REVIEW

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Library Selection and Directed Evolution Approaches to Engineering Targeted Viral Vectors

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ABSTRACT: Gene therapy, to delivery of genetic material to a patient for therapeutic benefit, has significant promise for translating basic knowledge of disease mechanism into biomedical treatments. The clinical development of the field has been slowed, however, by the need for improvements in the properties and capabilities of gene delivery vehicles. Vehicles based on viruses offer the potential for efficient gene delivery, but because viruses did not evolve to serve human therapeutic needs, many of their properties require significant improvement, including their safety, efficiency, and capacity for targeted gene delivery. Since viruses are highly complex biological entities, engineering such properties at the molecular level can be challenging. However, there has been significant progress in developing approaches that mimic the mechanisms by which viruses arose in the first place. In particular, library-based selection, the generation of one diverse genetic library and selection for new properties, and directed evolution, based on the multiple rounds of library generation and selection for iterative improvement of function, have strong potential in engineering novel properties into these complex biomolecular assemblies. This review will discuss progress in the application of peptide display, library selection, and directed evolution technologies toward engineering vectors based on retrovirus, adeno-associated virus, and adenovirus that are capable of targeted delivery to specific cell types. In addition to creating biomedically useful products, these approaches have future potential to yield novel insights into viral structure–function relationships.

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Introduction

Gene therapy is the delivery of genetic material to the cells of an individual for therapeutic benefit (Morgan and Anderson, 1993; Mulligan, 1993). A number of approaches are under development to apply therapeutic gene delivery to a variety of disorders, including hemophilia, anemia, α-1-antitrypsin deficiency, neurodegenerative disorders, cancer, AIDS, and a number of other genetic diseases. The past few years have witnessed the first hints of success in clinical trials: the partial cure of hemophilia B through the adeno-associated viral (AAV) vector-mediated delivery of a gene encoding Factor IX to several patients (Kay et al., 2000), evidence for success in treating heart disease by stimulating angiogenesis (Harris et al., 1992; Isner and Asahara, 1999; Marquart et al., 1980; McKirnan et al., 2000), the use of an oncolytic adenoviral vector for the treatment of cancer (Khuri et al., 2000), and initial signs of therapeutic efficacy in a cystic fibrosis clinical trial involving AAV (Moss et al., 2004). As the recent completion the human genome sequence will progressively aid efforts to understand disease at a molecular level and facilitate the identification of disease targets (Marshall, 2000), gene therapy has the potential to serve as a direct conduit of this basic scientific information into human benefit. Furthermore, the recent discovery of RNAi offers a new, powerful class of cargos for a number of disease applications (McCaffrey et al., 2002).

This significant promise, however, is tempered by tragic events in a clinical trial for a severe-combined immunodeficiency. The administration of a therapeutic vector cured fifteen patients of their disease and will therefore significantly extend their lifespan; however, three patients later contracted a lymphoproliferative disorder due to the retroviral vector used for the study (Cavazzana-Calvo et al., 2000). These events emphasize the fact that significant progress must still be made before gene therapy strategies become therapeutic realities.

Gene delivery vehicles can be roughly divided into two classes, viral and non-viral, with many complementary advantages and disadvantages. Non-viral vehicles are

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arguably safer since they lack viral components, though numerous viral vectors (such as retrovirus, lentivirus, and adeno-associated virus, discussed below) can be fully stripped of the viral gene cargos (Verma and Somia, 1997). In addition, viruses have evolved numerous strategies to successfully infect cells, and vehicles based upon them are thus more efficient than their non-viral counterparts. Finally, and importantly for this review, however, the properties of synthetic vehicles can be readily manipulated at the molecular level, since synthetic chemistry can readily be employed to systematically vary properties such as polymer or lipid composition and molecular weight. By contrast, viruses are highly complex and semi-optimized products of millions of years of evolution, and attempts to modify their intricate properties are more likely to compromise rather than enhance their delivery capabilities. However, viruses have evolved in nature for their own ends and not to serve as optimized human therapeutics, and the properties of viral vectors often require changes to enhance their safety and efficiency. Therefore, it will be crucial to develop approaches to engineer the properties of viruses, and particularly the capsid and/or envelope proteins that mediate their gene delivery, at the molecular level to overcome their shortcomings.

The field of protein engineering has been highly successful in the application of rational design (Dwyer et al., 2004), as well as library selection and directed evolution (Boder and Wittrup, 1997; Daugherty et al., 2000; Stemmer, 1994; Zhao and Arnold, 1997) approaches, to both enhance the function of and to engineer novel function into numerous biomolecules, in particular antibodies and enzymes. However, there are particularly formidable challenges in engineering viruses, which are large complexes of proteins, nucleic acids, and (in some cases) lipids that are intermediate in complexity between proteins and cells. For example, these are highly ''constrained'' systems both structurally and functionally. Structurally, capsid proteins must maintain the ability to self-assemble into complex virion particles, placing inherent limitations on the engineering of individual subunits (Xie et al., 2002). Furthermore, viruses have evolved their genomes for shorter lengths, often taking advantages of alternative splicing and alternative reading frames to express multiple proteins from the same nucleic acid sequence (Fields et al., 2001). In light of this high ratio of biological function per nucleotide, when engineering one function, care must therefore be taken to avoid impairing others. Finally, viral properties whose molecular basis is distributed throughout the primary sequence of the capsid, such as virus–cell interactions (Chiorini et al., 1999; Opie et al., 2003) and antibody neutralization (Moskalenko et al., 2000; Wobus et al., 2000), will be extremely challenging to rationally re-design, even with the availability of AAV structures (Xie et al., 2002). Given these numerous complexities and constraints, library selection and directed evolution are particularly well suited for re-engineering viruses.

Library selection is defined as the initial generation of a genetically diverse collection of genes, followed by the phenotypic selection of desirable variants that serve as the final products. Directed evolution involves repeated rounds of genetic diversification/mutation and phenotypic selection, such that the products of each selection are used as the substrates for the next round of genetic diversification and selection. One important feature of viruses for both approaches is that they are naturally amenable to the generation of ''linked'' libraries, where the protein being selected for a novel property or phenotype is physically linked to the genetic information that encodes it or the genotype. As a result, large libraries can be subjected to selection as a pool, and the genotype encoding the desirable proteins and properties can be recovered by sequencing the genomes of the final virion products. Very large libraries can therefore be screened in parallel, a feature that underlies the power of phage display (Smith, 1985). A number of studies have utilized peptide display and viral library selection to engineer several promising viruses, including retrovirus, AAV, and adenovirus, for targeted gene delivery to specific cell types. Furthermore, very recently directed evolution has been employed to enhance the properties of AAV.

Retroviral Vectors

Retroviruses, including lentiviruses, are enveloped viruses with a diploid, positive-sense RNA genome (Fields et al., 2001). They initiate infection by binding to the cell surface through interactions between the viral envelope proteins and cellular receptors. Following membrane fusion with either the cell surface membrane at a neutral pH, or with intracellular endosomes at an acidic pH, their capsid core is liberated from the viral particle into the cytoplasm. The diploid RNA genome is reverse transcribed to a double-stranded DNA genome, which then traffics to the nucleus, integrates into the host cell genome, and initiates viral gene expression with the aid of cellular factors.

Gene delivery vehicles based on retroviruses were first generated by Mulligan, Baltimore, and co-workers through the placement of viral genes onto separate pieces of DNA that were then used as helpers to package a viral genome containing a recombinant gene cargo (Cepko et al., 1984; Mann et al., 1983). Following relatively efficient delivery to cells, retroviruses integrate their genomes into host chromosomes and thereby offer the potential for stable, long-term gene expression. Retroviral vectors can also accommodate relatively large inserts up to 8 kb (Verma and Somia, 1997), enabling incorporation of moderately sized promoter elements within their genomes for controlled expression of therapeutic genes. Furthermore, vectors based on lentiviruses offer the added advantage of efficient delivery to non-dividing cells (Naldini et al., 1996).

Since they bind to a specific cell surface receptor, envelope proteins are the primary determinants of the cell specificity or tropism of a retrovirus, though additional tissue-specificity can be garnered through the use of promoter or enhancer elements within the virus genome to direct

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transgene expression (Robson and Hirst, 2003; Romano, 2004). Pseudotyping, the packaging of vectors with any of a number of heterologous viral envelope proteins rather than the retroviral envelope, can offer the ability to change or extend cell tropism (Cronin et al., 2005). However, pseudotyping is still restricted to a relatively limited collection of viral envelope options.

Alternatively, the genetic fusion of antibody fragments or ligands to the viral envelope (Benedict et al., 1999; Cosset et al., 1995; Russell et al., 1993), as well as non-covalent attachment of adaptor molecules composed of the viral receptor fused to a targeting ligand (Snitkovsky and Young, 1998), have been employed to target retroviruses to new receptors. However, while genetic modifications of envelope proteins often succeed in shifting viral-binding specificity, they often come at the cost of a severe drop in viral infectivity (Cosset et al., 1995). To minimize the disruptive effects of large polypeptide insertions on envelope functions such as membrane fusion, shorter receptor-binding motifs have been rationally inserted into permissive sites within envelope proteins. However, with few exceptions (Hatziioannou et al., 1999; Patterson et al., 1999; Russell and Cosset, 1999), an incomplete understanding of the complicated structure–function relationships of viral envelopes still limits our ability to rationally engineer targeted and efficient envelope proteins. For example, the binding of the envelope protein to a cell surface receptor often triggers viral fusion with the cell membrane, and the complex fusion domains can be widely distributed throughout an envelope protein (Kayman et al., 1999; Li et al., 1993; Martinez and Wertz, 2005; Schlehuber and Rose, 2004). Swapping binding specificity without compromising the actuation of fusion is challenging. Furthermore, all such rational design strategies for targeting viruses do not allow targeting of novel or unknown cell surface receptors.

A promising alternative approach is to exploit the natural algorithm responsible for the creation of viruses: evolution. Large pools of viral envelope mutants can be generated using a number of approaches, including randomly shuffling gene templates from different parent viruses (Soong et al., 2000), inserting random peptide sequences at a specific location (Bupp and Roth, 2002, 2003; Bupp et al., 2005, 2006), or inserting a defined peptide into random locations (Yu and Schaffer, 2006a, 2006b). The resulting envelope mutant genetic library is inserted into recombinant viral genomes, and each envelope protein mutant is packaged to generate virus particles encompassing the genome that encodes it. The resulting library can be selected for specific properties, such as the ability to bind a specific receptor or infect a particular cell type, and sequencing of selected variants can subsequently be conducted to analyze the successful or ''fittest'' clones (Fig. 1). Such approaches are especially attractive when detailed information on the repertoire of surface molecules on target cells, as well as intricate structure–function relationship information on the envelope protein, are not available, situations that severely limit rational design approaches. Therefore, library-based selection and directed evolution approaches are particularly well suited to overcoming these challenges, and these efforts have been designed to interface with two cellular targets to achieve specificity: cell surface receptors and membrane-bound or secreted proteases.

Cell Receptor-Specific or Transductional Targeting

Some of the earliest efforts to apply library selection approaches to target retroviral vectors involved libraries where short, random peptide sequences were genetically substituted into the receptor-determining region (RDR) of feline leukemia virus subgroup A (FeLV-A) envelope proteins followed by selection on target cells (Table I) (Bupp and Roth, 2002, 2003; Bupp et al., 2005, 2006). In contrast to MLV envelope proteins that require changes in both variable regions of their receptor-binding domain (VRA and VRB), modification of only the VRA domains of FeLV envelope proteins allows modulation of viral tropism without severe structural perturbation (Bupp and Roth, 2002). An envelope mutant library where 10 randomized amino acids were inserted into VRA was singly passaged on a feline cell type, resulting in the selection of a fully functional envelope protein variant. Surprisingly, the selected variant was capable of mediating efficient gene delivery to D17 canine osteosarcoma cells compared to four other cell lines (Bupp and Roth, 2002). Selection of a similarly constructed but larger scale library on a specific cell type, Ras-transformed 143B human osteosarcoma cells, yielded a variant that exhibited 50-fold to 1,000-fold higher titers on 143B and 293T cells compared to other 12 cell lines (Bupp and Roth, 2003). The former mutant utilized a FeLV-C receptor to dock to the cell surface, whereas the latter used a novel but unidentified receptor distinct from the parental envelope protein receptor (Bupp and Roth, 2002, 2003). These studies demonstrate that even without information on target receptors, random peptide display library selection can yield envelope protein variants that allow retroviral vectors targeting to specific cell types.

Subsequent work by Bupp et al. (2005) demonstrated that the degree of cell specificity of the selected envelope protein variants varied depending on cell type used during the library selection. While the D17 cell-specific envelope protein variants were obtained when they used D17 cells for library selection, variants of broader tropism were isolated when AH297 feline cells were employed (Bupp et al., 2005). Furthermore, a library, whose individual viral particle variants contained/displayed both the same random peptide library as well as amphotropic murine leukemia 4070A virus envelope proteins on their surfaces, yielded an interesting result after selection on 4070A-infected PC-3 human prostate cells. The envelope protein variants that emerged from this selection apparently cooperated with 4070A envelope proteins to infect PC-3 cells, such that pre-infection of these cells with wild-type 4070A retrovirus prior

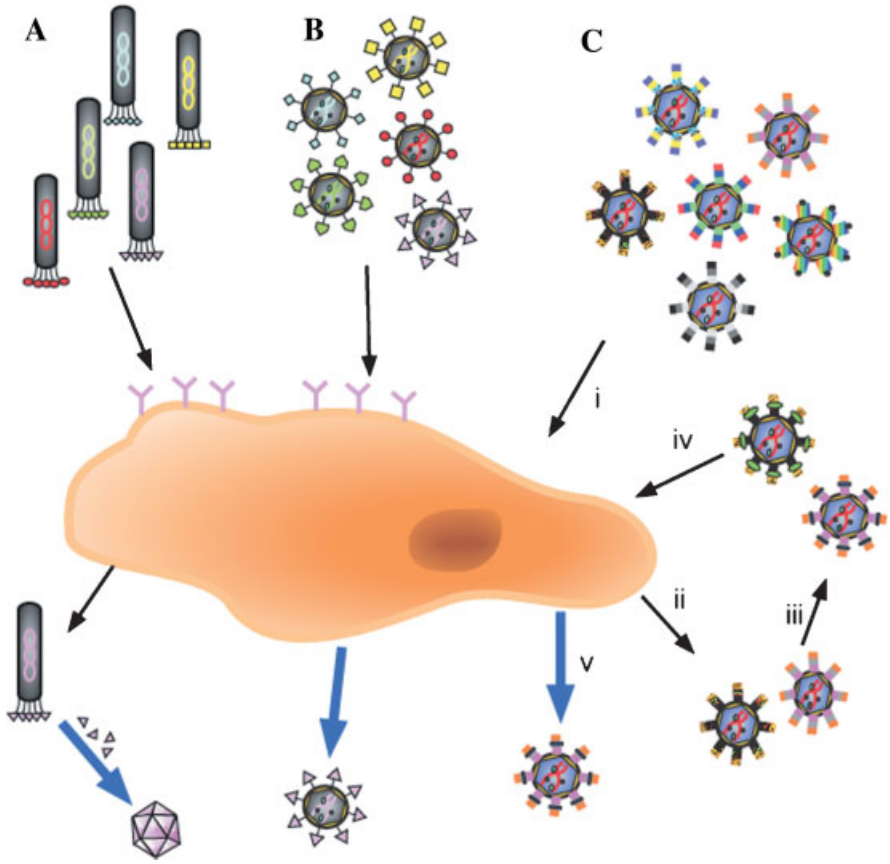

Figure 1. Library selection and directed evolution approaches have included three classes of viral-engineering methods. **A**: Phage display is first conducted to select peptides capable of binding a specific receptor, cell surface, or tissue. The selected peptides are then inserted into the viral attachment protein in an attempt to generate a targeted virus. **B**: Alternatively, a peptide library can be directly inserted into the viral vector surface, and subsequent selection on cells can yield a targeted vector. **C**: Directed evolution is composed of multiple rounds of library generation and selection. After the first library is generated, steps include: (i) cellular infection with libraries, (ii) cellular selection of the fittest, (iii) new library generation through mutagenesis, (iv) infection with newly generated libraries, (v) and after multiple rounds of i–iv, a final isolation and validation of a successful variant.

Table I. Summary of library selection and directed evolution approaches for engineering retroviral and adeno-associated viral vectors.

Virus	Approach	Results	References
Retrovirus	Random peptide insertion	Selected a FeLV-A envelope protein mutant mediating gene delivery to canine osteosarcoma cells	Bupp and Roth (2002)
Retrovirus	Random peptide insertion	Selected a FeLV-A envelope protein mutant mediating the specific infection of human osteosarcoma cells	Bupp and Roth (2003)
Retrovirus	Random peptide insertion	Showed the dependency of the cell specificity of selected FeLV-A envelope protein mutants on the cell types used during library selection	Bupp et al. (2005)
Retrovirus	Single round shuffling	Selected a chimeric MLV envelope protein mutant mediating the infection of CHOK1 cells	Soong et al. (2000)
Retrovirus	Random peptide insertion	Selected peptide substrates that can be cleaved by tumor-associated MMP-2	Schneider et al. (2003)
Retrovirus	Random peptide insertion	Selected protease substrate linkers that allowed the specific infection of MMP-2 positive cancer cells	Hartl et al. (2005)
AAV	Random peptide insertion	Selected random peptide-display AAV libraries highly specific to primary human coronary artery endothelial cells	Muller et al. (2003)
AAV	Random peptide insertion	Selected AAV mutants with a significantly enhanced transduction efficiency for non-permissive cell lines compared to wild-type AAV2	Perabo et al. (2003)
AAV	Directed evolution	Evolution of AAV capsid libraries with altered affinity to heparin and the capability to enhance gene delivery in the presence of anti-AAV serum	Maheshri et al. (2006)
AAV	Single round mutagenesis	Selected AAV capsid mutants with the ability to evade from human antibody neutralization	Perabo et al. (2006)

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to addition of the novel viruses competed away most but not all of the latter's infectivity (Bupp et al., 2006).

Both rational ligand insertion and random peptide display require knowledge of insertion sites that do not compromise envelope function and are exposed on the protein surface for receptor binding (Bupp and Roth, 2002, 2003; Bupp et al., 2005, 2006). However, if information on such permissive insertion sites is not available, it can be also newly obtained by transposon-based scanning of envelope proteins (Rothenberg et al., 2001; Yu and Schaffer, 2006b). The insertion and excision of a bacteriophage Mu transposase cassette into cDNA encoding the Moloney murine leukemia virus (MoMLV) envelope protein resulted in the duplication of five amino acids at a random insertion site. Subsequent selection of the resulting mutant library for the ability to mediate viral replication in culture led to the identification of multiple sites permissive for the five amino acid insertion (Rothenberg et al., 2001). More recently, a 13 amino acid sequence, including a 6 histidine peptide, was randomly inserted into envelope proteins of vesicular stomatitis virus (VSVG), and the selection of the resulting library for the ability to mediate viral replication in culture and for the capacity to bind an immobilized metal column led to the identification of several permissive insertion sites on the outer surface of VSVG (Yu and Schaffer, 2006b). These hexa-histidine mutant VSVG envelopes offer the capability for rapid and highly effective purification of viral vector. Furthermore, the subsequent insertion of targeting peptides into these surface-exposed sites can shift viral specificity (Lim, Yu, and Schaffer, unpublished work). Finally, comparison with recently elucidated crystal structures of viral proteins can yield structural biology insights into regions of a protein surface that can tolerate exogenous amino acid sequence insertions (Roche et al., 2007).

An alternative to peptide and domain insertions is gene shuffling, where a number of closely related "parent" sequences are shuffled to generate large libraries of chimeric genes. Such family shuffling can engender variants with novel properties not possessed by any of the parents. Soong et al. (2000) first generated a random chimeric retroviral envelope library from six distinct MLV parent sequences and selected it for a novel function, the ability to support murine leukemia virus (MLV) infection of Chinese hamster ovary (CHOK1) cells, normally non-permissive to MLV infection. Even though detailed mechanisms involved in the new virus tropism, such as the cell surface receptor utilized by the new variants, were not explored, this work showed a great potential of gene shuffling to generate viral envelope proteins with novel and desirable properties. This approach was subsequently applied to generate envelope proteins with greater stability (Powell et al., 2000).

Cell Protease-Specific Targeting

It is known that cells express a specific pattern of proteases (Curran and Murray, 1999), which has led to a novel approach for targeting retroviral vectors (Buchholz et al., 1998; Hartl et al., 2005; Schneider et al., 2003). In early efforts to genetically fuse targeting ligands to the viral envelope protein for receptor-specific delivery, it was observed that some ligand domains when genetically attached to the envelope protein blocked viral entry at post-binding steps (Cosset et al., 1995). Subsequent work fused these virus entry-blocking ligands to envelope proteins via an intervening protease substrate peptide/linker to create environmentally responsive, or "smart," vectors activated by cell-specific proteases, such that proteolytic removal of the blocking ligand yielded an infectious vector (Buchholz et al., 1998; Hartl et al., 2005; Schneider et al., 2003). However, the identification of protease-specific substrate sequences is critical to the success of this approach. Buchholz et al. (1998) first used a library of randomized protease substrate linkers to find substrate sequences that could be specifically cleaved by proteases expressed by cancer cell lines. They inserted randomized seven amino acid linkers between an inhibitory EGF domain, known to direct cell-bound viruses to lysosome-mediated degradative pathways (Cosset et al., 1995), and the *N*-terminus of ecotropic MoMLV envelope proteins. They subsequently screened the resulting library for the ability to infect fibrosarcoma HT1080 cells that over-expressed EGF receptors. While the selected peptide linker substrates were likely cleaved by ubiquitously expressed furin-like proteases, rather than cancer-associated ones, this work established the utility of the approach (Buchholz et al., 1998).

A subsequent study also attempted to target a key class of tumor-associated proteases, matrix metalloproteases (MMPs), on human cells. Schneider et al. (2003) constructed a protease substrate linker library that connected EGFs to amphotropic MLV envelope proteins and screened the library on HT1080 (human fibrosarcoma) cells. The selected substrates were cleaved specifically by a tumor-associated MMP-2. However, because the inhibition of infection by such EGF-presenting retroviruses relies upon high EGF receptor expression in cells (Cosset et al., 1995), these viruses can infect cells with low or no EGFR expression regardless of the cleavage of the EGF-blocking domain, thereby compromising specificity. Therefore, another virus entry-blocking ligand has been developed to create protease-activatable vectors (Hartl et al., 2005). Using CD40 ligand as the domain to block amphotropic MLV envelope-mediated infectivity, Hartl et al. (2005) successfully isolated specific MMP-2 substrate peptides from a library, which ultimately allowed them to target MMP-2 positive cancer cells.

Adeno-Associated Virus

AAV, a member of the *Dependovirus* family, has a 4.7 kilobase single-stranded DNA genome with two open reading frames, *rep* and *cap*, encoding four proteins necessary for genome replication and three structural proteins that self-assemble to form the viral capsid, respectively. AAV vectors

are non-pathogenic, and the majority of the human population has been exposed to various natural human AAV variants or serotypes (e.g., >80% have been exposed to AAV serotype 2), with no known deleterious effects (Berns and Linden, 1995). The inherent safety of the virus is thus an advantage for gene delivery vehicles based on AAV (Samulski et al., 1989). In addition, these vehicles have the capacity for efficient gene delivery to both dividing and non-dividing cell types, such as muscle (Fisher et al., 1997), brain (Kaplitt et al., 1994), and retina cells (Flannery et al., 1997). Due to these advantages, AAV vectors have been increasingly explored in clinical trials (Flotte et al., 2004; Kay et al., 2000).

Despite these advantageous properties, AAV suffers from several shortcomings, including the inability to target delivery to specific cell types (Muzyczka and Warrington, 2005), inefficient gene delivery to a number of "non-permissive" cell types (Hughes et al., 2002; Ponnazhagan et al., 1996; Smith-Arica et al., 2003; Stacchini et al., 1999), a limited packaging insert size (Dong et al., 1996), and the prevalence of pre-existing immunity to human AAV serotypes in the human population (Moskalenko et al., 2000; Sun et al., 2003).

Transductional Targeting of AAV

There have been significant efforts to engineer AAV to overcome its challenges. For example, numerous studies have attempted to enhance selective AAV vector gene delivery to specific cell types via altering binding affinity to receptors expressed on the target cells. Several such strategies to modify AAV tropism include: (i) association with "adaptor" bispecific antibodies, with one arm directed against AAV and the other against a target receptor (Bartlett et al., 1999), (ii) chemical or metabolic conjugation of biotin to the virus, followed by the addition of avidin and a biotinylated-targeting molecule (Arnold et al., 2006; Ponnazhagan et al., 2002), and (iii) genetic modification of the viral capsid structures through the insertion of defined peptide sequences (Girod et al., 1999; Grifman et al., 2001; Nicklin et al., 2001; Work et al., 2004). These rational design approaches have resulted in moderately selective receptor-mediated gene delivery and demonstrated the strong potential for engineering the AAV capsid for targeting. However, such capsid modifications can result in significantly reduced viral infectivity. Furthermore, in many cases one may not know a suitable, specific receptor to exploit for targeted delivery to a given cell type, and binding to the cell surface may not be the sole determinant of tropism (Hansen et al., 2000). Therefore, complementary efforts may be necessary to aid such rational design approaches, such as ones based on library selection approaches.

A defined peptide insertion requires knowledge of the appropriate ligand to utilize for targeted delivery via a given receptor or to a specific cell. Phage display technology has been applied to select peptides that bind a target cell, and the resulting peptides have subsequently been genetically inserted into AAV with some success (White et al., 2004; Work et al., 2004). However, it has also been demonstrated that the same peptide can behave differently when inserted into different regions on the AAV capsid surface, presumably due to local conformational differences (Grifman et al., 2001; Shi et al., 2001; Wu et al., 2000). Accordingly, peptides selected for the ability to mediate phage binding to a given cell type can suffer losses in targeting capacity when incorporated into AAV (Work et al., 2006). It is therefore arguable that it would be preferable to select for novel peptide specificity in the context in which it will be ultimately utilized, that is, directly within the AAV capsid.

In light of these challenges with rational AAV design, there have been an escalating number of studies that have attempted to engineer the virus in a manner analogous to nature's approach: library selection and directed evolution. Random peptide display library approaches have yielded significant progress in engineering vectors that target desired cell types. Specific loop regions that protrude from the external virion surface have been identified as receptor-binding regions, and specific sites on these loops are amenable to the insertion of exogenous peptides. In particular, amino acid position 587 of the AAV serotype 2 capsid, which is required for binding to the viral receptor heparan sulfate and had previously been demonstrated to tolerate defined peptide insertions (Girod et al., 1999; Xie et al., 2002), has been recently employed to generate random peptide display libraries (Muller et al., 2003; Perabo et al., 2003).

Muller et al. generated AAV2 libraries with high diversity by genetically inserting random seven amino acid peptide sequences at the 587 position, and the resulting AAV library was selected for transduction of human coronary artery endothelial cells. After several rounds of selection by passaging virus on the target cell, at least three prominent consensus peptides were enriched in the selected mutants, implying that these motifs enhance efficiency on the target cell (Muller et al., 2003). Further analysis revealed that these selected variants mediated a 4-fold to 40-fold enhanced reporter gene expression in the target cell, as well as an 80–99.8% reduction of viral-mediated gene delivery in a non-endothelial control cell line (HeLa), compared to the wild-type AAV2 capsid, thereby demonstrating not only improved efficiency but also selectivity toward the target cells.

Perabo et al. (2003) also produced AAV2 libraries carrying random seven amino acid insertions at position 587 (of the AAV2 capsid), and mutants were selected for the ability to infect cell lines ordinarily non-permissive to AAV infection. While the selected variants mediated enhanced transduction of target cells non-permissive to wild-type AAV2 (the human megakaryocytic cell line M-07e and B-cell chronic lymphocytic leukemia B-CLL cells) (Ponnazhagan et al., 1996; Stacchini et al., 1999), the ability of the selected mutants to transduce cells expressing heparan sulfate (human colon carcinoma CO-115 cells) was reduced approximately 12–50%, implying enhanced specificity toward the target cell types.

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These random peptide display approaches offer the potential for better results compared to the rational insertion of single, defined peptides for targeted gene delivery. Furthermore, it can be noted that because the random peptides are selected for their ability to enhance the full process of AAV infection, it is conceivable that selected peptide could act at known transduction barriers beyond the cell surface, such as endo-lysosomal trafficking (Hansen et al., 2000) or viral unpackaging (Thomas et al., 2004). However, it is unlikely that selection of a library with modification at a single capsid site can overcome problems distributed throughout the AAV capsid, a challenge that directed evolution has recently been shown to address.

Directed Evolution of AAV

The peptide display efforts demonstrate the utility of library selection approaches for vector engineering. By contrast, directed evolution involves the iterative application of multiple rounds of library generation (i.e., genetic diversification) and selection. Directed evolution has recently been applied to address another challenge with AAV vectors: preexisting immunity. The majority of the human population has previously been exposed to numerous serotypes, and as a result a large fraction of the future potential patient pool harbors neutralizing antibodies that can greatly reduce gene transfer *in vivo* (Sun et al., 2003). Furthermore, even serotypes that activate distinct sets of B cells can still share T cell epitopes (Mingozzi et al., 2007), also a challenging problem to address.

One approach to overcome the former, neutralizing antibody problem is to mutate epitopes to reduce neutralizing antibody affinity; however, identifying a set of mutations that both reduces antibody neutralization and maintains high viral infectivity is highly challenging given the vast number of possible capsid mutants. Maheshri et al. (2006) have applied directed evolution, utilizing error prone PCR and staggered extension process, to generate a large and diverse ($\sim 10^7$) AAV *cap* gene library for subsequent selection of variants with novel function. The resulting AAV variant library exhibited considerable diversity in properties, as indicated by a broad range of affinities for heparin (a proteoglycan similar to the AAV2 receptor, heparan sulfate). This library was then iteratively mutagenized and passaged on 293 cells in the presence of increasing concentrations of anti-AAV polyclonal serum, resulting in the evolution of AAV mutant vectors capable of both efficient cell infection and evasion of neutralizing antibodies. Two key amino acid sequence changes, N587I and T717A, were primarily responsible for escaping from the neutralizing antibodies, potentially implicating these regions as functionally important. Importantly, two rounds of evolution were required for variants to accumulate these two key mutations, highlighting the advantages of multiple rounds of evolution. The former mutation lies in the heparin-binding domain, and the latter has not been associated with a known function, though both have been associated with B cell epitopes (Moskalenko et al., 2000; Opie et al., 2003). Importantly, two novel variants evolved *in vitro* to evade antibodies mediated efficient delivery *in vivo* in the presence of neutralizing serum levels 1–3 orders of magnitude higher than those needed to completely neutralize wild-type capsid. This result demonstrates that directed evolution can also serve as a valuable tool for viral engineering. Furthermore, in general analysis of the resulting variants may yield further insights into viral structure–function relationships, which can provide a future basis for additional rational design.

In an analogous library selection strategy involving one round of mutagenesis and subsequent selection, Perabo et al. (2006) subjected AAV variants with random capsid mutations to human serum neutralization and revealed that the mutations R459G, N551D, as well as a R459K-N551D double mutation, were largely responsible for evading from human antibody neutralization. A 3.3-fold to 5.5-fold higher serum concentration was required to reduce the mutant virus infectivity by 50% on HeLa cells compared to wild-type capsid.

Adenoviral Vectors

Library selection techniques have also been applied to engineer novel adenoviral vectors (Glasgow et al., 2006). As with AAV, rational approaches for adenoviral vector targeting include bispecific antibodies (Reynolds et al., 2000; Wickham et al., 1996) and incorporation of defined peptides into the exposed HI loop of the adenovirus 5-fiber knob (Nicklin et al., 2004; Work et al., 2004). Although these approaches allowed for tropism alterations, in some cases the peptide ligands suffered a loss of binding specificity to the target cells once incorporated into the viral capsid (Magnusson et al., 2002). Several studies have employed an innovative approach to select novel targeting peptides that have an improved chance of working in adenovirus, the construction of ''context-specific'' phage libraries in which a random peptide is displayed in the context of the HI loop of the adenovirus knob (Ghosh and Barry, 2005; Pereboev et al., 2001). These phage libraries were panned on target cells, leading to the identification of selected clones with high affinity to the target cell types. Subsequent genetic insertion of the selected peptide sequences onto the knob domain of adenovirus type 5 resulted in adenoviral vectors with enhanced specificity for the target cells. This approach further demonstrates the need to attempt to evolve novel functionalities in the context in which they will ultimately be used.

Conclusions

Several principles emerge from this collective work. Viruses, nature's solution to the gene delivery problem, have substantial potential to be safe and efficient gene delivery vehicles, but their properties, such as the capacity for

targeted delivery, need to be engineered. This class of problems can therefore serve as a model system for developing new approaches to engineer protein complexes on the 10s–100s nm size scale. Viruses are particularly well suited for library and evolution-based engineering methods, both because rational design is challenging for protein and nucleic acid assemblies of their structural and functional complexity and because their inherent linkage between the genotype and phenotype naturally lends itself to these approaches. A number of library-based approaches have been applied to engineering viral vectors for targeted delivery, including phage display-based selection of a targeting peptide that is later incorporated into the vector, single genetic diversification and library selection of the viral vector, and finally directed evolution composed of iterative rounds of genetic diversification and selection. Of these, the last approach, which most closely mimics the process of natural viral evolution, arguably has the most significant and untapped potential for engineering-enhanced vector properties, via progressive or iterative improvements. Finally, in some situations, it may be possible to gain fundamental new information on virus structure–function relationships, including the structural plasticity and functional importance of specific regions to the viral infection process. Therefore, library-based approaches promise to yield advances in both fundamental virology and biomedical application.

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