperature. After blocking, tissue sections were incubated with primary antibodies for 72 hours at 4°C. The primary antibodies used were as follows: mouse anti-Nestin (1:500 dilution, Abcam, Cambridge, MA), rabbit anti-Sox2 (1:250 dilution, Millipore, Billerica, MA), mouse anti-NeuN (1:100 dilution, Millipore), guinea pig anti-Doublecortin (1:1000 dilution, Millipore), mouse anti-Glial Fibrillary Acidic Protein (1:1000 dilution, Advanced ImmunoChemical, Long Beach, CA), rabbit anti-Glial Fibrillary Acidic Protein (1:1000 dilution, Abcam), rabbit anti-S100β (1:1000 dilution, Abcam), and chicken anti-Green Fluorescent Protein (1:1000 dilution, Abcam). Tissue sections were washed again three times for 15 minutes each in phosphate buffered
saline, then blocked in a solution containing 3% donkey serum and 0.3% Triton X-100 for 1 hour at room temperature. After blocking, tissue sections were incubated with AffiniPure donkey anti-mouse, rabbit, guinea pig, and chicken secondary antibodies (1:250 dilution, Jackson ImmunoResearch, West Grove, PA) and 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (Invitrogen) for 2 hours at room temperature. Tissue sections were washed three more times in phosphate buffered saline and mounted onto slides then coverslipped. Images of the sections were taken using an LSM 710 laser scanning confocal microscope (Zeiss, Thornwood, NY). Quantification of the infected cells within the sections was performed using an Axio Imager.M1 microscope and Analysis System (Zeiss) and Stereo Investigator Analysis Software, version 8.26, (MBF Bioscience, Williston, VT). Cells were scored using the following markers: Type I NSCs (nestin+/GFAP+), Type II NSCs (nestin+Sox2+), neurons (NeuN+ or DCX+), astrocytes (GFAP+/S100β+).

**Systemic Injections**

10¹⁰ viral genomes of recombinant AAV2, AAV6, or r3.45 vectors encoding luciferase were administered via tail vein injection into 9 week old female Balb/c mice. Four weeks post-injection, mice were perfused with .9% saline solution. Heart, liver, lungs, kidney, spleen, brain, spinal cord, and hind limb muscle were harvested and flash-frozen for *in vitro* luciferase analysis.

**In Vitro Luciferase Analysis**

Frozen tissue samples were homogenized and resuspended (approximately 50 mg of tissue) in reporter lysis buffer (Promega, Mannheim, Germany) for *in vitro* luciferase analysis. Lysate containing luciferase was clarified by centrifugation for 10 minutes at 10,000g. To assay the samples, 20 µL of the lysate was added to 100 µL of the luciferase assay buffer, mixed, incubated for 5 minutes, and placed in the luminometer. The signal was integrated for 30 seconds with a 2 second delay and was reported in Relative Light Units (RLU) detected by a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). The luciferase signal was normalized to the total protein content determined by a bicinchoninic acid assay (Pierce, Rockford, IL).

**Results**

*AAV Variant r3.45 Enables Increased Specificity Towards hESC-derived Neural Stem Cells In Vitro*

A previous report by Jang *et al.* described the creation of a novel AAV2 variant r3.45, which contained an LATQVGQKTA insertion at amino acid 587 and a V719M mutation. The variant mediated enhanced gene delivery to rat, mouse, and human neural stem cells *in vitro* compared to several wild-type AAV serotypes; however, efficiency is distinct from selectivity. To test the specificity of r3.45 for neural stem cells, a human embryonic stem cell-derived culture containing a mixture of cells including neural stem cells, neurons, and astrocytes was infected with variant r3.45, wild-type AAV2 (parental serotype), or wild-type AAV6 (best wild-type serotype *in vitro*) vectors carrying GFP cDNA. Cells were infected at an MOI of 10,000, and transduction was assessed 48 hours post-AAV administration. Of the cells infected in each culture, the percentage staining positive for the neural stem cell marker nestin was significantly
higher for variant r3.45 compared to wild-type AAV2 and AAV6 (Figure 1). Although other cell types in the culture were infected, only specificity towards NSCs and mature neurons was examined. Furthermore, variant r3.45 was the only virus to display selectivity towards neural stem cells compared to neurons (Figure 1). In conjunction with the *in vitro* data reported by Jang *et al*., this experiment shows that variant r3.45 is capable of generalized neural stem cell infection, but selectivity towards neural stem cells compared to neurons.

![Figure 1](image)

**Figure 1.** Specificity Towards hESC-derived Neural Stem Cells In Vitro Mediated by Various AAV Serotypes. (a) Representative images of areas of hESC-derived neuronal differentiation cultures containing neural stem cells (top row – red) or mature neurons (bottom row – red), 48 hours post-infection with recombinant AAV2 (first column), AAV6 (second column), or r3.45 (third column) vectors expressing GFP (green). (b) The percentage of GFP+ cells co-staining for Nestin or MapIIb was quantified to determine the specificity of each viral vector. Error bars indicate standard deviation (n=3), * = p < 0.01, ** = p < 0.005. AAV, adeno-associated virus; GFP, green fluorescent protein; hESC, human embryonic stem cell.
**AAV Variant r3.45 Enables Increased Specificity Towards and Infectivity of Adult Neural Stem Cells in the Rodent Brain**

Several AAV variants created via *in vitro* directed evolution systems have demonstrated success when translated to an *in vivo* model.\(^{[17, 18]}\) Since r3.45 has exhibited infectivity of and selectivity towards neural stem cells *in vitro*, we investigated its transduction properties *in vivo*. As shown by Koerber *et al.*, variants selected against rat astrocytes in culture were more efficient at transducing rat astrocytes *in vivo* than variants selected against human astrocytes (although the human astrocyte variant was still more efficient than wild-type AAV2).\(^{[17]}\) As variant r3.45 was evolved to infect adult rat hippocampal neural stem cells *in vitro*, r3.45 was first administered to the dentate gyrus of the rat hippocampus via intracranial injection. Compared to wild-type AAV2, AAV4, and AAV6, a considerably higher percentage of cells infected by variant r3.45 expressed the neural stem cell marker nestin (Figure 2a, 2b). Specifically, approximately 65% of the cells infected by variant r3.45 were neural stem cells, compared to approximately 33% neurons and 1% astrocytes (Figure 2b), demonstrating that variant r3.45 selectively infects neural stem cells. Variant r3.45 also infected a larger fraction of the resident neural stem cells in the subgranular zone (approximately 60%) than any of the wild-type AAV serotypes tested, indicating that it is also capable of efficient transduction of neural stem cells *in vivo* (Figure 2c).
Figure 2. Specificity Towards and Infectivity of Adult Neural Stem Cells in the Rat Brain Mediated by Various AAV Serotypes. (a) Representative images at low (20x) and high (100x) magnification of the rat dentate gyrus three weeks post-injection of recombinant AAV2 (first column), AAV4 (second column), AAV6 (third column), or r3.45 (fourth column) vectors expressing GFP (green). Brain sections were co-stained with Nestin (top row – red), NeuN (middle row – red), or GFAP (bottom row – red) and DAPI (blue), and infected cells of each type are marked with a white arrow. (b) The percentage of GFP+ cells co-staining for markers of each cell type was quantified to determine the specificity of each viral vector. (c) The percentage of Nestin+ cells infected by each viral vector was quantified to determine the infectivity of neural stem cells. Error bars indicate standard deviation (n=3), * = p < 0.01, ** = p < 0.005, *** = p < 0.001. AAV, adeno-associated virus; GFP, green fluorescent protein.

Based on these results in rat brain in vivo, administration to the mouse dentate gyrus of the hippocampus via intracranial injection was also investigated. Three weeks post-AAV administration, variant r3.45 selectively transduced murine neural stem cells relative to wild-type AAV2 and AAV6, but it appeared to transduce neurons and neural stem cells with similar selectivity (Figure 3a, 3b). In the developing brain, neural stem cells expand the stem cell pool through symmetric self-renewal, then generate neurons and glial cells through asymmetric cell division to form one neuron (or glial cell) and one stem cell. However, since NSCs continuously undergo differentiation into neurons over a period of days to weeks, it is possible that GFP+ neurons visualized after 3 weeks originated from cells that were NSCs at the time of AAV injection. Therefore, to determine whether this change in specificity was due to variant r3.45 being more specific for rat neural stem cells or the more rapid stem cell differentiation time scale in mice, animals were injected with BrdU to label proliferating stem cells that subsequently differentiated. Of the infected neurons present three weeks post-injection, a significantly higher percentage were BrdU+ (Figure 3c), indicating that these cells could have been neural stem cells at the time of infection. To explore the timing of gene expression and NSC differentiation in greater detail, mice were sacrificed three days, one week, and two weeks post-injection of r3.45. At all three earlier time points, a significantly higher percentage of neural stem cells was infected relative to neurons (Figure 3d), providing more evidence that variant r3.45 specifically infects neural stem cells in the mouse hippocampus. Consistent with the results from administration to rats, variant r3.45 infected approximately 38% of mouse neural stem cells in the subgranular zone in vivo, a larger percentage than the wild-type AAV serotypes tested, showing that efficient transduction of neural stem cells is conserved across rodent species (Figure 3e).
Specificity Towards and Infectivity of Adult Neural Stem Cells in the Mouse Brain Mediated by Various AAV Serotypes. (a) Representative images at low (20x) and high (100x) magnification of the mouse dentate gyrus three weeks post-injection of recombinant AAV2 (first column), AAV6 (second column), or r3.45 (third column) vectors expressing GFP (green). Brain sections were co-stained with Nestin (top row – red), NeuN (middle row – red), or GFAP (bottom row – red) and DAPI (blue), and infected cells of each type are marked with a white arrow. (b) The percentage of GFP+ cells co-staining for markers of each cell type was quantified to determine the specificity of each viral vector. (c) The percentage GFP+ cells co-staining for NeuN (a neuronal marker) or NeuN and BrdU (a cellular division marker) were analyzed to determine the amount of GFP+ neurons that had differentiated from infected neural stem cells prior to sacrifice. (d) The percentage of GFP+ cells co-staining for markers of each cell type was quantified to determine the specificity of variant r3.45 three, seven, and fourteen days post-injection. (e) The percentage of Nestin+ cells infected by each viral vector was quantified to determine the infectivity of neural stem cells. Error bars indicate standard deviation (n=3), * = p < 0.01, ** = p < 0.005, *** = p < 0.001. AAV, adeno-associated virus; GFP, green fluorescent protein.

In Vivo Localization of AAV Variant r3.45 Following Intravenous Administration

To date, only one AAV serotype has demonstrated the capacity to cross the blood-brain barrier in adult mammals following intravenous administration.[25] Foust et al. showed that AAV9 could cross the blood-brain barrier in neonatal and adult mice. However, the route of administration appeared to alter the tropism of the virus. For example, direct hippocampal injection of AAV9 resulted in high neuronal transduction, while intravenous injection of AAV9 resulted in astrocyte and vascular transduction.[25] Given the specific and efficient neural stem cell tropism following intracranial injection of r3.45, tropism was assessed following intravenous injection to determine if neural stem cells could still be reached. The tissue localization data for AAV2 and AAV6 agree well with reported biodistribution in mice.[26] r3.45 did not reach the brain or spinal cord at a higher level than wild-type AAV2 or AAV6 (Figure 4). Interestingly, however, liver and heart transduction was improved over AAV2. The 7mer peptide insertion in the heparin-binding domain of r3.45 was shown to alter the capsid’s affinity to heparin sulfate,[16] shifting the affinity of r3.45 from the wild-type AAV2 affinity towards the AAV6 heparin sulfate affinity. This may help to explain increase in heart, lung, and muscle tropism by r3.45 compared to AAV2, as the gene expression in these tissues by r3.45 appears to be higher than AAV2 but lower than AAV6.
**β-Catenin Increases Neurogenesis Through Both Proliferation and Differentiation of Neural Stem Cells**

The Wnt signaling pathway functions in cell-cell communication in the both the embryo and adult and has been shown to play a role in stem cell proliferation and differentiation during development and healing. The canonical Wnt pathway involves the activation of β-catenin, which translocates to the nucleus following activation to act as a transcriptional cofactor of key transcriptional targets. Previous work has identified the canonical Wnt pathway as the principal regulator of adult hippocampal neurogenesis, although did not identify whether the signaling pathway functioned through proliferation or differentiation of NSCs. Due to the ability of r3.45 to specifically and efficiently infect NSCs in the mouse hippocampus, we used this vector to deliver constitutively-active β-catenin to NSCs in the mouse hippocampus to study the mechanism of action through which activation of the β-catenin pathway increases neurogenesis. Increases in both proliferation and differentiation (although not large) appear to have a cumulative effect to significantly increase the number of new mature neurons in the brain (Figure 5), indicating that the Wnt pathway functions through both mechanisms to induce neurogenesis. In addition, this study acts as a proof-of-principle that r3.45 can be used to deliver transgenes that can induce changes in the NSC population in the hippocampus.
Figure 5. Proliferation and Differentiation of Neural Stem Cells Activated by β-Catenin. Representative images at low (20x) magnification of the mouse dentate gyrus three weeks post-injection of recombinant r3.45 vectors expressing (a) GFP (green) or (b) constitutively active β-Catenin-2xFLAG (green). Brain sections were co-stained with NeuN (blue), and EdU (red), and infected cells co-staining for NeuN and EdU are marked with a white arrow. (c) Infected cells (FLAG+, β-Catenin condition; GFP+, control condition) co-staining for EdU (a cellular division marker), NeuN (a neuronal marker) or EdU and NeuN and were analyzed to determine the degree to which β-Catenin stimulates proliferation and differentiation of neural stem cells. Error bars indicate standard deviation (n=3), * = p < 0.01, ** = p < 0.005. GFP, green fluorescent protein.

Discussion

Adult neural stem cells, defined by the capacities for self-renew and differentiate into mature neural cell types of the brain, play an important role in neurogenesis throughout the life of an organism. Specific and efficient gene delivery to NSCs in vivo provide an opportunity to better study basic mechanisms that regulate the quiescence, proliferation, self-renewal, and differentiation of the NSCs in their in vivo environment, as well as make NSCs an attractive gene therapy target to treat CNS injury or disease. The attempts made to deliver genes to neural stem cells in vivo using polymer-DNA complexes or lentiviruses have had limited success.[5-7] AAV gene vectors have become a more frequent choice for clinical gene delivery applications due to recent success in clinical trials to treat retinal degeneration.[29-32] AAV variant r3.45 was reportedly capable of efficient transduction of rat, mouse, and human neural stem cells in vitro.[16] Evidence exists of previous AAV variants developed by in vitro directed evolution systems that also have demonstrated success when translated to in vivo models of gene delivery to astrocytes and retinal glial and evasion of neutralizing antibodies.[17,18]

In a human embryonic stem cell-derived culture containing a mixture of cells including neural stem cells, neurons, and astrocytes, the percentage of cells infected by variant r3.45 that were neural stem cells was significantly higher compared to wild-type AAV. Upon characterization of the in vivo properties of r3.45, it was discovered that this variant is also capable of efficient and specific infection of neural stem cells in both the adult rat and mouse brains. Approximately 65% of the cells infected in the rat hippocampus by variant r3.45 were neural stem cells three weeks post-injection, and approximately 60% of neural stem cells were transduced. This trend continued in the mouse brain, where approximately 38% of neural stem cells were transduced in the hippocampus three weeks post-injection.

In conclusion, AAV variant r3.45 has demonstrated efficient and specific transduction of adult mouse, rat, and human neural stem cells, both in vitro and in vivo. The
characterization of variant r3.45 has lead to the discovery of a gene delivery vector that could enable further studies of neurogenesis in the adult brain. Furthermore, the ability to efficiently transduce neural stem cells may allow their use in novel gene therapy and cell replacement therapy applications.

References
CHAPTER 7

ENHANCED GENE TARGETING OF ADULT AND PLURIPOTENT STEM CELLS USING EVOLVED ADENO-ASSOCIATED VIRUS

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Abstract
Efficient approaches for the precise genetic engineering of stem cells can enhance both basic and applied stem cell research. Adeno-associated virus (AAV) vectors have demonstrated high efficiency gene delivery and gene targeting to numerous cell types, and AAV vectors developed specifically for gene delivery to stem cells have further increased gene targeting frequency compared to plasmid construct techniques. This chapter details the production and purification techniques necessary to generate adeno-associated viral vectors for use in high efficiency gene targeting of adult or pluripotent stem cell applications. Culture conditions used to achieve high gene targeting frequencies in rat neural stem cells and human pluripotent stem cells are also described.

1. Introduction
Gene targeting manipulates an endogenous gene through the use of homologous recombination (HR) to swap a modified sequence in place of the host DNA. This engineering of a human stem cell’s genome by exploiting the natural ability of cells to perform homologous recombination has broad applications and implications, including safe harbor integration of genes for basic or therapeutic application, creating in vitro models for investigating human development and disease, and high-throughput drug discovery and toxicity studies.[1,2] Furthermore, high efficiency gene targeting to stem cells can aid in the study of specific genes involved in stem cell self-renewal and differentiation into therapeutically relevant lineages.

Adeno-associated virus (AAV) is a nonpathogenic, nonenveloped virus containing a 4.7 kb single-stranded DNA genome. Recombinant versions of AAV can be created by replacing the viral genome with a gene or sequence of interest and providing the viral genome in trans along with helper viral genes during vector production.[3] The resulting recombinant vectors can efficiently deliver a transgene and safely mediate long-term gene expression in dividing and nondividing cells of numerous tissues.[4] Such AAV-based vectors have proven safe, efficient, and recently very effective for clinical applications,[5-7] making them an attractive option for both basic and translational research. Importantly, Russell and colleagues have discovered that the secondary
structure of AAV vector genomes carrying gene targeting constructs can mediate HR with target loci in a cellular genome at efficiencies $10^3$-$10^4$-fold higher than genes of interest delivered using plasmid constructs.\[^8\] This capacity would be further enabled through the development of vectors that can deliver these gene targeting constructs to the nuclei of target cells with high efficiency.

Using directed evolution, we have created AAV variants capable of enhanced gene delivery and gene targeting in both adult and pluripotent stem cells. In particular, Jang et al. developed an AAV2 variant that demonstrated enhanced gene delivery and targeting in rat neural stem cells (NSCs). Additionally, this variant exhibited increased transduction of murine NSCs, human fetal NSCs, and human embryonic stem cell-derived neural progenitor cells,\[^9\] which should additionally translate to enhanced gene targeting frequencies in these cell types. Furthermore, Asuri et al. developed an AAV variant that demonstrated enhanced gene targeting within several human embryonic stem cell (hESCs) and human induced pluripotent stem cell (hiPSCs) lines.\[^10\] Here, we describe the techniques required for efficient gene targeting of neural stem cells or pluripotent stem cells using these AAV vectors.

The materials and methods presented in the following sections detail the production and purification of high titer AAV, as well as culture conditions for rat neural stem cells and human pluripotent stem cells that will enable high efficiency gene targeting. Another important aspect of the high efficiency gene targeting – the design of the gene targeting construct – will not be discussed. Research has shown that use of gene targeting constructs with longer homology arms and placement of a mutation in the center of the gene targeting construct, along with the secondary structure of the AAV inverted terminal repeats that facilitate the recruitment of host cell recombination or repair enzymes, can increase the frequency of gene targeting events during AAV-mediated gene targeting.\[^11,12\] The reader is referred to these valued references for advice in designing new gene targeting constructs for their specific applications.

When designing gene targeting constructs, one important consideration is the downstream selection of correctly targeted stem cells. That is, even high efficiency gene targeting will require the use of some manner of selection to distinguish the modified cells from cells in which the construct has incorrectly integrated, and the options available for selection depend on the function of the gene being targeted. Some genes encode products whose loss can be readily detected. As one example, the \(\text{PIG-A}\) gene, located on the X chromosome, is required for several cell surface glycosyl-phosphatidylinositol-anchored proteins (GPI-APs) to attach to the cell membrane. Using gene targeting to knock out the function of this gene results in targeted cells that can be selected by fluorescence-activated cell sorting of cells deficient for GPI-APs, such as CD59.\[^13\] Genes whose targeting does not result in a straightforward molecular marker or phenotype require the insertion of genes to select the positively targeted cells, and the most common selection is the insertion of an antibiotic resistance gene, such as genes that confer resistance to puromycin or neomycin.\[^14,15\] These resistance genes can be inserted such that they replace one or more exons of the host target gene, such that the targeting construct offers the dual functionality of gene knock out and a positively selectable marker.\[^14,15\] Furthermore, a negative selection marker can be appended outside of the homology arms to eliminate cells in which the construct was inserted non-specifically.\[^14\] As a common example of a negative selectable marker, herpes simplex virus thymidine
kinase (HSV-tk) converts the pro-drug gancyclovir into a cytotoxic product. The selected cell population can be further analyzed to determine both the on-target and off-target integration events. To confirm on-target integration in a cell population, Southern blot and polymerase chain reaction (PCR) are effective screening strategies. To determine the frequency of off-target integration in a cell population, PCR can be used to amplify an end of the AAV donor construct between the homology arm and the AAV ITR. This region of the construct will not be inserted into the genome during homologous recombination, and thus represents the presence of off-target integration. The amount of PCR product can be compared to a standard curve representing various frequencies of integration to quantify the frequency of off-target integration events, as demonstrated by Asuri et al. AAV thus represents an effective DNA donor, but gene targeting efficiency can be further enhanced by improving the properties of the acceptor locus. For example, introducing DNA damage such as a double stranded breakage at the target site can greatly enhance homologous recombination, and the co-administration of site-specific DNA nucleases, such as zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs), has been shown to substantially enhance the efficiency of plasmid DNA gene targeting constructs. As we have shown, cDNA encoding ZFNs or TALENS also can be packaged into AAV capsids, and their delivery along with AAV genomes containing gene targeting constructs can lead to even higher gene targeting efficiencies. Since ZFNs and TALENs must be designed to target a specific site within the host genome, the reader is referred to other references for more information in designing nucleases for their specific applications.

2. Materials

2.1 Adeno-Associated Virus Production

2.1.1 Viral Packaging

1. HEK 293T cells (ATCC, Manassas, VA)
2. Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Medium containing fetal bovine serum can be stored at 4°C for up to one month.
3. 2x HEPES-Buffered Saline (HeBS) Solution: 280 mM NaCl, 1.5 mM Na_2HPO_4, and 50 mM HEPES in water. Adjust pH to 7.10. Solution should be sterilized using a .22 µm filter in tissue culture hood and can be stored at room temperature.
4. 2.5 M CaCl_2 Solution: Solution should be sterilized using a .22 µm filter in tissue culture hood and can be stored at room temperature.
5. Sterile tissue culture water
6. Plasmids containing adenovirus helper genes, AAV rep and cap genes, and gene of interest between AAV ITRs. For example, plasmid pHelper (containing the adenovirus helper genes), plasmid pXX2 (containing AAV2 rep and cap genes), and plasmid t37GFP (containing a truncated GFP transgene-IRES-puromycin resistance cassette between AAV ITRs) were used by Asuri et al. to package AAV2 to evaluate the gene targeting efficiency of wild-type and novel AAV serotypes.
7. Lysis Buffer: 50 mM Tris Base and 150 mM NaCl in water. Adjust pH to 8.2-8.5 using HCl. Solution should be sterilized using a .22 µm filter in tissue culture hood and can be stored at room temperature.
8. Benzonase Nuclease: Dilute benzonase nuclease (Sigma Aldrich) to 1 U/µL in lysis buffer. Dilution can be stored at 4°C.

2.1.2. Viral Purification: Iodixanol Density Gradient Centrifugation (see Note 1)

1. 10x PBS-MK: 10x PBS solution containing 10 mM MgCl₂ and 25 mM KCl. Solution should be sterilized using a .22 µm filter in tissue culture hood and can be stored at room temperature.
2. 1x PBS-MK with 2 M NaCl: Solution should be sterilized using a .22 µm filter in tissue culture hood and can be stored at room temperature.
3. 54% iodixanol solution: 9 volumes of OptiPrep™ Density Gradient Medium – 60% Iodixonal Solution (Sigma Aldrich, St. Louis, MO) and 1 volume of 10x PBS-MK. OptiPrep Medium is sterile, so solution can be mixed in tissue culture hood. Solution can be stored at room temperature and should be protected from light.
4. 40% iodixanol solution: 4 volumes of 54% iodixanol solution and 1.4 volumes of 1x PBS-MK.
5. 25% iodixanol solution: 2.5 volumes of 54% iodixanol solution and 2.9 volumes of 1x PBS-MK.
6. 15% iodixanol solution: 1.5 volumes of 54% iodixanol solution, 2.7 volumes of 1x PBS-MK with 2 M NaCl and 1.2 volumes of 1x PBS-MK.
7. Optional: Phenol red (0.01 µg/mL) can be added to 54% and 25% iodixanol solutions to enable easier identification of the layers.
8. OptiSeal polyallomer centrifuge tubes, capacity 4.9 mL (Beckman Coulter, Brea, CA)
9. 21G1½ needles
10. 1 mL sterile syringes
11. Optima L-Series Preparative Ultracentrifuge (Beckman Coulter)
12. VTI 65.2 fixed angle ultracentrifuge rotor (Beckman Coulter)
13. Sterile 15 mL conical tubes (BD, Franklin Lakes, NJ)

2.1.3. Viral Purification: Buffer Exchange and Concentration

1. PBS with 5% Tween-20: Solution should be sterilized using a .22 µm filter in tissue culture hood and can be stored at room temperature.
2. PBS with 0.001% Tween-20: Solution should be sterilized using a .22 µm filter in tissue culture hood and can be stored at room temperature.
3. Amicon Ultra-15 Centrifugal Filter Devices (Millipore, Billerica, MA)
4. J-Series High Speed Centrifuge (Beckman Coulter)
5. JLA 16.250 fixed angle high speed centrifuge rotor (Beckman Coulter)
6. Sterile 1.7 mL microcentrifuge tubes (Eppendorf, Hauppauge, NY)

2.1.4. Viral Titering: Extraction of Viral Genome from Protein Capsid and qPCR Analysis of Viral Titer

1. 10x DNase Buffer: 100 mM MgCl₂ and 250 mM Tris-HCl (pH 7.4) in water. Solution should be sterilized using a .22 µm filter in tissue culture hood and can be stored at room temperature.
2. DNase I: Dissolve DNaseI (Roche, Indianapolis, IN) to a concentration of 10 U/uL in buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, and 50% (w/v) glycerol. DNase solution can be stored at -20°C.

3. 2x Proteinase K Buffer: 10 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), and 20 mM NaCl and in water. Solution should be sterilized using a .22 µm filter in tissue culture hood and can be stored at room temperature.

4. Proteinase K (New England Biolabs, Ipswich, MA): Use in supplied concentration. Proteinase K can be stored at -20°C.

5. 2x iCycler mix: 200 µL 10x PCR mix without Mg²⁺, 120 µL 50 mM MgCl₂, 40 µL 10 mM dNTP mixture (see below), 640 µL molecular grade water.

6. 10 mM dNTP mixture: Dilute from 100 mM stock solutions of dATP, dCTP, dGTP, and dTTP (Invitrogen, Carlsbad, CA). Aliquots can be stored at -20°C.

7. Primers against gene of interest

8. 1 µM Fluorescein: Dilute stocks of 100 µM Fluorescein (Bio-Rad, Hercules, CA). Aliquots can be stored at -20°C.

9. 40x SYBR: Dilute from 10,000x SYBRGreen stock (Invitrogen). Aliquots can be stored at -20°C.

10. Taq/Antibody mixture: Mix equal amounts of Taq DNA Polymerase (New England Biolabs) and JumpStart Taq Antibody (Sigma Aldrich). Incubate on ice for 20 minutes. Aliquots can be stored at -20°C.

11. 20 ng/µL linearized plasmid standard containing gene of interest

12. iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad)

2.2 Rat Neural Stem Cell Gene Targeting

1. Tissue culture plates previously coated with 10 µg/mL poly-L-ornithine (Sigma Aldrich) and 5 µg/mL mouse laminin (Invitrogen). See Peltier et al. for a description of the plate coating protocol.[19]

2. NSC proliferation medium: DMEM/F12 medium (Invitrogen) containing N-2 supplement (Invitrogen) and 20 ng/mL recombinant human fibroblast growth factor 2 (Peprotech, Rocky Hill, NJ). Medium containing supplements can be stored at 4°C for up to one month.

3. Accutase (Innovative Cell Technologies, San Diego, CA)

2.3 Human Pluripotent Stem Cell (hPSC) Gene Targeting

1. Tissue culture plates previously coated with BD Matrigel™ hESC-qualified Matrix (BD). See Stover and Schwartz for a description of the plate coating protocol (see Note 2).[20]

2. hPSC proliferation medium: X-Vivo medium (Lonza, Norwalk, CT) supplemented with 80 ng/ml FGF-2 (PeproTech, Rocky Hill, NJ) and 0.5 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN) or mTeSR1 maintenance medium (Stem Cell Technologies, Seattle, WA). Medium containing supplements can be stored at 4°C for up to one week.

3. Collagenase, Type IV (Invitrogen)

4. Rho kinase inhibitor (ROCK inhibitor, Y-27632; CalBioChem, San Diego, CA)
3 Methods
3.1 Adeno-Associated Virus Production

3.1.1 Viral Packaging

1. Plate HEK 293T cells in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin on 10 cm or 15 cm plates two days prior to transfection. Cells should be 80-90% confluent for transfection.

2. Change media 5-7 hours prior to transfection.

3. Prepare HeBS and DNA/CaCl$_2$ solutions according to Table 1.

<table>
<thead>
<tr>
<th>15 cm plate</th>
<th>10 cm plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x HeBS</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>2.15 mL</td>
</tr>
<tr>
<td>AAV rep and cap plasmid</td>
<td>15 µg</td>
</tr>
<tr>
<td>Adenovirus helper plasmid</td>
<td>15 µg</td>
</tr>
<tr>
<td>gene of interest plasmid</td>
<td>15 µg</td>
</tr>
<tr>
<td>2.5 M CaCl$_2$</td>
<td>300 µL</td>
</tr>
</tbody>
</table>

Table 1: AAV Transfection Solutions

4. Add DNA/CaCl$_2$ solution dropwise to HeBS solution. Pipet entire volume 2-3 additional times to completely mix solutions.

5. Incubate transfection solution at room temperature for 2-3 minutes.

6. Add transfection solution dropwise to cells.

7. Change media 8-12 hours post-transfection.

8. Virus can be harvested from cells 48-72 hours post transfection. To harvest virus, manually dissociate cells from tissue culture plate and pipet media and cells into conical tubes.

9. Centrifuge at 1,160g for 2.5 minutes to pellet cells.

10. Remove media and resuspend cells in 2 mL of lysis buffer for each 15 cm plate (or 1 mL of lysis buffer for each 10 cm plate).

11. Freeze/thaw samples three times using a dry ice/ethanol bath and a 37°C water bath.

12. Add 10 U of Benzonase Nuclease per mL of cell lysate. Incubate samples at 37°C for 30 minutes.

13. Centrifuge at 10,000g for 10 minutes.

14. Transfer supernatant to new tubes.

15. Optional: add 2 µL 5% Tween-20/mL lysate to decrease aggregation of viral particles.

16. Store supernatant at 4°C until purification.

3.1.2 Viral Purification: Iodixanol Density Gradient Centrifugation
1. Underlay 1200 µL of 15% iodixanol solution with 700 µL of 25% iodixanol solution, 600 µL of 40% iodixanol solution, and 600 µL of 54% iodixanol solution.

2. Overlay each gradient with 1.6 mL of crude lysate from 3.1.1, step 16 (see Note 3).

3. Weigh tubes to ensure that they are properly balanced. PBS can be added as necessary to balance tubes.

4. Seal tubes using the black caps provided with the tubes.

5. Centrifuge at 174,000 g for 2 hours at 18°C. To prevent disruption of the iodixanol gradient, set the ultracentrifuge to slow acceleration and deceleration.

6. Puncture tube using 21G1½ needle attached to 1 mL syringe at the interface between the 40% and 54% iodixanol solutions.

7. Collect the bottom 4/5 of the 40% iodixanol solution and the top 1/5 of the 54% iodixanol solution (see Note 4).

8. Store collected fractions in sterile 15 mL conical tubes at 4°C until further purification.

3.1.3 Viral Purification: Buffer Exchange and Concentration

1. Incubate Amicon Ultra-15 centrifugal filter in PBS with 5% Tween-20 at room temperature for 20 minutes. Following incubation, wash filter once using PBS with 0.001% Tween-20.

2. Dilute collected iodixanol fractions to 15 mL in PBS with 0.001% Tween-20 and apply diluted iodixanol solution to Amicon filter.

3. Centrifuge at 3,000g for 15-30 minutes (until solution has been concentrated to less than 2 mL).

4. Add PBS with 0.001% Tween-20 to 15 mL and mix well.

5. Repeat steps 3-4 until all iodixanol has been eliminated. This has occurred when solution is clear and viscosity is similar to PBS with 0.001% Tween-20 solution.

6. On the final centrifugation step, concentrate virus solution to 200-300 µL.

3.1.4 Viral Titering: Extraction of Viral Genome from Protein Capsid and qPCR Analysis of Viral Titer

1. For each virus sample to be tested, combine 10 µL of virus stock solution with 34.5 µL of water, 5 µL of 10x DNase Buffer, and 0.5 µL of DNase I to create a 50 µL total volume.

2. Incubate at 37°C for 30 minutes.

3. Incubate at 56°C for 10 minutes to deactivate DNase I.

4. Add 50 µL of 2x Proteinase K buffer and 10 µL of Proteinase K to each virus sample for a 110 µL total volume.

5. Incubate at 37°C for 1 hour.

6. Incubate at 95°C for 20 minutes to deactivate Proteinase K.

7. Create 10-fold serial dilutions of linearized plasmid standard between 0.2 ng/µL and 0.02 pg/µL for use as a standard curve.

8. Solutions resulting from viral purification are typically high titer (10^{11}-10^{12} viral genomes per mL), so the purified DNA solution should be
diluted 1:10 before analysis in order to ensure that samples fall within the linear range of standards.

9. Prepare qPCR master mix according to Table 2.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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<tbody>
<tr>
<td>2x iCycler Mix</td>
<td>100 µL</td>
</tr>
<tr>
<td>10 µM Sense Primer</td>
<td>2 µL</td>
</tr>
<tr>
<td>10 µM Antisense Primer</td>
<td>2 µL</td>
</tr>
<tr>
<td>1 µM Fluorescein</td>
<td>2 µL</td>
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<tr>
<td>40x SYBR</td>
<td>2 µL</td>
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<tr>
<td>Molecular Grade Water</td>
<td>40 µL</td>
</tr>
<tr>
<td>Taq/Antibody Mixture</td>
<td>2 µL</td>
</tr>
</tbody>
</table>

Table 2: qPCR Master Mix (for 10 samples)

10. Combine 15 µL of Master Mix and 5 µL of standard or diluted viral sample.

11. Run PCR protocol according to Table 3.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Step</th>
<th>Repeat</th>
<th>Temperature</th>
<th>Time (minutes)</th>
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<tr>
<td>1</td>
<td>1</td>
<td>1x</td>
<td>95°C</td>
<td>5:00</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>40x</td>
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<td>0:30</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>60°C</td>
<td>0:30</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>72°C</td>
<td>0:10</td>
</tr>
</tbody>
</table>

Table 3: iCycler Reaction Protocol

12. Threshold cycle values for standards and viral samples can be plotted to determine the genomic titer of each sample.

3.2 Rat Neural Stem Cell Gene Targeting (see Note 5)

1. Seed NSCs onto poly-L-ornithine/laminin-coated 24-well tissue culture plates at a density of 50,000 cells per well 24 hours prior to AAV infection.
2. Add AAV gene targeting vector to NSCs at a genomic multiplicity of infection (MOI) of 5 x 10^{5}.
3. Change media 24 hours post-infection.
4. Infected NSCs can be analyzed (or antibiotic selection, for example neomycin or puromycin, can begin) 72 hours post-infection.

3.3 Human Pluripotent Stem Cell Gene Targeting (see Note 6)

1. Seed hPSCs onto Matrigel-coated 12-well tissue culture plates at a density of 10^{5} cells per well 24 hours prior to AAV infection. hPSCs should be seeded as small colonies to increase AAV transduction. 10 µM ROCK inhibitor can be added to hPSC culture to increase cell survival in small colony or single cell culture conditions.
2. Add AAV gene targeting vector to hPSCs at a genomic MOI of 10^{5}.
3. Change media 24 hours post-infection.
4. Infected hPSCs can be analyzed (or antibiotic selection can begin) 48 hours post-infection.
4 Notes

4.1 The use of a viral purification protocol involving 1) iodixanol density gradient centrifugation and 2) buffer exchange and concentration results in a high titer virus solution that can be applied to sensitive in vitro cell culture systems (such as stem cell cultures) that will not result in cell toxicity. In addition, this virus solution is safe for in vivo administration.

4.2 The use of feeder layers may decrease the gene targeting frequency of the system, as a portion of the viral vectors may bind to and infect feeder cells instead of the hPSCs.

4.3 Tubes must be completely full to prevent deformation during centrifugation.

4.4 Contaminating proteins remaining from cell lysate will be concentrated in a band at the interface between the 25% and 40% iodixanol solutions. Do not collect these proteins.

4.5 General protocols for the maintenance of rat NSCs should be used to expand NSC cultures until the amount of cells needed for gene targeting has been obtained. See Peltier et al. for a complete protocol of rat NSC expansion and passaging.[19] Follow passaging protocol for cell seeding for gene targeting experiments.

4.6 General protocols for the maintenance of hPSCs should be used to expand hPSC cultures until the numbers of cells needed for gene targeting have been obtained. See the mTeSR technical manual for a complete protocol of hPSC maintenance and passaging for proliferation. Follow passaging protocol for cell seeding for gene targeting experiments.

Acknowledgements
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References
APPENDIX A. SUPPLEMENTAL DATA FOR CHAPTER 4

Supplementary Table and Figure

<table>
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<th>Human Serum Sample</th>
<th>~ NAB titer</th>
<th>Human Serum Sample</th>
<th>~ NAB titer</th>
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<tr>
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</table>

Table A1. Neutralizing Antibody Titers of Individual Human Serum Samples. Neutralizing antibody (NAb) titers for each sample are reported as the reciprocal of the volume fraction of serum necessary to reduce infectivity to 37% of the value measured in the absence of serum. Three sera pools (α = A + F + G, β = B + H + M, and γ = I + J + N) were then generated by mixing equivolume amounts of three individual serum samples.

Figure A1. Generation of Human Antibody Evaders. a) Four viral clones selected after three rounds of selection against the low stringency α pool demonstrate enhanced resistance to 1 µL of α serum at MOI of 1. Two additional rounds of diversification (i.e. mutagenesis and DNA shuffling) and selection (3 rounds of increasing serum amounts) resulted in significantly enhanced viral recovery in the presence of large amounts of highly potent b) β and c) γ pools. d) Additionally, two viral clones (1.45 and γ4.3) demonstrate 1.23- and 3.10-fold enhanced resistances to a highly diverse pool of pre-existing antibodies present with pooled human intravenous immunoglobulin (IVIg) from ~100,000 individuals compared to wild-type AAV2.

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APPENDIX B. SUPPLEMENTAL DATA FOR CHAPTER 5

Supplementary Methods

Isolation of Viral Genomes from hESCs
Following removal of stromal cells, HSF-6 hESCs were washed twice with cold PBS and resuspended in buffer containing HEPES, MgCl₂, KCl, and DTT. Cells were then incubated on ice for 15 minutes before adding 0.2% Triton X-100. Cell debris was pelleted at 6500 rpm for 5 minutes, and the supernatant containing viral DNA was analyzed via quantitative PCR.

PCR Assay for Assessing Frequency of Random Chromosomal Integration of AAV1.9 Vectors
HSF-6 hESCs harboring GFPΔ35 infected with AAV1.9 vectors encoding the donor plasmid and/or the GFP-specific ZFNs. were cultured for 14 days. The cellular genomic DNA was extracted with the QIAamp DNA Micro Kit (Qiagen), and subjected to PCR using – 5’-CCAGGGCAAGGGTCTGGGCAGC-3’ and 5’-GCATAGGCATCAGGGGCTGTTG-3’ primers to specifically amplify a 822 bp fragment partially spanning the AAV viral genome that follows the puromycin resistance gene (Figure B3a). Naïve hESC DNA samples spiked with different amounts of AAV1.9 donor plasmid was also subjected to PCR using the same primers for calibration. The resulting PCR reaction products were then analyzed using gel electrophoresis to assess the extent of random chromosomal integration.
**Supplementary Figures**

**Figure B1.** Viral Genomes Present Following AAV Infection. (a) Representative flow cytometer histograms of GFP fluorescence intensity for HSF-6 hESCs infected with AAV2-GFP, AAV6-GFP, or AAV1.9-GFP show that cells infected with AAV1.9-GFP have higher average fluorescence. (b) The average number of viral genomes per cell, quantified by qPCR, increases during infection with AAV1.9. Error bars indicate standard deviation (n=3), * = p < 0.01. ND = not detectable by qPCR.

**Figure B2.** AAV2 Mediated Correction of a Non-functional GFP Expressed in HEK293Ts and NT2s. Gene targeting frequencies assessed as the percentage of GFP positive cells measured via flow cytometry 72 hours post infection. Error bars indicate standard deviation (n=3), * = p < 0.01.
**Figure B3.** Representative Analysis of Residual AAV1.9 Genomes. (a) Arrows indicate the primers used in the PCR assay (b) Summary of the PCR and the gel electrophoresis experiments to assess the extent of residual AAV1.9 vectors in hESCs in the presence and absence of ZFN mediated DSBs.