

Genetic Targeting of Adenovirus Vectors Using a Reovirus σ 1-Based Attachment Protein

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Targeting adenovirus vectors (AdV's) for selective transduction of specific cell types requires ablation of native adenovirus tropism and introduction of a unique target-binding moiety. To bring these requirements within reach, we developed a novel strategy to target AdV's genetically that relies on replacement of the entire adenovirus fiber protein with a fusion molecule comprising the virion-anchoring domain of fiber and the oligomerization domain of reovirus attachment protein σ 1. The chimeric molecule forms trimers, is transported to the nucleus, and assembles onto the adenovirus capsid. In contrast to previously reported genetically targeted vectors, the AdV presented herein propagates efficiently without a requirement for complementing fiber. Due to ablation of the native adenovirus tropism, the infectivity of this AdV was at least 35-fold reduced on 293 cells. Importantly, a His tag incorporated into the chimeric attachment protein conferred His-tag-dependent tropism to the AdV, which resulted in a 12- to 40-fold greater transduction efficiency on two different cell lines expressing a His-tag-binding receptor. In addition, the infection efficiency was strongly reduced by preincubation with a His-tag-specific Ab. Thus, this σ 1-based chimeric attachment molecule provides a promising new platform for the generation of truly targeted AdV's.

Key Words: adenovirus, targeting, reovirus, fiber, sigma1, gene therapy

INTRODUCTION

Human adenoviruses, in particular serotypes 2 and 5, are widely applied as vectors for gene delivery. These viruses have many potential therapeutic benefits, including easy propagation to high titers, efficient infection of dividing and nondividing cells, and relatively limited toxicity in humans. However, the *in vivo* utility of adenovirus vectors (AdV's) is limited by their promiscuous tropism, which leads to efficient sequestration of administered AdV's in undesired tissues, thereby limiting the fraction of the AdV dose available for target cell transduction. The tropism of adenovirus types 2 and 5 is defined by three physically distinct receptor-binding interactions. The primary attachment of adenovirus to host cells is mediated by an interaction of the C-terminal knob domain of adenovirus fiber with CAR [1–3]. A second receptor-binding site is localized to the penton base and mediates virus interaction with α_v integrins [4–7]. A third receptor-binding site is localized to the third β -spiral repeat in

the fiber shaft and mediates binding to heparan sulfate glycosaminoglycans (HSG) [8,9]. Although CAR is the principal adenovirus attachment receptor, all three binding sites contribute significantly to the tropism of adenovirus *in vivo* [10–13].

The requirement for fiber in the interaction of adenovirus with host cells has directed most AdV targeting strategies to exploit this capsid protein as a portal for development of new cellular affinities (for reviews see [14,15]). Among these approaches, the one-component targeting strategy based on genetic modification of the fiber gene is the most well-defined and effective method of generating targeted vectors. The most straightforward approach, comprising the addition of targeting epitopes to the C-terminus of fiber, has been applied successfully but is limited to linear peptides of ~20 to 25 residues [16–19]. Another approach is to incorporate inserts into the HI loop of the fiber knob [20–22]. This site tolerates introduction of epitopes larger than 100 residues without substantially affecting

propagation and infectivity of the resulting AdV [23]. However, insertion of complexly folded and consequently more selective ligands appears to disturb trimerization of the fiber and prevent subsequent incorporation of fiber into the adenovirus capsid.

To circumvent these constraints and broaden the range of targeting epitopes, chimeric spike molecules have been developed in which the fiber knob domain alone or in combination with the fiber shaft domain has been replaced with an exogenous trimerization domain and a receptor-binding moiety [24–26]. In several cases, these artificial spike molecules trimerized efficiently and conferred new tropism to the AdV. Although these studies support the feasibility of this strategy, the applicability of this approach is limited by the impaired propagation efficiency of these vectors, which requires complementation with wild-type fiber or reintroduction of the fiber gene in the AdV genome for efficient vector production [24,26–28].

To construct targeted AdV's that do not require wild-type fiber complementation, we explored whether the reovirus attachment protein, $\sigma 1$, could serve as an alternative basis for an artificial AdV attachment protein. Reovirus $\sigma 1$ trimerizes efficiently and shows remarkable structural and functional similarities with the adenovirus fiber [29]. The $\sigma 1$ crystal structure reveals a fibrous tail and globular head, which closely resemble the structure formed by the fiber shaft and knob domains, respectively. In addition, $\sigma 1$ and fiber are similarly organized in the localization of several functional regions (Fig. 1). Notably, however, the two molecules differ in the location of their trimerization-determining region. In fiber, this region colocalizes with the main tropism-determining region to

the knob domain, whereas in $\sigma 1$ the trimerization and tropism-determining regions are localized to separate domains, i.e., the T(ii) and the head domain, respectively. This physical separation of functional regions over different structural domains suggests that native reovirus tropism, which is defined mainly by an interaction with the junction adhesion molecule-A (JAM-A), can be ablated by deletion of the head domain without affecting trimerization [30]. In support of this contention, replacement of the 334 C-terminal residues of $\sigma 1$ with the 291-residue chloramphenicol acetyltransferase (CAT) protein resulted in a fusion protein that trimerized efficiently and was incorporated into the reovirus capsid [31–33]. CAT enzymatic activity was preserved, suggesting that the fusion did not impose constraints on proper folding of the enzyme.

In this study, we report a new platform for genetically targeted AdV's that is based on replacement of the fiber molecule by an artificial fusion protein containing the T(ii) domain of reovirus $\sigma 1$. This molecule lacks CAR- and HSG-binding-sites to diminish native AdV tropism and provides target specificity through an incorporated prototype binding moiety. Introduction of sequences encoding this fusion molecule into the AdV genome allowed efficient propagation of the vector and resulted in high-titer vector production. The infection profile of the genetically targeted AdV was defined mainly by the incorporated $\sigma 1$ -based fusion molecule. Therefore, this new strategy of adenovirus targeting is a promising prelude to the generation of a truly targeted vector that lacks the promiscuous native AdV tropism and is equipped with a therapeutically relevant and specific binding affinity.

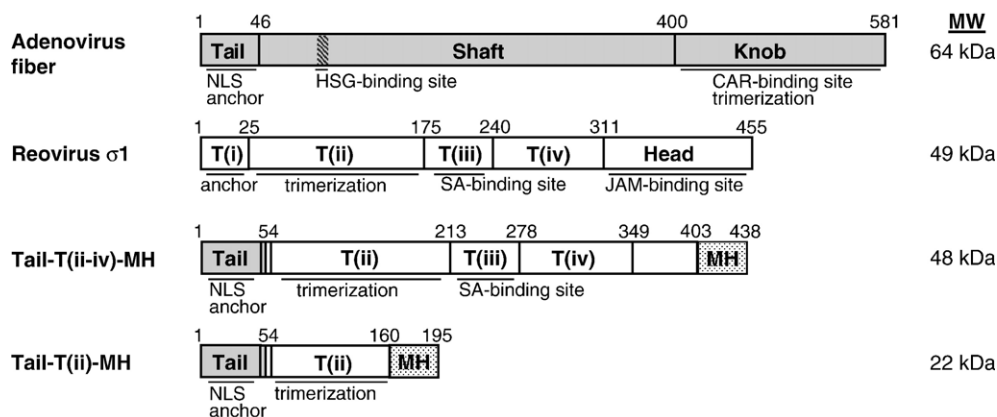


FIG. 1. Schematic representation of adenovirus fiber (serotype 5), reovirus $\sigma 1$ (type 3 Dearing), and the fiber- $\sigma 1$ fusion proteins Tail-T(ii-iv)-MH and Tail-T(ii)-MH. The fiber molecule contains three regions: the N-terminal tail domain, the shaft domain, and the C-terminal knob domain. The $\sigma 1$ molecule contains five regions: the T(i), T(ii), T(iii), and T(iv) domains, which form the fibrous tail, and the C-terminal head domain. The fiber- $\sigma 1$ fusion protein Tail-T(ii-iv)-MH consists mainly of $\sigma 1$ domains, but lacks the T(i) domain, which is replaced by the adenovirus tail domain. In addition, this fusion lacks most of the head domain, including the JAM-A binding site, which is replaced by Myc and His tags (MH). The fiber- $\sigma 1$ fusion protein Tail-T(ii)-MH is similar to Tail-T(ii-iv)-MH, but the contribution of $\sigma 1$ is limited to the major part of the T(ii) domain. The numbers of relevant amino acids and the locations of functional regions are indicated. The predicted molecular weights (MW) are shown in kilodaltons. NLS, nuclear localization signal; SA, sialic acid.

RESULTS

Design of $\sigma 1$ Fusion Proteins

The success of genetically targeted AdV's relies on development of fiber-like molecules that are ablated for native binding and can incorporate large and complex ligands without loss of trimeric quaternary structure. The capacity of the reovirus $\sigma 1$ protein to tolerate extensive modifications prompted us to design two different fusion proteins comprising key $\sigma 1$ domains (Fig. 1). The first $\sigma 1$ fusion protein, designated Tail-T(ii-iv)-MH, consists of the N-terminal 54 residues (tail domain) of fiber and the T(ii), T(iii), and T(iv) domains of $\sigma 1$. The fiber tail domain mediates transport of fiber into the nucleus and incorporation of the molecule into the adenovirus capsid. We reasoned that the $\sigma 1$ domains included in Tail-T(ii-iv)-MH would facilitate trimerization but lack interactions with reovirus receptor JAM-A. A second fusion protein, designated Tail-T(ii)-MH, differs from Tail-T(ii-iv)-MH in that it lacks the sialic acid-binding T(iii) domain and the T(iv) domain. Thus, this construct is incapable of binding to all known reovirus receptors.

To redirect the $\sigma 1$ -fusion proteins to a specific model receptor, we introduced six consecutive histidine residues (H) at the fusion protein C-terminus. The targeting peptide binds selectively to an artificial model receptor, consisting of an anti-His single-chain antibody linked to

the transmembrane domain of the platelet-derived growth factor receptor (HissFv.rec). Introduction of HissFv.rec into 293 cells (293.HissFv.rec) or CHO cells (CHO- α His) results in surface expression of the receptor [34,35]. We also introduced a Myc-epitope tag (M) adjacent to the His tag to facilitate detection of the fusion proteins.

Functional Characterization of the $\sigma 1$ Fusion Proteins

To enable functional characterization of the fusion attachment proteins, we introduced plasmids encoding Tail-T(ii-iv)-MH and Tail-T(ii)-MH into 293T cells by transient transfection. Following 48 h incubation to allow protein expression, we prepared cell lysates and subjected them to immunoblotting (Fig. 2A). Under denaturing conditions, both fusion proteins appeared as a single species of the expected size, ~48 kDa for Tail-T(ii-iv)-MH and ~22 kDa for Tail-T(ii)-MH. Under nondenaturing conditions, Tail-T(ii-iv)-MH migrates as an oligomer, similar to the adenovirus fiber. However, the apparent molecular weight of Tail-T(ii-iv)-MH was larger than expected for a homotrimer. This finding is analogous to the slower migration profile of trimerized fiber, which exhibits a larger apparent molecular weight as result of partial unfolding of the N-terminus [36]. Apart from a partially unfolded N-terminus, the slower migra-

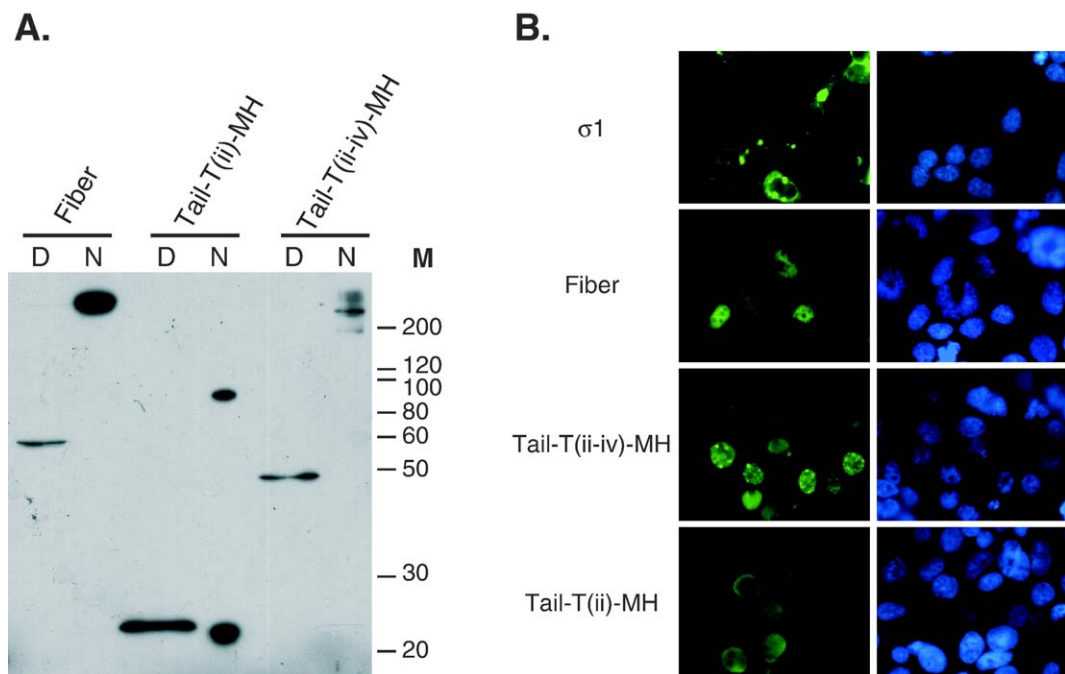


FIG. 2. Analysis of trimerization efficiency and nuclear localization of the fiber- $\sigma 1$ fusion proteins. (A) Native (N) and denatured (D) cell lysates of 293T cells transfected with plasmids encoding fiber, Tail-T(ii)-MH, or Tail-T(ii-iv)-MH were resolved by SDS-PAGE and analyzed by immunoblotting using fiber tail-specific MAb Ab4. Molecular weight markers (M) are indicated in kilodaltons. (B) Immunofluorescence of 293T cells transfected with plasmids encoding $\sigma 1$, fiber, Tail-T(ii-iv)-MH, and Tail-T(ii)-MH. The left shows protein staining detected by using the $\sigma 1$ head-specific MAb 9BG5 for reovirus $\sigma 1$ and MAb Ab4 for the other proteins. The right shows nuclear staining of the same cells detected by using Hoechst 33342.

tion observed for Tail-T(ii-iv)-MH might also be explained by partial unfolding of the C-terminus, as a consequence of the absence of a minor trimerization domain located in the $\sigma 1$ head [37]. In contrast to Tail-T(ii-iv)-MH, Tail-T(ii)-MH did not form oligomers as efficiently as fiber. Although a distinct fraction of the protein migrated as an oligomer, most of the expressed protein was found in monomeric form using nondenaturing conditions. Similar to fiber and Tail-T(ii-iv)-MH, the multimeric Tail-T(ii)-MH is presumed to be trimeric, with the larger apparent molecular weight perhaps due to a partially unfolded tail domain.

To determine the intracellular distribution of Tail-T(ii-iv)-MH and Tail-T(ii)-MH, we transfected 293T cells with fusion protein-encoding plasmids and imaged them 48 h after transfection by immunofluorescence microscopy (Fig. 2B). As anticipated, the parental $\sigma 1$ and fiber proteins were detected in the cytoplasm and nucleus of transfected cells, respectively, in accordance with the intracellular compartments accommodating reovirus and adenovirus assembly. Both Tail-T(ii)-MH and Tail-T(ii-iv)-MH were found predominantly in the nucleus, which confirms that the nuclear localization signal residing in the fiber tail domain directed import of the fusion proteins into the nuclear compartment.

Generation and Propagation of the Genetically Targeted AdV's

To investigate whether the fusion proteins are incorporated into adenovirus particles and yield AdV with newly directed tropism, we replaced the fiber gene with sequences encoding either Tail-T(ii-iv)-MH or Tail-T(ii)-MH in the genome of an AdV engineered to express green fluorescent protein (GFP) and luciferase marker genes in the E1 region. We transfected the resulting vectors, pAdG.L.Tail-T(ii-iv)-MH and pAdG.L.Tail-T(ii)-MH, into 293HissFv.rec cells [35] to generate AdG.L.Tail-T(ii-iv)-MH and AdG.L.Tail-T(ii)-MH, respectively. Despite numerous attempts, AdG.L.Tail-T(ii-iv)-MH could not be rescued. In contrast, AdG.L.Tail-T(ii)-MH was easily generated and efficiently propagated using 293HissFv.rec cells.

To characterize the propagation efficiency of AdG.L.-Tail-T(ii)-MH in comparison to AdG.L., which expresses wild-type fiber, we infected 293HissFv.rec cells at low m.o.i. and monitored virus replication for 10 days by measuring luciferase expression (Fig. 3). During the observation interval, AdG.L. and AdG.L.Tail-T(ii)-MH produced similar luciferase expression profiles, suggesting similar propagation efficiencies. In addition, three independent CsCl-purified preparations of AdG.L. and AdG.L.Tail-T(ii)-MH yielded similar quantities of viral particles (vp) (10^{12} – 10^{13} vp/20 T182 flasks). However, the ratio of virus particles to infectious units (IU) determined on 293HissFv.rec cells was approximately 70-fold higher for AdG.L.Tail-T(ii)-MH than for AdG.L. (5380 ± 1795

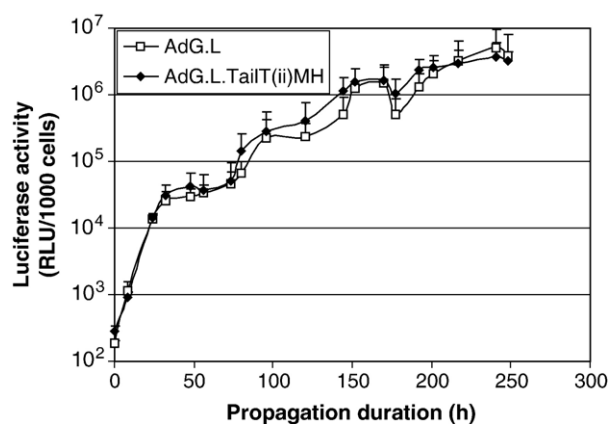


FIG. 3. Propagation efficiency of AdG.L. and AdG.L.Tail-T(ii)-MH following infection of 293.HissFv.rec cells. Cells were infected with either AdG.L. or AdG.L.Tail-T(ii)-MH at an m.o.i. of 0.004 IU/cell. At the indicated times after infection, luciferase expression was assessed as an indicator of AdV propagation. The results are expressed as the average values of an experiment performed in triplicate. Error bars indicate standard deviations.

SEM vs 77 ± 66 SEM), suggesting that the infectivity of AdG.L.Tail-T(ii)-MH on 293HissFv.rec cells is inferior to that of AdG.L.

Characterization of AdG.L.Tail-T(ii)-MH Virions

To assess the capsid composition of AdG.L.Tail-T(ii)-MH particles, we used SDS-PAGE to resolve AdG.L.Tail-T(ii)-MH structural proteins. CsCl-purified preparations of AdG.L.Tail-T(ii)-MH and Ad $\Delta 24$, a control vector expressing wild-type proteins, showed similar compositions of capsid proteins (Fig. 4A). However, AdG.L.Tail-T(ii)-MH contained an additional band, which likely represents the 22-kDa Tail-T(ii)-MH fusion protein. Since protein IIIa and fiber show similar migration properties using these gel conditions, the absence of fiber in AdG.L.Tail-T(ii)-MH could not be confirmed using this assay. Consequently, we investigated the incorporation of Tail-T(ii)-MH into virus particles further by immunoblotting using a fiber-specific monoclonal antibody (MAb) (Fig. 4B). A MAb specific for the fiber tail detected the 64-kDa wild-type fiber on control vector AdG.L. particles and the 22-kDa Tail-T(ii)-MH fusion molecule on AdG.L.Tail-T(ii)-MH particles. Only the Tail-T(ii)-MH fusion protein was detected using a Myc-specific MAb, confirming that Tail-T(ii)-MH is efficiently and exclusively incorporated into the genetically modified AdV. Notably, the band corresponding to Tail-T(ii)-MH was less intense than the band corresponding to fiber, which suggests somewhat less efficient encapsidation of the fusion protein relative to that of native fiber.

Infectivity of AdG.L.Tail-T(ii)-MH

To assess the effect of removal of the fiber knob and shaft domains on the infectivity of the newly derived genetically targeted AdV, we compared the infectivity of

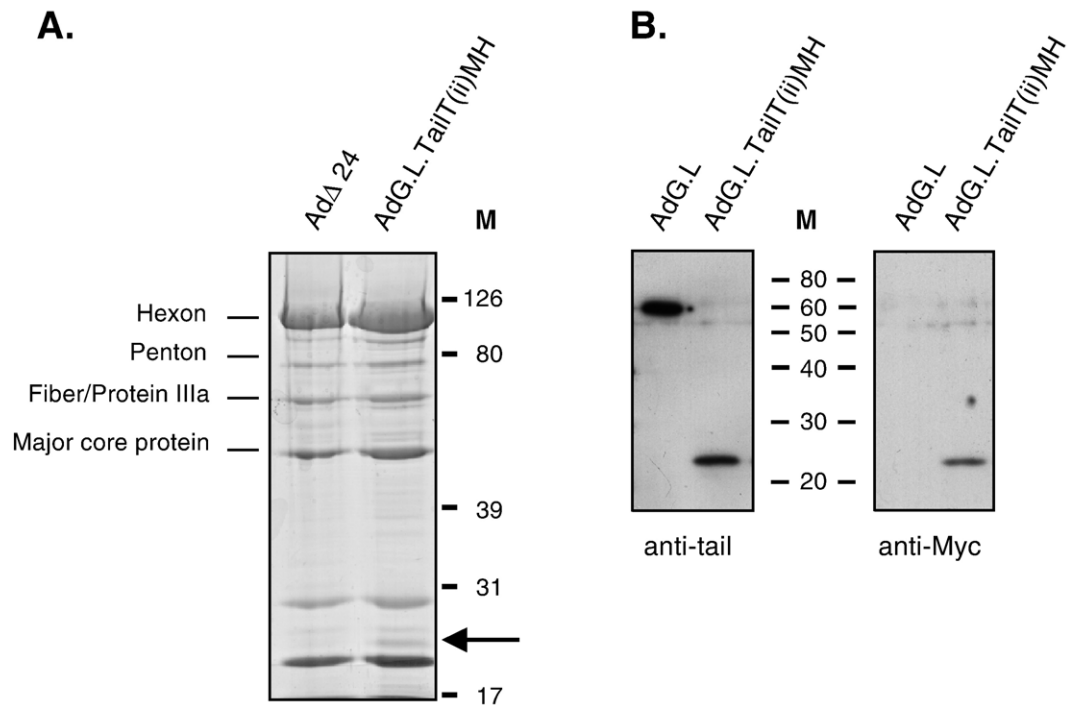


FIG. 4. Incorporation of Tail-T(ii)-MH into the adenovirus capsid. CsCl-purified particles of AdG.L.Tail-T(ii)-MH or a wild-type fiber-containing adenovirus were denatured and resolved by SDS-PAGE. (A) Capsid proteins of 1.2×10^{11} particles were visualized by staining with Coomassie blue. The arrow indicates the location of the Tail-T(ii)-MH fusion protein. (B) Purified particles (5×10^9) were resolved by SDS-PAGE and transferred to PVDF membranes. Blots were incubated with either tail-specific MAb (left) or Myc-specific MAb (right), and protein bands were visualized using ECL Plus. Molecular weights in kilodaltons of marker proteins are indicated (M).

AdG.L.Tail-T(ii)-MH to that of control vector AdG.L following adsorption of 293 cells and 293HissFv.rec cells using both GFP expression (Fig. 5A) and assessment of

luciferase activity (Fig. 5B). Transduction efficiency of AdG.L.Tail-T(ii)-MH after infection of 293HissFv.rec cells was clearly enhanced in comparison to that following

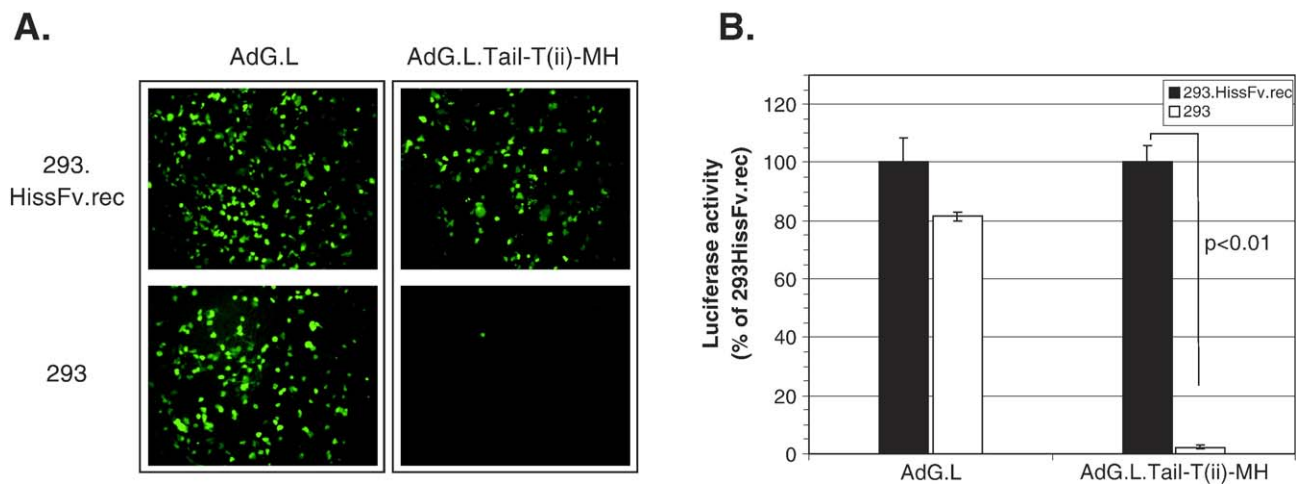


FIG. 5. Detargeting effect of AdG.L.Tail-T(ii)-MH. Infection efficiency of AdG.L.Tail-T(ii)-MH was analyzed using the nontarget cell line 293 and the target cell line 293.HissFv.rec. Both cell lines were infected with either AdG.L or AdG.L.Tail-T(ii)-MH at an m.o.i. of 0.5 IU/cell. Following 48 h incubation, transduction efficiency was evaluated by (A) analysis of GFP expression using fluorescence microscopy and (B) measurement of luciferase expression using a chemiluminescence assay. The averaged luciferase activity of three independent experiments is presented as the percentage of the activity found after infection of 293HissFv.rec cells. Error bars indicate standard deviations.

infection of 293 cells, while the transduction efficiency of AdG.L was similar after infection of both cell lines (Fig. 5A). Quantitation of this effect using luciferase expression showed that transduction efficiency by AdG.L.Tail-T(ii)-MH was about 40-fold greater after infection of 293.HissFv.rec cells than after infection of 293 cells. Importantly, upon infection of 293 cells the detargeting effect of AdG.L.Tail-T(ii)-MH resulted in a transduction efficiency that was at least 35-fold lower than that of the AdG.L control vector.

To confirm that transduction by the genetically targeted and control vectors was dependent on receptor-binding activities attributable to the respective attachment proteins we incubated both AdV's with either anti-knob Ab or anti-His-tag Ab prior to inoculation of 293.HissFv.rec or 293 cells (Fig. 6A). Anti-knob Ab

diminished transduction of both 293.HissFv.rec and 293 cells by AdG.L. In sharp contrast, anti-knob Ab had no effect on transduction of 293.HissFv.rec cells by AdG.L.-Tail-T(ii)-MH. Conversely, anti-His-tag Ab did not affect transduction by AdG.L after infection of either cell type, but this Ab reduced AdG.L.Tail-T(ii)-MH transduction of 293.HissFv.rec cells by ~90%. In addition, we analyzed the infectivity of both AdV's on CHO and CHO- α His cells, which lack CAR expression (Fig. 6B). As expected, transduction efficiency of AdG.L was similarly low on both cell lines. In contrast, AdG.L.Tail-T(ii)-MH exhibited a 12-fold increased transduction efficiency on CHO- α His cells in comparison to CHO cells. Moreover, AdG.L.Tail-T(ii)-MH transduced the CHO- α His cells significantly better than AdG.L. Together, these findings demonstrate that transduction by AdG.L.Tail-T(ii)-MH is principally defined by the Tail-T(ii)-MH protein and the artificial His-tag binding receptor.

DISCUSSION

In this study, we developed a genetically targeted AdV by replacing the adenovirus fiber molecule with Tail-T(ii)-MH, a newly designed σ 1-based chimeric attachment protein. This novel attachment protein contains the N-terminal tail domain of fiber fused to the T(ii) domain of reovirus σ 1 for proper trimerization, a Myc-epitope tag to facilitate detection, and a C-terminal His tag as an artificial model receptor-binding moiety. Thus, it lacks all known receptor-binding sites in adenovirus fiber and reovirus σ 1. Tail-T(ii)-MH localizes to the nucleus and forms trimers, although trimer formation is less efficient than that observed for wild-type fiber. Introduction of Tail-T(ii)-MH-encoding sequences into the adenovirus genome in place of the fiber gene resulted in the genetically targeted AdV AdG.L.Tail-T(ii)-MH. This AdV efficiently encapsidated Tail-T(ii)-MH and was easily propagated on 293.HissFv.rec cells.

Attempts failed to generate a similar virus, AdG.L.Tail-T(ii-iv)-MH, encoding a chimeric attachment protein additionally comprising σ 1 domains T(iii) and T(iv). Tail-T(ii-iv)-MH protein trimerized and translocated to the nucleus efficiently. Moreover, transient expression of Tail-T(ii-iv)-MH in 293T cells infected with a fiberless AdV yielded AdV with a His-receptor-targeted transduction profile (data not shown). Thus, Tail-T(ii-iv)-MH was fully functional as an AdV targeting molecule. It remains, therefore, unresolved why AdG.L.Tail-T(ii-iv)-MH could not be made. Perhaps the T(iii)-T(iv) fragment comprises a domain with negative impact on the adenovirus life cycle when expressed from the viral genome. Alternatively, the T(iii)-T(iv) encoding DNA region could have a destabilizing effect on the viral genome.

Use of a σ 1-based attachment protein as an AdV targeting platform confirms recent reports that used σ 1 to confer reovirus tropism to an AdV [38,39]. Notably, the

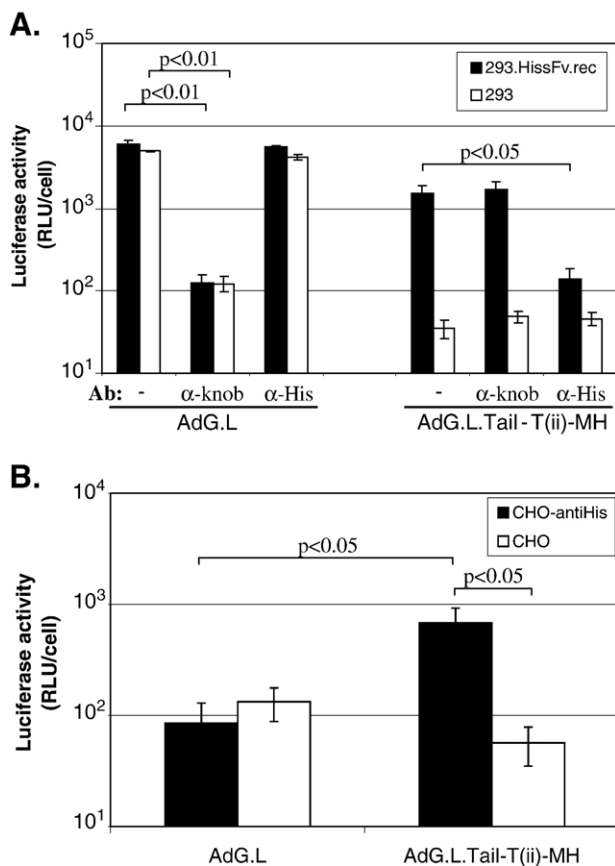


FIG. 6. Analysis of the infection specificity of AdG.L.Tail-T(ii)-MH. (A) AdG.L and AdG.L.Tail-T(ii)-MH were incubated in the presence or absence of 300 ng of knob-specific Ab or His-specific Ab at room temperature for 2 h prior to infection of either 293 or 293.HissFv.rec cells at an m.o.i. of 0.5 IU/cell. (B) The cell lines CHO and CHO- α His were infected with AdG.L or AdG.L.Tail-T(ii)-MH at an m.o.i. of 5 IU/cell. Following 48 h incubation, transduction efficiency was assessed by luciferase expression. The results are expressed as the average luciferase activity for three experiments. Error bars indicate standard deviations.

AdV's with reovirus tropism were produced in the presence of complementing fiber, whereas AdG.L.Tail-T(ii)-MH was efficiently produced in the absence of complementing fiber. In addition, the deletion of all known receptor-binding sites in $\sigma 1$ and addition of a model receptor-binding moiety, as is accomplished in Tail-T(ii)-MH, extend the applicability of this $\sigma 1$ -based attachment protein from broad range reovirus tropism to specific targeting toward receptors of choice.

The targeting profile of the generated AdG.L.Tail-T(ii)-MH is similar to that of another genetically targeted AdV, Ad5LucFF/6H, which contains an Ad5fiber-T4fibritin chimera with a His tag at the C-terminus [26]. However, AdG.L.Tail-T(ii)-MH displays a major advantage in propagation efficiency. The propagation of Ad5LucFF/6H and all other previously described genetically targeted AdV's that express knobless fibers with foreign trimerization domains was either impaired or required complementation with wild-type fiber [24–26,28]. In contrast to these AdV's, propagation of the genetically targeted AdV expressing Tail-T(ii)-MH was efficient and resembled that of a control vector expressing wild-type fiber. These features allow the generation of high-titer and well-characterized virus preparations, which will simplify the production of genetically targeted AdV's under GMP conditions, a prerequisite for clinical application. In addition, propagation of this AdV without the requirement for complementation with fiber avoids the risk of reintroducing the fiber gene into the adenovirus genome through recombination. In fact, we have observed such a recombination event during propagation of an AdV with the knobless fiber molecule TSFLCmychis [24] using wild-type fiber-complementing 211B cells [40] (unpublished data). Furthermore, the high propagation efficiency of AdG.L.Tail-T(ii)-MH also makes this type of targeting feasible for implementation in conditionally replicating AdV's (CRAds), which are promising anti-cancer agents due to their tumor-selective replication and cell killing. Since many primary cancer cells express only low levels of CAR, a CRAd engineered with a well-defined tumor-targeting attachment protein may lead to improved efficacy for this type of therapy.

Although the propagation of AdG.L.Tail-T(ii)-MH was efficient and led to high yields of virus particles, its infectivity on 293HissFv.rec cells was less than that of control vector AdG.L. It is possible that the reduced trimerization efficiency of Tail-T(ii)-MH in comparison to wild-type fiber and the less than complete incorporation of the chimeric attachment molecule onto the adenovirus capsid resulted in diminished infectivity. Alternatively, there may be differences in the efficiency of viral entry via engagement of CAR versus the artificial His-tag binding receptor, since differences in receptor density, affinity, and internalization efficiency all may affect infectivity. We are currently performing experiments to

optimize Tail-T(ii)-MH trimerization and encapsidation onto particles to distinguish between these possibilities.

Although the evaluation of the presented AdV targeting approach using a $\sigma 1$ -based attachment protein is limited to the introduction of a relative short linear peptide, we think it possible that larger and more complexly folded ligands can be incorporated into this attachment molecule. In a previous study, chloramphenicol acetyltransferase was fused to the T(i)–T(ii) region of $\sigma 1$, yielding a protein that forms trimers, incorporates onto the reovirus particle, and maintains enzymatic activity [33,38]. Although this finding suggests that the $\sigma 1$ -based trimerization scaffold allows considerable flexibility in targeting options, the assembly of adenovirus particles in the nucleus poses important limitations. Consequently, the choice of targeting moieties applicable for use in genetically targeted AdV's excludes ligands that require posttranslational modifications that would preclude nuclear import.

The approach reported herein also could be extended to abolishing fully native adenovirus tropism by ablating the integrin-binding site residing on the penton base [13]. This modification would remove the last known adenovirus receptor-interaction site and should lead to an AdV that does not actively attach to nontargeted cells. Possibly, this might reduce the limited residual transduction efficiency of AdG.L.Tail-T(ii)-MH on 293 cells even further. In combination with a target-selective binding moiety, this modification might bring the generation of a truly targeted AdV within reach. Obviously, this implies that generated AdV's could not be propagated using generally employed packaging cells, i.e., 293 and 911 cells. As illustrated in this study, the simple addition of the physiologically irrelevant His tag to the attachment molecule and subsequent propagation using 293.HissFv.rec cells easily circumvented this problem. Thus, the chimeric attachment protein based on the tail domain of fiber and the trimerization domain of $\sigma 1$ provides a promising new approach for AdV targeting, which opens new avenues for AdV-based gene delivery.

MATERIALS AND METHODS

Cell lines. The Ad5 E1-transformed human embryonic kidney cell line 293 and the Chinese hamster ovary cell line CHO were purchased from the American Type Culture Collection. Human 293T cells were derived from 293 cells by stable transfection with an SV40 large-T antigen expression plasmid [41]. The cell line 293.HissFv.rec, another derivative of 293 that stably expresses an artificial His-tag binding receptor [35], was kindly provided by Dr. J. T. Douglas (University of Alabama, Birmingham, AL, USA). The cell line CHO- α His, a derivative of CHO that stably expresses an artificial His-tag-binding receptor [34], was kindly provided by Dr. T. Nakamura (Mayo Clinic College of Medicine, Rochester, MN, USA). These five cell lines were maintained in F12-supplemented Dulbecco's modified Eagle medium supplemented to contain 10% FCS and antibiotics (Gibco BRL, Life Technologies B.V., Breda, The Netherlands). Medium used for

293T, 293.HissFv.rec, and CHO- α His cells was supplemented to contain 300 μ g/ml G418.

Construction of expression plasmids and adenoviral vectors. The Ad5 fiber expression construct pCMV.tpl.Fiber was generated using PCR. First, the Ad5 fiber gene was amplified using primers that flank the fiber-encoding sequence. The resulting 1.8-kb PCR product was blunted and cloned into *EcoRV*-digested pcDNA3 (Invitrogen, San Diego, CA, USA) generating pCMV.Fiber. The tripartite leader (tpl) was amplified from pMad5 [42] using the primers 5'-CTCGAATTCACCTCTCTCCGCATCGCTG-3' and 5'-CAGGAATCTTGGCAGCTGTGACTGGTTAG-3'. The resulting 203-bp PCR fragment was digested with *EcoRI* (underlined) and inserted into the unique *EcoRI* site of pCMV.Fiber between the cytomegalovirus promoter (CMV) and the fiber-encoding sequence. The Tail-T(ii-iv)-MH expression vector pCMV.tpl.Adtail- σ 1 Δ Head-MH was generated by isolating the Tail-T(ii-iv)-encoding sequence from the expression construct pCMV.tpl.Adtail-Sigma1 (Schagen *et al.*, manuscript in preparation) by digestion with *BglII*, Klenow fill-in of the sticky ends, and digestion with *MfeI*. The resulting 2.2-kb fragment was inserted into the 4.7-kb backbone of pCMV-(B⁻)-TSFLC-MycHis, containing a blunted *BspEI* end and a sticky *MfeI* end. The backbone pCMV-(B⁻)-TSFLC-MycHis plasmid was generated by digestion of pCMV-TSFLC [24] with *EcoRV* and *KpnI*, recircularization, and subsequent digestion with *BamHI* and *XbaI* for insertion of a *BamHI*- and *XbaI*-digested, 113-bp PCR fragment. This fragment was amplified from pcDNA3.1(-)/Myc-His/LacZ (Invitrogen) using the primers 5'-GCGAAATGGATTTTGCATCGAGCT-3' and 5'-GGCTCTAGACATATGTTTATTAATGATGATGATGATGGTCGACGG-3' and contained Myc and His tags and an *NdeI* (underlined) restriction site directly following the polyadenylation signal. The Tail-T(ii)-MH-encoding construct pCMV.tpl.Adtail- σ 1T(ii)-MH was generated by digesting pCMV.tpl.Adtail-Sigma1 with *BclI*, Klenow fill-in, and redigestion with *MfeI*. The Tail-T(ii)-encoding 1.5-kb fragment was inserted between the blunted *BspEI* end and sticky *MfeI* end of the 4.7-kb backbone of pCMV-(B⁻)-TSFLC-MycHis. Sequence of the inserts was confirmed by automated sequencing.

The expression constructs pCMV.tpl.Adtail- σ 1 Δ Head-MH and pCMV.tpl.Adtail- σ 1T(ii)-MH were used as donor plasmids for the generation of pAdG.L.Tail-T(ii-iv)-MH and pAdG.L.Tail-T(ii)-MH. The fusion molecule-encoding sequences were released from the donor plasmids with *NdeI* and cloned into *NdeI*-linearized pBr/Ad.BamRAFIIB [43]. The resulting constructs were used to introduce Tail-T(ii-iv)-MH and Tail-T(ii)-MH via recombination into pAdEasy-1 [44], which generated pAdEasy.Adtail- σ 1 Δ Head-MH and pAdEasy.Adtail- σ 1T(ii)-MH, respectively. Both constructs were recombined with pAdTrack.CMV.Luc, which was constructed by digestion of pABS.4-CMV-Luc [45] with *XbaI* and *SwaI*, isolation of the luciferase-encoding fragment, and insertion into the *XbaI*- and *EcoRV*-digested pAdTrack-CMV [44]. The recombination generated the full-length genome of the AdV's AdG.L.Tail-T(ii-iv)-MH and AdG.L.Tail-T(ii)-MH. These vectors contain GFP and luciferase reporter genes in place of the E1 region and the Tail-T(ii-iv)-MH- or Tail-T(ii)-MH-encoding sequences in place of the fiber gene. Control vector AdG.L was obtained by recombination of pAdEasy-1 and pAdTrack.CMV.Luc.

Analysis of recombinant spike and fiber proteins. Expression constructs pCMV.tpl.Fiber, pCMV.tpl.Adtail- σ 1 Δ Head-MH, and pCMV.tpl.Adtail- σ 1T(ii)-MH were introduced into 293T cells using Lipofectamine Plus (Invitrogen Life Technologies, Breda, The Netherlands) according to the manufacturer's instructions. For analysis of protein expression and capacity to form oligomers, transfected cells were lysed using reporter lysis buffer (Promega, Madison, WI, USA), and lysates were either incubated at 95°C for 5 min in denaturing sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 2.5% β -mercaptoethanol) or kept on ice in native sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.1% SDS). On the basis of protein content, 1 μ g of total cell lysate was used for the native Tail-T(ii-iv)-MH sample and 10 μ g was used for the denaturing sample. In the case of Tail-T(ii)-MH, 3 μ g of total cell lysate was used for both samples, and in the case of fiber, 50 μ g of cell lysate was used for both samples. Samples were resolved by SDS-10%

PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Recombinant proteins were detected using the fiber tail-specific MAb Ab4 (Neomarkers, Fremont, CA, USA) and visualized using chemiluminescence following incubation of the membranes with rabbit anti-mouse immunoglobulin G conjugated to horseradish peroxidase (R α M HRP; Dako, Glostrup, Denmark) and Lumilight^{plus} (Roche, Almere, The Netherlands).

For analysis of cellular localization of the recombinant proteins, cells were fixed with methanol:acetone (1:1) 48 h after transfection. Fiber and fusion proteins were detected using MAb Ab4, and σ 1 was detected using σ 1 head-specific MAb 9BG5 [46]. Fluorescein-isothiocyanate-labeled rabbit anti-mouse immunoglobulin G (R α M-FITC; Dako) was used as the secondary antibody. Nuclear DNA was stained using 1.2 ng/ μ l Hoechst 33342 (Sigma, St. Louis, MO, USA).

Propagation and analysis of AdV's. The *PacI*-linearized adenovirus genomes pAdG.L.Tail-T(ii-iv)-MH and pAdG.L.Tail-T(ii)-MH were transfected into 293.HissFv.rec cells using Lipofectamine Plus (Invitrogen Life Technologies) according to the manufacturer's instructions. Virus progeny was propagated up to the scale of 20 T182 flasks using 293.HissFv.rec cells. Generation and propagation of the control vector AdG.L were facilitated using 293 cells. The final virus harvests were purified by two successive rounds of CsCl centrifugation, dialyzed against 10 mM Hepes, pH 7.4, 10% glycerol, and 1 mM MgCl₂, and stored in 50- to 100- μ l aliquots at -80°C. The virus particle yield was determined by OD₂₆₀ following denaturation of the virus in PBS, 1% SDS, and 1 mM EDTA (pH 8.0) at 55°C. The infectious unit titer was determined by serial dilution of the virus stock and infection of 293.HissFv.rec cells. Following 48 h incubation, infected cells were visualized using the Adeno-X rapid titer kit (BD Biosciences, Palo Alto, CA, USA).

To examine the propagation efficiency of AdG.L and AdG.L.Tail-T(ii)-MH, 293.HissFv.rec cells were seeded at a density of 5×10^4 cells/well in 96-well plates and infected at an m.o.i. of 0.004 IU/cell. At various intervals, cells were lysed using 50 μ l reporter lysis buffer (Promega), and luciferase activity was measured by chemiluminescence (Promega) using a Berthold luminometer (Berthold, Bad Wildbad, Germany).

To determine whether the Tail-T(ii)-MH attachment protein was incorporated onto the adenovirus capsid, CsCl-purified virions were incubated at 95°C for 5 min in denaturing sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 2.5% β -mercaptoethanol) and resolved by SDS-10% PAGE. Viral proteins were either visualized using Coomassie blue staining or transferred to PVDF membranes, incubated with MAb Ab4 or mouse Myc-specific MAb 9E10 as the primary antibody, and visualized using R α M-HRP (Dako) and Lumilight^{plus} (Roche).

Infectivity of AdG.L.Tail-T(ii)-MH. One day prior to infection, 293 and 293.HissFv.rec cells were seeded at a density of 5×10^4 cells/well and CHO and CHO- α His cells at a density of 2×10^4 cells/well in 96-well plates. AdG.L.Tail-T(ii)-MH and AdG.L were incubated in the presence or absence of 300 ng anti-knob antibody (1D6.14) [47] or anti-His antibody (penta-His Ab; Qiagen, Hilden, Germany) at room temperature for 2 h. Preincubated mixtures were added to cells and replaced 2 h later with fresh medium. After 48 h incubation, GFP expression was assessed using fluorescence microscopy. After analysis of GFP expression, cells were lysed, and luciferase activity was determined.

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REFERENCES

- Law, L. K., and Davidson, B. L. (2005). What does it take to bind CAR? *Mol. Ther.* **12**: 599–609.
- Kirby, I., et al. (2000). Identification of contact residues and definition of the CAR-binding site of adenovirus type 5 fiber protein. *J. Virol.* **74**: 2804–2813.
- Roelvink, P. W., Mi Lee, G., Einfeld, D. A., Kovessi, I., and Wickham, T. J. (1999). Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science* **286**: 1568–1571.
- Mathias, P., Wickham, T., Moore, M., and Nemerow, G. (1994). Multiple adenovirus serotypes use alpha v integrins for infection. *J. Virol.* **68**: 6811–6814.
- Li, E., et al. (2001). Integrin alpha(v)beta1 is an adenovirus coreceptor. *J. Virol.* **75**: 5405–5409.
- Wickham, T. J., Filardo, E. J., Cheresch, D. A., and Nemerow, G. R. (1994). Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. *J. Cell Biol.* **127**: 257–264.
- Wickham, T. J., Mathias, P., Cheresch, D. A., and Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* **73**: 309–319.
- Dechecchi, M. C., et al. (2001). Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. *J. Virol.* **75**: 8772–8780.
- Dechecchi, M. C., Tamanini, A., Bonizzato, A., and Cabrini, G. (2000). Heparan sulfate glycosaminoglycans are involved in adenovirus type 5 and 2-host cell interactions. *Virology* **268**: 382–390.
- Koizumi, N., et al. (2003). Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. *J. Virol.* **77**: 13062–13072.
- Smith, T. A., et al. (2003). Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice. *Hum. Gene Ther.* **14**: 777–787.
- Nicklin, S. A., Wu, E., Nemerow, G. R., and Baker, A. H. (2005). The influence of adenovirus fiber structure and function on vector development for gene therapy. *Mol. Ther.* **12**: 384–393.
- Einfeld, D. A., et al. (2001). Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. *J. Virol.* **75**: 11284–11291.
- Krasnykh, V. N., Douglas, J. T., and van Beusechem, V. W. (2000). Genetic targeting of adenoviral vectors. *Mol. Ther.* **1**: 391–405.
- Barnett, B. G., Crews, C. J., and Douglas, J. T. (2002). Targeted adenoviral vectors. *Biochim. Biophys. Acta* **1575**: 1–14.
- Michael, S. I., Hong, J. S., Curiel, D. T., and Engler, J. A. (1995). Addition of a short peptide ligand to the adenovirus fiber protein. *Gene Ther.* **2**: 660–668.
- Wickham, T. J., et al. (1997). Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. *J. Virol.* **71**: 8221–8229.
- Hong, J. S., and Engler, J. A. (1996). Domains required for assembly of adenovirus type 2 fiber trimers. *J. Virol.* **70**: 7071–7078.
- Wickham, T. J., Roelvink, P. W., Brough, D. E., and Kovessi, I. (1996). Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. *Nat. Biotechnol.* **14**: 1570–1573.
- Nicklin, S. A., et al. (2001). Ablating adenovirus type 5 fiber-CAR binding and HI loop insertion of the SIGYPLP peptide generate an endothelial cell-selective adenovirus. *Mol. Ther.* **4**: 534–542.
- Krasnykh, V., et al. (1998). Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J. Virol.* **72**: 1844–1852.
- Dmitriev, I., et al. (1998). An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J. Virol.* **72**: 9706–9713.
- Belousova, N., Krendelchikova, V., Curiel, D. T., and Krasnykh, V. (2002). Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein. *J. Virol.* **76**: 8621–8631.
- van Beusechem, V. W., et al. (2000). Recombinant adenovirus vectors with knobless fibers for targeted gene transfer. *Gene Ther.* **7**: 1940–1946.
- Magnusson, M. K., Hong, S. S., Boulanger, P., and Lindholm, L. (2001). Genetic retargeting of adenovirus: novel strategy employing “deknobbing” of the fiber. *J. Virol.* **75**: 7280–7289.
- Krasnykh, V., Belousova, N., Korokhov, N., Mikheeva, G., and Curiel, D. T. (2001). Genetic targeting of an adenovirus vector via replacement of the fiber protein with the phage T4 fibritin. *J. Virol.* **75**: 4176–4183.
- Pereboeva, L., Komarova, S., Mahasreshti, P. J., and Curiel, D. T. (2004). Fiber-mosaic adenovirus as a novel approach to design genetically modified adenoviral vectors. *Virus Res.* **105**: 35–46.
- Belousova, N., et al. (2003). Genetically targeted adenovirus vector directed to CD40-expressing cells. *J. Virol.* **77**: 11367–11377.
- Chappell, J. D., Prota, A. E., Dermody, T. S., and Stehle, T. (2002). Crystal structure of reovirus attachment protein sigma1 reveals evolutionary relationship to adenovirus fiber. *EMBO J.* **21**: 1–11.
- Barton, E. S., et al. (2001). Junction adhesion molecule is a receptor for reovirus. *Cell* **104**: 441–451.
- Leone, G., et al. (1991). The N-terminal heptad repeat region of reovirus cell attachment protein sigma 1 is responsible for sigma 1 oligomer stability and possesses intrinsic oligomerization function. *Virology* **182**: 336–345.
- Leone, G., Mah, D. C., and Lee, P. W. (1991). The incorporation of reovirus cell attachment protein sigma 1 into virions requires the N-terminal hydrophobic tail and the adjacent heptad repeat region. *Virology* **182**: 346–350.
- Mah, D. C., Leone, G., Jankowski, J. M., and Lee, P. W. (1990). The N-terminal quarter of reovirus cell attachment protein sigma 1 possesses intrinsic virion-anchoring function. *Virology* **179**: 95–103.
- Nakamura, T., et al. (2005). Rescue and propagation of fully retargeted oncolytic measles viruses. *Nat. Biotechnol.* **23**: 209–214.
- Douglas, J. T., et al. (1999). A system for the propagation of adenoviral vectors with genetically modified receptor specificities. *Nat. Biotechnol.* **17**: 470–475.
- Mitraki, A., et al. (1999). Unfolding studies of human adenovirus type 2 fiber trimers: evidence for a stable domain. *Eur. J. Biochem.* **264**: 599–606.
- Leone, G., Maybaum, L., and Lee, P. W. (1992). The reovirus cell attachment protein possesses two independently active trimerization domains: basis of dominant negative effects. *Cell* **71**: 479–488.
- Mercier, G. T., et al. (2004). A chimeric adenovirus vector encoding reovirus attachment protein sigma1 targets cells expressing junctional adhesion molecule 1. *Proc. Natl. Acad. Sci. USA* **101**: 6188–6193.
- Tsuruta, Y., et al. (2005). Reovirus sigma1 fiber incorporated into adenovirus serotype 5 enhances infectivity via a CAR-independent pathway. *Biochem. Biophys. Res. Commun.* **335**: 205–214.
- Von Seggern, D. J., Kehler, J., Endo, R. I., and Nemerow, G. R. (1998). Complementation of a fibre mutant adenovirus by packaging cell lines stably expressing the adenovirus type 5 fibre protein. *J. Gen. Virol.* **79**: 1461–1468.
- DuBridge, R. B., et al. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol. Cell. Biol.* **7**: 379–387.
- Toes, R. E., et al. (1997). Protective anti-tumor immunity induced by vaccination with recombinant adenoviruses encoding multiple tumor-associated cytotoxic T lymphocyte epitopes in a string-of-beads fashion. *Proc. Natl. Acad. Sci. USA* **94**: 14660–14665.
- Havenga, M. J., et al. (2001). Improved adenovirus vectors for infection of cardiovascular tissues. *J. Virol.* **75**: 3335–3342.
- He, T. C., et al. (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* **95**: 2509–2514.
- Carette, J. E., et al. (2005). Replication-dependent transgene expression from a conditionally replicating adenovirus via alternative splicing to a heterologous splice-acceptor site. *J. Gene Med.* **7**: 1053–1062.
- Burstin, S. J., Spriggs, D. R., and Fields, B. N. (1982). Evidence for functional domains on the reovirus type 3 hemagglutinin. *Virology* **117**: 146–155.
- Douglas, J. T., et al. (1996). Targeted gene delivery by tropism-modified adenoviral vectors. *Nat. Biotechnol.* **14**: 1574–1578.