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Genetic Targeting Strategies for Adenovirus

Sam C. Noureddini[†] and David T. Curiel*[,‡]

VectorLogics, Inc., 550 11th Street South, Birmingham, Alabama 35294, and Division of Human Gene Therapy, Departments of Medicine, Pathology and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, 901 19th Street South BMR2 502, Birmingham, Alabama 35294

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Abstract: Adenovirus serotype 5 (Ad5) continues to be regarded as a gene delivery vehicle of high utility for a variety of clinical applications. However, targeting of the virus to alternate, non-native receptors has become a mandate for many gene therapy approaches, as inefficient viral transduction of target tissues has proven detrimental to the utility of Ad5. Thus, various targeting strategies have been endeavored to the end of highly specific cellular transduction, including that of genetic manipulation of the viral capsid. Modification of the tropism-determining fiber protein and other capsid locales has allowed vectorologists to develop vectors that are highly superior to the first-generation adenoviruses employed for gene therapy. Herein, the various genetic targeting strategies for Ad5 are reviewed, and the various schemas in which targeted transduction has been achieved with tropism-modified vectors are outlined.

Keywords: Adenovirus; gene therapy; target cell; gene transfer; review

Introduction

In recent decades, the biomedical community has been engaged in the development of novel and progressive means of combating human disorders via targeted interventions. This shift from the established to the nonconventional has arisen, in part, due to an increased focus on the molecular origins and pathology of disease. It is through this paradigm that one such treatment modality, gene therapy, has emerged as a potentially useful approach for the treatment of a myriad of human disorders. This strategy entails the introduction, to the cell, of genetic material whose subsequent translational products address the therapeutic outcome. Indeed, this therapeutic model would exploit the increasing repertoire of genes for which functions have been defined.

Critical to realizing the therapeutic potential of gene therapy is the achievement of safe and efficient delivery of genes to target cells. The NIH-commissioned Orkin−Motulsky report on gene therapy concurs with this notion by stating, "To confront the major outstanding obstacles to successful somatic gene therapy, a greater focus on basic aspects of gene transfer is required. Such efforts need to be applied to improving vectors for gene delivery and directing gene transfer to specific cell types."[1] Although many types of vectors have been studied to achieve this end, the adenovirus (Ad) serotype 5 possesses the evolutionary ability to effectively transfer double-stranded DNAs to a multitude of cell types, and has a well-characterized viral genome, which provides for the relative ease of capsid manipulation for cell-specific targeting. This review will focus on genetic targeting strategies for Ad5, and will outline the means by which these vectors can be evaluated in vivo to determine their overall efficacy as gene therapy reagents. While this review addresses specifically the area of Ad-capsid engineering to the end of targeting, we would also highlight the other review articles which cover this topic in addition to other relevant subject matter.[2−5]

* Corresponding author. UAB−Gene Therapy Center, 901 19th Street South BMR2 502, Birmingham, AL 35294. Phone: (205) 934-9515. Fax: (205) 975-7476. E-mail: curiel@uab.edu.

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[‡] University of Alabama at Birmingham.

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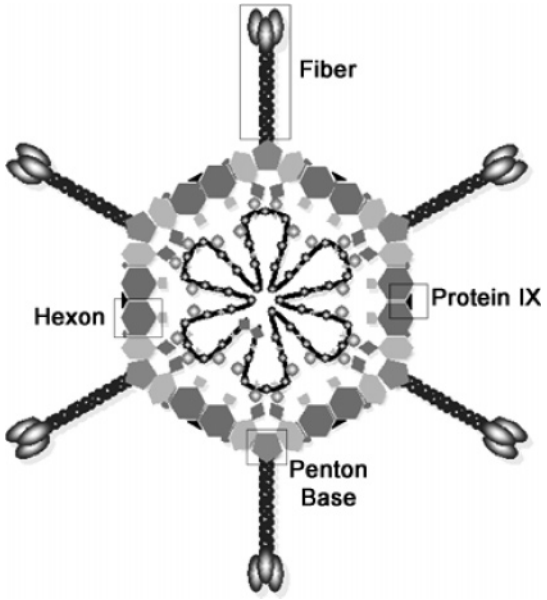

Figure 1. Cross section of native adenovirus serotype 5 capsid. A generalized graphical displaying the major structural proteins of the Ad5 capsid is shown. The locales that have previously been exploited for genetic modification have been indicated: fiber, protein IX, penton base, and hexon.

Ad5 Basic Biology, a Rationale for Targeting. The human adenovirus serotype 5 (Ad5), a species C member of the family *Adenoviridae*, is an icosahedral viral particle housing a 36 kb, double-stranded DNA genome (Figure 1). Wild-type Ad5, devoid of any modification, has a well-characterized ability to transduce both dividing and non-dividing tissues in vivo, and has demonstrated a relative ease in laboratory production and purification. These characteristics have justified using Ad5 as a vector system, with subsequent genetic engineering of the capsid components of the virus for highly specific gene therapy applications.

Adenoviral cellular entry, now a well-understood process, occurs via a two-step mechanism and provides as a rational point of departure for Ad tropism and modification. The globular knob domain, located at the distal end of the fiber homotrimers extending from the twelve capsid vertices, binds to its native cell-surface receptor, coxsackie and adenovirus receptor (CAR).[6−9] Following attachment, the Arg-Gly-Asp (RGD) motifs of the capsid penton base interact with cell surface integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. This secondary step initiates viral endocytosis within a clathrin-coated vesicle, with subsequent viral release into the cytoplasm resulting in nuclear translocation and viral replication. However, it is at the step of primary attachment that the wild-type virus proves to be inadequate for many gene therapy approaches, as many tissues and cells of interest, e.g., cancer cells, express little or no CAR on their surfaces. Thus, the rationale for Ad targeting is rooted in developing vector systems that not only are capable of recognizing alternate receptors and tissue types but also are completely removed of their native tropism, making these vectors truly "retargeted" species. Targeting of this nature has all but certainly become a mandate for many gene therapists, as efforts to achieve Ad targeting has steadily increased in recent years, yielding many new strategies to optimize these vector systems.

Genetic Targeting Strategies

To achieve the vector targeting central to the success of many Ad-based gene therapy applications, several genetic targeting strategies have been proposed. All of these strategies embody the manipulation of the Ad genome, subsequently modifying one of the Ad capsid proteins. The desired result is a single-component vector capable of achieving targeted cellular transduction via non-native receptors. Such capsid locales exploited for genetic engineering include the capsid fiber, hexon protein, penton base, and protein IX (pIX).

Fiber Engineering. (1) Serotype Fiber/Knob Chimerism. As the primary determinant of viral tropism, the trimeric capsid fiber protein represents the most logical site for genetic engineering for targeted transduction. Because trimerization of the fiber is a requisite for capsid incorporation, and thus cannot be perturbed, one such approach seeks to capitalize on the structural homology present between Ad5 and other Ad serotypes. Because this high degree of structural similarity exists in the fiber protein, especially in the fiber tail (the component of fiber−capsid attachment), chimeric vectors employing whole fibers or even the distal knob proteins from other Ad serotypes represent an efficient and natural means of achieving retargeting. Of course, this approach is relevant to those Ad serotypes whose native tropism is associated with receptors other than CAR, with this mode of serotype fiber/knob "switching" serving to achieve the linked targeting goals of CAR-tropism ablation and alternate receptor recognition (Figure 2).

Successful Ad fiber pseudotyping was achieved by Gall and colleagues, via replacement of the Ad5 fiber with the Ad7 fiber, resulting in the tropism redirection of the vector

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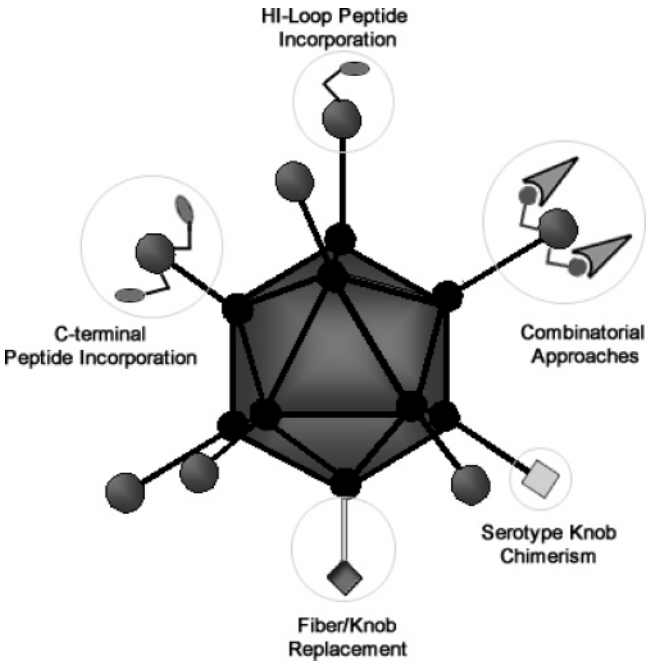

Figure 2. Genetic modifications endeavored for the fiber protein. This figure demonstrates, schematically, the various Ad targeting strategies embodying genetic modification of the fiber protein. Peptide incorporation to the HI-loop and C-terminus of the knob, serotype knob chimerism, fiber/knob replacement, and combinatorial approaches involving separate targeting molecules is shown.

to CAR-deficient tissues.[10] Additionally, chimeric Ad5 vectors employing alternate serotype capsid fibers were shown to effectively transduce human CD34-positive cells with fibers from Ad35,[11] and synovial tissues, vascular endothelial cells, and smooth muscle cells with Ad16 fibers.[12,13] Transduction of these tissues by unmodified Ad5 was previously shown to be inefficient for stringent application. In a slightly different approach, Krasnykh and colleagues employed only the globular knob protein from Ad3 for their chimeric Ad5 vector,[14] resulting in increased transduction of ovarian cancer cells[15] and squamous cell carcinomas,[16] again, a very significant improvement over native Ad5. In the aggregate, however, this vector strategy is limited to the tropic behavior of the characterized serotype adenoviruses, thus limiting the repertoire of candidate targets to which theses chimeric vectors can be directed.

(2) Targeting Ligand Incorporation. An alternate approach to vector targeting entails the direct genetic incorporation of targeting peptides into the Ad5 fiber knob (Figure 2). Although deviating from the concept of "true retargeting", as the native knob structure is retained, and thus, theoretically retains CAR affinity, these vectors may be tropism expanded and/or infectivity enhanced, a critical dynamic for many gene therapy applications. The necessary considerations critical for such an approach involve utilization of peptides that (1) retain function when in the context of the knob, (2) do not prove deleterious to fiber structure/trimerization, and (3) are devoid of major cytosolic post-translational modifications. This final consideration is necessary due to the fact that the fiber protein, while translated in the cell cytosol, is routed to the reducing environment of the nucleus for virion assembly.

Initially, the solvent-exposed carboxy terminus of the knob protein was exploited for ligand incorporation, due to the fact that genes coding for various targeting motifs could easily be incorporated at this terminal locale. Integrin-binding RGD peptides, as well as poly-lysine peptides, were introduced at this site and provided for efficient transduction of cell lines previously refractory to Ad infection.[17,18] However, as various groups concluded, constraints relative to the size of ligands that can be incorporated into the C-terminus led to analysis of alternate fiber knob locales, and ultimately, to the knob HI-loop.[19] Because this dual β-sheet motif is exposed on the surface of the knob protein, it was believed that HI-loop-incorporated targeting ligands would be readily accessible to their respective cellular targets. Krasnykh and

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colleagues successfully incorporated heterologous peptides at this locale,[20] with Belousova and colleagues further demonstrating that the HI-loop could tolerate up to 100 amino acid insertions without affecting fiber and virion integrity.[21] Dmitriev and colleagues constructed an Ad5 vector containing the RGD motif at the HI-loop locale,[22] which was later shown to be effective in transducing CAR-deficient ovarian cancer cell lines,[23] primary tumor material,[24] and carcinomas of the ovary, pancreas, and head and neck.[25] Additionally, the endothelial cell-binding SIGYLPLP peptide[26] and various phage-derived peptides have been incorporated into the HI-loop, displaying affinity for vascular endothelial cells,[27] various cancers,[28] vascular smooth muscle,[29] and transferrin receptor.[30] Despite these findings, genetic modifications to the native Ad5 fiber knob have proven to be very difficult to progress further, as the repertoire of incorporable ligands has been limited to simple peptides, further compounded by the necessity of a further modification to ablate CAR tropism.

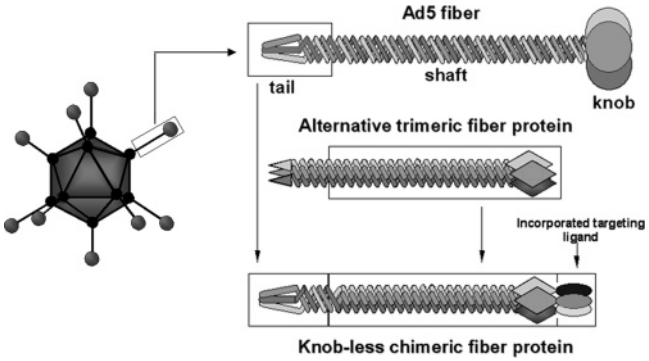

Figure 3. Schematic representation of knobless fiber replacement. The strategy for replacement of the native fiber protein with an alternate, knobless fiber platform is shown. A chimeric fiber is constructed via the genetic fusion of the N-terminal tail domain of the native fiber with the trimeric knobless fiber protein. Subsequent C-terminal incorporation of a specific targeting ligand yields targeted fiber proteins, capable of associate incorporation into the Ad capsid.

(3) Fiber Replacement. In view of the structural limitations encountered with modifying the native fiber knob, strategies entailing a more aggressive and elaborate approach to capsid engineering have resulted in some elegant targeting advances. Studies establishing the feasibility of developing fiberless Ad vectors[31−33] subsequently led to various hypotheses directed toward the replacement of the Ad5 knob, or fiber protein en toto. Indeed, this represents an attractive approach, as fiber modifications of this type would accrue the benefits of a larger repertoire of candidate ligands, and would embody the complete ablation of native CAR tropism (Figure 3).

After establishing the criteria that the alternate knob or fiber platform should confer trimerization and be compatible with the Ad5 fiber tail region for capsid incorporation, several fiber replacement strategies were endeavored. van Beusechem and colleagues developed an Ad vector with the knob protein replaced by the trimerizing domain of the Moloney murine leukemia virus envelope glycoprotein.[34] Magnusson and colleagues utilized the trimerizing neck region peptide of

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human lung surfactant protein D in a knobless fiber platform employing RGD, targeted to cell-surface integrins.[35]

Additionally, Barry and colleagues succeeded in developing a targeted, knobless Ad vector employing the sigma1 attachment protein of the mammalian reovirus, exhibiting targeted transduction to cells expressing the junction adhesion molecule-1.[36] Previously, our group has utilized the bacteriophage T4 fibritin protein for fiber replacement, replacing not only the Ad5 knob but also the majority of the shaft region, with direct N-terminal fusion of the artificial fiber to the Ad5 tail domain. A vector employing this fiber−fibritin (FF) chimera with a C-terminal six histidine (6-His) targeting motif exhibited highly efficient gene transfer to cells expressing artificial 6-His receptors, while exhibiting no CAR tropism.[37] Belousova and colleagues furthered this work by incorporating trimeric CD40 ligand to the FF-chimeric platform, exhibiting highly efficient gene transfer to CD40 positive dendritic cells, thus representing the most structurally complex capsid-incorporated targeting ligand to date.[38] Indeed, the genetic coupling of a complex targeting moiety such as this has allowed vectorologists to abandon the notion that only simple peptides are capsid-compatible, as many groups are now working toward incorporating larger physiological targeting ligands with rational therapeutic end points in mind.

Alternate Capsid Locales. (1) Hexon and Penton Base. Additional locales in the Ad5 capsid that have been exploited for genetic engineering include the hypervariable regions of the capsid hexon protein, as well as the penton base. Vigne and colleagues were able to demonstrate the feasibility of hexon-incorporated targeted ligands, by incorporating an integrin-binding RGD ligand at hypervariable region 5 (HVR5).[39] Vectors employing this modification exhibited enhanced infectivity of α_v integrin-positive cell lines.

Because in certain Ad serotype types the capsid penton base has been shown to interact with surface integrins directly to initiate transduction,[40,41] this locale has been utilized for the incorporation of targeting ligands in vectors derived from Ad5. Einfeld and colleagues constructed an Ad vector containing a linear peptide, hemagglutinin (HA), genetically fused to the penton base.[42] This group determined that not only was the structural integrity of these viruses unaffected but also these vectors were able to transduce cell lines expressing an artificial, HA-recognizing receptor.

(2) pIX: Novel Imaging and Targeting Locale. Recently, alternate capsid components have been examined as candidate locales for ligand incorporation, including the minor capsid protein IX (pIX). pIX is a so-called "cement" protein which stabilizes hexon−hexon interactions on the surface of the Ad capsid, contributing to the overall structural integrity of the virion.[43,44] Because the C-terminus of pIX is exposed on the surface of the capsid,[45] efforts have been focused toward incorporating ligands at this site to achieve vector targeting. Dmitriev and colleagues established the feasibility of this approach by incorporating peptides such as poly-lysine and FLAG-tags at the pIX locale, resulting in tropism redirection to cell-surface heparan sulfates.[46] Additionally, Vellinga and colleagues incorporated the integrin-binding RGD motif fused to an α-helical spacer on pIX, which exhibited enhanced infectivity to cells previously refractory to CAR-mediated transduction.[47]

Additionally, pIX has been shown to have utility for the growing field of in vivo imaging (Figure 4). Pioneered by Le and colleagues, a pIX-green fluorescent protein fusion

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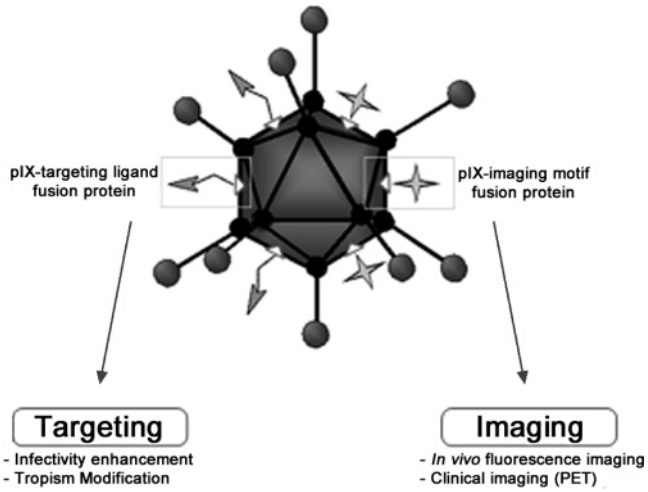

Figure 4. Genetic modifications of protein IX. pIX has been exploited for targeting strategies to the end of infectivity enhancement and tropism modification. Additionally, pIX has been exploited for both in vivo and clinically relevant imaging applications, with the incorporation of such imaging motifs as green fluorescent protein and herpes simplex virus-1 thymidine kinase.

protein has been constructed, which, once assembled into mature Ad virions, renders the capsid autofluorescent.[48,49] Very recently, Li and colleagues have incorporated other imaging motifs at this locale, including firefly luciferase and red fluorescent protein (manuscript under review). Additionally, the incorporation of herpes simplex virus-1 thymidine kinase on pIX by this same group has enabled vector imaging via the well-established positron emission tomography (PET) scan.[50] Developments such as these have a profound impact not only on the imaging field but on the vector targeting community as well, as there now stands the potential for the genetic incorporation of large, complex ligands on pIX. The body of work examining the utility of pIX as a capsid-engineering locale is rapidly growing, as many vectorologists are attempting to incorporate a wide repertoire of ligands at this site.

Combinatorial Approaches. Two-Component Targeting via Genetic Engineering. Targeting approaches employing the use of separate recombinant proteins to reroute Ad vectors represent the historical bases for the genetic, single-component targeting strategies described thus far. However, there have been recent developments establishing the utility of capsid-modified vectors in conjunction with other targeting molecules. This strategy entails the genetic capsid incorporation of a particular ligand exhibiting affinity for an external targeting molecule, to the goal of constructing targeted vector complexes. For example, various groups have employed the Fc-binding domains of *Staphylococcus aureus* protein A to achieve targeting via immunoglobulin (Ig) mediated transduction. Volpers and colleagues[51] and Henning and colleagues[52] have demonstrated the utility of incorporating these Fc-binding ligands to the HI-loop of fiber or into a knob-deleted fiber platform, respectively. Both groups were able to demonstrate that transduction of CAR-negative cell lines could be achieved via antibodies directed against specific cellular receptors. Additionally, our group incorporated a similarly derived Fc-binding ligand at the C-terminus of the fiber protein, and demonstrated that a highly purified targeting complex between the virus and an Fc-single chain antibody fusion protein could achieve highly specific targeting to alternate receptors, such as CD40.[53]

Barry, Campos, and colleagues have designed vector systems exploiting the high affinity biotin−avidin interaction. Incorporation of a biotin acceptor peptide (BAP) into the C-termini of either the fiber protein[54] or the pIX protein[55] has allowed for the metabolic biotinylation of these vectors during propagation via the endogenous ligases present in 293 cells. Targeting complexes were then formed between these vectors and biotinylated antibodies using high affinity tetrameric avidin molecules. In addition to vector targeting, this approach was subsequently used for ligand screening applications, as well as in an alternate viral purification approach.[54]

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In Vivo Analysis/Model Systems. In Vivo and in Situ Evaluation. With the advent of novel developments in adenoviral vectorology, specifically in the realm of genetic retargeting of the virus, serious questions and concerns remain in regard to the clinical utility of these targeted Ad vectors. Thus, the development of stringent model systems has become a mandate not only for the evaluation of genetically targeted Ad vectors but also for the validation of employing targeted versus nontargeted Ads. Indeed, many groups have become engaged in altering the capsid to the end of evading host immunity and liver sequestration in vivo, but the number of adequate model systems to evaluate the efficacy of targeted Ads is few.

Everts and colleagues recently reported the employment of a mouse model transgenic for the native human Ad receptor, hCAR, to achieve successful induction of a specific tumor associated antigen in the pulmonary vasculature.[56] The group utilized a CAR-tropic Ad vector, with expression of the tumor associated carcinoembryonic antigen (CEA) driven by the endothelial-specific flt-1 promoter. After systemic administration via the tail vein, the group reported successful induction of CEA in the mouse lung, followed by efficient targeting to CEA via an Ad-adapter targeting complex. Importantly, this study was able to establish the feasibility of utilizing transient transgenic animal models for the evaluation of targeted Ads in vivo.

de Gruijl and colleagues have established a stringent in situ model system for the evaluation of dendritic cell (DC) targeted Ad vectors via the employment of human skin explants. Initially, the resident cutaneous DCs in these patient-derived tissues were shown to be efficiently transduced by an Ad employing a bispecific fusion protein targeted to the CD40 receptor on the DC surface.[57] Recently, in collaboration with our group, a single-component genetically targeted Ad employing CD40 ligand (CD40L) and coding for CEA was shown to effectively transduce DCs in situ, and stimulate membrane presentation of the antigen.[58] Currently, we are employing other vectors in this stringent in situ model to establish the feasibility of utilizing our targeted Ad as an immunotherapeutic for cancer.

Conclusion

Adenovirus-based vectors remain as some of the most useful gene delivery vehicles for a variety of clinical contexts. As first generation Ad vectors were found to be inadequate for specific applications, highly improved vector systems have now been realized via the targeting strategies described herein. Genetic engineering of the adenoviral capsid, to the end of tropism modification, has vastly expanded the utility of Ad for gene therapy, as these new capacities may allow for efficient targeting in vivo. Again, with the establishment of novel, model systems offering stringent vector efficacy analysis, vectorologists will be able to more effectively determine the preclinical index of targeted Ad vectors. Likewise, the ability to obtain useful clinical data in the context of in vivo imaging will assist in understanding basic questions about the biology of targeted Ads in situ, fostering the further enhancement of these targeted therapeutics. However, despite the boundaries encountered with more elaborate and complex capsid modifications, progress in the area of genetic targeting has been steady, suggesting that the development of targeted vectors for clinical application is a realistic, highly attainable goal.

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