RICE UNIVERSITY

Ligand Discovery and Applications for Vector Targeting

by

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Abstract

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Adenoviral (Ad) and adeno-associated viral (AAV) vectors have great promise as gene delivery vehicles for gene therapy and genetic immunization. However, these vectors can non-specifically target tissues and cell types in vivo. Redirected targeting of these vectors by the addition of cell-specific ligands would improve the therapeutic efficacy and safety of these vectors by reducing the effective dosage needed for gene therapy. Phage display technology has been exploited to discover novel cell-specific ligands for vector targeting. However, these ligands are selected in the context of phage and translation of the ligands back into the viral capsid can ablate viral assembly and function or inactivate the targeting function of the ligand itself.

To circumvent this ligand-vector compatibility problem, a novel approach to identify cell-specific ligands is described. We have introduced structural “context” onto filamentous bacteriophage and generated random peptide libraries within these contexts for use in ligand selection. The HI loop of the adenoviral capsid was displayed on phage and a random peptide library was generated within this scaffold and used to identify cell-specific ligands against mouse skeletal muscle in vitro. A cell-specific peptide ligand,
designated 12.51, was incorporated back into Ad capsid and the redirected Ad vector improved targeting in vitro, suggesting the viability of this approach for ligand discovery. This "context"-based approach was extended towards generating random peptide libraries within streptavidin protein for ligand selection.

In addition, a system for conjugation of targeting ligands to the AAV capsid based on the streptavidin-biotin interaction, has been developed. A biotin acceptor peptide was engineered into the AAV capsid and resulted in the development of vectors that are metabolically biotinylated during production in cell lines. This avidin-biotin technology was previously utilized for construction of metabolically biotinylated Ad vectors. However, Ad vectors are extremely immunogenic compared to AAV and may not be suitable for in vivo applications. We constructed metabolically biotinylated AAV vectors and demonstrated proof-of-principle targeting in vitro using various biotinylated ligands. Eventually, streptavidin-context ligands can be conjugated to biotinylated vectors for targeted delivery.
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Chapter I

Thesis Overview and Specific Aims

Gene therapy has developed as an attractive therapeutic approach for the treatment of a large number of inherited and acquired diseases (296). Successful gene therapy involves effective therapeutic gene delivery to correct or replace defective genes (277). Inefficient transfer of genetic material may limit clinical efficacy of cancer gene therapy, genetic immunization, and gene therapy against a myriad of diseases (295). Consequently, it is necessary to generate targeted vectors that have the ability to transduce target cells or tissues while avoiding non-specific cells and possible uptake by immune cells or any other cell that may limit the therapeutic effect and/or cause an innate or adaptive immune response.

Viruses have naturally evolved to infect cells and have therefore been investigated and engineered as vectors for gene delivery. Viral vectors such as adenovirus and adeno-associated virus have been used as gene delivery vectors for clinical treatment of cancers and monogenic diseases such cystic fibrosis and hemophilia, respectively (277). However, these vectors have natural tropism towards cells expressing native receptors towards these vectors. In addition, these vectors may elicit an immune response upon administration. One approach to re-target vectors is to genetically modify the protein coat, or capsid, of the virus with cell-specific ligands to re-target vectors to the cells by an alternative ligand-receptor pathway. True targeting can be achieved by combining the
addition of new ligands to the virus with the ablation of native viral ligands already present in the viral capsid.

One limitation to this approach is the lack of cell-specific ligands with which to modify the vectors. One technology to generate and identify potential ligands utilizes peptide-presenting peptide libraries to screen billions of peptide ligands for their ability to bind or enter mammalian cells. While cell-targeting peptides can be identified using phage libraries, these ligands are selected in the structural context of bacteriophage and frequently do not function when translated the viral capsid because either the ligand destroys virus functions or the ligand cannot function in this new protein context (201, 308).

In order to circumvent this problem of virus-ligand compatibility, our work focused on the development of a novel technology to identify ligands that would be more compatible for engineering back into adenovirus and adeno-associated virus vectors. Instead of selecting peptides in the context of a phage protein, we introduced protein structures from the virus into the phage and selected peptides with these “context-specific” libraries. We hypothesized that ligands selected in these “contexts” will be functional when translated into adenoviral and adeno-associated viral vectors. This hypothesis was tested for its ability to enhance vector targeting to muscle and other cells in vitro without ablating virion assembly or function and without impairing the cell-binding ability of the selected peptide ligand.
Specific Aims

Specific aims for the work are the following:

1. To develop “context-specific” random peptide libraries presented on filamentous bacteriophage and identify peptide ligands that bind target cells.

2. To functionally display streptavidin on phage and generate random peptide libraries within this structural context for use in ligand selection against target cells \textit{in vitro}.

3. To translate selected peptides from Specific Aim 1 into the adenoviral capsid and test the resulting modified Ad vectors for targeting against muscle cells \textit{in vitro}.

4. Development of a metabolically biotinylated adeno-associated virus (AAV) vector for targeted delivery and demonstration of proof-of-principle of targeting using biotinylated ligands. Ligands generated from the streptavidin library (Specific Aim 2) will be conjugated to the biotinylated AAV vector for targeting.

The thesis is organized as follows. Chapter 2 provides background of adenovirus biology, structure, life cycle and assembly, targeting strategies and background of filamentous phage biology, life cycle, structure, various vectors for display, and applications of phage. Chapter 3 will discuss the development of the HI phage library to generate cell-targeting ligands against muscle cells and engineering of peptide-selected
modified Ad vectors to achieve targeting \textit{in vitro} and \textit{in vivo}. Chapter 4 will focus upon progress towards building a streptavidin-display library. The work in chapter 5 will focus upon the development and evaluation of biotinylated AAV vectors as an alternate targeting strategy with biotinylated ligands via an avidin bridge. Chapter 6 will discuss conclusions of this work and future directions of the research.
Chapter II

Background

2.1 Gene Therapy

Gene therapy is a promising molecular approach towards the treatment of inherited or acquired disease. Genetic material is introduced into a cell, tissue, or organ to cure disease or to slow the progression of the disease. Gene-based therapy can be utilized towards delivery of a corrected or replacement gene towards a wide variety of genetic disorders, such as hemophilia, muscular dystrophy, sickle cell anemia, among others (295). Cancer therapy can be treated by suicide genes, proapoptotic genes or tumor suppressor genes (295). Genetic immunization has been developed as an alternative to protein-based therapies for humoral and cellular immunity (295). While these therapies are promising, they are dependent on the efficient and successful delivery of genes into the cells, tissues, and organs (277). While naked DNA injection has shown in vivo transgene expression, but the level of transduction, or delivery and expression, is low (277). It is therefore necessary to develop delivery vectors for efficient gene transfer.

Current research has focused upon the development of two categories of vectors: non-viral and viral vectors. Nonviral vectors consist of naked DNA entrapped in liposomes (cationic lipids mixed with DNA), nanoparticles, and other means. Polycationic molecules like polyethyleneimene and polylysine can complex to the DNA by charge interactions and form polypelexes, protecting the DNA from nuclease
degradation and to enhance cell binding and entry. In addition, cationic liposomes can complex with DNA and deliver it to the cell by membrane fusion. These nonviral vectors probably will not suffer from immunological or toxicity issues. However, there are several barriers from delivery into the cell. Intracellular trafficking events, such as endosomal escape, cytosol transport, and nuclear delivery are obstacles towards efficient delivery.

Viral vectors have naturally evolved for efficient delivery of genetic material into non-dividing and dividing cells and been studied and developed as gene delivery vehicles. Viruses take advantage of their natural ability to infect a host and deliver their genetic payload into the cell for viral DNA replication and viral packaging and assembly. Wild-type viruses are deleterious to the cell during production and can be detrimental or pathogenic towards the host. To convert viruses into more benign delivery vehicles for safe gene therapy, it is necessary to remove those genes and proteins from the virus that are involved with toxicity and pathogenicity. Typically, viral vectors have been genetically re-engineered to be replication-deficient, but still effective at delivering genes into cells on a transient or more permanent basis depending on the biology of the virus.

2.2 Viral Vectors: Retrovirus and Herpes Virus

Retroviruses

The retrovirus section is adapted from Goff et al (89). The first virus developed as a gene therapy vector was murine leukemia retrovirus. Retroviruses are enveloped RNA viruses and contain two copies of the viral RNA genome. The viral RNA generally encodes for three proteins, gag, pol, and env and is flanked by inverted
terminal repeats. The gag gene encodes viral core proteins including matrix and nucleocapsid that are generated by the cleavage of the gag precursor protein. The pol gene encodes for the viral enzymes protease, reverse transcriptase, and integrase also produced by protease cleavage of the pol protein precursor. The env gene encodes the envelope protein, that mediates cell binding and cell entry. After cell binding, the virus fuses to the plasma membrane and viral RNA genome enters the cell and move to the nucleus. The viral RNA genome is then converted into double stranded viral DNA by reverse transcriptase enzyme encoded by the pol gene. The viral proteins, in concert with the viral DNA, form a complex and translocate into the nucleus. Integrase helps integrate viral DNA into the host genome. Transcription factors (from LTRs) allow for new viral transcripts and particles assembly at the membrane. Retrovirus vectors are constructed with out any viral genes present in the vector for safety. The missing viral proteins are generally supplied in trans by a genetically modified producer cell line, providing essential proteins for viral assembly. The integrative ability of these vectors into the host genome is quite attractive because it allows for long-term transgene expression. However, chromosomal integration has inadvertent effects limiting the success of retroviral vectors towards therapy. For example, patients treated for severe combined immunodeficiency disorder (SCID) by an ex vivo retroviral transduction with the common gamma chain gene were cured of this genetic disease, but later a subset of these patients developed a leukemia-like syndrome due to activation of a protooncogene due to vector integration next to the gene (37). In addition, these vectors are limited for in vivo therapy by their production in low titers and inefficient purification.
Herpes Virus

Literature about herpes virus (HSV) is reviewed in (229). Herpes viruses are large, enveloped DNA viruses (with ~150 kb) double stranded DNA genomes. They are attractive due to their potential to very large amounts of DNA and their ability to infect neurons and other cells. HSV is 200 nm and consists of envelope, tegument, capsid and a double-stranded DNA viral genome. The envelope contains 12 glycoproteins essential for viral entry. Tegument is the protein layer between the viral capsid and envelope and is responsible turning off host protein synthesis and activation of early viral gene expression and viral assembly functions. Vectors have developed that removes lytic genes and can persistent long-term in non-dividing cells, especially in neurons. Recombinant vectors, deleted of cis viral elements, have a capacity for large amount of foreign DNA (~30 kb). The essential alpha genes needed for early gene activation are deleted and are provided in trans, enabling replication-deficient vector production. However, these vectors still contain large viral genes and viral gene products induce cytotoxicity and immune responses. Also, these vectors are limited by their transient expression. They are most effective towards therapy of neurological diseases, glioblastoma, and nerve injury.

2.3 Adeno-Associated Virus Vectors

Adeno-associated virus (AAV) is a non-enveloped single stranded 4.7 kb DNA icosahedral virus from the Parvoviridae family (278). There have been over 100 serotypes isolated (84, 85). Background in this chapter will focus upon studies with AAV serotype 2 (AAV2). AAV latently infects cells and need helper functions provided by
helper viruses such as adenovirus or herpes virus for replicative infection (7, 278). AAV vectors have been utilized in clinical gene therapy trials towards the treatment of many diseases. As a vector, it can transduce a variety of nondividing tissues in vivo for long-term expression. This can be attributed in part due to the ability of the viral genome to integrate into the host genome in rodent models, preferably at chromosome 19 (243). Also, the virus does not elicit an inflammatory or immune response in vivo (42, 123). Cytotoxic T lymphocyte response is less against AAV vectors compared to other viral vectors like adenovirus (42, 110, 123, 323). The virions are 20-25 nm in diameter that consist of two open reading frames (ORF) denoted as rep and cap, flanked by two inverted terminal repeats (ITR) (278). Typically, the vector consists of inverted terminal repeats flanking the gene of interest. The genes encoding viral proteins needed for replication, structure, and packaging and assembly are provided in trans from helper plasmids that cannot be packaged into virions. The lack of viral genes in the vector produces no viral gene products that can elicit an immune response (42). Also, AAV does not transduce dendritic cells, which are the main antigen processing and presenting cells for the immune system, which may also reduce immune responses against the vectors and its transgene products (123).

The genetic map of AAV has principally been derived from AAV2 (109). There is an open reading frame (ORF) on the right side of the genome that encodes for the three capsid proteins of the virus, VP1, VP2, and VP3. Alternative splicing of the mRNA encodes for the three transcripts of the capsid proteins. On the left side of the genome, there is another ORF that encodes for the rep, or the replication proteins Rep78, Rep68, Rep52, and Rep40, involved in viral DNA replication. Mutations eliminating Rep68 and
Rep78 resulted in inhibition of DNA replication. In addition to a regulatory role in DNA replication, Rep68 and Rep78 transactivates AAV gene expression (139) and is essential for DNA replication and rescue of the viral genome (109). Rep52 and Rep40 play a role in DNA encapsidation. Mutagenesis studies of Rep52 and Rep40 showed that DNA was able to replicate but the genome was not encapsidated (138, 171, 224). The ITRs are necessary in cis for DNA encapsidation (321).

Depending on the serotype, AAV uses various receptors for cell attachment and entry. For AAV2, the primary receptor for entry is heparan sulfate proteoglycan (284) and secondary receptors fibroblast growth factor receptor 1 (214) and alpha v beta 5 integrin (283) have been suggested as possible co-receptors needed for receptor-mediated endocytosis of the virion (16). Other serotypes such as AAV4 and AAV5 use sialic acid as the primary receptor for cell entry (125, 302). Upon cell entry, the virion is transported to the nucleus via endosomal pathway and release into the nucleus (16, 64).

Upon nuclear entry, the virus uncoats and the single stranded genome undergoes second-strand synthesis to produce the double stranded genome needed for replication and gene expression before viral encapsidation and assembly (75). The self-complementary sequences in the ITRs fold back, forming a hairpin structure containing the replication origins. The Rep proteins specifically bind sequences in the hairpin region, initiating DNA replication. The virus uses the host machinery and helper functions provided by the helper virus for transcription, gene expression, and DNA replication. After second-strand synthesis, the double stranded genome (+ and – strands) are separated into the + and – genomes, which are packaged into the capsid.
AAV2 was developed as a vector by removing the rep and cap genes and replacing them with the gene of interest. The rep and cap genes were trans-complemented by a packaging plasmid to rescue the viral genome. Virions can be produced by transfection of packaging plasmids into mammalian 293 cells (which provide E1 protein needed for DNA replication), including plasmids encoding the Ad helper proteins (E2, E4, VA RNA).

AAV vectors are promising for gene delivery due to their ability to integrate and low cytotoxicity. However, after a single injection of AAV, it elicits a humoral immune response against the capsid (110). Neutralizing antibodies are raised against the viral capsid upon administration, interfering with vector readministration (43, 117, 209). Consequently, it would be necessary to use higher vector dosage to obtain a therapeutic effect. To overcome this problem and improve transduction towards target cells, we can potentially modify the capsid for re-targeting by swapping with capsid proteins from other serotypes (215, 216) or direct modification of the capsid with ligands to improve targeting (88). Further literature and progress will be discussed later in Chapter 5.

2.4 Adenovirus

Human adenoviruses have been extensively studied in biology and have been utilized towards gene therapy as gene delivery vehicles. Adenoviruses were first isolated from primary cell cultures derived from human adenoid tissue, responsible for the degeneration and pathogenicity (237). They are part of the Adenoviridae family of viruses, which is subclassified into Mastadenovirus and Aviadenovirus (195). Aviadenovirus are restricted to viruses of birds, whereas are viruses originating from
human, porcine, simia, murine, ovine, equine, and canine viruses. There are currently 51 human serotypes, based upon their resistance to neutralizing antibodies and sequence homology (55). Antibodies react and bind against known epitopes against adenoviral proteins, namely hexon protein and the terminal knob protein of fiber (194). The serotypes are classified into six subgroups, based upon their ability to agglutinate red blood cells (235). Adenovirus is a nonenveloped icosahedral virus, 70-90 nm in diameter, encapsulating a linear, double-stranded DNA virion genome of 34-48 kb with an outer protein shell surrounding an inner nucleoprotein core. The outer protein shell is referred to as the capsid and is composed of 252 subunits, or capsomeres.

 Principally, Ad serotype 2 or 5 have been studied extensively and been developed as vectors and most of the information provided will be focused upon these vectors. First generation Ad vectors are deleted for their E1 gene that is essential for DNA synthesis and replication. In many cases, the E3 gene is also deleted to increase space for transgenes, since E3 is non-essential and is involved in immune evasion and other functions (deletion of the E1 and E3 genes allow for transgene insertion capacity of 8 kb). Once E1 and E3 are deleted, transgene expression cassettes are generally inserted into the E1 deletion site for vector expression. Since E1 is not present in the virus the first-generation vector will replicate in normal cells. To provide E1 functions in trans, most Ad vectors are grown in 293 cells that express and are transformed by the E1 genes (91). First-generation vectors can be produced to large titers of up to $10^{13}$ particles per mL by this approach and are able to transduce a wide variety of quiescent and dividing cells.

 While these vectors have a broad tropism for many (but not all) cell types, they also have several limitations. First generation vectors still carry ~27 kbp of viral genes
and leaky expression from these genes in transduced cells can result in cytotoxicity and immune attachment on the cells by host T cells (150, 153). To minimize these immune responses and obtain longer, more persistent expression, "gutless" or helper dependent Ad vectors have been generated that lack any of the adenoviral coding genes and only contain the inverted terminal repeats (ITRs) and the packaging signal as viral elements. Because it is "gutted", helper-dependent Ad vectors can accommodate substantially larger sizes of transgene cassettes up to 35 kbp in size. In addition, the lack of viral gene products, compared to first-generation vectors, reduces cytotoxicity and immunogenicity and extends the duration of genetic modification in vivo (176, 180). Because all of the viral genes have been gutted from the vector, the missing viral proteins have to be supplied in trans by a helper virus.

One of the main limitations of first-generation and helper-dependent Ad vectors is the acute, immediate toxicity and inflammation of the vectors in vivo. Upon systemic administration of the vectors, macrophages in the liver, known as Kupffer cells, uptake the vectors and activate pro-inflammatory response such as cytokine release (150, 287, 323). Studies have shown that removal of the Kupffer cells by drug administration prior to Ad delivery reduced vector-related toxicity and reduced humoral immunity against the capsid proteins (255, 258). Delivery using helper-dependent vectors elicited an immune response, suggesting the capsid proteins are responsible for eliciting an immune response (153). Also, due to the uptake of Ad vectors by Kupffer cells, increased doses of vector have to be administered to achieve a therapeutic effect (287). However, increased dosage will result in a deleterious effect in vivo. In the case of Jesse Gelsinger, he was given a high dosage of Ad vector for treatment of ornithine transcarbamylase and subsequently
died several days later due to the severe elicited immune response (220, 221).

Consequently, it is necessary to develop Ad vectors that can transduce the target site while circumventing the non-specific cells that exacerbate these innate immune responses.

2.4.1 Adenovirus Capsid Proteins and Genome

The adenovirus particles are 150 MDa in mass, comprised by approximately 13% DNA and 87% protein. The icosahedral capsid consists of 252 capsomeres, consisting of 240 hexons and 12 pentons (Fig. 2.1A) (73). There are at least nine viral capsids. Hexon (protein II), penton base (protein III) and fiber (protein IV) are designated as major capsid proteins (Fig. 2.1C). Proteins IIIa, VI, VIII, and IX are denoted as minor capsid proteins. The numbering designation was based upon increasing mobilities of the proteins of Ad2 particles on polyacrylamide gels. The other four proteins, protein V, protein VII, mu and the terminal protein TP, are packaged with the viral DNA in the viral core.

![Figure 2.1](image)

**Figure. 2.1: Current model of adenovirus major capsid proteins.** (A) Isosurface rendering of Fabry et al.’s 10Å wild type capsid, viewed along the icosahedral 3-fold axis. (B) Enlarged view of a single facet with the 5-fold, 3-fold, and 2-fold axes
marked by black pentagons, triangle, and ellipses, respectively. (C) Schematic of the facet. Pentamers are represented with cyan pentagons. Hexons are represented by hexons; those belonging to a group-of-nine (GON) are colored grey, those not are colored white. Protein IIIa is represented by red circles. The N-terminus of protein IX is represented by green ellipses with the C-terminus shown as purple ellipses.

From Campos et al. J. Virology in review.

Hexon (protein II) is the most abundant capsid protein. Ad2 hexon protein has 967 amino acids and three hexon monomers interact with each other, forming a trimeric protein referred to as a hexon capsomere. The 240 hexon capsomers comprise of 720 hexon monomers. The hexon trimers form each of the 20 triangular facets of the capsid (Fig. 2.1B and C). It has a pseudo-hexagonal shape due to the 3-fold repetition of two similar beta-barrel domains, V1 and V2, in the base of each subunit (8). From the beta-barrel structure, three of the loops wrap around each other to form the three tower domains at the top of the hexon (292). The intramolecular interaction gives hexon stability. It is likely that protein VI (217 amino acids), VIII (134 amino acids), and IX (139 amino acids) are associated with hexon (73).

Five copies of penton (protein III) form the penton base, found at each of the 12 vertices of the icosahedron (Fig. 2.1C). Electron microscopy showed that the penton base has a polygonal cross-section with a central 30 angstrom opening where fiber resides (280). The penton base and trimeric fiber form complexes at each of the 5-fold vertices of the capsid (281). Through dissociation studies, information about the capsid was garnered. Through dissociation under mild conditions, virions lose the penton base, then the hexons adjacent to the pentons, and then the group-of-nine (GON) hexons on the
facets, leaving the viral core (328). They suggest that a minor coat protein, protein IX, acts as a capsid “cement” and binds between hexons (Fig. 2.1C) (280). The penton base bridges hexons and protein VII of the core (280). The combination of the penton base and the fiber is called the penton capsomere.

The fiber is primarily responsible for the binding of the virus to the host cell (130) and the penton base is responsible for virus internalization (307). During the entry process, the penton base interacts with alpha v integrins to initiate receptor-mediated endocytosis via the RGD integrin-binding motif present in a loop region in the penton base (307). During fiber binding, the penton base protein undergoes surface rearrangement to enable its docking with integrins (251). The monomeric penton is similar to hexon with a jellyroll beta barrel domain at the base with two loop regions at the distal end. The trimeric fiber binds to the FN-PVYPY amino-acid sequence motif in penton base at the interface between jellyrolls and causes the conformational change in the penton base (328). Using cryo-EM, adenoviral interaction with integrins have been studied extensively. Co-purification of the Ad2 bound to purified integrin protein suggests that the penton base with the cell surface causes integrin receptors to cluster and aggregate, signaling activation of receptor-mediated endocytosis pathway (328). This is further corroborated by the activation of tyrosine kinase by penton base and cell adhesion promotion (44, 184).

Fiber (protein IV) is the major coat protein primarily responsible for initial cell attachment of the viral capsid. Fiber (protein IV; 582 residues) is a major coat protein extending from the penton base at each of the 12 vertices of the icosahedral virion. The fiber protein consists of N-terminal tail, a central shaft of vary repeating sequences
(different lengths for different serotypes), and distal, carboxy-terminal globular knob domain. Three monomers, or subunits, of fiber interact with five pentons to form the penton capsomere at each vertex (320). The N-terminus of the fiber binds to the penton base. The central shaft connects the N-terminus to the C-terminus. Also, it is suggested that shaft size can influence interaction of knob with its receptors (4, 253, 259, 316). The C-terminal head, a globular knob domain is the primary site for initial attachment of the virion to the host cell. X-ray crystallography by Xia et al provided insight into the fiber structure (320). The knob domain on the C-terminus of the fiber is trimeric, in agreement with previous stoichiometric analysis (291). The fiber knob contains eight-stranded beta barrels that form the fiber monomer. The fiber knob has central depression and three symmetric-related valleys. On the surface loops of the knob, fiber binds to its primary receptor, coxsackie-adenovirus receptor (CAR) (24). Virions deleted for fibers can be constructed but are very unstable, suggesting the importance of fiber towards virion stability and subsequent role in binding and delivery (300).

**Minor Coat Proteins**

The minor coat proteins are protein IIIa, VI, and IX. There is not much known about the structure and role of these proteins in virion assembly and packaging (279). It is thought that the minor proteins act as capsid “cement” to maintain the virion stability for its proper assembly. Protein IIIa (570 residues) is a monomer whose structure is unknown. However, through cryo-EM, the protein is on the exterior of the capsid between the facets of the hexons (279). Co-immunoprecipitation of pIIIa and protein VII suggests that protein IIIa domains interact with protein VII (279). It is suggested that the protein may act as a “rivet” to stabilize the area between two facets (279). Another
possible function of pIII has been revealed. During early Ad infection, the Ad major late promoter is very active and produces one mRNA (L1 52, 55K RNA). This transcript is alternatively spliced, leading to two predominant cytoplasmic: 52, 55K mRNA and IIIa mRNA. pIII mRNA transcript is only produced after DNA replication has begun (102, 103). Increased expression of pIIIa protein stimulated production of more pIII mRNA transcript but affects 52, 55K mRNA production but the mechanism is unclear.

Protein VI (206 residues) is on the inner capsid surface, where it anchors hexons on the inside of the surface of the capsid and connects the capsid to the viral core (281). The protein binds between the bases of its two adjacent hexons (73, 94, 281). Molecular reconstruction (162) suggests that the protein VI densities connect to the core (244, 245). In addition, the protein is said to act as a cement. The C-terminus and DNA are cofactors for the adenovirus protease (162). The protease is partially activated by DNA and fully by the C-terminus protein VI. Protein VI matures into a new form, which has enhanced binding to hexon. Burnett et al suggest that the hexon, DNA, and protein VI interact for stability (291). Protein VI may have a role of viral escape from the endosome during cell entry (309). It induces a pH-dependent disruption of the membrane and proteolysis occurs after endosomal lysis and cytoplasm entry.

Protein VIII (140 residues) is not well characterized. It is located in the inner surface of the triangular facets as dimers and interacts with adjacent hexons. It is synthesized as a precursor, like protein VI and IIIa, and processed proteolytically by adenaine (281). It is suggested to be highly disordered (281). Analysis of mutant viruses suggest that VIII may have a role in virion stability (152). The crystal structure of the intact bacteriophage PRD1 virion has an elongated protein, P30, that lies along the virus
(245). Abrescia et al hypothesize that the protein VIII may have a similar role in Ad. The elongated structure would be a feasible explanation for its absence from image reconstructions (281).

Protein IX is the smallest minor capsid protein (14.3 kD). Twelve molecules of protein IX are located at each of the 20 facets, resulting in 240 copies of pIX monomer. pIX forms stable complexes with the nine hexon capsomers, termed the group-of-nine (GON) (Fig. 2.1C). The GON form the central part of each facet of the icosahedron. Protein IX are located as trimers in the cavities between the hexons (80, 280, 281). The conserved leucine zipper domain in the C-terminus of the protein allows for the protein to self-associate (233, 294). While viruses defective in pIX can assemble, they are more instable/sensitive to temperature and they do not form the GON compared to wild-type viruses (28). It has thus been suggested the role of the protein as a capsid “cement”. However, recent work suggests an alternative location for pIX within the capsid. Through cryo-EM of pIX modified vectors, the pIX occupies the position where pIIIa is said to be in the classical model (Fig. 2.1C).

Core Structure

The core of the virion contains four proteins: protein V, VII, mu and the terminal proteins (TP). These proteins are basic, arginine-rich proteins that are in contact with viral DNA. The function of the mu protein (19 residues) is unknown. Protein VII (174 amino acids) is the major core protein and the viral DNA is wrapped around it, like a histone. Protein V (368 residues) binds to the penton base and acts a bridge between the core and capsid (41, 167). The terminal protein (671 amino acids) is attached at the ends of the viral DNA. They serve as primers for DNA replication and helps genome
attachment to the nuclear matrix (41). Virions also have 10 copies of the adenovirus protease, an endopeptidase that cleaves the structural preproteins into their mature form before viral assembly (93).

2.4.2 Adenoviral Life Cycle

Adenoviruses are linear, double-stranded genomes that are about 36 kb. At each end of the genome, there are inverted terminal repeat (ITR) of 100-140 bp to which TP are covalently linked (Fig 2.2). ITRs allow single strands of DNA to circularize by base-pairing the terminal sequences, allowing for replication of DNA. The genome has two origins of replication, each one present at one of the ITRs. Genes are encoded on both strands in a series of overlapping transcription units. The genome contains a cis-acting packaging signal about several hundred base pairs from the end of the genome. It is necessary towards encapsidation of the viral DNA (262).

![Diagram](image)

**Figure 2.2:** Organization of the Ad5 genome. Early regions are shown in white, intermediate genes in gray and late genes in dark. Adapted from (54).

The viral genome carries five early transcription units: E1A, E1B, E2, E3, and E4 (Fig 2.2). It has two delay early units (IX and IVa2) and one major late promoter (Fig
2.2) E1A is a transcription unit important towards other Ad early transcription units and causes the cell to enter S phase to provide an environment for viral replication (25). E1B encodes for proteins involved inhibition of apoptosis. E2 encodes proteins need for DNA replication. E3 encodes function to evade the host immune response and allow for persistence of host infection. E4 is responsible for a wide array of functions, including cell cycle regulation, mRNA transport and DNA regulation.

**Adenoviral Entry and Trafficking**

Initial attachment *in vitro* of Ad to cells is mediated by the distal, carboxy-terminal knob domain of fiber protein to the coxsackievirus and adenovirus receptor (CAR), which is a member of the immunoglobulin superfamily (24, 289). The high affinity interaction occurs between CAR and Ad from various subgroups. CAR is a type 1 transmembrane protein present in many tissues including heart, liver, lungs and brain. After initial docking, Ad internalization is mediated by a second protein-protein interaction. An exposed arginine-glycine-aspartic acid (RGD) sequence motif in the Ad penton base binds to heterodimeric cell surface receptors α, integrins and initiates clathrin-mediated endocytosis (307).

Receptor binding and internalization of Ad occurs within 5-10 minutes (Fig 2.3) (94). During uptake, Ad begins to disassemble, with the shedding of fiber proteins. Ad virion is encapsulated in an endosome and during uptake, the endosomal acidification results in the conformational change in the Ad capsid, resulting in the endosomal lysis and release of the fiber-shed virion into the cytosol. It has been suggested that Ad uses fiber as pH sensor and releases fiber and undergoes conformational change upon reaching an acidic pH and releases the peripentonal hexons, protein IIIa, and protein VI (94, 309).
Upon release the N-terminal amphipathic $\alpha$-helical domain of VI becomes free to disrupt the endosomal membrane (309). Endosomal escape occurs quickly, with most virions reaching the cytoplasm within 15-20 min. after infection (Fig 2.3).

From the cytoplasm, Ad virion is transported to the nuclear pore via dynein, a microtubule protein (9). At the nuclear membrane, Ad specifically binds to CAN/Nup214 receptor of the nuclear pore complex and histone H1, which imports the Ad genome into the nucleus (92). Typically, upon initial cell attachment, it takes ~30-60 minutes to reach the nucleus (Fig 2.3). Alternative receptors have been used for Ad attachment, such as vascular cell adhesion molecule I on vascular endothelial cells and heparan sulfate proteoglycan (45, 57, 58).
Figure 2.3: Events in adenovirus binding, entry, and cytoplasmic transport, provided courtesy of Dr. Campos.

**Adenoviral Life cycle: Replication**

The first viral transcription unit expressed is the E1A, which produces multiple mRNA. During infection, E1A trans-activates the other Ad transcription units (E1B, E2, E3, and E4) and induces the cell to enter S phase to allow for viral DNA replication in the host cell (25). E1A interferes with proteins of the retinoblastoma (Rb) pathway, inducing non-dividing cells to cycle (25). The E1A transcript proteins 289R and 243R bind to Rb, which releases its binding to E2F, a transactivating protein that promotes expression of
genes needed to drive the cycle to the S phase (186). Cell cycle deregulation induced by
E1A results in compensatory accumulation of the tumor suppressor p53 that will initiate
apoptosis during this aberrant cell cycle activation (186). However, adenovirus
counteracts with its E1B-55K protein that binds and inactivates p53 (247). Another
protein produced from the E1B transcript, E1B-19K can block downstream tumor
necrosis factor (TNF) from initiating apoptosis. These mechanisms prevent cell
apoptosis and allow for cell survival long enough to allow the production of maximum
viral yields (288), but ultimately the cell will die. E2 encodes for proteins needed in viral
DNA replication, including DNA polymerase, preterminal protein (pTP), and single
stranded DNA-binding protein (56). The viral DNA undergoes replication with the help
of these proteins. pTP binds to the ends of the Ad genome at the ITRs and serves as a
protein primer for the DNA polymerase (56). Replication occurs via a strand
displacement method mediated by the 72kd protein and DNA polymerase. E3 transcripts
encode for proteins that prevent the host immune response, allowing for infected cells to
persist. E3 products prevent antigen presentation and subsequent cell lysis, and also
inhibit TNF-alpha, Fas ligand, and TRAIL, which are factors involved in cell apoptosis
(22, 267). E4 encodes for proteins involved in cell cycle regulation; however, the
mechanism behind their functions is unclear.

The adenoviral late genes produced from the major late promoter and are spliced
into a complex array of mRNAs from this single transcript. These mRNAs encode the
L1 to L5 genes that produce the structural viral proteins and the proteins involved in
virion assembly. One of the products of the L4 region, the 100K protein, helps in hexon
trimerization and recruitment of ribosomes to viral mRNA for translation (38). After
DNA replication, MLP transcribes at high levels, allowing for sufficient production of structural and assembly proteins for virion assembly. Virion assembly occurs in the nucleus but the initial hexon trimerization occurs in the cytoplasm. The 100K protein associates with hexon and after trimerization, hexon trimers are translocated into the nucleus with the aid of pVI (313). The penton base and minor coat protein then associates to compose the viral capsid. It is unknown exactly how the viral genome and capsid assemble, but pulse-chase experiments and analysis of temperature-sensitive mutants suggest that genome encapsidation occurs by insertion of the viral DNA into the preformed capsids (69, 70). Proteins such L1-52/55K are necessary for DNA packaging and IVa2 binds to the Ad packaging signal (324-326). After assembly and DNA encapsidation, an adenoviral protease cleaves the immature structural proteins into their mature form to produce fully infectious virions (162). The process of assembly to cell lysis takes approximately 30-60 min post infection.

### 2.4.3 Adenoviral Targeting Strategies

Due to the dependence of Ad binding and entry of native cellular receptors, Ad delivery can be inefficient in vivo for a number of target tissues. Also, after systemic administration, most of the virus is sequestered in the liver. Following systemic Ad administration, 95% transduction occurs in liver hepatocytes (116). In addition, uptake by Kupffer cells results in the rapid clearance of virus, inactivation, degradation and release of inflammatory cytokines (149, 255, 257). To overcome virus clearance, increasing doses need to be administered to achieve transgene expression. However, increased number of particles results increased viral gene products and capsids, triggering
innate and adaptive immune response. To achieve suitable transgene expression and delivery to target cells while avoiding non-specific cells, it will likely be necessary to design safe vectors that possess targeting ability combined with ablation for natural tropism. The following will describe various approaches towards targeted delivery.

**Conjugate or "Adaptor"-Based Targeting**

Genetic capsid modification is limited by the size of inserted peptide. Also, genetic engineering of the ligand into the capsid is limited the Ad assembly process. Ad assembly in the cytosol occurs in a reductive environment, possibly leading to improper folding of disulfide-bearing ligands during assembly. One targeting approach that circumvents this problem involves the formation of a molecular bridge or conjugate between Ad vector and an alternative cell surface receptor. In this case, natural CAR binding is circumvented by using "bi-specific" molecules that retarget Ad to an alternative cell surface receptor. Approaches have been used using bi-specific antibodies against knob target folate receptor, epidermal growth factor receptor, and fibroblast growth factor (61, 65, 104).

**Chemical Modification of the Capsid**

Another targeting approach involves the direct chemical modification of the Ad capsid for redirected targeting. The primary amines present on the capsid can be modified with chemically reactive groups like poly (ethylene) glycol (PEG) or biotin conjugated to antibodies, peptides, growth factors like fibroblast growth factor or epidermal growth factor for targeting (175, 196, 230). In addition, molecules like PEG
coat the virion and the immunogenic capsid, allowing for longer circulation of Ad _in vivo_ and reduced immune response (49, 50, 176).

**Fiber Swapping with Other Serotypes (Pseudotyping) and Fiber Replacement/Deletion**

Another approach towards targeting utilizes the fiber protein from other adenovirus serotypes that recognizes natural receptors other than CAR. Fibers from Ad3, Ad11, Ad35 serotypes from subgroup B family have been swapped with Ad5 fiber to create "pseudotyped" hybrid vectors (256, 260). They have been utilized for targeting towards cells such as hemopoetic cells expressing CD80/CD86 and CD46 receptors, in lieu of CAR (81, 256, 260). Fibers from other serotypes such as Ad16 and Ad17 have been swapped for targeting towards CAR-negative endothelial and smooth muscle cells and airway epithelial cells, respectively. However, this strategy is not true targeting since it exploits natural receptors present on the cell that are permissive to the fiber protein. Besides fibers from Ad serotypes, other viruses that have similar structure to fiber have recently been exploited to create a novel pseudotyped vector. Mercier _et al_ swapped Ad5 fiber with a similarly structured sigma 1 protein from reovirus and demonstrated targeting towards cells via sialic acid and junction adhesion molecule (JAM). The vector is currently being tested as a vaccine for mucosal immunity, since the reovirus naturally infects cells in the mucosa (172). Other approaches in fiber replacement have involved ablation of the CAR-binding knob domain of fiber and replacement with ligands able to trimerize and retarget. Fibrin trimeric protein from T4 bacteriophage fused to CD40 ligand can trimerize and was shown to retarget human dendritic cells (20, 120). Other approaches to de-"knob" the fiber have involved
incorporation of other trimerization signals coupled with RGD-integrin binding motif for targeting and proteolytic cleavage of the knob domain from ligand-fiber fusions for targeting and removal of the native CAR tropism due to the knob domain (112, 159, 160).

Genetic Modification of the Capsid

Retargeting of Ad vectors can be achieved by incorporation of specific receptor ligands directly into the capsid by genetic engineering. Structural data have revealed several locations on the viral capsid amenable for ligand insertion. In the fiber, there are two principal locations that have been shown to tolerate ligands without impairing fiber assembly or function: the C-terminus of the fiber and the HI loop. The C-terminus is exposed on the capsid surface and ligands such as RGD and polylysine have been displayed for targeting in vitro and in vivo (308). However, other ligands have proven ineffective at this location (308, 316). The HI loop is an exposed loop flanked by the designated H and I beta sheets in the trimeric knob domain used for capsid modification for retargeting. Krasnykh et al inserted a heterologous peptide in the HI loop and was able to tolerate peptide insertion without disrupting fiber trimerization (135). Peptides up to 100 amino acids have been tolerated into the HI loop without affecting virion formation (21). RGD motif has been incorporated into the HI loop and Ad displaying RGD in the HI loop exhibited enhanced transduction compared to unmodified vectors, suggesting the ability to retarget. This retargeted vector has been used towards carcinomas of the ovary, pancreas, colon and head and neck (18). Other peptides, such as the C-terminus of the Fc-binding domain of Staphylococcus aureus Protein A and anti-CD40 single chain antibody, have been used towards targeting (134). Phage selected
peptides against the vasculature have been used as ligands in the HI loop for Ad targeting (190, 315).

Other locations on the Ad capsid have been exploited for ligand insertion for retargeting. Hexon, penton base, and pIX proteins have recently been investigated as potential locales for genetic engineering. Vigne et al displayed RGD into the hypervariable region 5 (HVR5), a solvent-exposed loop, and demonstrated fiber-independent transduction to low-CAR expressing vascular smooth muscle cells (298). Since penton base interacts with integrins towards virus internalization, it has been used for incorporating ligands. A linear peptide, hemagglutin (HA), was engineered into the penton base and preliminary work suggested Ad modified vectors could transduce an artificial cell line expressing the HA-receptor (71). Minor coat protein IX was considered for ligand insertion due to the high expression of pIX monomer protein. Ligands fused to the surface-exposed C-terminus would be expressed at 240 copies, allowing for a high avidity interaction between ligand and receptor. Dmitriev et al initially demonstrated CAR-independent binding to heparan sulfate moieties by inserting polylysine or FLAG motifs onto the pIX C-terminus (62). Vellinga et al incorporated RGD peptide with various length spacers for improved display of peptide on pIX for targeting (293). However, one of the main limitations in genetic modification of the capsid is the peptide ligand. It is unknown whether a ligand can be tolerated in the capsid locale without ablating viral function and assembly. Conversely, the virus may inactivate the function of the ligand once it is incorporated into the structural context of the capsid protein.
2.5 Filamentous Bacteriophage for Peptide Display

2.5.1 Filamentous Bacteriophage Biology

One of the most important issues in retargeting is identifying ligands specific to the target cell. Phage display technology has developed as a high throughput approach for generating cell specific peptides or antibodies as potential ligands (13, 53, 66). One class of bacteriophage are filamentous bacteriophage (Fig 2.4). Filamentous bacteriophage (phage) are 880 nm long viral particles that are able to infect bacteria expressing F-pilus receptor. The virion has a single-stranded genome encapsulated by a long protein cylinder. There are mainly M13, f1, and fd phage in the filamentous bacteriophage class. They have been sequenced and have approximately 98% homology. They have similar sequence and similar behavior towards infection and assembly and are referred to the Ff phage.
Figure 2.4: Electron micrograph (above) and cartoon (below) of filamentous bacteriophage with coat, assembly and replication proteins denoted (adapted from (222)).

**Phage Proteins**

pIII, pVI, pVII, pVIII, and pIX are the capsid proteins, or protein coat, of the phage particle (Fig 2.4). They reside in the membrane, until the phage is assembled. pVIII is the major capsid protein and is the most abundant protein on phage. There are 2700 copies of pVIII protein. It is a 73 amino acid consisting of a 23 N-terminal precursor protein and a mature 50 amino acid protein. Before phage assembly, the 23 N-terminal signal sequence is cleaved once the pVIII protein is inserted into the membrane (261). The 50 amino acid mature pVIII protein has its hydrophobic region (residues 20-40)
embedded in the membrane, with the N-terminus of the protein in the periplasm and the C-terminus in the cytoplasm. From crystallography, pVIII appears to have an alpha helical conformation (except for the C-terminus residues). The pVIII monomers overlap, forming a tight bundle. The carboxy-terminal 10-13 residues form the inside wall of the particle. This region has four positively charged lysine residues, which form one face of an amphiphilic helix. These positive charges interact with the negative charge from the sugar phosphate backbone of the phage DNA (97, 166).

The other four proteins constitute the minor capsid of the phage. They reside in the inner membrane prior to phage assembly. On one end of the phage are approximately five copies of hydrophobic pVII and pIX capsid proteins. They are located at the end where phage assembly initiates. However, it is unclear of the structure of pVII and pIX proteins or how they assemble or interact with the pVIII protein cylinder. It has been hypothesized that pVII is burrowed, in contact with the phage DNA, while pIX is exposed on the phage surface (161). This is supported by the observation that antibodies interact with pIX but not pVII (72). pVI and pIII are the capsid proteins located at the other end of the phage. pIII is a 424 amino acid protein, consisting of a 18-amino acid N-terminal signal sequence that is removed upon insertion of the mature 406 amino acid protein into membrane (with the help of the bacterial Sec system). pIII consists of three domains, denoted as N1, N2, and CT. The N1 domain is required during phage infection for DNA translocation into the host cytoplasm and insertion of the coat proteins into the membrane (23). The N2 domain (residues 87-217), specifically the outer rim of the N2 domain, is responsible for phage binding to the F pilus (59). While the phage is not in the infection or assembly process, the N1 and N2 domains form intramolecular disulfide
bonds (23). The structure of these domains has been visualized by NMR spectroscopy and X-ray crystallography. The carboxy-terminal CT domain helps in the formation of a stable phage particle (157). It is suggested that the CT domain of pIII, along with pVI, interacts with pVIII to cap one end of the phage particle (218). These domains are necessary towards the assembly and function of infectious phage. Removal of these domains by protease treatment has been shown to produce noninfectious phage.

pII, pX, and pV are phage proteins involved in phage DNA synthesis and packaging. During phage infection, the single stranded genome is converted to a double-stranded genome. From the double-stranded DNA, all the phage proteins are translated, including pII, pX, and pV. pII nicks the + strand in the intergenic region, leading to the DNA undergo "rolling circle" amplification and circularize the (+) strand, making more double stranded DNA; more DNA leads to produce more phage proteins (5, 96).

pIV, pI, and pXI are proteins involved in phage assembly. pIV has a 21-amino acid N-terminal signal peptide and is translocated into the periplasm (222). About 12-14 pIV molecules integrate on the outer membrane, with the N-terminus located in the periplasm (128). From scanning electron microscopy, the pIV multimer is cylindrical and forms a pore/channel (151). pI and pXI do not have a signal peptide sequence are inserted into the cytoplasmic membrane with the help of the SecA bacterial system (222). It is suggested that pI and pXI interact to form a channel, which possibly interacts with the pIV multimer channel, forming a complex where there are channels spanning the outer and cytoplasmic membrane (74, 222, 238).
Phage Life Cycle: Infection

The basic process of phage infection is attachment of the phage tip to the tip of the F pilus of E. coli bacteria (containing the F conjugative plasmid) as a receptor. The pilus is encoded by the tra operon on the F conjugative plasmid. During phage infection, the phage attaches to the F pilus and is brought to the membrane with the depolymerization of the F pilus. Here, the phage capsid proteins integrate into the membrane and phage DNA translocates through the membrane and into the cytoplasm of the host.

Phage infection of E. coli is a two-step process involving phage interaction with the F pilus and bacterial TolQ, TolR, and TolA (tolQRA) cytoplasmic membrane proteins. The Tol proteins are located in the cytoplasmic (inner) membrane. They form a complex due to the interactions of their transmembrane regions (142). TolQ spans the membrane three times (297), where TolaA and TolR span the membrane one time, while most of their residues are exposed in the periplasm (147). TolA has three domains, separated by a Glycine-rich region. D1 is anchored on the cytoplasmic membrane at its N-terminus, connected to the alpha-helical D2, which spans the periplasm, and connected to domain 3 (D3), which is carboxy-terminal domain of ~108 residues tethered to the outer membrane (147). The F pilus is a protein tube that is assembled and disassembled by the polymerization and depolymerization of pilin subunits from the bacterial inner membrane (78). Pilus depolymerization, or retraction, pulls in the donor into the recipient bacteria and helps the DNA transfer from infector to host (119). Genes from the tra operon encode for the tolQRA proteins necessary for its structure, assembly and disassembly (79). The outer rim of the N2 domain of pIII interacts with the tip of the F
pilus (59). Upon binding, the pilus retracts and the pilin subunits depolymerizes, bringing the phage to the periplasm. N2 binding to the F pilus releases N1, which is complexed to N2. N1 domain interacts with the third domain of TolA. TolA acts a co-receptor, binding the N1 domain of pIII. The phage is ready for translocation of its DNA into the cytoplasm, but the process of translocation is unclear. It is hypothesized that the pVIII, pVII, and pIX disassemble and insert in the cytoplasmic membrane (154, 174). However, TolQ, TolR, and TolA are necessary for DNA translocation. Mutagenesis of these genes ablated uptake of the phage DNA into the cytoplasm (174).

**Phage Life Cycle: Synthesis and Assembly**

Phage assembly occurs at the junction where the inner and outer membrane are in closest contact (154). The assembly process involves the capsid proteins, along with the assembly proteins. Upon translocation of the single stranded phage DNA (+ viral strand) into the cytosol, the complementary DNA strand (-) is synthesized with help of bacterial enzymes (154). This double stranded, closed circular DNA, is known as replicative form (RF) DNA. RF DNA serves as a template for transcription and translation of all the phage proteins. The RF DNA can be used to synthesize more single stranded viral DNA. In addition, RF DNA can produce pV protein, a DNA-binding protein that prevents the conversion of single-stranded DNA into RF DNA. pV acts a feedback protein to regulate single stranded DNA production for eventual DNA encapsulation and viral assembly. pV, as a dimer, binds ss DNA and the genome is wrapped around a cluster of pV monomers (101, 197). The pV-DNA complex has a 78-nucleotide packaging signal at one end of the complex (17). The formation of the pV-DNA complex is the initial step
towards phage assembly. It has been proposed that pVII and pIX, along with the first few residues of pVIII, interact with the packaging signal to initiate phage assembly (239). This end of the phage extrudes out of the bacterium first. It is hypothesized that the pVII and pIX proteins organize and form the tip of the phage capsid around the packaging signal (239). The assembly proteins pI, pIV, and pXI interact before the formation of the pIX-pVII end of the phage. Mutants defective in genes encoding for pIX and pVII were still able to form the assembly sites, as evidenced by electron microscopy (155). After initiation, the phage begins its elongation process. The pV dimers are removed from the complexed DNA by ATP hydrolysis and the reductive protein thioredoxin and replaced with pVIII as the DNA is extruding out the pI/pXI/pIV pore complex (240, 241). pI is said to catalyze these reactions. The positive-charged C-termini residues of pVIII interact with the phage DNA while the pVIII transmembrane domains interact with each other to encapsulate the DNA. Since the structure of the DNA is different in the pV-DNA complex compared to the DNA being extruded through the channel, it is suggested that the DNA undergoes a conformational change to interact with pVIII (222). pI and pXI have similar residues compared to the 10 C-termini residues of pVIII. It is hypothesized that pI and pXI orient the DNA to interact with pVIII (222). As the DNA extrudes out, pVIII proteins from the cytoplasmic membrane interact with the DNA and encapsulate the DNA, while being extruded out (222). When the end of the DNA, pIII and pVI are added to the end of the phage, signaling the termination of phage assembly. From site-directed mutagenesis by Rakonjac et al, it was found that the carboxy-terminal 93 residues of the CT domain are necessary for phage release from the bacteria (219). Rakonjac et al has proposed that pVI and pIII interact with the end of pVIII to form a
complex; then the CT domain undergoes a conformational change, allowing the phage to be released from the bacteria (218). From the structure, the CT domain is initially anchored to the inner membrane. However, on the phage particle, the CT domain is reoriented to attach first to the end of the phage particle. Consequently, it is suggested that the CT domain undergoes a conformational change (218).

2.5.2 Phage Display as Vectors

All of the exposed phage capsid proteins are theoretically amenable for peptide or protein display. The phage pIII, pVIII, and pVI proteins are flexible structures on the surface protein coat that can be modified on their termini to display peptides or antibodies. The most commonly used locations for display are the pIII and pVIII proteins. Smith et al classified phage vectors based on the following: (1) display on pIII or pVIII, (2) whether the peptide was displayed on some or all copies of the capsid, and (3) the displayed peptide is engineered in the phage genome or is provide separately, in trans, by a separate genome (i.e. phagemid) (272). Principally, there are three types of phage libraries: a phage vector, where peptide is encoded in a near wild-type phage genome that forms plaques; a selectable phage vector, where antibiotic selection is introduced so it grow like a plasmid and forms colonies rather than plaques; a phagemid vector, where the peptide is fused to the capsid protein in a plasmid that carries the phage (-) strand origin of replication, along with antibiotic selectable marker. Phagemids produce the protein, and with the help of helper phage in trans, recombinant phage is produced.

Types of Phage Display Vectors:
Wild-type phage vectors are simple vectors encoding for the phage genome. There are restriction sites introduced between the carboxy-terminus of the signal sequence and the N-terminus of the mature protein. The genome is not altered with the incorporation of the peptide. During production, the peptide will be displayed on the N-terminus of the mature capsid protein. Typically, the phage vector has insertions introduced into pIII. While pIII can tolerate large insertions, it is difficult to display all proteins (127). Large proteins, such as antibody fragments (Fab) or single chain antibodies (scFv), fused to pIII may misfold during synthesis or get degraded due to proteases (212). Also, peptide or protein display may inhibit pIII protein production and infectivity to the F pilus (200). pVIII-based display vectors can typically tolerate a smaller number of residues without impairing capsid assembly and function and thus, is unable to tolerate large peptide/protein sequences (98, 213). However, recently, random mutagenesis of the pVIII protein, fused to streptavidin or human growth hormone proteins, allowed for improved display of these proteins on pVIII (268, 269). This suggests that the structure can be mutagenized to tolerate large peptides or proteins without interfering with pVIII assembly (268, 269). Peptides up to 6-8 amino acids were shown to be displayed on pVIII phage vectors (118, 213). The phage vector is similar to wild-type phage in its infectivity and production, up to $10^{13}$-$10^{14}$ particles/mL. The vector has no antibiotic marker for selection, leading to possible wild-type contamination. The vector can display peptides on some of the capsid (the other capsid proteins are wild type) or display peptides on all the capsid molecules. Commercially available vectors include the M13KE (NEB), type 3 and type 8 vectors.
Phage "crippled" vectors arose due to the poor infectivity and phage production of EcoRI-pIII fusion as seen by Smith et al (272). Phage growth was reduced 100 fold, compared to wild type phage and the activity was reduced to 50 particles per plaque forming units (i.e. ratio of how many phage particles it takes for one infective particle). Consequently, phage vectors such as fd-tet were developed that could be propagated like a plasmid, with the help of an antibiotic selectable marker.

**Phagemid Vectors for Phage Display:**

Phagemid vectors are another alternative for phage display of peptides and proteins (68). Phagemids are plasmids that contain a phage origin of replication and antibiotic selection marker. The phagemid DNA can be encapsidated by the phage capsid. The phagemid does not contain the whole phage genome. It only contains the peptide or protein fused to the pIII or pVIII protein for display. Consequently, for phage production, they need to be superinfected with wild-type phage as a helper. This "helper" phage will provide all the necessary phage proteins needed for assembly and packaging. Cells containing phagemid DNA and infected with helper phage will produce both recombinant phage containing the phagemid DNA and helper phage. To minimize helper phage contamination, several groups have constructed helper phage with defective origin of replication or packaging signal (68). For pIII display, it is known that the N-terminal domain is important towards infectivity with the F-pilus and the carboxy-terminal residues interact with pVI to cap the phage particle during assembly (219). Also, the presence of pIII in the bacterium prevents the cell from being infected again by another filamentous phage. The pIII confers immunity towards the cell against superinfection. In the construction of phagemid vectors, it is subsequently necessary to delete the N-
terminal region of pIII in the phagemid pIII fusion. Otherwise, the pIII produced by the phagemid will prevent infection by the helper phage. Various phagemid vectors have been designed to display pIII fusions (11, 113). In order for proper assembly, infection, and production, the phage incorporates 3-5 copies wild type pIII, along 0, 1, or 2 copies of the pIII fusion. It cannot display all fusion on pIII; otherwise, the phage will have reduced infectivity and assembly (218). pVIII has also been used for display in phagemid vectors. However, it is difficult to express large proteins such as antibody Fab fragments and the instability of trying to express these proteins may result in their deletion during packaging (126). This can be attributed to the size-limiting capacity of pIV pore complex, which may prevent large pVIII fusions from passing (163).

Subsequently, pVIII display is limited to approximately 6-8 residues. However, pVIII display may be an attractive locale for display due to the high number of copies (~2700 copies) that can display fusion proteins. Consequently, pVIII binding and selection can be mediated by avidity interactions with the target. Other work has sought to engineer larger peptides or proteins onto the pVIII capsid and mutate the pVIII protein itself to improve functional display. Large proteins, such as 64 kD streptavidin and fibroblast growth factor, have been displayed on pVIII and the capsid protein was mutated to improve display of the molecule (213, 269).

2.5.3 Phage Display of Peptides and its Applications

Phage developed as a potential cloning vehicle due to the membrane-based nature of its assembly. As the DNA is extruded out from the pI-pXI-pIV pore complex, capsid proteins assemble around the DNA. Consequently, there is no limitations to amount of
DNA that can be packaged into the phage capsid. The principle of capsid assembly from the membrane also allows for the display of foreign peptides, or chimeric peptides into the phage display. As long as a peptide is fused to the periplasmic part of a capsid protein, it should be able to be translocated. Smith *et al* were able to achieve the display of foreign peptides on phage (272). They displayed fragments of the EcoRI endonuclease on the N-terminus of pIII. A mixture of wild type and recombinant phage displaying the fragment were produced. Recombinant phage was purified by binding the phage mixture to a dish adsorbed with EcoRI antibody. After several washes, phage was eluted with acid and amplified by bacterial infection. The phage was able to undergo several rounds of affinity purification, or “panning”, enriching the population displaying EcoRI fragment 1000-fold (272). From this work, it was established that large random peptide libraries could be displayed on phage capsid, where each phage particle displays a unique, specific peptide. In addition, there is a direct correlation between the genotype and phenotype of the phage peptide. Consequently, it is possible to determine the sequence of a displayed peptide. Cwirla *et al* initially generated the first random peptide phage libraries to identify ligands (53, 60). Approximately $10^8$ random peptides (also known as library members) were displayed on pIII and was panned a monoclonal antibody for several rounds, resulting a peptide that demonstrated enriched affinity towards the antibody (53). This study confirmed that from a library of a wide repertoire of peptides, functional ligands could be selected. Phage display has developed for many applications, such as epitope mapping for antibodies, mapping protein-protein interactions (199), generation of single-chain antibodies (164), and cDNA expression screening (121).
Antibody Libraries:

One of the most important applications for phage display developed recently is the generation of antibody libraries on the phage capsid for the identification of possible antibodies or single chain antibodies against antigens (164, 165). This technology is very attractive to identify and generate antibodies against antigens from a large and diverse repertoire of antibody structures. This approach bypasses other technology such as hybridoma production and immunization. Briefly, in the immune system, upon exposure to an antigen, unarranged V genes in a germ cell undergoes rearrangement, creating a set of B cells displaying a single antibody against an antigen. Upon binding to antigen, the cells will secrete antibody and also differentiate to memory cells present in lymph nodes, spleen, and bone marrow. Upon further binding to antigen, the memory cells undergo hypermutation and produce antibodies that exhibit improved affinity towards the antigen. This process of affinity maturation occurs with subsequent antigen binding. Work has been done to immortalize these antigen-immunized B cells by fusion to myeloma cells to generate monoclonal antibodies. However, this process is expensive and laborious. Subsequent work has focused upon generating antibodies in vitro (164, 165). Phage has been exploited because of its ability to mimic the B cell and produce affinity matured antibody fragments on the phage capsid. Phage displays antibody fragments of differing affinities to antigen. Upon binding to antigen, specific binders with affinity are selected and after several rounds of selection, antibody fragments are selected that exhibit enhanced (enriched) binding. The first instance of generating antibodies from phage display was demonstrated by Marks et al (164, 165). Patient lymphocytes were isolated
and antibody genes (the light and heavy variable chains of antibodies) were PCR amplified and cloned onto pIII. Further studies involving selection and panning against antigens such as turkey-egg lysozyme and bovine serum albumin resulted in isolated variable heavy and light chains similar to those found in germ-line antibodies that displayed uM affinity towards antigens (46, 113). Targets have included surface markers of lymphocytes, tumor cells, and red blood cells (46, 113, 165, 250).

**Peptide-presenting Libraries:**

Peptide libraries and proteins, such as antibodies and cytokines, have been displayed on pIII and pVIII (252, 311). Jespers et al demonstrated the ability of pVI C-terminus as a location to display cDNA libraries for ligand selection (121). pIX capsid protein has recently been utilized towards peptide display (82, 83). Initially, Smith et al utilized phage display to affinity purify protein fragments that were coded by cDNA. However, the technology has developed to display random libraries on phage for selection of peptides and antibodies against antigens, receptors, or other biological targets. Cwirla et al first utilized the random peptide library to identify possible ligand (53). The library was panned against the antibody against N-terminus of beta-endorphin to identify ligands that demonstrated specificity against the antibody (53). This technology has since been used to display and identify ligands that target proteins. For example, phage display has been used to identify ligands that specifically bind to α_{IIBβ3}, α_{15β1}, and α_{iβ3} integrins (108, 132). RGD and non-RGD binding motifs were found to bind these and other integrins. Peptide libraries have been used to target growth factor receptors (53, 67). Cytokine binding to growth factor receptors elicits cellular responses...
involved in cancer development and the inflammatory response. Peptides have been identified as competitors with cytokines towards growth factor receptors, such as erythropoietin receptor and thrombopoietin receptor have been identified (51-53, 67, 148). Peptide-presenting phage libraries are an attractive approach towards generating and identifying ligands.

**Peptide Selection from Phage Libraries Against Mammalian Cells:**

In our laboratory, we have exploited phage display technology and have developed a technology to identify peptide ligands against mammalian cells *in vitro* for cell-specific targeting (13). Large libraries up to $10^{10}$ random peptides can be displayed on phage. They can interact with a variety of cell types to generate peptides that demonstrate binding affinity towards the cell. By undergoing repeated rounds of affinity selection, we can decrease the number of ligands from $10^8$-$10^9$ to one or a few peptides. Figure 2.5 is a schematic of peptide selection from phage libraries against mammalian cells. Briefly, phage are allowed to bind to the target cell, and non-specifically bound phage are washed off while bound phage are collected and re-amplified in bacteria. The collected phage undergo repeated steps of binding and amplification until one or a few peptides are selected after sequencing of phage clones. This technology is a powerful approach to select potential cell-targeting ligands from a large library of peptides ($10^8$-$10^{11}$ peptides) without having prior knowledge of the cell biology (i.e. cell surface receptors). Selected peptides containing the RGD integrin binding sequence have been shown to bind and internalize to cells (105). In addition, selected antibodies from phage libraries have been shown to internalize to targeted cells (19). Peptides generated from
these libraries have the ability to bind and internalize, indicating the feasibility of identifying random ligands for cell targeting of gene therapy vectors. We can take advantage of phage display technology to select functional peptide ligands against muscle cells.

Figure 2.6: Cartoon for “biopanning”. Method for selection of peptides against mammalian cells using phage library, provided courtesy of Michael A. Barry.

Work by others have shown the use of phage libraries to select ligands *in vivo* against the vasculature (100, 207, 208) and even the ability to use phage display as a vector for gene delivery (141).
2.5.4 Issues of Ligand-Virus Compatibility

While peptide ligands can be selected from phage presenting random peptide libraries, these peptides have been selected in the context of a bacteriophage. Without this scaffold, ligand function can be inactivated. Barry et al reported a fibroblast-binding peptide (denoted 20.2) that exhibited binding while displayed on pIII phage protein. However, the synthetic version of the peptide did not demonstrate any binding to fibroblasts (13). The peptide ligand only works in the context of the phage pIII protein.

Our objective is to develop ligands and translate them into viral vectors for muscle cell targeting. In regards to ligand modification in viral vectors, while some research has indicated improved targeting with ligand insertion (63, 87, 99, 173, 187, 189, 227, 263, 290, 317, 319), other research has indicated that ligand insertion can ablate viral activity and can actually eliminate the function of the ligand. Incorporation of varying polypeptide ligands onto the c-terminus of Ad fiber prevents fiber trimerization and subsequent viral formation (308). In our laboratory, we have observed similar effects of polypeptide addition on fiber trimerization (Figure 2.6). While previous research has indicated that insertions into the adenoviral HI loop are tolerated better, incorporation of polypeptide ligands containing RGD sequences of varying lengths into the adenoviral HI loop can affect virus assembly (21). Viral production and transduction decreased with increasing length of RGD inserts (23, 33, 43, 53, 63, and 73 amino acids) (21). Similarly, insertions of peptides into surface-exposed loop domains of AAV-2 capsid can prevent AAV-2 assembly (319). Consequently, despite possible selection of a suitable ligand, it may not be compatible when translated into viral vectors. The ligand may ablate viral function or assembly and the insertion of the ligand may inactivate the ligand itself. It is
therefore necessary to develop random peptide libraries in a structural context that avoid these problems of ligand-scaffold compatibility.

![Western blot of varying length polypeptides on c-terminus of fiber. (d) stands for protein samples in denatured conditions and (n) stands for samples treated under native conditions. Fiber trimerization is present in wild type, +18 and +82 amino acid insertion. Trimerization is ablated at +25 and +135 amino acid insertion. Monomer is present in all samples (blot courtesy of B. Parrott).](image-url)
Chapter III

“Context-specific” Peptide-presenting Phage Libraries for Adenoviral Targeting

ABSTRACT

Production of cell-targeting vectors in part involves the addition of new targeting ligands to the vector to mediate binding to the cells of interest. For viral vectors, the ideal approach is to genetically engineer new ligands into the capsid proteins of the virus to generate a single agent to mediate therapy. While this is ideal, this insertion of an exogenous ligand from one structural context into the differing structural context of a capsid protein can ablate the function of the ligand or disrupt viral assembly and function. To address this context problem for adenoviral vectors, we have engineered a “context-specific” peptide-presenting phage library. We have displayed a twelve amino acid (12-mer) random peptide library between the H and I sheets of the fiber protein of adenovirus type 5 (Ad5) on the pIII protein of fd bacteriophage. This library was used for peptide selection against C2C12 mouse skeletal muscle cells. Five rounds of selection combined with four rounds of clearing on non-target cells selected one primary peptide designated 12.51, which bound target C2C12 cells approximately 100-fold better than the positive control RGD peptide. Translation of HI 12.51 back into the fiber protein produced a ligand-modified adenoviral vector that mediated 14-fold better transduction of target C2C12 cells. These data suggest context-specific peptide-presenting libraries may allow
selection of compatible peptide ligands for functional translation into viral vectors for re-targeting.

3.1 INTRODUCTION

Adenoviruses are arguably one of the most robust vectors for gene delivery. The attraction for the use of these viruses as gene delivery vectors stems from their ability to transduce a wide range of dividing and non-dividing cell types. Adenovirus types 2 and 5 bind and enter cells through a multi-step process usually involving the combined interactions of the fiber and penton base proteins with cellular receptors. The trimeric fiber protein forms a shaft structure at the vertices of the virus icosahedron and is thought to mediate initial cell binding (114). Cells lacking the fiber receptor (the coxsackie-adenovirus receptor-CAR) are relatively resistant to infection (24). Alternate interactions are mediated by an RGD motif in the penton base that binds to \( \square \), integrins. For most cells, this binding is thought to initiate virus internalization after fiber binding (307). However, in cells lacking CAR, penton base mediates both cell binding and entry, albeit at one half the level as in cells bearing both receptors (114). Recent work has also identified a third ligand for cell binding in the fiber protein that appears to mediate cell-binding via cellular heparin sulfate proteoglycans (274). Studies have demonstrated that intravenous injection of adenoviral vectors into mice and primates results up to 70% of total gene delivery occurring in the liver, mainly in Kupffer cells (115, 286). This high level of gene delivery is likely due to the easy access the vector has to liver cells from the blood due to the presence of large fenestrations in the vasculature of the liver.
Adenoviral vectors also transduce a variety of non-dividing cells including myoblasts and myotubes. First generation adenoviral vectors carrying a 6.3 kilobase pair mini-dystrophin gene mediated expression in as many as 50% of myotubes after direct injection into the muscle bed of mdx mice (217). This promising result was observed in very young (5-7 day old) mice and used fairly large amounts of adenovirus (>10⁶ plaque forming units). Subsequent work showed this high efficiency delivery occurred only in very young mice (6). This decreased transfection with age appears to be due in part to the loss of the CAR receptor on the muscle cells (35, 254).

One approach to avoid these problems is to develop cell-targeting vectors by replacing the promiscuous or ineffective cell-binding native ligands present on vectors with cell-specific ligands to generate vectors with enhanced cell specificity and activity. Proof of principle for this approach for muscle has been demonstrated using Ad vectors modified with ligands like pollylysine (29).

While pollylysine does increase transduction, this ligand is non-specific and increases transduction on many cell types. To identify other ligands for vector targeting, we and others have applied peptide-presenting phage libraries to select cell binding peptides (reviewed in (14)). While many cell-binding peptides can be identified from phage libraries, two unpredictable problems can arise when inserting ligands into capsid proteins: 1) insertion of the ligand can destroy capsid and vector function (193, 308) and 2) the ligand may fail when translated into the heterologous structure of the virus (191). These “context” problems are fundamental, since an ideal candidate peptide ligand may be identified, but cannot be applied because the ligand destroys the vector or the vector destroys the ligand. This translation problem stems in part from the fact that peptides
isolated from phage libraries are selected in the protein structural context of the phage pIII protein and are then translated into differing protein structure of a viral capsid protein.

Given this “context” problem, we have engineered viral “context-specific” phage libraries by introducing the H and I sheets of the adenovirus knob domain on to the pIII protein of filamentous bacteriophage. A 12 amino acid (12-mer) random peptide library was constructed by insertion between the H and I sheets in the normal position of the HI loop. Selection of this HI loop context-specific peptide library against C2C12 myoblasts with pre-clearing against non-target cells generated a peptide designated 12.51 with binding substantially better than that of the positive control integrin-binding ligand RGD. When this peptide was translated back into the knob domain of an Ad5 vector, this vector was functional, and 12.51 mediated improved muscle cell transduction, compared to wild type Ad5. These data suggest context-specific phage libraries may be used to identify compatible peptide ligands for viral vector targeting.

3.2 MATERIALS AND METHODS

Cells. C2C12 mouse myoblasts were purchased from American Type Tissue Culture (location). C2C12 cells were maintained in D-MEM supplemented with 10% fetal bovine serum (FBS) with antibiotic/antimycotic. C2C12 myotubes were grown in D-MEM supplemented with 2% FBS and AbAm. 293, Chinese Hamster Ovary (CHO), HeLa, DU-145, MDA-MB-231, and Hepa 1-6 cells were purchased from American Type
Tissue Culture and maintained in RPMI or D-MEM supplemented with 10% FBS and antibiotic/antimycotic.

Construction of Phagemids with Test Peptides. pUC118HI-BAPpIII and pUC118HI-RGD-pIII were constructed as phagemids expressing the 14-amino acid biotin acceptor peptide and RGD integrin-binding motif, respectively, within the H and I sheets of the adenoviral knob on the N-terminus of the pIII phage protein. HI-BAP displaying a biotin acceptor peptide (BAP) and HI-RGD displaying this integrin-binding motif were PCR amplified from prAAV-HI-BAP and prAAV-HI-RGD. The cassettes were digested with BamHI and Xba I restriction endonucleases and ligated to BamHI/Xba I insertion site of pUC118knobpIII placing the H and I sheets between the pIII secretory leader and pIII. Phagemids were transformed in XL-1 Blue electrocompetent cells (Stratagene, La Jolla, CA). Colonies were grown to mid-log phase, infected with VCSm13 kanamycin-resistant helper phage (Stratagene, La Jolla, CA) and grown overnight. Cultures were centrifuged and the supernatant was precipitated with PEG 8000/20% NaCl for 1 h. The phage supernatant was centrifuged and the resulting phage pellet was resuspended in 1 mL Hanks’ Buffered Salt Solution with 0.1% bovine serum albumin (HBSS-BSA). Phage titers were estimated by incubation of serial tenfold dilutions of this sample with 100 μl of concentrated ARI 292 cells for 10 min at 37°C. The cells were then plated onto 6 well YT-AMP plates and grown overnight. Sample phage titers were then calculated from the number of phage/bacteria colonies and the appropriate dilution factor.
Functionality of Peptides within Phage Context. 10⁸ HI-BAP phage were enzymatically biotinylated with 10 μL BirA biotin protein ligase (Avidity, CO), in the presence of 100 μM d-biotin. Phage were then analyzed by western blots detected with anti-pIII antibody or Neutravidin-horseradish peroxidase conjugate (Pierce, CA) at a 1:2500 dilution. Cell binding of HI-RGD phage was assessed by binding to the integrin-expressing cell line, 293A. Cells were grown to 80% confluency and VCSm13, HI-BAP phage, and HI-RGD phage were added for 1 h at 37°C. The cell-associated fraction was collected and_titered as described in (13).

Construction of Phage Library. Library cloning was adapted from Cwirla et al ((53). Oligonucleotides encoding a 12-mer random peptide library flanked by the H and I β sheets of the adenoviral HI loop were phosphorylated with T4 polynucleotide kinase (New England BioLabs). The 5' library olio Hsheet12merlib (5'-CTCAAGGATCCCTGCATCCGGCTCCGCACCTGTAACACTAACCATTACACTANNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKGGTGCAAC-3') was annealed to a complimentary oligo at its 3' end, Isheet3' (5'-CTCATCTAGAGCATGATGATGATCCGCGCCGTCGATC-3'). The complementary strand was filled in using Sequenase (USB, Cleveland, OH). These annealed oligos were then cut with BamHI and XbaI and were ligated at a 1:5 mass ratio with 20 μg of pUC118knobpIII phagemid vector digested with BamHI and XbaI. The ligation was purified by phenol/chloroform extraction and was transformed into XL-1 Blue electrocompetent
cells. Transformation efficiency was determined by titration and library diversity was confirmed by sequencing. Phage was prepared as described above.

**Phage Library Selection.** Peptide selection was performed as described in (13, 285). Direct affinity selection of phage without clearing was performed essentially as described in (13) with the following variations. Briefly, each cell line was grown to confluence in 60 mm dishes. The cells were then washed once with serum-free RPMI and incubated in serum-free medium for 2 h prior to incubation with phage. In the first round of panning, approximately 10 library equivalents (10⁸ phage) were added to target cells in a total volume of 2 ml HBSS-BSA. The phage were then incubated with the cells for 1 h at 37°C with 100 μM chloroquine with protease inhibitor cocktail (Complete™ Protease Inhibitors, Roche). The cells were washed 6 times with 5 ml of room temperature HBSS-BSA. The cells were then incubated for 10 min on ice with 2 ml of 0.1 M HCl pH 2.2 (by glycine). This acid-eluted fraction of phage was saved and neutralized with 400 μl of 1 M Tris pH 8. The cells were lysed in 1 ml of 30 mM Tris pH 8, 1 mM EDTA for 1 hour on ice. The cell debris was scraped from the plate, vortexed briefly and saved as the cell-associated phage fraction. Phage from each fraction were then amplified as described (13). For each subsequent round of panning, portions of purified phage were re-applied to the target cells and panning was carried out as described while maintaining fraction specificity (e.g. when an acid-eluted fraction of phage was initially recovered, then only the acid fraction was amplified in all subsequent rounds).
Phage Library Selection With Clearing. To attempt to remove promiscuous cell-binding peptides from the peptide selection, the phage libraries were bound to non-target competitor cells (Figure 3.1). Phage libraries were first selected for one round against the target cells as described above to enrich the peptide population with cell-binding peptides (approximate 10,000-fold enrichment). At each round of selection after the first round, the phage population from the prior round was pre-bound to non-target competitor cells grown in monolayer in 60 mm dishes for one hour at 37°C. For this study, phage were cleared in mass on a mixture of HeLa, 293, DU-145, Hepa 1-6, CHO, MDA-MB-231, and RAW264.7 cells where $1 \times 10^6$ of each cells was mixed and used for clearing. Once the phage population had incubated with the non-target clearing cells, the supernatant containing the specific binders was recovered and transferred to the target C2C12 myoblasts. These cleared phage were incubated for 1 hour at 37°C on the target cells and cell-binding phage were recovered and amplified as described above. Clearing and affinity panning were repeated for all rounds after round one.
Fig 3.1: Cartoon of selection strategy with clearing.

**Sequencing Selected Phage.** After five rounds of panning, individual colonies of phage-infected bacteria were isolated from random sites on plates and each colony was grown overnight in 3 ml YT-AMP (YT media with 50 μg/ml ampicillin). Phage DNA was isolated from this solution on M13 DNA purification columns (Qiagen, CA). The DNA was PCR amplified using M13 forward and reverse primers and prepared for sequencing.

**Phage Binding Comparison.** Indicated phage clones and libraries were grown up in liquid culture and purified. To test for cell binding, equal numbers of each phage (10⁸) were diluted into a master solution of HBSS-BSA immediately prior to each experiment and aliquots of this solution were added to approximately 10⁵ target cells in 24 well plates
for up to 1 h at 37°C as described in (13). The cell-associated phage were recovered and their titers were estimated as described above.

**Peptide Re-targeting of Adenoviral Vectors.** One of the selected peptides, designated 12.51, was genetically engineered into the HI loop of pL29FiberStop expression vector by site directed mutagenesis, using the Quik Change Site-directed Mutagenesis kit (Qiagen, CA). Briefly, PCR product was amplified using phosphorylated oligos encoding the 12.51 peptide. After DpnI digestion, the PCR products were gel purified, self-ligated, and transformed in XL-1 electrocompetent cells. Successful clones were grown up, and the vector was digested with Bgl II and Kpn I and the resulting insert containing the 12.51 peptide and a downstream zeocin resistant cassette and E4 sequence was recombined into pAd-Easy backbone expressing the dsRed2 red fluorescent protein using the Red recombinase system as in (33, 172). The resulting modified plasmid pAd-HI-12.51 was transformed into XL-1 Blue electrocompetent cells. After confirmation by PCR screening, DNA was purified using a QiaFilter Midi kit (Qiagen). Adenoviral production was followed according to manufacturer’s recommendations (Stratagene, CA). Briefly, pAd-HI-12.51 and wt pAd were digested with PacI and 5 μg of each were transfected into 10 cm tissue culture plate seeded with 293A cells grown to 70% confluency, using Lipofectamine (Invitrogen, CA). About 14 days post-transfection, plaque formation was observed and plaques were picked, subjected to small-scale amplification. Large-scale amplification was done using a cell factory (Corning, NY). After cytopathic effect was observed, cell lysate was collected and subjected to three freeze-thaw cycles. After centrifugation, viral supernatant was purified by two rounds of
cesium chloride (CsCl) density gradient ultracentrifugation at 20,000 rpm for 3 h. Collected viruses were desalted using DG10 desalting columns (BioRad, CA). The genomic titer of the viral particles was quantitated by real-time PCR (Rotorgene, Corbett, Sydney, AUS) using Stratagene MasterMix, following manufacturer's recommendations.

**Adenovirus Transduction on Muscle Cells.** C2C12 myoblasts were grown to 70% confluency on 12-well plates. C2C12 myotubes were differentiated from low passage (passage 1) C2C12 myoblasts to 50% confluency in the presence of DMEM+ 2% horse bovine serum in 24 well plates. On the day of transduction, media was removed and replaced with serum-free media for 2 h to clear receptors. For C2C12 targeting, media was then removed and wt Ad and Ad-HI-12.51 were added at MOI=1000 for 60 min. in serum-free media. The media were then aspirated and cells were refreshed with serum-containing media. Transduction was visualized by fluorescence microscopy (Olympus, WA) and was assessed 48 hours post-transduction by flow cytometry (Becton-Dickinson, CA).

### 3.3 RESULTS

**Display of Peptides within the Fiber HI Loop on Phage.** A biotin acceptor peptide (BAP) and RGD integrin-binding motif were incorporated into the HI loop of the Ad5 fiber as negative and positive control cell-binding peptides, respectively ((202) and data not shown). The H and I sheets along with intervening peptides were then subcloned for display on the C-terminus of the mature pIII protein on fd bacteriophage (phage designated HI-BAP and HI-RGD). To determine the functionality of the displayed
peptides, HI-BAP was biotinylated with the biotin protein ligase, BirA. Western blotting of HI-BAP and HI-RGD detected with neutravidin and anti-pIII demonstrated that this peptide was biotinylated and therefore functional in the context of the 29 kDa HI-BAP pIII protein (data not shown). To test the functionality of HI-RGD phage for cell binding, phage were tested for their ability to bind integrin-positive 293 cells as in (13). HI-RGD phage bound the cells 60 and 200-fold better than negative control VCSm13 and HI-BAP phage, respectively (Figure 3.2). These data demonstrate that both peptide ligands are functionally displayed within the HI loop on phage and suggest this HI loop “context” can be used as a scaffold for random peptide display on phage.

Fig 3.2: Phage Binding of HI-RGD on Integrin-expressing Cell Line. HI-RGD phage was bound to integrin-expressing human embryonic kidney 293A cell line,
compared to control VCSm13 helper phage and HI-BAP phage. $10^8$ input phage of each was bound to 293A cells for 1 h and collected cell fractions were titered on YT-AMP plates in serial tenfold dilutions. Results are of statistical significance with *P < 0.05.

**Selection of random peptides against C2C12 mouse myoblasts.** Semi-random oligonucleotides bearing twelve NNK codons were inserted into the HI loop between the H and I sheets in place of RGD in pUC118 HI-RGD as in (53). The ligation was then transformed into XL-1 bacteria by electroporation followed by infection with helper phage VCSM13 to generate a 12-mer peptide library with approximately 2X10^8 members. Diversity of the library clones was confirmed by sequencing of randomly-picked phagemid clones (Table 3.1).
<table>
<thead>
<tr>
<th>Sequence of H strand</th>
<th>Sequence of 12-mer clones</th>
<th>Sequence of I strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVTLTITL</td>
<td>FCPESQVKWLTLL</td>
<td>GAYYSMSFSW</td>
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<tr>
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<td>KWQVFFHSEDVG</td>
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</tr>
<tr>
<td>PVTLTITL</td>
<td>VTVKMLLDWLAA</td>
<td>GAYYSMSFSW</td>
</tr>
<tr>
<td>PVTLTITL</td>
<td>LRADSVELYIYG</td>
<td>GAYYSMSFSW</td>
</tr>
<tr>
<td>PVTLTITL</td>
<td>GMAELGGGLEWGM</td>
<td>GAYYSMSFSW</td>
</tr>
<tr>
<td>PVTLTITL</td>
<td>ARADRELTKDSW</td>
<td>GAYYSMSFSW</td>
</tr>
<tr>
<td>PVTLTITL</td>
<td>RDGCQSPGLGRG</td>
<td>GAYYSMSFSW</td>
</tr>
<tr>
<td>PVTLTITL</td>
<td>TSGTLRRSVYIG</td>
<td>GAYYSMSFSW</td>
</tr>
<tr>
<td>PVTLTITL</td>
<td>GEDKMRAWRNFK</td>
<td>GAYYSMSFSW</td>
</tr>
</tbody>
</table>

This 12-mer peptide-presenting phagemid library was selected in vitro against C2C12 mouse myoblasts as in (13, 285) with several modifications. To reduce the number of promiscuous peptides that were selected against C2C12 myoblasts, a clearing strategy was applied in which the phage were first adsorbed onto non-target cells before transferring the supernatant of this reaction to the target muscle cells. After five rounds
of selection, ten phage clones were picked and sequenced to assess the repertoire of selected peptides (Table 3.2). 30% of the clones were identical to the 12.51 sequence, 20% were identical to 12.52 sequence, and 1 clone displayed the 12.53 sequence. The remaining clones either had a frame shift, truncation, or deletion in the sequence which are frequently observed when selecting phagemid libraries (36, 90, 122, 276).

Nevertheless, observation of the same peptide in multiple clones indicated that these peptides were likely selected for their cell binding abilities (13, 285). BLAST of the 12.51, 12.52, and 12.53 sequences for similar proteins did not generate any matches with known proteins. These observations are consistent with our previous work with long peptide libraries where no significant data has been obtained by alignment of peptides to the nucleic acid and protein databases (13, 285). It is interesting to note that these peptides have basic residues and positively charged, possibly interacting with generally negative charged cells.

**Table 3.2: Sequences of selected peptides against C2C12 myoblasts after 5 rounds of selection with 4 rounds of pre-clearing**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.51</td>
<td>TARGEHKEEELI</td>
<td>3/10</td>
</tr>
<tr>
<td>12.52</td>
<td>LRQTGAASAVWG</td>
<td>2/10</td>
</tr>
<tr>
<td>12.53</td>
<td>ARRAQTQWRGLE</td>
<td>1/10</td>
</tr>
<tr>
<td>Frame Shift</td>
<td></td>
<td>1/10</td>
</tr>
<tr>
<td>Deletions/Truncations</td>
<td></td>
<td>3/10</td>
</tr>
</tbody>
</table>
Cell Binding of 12.51 Selected Peptide on Phage. Since the 12.51 sequence was most frequently displayed from the selected clones, the 12.51 phage was tested for its ability to bind C2C12 cells as compared to negative control phage, such as HI-BAP and HI-RGD (285). Phage were bound to C2C12 myoblasts and were collected and titered according to previous work (13). 12.51 phage-selected peptide bound C2C12 myoblasts approximately 100 and 1000 fold better than HI-RGD and HI-BAP phage, respectively (Figure 3.3).

![Chart showing binding ratios of BAP, RGD, and 12.51 phage to C2C12 myoblasts.]

Figure 3.3: Binding of C2C12-selected phage to target C2C12 myoblasts. The indicated peptide-presenting phage clone 12.51 and control phage were bound on C2C12 and phage binding was quantitated titering phage as previously mentioned.
**Phage Pool Binding against C2C12 Myoblasts.** During selection, amplified phage from each successive round of biopanning should exhibit increased affinity towards their target. With each round of selection, the phage population should be enriched for cell-specific binders and consequently, will demonstrate improved binding against the target cell with increasing rounds of selection. To test the selected phage for enrichment, phage pools from each selected round and negative control phage were added to C2C12 myoblasts for cell binding. Phage-bound cell fractions were collected and titered with bacteria for phage activity. The input phage titer was compared to the output titer and was determined for each round of selected phage. As seen in Figure 3.4 (below), there is increased phage binding with each successive round of selection and better binding compared to negative control phage displaying a biotin acceptor peptide domain. This suggests that specific phage binders against C2C12 cells are enriched and selected peptides demonstrate improved affinity towards target cells. It is interesting to note that phage collected from rounds 4 and 5 of selection bind better to C2C12 than phage displaying the RGD integrin-binding motif. Potential selected peptides may have improved affinity compared to RGD-integrin interaction.
Figure 3.4: Phage Binding of Phage Pools from Selected Rounds against target C2C12 myoblasts. Phage collected from each round were bound to C2C12 myoblasts to assess binding and enrichment for selected phage. Phage from each round were compared with negative control phage displaying biotin-acceptor peptide (BAP) and phage displaying the RGD integrin-binding motif (designated as RGD). Phage bound to the cells (output) were titered and compared to the input phage (equal phage added to each).

Re-Targeting of Adenoviral Gene Therapy Vectors with Muscle-selected Peptides.
Given that 12.51 appeared to bind C2C12 cells, this peptide was genetically engineered in between the H and I sheets of the Ad5 fiber and this modified fiber gene was recombined into a dsRed2 red fluorescent protein-expressing Ad5 genome using Red recombinase system (Figure 3.5) (33, 172).
Figure 3.5: Translation of selected 12.51 peptide into Ad vector. 12.51 peptide flanked by H and I β sheets were cloned into pL29FiberStop shuttle construct by site-directed mutagenesis. After BglII/KpnI digestion, the resulting fragment was recombined into the pAd Easy backbone by red recombinase system and recombinants were selected on Zeocin resistant plates.

This recombinant Ad5 was then transfected into 293 cells and formed plaques within 14 days (Figure 3.7). Given that the virus formed plaques at the same rate as unmodified Ad5, this suggested that the 12.51 peptide was tolerated within the viral context and did not disrupt viral assembly and function. Large scale amplification and purification yielded normal amount of viral particles.

Peptide-Modified Ad Vectors: Assembly and Function. After peptide selection, peptides were genetically engineered directly into the HI loop in the Ad genome. The modified Ad genomes were transfected into 293 mammalian cells for virion production. In order to determine the compatibility of the engineered ligands in the trimeric fiber protein, we transfected 12.51 and 12.52 modified-fiber expressing plasmids and looked at fiber expression from a Western blot under native and denatured conditions. Samples were either prepared following non-denaturing or denaturing conditions and were electrophoresed on a SDS-polyacrylamide gel. Samples were transferred onto a PVDF membrane and probed with antibody against monomer and trimeric fiber proteins and secondary HRP conjugate to look for fiber expression. Figure 3.6 is Western blots of 12.51 and 12.52 peptides engineered into the fiber protein. Under denatured conditions
(Figure 3.6, left), there are bands present around ~66 kD for wild type fiber (leftmost) and fiber-12.51 and fiber-12.52. This is expected and suggests that the fiber does not interfere with fiber expression. Under native conditions (Figure 3.6, right), there are bands present at ~180 kD for wild type fiber and the peptide-modified fibers. This suggests that the peptides do not ablate fiber trimerization and the fiber can properly assemble with the incorporated peptides.

Figure 3.6: Western blots of fibers modified with 12.51 and 12.52 selected peptides under denatured (a) and native (b) conditions. Samples were loaded and electrophoresed on 7.5% SDS-polyacrylamide gels. After transfer onto a PVDF membrane, the blots were probed with mouse RDI antibody against fiber monomer and trimers (1:400 dilution in TBST) and a secondary goat-anti mouse-HRP
conjugate (1:10000 dilution). Blots were exposed to chemiluminescent substrate and were developed on X-ray film. In (a), 12.51 and 12.52-modified fibers express monomers (arrow) and more importantly in (b), these modified fibers are able to form trimers (arrow) like wild type fiber (2nd column).

We also looked at plaque formation of the modified virions to determine if peptides can be tolerated and not ablate viral assembly and growth. After transfection of wild type and modified Ad genomes into 293 cells, plates were overlaid with agar to allow for plaque formation (cell lysis and viral spread, indicative of viral growth). As visualized by fluorescent microscopy (Figure 3.7), Ad wt and Ad-HI-12.51 both exhibit plaque formation, as evidenced by the holes formed of cell lysis by virus. 12.52-modified Ad also demonstrates virion formation (data not shown).

![Fluorescent micrographs of plaque formation of wt Ad (left) and Ad-HI-12.51 (right) expressing DsRed reporter protein. Linear Ad genomes were transfected in 293 cells and after three weeks, cells were overlaid with agar to allow for viral-based cell lysis and viral spread. As evidenced by fluorescent microscopy,
there are holes (indicative of cell lysis) flanked by surrounding cells expressing red fluorescent protein (indicative of viral spread).

**Targeted Transduction of Ad-HI-12.51 against C2C12 myoblasts.** To assess transduction, unmodified Ad and Ad-HI-12.51 were added to C2C12 myoblasts and transduction was assessed 48 hours later. In this case, Ad-HI-12.51 mediated approximately 14-fold increase in transduction to C2C12 myoblasts compared to unmodified Ad (Figure 3.8A and 3.8B), as evidenced by flow cytometry. These data suggest that translation of this “context”-selected peptide back into its native context in the adenoviral fiber can enhance transduction to target cells.
Figure 3.8: Transduction of mouse C2C12 myoblasts. Wild type Ad5 or Ad-HI-12.51 expressing red fluorescent protein was applied to the indicated C2C12 myoblasts. Cells were transduced with 1000 particles/cell and transduction was analyzed 48 hours later by fluorescent microscopy (A) and flow cytometry (B). Flow cytometry results are expressed as percent positive of cells transduced by respective virus after 60 minutes incubation. Standard deviation of each is indicated. Results are statistically significant with *P<0.05.

Ad-HI-12.51 Targeting against C2C12 Myotubes. Ad-HI-12.51 was also tested against C2C12 myotubes to determine the ability of the 12.51 peptide to target differentiated muscle cells that are indicative of the muscle environment in vivo. At particle to cell ratios of 1000, Ad-HI-12.51 mediated four-fold higher transduction compared to unmodified Ad5 (Figure 3.9), as indicated by flow cytometry. While the
level of transduction is low, this difference is significant. The small level of transduction can possibly be attributed due to the peptide being selected against non-differentiated C2C12 myoblasts and low level expression of the unknown targeted receptor.

Nevertheless, these data suggest the potential of 12.51 may have utility in increasing transduction of both non-differentiated and differentiated muscle cells.

A.

B.
Figure 3.9: Targeted Transduction of C2C12 Myotubes with Ad-HI-12.51. Wild type Ad or Ad-HI-12.51 expressing dsRed reporter protein was added to differentiated C2C12 cells (myotubes). Cells were transduced with 1000 particles per cell and assessed for transduction by fluorescent microscopy (A) and flow cytometry (B) 48 hours later. Flow cytometry results are expressed as percent positive of cells transduced by respective virus after 60 minutes incubation. Standard deviation of each is indicated. Results are statistically significant with \*\*\* \( P < 0.01 \).

Ad-HI-12.51 Specificity Towards Non-Specific Cells. We wanted to determine if 12.51-modified Ad non-specifically targets other cells \textit{in vitro}. Wild type Ad and Ad-HI-12.51 were incubated on target C2C12 cells, A431, and Raw264.7 macrophage cells. Targeting was assessed 48 hours post-transduction by fluorescence microscopy. As seen in Figure 3.10 below, Ad-HI-12.51 targets C2C12 myoblasts and exhibits no tropism towards A431 or Raw264.7 cells. While wild type vector also does not transduce these cells, it does not transduce C2C12 myoblasts, possibly due to low levels of CAR expression on these cells. Independent of CAR, Ad-HI-12.51 is able to transduce refractory C2C12 cells and not other similarly refractory cells, suggesting the specificity of Ad-HI-12.51 is towards C2C12 myoblasts.
Figure 3.10: Transduction of Ad-HI-12.51 against various cell types. Ad-HI-12.51 was added to target C2C12 cells and non-specific A431 and Raw264.7 cells to test specificity of the peptide selected vector. Transduction was assessed 48 hours post-incubation for DsRed reporter protein expression by fluorescence microscopy.

Re-targeting to human skeletal muscle in vitro using Ad-HI-12.51. Ad-HI-12.51 was also tested against human skeletal muscle cells to determine the cross-species ability of the murine-selected 12.51 peptide to target human skeletal muscle cells (hSkMCs). Wild type Ad and Ad-HI-12.51 were added at various particle to cell ratios to hSkMCs and incubated for 60 min. Transduction was assessed 48 hours later by fluorescent microscopy (Figure 3.11). As seen in Figure 3.11, there is transduction of Ad wt and Ad-HI-12.51 by the levels of red fluorescence expression at particle to cell ratios (designated
here as MOI) of 100, 200, and 500. There is no significant difference between wild type and peptide-modified transduction. This can be due to the high level of CAR receptor expression present on hSkMCs, resulting in background transduction (270). It is unclear if the peptide facilitates increased transduction to cells from different species and subsequent reporter expression.

Figure 3.11: Targeted transduction of human skeletal muscle cells with wild type Ad and Ad-HI-12.51 at various particle to cell ratios (denoted here as MOI). Cells were incubated with Ad wild type or Ad-HI-12.51 for 60 min and transduction was assessed for red fluorescence by fluorescent microscopy 48 hours later.
3.4 DISCUSSION

Adenoviruses have developed as attractive method for gene delivery due to their ability to transduce a wide variety of dividing and non-dividing cells. However, their ability to successfully target a given cell type is limited by its evolution to infect a multitude of cell types. As one approach to develop more specific vectors, we and others have used peptide-presenting phage libraries to identify cell-binding peptides to be engineered into gene therapy vectors (13, 168, 191, 231, 232, 285, 305). However, two problems can arise from incorporation of selected peptide ligands into viral vectors for retargeting. The selected peptide ligand can ablate viral assembly or function and the virus can debilitate the binding and/or internalization ability of the peptide ligand. In order to circumvent this ligand compatibility problem, in this work, we generated a “context-specific” filamentous phage library in which random peptides were displayed between the H and I sheets from the Ad5 fiber protein, a location previously used on the virus for ligand display (225). By this approach, we demonstrate the selection and functional translation of a skeletal muscle cell binding peptide back into Ad5 vectors to increase transduction of these cells.

Previous work reported the display of the intact knob domain from the fiber on filamentous phage (211). In our hands, similar display of intact knob phage was toxic to bacteria and with only 1 out of 100 displaying the knob (14). We speculate that this toxicity was due to incompatibility of knob during its secretion from the bacteria on the pIII protein. More recent work has displayed intact knob peptide libraries on lysogenic lambda phage rather than the secreted filamentous phage (77). In this case, a $2 \times 10^5$ member semi-random 14-mer peptide library was displayed in the HI loop of knob.
Selection of this library against NIH 3T3 cells selected peptides that increased transduction on the 3T3 cells when translated into the virus by 100-fold (77). The advantage of this lambda library approach is that legitimate knob trimers can be displayed on the phage. The advantage of the filamentous phage display used in our work is that substantially larger peptide libraries can be generated to theoretically allow more peptide ligands to be screened to perhaps find higher affinity ligands (14).

Our data suggests that "context-specific" peptide libraries can be used to identify ligands that are compatible when translated back into the viral capsid context. Ad-HI-12.51 exhibited 14-fold increase in transduction compared to wild type Ad in vitro. In our studies, we observed poor transduction of mouse muscle cells by unmodified Ad5 which is likely due to the low level of CAR expression on these cells (129). Consequently, the efficacy of Ad transduction is limited. Upon incorporation of selected 12.51 peptide into the Ad HI loop, we observed significant increase in transduction.

When tested on human skeletal muscle cells, Ad-HI-12.51 mediated increased transduction relative to unmodified Ad5, particularly at lower particle to cell ratios, suggesting the broad tropism of 12.51 to target similar receptors. However, at higher ratios, both vectors mediated substantial increases in transduction, suggesting that CAR or other Ad receptor levels may be higher on the human cells in contrast to the mouse cells. The native tropism of Ad vectors will need to be ablate to prevent non-specific transduction and create a truly targeted vector (71). It will be necessary to design vectors ablated for CAR, heparin, and intergrin binding (57, 71, 271, 306). Work is underway to display 12.51 and the other peptides in the context of Ad vectors ablated for CAR,
heparin, and integrin binding to determine if these will mediate increased transduction of both mouse and human skeletal muscle cells.

While we are able to demonstrate improved targeting of Ad vectors in vitro with selected 12.51 peptide, the level of transduction is not high and may be the result of poor affinity of the ligand towards the target muscle cells. Work by others have improved the affinity of ligands such as antibody fragments by affinity maturation (185). Antibody fragments selected by phage display were randomly mutagenized and re-panned against immobilized antigen target and selected, “matured” antibody fragments exhibited improved affinity towards the antigen compared to the originally selected fragment (185). Adapting this approach, we can improve the affinity of the 12.51 peptide and potentially improve the targeted transduction efficiency of the ligand-modified Ad vectors. Besides affinity-based approaches, targeting can possibly be enhanced by improving the avidity of ligand-modified vectors. The 12.51 peptide was engineered into the HI loop domain of fiber protein and is expressed in 36 copies of modified fiber. Other locales with higher number of copies in the viral capsid such as hexon and penton can be investigated for insertion and increased display of selected peptides. Work by others has incorporated various peptides into the C-terminus of pIX, hexon and penton for ligand display and targeting (62, 293, 299, 306). These proteins are displayed with higher valency and selected peptides can be engineered into these locales to improve targeting by increased avidity.

Peptides have been selected as functional ligands against myoblasts by biopanning techniques. However, BLAST analysis suggested that these peptides are unknown proteins. While using the biopanning approach against cells is advantageous
because it is not necessary to have prior knowledge of cell surface receptors, the ligand-
receptor interaction is unknown. Future work can involve determination of ligand-
receptor interaction by isolation of the receptor from membrane proteins via ligand-
affinity chromatography, as previously demonstrated (133).

In summary, we demonstrate proof of principle for the use of relatively large
repertoire “context-specific” peptide-presenting phage libraries as a potential approach to
generate and identify compatible ligands when incorporated back into the viral capsid for
retargeting. We are currently testing this library for its utility in selecting novel peptides
against human skeletal muscle cells and other target cells for gene therapy.
Chapter IV
Streptavidin-Context Library

4.1 Introduction

Avidin is a 64 kDa tetrameric glycosylated chicken egg white protein that binds to biotin (also known as vitamin H) with the strongest known non-covalent interaction found in nature (95). The biotin-avidin interaction has an affinity coefficient of $K_a = 10^{14} - 10^{15}$ M, which is $10^6$-fold stronger than the nanomolar affinity of an antigen-antibody interaction (95, 234, 310). A $K_a$ of $10^{15}$ M corresponds to a free dissociation energy of 87.9 kJ/mol (226), roughly one-quarter the strength of a typical C-C bond (30). The strong and specific interaction between avidin and biotin has been exploited as a technology for a wide array of biological applications, such as protein purification, molecular labeling, imaging, and drug targeting, protein targeting, and gene therapy vector targeting (34, 156, 181, 182, 201, 273) (12). This technology has been expanded towards targeting applications such as conjugation to polyethylene glycol for tumor targeting (32), coupling to small ligands for gene transfer (136) and targeted drug delivery (183, 249).

Biotin is a naturally occurring cofactor for metabolic enzymes known as the biotin-dependent carboxylases. These carboxylases remove or transfer carboxyl groups to and from metabolites and are important in many metabolic processes (40). Biotin is covalently attached to a specific lysine residue of a highly conserved sequence of the apocarboxylase domain of the carboxylase enzymes (40). Biotin attachment to the
transcarboxylases is mediated by enzymes called biotin-protein ligases (BPLs). BPLs present in mammalian cells, prokaryotes and \textit{S. cerevisiae} have naturally diverged from each sources (242). Holocarboxylase synthetase (HCS) is the mammalian BPL that can recognize and biotinylate apocarboxylase domains (146).

Biotin attachment to carboxylases by BPLs is a post-translational modification. In mammalian cells, HCS biotinylates the four mammalian apocarboxylases: acetyl-CoA, methylcrotonyl-CoA, propionyl-CoA, and pyruvate carboxylases (131). These carboxylases have a role in the biotin-dependent metabolism and regulation of gene expression (275).

Biotinylation of proteins has been achieved by multiple means. Originally, biotin labeling of proteins was done \textit{in vitro} by chemical modification. Cross-linking agents have been used to covalent attach biotin to proteins (47). However, there are several drawbacks to this approach. Chemical biotinylation is not site-specific and may ablate function of the biotinylated protein due to random chemical modification of primary amine groups (282). In addition, proteins need prior purification before biotinylation (282). Studies have shown that chemically-biotinylated proteins can be unstable in a position- and reagent-dependent fashion (27).

An alternative to chemical biotinylation is site-specific metabolic biotinylation of proteins \textit{in vivo} through genetic fusion of biotin-accepting apocarboxylase domains (also known as biotin acceptor peptide (BAP) domains). Initial work by Cronan \textit{et al} demonstrated that the 1.3S subunit of the 123 amino acid \textit{Propionibacterium shermanii} transcarboxylase domain (PSTCD) was post-translationally biotinylated (48, 203, 205). PSTCD fused to other proteins are site-specifically biotinylated at the E-amino group of
the lysine residue at position 89 by endogenous BPLs like *E. coli birA* or human HCS (48, 203, 204). In our laboratory, we demonstrated the ability to tag green fluorescent protein and adenoviral knob protein with various lengths of the PSTCD domain and biotinylate these fusion proteins in mammalian cells and in mice via endogenous BPLs (203).

We were able to truncate the 123 amino acid residues of the PSTCD domain into 63 and 70 amino acid C-terminal residues and are sufficiently metabolically biotinylated *in vivo* in mammalian cells, without the addition of exogenous BPLs (16). The NMR solution structure of the PSTCD (223) reveals that these 70 residues fold into a compact \( \beta \)-sandwich structure, with the N and C termini in close proximity on the end opposite the biotinyl-lysine residue (Figure 4.1). The ability to truncate the PSTCD domain and maintain its ability to be enzymatically biotinylated is important towards the development of metabolically biotinylated vectors for gene targeting.

In our laboratory, we are interested in taking advantage of the biotin-avidin system and utilizing it towards the development of metabolically biotinylated gene therapy vectors for targeted gene delivery. To accomplish vector targeting, there are several approaches to improve targeting to specific cells. Cell-specific ligands can be genetically engineered directly into surface exposed regions of the viral capsid for ligand display and modify vector tropism. However, insertion of ligand directly into the viral capsid may ablate virion assembly and/or function and conversely, the ligand may be
Figure 4.1: Ribbon diagram of the C-terminal 70 amino acids of the BAP from *P. shermanii* transcarboxylase domain, from (223).

inactivated upon incorporation into the capsid. Ad assembly in the cytosol occurs in a reductive environment, possibly leading to improper folding of the ligand during assembly due to the inability to form proper disulfide bonds. In addition, direct genetic capsid modification is limited by the size of inserted peptide. Another approach that avoids these limitations is the formation of a molecular bridge or conjugate between Ad vector and an alternative cell surface receptor. The native virus-cell binding is
circumvented by using "bi-specific" molecules that retarget Ad to an alternative cell surface receptor.

To apply metabolic biotinylation for vector targeting, our approach involved the combination of genetic engineering and conjugate targeting approaches. We engineered the small 70-aa PSTCD domain into various locales of the adenoviral capsid such as the C-terminus of the fiber, pIX minor protein, and hexon protein (34, 202). We were able to produce PSTCD-capsid modified virions that were metabolically biotinylated during virus production in mammalian 293 cell line by endogenous BPLs (34, 202). These biotinylated virions served as a platform to conjugate biotinylated ligands such as antibodies and peptides, via a tetrameric avidin bridge, for vector targeting and screening. Targeting was demonstrated against erythroid leukemia cells, mouse myoblasts, dendritic cells, and liver cells in vitro (34, 202). Various ligands were screened as potential cell-specific ligands by conjugation to the vectors against dendritic cells (202). The biotin-avidin system has been utilized towards development of a novel vector targeting strategy.

However, there are some improvements that could be made in the metabolically biotinylated adenoviral vector system. Currently, this technology is a three-component system. Biotinylated ligands bind to molecular tetrameric avidin and this complex is conjugated to the biotinylated adenovirus. While the three-component system works, we would like to simplify the system to apply it as a two-component system to limit potential problems towards targeting. One way to reduce the number of the components is to fuse ligands to directly to avidin rather than complexing them via the biotin-avidin interaction. Extensive research has focused upon the production of avidin-ligand fusions for affinity purification and biotin-based applications. Beta-galactosidase, glutathione S-transferase,
and mannose 6-phosphate receptor proteins have been successfully fused to avidin on either N- or C-terminus of the protein for functional ligand display and targeting (1-3, 124, 140). Recent work by Lehtolainen et al demonstrated fusion of macrophage scavenger receptor and avidin for and successful conjugation to biotinylated drug molecules for targeting (145). However, ligand-avidin fusions are limited by successful folding of the both the ligand and the tetrameric avidin. It is difficult to predict which ligand will or will not impair avidin assembly and vice versa, if the ligand will be able to fold properly. It is necessary to identify ligands that will be compatible with avidin.

There are also other drawbacks to the three-component metabolically biotinylated vector system. It is possible that the biotinylated ligands could bind to all four binding regions in the avidin, preventing successful conjugation of vector to an available site on the avidin protein. Consequently, it would be attractive to develop a system where we can display ligands on avidin without occupying the biotin binding sites needed for biotinylated vector conjugation.

In Chapter 3, we developed phage-presenting random peptide libraries in an adenoviral structural context for functional ligand selection. We hypothesized that context-selected peptides can be genetically engineered into viral vectors without disrupting viral function or formation or possibly inactivate the ligand itself. We were able to demonstrate that functional ligands could be selected in the adenoviral HI loop structural context on phage and be incorporated into the viral capsid for Ad re-targeting against skeletal muscle. In this chapter, we describe work to expand the scope of “context-specific” libraries to target biotinylated vectors by engineering peptide libraries in an avidin structural context.
We could attempt to display peptides in avidin, however, this eukaryotic glycosylated protein is difficult to produce in bacterial systems needed for phage display. In contrast, streptavidin is a protein produced by *Streptomyces avidinii*, is similar to avidin except that it is non-glycosylated and neutrally charged, compared to avidin (high isoelectric point pI ~10.5) (39). Streptavidin exhibits less background binding than avidin and is used in many applications instead of avidin. Reports have indicated engineering functional peptides into the domain of streptavidin for cell binding (170). McDevitt *et al* engineered functional RGD-integrin binding peptides into accessible loop domains on streptavidin (Figure 4.2). From X-ray crystallography, they were able to incorporate RGD motif into a known exposed loop flanked by opposing β strands at amino acid residues 65, 66, and 67 of streptavidin, and exhibited cell binding without compromising its biotin binding properties (143, 169). The RGD modified streptavidin could bind endothelial cells and was inhibited by RGD competitive peptide, suggesting the RGD motif in streptavidin was responsible for cell binding and not the streptavidin molecule itself (169). In addition, the RGD peptide did not disrupt streptavidin assembly and was able to bind to biotic, as demonstrated by ELISA (169). Structurally, the loop domain where RGD was inserted is not close to the biotin-binding site (143). This suggests that peptides can be tolerated in specific locales of streptavidin without ablating the streptavidin-biotin interaction.
Figure 4.2: Three-dimensional structure of streptavidin. Yellow colored portions of blue-colored loops are sites for insertion of RGD peptides (McDevitt et al, 1999).

Our work focuses upon displaying streptavidin on phage and developing random peptide libraries within the streptavidin structural context for use in ligand selection. Previous work has demonstrated that tetrameric streptavidin can be functionally displayed on phage. We have engineered streptavidin on the N-terminus of pVIII phage protein and generated a random peptide library within this scaffold at the accessible loop domains of streptavidin (amino acid sites 65, 66, and 67). We have demonstrated functional streptavidin display and maintenance of biotin-binding ability. Progress has made towards selection against mouse skeletal muscle for targeting and current and future work towards ligand identification and targeting will be discussed.
4.2 Materials and Methods

Cloning of Streptavidin into p8v2 Phagemid. Streptavidin (SA) gene was gene built using a protocol adapted by S. Johnston. Overlapping oligonucleotides encoding for the core streptavidin with a c-myc epitope tag located in amino acid residue 65, 66, and 67 were PCR amplified and subcloned into the pCR2.1TOPO cloning vector (Invitrogen, CA). The resulting pCR2.1TOPO-SA plasmid was cut with BamHI/XbaI restriction endonucleases to cut out the SA insert and cloned into the similarly digested phagemid p8v2 (cut with the same endonucleases). Phage preparation is as described in Chapter 3. Briefly, resulting phagemid p8v2SA was grown in LB media with selective antibiotic and infected with helper phage at a MOI = 10. Phage was precipitated with 0.2 volume PEG 8000/20% 2.5M NaCl and resuspended with 1 mL HBSS-0.1% BSA. Phage was titered in ten-fold dilutions, as described before.

Library Construction. 20-mer random peptide-encoding libraries were incorporated into the phagemid adapted from the protocol by McDevitt et al (McDevitt et al, 1999). Briefly, (NNK)20 oligonucleotides were purchased from Operon Technologies (Operon, CA) and 5’ phosphorylated with T4 polynucleotide kinase (Invitrogen, CA). They were cloned into p8v2 following the methods described above. Ligation products were screened by PCR and transformed into XL-1 MRF’ electrocompetent cells. Phage were grown and prepared as described before.

Western Blot. Samples were probed for streptavidin display on phage. Phage samples were denatured in Lamelli’s SDS-protein loading buffer and were loaded on 7.5% SDS-
polyacrylamide gels. After electrophoresis, the samples were transferred onto a PVDF membrane using a BioRad trans-blot apparatus. Samples were detected with a 1:1000 dilution of rabbit anti-streptavidin serum primary antibody. The blots were incubated with 1:10000 dilution goat anti-rabbit-HRP conjugate and detected using chemiluminescent substrate.

**Direct ELISA for Streptavidin Display.** To quantify streptavidin display, we did direct binding phage ELISAs. Briefly, phage samples were diluted four-fold in HBSS-0.1% BSA and 100 uL volumes of each sample were adsorbed on 96-well microtiter plates for 2 h at room temperature, with gentle shaking. Wells were blocked with 5% milk in TBST for 2 h to prevent non-specific binding. After several washes, samples were incubated with either 1:1000 dilution of rabbit anti-streptavidin antibody (to look for SA display) for 1 h, with gentle shaking. After several washes, samples were probed with a secondary anti-rabbit-HRP conjugate against the primary antibody. After incubation, TMB substrate was added to produce a colorimetric reaction. The reaction was stopped after 30 min with 1.8 M H₂SO₄ and plates were read for absorbance at 450nm using a plate reader and analyzed with HTSoft software.

**Capture ELISA for Biotin Binding.** To look at biotin binding, we performed a capture ELISA. 96-well microtiter plates were adsorbed with biotinylated antibody at a concentration of 100 ng/well for 2 h at room temperature with gentle shaking. After multiple washes with PBS, plates were blocked for non-specific protein adsorption with 5% milk solution in Tris-buffered saline with Tween detergent (TBST) for 2 h at room
temperature. After blocking, negative control phage, streptavidin display and streptavidin-context library on phage were adsorbed on the wells for 2 h at room temperature with gentle shaking. After repeated washes, samples were detected with 1:1000 dilution of rabbit anti-streptavidin antibody and a reactive HRP conjugate for 1 h each. Wells were incubated with TMB substrate and the colorimetric product was read as described above.

**Peptide Selection.** Phage were panned for peptide selection following methods previously described in Chapter 3. Briefly, C2C12 mouse myoblasts were cultured on 60mm plates until 90-100% confluence in serum-rich DMEM media. Prior to binding, media will be aspirated and replaced with serum-free DMEM to clear receptors. After 2 h media will be replaced with 2 mL HBSS-0.1%BSA and $10^8$ streptavidin-library phage. Phage will be incubated for 1 hour at 37°C. After five washes with HBSS-0.1%BSA, 2 mL 0.1 N HCl-Glycine (pH = 2.2) was added and weakly bound phage was eluted and neutralized with 225 uL 2 M Tris. After acid elution, the cell fraction was harvested and resuspended in 1 mL 30 mM Tris (pH = 8.0). The cell fraction was used to infect XL-1 Blue bacteria (Stratagene, CA) and was co-infected with VCSm13 helper phage (Stratagene, CA) to amplify the phage peptides collected in the cell fraction. The amplified culture was grown overnight and phage was precipitated as described before in (Chapter 3). The amplified phage was subsequently used for the next round of selection. Samples were sequenced as described before in Chapter 3.
4.3 Results

**Streptavidin Display on Phage.** To determine if streptavidin could be functionally displayed on phage, streptavidin was cloned onto the N-terminus of pVIII protein in a phagemid vector. The resulting construct was packaged in conjunction with a helper phage and the resulting phage particles were evaluated by western blot (Figure 4.3). There was a band present at ~20 kD, which is the expected band size of streptavidin fusion to the phage protein.

![Western blot](image)

**Figure 4.3:** Western blot of phage display of streptavidin. Various samples displaying streptavidin on phage were electrophoresed on 10% SDS-polyacrylamide gels. Samples were transferred onto a PVDF membrane and probed with 1:1000 dilution of rabbit anti-streptavidin primary antibody and 1:10000 goat anti-rabbit-HRP secondary antibody. Blots were incubated with sensitive chemi- luminescent substrate and developed on film. All samples express streptavidin at expected size of ~20 kD (as indicated by arrow).
To determine if the displayed streptavidin was functional, SA-display phage and negative control phage were adsorbed onto microtiter plates at various dilutions. They were then probed with either streptavidin-specific antibodies and reacted with TMB substrate, resulting in a colorimetric product. Figure 4.4 below shows SA display of the phage at a titering effect. At high phage concentrations, SA was detected by ELISA for SA expression, compared to negative control. We later examined phage displaying streptavidin for biotin binding by capture ELISA.

![Graph showing absorbance at 450nm against ten-fold dilution](image)

**Figure 4.4: Direct ELISA for Streptavidin Display on Phage.** Phage displaying streptavidin on pIII were diluted ten-fold and adsorbed on microtiter plates and compared to control and a positive control (streptavidin protein at ten-fold dilutions). Phage were probed with rabbit anti-streptavidin (anti-SA) antibody at a 1:1000 dilution (in TBST) and secondary donkey anti-rabbit-HRP conjugate (1:10000) dilution. Samples reacted with TMB substrate to produce a colorimetric reaction. The reaction was stopped with 1.8 M sulfuric acid and absorbances were read at 450nm using a plate reader.
**Streptavidin Library Production and Functional Display.** After demonstrating that streptavidin could be functionally displayed on phage, we incorporated a 20-mer peptide library into the surface-exposed loop of streptavidin, previously used for RGD peptide insertion (169). During the building of the streptavidin gene, we incorporated a c-myc epitope into the loop domains of streptavidin (around residues 65, 66, and 67) with flanking restriction endonuclease sites for library oligonucleotide insertion. Oligonucleotides coding for random 20-mer peptides were cloned into the phagemid, transformed into bacteria, and were packaged with helper phage. After titering of phage, we were able to determine the library had approximately 2x10⁹ members.

To determine if incorporation of the 20-mer peptide library disrupted streptavidin display, we performed a direct ELISA to detect streptavidin expression. As seen in Figure 4.5, SA is expressed and suggests that library peptides do not hinder functional SA display.

![Absorbance (560nm) vs Fold Dilution Graph](image)
Figure 4.5: Direct ELISA of streptavidin-context phage library for streptavidin detection. Phage were adsorbed on microtiter plates at various dilutions and probed with streptavidin antibody (rabbit anti-SA at 1:1000 dilution in TBST) for functional streptavidin display.

We wanted to evaluate the biotin binding properties of the streptavidin on phage and the streptavidin-context phage library. We were able to detect binding by a capture ELISA. Microtiter plates were adsorbed with biotinylated antibody and phage were bound to the biotinylated antibody-coated plates. After repeated washes before and after binding, samples were detected with streptavidin-specific antibody and reacted with TMB substrate to form a colorimetric product. This reaction was stopped at read for absorbance at 450nm using a plate reader. As seen in Figure 4.6, there was biotin binding of both streptavidin on phage and the streptavidin-context library, compared to the negative control. The streptavidin-biotin interaction was detectable, suggesting that the streptavidin-context ligands can maintain their biotin-binding properties needed for eventual conjugation to biotinylated vectors for targeting.
Figure 4.6: Biotin binding of streptavidin-context library by capture ELISA. Phage, including negative control phage, positive control phage displaying streptavidin (denoted SA phage), and streptavidin-context library (SA library), were captured on microtiter plates precoated with biotinylated antibody (100 ng/well). Samples were detected with primary anti-streptavidin antibody (1:1000 dilution) and a secondary goat anti-rabbit-HRP conjugate (1:10000 dilution). Samples were reacted with TMB substrate and the colorimetric reaction was read using a plate reader with an absorbance filter at 450nm.

**Peptide Selection Against C2C12 Myoblasts.** The goal of the SA-library was to identify ligands in the structural context of streptavidin for eventual application towards targeted delivery. Towards this end, we used the streptavidin library and selected against C2C12 mouse myoblasts for three rounds. However, we were unable to collapse the library of $2 \times 10^9$ peptides into a set of a few, repeated peptides or even determine a consensus sequence. It is necessary to continue selection in order to determine a set of a few ligands for potential targeting. However, the selected rounds were able to maintain their biotin binding properties (data not shown).

**4.4 Discussion**

The focus of our work was to develop a streptavidin-context phage library to generate and identify potential peptide ligands. The goal of this work would be applied to conjugate these streptavidin-context ligands to previously developed metabolically biotinylated Ad vectors and metabolically adeno-associated viral vectors (Chapter 5).
towards vector re-targeting. In the current state of metabolically biotinylated vectors, we need to conjugate biotinylated ligands to the vectors via a tetrameric avidin bridge or use avidin-fused ligands. The three-step complexing process for vector targeting may be too cumbersome for effective targeting and the avidin bridge may be saturated by either biotinylated ligand or biotinylated vector, preventing effective conjugation of vector to ligand. In addition, ligands fused to the N- or C-terminus may ablate proper avidin/streptavidin assembly or may inactivate the ligand itself. In order to circumvent the problem of ligand-avidin compatibility, we have adapted the “context-specific” library approach from Chapter 3 to generate and identify compatible ligands in the structural scaffold of streptavidin on phage that can be complexed directly to biotinylated vectors for targeting. McDevitt et al. discovered locales on streptavidin that could tolerate peptide insertion while maintaining biotin binding properties (169). We took this finding and generated a random peptide library on the surface exposed loops of SA and displayed SA on phage. We were able to functionally display SA on phage and generate a $\sim 10^9$ member library within streptavidin context on phage. The SA library was able to display streptavidin and maintain biotin binding.

Current and future work involves selection of peptide ligands from the SA library. After confirmation of cell-specific binding, the SA-context ligand will be produced in a bacterial expression system (246) and purified by affinity separation, taking advantage of reversible biotin-monomeric avidin interaction ($K_d = 10^7$)(204). For our work, after ligand production and purification, the ligand can be directly complexed to biotinylated vectors and evaluated for targeting in vitro and in vivo. The streptavidin-context ligands can be used towards a wide array of applications. This can replace avidin/SA fusion
ligands because there will be no compatibility issues. There will be no problems predicting proper folding of ligand and/or avidin or streptavidin. In addition, the cell-targeting ligands can be complexed to other biotinylated molecules for biological applications, such as labeling, DNA and drug targeted delivery, and imaging (10, 111, 144, 183, 228, 236, 248, 301).
Chapter V

Construction of a Metabolically Biotinylated Adeno-Associated
Virus Vector as a Platform for Ligand Screening and
Targeting

5.1 Introduction

As shown in Chapter 3, we hypothesized that introduction of structural context onto filamentous bacteriophage can improve selection of functional peptide ligands from phage display. Currently, with our adenoviral-based HI library, we selected peptide ligands that can be utilized to enhance transduction of adenoviral vectors towards its target. However, one of the limitations of this approach is that for each selected peptide ligand, it has to be genetically engineered into the viral capsid each time for generation of a re-targeted adenoviral vector. This can be a time-consuming and laborious approach for making targeted gene delivery vectors. One possible approach is to circumvent this problem is to genetically engineer or adaptor or molecular conjugates onto the viral capsid that can serve as a platform to conjugate various ligands, including selected peptides, for targeting.

From our previous chapter, we have generated a streptavidin-context specific library to generate potential cell-specific ligands that are compatible within the structure of streptavidin. After selection, the eventual goal is to produce and conjugate these context-based ligands via a biotin-based platform for vector targeting. Biotin-avidin
technology has been used for a wide array of biomedical and molecular biology applications, such as immunoblotting and purification. This non-covalent interaction is the strongest present in nature ($K_d = 10^{-15}$).

Previous work in the laboratory demonstrated that metabolically biotinylated adenoviral vectors could be used as a versatile platform to conjugate ligands for screening and targeted delivery. In this work, we utilized the 1.3S subunit of *Propionibacterium shermanii* transcarboxylase domain (PSTCD) protein that is naturally biotinylated in *Escherichia coli* and *Saccharomyces cerevisiae* at lysine 89 by a biotin protein ligase. PSTCD domains were truncated to 63 and 70 amino acids by N-terminal deletion and were shown to be metabolically biotinylated in mammalian cells by biotin protein ligase, holocarboxylase synthetase (203). One PSTCD domain was incorporated onto the C-terminus of Ad fiber and was metabolically biotinylated in mammalian cells during virion production without the addition of exogenous biotin protein ligase (202). We were able to produce functional biotinylated adenovirus that was useful in vector targeting and ligand screening by conjugation to biotinylated ligands to the virus via a tetrameric molecular avidin bridge. These vectors could be purified by reversible binding to monomeric avidin ($K_d = 10^{-7}$). The biotinylated Ad vector is an attractive approach to screen cognate ligands against specific cell surface receptors.

In our laboratory, we currently use adenoviral vectors for targeting. However, alternate viral vectors can be used for other applications in vector targeting. Even though adenoviral vectors can transduce a wide variety of cells and can incorporate transgenes of large size, they are immunogenic *in vivo*. Even though Ad can transduce nondividing cells successfully, the success is short-term and limited due to the clearance of the Ad
vectors by Kupffer cells, the resident macrophages in the liver (150, 255). Studies report that upon Ad administration, proinflammatory regulatory molecules cytokines and chemokines are induced, leading to the innate immune response (257, 258). The viral gene products and the capsid proteins of Ad vectors also elicit an adaptive immune response (150, 153).

Depending on the disease, it may be necessary to utilize non-immunogenic, stable vectors for in vivo therapy. One such vector, adeno-associated virus (AAV), is not immunogenic and may be more appropriate for stable, long term in vivo therapy. AAV is a small (20-25 nm diameter), non-enveloped virus with a 4.5 kilobase, single-stranded genome that is able to integrate into the infected cell at human chromosome 19 (150). It consists of two main classes of proteins, the Rep or replication proteins and the Cap or capsid (protein coat) proteins that encapsulate the AAV genome. AAV, compared to Ad, is not able to transduce dendritic cells, which are the main immunological cells involved in antigen processing and presentation for cellular immune response (123). Thus, AAV infection does not elicit a cellular immune response and is very attractive as a vector-based approach for targeting in vivo.

The AAV capsid is icosahedral, consisting of three main capsid proteins: VP1, VP2, and VP3. These proteins are encoded on the same open reading frame (ORF) but have different transcripts due to alternative mRNA splicing. They are approximately 87, 72, and 60 kD and there are a total of 60 capsid subunits, with a molar capsid ratio of 1:1:18 respectively. From X-ray crystallography of AAV2, it is known that the icosahedral capsid has three-fold symmetry (322). Through structural studies and site-directed mutagenesis studies of the AAV capsid, several sites were mapped to have
interactions with cell hosts or are antigenic regions, indicative of surface exposed regions (216, 264, 312). Studies determined that these locations could possibly tolerate peptide insertions without disruption to the AAV capsid or AAV life cycle.

Two different strategies have been used towards AAV targeting against cell surface receptors. One approach is to use an adaptor molecule such as a glycoside molecule or a bispecific antibody, which is bound to the viral surface and binds to a specific cell surface molecule (31). Another approach is to genetically engineer a ligand directly into the capsid for direct targeting. With the first approach, it is not necessary to know the capsid structure to achieve targeting. Work has been done to conjugate a bispecific antibody to AAV vectors for redirected targeting against a cell surface receptor expressed on megakaryocytes (15). However, the adaptor molecule must stably bind to the vector and be able to specifically bind to receptors. The second approach is to directly engineer cell-specific ligands directly into viral capsid for targeting. This approach has been investigated extensively for vector targeting. However, it is necessary to determine suitable locations for peptide insertion and upon insertion, the peptide cannot destabilize the capsid and prevent virion assembly. Through sequence alignment between AAV-2 and other related parvoviruses, mutagenesis studies of the AAV-2 capsid, and the atomic structure of the capsid, various locations were found to possibly tolerate ligand insertion. Girod et al first demonstrated successful incorporation peptides into the viral capsid for targeting (88). Through sequence alignment, they located residues 261, 381, 447, 534, 573, and 587 on the AAV2 capsid as sites expected to be exposed on the capsid surface (88). They inserted a 14-amino acid peptide containing the RGD-integrin binding motif, into the 587 location of the viral capsid and were able to
target mouse melanoma and rat schwannoma cell lines, which are non-permissive to wild-type AAV. Others have inserted other peptides into the 587 location for targeting to the endothelium, vasculature, and erythroid cells (188, 304, 315). Mutagenesis studies of the AAV2 capsid identified several novel regions that could tolerate ligand insertion for targeting. These positions were 34 (in the VP1), the N-terminus of VP2 (138), and 266, 328, 447, 522, 553, 591, and 664 (VP3) (318). The atomic structure of AAV2 and epitope mapping for neutralizing antibodies confirms these locations as surface exposed regions, making them possible locations for peptide insertions (117, 312, 322). However, Shi et al and others showed that at various locations, different peptides affected virion assembly and production (216, 264). While one peptide would be well tolerated and titers would be similar to wild-type AAV particles, insertion of different peptide ligands created non-infectious particles with lower titers (216, 264). Ligand toleration in the capsid was dependent on the peptide sequence and is a rate-limiting step towards successful targeting.

To combine both targeting approaches, we proposed to genetically engineer the PSTCD domain into various locales on the capsid and generate capsid-modified AAV2 virions that would be metabolically biotinylated in mammalian 293 cells. The biotinylated AAV2 could then serve as a platform to conjugate ligands for screening and targeting. To demonstrate proof of principle, the biotinylated vector targeting system could be tested as a three-component system: the metabolically biotinylated vector, conjugated to a molecular tetrameric avidin bridge, which would then be complexed to a wide array of biotinylated ligands (Figure 5.1). Eventually, the goal would be to condense this into a two-component system; with the selection of ligands from the
target mouse melanoma and rat swannoma cell lines, which are non-permissive to wild-type AAV. Others have inserted other peptides into the 587 location for targeting to the endothelium, vasculature, and erythroid cells (188, 304, 315). Mutagenesis studies of the AAV2 capsid identified several novel regions that could tolerate ligand insertion for targeting. These positions were 34 (in the VP1), the N-terminus of VP2 (138), and 266, 328, 447, 522, 553, 591, and 664 (VP3) (318). The atomic structure of AAV2 and epitope mapping for neutralizing antibodies confirms these locations as surface exposed regions, making them possible locations for peptide insertions (117, 312, 322). However, Shi et al and others showed that at various locations, different peptides affected virion assembly and production (216, 264). While one peptide would be well tolerated and titers would be similar to wild-type AAV particles, insertion of different peptide ligands created non-infectious particles with lower titers (216, 264). Ligand toleration in the capsid was dependent on the peptide sequence and is a rate-limiting step towards successful targeting.

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cloned into the 587 location of AAV2 capsid. Plasmid DNA purification columns were purchased from Qiagen (Chatsworth CA). Lipofectamine PLUS and antibiotic/antimycotic were bought from Invitrogen (Carlsbad CA). The pTRUF5-SC and pLM45 were kindly provided by Ken Warrington (University of Florida). pXX6 plasmid was provided courtesy of R. Jude Samulski (University of North Carolina). Oligonucleotides were synthesized and purchased from Operon (Alameda CA). Avidin-HRP secondary antibody conjugate was bought from Vector Laboratories (Burlingame CA). SuperSignal chemiluminescent substrate was purchased from Pierce (Rockford, IL). Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (UT).

**Cells.** Human embryonic kidney 293 cells were purchased from American Tissue Cell Culture (ATCC, Bethesda, MD). SKOV-3 ovarian cancer cell line and HEK 293s were maintained in DMEM supplemented with 10% FBS and antibiotic/antimycotic. K562 leukemia cell lines were maintained in RPMI supplemented with 10% FBS and antibiotic/antimycotic.

**Methods**

**Cloning of BAP domain into the AAV capsid domains.** The PSTCD domain was PCR amplified from Pinpoint-Xa (Promega, CA) using primers PSTCD-EagI (5’-GGAGGACGGCGGAGGAAGCGGAGGCGAGATTCCCG CCG-3’) and PSTCD-MluI (5’-GGAGCGACCGCGTGAGGAGAAGCCCGATCTTTGAT GAGCCCTGACC-3’) with flanking EagI and MluI restriction endonuclease sites,
respectively. The PCR product was amplified and purified with Qiagen Gel Extraction purification kit. The subsequent PCR product was digested with EagI and MluI and ligated to pIM45-588 (provided courtesy of Ken Warrington, University of Florida), a plasmid that encodes AAV replication (rep) and capsid (cap) proteins, at the 587 location of the VP3 capsid (88). The resulting plasmid is called pIM45-PSTCD.

**Virus Production and Purification.** Recombinant wild-type AAV2 (wt AAV2) and mosaic biotinylated AAV (b-AAV2) were produced according to a triple transfection protocol adapted from Warrington et al. Briefly, three 150 mm² plates were seeded with HEK 293 cells so that they would be 70-80% confluence on the day of transfection. DNA was mixed in an equimolar 1:1:1 ratio; 45 ug of pTRUF5-SC, 45 ug pIM45/pIM45-BAP, and 135 ug pXX6 were mixed with 240 uL PLUS reagent and resuspended with 3 mL serum-free DMEM. The DNA-PLUS complex was then added to 3 mL serum-free DMEM supplemented with 300 uL Lipofectamine and incubated for 15 minutes at room temperature. The Lipofectamine-PLUS complexes were added to the plates equally in a dropwise manner. The cells were refreshed with serum-rich DMEM and incubated for 60-72 hours at 37°C with 5% CO₂. Approximately 72 hours post-transfection, cells were harvested and collected in 5 mL Tris-HCl (pH= 8.0; 150 mM NaCl). The cell lysate was subjected to three rounds of freeze-thaw in an ethanol-dry ice bath, alternating with a 37°C water bath. The supernatant was centrifuged at 4000 rpm for 15 min and was treated with DNAse I (250 U/mL; Roche) for 30 min. at 37°C to remove residual DNA. The viral lysate was then ready for iodixanol step gradient centrifugation for virus purification, as adapted from Zolothukin et al (327). Briefly, 5 mL viral lysate was
transferred into 12 mL Seton centrifuge tubes. 1.5 mL of 15% iodixanol (OptiPrep, Sigma) and 1 M NaCl in PBS-MK buffer (1x phosphate buffered-saline (PBS), 1 mM MgCl₂, and 2.5 mM KCl), 1.5 mL of 25% iodixanol in PBS-MK buffer containing Phenol Red (2.5 µL of a 0.5% stock solution per ml of the iodixanol solution); 5 mL of 40% iodixanol in PBS-MK buffer; and 5 mL of 60% iodixanol in PBS-MK buffer containing Phenol Red (0.01 µg/mL). Tubes were centrifuged at 20,000rpm for 11 h at 4°C in a Ti641 rotor (Sorvall). Afterwards, the 1 mL 40% fraction was collected, containing the purified virus.

**SDS-PAGE and Western Blot Analysis.** Iodixanol-purified AAV virions were boiled in Lamelli’s protein loading buffer and boiled at 95°C for 5 minutes. The samples were electrophoresed on a 7.5% tricine-based polyacrylamide gel. Samples were transferred onto a PVDF membrane using a BioRad Trans-Blot semidry transfer cell (Hercules, CA). Blots were blocked with 5% milk-TBST (10 mM Tris, 150 mM NaCl, 0.5% Tween 20, pH = 8.0) overnight to minimize non-specific protein adsorption. Blots were probed with gentle shaking for 1 h at room temperature with 1:2500 neutravidin-horseradish peroxidase or a primary mouse B1 ascites (against AAV capsid proteins) at a 1:1000 dilution (in TBST) coupled with a secondary goat anti-mouse-HRP antibody conjugate at a 1:10000 TBST dilution. Horseradish peroxidase conjugates were detected with SuperSignal chemiluminescent substrate and were developed using Kodak BioMax film.

**ELISA for Capsid Biotinylation.** Biotinylated maltose binding protein (MBP-biotin, Avidity) with a known MBP: biotin ratio of 1:1 was diluted to 1 µg/mL in PBS and
amounts ranging from 1-12 ng were immobilized on a flat-bottom microtiter plate. Serial
two-fold dilutions ranging from $2 \times 10^9$ viral particles to $1.93 \times 10^9$ viral particles were
diluted in phosphate buffered saline (PBS) and 100 uL of each vector were adsorbed on
the wells for 2 h at room temperature, with gentle shaking. After several washes with
PBS, plate was blocked for non-specific protein adsorption with TBST with 5% milk for
2 h at room temperature. After extensive washing, the wells were incubated with 100 uL
of 1:2500 dilution of Neutravidin-HRP conjugate. The colorimetric reaction was
detected with tetramethylbenzidine substrate (TMB) for 30 min at room temperature.
The reaction was stopped with 50 uL 1.8 M sulfuric acid and the plate absorbance was
read with a 450 nm absorbance filter with an automated plate reader. Different amounts
of biotin were quantified for neutravidin-HRP conjugate. From the known amounts of
adsorbed MBP-BAP and the molecular weight of the biotinylated protein, a standard
curve was generated of biotin amount against absorbance at 450 nm. Absorbance
measurements of vectors were compared against the standard curve to determine and
quantify the number of biotins molecules per capsid per vector.

**Targeting of Metabolically Biotinylated Vectors.** Approximately $10^6$ K562 cells were
washed three times with HBSS-BSA (Hanks' Buffered Salt Solution with 1% bovine
serum albumin with antibiotic/antimycotic) and resuspended in 100 uL HBSS-BSA. For
heparin competition experiments, cells were incubated with 5 ug/mL heparin for 30 min
before targeting. Cells were incubated with 100 uL biotinylated ligands (isotype control,
biotinylated anti-CD59, anti-CD71, transferrin and biotinylated cholera toxin subunit B)
for 30 minutes at 4°C. After incubation, cells were washed three times with HBSS-BSA
and centrifuged before each wash. Cells were incubated with 1 mL of HBSS-BSA with neutravidin (0.01 ng per cell). Incubation and washes were repeated as mentioned above. Finally, recombinant wild-type AAV-2 and biotinylated mosaic AAV-2 were added at a particle/cell ratio of 10000 in 1 mL HBSS-BSA and incubated at 4°C for 1 h. Cells were washed and resuspended in 2 mL serum-rich RPMI and incubated at 37°C. After 48 hours, EGFP expression (a measure of transduction) was analyzed by flow cytometry, using the FACScan (Becton Dickinson, Franklin Lakes, NJ).

5.3 Results

Construction of a Metabolically Biotinylated Mosaic Vector. In early work, we constructed AAV-2 vectors in which the 70 amino acid PSTCD BAP and a smaller 14 amino acid BAP were introduced into the 587 location of the VP3 capsid. However, when every VP3 was produced with this modification, we were not able to produce any virus (data not shown). Previous reports have suggested that the 587 loop domain can only tolerate small peptides, such as L14 and phage library-selected 7-mer peptides (88, 99, 315). Alternative locales were investigated for possible PSTCD insertion. Reports suggested the N-terminus of the VP2 protein as a possible location for peptide insertion. Warrington et al and Lux et al fused green fluorescent protein, a 27 kD protein, onto the N-terminus of VP2 protein and were able to produce functional AAV-2 capsid-modified virions used for imaging and cell-trafficking applications (158, 303). Based on this, we were able to genetically engineer the PSTCD domain onto the N-terminus of VP2 and produce virions. After triple transfection and harvest of cell lysates, virus was purified using iodixanol gradient step purification. Assay of capsid expression and biotinylation
of virions with PSTCD domain fused onto the VP2 N-terminus by western blot analysis demonstrated that the VP2-PSTCD fusion was expressed, incorporated incorporated into virions, and biotinylated (Fig. 5.2).

Figure 5.2: Western blots of capsid expression (left) and biotinylation (right) of rAAV-VP2-PSTCD vectors. For capsid expression, rAAV wild type and rAAV-VP2-PSTCD vectors were probed with B1 primary antibody (1:3000 dilution in TBST) against all capsid proteins with a secondary goat anti-mouse-HRP conjugate (1:10000 dilution). Note the shift in VP2 band, due to its fusion to the PSTCD domain at its N-terminus. Capsid biotinylation was probed with 1:2500 neutravidin-HRP conjugate. The sample on the left is a positive control at ~74 kD and the band on the right is the VP2-PSTCD fusion at ~80 kD.

However, when tested with ligands established to be functional with biotinylated adenovirus, we were unable to redirect targeting of metabolically biotinylated rAAV2 (Figure 5.3). Recent cryo-electron microscopy studies indicate that the VP2 capsid proteins are naturally located at the twofold symmetry axis inside the capsid and that their N-terminus (where the PSTCD was added), is buried inside the viral icosahedron (137).
<table>
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<th>VP2-PSTCD</th>
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Figure 5.3: Fluorescent micrographs of K562 Targeting using rAAV-VP2-PSTCD vector. K562 cells were targeted with biotinylated ligands (isotype control or anti-CD59 antibody) bound to wild type rAAV, biotinylated rAAV-VP2-PSTCD, or biotinylated, pre-heated rAAV-VP2-PSTCD via tetrameric avidin. Targeted transduction of vectors is evaluated by green fluorescent protein (GFP) reporter expression by microscopy.

Given this, we constructed a metabolically biotinylated AAV2 mosaic vector in which both wild-type and biotinylated VP3 proteins are assembled with the rationale that display of fewer BAPs might relieve the previously observed disruption of virion production. As an alternative approach to expand cell tropism, Rabinowitz *et al*
combined capsid proteins from various AAV serotypes at different ratios to produce AAV hybrid vectors displaying a mixture of various capsids (215). They were able to transduce cells that were previously non-permissive towards AAV2 transduction (106). Gigout et al adapted that strategy and developed mosaic vectors that combined wild type, non-mutated capsid proteins with mutant capsids displaying peptides that confer targeting towards specific cells. They were able to make mosaic vectors that expressed wild type capsid and mutant capsids that contain immunoglobulin-binding Z34C fragment of protein A, which enabled ligand conjugation for redirected tropism against MO7e and Jurkat cells in vitro (86).

Adapting the strategy by Gigout et al, we constructed a biotinylated mosaic AAV vector with wild type and PSTCD-modified mutant capsids for redirected targeting. The PSTCD domain was engineered into the 587 location of the AAV2 ORF of a recombinant plasmid that encodes for rep and cap proteins and transfected at an equimolar ratio with the wild type rep cap plasmid, vector plasmid and Ad helper expressing plasmid for virion production. Recombinant wild type and biotinylated mosaic vectors were produced in mammalian 293 cells. After purification on iodixanol gradients, the virions were analyzed by western blot with anti-AAV-2 antibodies (Figure 5.4). For wild-type AAV-2, protein bands were observed at ~87, 72, and 60 kDa after probing with AAV antisera indicating that wild type VP1, VP2, and VP3 proteins were produced. In contrast, the AAV VP3-PSTCD mosaic displayed six bands.
Figure 5.4: Western blot of capsid expression of wt and PSTCD-mosaic AAV vectors. Virions were produced from triple transfection of AAV plasmids and purified by iodixanol gradient ultracentrifugation. Virions were denatured at 95°C and loaded on SDS-polyacrylamide gels. Samples were transferred onto a PVDF membrane and probed with B1 primary antibody (from mouse ascites) against all capsid proteins at a 1:3000 dilution in Tris-buffered saline (TBST). A 1:10000 dilution of goat anti-mouse horseradish peroxidase (HRP) secondary antibody conjugate was added, and blot was detected using a chemiluminescent substrate against the HRP. Wild type AAV vector (column on left) produces three bands, at ~87, 72, and 60 kD, indicative of VP1, VP2, and VP3 proteins, respectively. Note the ratio of intensity of the bands, which is relative to the 1:1:8 molar ratio of the capsid proteins on the protein subunit. AAV VP3-PSTCD mosaic vector (column on right) displays six bands. Three of the bands are typical of the wild-type capsid proteins (as indicated). There are three other bands, which are the expected sizes of the VP-PSTCD fusion proteins. The ~8 kD PSTCD domain was cloned into the 587 domain of the AAV2 ORF. Since all three capsid proteins are read from the same ORF (but different transcripts), there will be three PSTCD fusion proteins.
Three bands corresponded to the sizes of the wild type VP1, VP2, and VP3 capsid proteins, whereas the other three bands corresponded to these proteins with an ~8 kDa increase in size due to the addition of the 70 amino acid PSTCD domain. The 1:1 stoichiometry between wild type and mutant capsids was maintained, as evidenced by western blot. The PSTCD domain appeared to be tolerated by the capsid, in conjunction with wild-type capsid expression and encapsidation into the virions. Samples were also analyzed by western blot to determine capsid biotinylation by detection with neutravidin-HRP. In this case, only three of the bands were biotinylated corresponding to VP1-PSTCD, VP2-PSTCD, and VP3-PSTCD (Figure 5.5). These data demonstrated that the PSTCD-modified mosaic AAV virus was metabolically biotinylated during virion production in 293 cells to form functional virions.

**Biotin Quantification of AAV Capsids.** To further characterize the biotinylated mosaic AAV2, we quantitated capsid biotinylation by ELISA. Biotinylated virions were adsorbed at varying concentrations on microtiter plates and were detected with avidin-HRP. Biotins per capsid were quantitated from a standard curve of biotinylated maltose-binding protein (Figure 5.6). There are approximately 11 biotins per capsid for biotinylated mosaic AAV2, compared to 0.5 biotins/capsid for the previously tested rAAV-VP2-PSTCD and roughly 0.0 biotins/capsid for rAAV wt. This was expected, since there can theoretically only be a maximum of 6 biotins on the VP2 subunits (maximum 6 VP2 subunits), compared to 30 biotins/capsid for the mosaic vector (assuming 1:1 wild type: PSTCD capsid ratio and 60 total capsid subunits). Compared to
the expected stoichiometry of capsid proteins, the estimated number of biotins per capsid was approximately 16% of the maximum number of biotins possible on the capsid.

Figure 5.5: Western blot of biotinylation of PSTCD-mosaic AAV vectors (left column) and positive control (right column). Virions were produced from triple transfection of AAV plasmids and purified by iodixanol gradient ultracentrifugation. Virions were denatured at 95°C and loaded on SDS-polyacrylamide gels. Samples were transferred onto a PVDF membrane and probed with 1:2500 dilution of neutravidin-HRP conjugate, and blot was detected using a chemiluminescent substrate against the HRP. AAV VP3-PSTCD mosaic vector displays three bands, at the expected sizes of the PSTCD fusion capsid proteins. There are three other bands, which are the expected sizes of the VP-PSTCD fusion proteins. The expected sizes of the fusion proteins are ~95 kD, 80 kD and 68 kD. The virion maintains the 1:1:8 molar stoichiometry of the capsid proteins. The expected size of the positive control is ~69 kD.
Figure 5.6: Biotin quantification of capsids. Various AAV vectors, including wild type rAAV, rAAV-VP2-PSTCD (heat-treated and not heat-treated), and mosaic, were quantified for number of biotins per each capsid by ELISA. Vectors were diluted in two-fold dilutions and were probed with 1:2500 dilution of neutravidin-HRP conjugate to detect accessible biotins on the vector. These values were graphed and compared to a standard curve of known amounts of biotinylated maltose binding protein.

Targeting of Metabolically Biotinylated AAV2 Mosaic Vectors. Previously, Wojda and Miller used biotinylated polyethyleneimine-DNA vectors to target K562 cells (314). In our laboratory, we have previously used K562 as a system to target biotinylated hexon, fiber, and πIX-modified Ad vectors using a panel of ligands (34, 201). K562 cells express high levels of CD59, a glycosyl phosphatidyl inositol (GPI) linked complementary
regulatory protein, CD71, the transferrin receptor, and ubiquitous cell surface ganglioside \( G_{mi} \) (binds to cholera toxin subunit B).

To test if the mosaic vector could be retargeted, it was tested by targeting it to various specific cell surface receptors on K562 cells using biotinylated ligands. 10^6 cells were washed and incubated with biotinylated anti-CD59 or anti-CD71 antibodies, biotinylated transferrin or biotinylated cholera toxin subunit B ligands and then conjugated to biotinylated AAV2 via a tetrameric netravidin bridge. Compared to wild-type control, there is approximately a 1.5-fold increase in transduction via addition of biotinylated cholera toxin subunit B and biotinylated transferrin (Figures 5.7 and 5.8). While this was a significant increase, the level of increased transduction was not comparable to the 10 and 100-fold increases previously observed with the same ligand targeting metabolically biotinylated adenoviral vectors.

In all cases, there was a relatively high level of background transduction with the negative and isotype controls (Figures 5.7 and 5.8). We hypothesized that this might be due to the presence of the native heparin binding function on the AAV vectors (Arginine-585 and Arginine-588) that are normally used by the virus for transduction. To test if this background could be reduced (and increased the ratio of targeting efficiency), the AAV vectors were incubated with heparin at 5 ug/mL for 30 minutes prior to cell binding to saturate their heparin binding motifs and inhibit their natural tropism. Heparin competition did indeed ablate background transduction by both the wild type and mosaic vectors (Figure 5.9). Unfortunately, this also ablated the increased transduction mediated by the biotinylated ligands. This suggests that while the new ligands may enhance transduction, the AAV virions may still be using the heparin may to assist in infection.
Figure 5.7: K562 targeted transduction of biotinylated mosaic AAV2 using biotinylated cholera toxin subunit B ligand. K562 cells were incubated with biotinylated isotype control antibody or biotinylated cholera toxin subunit B ligand (or not incubated at all— noted as virus) and conjugated to biotinylated mosaic AAV2 at 4°C. Transduction was assessed 48 hours later for green fluorescent protein (GFP) expression by flow cytometry.
Figure 5.8: Ligand Screening. Comparison of ligand-targeted transduction of biotinylated mosaic AAV vectors against K562 cells by flow cytometry. Cells were incubated with various biotinylated ligands and were bound to biotinylated AAV mosaics via a tetrameric avidin bridge. Targeted transduction was compared to untargeted vectors. Transduction of targeted delivery was assessed by evaluating percent positive cells expressing GFP by flow cytometry.
<table>
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<th>virus</th>
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**Figure 5.9:** Comparison of K562 transduction using various ligands in the absence or presence of heparin by fluorescence microscopy. Ligands, such as biotinylated anti-CD59, anti-CD71, and cholera toxin subunit B, were conjugated to AAV PSTCD mosaic vectors for targeting towards relatively non-permissive K562 erythroid leukemia cells at a particle to cell ratio of 10000. Targeting was performed against cells with or without heparin (5 ug/mL) to inhibit native cell binding. Transduction was assessed 48 hours later by green fluorescent protein reporter expression.
5.4 Discussion

Previous work in our laboratory has shown the utility of metabolically biotinylated Ad vectors towards targeting, ligand screening and purification. However, the efficacy of adenoviral vectors is restricted due to their innate immunogenicity. Adenoviruses expressing the LacZ gene were shown to elicit cellular and humoral immune response after delivery to the lung, liver, and muscle (123). This can be attributed to the ability of Ad vectors to efficiently transduce dendritic cells, which process and present antigen responsible for eliciting an immune response (178-180). Also, Ad uptake by Kupffer cells in the liver reduces the efficacy of Ad vectors for transduction. AAV vectors do not transduce dendritic cells efficiently, leading to poor immune response (76, 123). Consequently, AAV vectors are an attractive approach for in vivo therapy.

We took advantage of our metabolically biotinylated technology and developed various capsid-modified biotinylated AAV vectors. In our initial attempt, we incorporated the synthetic 14-amino acid biotin acceptor peptide into the 587 domain of the VP3 capsid to generate a biotinylated vector. However, while we were able to produce virions, we were unable to redirect cell tropism (data not shown). We were able to only display two biotins per capsid, suggesting that the targeting was limited by the inefficient biotinylation of the virions. To improve upon capsid biotinylation, we engineered the 70-aa PSTCD domain into the 587 domain to generate more efficiently biotinylated virions. However, we were not able to make any virions, most likely due to the large insert size of the PSTCD domain. Previous research has suggested only small peptides could be functionally incorporated in the VP3 domain (88, 100, 177, 210, 264-
266, 315, 318). The next approach was to insert the PSTCD domain onto the N-terminus of the VP2 protein. Previous work by Warrington et al suggested that the 138 position of the VP2 capsid can tolerate large insertions, such as green fluorescent protein and make functional virions with titers similar to wild type (303). While we were able to express PSTCD on the N-terminus of VP2 and make biotinylated virions, they were unable to target various cell lines, such as Jurkat and K562 cells. Quantification of biotins on the viral capsid suggested that the VP2-modified vector is poorly biotinylated and may not redirect tropism. This can be attributed due to the low number of VP2 proteins (about 3-6 per virions). Recent results published by Bleker et al and Kroneneberg et al proposes that VP1 and perhaps VP2 are naturally burrowed inside the capsid and not exposed on the surface (26). Cryo-electron microscopy indicates that globular proteins, attributed to VP1 and VP2, are located under the surface of the capsid. Further analysis shows that under mild heat treatment to 65°C, the VP1 phospholipase domain essential for AAV2 infection is exposed to the surface and facilitates AAV2 infection (26). It is suggested that the VP2 protein is also exposed upon heat treatment. From Western blot and ELISA, we noticed that VP2-PSTCD exhibits better expression of biotinylation upon heat treatment. From ELISA, there is a four-fold improvement of biotin display and we are able to capture biotinylated virions using streptavidin beads upon heat treatment. We speculate from the data that the VP2 region is not exposed until infection or upon heat treatment. It will be necessary, though, to perform structural studies using cryo-electron microscopy or even X-ray crystallography to determine the exact structure of VP2. The issues of location and surface display of the capsid and ligand insertion size are considerations in engineering a targetable vector.
Nevertheless, we utilized another method in our attempts to redirect cell tropism using biotinylated AAV vectors. An alternative approach is to display a mixture of wild type and mutant capsid proteins on the AAV capsid. Gigout et al were able to generate mosaic AAV vectors that displayed wild type capsid and L4 and L5 peptides on the 587 location of the AAV modified capsid at various ratios. They were able to target refractory Jurkat and MO7e erythroid cells in vitro (86). Adapting their approach, we developed metabolically biotinylated AAV mosaic vectors for re-targeting. The PSTCD domain was engineered into the 587 locale and we transfected equimolar ratios of wild type and PSTCD-modified capsid encoding plasmids to produce mosaic biotinylated virions. This approach demonstrates the ability of the 587 region to accept larger peptides, compared to previous results, for functional display. We were able to conjugate biotinylated transferrin and biotinylated cholera toxin to the biotinylated AAV mosaic and target K562 cells, to demonstrate proof of principle. We were able to achieve gain of function with the genetic incorporation of the PSTCD domain into the VP3 capsid protein. However, in the presence of heparin, transduction was ablated in wild type and mosaic vectors. Heparan sulfate proteoglycan is the primary receptor in AAV2-cell binding. We would speculate that the mosaic vector is primarily dependent on heparin sulfate for binding due to its wild-type proteins and perhaps uses the targeting ligand as a secondary receptor. Previous work showed how insertion of small peptides enhanced transduction but how they were not primary receptors towards targeting. Wu et al inserted serpin into various locales in the AAV2 ORF and demonstrated improved targeting towards target cells that is still dependent on heparin sulfate proteoglycan for receptor binding (318). Nevertheless, the conjugation of some cell-specific ligands can
enhance delivery to target cells. Ligand screening of biotinylated anti-CD59 and anti-CD71 antibodies with the mosaic vector demonstrated no gain of transduction compared to transferrin and cholera toxin subunit ligands, minimizing the vector interaction with an alternate cell surface receptor pathway. All receptors for the ligands are highly expressed in K562 cells. It is unclear why antibodies are not suitable ligands for targeting. Perhaps the biotinylated antibodies are too large (~150 kD) compared to the transferrin and cholera toxin subunit ligands and may somehow interfere with vector-cell binding. Also, it is interesting to note that transferrin and anti-CD71 both bind to transferrin receptor. The difference in transduction may suggest that the virus binds to different “regions” on the receptor. While we have achieved improved targeting with biotinylated mosaic vectors, it will be necessary to develop a “true” targeting AAV vector and abolish the natural tropism of the vector towards cells by creating vectors mutated against heparin sulfate binding and possibly, integrin and fibroblast growth factor receptor binding, which have been suggested as secondary co-receptors in AAV2 attachment.

With the development of biotinylated AAV2 mosaic vector for targeting in vitro, we can utilize this technology towards in vivo applications. In addition, biotin-conjugated ligands, such as peptides or antibodies, can be screened as potential ligands using this vector technology, as previously demonstrated (202). From our previous chapter, we can possibly generate new peptide ligands from the streptavidin-scaffold library that are compatible in the streptavidin context and directly conjugate these context-based ligands to biotinylated AAV2 for targeting. This approach would eliminate the need for three components in the targeting schema: the biotinylated ligand,
tetrameric avidin bridge and biotinylated vector. These ligands will be functional upon conjugation to biotinylated virions for functional targeting.
Chapter VI

Conclusions and Future Directions

This work focused upon the development of "context-specific" phage peptide libraries towards ligand discovery and construction of ligand-modified adenovirus (Ad) and adeno-associated viral (AAV) vectors for redirected targeting. To avoid the issues of ligand-virus compatibility, we proposed engrafting adenoviral and streptavidin structural context on filamentous bacteriophage and generating random phage peptide libraries within this context to generate ligands that can be identified during biopanning. Since these ligands are selected in a similar structural context that they are engineered into for redirected targeting, the ligand should confer cell-specificity to the modified vectors without impairing ligand function or destroying viral function. We were able to develop an adenoviral HI loop random phage peptide library on phage and select several cell-binding peptide ligands against C2C12 mouse myoblasts in vitro. One peptide, designated 12.51, was engineered back into the viral capsid and the redirected modified Ad vector demonstrated improved targeting in vitro against mouse myoblasts and myotubes. In our laboratory, we have previously developed metabolically biotinylated Ad vectors as a vehicle for targeting and ligand screening. Towards the development of compatible ligands for conjugation to these vectors, we developed a streptavidin-context library, which displays random peptide libraries within the scaffold of streptavidin on phage. The library is able to functionally display streptavidin and maintain biotin binding. Peptides generated from the SA library can be complexed to metabolically biotinylated Ad and AAV vectors for redirected targeting. Metabolically biotinylated
AAV vectors were also constructed as an alternative vector for targeted delivery. AAV is non-immunogenic compared to Ad vectors and may be more suitable for targeted delivery in vivo. To demonstrate proof of principle, we were able to produce mosaic biotinylated AAV and enhance targeting using biotinylated transferrin and cholera toxin subunit B ligands in vitro. Biotinylated AAV served as a suitable platform for ligand screening and may be ideal for targeting involving complex ligands, such as antibodies, growth factors, or carbohydrates.

The HI “context-specific” library was used to select several peptides against myoblasts in vitro. While only one peptide was tested viral targeting, we have engineered another peptide, designated as 12.52, into the viral capsid. Upon incorporation of the peptide into the HI loop, we have demonstrated by Western blot that fiber tolerates the peptide and is able to functionally trimerize. The 12.52-modified Ad genome (Ad-HI-12.52) is able to produce virions in mammalian 293 cells. However, the growth rate of the denoted Ad-HI-12.52 virus is slightly slower than the Ad-HI-12.51 (data not shown). In addition to selection against myoblasts, we have selected a peptide against breast cancer cell line MDA 231. The peptide exhibits significantly enhanced binding to cells, compared to negative control phage and is being investigated for viral targeting. This demonstrates the advantage of the HI library technology. It can be utilized against various cell targets to identify potential cell-specific ligands. The selected peptides engineered into the HI loop are displayed in 36 copies of the fiber. We can engineer these peptides into other locales of the capsid, such as hexon and pIX (720 and 240 copies, respectively), for display at higher copy number. Work using lower affinity peptides such as poly-lysine and RGD in hexon and pIX have been shown to
enhance transduction (62, 293, 298). By this approach, we can investigate the role of avidity towards improved targeting and also determine if selected peptides are context-independent and can be inserted into any similar structural context. Currently, we have incorporated 12.51 peptide into the hypervariable region 5 loop of hexon protein of the Ad capsid for increased valency and enhanced targeting.

Phage display is an attractive technology to generate and identify cell-specific ligands but is limited by the affinity of selected ligands towards their targets. While we were able to significantly enhance transduction with 12.51 peptide, it would be attractive to improve upon targeted transduction. Selected peptides have demonstrated cell-specific and enriched binding but are not necessarily high affinity ligands. HI and SA library peptides are displayed on pIII phage protein, which is only expressed 3-5 copies per phage. There is low valency of peptide display. This suggests that selected binders will demonstrate highest affinity towards the cell target but work by others has suggested that selected peptides have micromolar affinity, orders of magnitude smaller than an antibody-antigen interaction (206). To improve peptide affinity, we can subject the peptide to affinity maturation and optimize the peptide for enriched binding. Winter et al were able to improve affinity of phage-selected antibody fragments by randomly mutagenizing the codons and re-panning to select further enriched binders that demonstrated ~10^3-fold improvement in affinity (185). This approach to improve ligand affinity may translate into enhanced vector targeting.

The development of biotinylated mosaic AAV as a targeted vector resulted from the necessity to have a non-immunogenic vector for in vivo gene therapy applications. We were able to utilize the metabolic biotinylation technology to demonstrate proof-of-
concept of targeting conjugates to our vector. Future work will involve the conjugation
of ligands selected from the SA library to the vector for redirected targeting. Previous
work has shown the size limitations in ligand insertion in various locales of the AAV
capsid (216, 264). The mosaic vector, which combines wild-type capsid proteins with
modified capsid proteins, allows for insertion of larger peptides and domains into the
capsid for potential targeting, trafficking, and imaging applications. The biotinylated
AAV mosaic vector is the first known vector to tolerate a domain (PSTCD domain) of
this size in the capsid and redirect targeting.

The goal in vector targeting is to incorporate cell-specific ligands to redirect
targeting by an alternative ligand-receptor interaction in lieu of the native tropism of the
vector. It is consequently necessary to ablate native tropism of the vectors for true
targeting. For Ad vectors, it will be necessary to ablate or mutate the regions involved in
CAR binding and RGD-based internalization (107, 192). In AAV vectors, it will be
necessary to mutate Arginine-585 and Arginine-588 residues responsible for primary cell
attachment via heparan sulfate proteoglycan (198, 284) and possibly the residues
involved in binding to secondary receptors fibroblast growth factor 1 and α, integrins
(214, 283).

We have been able to utilize phage display and adapt this technology towards
context-specific selection of ligands for viral vector targeting. Direct genetic capsid
modification and conjugate-based targeting vector approaches have potential applications
in vivo. With improvements made in the vector design towards safer and longer
therapeutic delivery, using tissue-specific promoters, and elements for long-term
integration, ligand-based targeting is attractive towards gene therapy for acquired and inherited diseases.
References


78. **Frost, L. S., and D. P. Bazett-Jones.** 1991. Examination of the phosphate in
    conjugative F-like pili by use of electron spectroscopic imaging. J Bacteriol

    and gene products of the transfer region of the F sex factor. Microbiol Rev

    polypeptide IX revealed as capsid cement by difference images from electron

    Lieber.** 2005. Localization of regions in CD46 that interact with adenovirus. J

82. **Gao, C., S. Mao, H. J. Ditzel, L. Farnaes, P. Wirsching, R. A. Lerner, and K.


84. **Gao, G., L. H. Vanden Berghe, M. R. Alvira, Y. Lu, R. Calcedo, X. Zhou, and
    J. M. Wilson.** 2004. Clades of Adeno-associated viruses are widely disseminated


86. **Gigout, L., P. Rebollo, N. Clement, K. H. Warrington, Jr., N. Muzychka, R.
    M. Linden, and T. Weber.** 2005. Altering AAV tropism with mosaic viral

87. **Girod, A., M. Ried, C. Wobus, H. Lahm, K. Leike, J. Kleinschmidt, G.
    Deleage, and M. Hallek.** 1999. Genetic capsid modifications allow efficient re-

88. **Girod, A., M. Ried, C. Wobus, H. Lahm, K. Leike, J. Kleinschmidt, G.
    Deleage, and M. Hallek.** 1999. Genetic capsid modifications allow efficient re-

89. **Goff, S. P.** 2001. p. 1871-1923
    In D. M. Knipe and P. M. Howley (ed.), Fields Virology, vol. 2. Lippincott
    Williams & Wilkins, Philadelphia.

90. **Goldman, E., M. Korus, and W. Mandecki.** 2000. Efficiencies of translation in
    three reading frames of unusual non-ORF sequences isolated from phage display.
    Faseb J 14:603-11.

    a human cell line transformed by DNA from human adenovirus type 5. Journal of

    and A. Helenius.** 1997. The role of the nuclear pore complex in adenovirus DNA

93. **Greber, U. F., P. Webster, J. Weber, and A. Helenius.** 1996. The role of the
    adenovirus protease on virus entry into cells. Embo J 15:1766-77.


