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Advanced targeting strategies for murine retroviral and adeno-associated viral Vectors

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Abstract Targeted gene delivery involves broadening viral tropism to infect previously nonpermissive cells, replacing viral tropism to infect a target cell exclusively, or stealthing the vector against nonspecific interactions with host cells and proteins. These approaches offer the potential advantages of enhanced therapeutic effects, reduced side effects, lowered dosages, and enhanced therapeutic economics. This review will discuss a variety of targeting strategies, both genetic and nongenetic, for re-engineering the tropism of two representative enveloped and nonenveloped viruses, murine retrovirus and adeno-associated virus. Basic advances in understanding the structural biology and virology of the parent viruses have aided rational design efforts to engineer novel properties into the viral attachment proteins. Furthermore, even in the absence of basic, mechanistic knowledge of viral function, high-throughput library and directed evolution approaches can yield significant improvements in vector function. These two complementary strategies offer the potential to gain enhanced molecular control over vector properties and overcome challenges in generating high titer, stealthy, retargeted vectors.

Keywords Adeno-associated virus · Lentivirus · Retrovirus · Targeting · Viral vector

1 Introduction

The two major classes of gene delivery vehicles, viral and synthetic, have in many senses complementary advantages and disadvantages. Synthetic vehicles have the significant benefit that their chemical compositions and properties can readily be varied and controlled, a capability that makes them very flexible particles to engineer. However, their delivery efficiencies are generally not yet high. In contrast, over evolutionary timescales, viruses have acquired numerous strategies to overcome gene transfer barriers and can therefore deliver their genetic cargo with high efficiencies. However, a number of their delivery properties are not yet optimized for human therapeutic use, for the simple reason that nature did not evolve them explicitly for this purpose. Therefore, it is desirable to engineer novel properties into viral vectors, but this goal can be challenging because they are very complex and intricate entities.TS[a]

Targeted gene expression is an attractive goal for gene delivery systems. It can be achieved by transductional targeting (the delivery of genes to specific cells) as well as transcriptional targeting (the use of promoters that mediate gene expression only in targeted cell types). This review focuses on the former approach, and readers are referred to several recent reviews for discussion of tissue-specific promoters [1, 2]. There are three potential goals of transductional targeting. First, the tropism or delivery range of vectors must sometimes be broadened to allow them to infect cells ordinarily resistant to transduction with the virus. Second, targeted delivery to *only* a specific cell type can be advantageous, since gene products that are therapeutic in some cellular settings can have side effects in others. This goal requires engineering a vector to reduce or eliminate its natural delivery properties and *replace* these properties with a novel, desired tropism. Finally, a vector can be enhanced to improve its stealth; in other words to reduce potentially undesirable interactions with cells or proteins including components of the immune system. It should be noted that these latter two goals, replacing tropism and vector stealth, have the advantage of potentially reducing the vector dosage needed for an application, which can lessen side effects as well as enhance the economics of gene medicines.

There has been success in targeted delivery by a number of viral vehicles, including the enveloped alphavirus, herpesvirus [3], retrovirus, and lentivirus, as well as the nonenveloped viruses adenovirus [4] and adeno-associated virus (AAV). This review will focus on one promising vector class from each category, retrovirus/lentivirus and AAV.

Three general strategies for engineering novel properties into viral vectors have been developed (Fig. 1), and all essentially strive to achieve a high level of control over the molecular properties of these vehicles. The first is to enhance vector properties through nongenetic approaches. These include using

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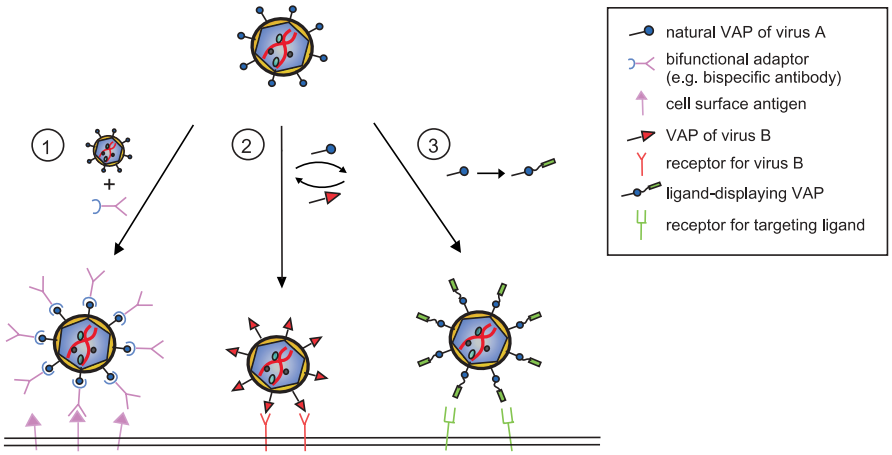

Fig. 1 Three approaches to targeting through the viral attachment protein (VAP). These include: 1) bridging with an adaptor molecule (nongenetic), 2) pseudotyping with a compatible viral capsid or envelope protein, and 3) genetic engineering of the VAP to insert new target cell specificity

bispecific antibodies, chemically cross-linking ligands to the viral surface to enhance binding to specific cell types, and grafting of polymers that resist protein adsorption to reduce interactions with the immune system. These latter two methods essentially attempt to merge the benefits of viral vectors with a major advantage of synthetic systems, the precise chemical control of vector properties. A second approach is to replace the viral attachment protein (VAP) of a vector with the corresponding VAP of another compatible virus to alter its tropism, known as pseudotyping. Although we will discuss numerous examples of these first two approaches, this review focuses mainly on the third promising strategy, the genetic engineering of viral attachment proteins for targeted gene delivery. If successful, this approach offers the highest potential for precise control over vector properties.

2
Retroviral and lentiviral vector targeting

The retroviridae are a family of enveloped viruses with a diploid, positive-stranded RNA genome. Retroviral vectors are very promising vehicles for delivering therapeutic genes to cells because they offer the advantage of stably integrating their genomic information into the chromosomes of their host's DNA. In addition, their simple gene composition has allowed vectors to be engineered to contain all viral functions needed to enter the cell but none of the viral gene sequences. Relatively recent work with this vector resulted in

the apparently permanent cure of children with SCID-X1 in the first successful gene therapy clinical trial [5]. Though two of nine children treated later experienced a severe adverse effect from the therapy [6], therapies based on retroviral vectors still comprise 28% of the clinical trials in progress today [7]. The results of the SCID-X1 trial further emphasize the need to develop safe and regulatable gene delivery vectors.

The majority of retroviral vectors in clinical trials are based on murine leukemia viruses (MLVs), a type-C simple retrovirus that is nonpathogenic to humans [8]. MLV genomes consist of three genes, *gag*, *pol*, and *env*, which encode all of the necessary proteins for the retrovirus to complete its life cycle. The *env* gene produces two protein subunits, transmembrane (TM) and surface (SU), which are cleaved from the same precursor and associate to form the Env protein, the VAP for MLV. This protein is directed into the endoplasmic reticulum where it is glycosylated and folded. Correctly folded proteins associate into homotrimers and are processed through the Golgi apparatus before export to the cellular membrane. As the virus assembles near the cell membrane, the envelope proteins concentrate at the site of budding through a mechanism that is not yet well understood.

During cellular entry, the envelope glycoproteins of the retrovirus mediate its attachment to the cell surface and subsequent fusion and insertion of the capsid into the cytoplasm. Since the envelope protein is the primary molecule that comes into contact with the cell surface, the majority of targeting efforts have been directed towards its modification and enhancement. The three strategies for engineering vector specificity discussed above (nongenetic modification, pseudotyping, and genetic engineering) have been applied to retroviruses. Success has been limited primarily by the fact that the binding of the natural retroviral envelope protein to its cell surface receptor triggers the fusogenic activity that mediates viral fusion and cell entry. This intimate coupling of binding and fusion makes it difficult to re-engineer the binding specificity without significantly compromising fusion, and thereby reducing viral titer. Therefore, engineering viral envelope proteins for novel specificity while fully retaining vector packaging and infection functionalities remains an important goal. Extensive investigations of different MLV envelope proteins have provided information on tolerable insertion sites and important regions for functionality [9–11], and these studies have provided a foundation for attempting the envelope protein modifications discussed below.

One major disadvantage of using simple retroviruses as gene therapy vectors is their inability to infect post-mitotic cells, a desired tissue target for many therapies. Much work has therefore been conducted to engineer complex retroviruses, lentiviruses and foamy viruses, which are able to transduce nondividing cells, to serve as gene therapy vehicles [12, 13]. Like all enveloped viruses, both of these classes of retroviruses also attach to their target cell through an envelope glycoprotein. One of the earliest strategies for targeting

consisted of packaging retroviral and lentiviral vectors with the viral attachment proteins of other enveloped proteins, (pseudotyping) [14, 15]. While this approach can successfully swap vector tropism with that of other viruses, in many cases it is not useful if there are no envelope proteins available to target delivery specifically to a desired cell population. Still, the success of pseudotyping has shown that retroviral envelope proteins are very modular, and foreign glycoproteins can be efficiently incorporated into fully infectious retroviruses and lentiviruses. Because of this feature, glycoproteins that are engineered for a desired function in one type of virus can often be readily interchanged and utilized in other enveloped viral vectors.

2.1 Direct targeting with retroviral glycoproteins by genetic and nongenetic approaches

Investigations in direct targeting began with the simple idea that if a vector could be engineered to attach to a cell through tissue-specific cell surface molecules, infection would predominantly occur in that tissue type. Strategies to promote such specific binding have included the genetic incorporation of ligands such as growth factors [16–18], peptides [19, 20], and single-chain antibodies [21–24]. In addition, nongenetic bifunctional adaptor molecules such as biotin-streptavidin or bispecific antibodies that bridge interactions between the virus and cell have been explored [25–29]. Furthermore, there is also a class of matrix-targeting vectors that incorporate collagen-binding domains that can direct the vector to extracellular matrix exposed during metastatic cancer [30–32]. While targeting matrix may concentrate vectors in the region of interest, it does not eliminate the possibility of infecting bystander cells. Much of this early work in direct targeting with retroviral vectors has been well reviewed [33, 34]. Some of this work demonstrated that enhanced binding of viruses to a desired cell type could be achieved; however, infection was also inefficient because the mutant envelope proteins fail to induce fusion [35]. The envelope protein has binding and fusion activities that are coupled in a complex and not fully elucidated mechanism; therefore, it has been difficult to re-engineer binding activity to a novel receptor target but maintain the same, efficient level of fusion [36]. In addition to inefficient cell entry, modified envelopes often reduce the packaging efficiency of the vectors, which again results in low overall titers. Limited success at targeting has been achieved by coexpressing the modified targeting envelope with the wild-type envelope in an attempt to improve fusion activity [16, 18, 21, 24]. The wild-type protein serves as an escort to the targeting protein to provide the means for fusion. However, this can only *broaden* rather than *replace* viral tropism since the wild-type proteins are free to interact with native receptors on nontargeted cells. Ideally, coexpression of a binding-defective envelope that can still trigger fusion would lead to more stringent targeting. This idea

of separating the mechanisms of binding and fusion is further explored below in the discussion of pseudotyping with pH-dependent glycoproteins. It is now clear that specific attention needs to be invested to ensure that the fusion mechanism of the virus is not impaired by envelope modifications.

Previous work identified a strategy of receptor co-operation whereby retroviruses expressing two different receptor binding domains linked by optimized proline-rich spacers can only infect cells expressing both retroviral receptors [37]. Martin et al. have applied this strategy by using single-chain antibodies that recognize high molecular weight melanoma-associated antigen (HMWMAA) and carcinoembryonic antigen (CEA) to target tumor cells [38]. The spacer length separating these dual targeting antibodies was optimized such that binding by both receptors was necessary to induce infection. It is proposed that binding of the first (targeting) receptor induces a conformational change in the proline spacer that allows binding of the second (viral/entry) receptor to occur. These vectors showed significant improvement in infection of tumors over previous strategies based on vectors that coexpressed the same scFv antibodies and wild-type Env [39–41]. Though the specificity and infection levels of these vectors are encouraging, low packaging efficiencies must be addressed before the strategy can be used clinically. An earlier version of the HMWMAA-targeting vector was the first to show selective transduction of targeted cells and reduced transduction of nontargeted cells in vivo [42]. However, these vectors transduced tissue at 10% of the efficiency of control vectors expressing wild-type Env. Future in vivo work will determine whether the combination of receptor cooperation and established binding strategies can increase transduction efficiency.

A nongenetic approach, the conjugation of targeting proteins to a virus after it has been packaged using receptor-ligand bridges, has been explored by Young and colleagues [43]. Earlier work showed enhanced infection of subgroup A avian leucosis viruses (ALV-A) when $EGFR^+$ target cells had been incubated with a bridge consisting of the soluble form of the ALVA receptor fused to EGF [44]. Another version of the system using ALV-B and a similar fusion protein had the improved feature of successfully allowing the virus, rather than the cells, to be preloaded with the targeting molecule [43]. This work also showed that the targeting virions could be produced directly from packaging cells by coexpression of the fusion protein. In addition, this group has had success using vascular endothelial growth factor (VEGF) and heregulin to target cells, thus establishing a broader applicability of this strategy [45, 46]. These bridge proteins, termed guided adaptors for targeted entry (GATEs), allow the native envelope-receptor interactions to be preserved since they are attached to the viral envelope protein rather than genetically incorporated into them. However, the stability of the protein-protein interaction between the virus and the bridge molecule is crucial to the success of this strategy in vivo. In addition, until the mechanism of binding-triggered fusion is better described, it is unclear whether fusion can be triggered by the

preloading and thus pose a toxicity threat to producer cells. It is important to note that the success of using EGF as a targeting molecule in this study may be due to the novel two-step cell entry mechanism of ALV that is still not completely understood, but seems to require both pH-independent binding and pH-dependent fusion events that are relatively uncoupled [47, 48].

One approach that combines a nongenetic bridging strategy that relies on post-packaging modifications as well as a genetically modified envelope protein utilizes antibody-antigen interactions to direct viruses to cells [27, 28]. To achieve this, envelope proteins are engineered to express the antibody binding domain of protein A. Prior to cellular infection, these viruses are incubated with monoclonal antibodies specific for the desired target cell. This modular system allows for several different cell types to be targeted without the need to re-engineer the vector. Preferential infection in the presence of targeting antibody with these modified viruses was first shown with vectors pseudotyped with the envelope protein of Sindbis virus, an alphavirus that utilizes pH-dependent fusion [29]. More recently, antibody-mediated targeting has been shown to be successful in replication-competent retroviruses with modified ecotropic and amphotropic Env proteins [49]. These envelopes incorporated the same protein A IgG-binding domain and were complexed with anti-human epidermal growth factor-like receptor-2 (HER2) antibodies to target HER2, a receptor overexpressed on 30% of breast cancer cells. Similar to previous direct targeting experiments with MLV envelope, viruses harboring only the chimeric envelope were able to bind to but not infect target cells. Infection was achieved by coexpressing wild-type envelope with the HER2 targeting envelopes. Significant enhancement of infection was seen on murine NIH3T3 cells engineered to overexpress HER2, but not on human mammary carcinoma cell lines despite evidence of enhanced binding in the presence of the anti-HER2 antibody. This discrepancy between the infection of engineered target cells and the natural target cells with the same receptor shows that cellular factors other than receptor expression must be explored in the development of targeting strategies. This difference underscores the importance of the need for more in vivo work in the development of targeting strategies.

An alternative pseudotyping method that utilizes incorporation of viral receptor proteins rather than viral envelope proteins was first shown in lentiviral vectors by Endres et al. [50]. This strategy takes advantage of the fact that virally infected cells express viral glycoproteins on their surface. Vectors that express the cellular receptor for the viral envelope protein are targeted to infected cells by exploiting the virus-cell binding in a reverse-directional manner. Endres et al. were able to selectively target HIV and SIV infected cell lines with lentiviral vectors pseudotyped with HIV and SIV receptor CD4 and coreceptor CXCR4 or CCR5 [50]. This idea has been further extended to create MLV vectors that are reverse-targeted to cells expressing RSV and MLV glycoproteins [51] and HIV-infected cells [52] by pseudotyping with the corresponding receptor(s). This approach has specific potential for anti-HIV

therapies. Successful HIV infection requires the binding of a coreceptor subsequent to attachment to the viral receptor CD4. Bittner et al. have shown that MLV and lentiviral vectors pseudotyped with a hybrid CD4/CXCR4 receptor can successfully transduce HIV Env-expressing cells [53].

Recent evidence of nonspecific, receptor-independent adsorption of retroviruses to cells suggests that earlier results that apparently demonstrated enhanced binding of targeting vectors may warrant re-examination [54]. Pizzato et al. attempted to target MLV vectors to ovarian cancer cells by fusing a single-chain antibody directed against the α folate receptor, which is overexpressed on ovarian cancer cells, to amphotropic and ecotropic MLV Env [55]. FACS analysis using fluorescently labeled anti-Env antibodies to detect the association of viruses with cells indicated that virions with ligand-incorporated glycoproteins had enhanced binding to target cells. Similar to previous targeting studies, this enhanced binding did not result in enhanced infection. However, further analysis by immunofluorescence microscopy showed that virions with wild-type envelope, ligand-incorporated envelope, or no envelope at all associated equally well with the cell surface. Furthermore, this group has also shown via confocal microscopy that a fluorescence increase detected by FACS, which could be interpreted as increased viral binding, is actually due to interactions between cells and a contaminant in the viral stock, soluble vesicles containing the SU protein [54]. Both results have major implications on other direct targeting studies that have reported enhanced binding based on FACS analysis but limited infectivity of target cells.

These results highlight several major issues that must be considered in the design of targeting retroviral vectors. First, if particles associate equally well with cells regardless of the presence of a targeting ligand, the inclusion of this ligand could be inconsequential unless the binding affinity and kinetics of this association are such that they favor actual infection. Further investigation of the mechanism of nonspecific retroviral binding may be required to aid the design of better ligands. Secondly, these results reiterate the fact that a productive ligand-receptor interaction does not necessarily catalyze viral fusion. The targeting vector must have a specific strategy designed to trigger viral entry. Depending on the results of these two issues, it may be more desirable to focus on mechanisms to trigger cell-specific fusion rather than cell-specific binding. Lastly, widespread nonspecific adsorption would increase the required therapeutic dosage and adversely affect the therapy's economics; therefore, it would be worth exploring ways to reduce nonspecific binding.

2.2
Direct targeting via genetic engineering of pH-dependent glycoproteins

Fusion of enveloped viruses with their target cells can be triggered by two mechanisms. Most retroviruses undergo pH-independent binding where fu-

sion is triggered by interaction with the cellular receptor upon binding. Other enveloped viruses employ a pH-dependent fusion mechanism that occurs inside the cells. Upon binding, these viruses undergo receptor-mediated endocytosis, and the subsequent reduction in endosomal pH triggers fusion between the virus and the endosome. Although retroviral binding and cell entry are intimately associated events in wild-type virions, work with recombinant HIV has shown that it is possible to decouple these two mechanisms and still generate fully infectious particles in vitro [56]. In general, however, because it has been difficult to engineer binding without compromising the fusion trigger of envelope proteins with pH-independent triggers, glycoproteins with uncoupled cell surface binding and pH-dependent fusion activities offer strong potential for engineering novel binding specificities.

Influenza hemagglutinin (HA) protein is an extensively studied fusion protein that undergoes a conformational change under acidic pH. Retroviruses were shown to specifically bind to target cells when pseudotyped with HA proteins from fowl plague virus fused to four different targeting ligands, including EGF, an anti-human MHC class I scFv, an anti-HMWMAA scFv, and an IgG Fc-binding polypeptide of protein A [57]. This binding could be abolished by the addition of neutralizing antibodies to the targeting ligand, thus confirming the mechanism of viral attachment. Some chimeric viruses showed greater selection for infecting appropriate target cells, but this effect was masked somewhat by the basal level of infection through the natural HA receptor, sialic acid. This approach therefore involved broadening rather than swapping viral tropism. A later improvement to this strategy was made by Lin et al. by coexpressing a fusion-defective, ligand-containing Moloney MLV Env protein with a mutant HA protein defective in its ability to bind sialic acid [58]. By completely separating the mechanisms of binding and fusion, these vectors showed a 10-fold increase in titer of targeted cell types over the control cell type. This effect was eliminated in the presence of competing soluble targeting ligand. In addition, this increase was found only in vectors coexpressing both envelope types and was not seen when mixing vectors expressing only one type. This indicates that the functions of binding and fusion cannot operate in *trans* across separate virions and suggests that fusion of these chimeras must be triggered by the internalized virus after binding.

The G glycoprotein from vesicular stomatitis virus (VSV-G) is commonly used to pseudotype retroviral and lentiviral vectors due to its broad tropism and the capacity of the pseudotyped particles to be concentrated by ultracentrifugation [15]. VSV-G pseudotyped viruses are internalized by the cell, and fusion between the virus and endosome occurs at approximately pH 6 [8]. Though VSV-G pseudotyped lentiviral vectors can be inactivated by human serum [59], recent evidence shows that this may be prevented by PEGylation of the vector, thus strengthening the opportunities for the use of this glycoprotein in vivo [60]. Attempts at targeting modifications to VSV-G have been limited, however, due to the lack of a three-dimensional crystal structure

and incomplete understanding of its fusion mechanism. Recently, MLV-based and HIV-1-based vectors pseudotyped with a modified VSV-G protein expressing a collagen binding domain of von Willebrand factor were shown to have increased attachment on a collagen-matrix while retaining their infectivity [61]. However, the ligand modifications resulted in a temperature-sensitive defect in the intracellular trafficking of the protein that could be restored if the viruses were packaged at the permissive temperature of 30 °C. Nonetheless, this work demonstrates the first successful modification to VSV-G that still allows for functional pseudotyping.

2.3
Inverse targeting by receptor sequestration and proteolytic cleavage

The lessons learned from early work in direct targeting led to a complementary approach that exploits the fact that retroviruses can remain bound to the surface of cells through a targeting ligand without being internalized by the cell. In inverse targeting, retroviral vectors with envelope proteins displaying high affinity ligands are blocked from infecting cells that express the cognate receptor due to the sequestration of the virus by that receptor. Cosset et al. showed that retroviral vectors with amphotrophic envelope proteins displaying EGF were able to bind to cells that expressed EGFR [17]. However, unlike the pH-dependent entry mechanisms described above for EGF-ALV Env vectors, fusion could not be triggered by the low pH environment that the bound virus may encounter during intracellular EGFR trafficking. Therefore, despite viral attachment, successful infection did not occur on $EGFR^+$, $Ram\text{-}1^-$ cells. The modified vectors were, however, able to infect $EGFR^-$ cells that displayed the virus' natural receptor, Ram-1, confirming infectivity of the vectors. Infection of $Ram\text{-}1^+$ cells was slightly reduced compared to wild-type, which is most likely attributed to steric effects of the incorporated ligand. This group has shown analogous results with virus that displays stem cell factor (SCF) [62]. This inverse targeting strategy offers benefits over initial direct targeting approaches because it allows the vector to exploit the virus' natural fusion pathway for cell entry. However, since this method relies on the absence of a specific receptor on the targeted cells, it is limited in scope and application.

The phenomenon of vector sequestration has led to another targeting strategy whose specificity is based upon cell surface proteases rather than receptors. Here, envelope proteins display a high-affinity ligand tethered to their N-terminus by a peptide containing a protease cleavage site. The target cells must express the proper receptor to enhance the binding of the virus to the cell surface. If the cell also expresses the appropriate protease, the high-affinity ligand is cleaved, and the virus is allowed to enter the cell through its natural receptor. This was first demonstrated by fusing EGF to amphotrophic MLV envelope via a factor Xa protease recognition site [63]. The modified vectors were able to bind EGF receptors on human cells in

vitro, but did not proceed with gene transfer until they were cleaved by factor Xa protease. Comparable results were found using similarly displayed EGF on spleen necrosis viruses (SNV), an avian retrovirus [64]. Additional work has shown parallel results for vectors fused to protease-cleavable insulin-like growth factor (IGF-I), albeit to a much lesser extent, suggesting that the success of this method may rely on the specific ligand chosen [65]. When used to pseudotype a lentiviral vector, the factor Xa-targeting, EGF-displaying envelope proteins were shown to be effective in altering the biodistribution of the vector in vivo [66]. Here, vectors that had EGF incorporated into their envelope had a lower infectivity in the liver (EGF-rich cells) than vectors with wild-type envelope. Infectivity levels were restored by competing with soluble EGF as well as by introducing factor Xa protease. In vivo studies using matrix-metalloproteinase (MMP) cleavable retroviruses have shown promising results targeting MMP-rich and carcinoembryonic antigen (CEA) expressing tumor xenografts in nude mice [67, 68].

Despite these promising results, there are two shortcomings that need improvement. Nonspecific infection of bystander cells can arise from the incomplete masking of the envelope's native binding activity, and large dosages of vector may be required to overcome the high levels of vector sequestration by nontargeted, $EGFR^+$ cells. A strategy that addresses both problems uses larger trimeric leucine zipper peptides or the trimeric C-terminal domain of CD40 ligand to sterically block infection of cells [69]. These peptides replace EGF in the previous strategy and are again linked to the amphotrophic envelope protein by a protease cleavage site. Infectivity of the modified vectors was low compared to a control and was restored upon addition of factor Xa protease. However, the incorporation rate of these bulky chimeric envelope proteins into virions during production was reduced and may present an obstacle when trying to achieve high packaging titers. Unless the packaging efficiency of these vectors can be improved, the benefit of sterically blocking infection of nontarget cells may not present as much of an advantage as the enhanced binding efficiency provided by a small targeting ligand.

2.4 Directed evolution methods for retroviral targeting

Though the above strategies have shown that vector retargeting is possible, they also illustrate the difficulty associated with predicting the modifications needed to impart new functionality while retaining the vector's ability to package and infect at high titer. One approach that circumvents the need for complex rational design is directed evolution. Directed evolution emulates the process of natural evolution by generating large libraries of mutants or variants and screening these libraries for improved function, an approach that has previously been applied with great success to enzyme and antibody engineering [70–72].

To explore the possibility of altering the tropism of a vector through directed evolution of the envelope gene, the DNA of a family of MLV *env* genes was shuffled and resulted in a clone that had an entirely new tropism not present in any of the parents [73]. As an additional example, phage display libraries have been used to search for optimal peptide sequences for cell specific binding [74, 75]. Since selection for binding activity is conducted in the context of a phage coat protein, however, it does not ensure that the binding specificity will be maintained when imported into a viral vector. In contrast, the identification of tolerable insertion sites in retroviruses has allowed the creation of retroviral display libraries that can be used to identify and select polypeptides with specific interactions in mammalian cells [9, 76]. After screening libraries for infectious mutants with desirable targeting features, the responsible modifications can be analyzed by sequencing the "successful" viral genomes. In the first application of this approach to retroviral targeting, the screening of a random display FeLV Env library resulted in the identification of a mutant with specificity for D17 canine osteocarcinoma cells, a tropism distinct from any FeLV subgroup [77]. The direct screening of random-display libraries also enables the discovery of mutants with altered tropism via unknown receptors. Bupp et al. were successful in identifying a mutant FeLV envelope protein that preferentially targets 143B cells and 293T cells via a novel, unidentified receptor [78].

The approach of using library and directed evolution in a mammalian cell context offers several major benefits. First, since the mutants are being screened directly for infectivity, vectors that have desired binding properties but unsuccessful entry properties, such as those in the early direct targeting literature, are not selected. Second, this approach greatly enhances the diversity of cell types that can be targeted since it can be applied to cells that are not yet well characterized. The studies utilizing random display libraries mentioned above each focused on identifying optimal virus-receptor interactions, but the strategy has also been shown to be successful in identifying optimized protease-activated retroviruses by screening libraries of protease cleavable peptides [79].

In summary, vectors with enhanced function must still be improved for high titer and stability during purification and storage before they will be able to be used clinically. However, the promising results of both rational and directed evolution approaches to vector targeting have laid the foundation for addressing these and other aspects of vector optimization.

3
Targeting adeno-associated viral vectors

AAV is a nonenveloped virus with a 4.7 kb single-stranded DNA genome, and it belongs to the family *Parvoviridae* and genus *Dependovirus* [80, 81]. Its

genome contains only two open reading frames flanked by short inverted terminal repeats (ITRs); however, the economical use of alternative splicing and start codons allows AAV to express seven partially-overlapping proteins from this short sequence. The first gene *rep* encodes four proteins necessary for protein replication (Rep78, Rep68, Rep52, and Rep40), and the second gene *cap* uses alternative splicing and start codons to express three structural proteins, VP1–3. VP3 is the shortest, whereas VP1 and VP2 include the entire VP3 sequence plus additional N-terminal extensions. Sixty subunits of VP1–3 self-assemble in an approximate stoichiometric ratio of 1 : 1 : 20 to generate the viral capsid. There are a number of known, relatively highly homologous, primate AAV serotypes [82, 83]. However, AAV2, which was first converted into a recombinant vector by Muzyczka et al. and Samulski et al. in the 1980s, is the best characterized and has received the most attention as a gene delivery vehicle [84–86]. The biology, highly promising gene delivery properties, and clinical application of AAV are discussed in more detail in another article in this volume (Grieger and Samulski).

The three general strategies for viral vector targeting have also been applied to AAV. First, as a nongenetic targeting approach, Bartlett et al. used a bispecific antibody to mediate the interaction between the AAV vector and a specific cell surface receptor expressed on human megakaryocytes [87]. The resulting vectors could transduce certain megakaryocyte cell lines at levels 70-fold above background, and the targeting was both selective and restrictive in that the endogenous tropism of the modified vectors was significantly reduced. In a second example, biotin was recently chemically crosslinked to the AAV2 capsid, and streptavidin was used as a bridge for the attachment of targeting ligands. Specifically, streptavidin genetically fused to the ligands epidermal growth factor or fibroblast growth factor-1 mediated a > 100-fold increase in gene delivery to cell lines ordinarily resistant to AAV gene delivery [88]. Finally, chemical approaches can be used to reduce the interaction of AAV with components of the immune system. We have recently crosslinked polyethylene glycol (PEG) to the surface of AAV2 to shield the virus from neutralization by serum antibodies (Lee, Kaspar, and Schaffer, submitted). In the future, PEG coating may be combined with targeting ligands as a chemical approach to replace AAV targeting specificity.

The delivery specificity of AAV2 can also be altered by vector pseudotyping, since different AAV serotypes bind to distinct receptors on cell surfaces [85, 89–92]. In particular, AAV2 binds to heparan sulfate as its primary receptor and FGFR and an integrin as secondary receptors [93–95]. It has also been shown that AAV3 also binds heparan sulfate, AAV4 and AAV5 bind to specific sialic acid linkages [96], and AAV5 binds to the PDGF receptor [97], whereas the receptors of other serotypes are not yet known. These serotypes are capable of packaging recombinant AAV2 genomes composed of a reporter gene flanked by the short AAV2 ITRs [98]. Consistent with the distinct cell binding properties of the parent serotypes, the different pseudotyped vectors

possess distinct gene delivery properties. For example, in the brain AAV2 selectively transduces neurons, AAV5 transduces both neurons and astrocytes, and AAV4 preferentially delivers genes to ependymal cells [92]. Different gene delivery properties have also been reported for the different serotypes in the retina, where AAV2 transduces phororeceptors, AAV5 capsid directs gene delivery to both retinal pigment epithelium (RPE) and photoreceptors, and AAV1 delivery is restricted to the RPE [99].

3.1 Targeting via genetic engineering of the AAV capsid

As with retroviral and lentiviral vectors, pseudotyping offers new vector tropism options. However, if no natural capsid that offers precisely the desired gene delivery properties is available, it would be desirable to engineer the AAV capsid for "custom" targeted gene delivery. In contrast to retrovirus, to date only direct targeting approaches have been applied for AAV. In addition, at the time that such work was initiated, the crystal structure for AAV2 was not available, making targeting via genetic engineering efforts challenging.

In the first AAV targeting effort, Yang et al. genetically fused a single chain antibody directed against the CD34 antigen, a marker of hematopoietic stem cells, to the N-terminus of VP2 [100]. This large insertion significantly increased selective gene delivery to a $CD34^+$ cell line, although the viral titers were low. This important study represented the first successful targeted AAV gene delivery. However, it also demonstrated that the use of smaller, less disruptive targeting moieties, as well as insertion into optimal locations on the capsid, may be required to enhance viral packaging and targeting efficacy. These constraints likely result from the need for the viral proteins to undergo a complex self-assembly process to generate the capsid, a process readily interrupted by insertional mutagenesis.

The next successful effort involved the incorporation of small targeting peptides into the capsid. By superimposing the AAV2 capsid sequence onto the known structure of canine parvovirus (CPV), Girod et al. chose six sites putatively on the viral surface for the insertion of a ligand containing the integrin-targeting amino acid sequence RGD. The insertion sites were predicted to lie on various surface loops of the VP structure, and one mutant infected cell lines that were resistant to infection by wild-type AAV2, but with a modest titer [101]. This successful insertion occurred at amino acid 587, based upon numbering of VP1, within the surface accessible loop 4 of the capsid protein.

Subsequent efforts involved widespread insertional mutagenesis to probe the capsid for sites exposed to the surface and tolerant of peptide insertions. One earlier study of basic AAV biology explored the effects of small peptide insertions on viral packaging and infectivity [102]. More recent studies have

comprehensively scanned the capsid to search for insertion sites for targeting peptides. Rabinowitz et al. employed linker insertional mutagenesis into several dozen sites of the vector and found several regions amenable to the insertion of small peptides [103].

In addition, Wu et al. generated 93 mutants by epitope tag or ligand insertion, or alanine scanning mutagenesis. This work identified numerous locations where insertion disabled the virus, as well as located regions on VP1 and VP2 where the insertion of a serpin receptor peptide ligand successfully altered viral tropism [104]. Interestingly, insertion of their targeting peptide within loop 4 was not tolerated, indicating that the optimal insertion site could be dependent on the specific identity of the targeting peptide. Furthermore, mutants defective in heparin binding were identified, which could aid future efforts to fully replace viral tropism. In another study, Shi et al. generated capsid mutants with peptide insertions at 38 locations, and the addition of the peptide fragment of luteinizing hormone to VP1 and VP2 enabled selective targeting to an ovarian cancer cell line [105]. Shi and Bartlett followed up on this work by inserting RGD peptides into several permissive sites they had previously identified. The resulting vectors exhibited significantly increased transduction to cell lines that express integrins but low levels of heparan sulfate [106].

While these comprehensive insertional mutagenesis efforts were in progress, Grifman again employed a comparison of the AAV and CPV to identify accessible regions on the viral capsid for the insertion of the tumor-targeting NGRAHA peptide that binds to CD13, a vascular receptor and regulator of angiogenesis [107]. Insertion of this motif in loop 4 of the capsid led to a 20-fold increase in delivery to several tumor cell lines. Finally, Baker used phage display to identify peptides that bound venous endothelial cells and inserted them into position 587 of the AAV2 capsid, the site previously identified by Girod et al. [101]. Several of the resulting vectors exhibited reduced accumulation in the liver and enhanced transduction of the vena cava in vivo [108].

In a modular approach that combined genetic and nongenetic targeting approaches, Ried et al. generated AAV2 vectors with capsids displaying immunoglobulin-binding domains borrowed from protein A [109], elegant work similar to that conducted with retrovirus [27, 28]. These vectors, when coupled to antibodies against receptors such as CD29, CD117, or CXCR4, specifically transduced human hematopoietic cell lines. Furthermore, this approach is highly modular in that it readily allows different antibodies to be employed.

These insertional mutagenesis and targeting studies identified numerous regions of VP1–3 exposed to the viral surface, findings that were further confirmed and substantiated by the solution of the AAV2 crystal structure [110]. Furthermore, as compared to the previously determined structures for CPV and insect parvovirus, the AAV2 crystal structure revealed the presence of prominent peaks on the viral surface at the three-fold axis of symmetry, and a cluster of positively charged residues on the side of this peak likely medi-

ates binding to the receptor heparan sulfate. The AAV2 structure, as well as the recently elucidated AAV5 structure [111], will likely aid further efforts to rationally design novel capsid properties.

3.2
Directed evolution and library methods for AAV targeting

The prior targeting work involved insertion of a defined targeting sequence into numerous locations on the viral surface. In contrast, a recent elegant library approach inserted random peptides into a single location on the viral surface to identify novel targeting sequences for specific cellular targets [112]. Specifically, a random seven amino acid peptide sequence was inserted into position 588 at the peak of the three-fold axis peak, again a previously identified insertion site [101]. In an approach analogous to phage display, the resulting library was selected for the ability to infect human primary coronary endothelial cells, cells nonpermissive for AAV infection, and variants with a consensus sequence emerged and were able to infect cells at levels as high as 630-fold higher than wild-type virus. The molecular mechanisms of AAV gene delivery are being progressively elucidated [113–117], knowledge that will enable further rational design. Until the viral structure-function relationships are fully elucidated, however, such library approaches offer a high-throughput means to solve problems in targeted delivery.

We have recently applied a directed evolution approach in order to address the problem of antibody neutralization of AAV. It is clear that there is significant sequence and functional diversity in the AAV capsid [82, 83, 92, 98], and the capsid is therefore potentially reasonably plastic and tolerant of point mutation. We have therefore applied a directed evolution approach involving random mutagenesis of the capsid and selection for variants that are not neutralized by serum containing anti-AAV antibodies. This approach has led to the generation of viral variants with mutations that render them largely resistant to antibodies that neutralize wild-type virus, and analysis of their sequences could yield further insights into the mechanism of viral cell entry (Maheshri et al., submitted). Furthermore, this approach of evolving novel "custom serotypes" can be applied to a variety of challenges in AAV gene delivery.

4
Summary

Significant progress has been made in the molecular engineering of viral attachment proteins for the three goals of broadening tropism, replacing tropism, and stealthing vectors to reduce unwanted molecular interactions. In efforts to fulfill these three targeting goals, the different design challenges

and constraints of enveloped and nonenveloped viruses have been addressed. Envelope proteins can be highly challenging to engineer since in many cases receptor binding directly triggers viral fusion; however, these proteins can typically tolerate relatively large genetic insertions since structurally they need only assemble into small multimers such as trimers. In contrast, capsid proteins must multimerize into highly organized structures, and even small peptides can readily disable viral function if not inserted into the correct sites. However, the binding and endosome disruption activity of these proteins are not typically intimately coupled, potentially making it somewhat easier to re-engineer binding specificities.

For both enveloped and nonenveloped vectors, there are three strategies for developing targeting vectors (Fig. 1), and each has characteristic advantages. Nongenetic targeting approaches offer the potential for highly modular systems, where a single vector product can be adapted with antibody or chemical prosthetics for specific applications. Future work must be conducted to fully explore the potential of this approach. In addition, pseudotyping with viral attachment proteins from related viruses offers a selection of viral tropism options, and it is possible that one of these options could match the precise needs of a given gene delivery application. Finally, genetic engineering offers the potential for rapid targeting, or stealthing, without the need for an extra prosthetic. However, different clinical applications may require the generation and large-scale production of different targeted vector variants. For genetic engineering strategies, rational design methods can be effective when a significant amount of structural information about the viral attachment protein is known. In parallel, library and directed evolution methods have the potential to yield useful vector products as well as yield new insights into viral structure and function, particularly for viruses for which little structural information is known. Major progress has been made in pursuing these strategies for targeting vectors, and future work will reveal whether they can solve the challenges that remain, particularly the generation of high titer vector variants with fully replaced tropism.

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