Current Advances and Future Challenges in Adenoviral Vector Biology and Targeting

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Abstract: Gene delivery vectors based on Adenoviral (Ad) vectors have enormous potential for the treatment of both hereditary and acquired disease. Detailed structural analysis of the Ad virion, combined with functional studies has broadened our knowledge of the structure/function relationships between Ad vectors and host cells/tissues and substantial achievement has been made towards a thorough understanding of the biology of Ad vectors. The widespread use of Ad vectors for clinical gene therapy is compromised by their inherent immunogenicity. The generation of safer and more effective Ad vectors, targeted to the site of disease, has therefore become a great ambition in the field of Ad vector development. This review provides a synopsis of the structure/function relationships between Ad vectors and host systems and summarizes the many innovative approaches towards achieving Ad vector targeting.

Keywords: Ad biology, Ad trafficking, Ad structure.

INTRODUCTION: A RATIONALE FOR AD VECTOR TARGETING

Adenoviruses (Ad) were first discovered in 1953, isolated from cultures of human adenoid tissues [Rowe *et al.,* 1953]. Since then, over 50 different serotypes of human adenoviruses have been isolated and characterized and the family *Adenoviridae* has been shown to be comprised of numerous non-human serotypes from a variety of mammalian, avian, reptilian, amphibian, and even fish species [Davison *et al.,* 2003, Shenk, 2001]. Ad virions consist of a ~26-40 kb linear dsDNA genome encased within a non-enveloped icosahedral particle.

Adenoviruses were one of the first vector systems to be developed. The use of Ad for the expression of foreign genes (transgenes) was actually more of a discovery than an intentional development. Stocks of live adenovirus vaccines propagated in monkey cell lines were found to be contaminated with simian virus 40 (SV40). Infection of tissue culture cells with this Ad vaccine resulted in the production of the SV40 T antigen, even after removal of the SV40 virions from the Ad stocks by immunodepletion. Analysis of this adenovirus revealed that the T-antigen gene from SV40 had recombined into the E3 region of the Ad genome. This demonstrated the possibility that Ad could carry foreign genes and express them as well as demonstrating the dispensability of the E3 genes for *in vitro* replication [Roy-Chowdhury & Horwitz, 2002].

The development of adenoviral vectors has substantially progressed since this initial discovery. Much of the early development was focused on "vectoring" Ads derived from the human serotype 5 (Ad5) and serotype 2 (Ad2). Vectors based on Ad5 can mediate high levels of transduction in a wide variety of both quiescent and proliferating cells. Transgene expression from Ad5 vectors is typically transient because the Ad5 genome does not integrate into host cell chromosomes. First-generation replication deficient Ad5 vectors have been developed by the deletion of the E1 genes, necessary for expression of E2 and late genes required for Ad DNA synthesis, capsid protein expression, and viral replication. Subsequent deletions included deletion of E3 genes involved in anti-host immunity that are dispensible for replication of the virus *in vitro*. Early work on the transformation of mammalian cells led to the creation of the 293 cell line, which was transfected with sheared adenovirus-type 5 (Ad5) genomic DNA and stably expresses the E1 genes [Graham *et al.,* 1977]. 293 cells trans-complement the E1 deletion of first generation vectors, allowing efficient production of replication deficient Ad vectors. Ad vectors can be grown to extremely high titers in the 293 cell line, with burst sizes typically between 10^3-10^4 viral particles (VP) per cell and final concentrations reaching 10^{13} VP/ml, after banding by CsCl density gradient centrifugation. The deletion of the E1 and E3 regions allows ~8 kb of foreign DNA to be inserted into the Ad vector genome, permitting the expression of transgenes in mammalian cells infected with the Ad vector. For a general review of Ad vectors, see [Volpers & Kochanek, 2004].

Although the first-generation Ad vectors are generally considered replication defective, there is some low level expression of viral antigens that limits the duration of transgene expression *in vivo*, due to elimination of transduced cells by the cellular immune system [McConnell & Imperiale, 2004]. To avoid eliciting unwanted cellular immune responses, helper-dependent Ad (HD-Ad) vectors, also called “gutless” vectors have been developed. These HD-Ad vectors are de-

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void of all viral coding sequences and consist of merely the *cis*-elements required for genome replication and encapsidation. Since all viral sequences are deleted, helper adenoviruses must be used during HD-Ad production to provide all the necessary functions in *trans* [Amalfitano, 1999]. The elimination of Ad antigen expression in HD-Ad vectors permits long-term episomal expression of genes in quiescent cells by avoiding problems associated with the cellular immune response against viral gene products [Palmer & Ng, 2005, Toietta *et al.,* 2005]. In addition, these vectors have a much higher packaging capacity of ~35 kb of foreign DNA, enabling the expression of large transgenes or the inclusion of human genomic regulatory elements [Palmer & Ng, 2005].

The use of Ad vectors for clinical gene therapy is widespread. As of July 2006, adenoviral vectors are used in 26% of the 1,192 current worldwide gene therapy clinical trials. Of the 301 clinical trials involving the use of Ad vectors, 76% are for the treatment of cancer with vascular disease and monogenic disorders following at 14% and 7% respectively (http://www.wiley.co.uk/genetherapy/clinical/).

Many clinical gene therapy applications of Ad vectors will require expression of therapeutic transgene in disseminated tissues throughout the body and systemic administration is the most logical route of delivery. Systemic delivery of Ad vectors results in a variety of rapid physiological responses including activation of innate immunity, induction of cytokines, inflammation, transient liver toxicity and thrombocytopenia. These innate host responses represent a major barrier to clinical gene therapy, and are believed to result in part from the uptake of vector particles by Kupffer cells, resident macrophages in the liver [Alemany *et al.,* 2000, Mok *et al.,* 2005, Muruve, 2004]. Studies with HD-Ad vectors have revealed that these innate responses are triggered by the actual Ad capsids, and are independent of the expression of any viral genes [Muruve *et al.,* 2004]. Direct interactions of Ad vector particles with target cells and/or immune cells (including Kupffer cells) can result in a variety of downstream effects [Liu *et al.,* 2003, Zhang *et al.,* 2001].

Signaling events during the course of receptor binding and endocytosis of Ad virions leads to activation of PI3K and ERK/MAPK pathways, causing NF-κB dependent activation of cytokine effector genes [Liu & Muruve, 2003, Shayakhmetov *et al.,* 2005c]. In murine models, intravenous delivery of Ad vector results in very rapid (peak levels reached by ~1 hr) induction of IL-1 and MIP-2 cytokines, followed by slightly slower increases in an assortment of additional cytokines including CXCL-1, TNFα, IL-5, IL-6, IP-10, MCP-1, IFNγ, IL-12, and RANTES. The IL-1 cytokine appears to be an early mediator of the cytokine cascade, as mice defective for IL-1 signaling have blunted innate immune responses and decreased cytokine production upon systemic Ad vector administration [Shayakhmetov *et al.,* 2005c]. The MIP-2 cytokine is a chemo-attractant that promotes leukocyte recruitment and infiltration. Among the migrating leukocytes, neutrophils have been shown to take up opsonized Ad vector particles through Fc receptors and complement receptor 1 [Cotter *et al.,* 2005]. This uptake by leukocytes then leads to further cytokine production and inflammation, priming the immune system for an adaptive response against the Ad vector. The complement branch of the innate immune system appears to play a larger role than just vector opsonization and clearance, studies have shown that complement deficient C3-knockout (KO) mice are defective in the induction of a number of cytokines including CXCL-1, IL-5, G-CSF, GM-CSF, and IL-6 [Kiang *et al.,* 2006]. TNFα also appears to be a modulator of acute toxicity as TNFα-deficient mice display decreased levels of IL-6 and reduced thrombocytopenia in response to Ad vector [Mane *et al.,* 2006].

Recently, the viral genome itself has been implicated as an activator of host innate responses against Ad vectors. Induction of type-1 IFNs in dendritic cells (DC) through a TLR9/MyD88-dependent pathway has been demonstrated in response to CpG motifs within the Ad vector genomes [Iacobelli-Martinez & Nemerow, 2007]. Type 1 IFNs are also induced by vector DNA in non-DC, through a TLR-independent pathway [Zhu *et al.,* 2007]. Activation of TLR/MyD88 signaling looks to be an important determinant of the *in vivo* innate immune response against Ad vectors as MyD88-KO mice exhibit significantly lower levels of CXCl-1, GM-CSF, G-CSF, IL-5, IL-6, IL-12, MIP-1, and MCP-1 plasma cytokines [Hartman *et al.,* 2007]. Interestingly, TLR/MyD88 signaling was also implicated in adaptive immunity, with MyD88-KO mice having a significantly lower titer of anti-[Ad] IgGs and reduced CTL responses against vector-encoded antigen [Hartman *et al.,* 2007]. Indeed, blockade of TLR/MyD88-induced IFN production with either anti-[IFN] neutralizing antibodies or use of IFN-receptor deficient mice shows increased vector persistence, increased transduction, and decreased vector-specific CTL activity [Zhu *et al.,* 2007]. The development of HD-Ad vectors with lower CpG content (ideally, consisting mostly of human genomic sequences) may help alleviate these genome-driven immune responses against the vectors.

In addition to triggering innate immune responses, the uptake of Ad vectors by Kupffer cells has been shown to cause a non-linear dose response, requiring the administration of high levels of Ad vectors for therapeutic levels of transgene expression [Tao *et al.,* 2001]. Injection of high amounts of Ad vectors can provoke severe acute inflammatory responses, compromising the safety of the patient [Marshall, 1999]. Targeting Ad vectors to the site of disease and reducing their native interactions with host cells and the innate immune system should therefore lead to safer and more efficacious vectors, reducing the dosage of vector required for an effective therapeutic response. The physical modification of Ad vectors with the goal of removing the native tropism and blocking natural capsid-host interactions, combined with the introduction of new ligands for the targeted transduction of specific host cells, is therefore a major objective in the field of Ad gene therapy. This review provides a summary of the many Ad targeting strategies as well as background on Ad capsid structure and the biology of cell entry.

ADENOVIRAL CAPSID STRUCTURE

Direct physical interactions between the Ad vector particle and host proteins, cells, and tissues determine all subsequent downstream events including cellular uptake, gene

transfer efficiency, and induction of innate immune responses. A comprehensive understanding of Ad capsid structure is therefore fundamental to the development and improvement of Ad vectors for clinical gene therapy.

The adenoviral virion is a non-enveloped icosahedral particle composed of 12 distinct polypeptides and a ~36 kilobase double stranded linear DNA. Electron microscopy provided the first visual picture of the icosahedral capsid architecture, revealing 252 distinct capsomeres and the presence of long fibers protruding from each of the twelve vertices [Horne *et al.,* 1959, Valentine & Pereira, 1965]. The bulk of the particles are ~900 Å in diameter with a molecular mass on the order of 150 MDa [van Oostrum & Burnett, 1985]. Detailed analyses of the structure and composition of the adenovirus virion [Stewart *et al.,* 1991, van Oostrum & Burnett, 1985], and the interactions between its components [Everitt *et al.,* 1973, Prage *et al.,* 1970] has resulted in a detailed model of the virion structure. The seven polypeptides comprising the capsid are hexon (II), penton base (III), fiber (IV), IIIa, VI, VIII and IX. Four additional proteins, V, VII, X (mu), and the terminal protein (TP) are associated with the DNA genome to form the core. The adenoviral protease is also present within the virion [Anderson, 1990].

Major Capsid Proteins

The major structural protein of the Ad5 capsid is the 105 kDa hexon (polypeptide II). The twenty interlocking facets of the icosahedron are built from twelve hexon trimers, resulting in a stoichiometry of 240 trimers per virion. Solution of the hexon crystal structure [Rux & Burnett, 2000] revealed a complex design whereby extended loop structures wrap tightly around one another to form a highly stable trimer. Hexon trimerization occurs in the cytoplasm of infected cells and requires the assistance of the adenovirus 100 kDa polypeptide [Cepko & Sharp, 1982]. The monomers trimerize in a manner as to form a pseudo-hexagonal base with three tower structures at the top of the molecule. These towers are clearly visible in cryoelectron microscopy (cryoEM) reconstructions of the capsid (Fig. **1A**). Analysis of the structure and sequence alignments to other Ad serotypes has revealed several hypervariable regions (HVR's) present on surface loops of the towers [Rux *et al.,* 2003]. The bottom of the hexon trimer features a central cavity facing towards the inner core of the particle.

Present at each of the twelve vertices are the penton complexes, each consisting of a pentameric penton base (polypeptide III, 63 kDa monomer) and a trimeric fiber (polypeptide IV, 62 kDa monomer) extending approximately 330 Å outwards on Ad2 and Ad5. The X-ray crystal structures of the full length Ad2 penton base [Zubieta *et al.,* 2005], and a truncated fragment of the trimeric Ad2 fiber shaft [van Raaij *et al.,* 1999], and the knob domain [Xia *et al.,* 1994] have been solved. Adenovirus fibers are modular in structure and consist of three domains, a short N-terminal tail that attaches the fiber to the penton base, a shaft domain comprised of a repeating triple β-spiral motif, and a globular knob domain required for trimerization of the monomers and primary receptor binding. Although conserved in structure, the shaft length is highly variable among Ad serotypes and is determined by the number of pseudo-repeats present, ranging from 6 repeats in subgroup B Ad3 and Ad7 to 22 repeats in subgroup C Ad5 and 23 repeats in subgroup A Ad12 [Rux & Burnett, 2004]. Recently, a KKTK (lysine-lysine-threonine-lysine) motif in the third repeat of the shaft of Ad2 and Ad5 has been suggested as a potential heparan sulfate binding ligand, important for *in vivo* biodistribution and bioactivity [Smith *et al.,* 2003]. Interestingly, Ad5 fibers appear to have a flexible kink corresponding to a non-consensus sequence present within this third pseudo-repeat [Mitraki *et al.,* 2002, Wu *et al.,* 2003]. This flexibility of the fiber has been shown to be important for the cellular uptake of Ad vectors.

The crystal structure of the penton base revealed the answer to the 3-fold/5-fold symmetry mismatch that occurs when the trimeric fiber docks onto the pentameric base. Solution of the penton base structure with a short N-terminal peptide from the fiber tail shows that five binding sites exist between each penton base subunit but steric hindrance imposed by the trimeric structure of the fiber allows only 3/5 of the sites to be filled, resulting in one fiber per base [Zubieta *et al.,* 2005]. The structure has also confirmed the presence of a flexible surface exposed loop containing an arginine-glycine-aspartic acid (RGD) motif, which has been shown to bind cell surface integrins and facilitate endocytic uptake [Chiu *et al.,* 1999, Stewart *et al.,* 1997, Wickham *et al.,* 1993].

Minor Capsid Proteins

Detailed analysis of the Ad2 capsid structure by cryoEM and particle reconstruction [Stewart *et al.,* 1991] combined with the crystal structure of the Ad2 hexon [Athappilly *et al.,* 1994] was used to construct a quasi-atomic model of the three-dimensional Ad2 capsid. Difference imaging and subtraction of the hexon density map away from the cryoEM map of the capsid resulted in a density map attributable to minor capsid components [Stewart *et al.,* 1993]. Proteins IIIa, VI and IX were then assigned to specific densities within the three-dimensional map based on known masses and copy numbers, predicted protein volumes, and biochemical data [Colby & Shenk, 1981, Stewart *et al.,* 1993, van Oostrum & Burnett, 1985]. None of the minor capsid protein structures have been determined. All of the minor capsid proteins with the exception of IX are processed by the viral protease to yield the mature chains present within and necessary for infectious virions [Shenk, 2001].

Each facet of the virion is formed from twelve hexon trimers. Central to each facet are groups-of-nine (GON) hexons that remain associated after heat or pyridine disruption [Colby & Shenk, 1981, Prage *et al.,* 1970]. Protein IX, a small 14 kDa polypeptide present at 240 copies per virion [van Oostrum & Burnett, 1985], is believed to function as a cement protein, stabilizing the GON structure. Indeed, although IX is not necessary for encapsidation, virions devoid of IX are less thermostable than wild type virions and no GON's are seen after controlled disruption [Colby & Shenk, 1981]. Electron microscopy studies and difference imaging of purified GON's have identified density assigned as IX, present as four small trimers positioned between the bases of the hexon trimers central to the GON [Furcinitti *et al.,* 1989]. The C-terminus of IX is exposed on the surface of the virion

[Akalu *et al.*, 1999], despite the assignment of IX to an inaccessible position well below the surface of the hexon trimers.

Protein IIIa, a 63 kDa monomeric protein present at 60 copies per virion, was originally assigned to a position on opposite and adjacent sides of the 2-fold axis of symmetry just outside the peripentonal hexons, apparently functioning to rivet the interlocking facets together [Stewart *et al.*, 1993]. Two independent cryoEM reconstructions of Ad virions devoid of protein IX (AdΔIX) have revealed a correlation between this assigned IIIa density and protein IX; AdΔIX virions were always lacking the IIIa density [Fabry *et al.*, 2005, Scheres *et al.*, 2005]. More recent cryoEM studies in our laboratory on Ad5 vectors displaying either biotin acceptor proteins (BAPs) or EGFP on the C-terminus of protein IX shows the added BAP/EGFP density appearing above the density assigned as protein IIIa, strongly suggesting that this density may be attributable to the C-terminal portion of protein IX [Marsh *et al.*, 2006]. This observation, taken together with the observed loss of putative IIIa density upon IX deletion argue strongly that assigned IIIa density is actually protein IX.

Another very recent high-resolution cryoEM reconstruction of the Ad capsid has enabled the visualization of alpha-helical densities of the capsid proteins [Saban *et al.*, 2006]. Protein structure prediction algorithms, in combination with the cryoEM density map data and docking of the crystal structures of hexon and penton base has resulted in the reassignment of several of the minor capsid proteins, including protein IX. The new model (Fig. **1B**) is in agreement with our Ad-IX-EGFP reconstruction [Marsh *et al.*, 2006] and has four trimers of the N-terminal portion of protein IX residing between hexons of the GON with the C-terminal portions present as four-helix bundles at the position originally assigned as IIIa [Saban *et al.*, 2006]. This exposed position of the C-terminus of protein IX is consistent with its exposed display of peptides and proteins from the Ad virion [Campos & Barry, 2006, Dmitriev *et al.*, 2002, Li *et al.*, 2005, Vellinga *et al.*, 2004].

The recent high-resolution reconstruction revealed clusters of alpha-helical density underneath the penton base and peripentonal hexons, at the five-fold axes of symmetry. These clusters are assigned as protein IIIa, a protein predicted to have a high alpha-helical content, and the assign-

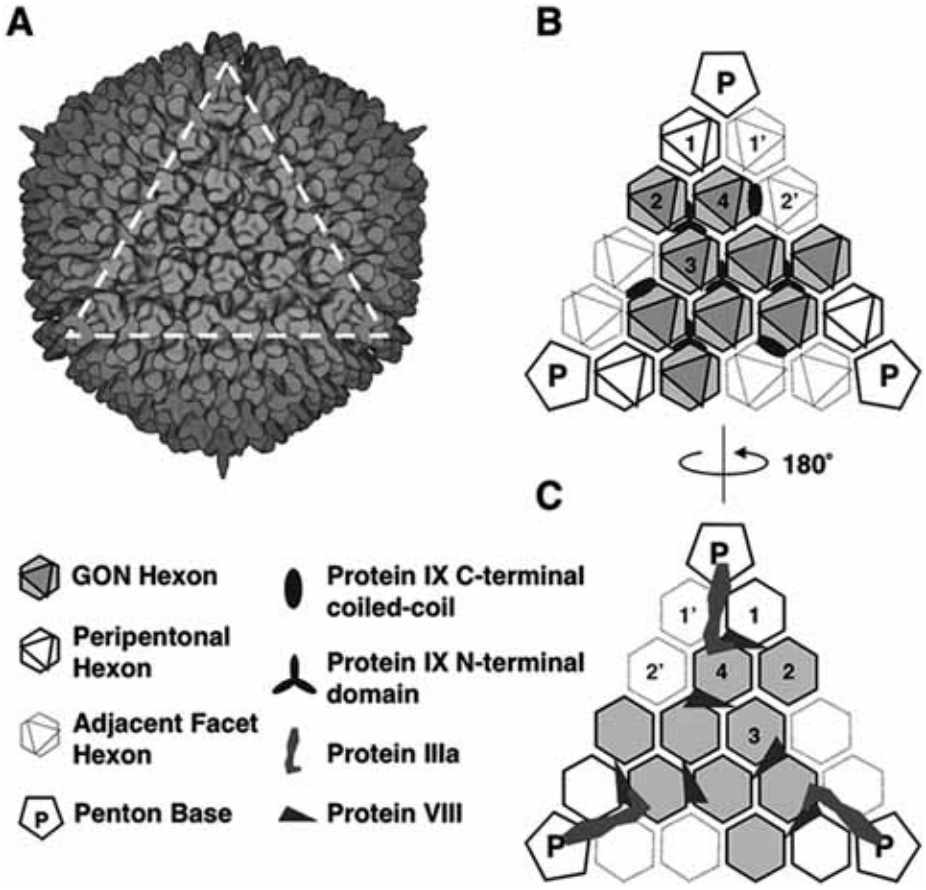

Fig. (1). (A) CryoEM reconstruction of the Ad5 virion, viewed along the three-fold axis of symmetry at ~21 Å resolution. The flexible fibers are averaged out during the reconstruction and only a small portion can be visualized protruding from each penton base. The central facet of the icosahedron is outlined. **(B)** Schematic of outer surface of the central facet, indicating the positions of the GON, peripentonal, and adjacent facet hexons as well as the position of the N- and C-terminal domains of protein IX. The four unique hexons of each asymmetric unit are designated by the numeric labels H1, H2, H3, and H4. H1 designates peripentonal hexons, H2 hexons lie along the two-fold axis, H3 hexons surround the three-fold axis and H4 are at the fourth nonequivalent position. Hexons labeled H1' and H2' are from the adjacent facet. **(C)** Schematic of the inner surface of the same facet, indicating the positions of proteins IIIa and VIII. Hexon labeling is the same as in panel **(B)**.

ment is consistent with the biochemical copy number of 60 molecules per virion [Saban *et al.,* 2006, van Oostrum & Burnett, 1985] (Fig. **1C**). The reassignment of IIIa to an internal location underneath the capsid shell is also in good agreement with other biophysical and immuno-electron microscopy studies concluding an internal position for IIIa [Newcomb & Brown, 1988, Scheres *et al.,* 2005].

Protein VIII, a small 15 kDa polypeptide present at 120 copies per virion, is tightly associated with hexons [Sundquist *et al.,* 1973]. Until recently, its precise location within the capsid has remained elusive. Now two independent cryoEM reconstructions have assigned VIII to six elongated densities underneath each of the twenty icosahedral facets [Fabry *et al.,* 2005, Saban *et al.,* 2006]. Three of the six densities surround the three central hexon trimers in the middle of the facet. The other three densities are aligned along the peripentonal hexons, in close association with the helical clusters assigned as protein IIIa (Fig. **1C**).

Protein VI, a 23 kDa polypeptide present at ~360 copies per virion, has been identified as the lytic factor necessary for endosomal membrane disruption during the early stages of infection [Wiethoff *et al.,* 2005]. Immature protein VI also functions as a nuclear shuttle to bind and import hexon trimers into the nucleus during particle assembly [Wodrich *et al.,* 2003]. Protein VI was originally localized to regions underneath and between the five peripentonal hexon trimers and the penton base at each vertex. Six molecules of VI, present as trimers of dimers were thought to be associated with each of the peripentonal hexon trimers, stabilizing the vertex regions [Stewart *et al.,* 1993]. The more recent reconstruction has since reassigned this density as proteins IIIa and VIII (Fig. **1C**). The current model has protein VI residing at partial occupancy (and therefore poorly resolved by cryoEM) within the central cavities, beneath each of the hexon trimers [Saban *et al.,* 2006]. Residence at this position agrees well with its function as a nuclear import shuttle and with biochemical data mapping the protein VI-interacting hexon residues to this cavity [Wodrich *et al.,* 2003]. Apparently protein VI is masked underneath the hexons until low pH from endosomal acidification triggers the release of vertex components (fiber, penton base, IIIa, and peripentonal hexons), exposing some protein VI and allowing it to lyse the endosomal membrane [Wiethoff *et al.,* 2005].

The importance of these so-called "minor" capsid proteins is exemplified in a temperature sensitive mutant of Ad2 called *ts*1. This mutant has a point mutation in the viral protease, resulting in poor encapsidation of protease and no cleavage of the minor capsid proteins IIIa, VI, and VIII as well as core proteins VII and X [Hassell & Weber, 1978, Rancourt *et al.,* 1995]. The consequences of this lack of proteolysis is a hyper-stable particle, severely defective for uncoating. *ts*1 particles bind and enter cells normally, but fail to escape the endosomal compartment and eventually get degraded in lysosomes [Hannan *et al.,* 1983].

Core Structure

The 36 kilobase Ad genome is associated with proteins V, VII, X (mu) and the terminal protein forming a condensed core structure. CryoEM reconstructions of Ad virions show a featureless density present underneath the highly ordered capsid [Stewart *et al.,* 1991]. Early studies on the structure of the nucleoprotein core have resulted in different models for core organization. Nuclease digestion experiments suggest that core proteins may form nucleosome-like structures, protecting 150-200 bp fragments of DNA from enzymatic digestion [Corden *et al.,* 1976]. Other studies suggest a model whereby the core is organized into 12 lobes present under each of the vertices of the capsid, as visualized by ion-etching and electron microscopy of sarkosyl treated cores [Brown *et al.,* 1975, Newcomb *et al.,* 1984].

Biochemical analysis of core protein interactions supports a model where 840 molecules of the 19 kDa protein VII and ~100 molecules of the 4 kDa protein X (mu) are tightly associated with the genomic DNA [Chatterjee *et al.,* 1985, van Oostrum & Burnett, 1985]. Two copies of the 55 kDa TP are covalently linked to the ends of each genome, functioning as protein primers during replication. Protein V, a 42 kDa protein present at 160 copies per virion, has a weaker interaction with the genome and is thought to form a coating around the tight DNA-VII-X nucleoprotein core. Protein V interacts with both core proteins VII and X as well as dimers of capsid protein VI [Chatterjee *et al.,* 1985, Matthews & Russell, 1998], thereby linking the core to the outer capsid. Although the 23 kDa viral protease is not considered a core protein, it is thought to be present at ~10-12 copies within the capsid. In addition to several of the capsid proteins, core proteins VII, X, and TP are cleaved into mature forms by the Ad protease [Shenk, 2001].

ADENOVIRUS BINDING, ENTRY, AND TRAFFICKING

Although not the sole determinant of viral tropism, the initial events of binding and entry into host cells are essential to the viral life cycle. Viruses have evolved to exploit many different cell-surface molecules for attachment and endocytic uptake [Baranowski *et al.,* 2001, Smith & Helenius, 2004]. *In vitro* work has demonstrated the initial attachment of Ad5 is mediated by the high affinity binding (K_d = 15 nM) of the distal knob domain of the adenoviral fiber to cell surface coxsackievirus B and adenovirus receptor (CAR), a type 1 transmembrane protein in the immunoglobulin superfamily [Bergelson *et al.,* 1997, Howitt *et al.,* 2003, Kirby *et al.,* 2000]. Most subgroups of adenoviruses, with the exception of subgroup B, have members with CAR-binding fibers. Subgroup B Ads have been shown to utilize either CD46, CD80/CD86, an unidentified glycoprotein "receptor X", or combinations thereof as cellular receptors [Gaggar *et al.,* 2003, Marttila *et al.,* 2005, Short *et al.,* 2004, Tuve *et al.,* 2006]. Sialic acid is also involved in the uptake of some subgroup D members [Wu *et al.,* 2003].

In addition to CAR, heparan sulfate proteoglycans (HSPGs) have been shown to promote Ad5 virion attachment to certain cell types [Dechecchi *et al.,* 2001]. A putative heparin binding lysine-lysine-threonine-lysine (KKTK) motif within the non-consensus third pseudo-repeat of the fiber shaft is thought to mediate this interaction [Smith *et al.,* 2002, Smith *et al.,* 2003], however more recent data suggests that mutation of this KKTK motif affects more than just HSPG binding and may perturb the folding and/or conformation of the fiber knob [Bayo-Puxan *et al.,* 2006]. Additional

work has shown that mutation of the KKTK motif may drastically alter the properties of the fiber, resulting in particles that bind and enter cells normally but exhibit altered trafficking, similar in phenotype to the *ts*1 mutant [Kritz *et al.,* 2007]. These results strongly suggest that the KKTK mutant defects result from a post-entry block rather than from a defect in HSPG binding. It should be noted that he KKTK motif within the Ad5 fiber shaft has not been formally been shown to bind heparin or HSPGs.

Two common routes of entry into cells are clathrin- and caveolae/lipid raft-mediated endocytosis. Clathrin-mediated entry routes have been well characterized for viruses like adenovirus, Semliki Forest virus, and influenza viruses [Smith & Helenius, 2004]. The clathrin-dependent endocytic route is typically characterized by the formation of clathrin-coated pits upon virus binding. These pits eventually internalize resulting in intracellular coated vesicles containing virions. These vesicles then enter the endosomal pathway, gradually maturing through acidification and eventually leading to the lysosomal compartment [Mellman, 1992]. The unique environment of the endosomal compartment acts as a stimulus for many viruses that enter through this route, triggering irreversible conformational changes in the viral capsid that lead to processes like uncoating and endosomal escape that are critical for infection [Smith & Helenius, 2004, Wiethoff *et al.,* 2005]. Secondary interactions between exposed arginine-glycine-aspartatic acid (RGD) motifs present within surface loops of the penton base and cell surface $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, or $\alpha_3\beta_1$ integrins promotes uptake through clathrin-mediated endocytosis [Li *et al.,* 1998a, Salone *et al.,* 2003, Wickham *et al.,* 1993]. This classical model of Ad5 uptake (Fig. **2**) has recently been elaborated and new data suggests that in addition to endocytosis, Ad triggers other uptake mechanisms like micropinocytosis [Meier *et al.,* 2002]. The presence of cholesterol has also been shown to be important both for Ad uptake and the latter process of endosomal escape [Imelli *et al.,* 2004].

Cellular endocytic processes and signal transduction pathways are tightly intertwined and successful viral uptake often requires the induction of proper signaling cascades [Greber, 2002]. Numerous signaling events have been associated with both clathrin and caveolae/lipid raft endocytosis, using a high-throughput siRNA assay a recent study has identified 210 kinases that regulate endocytosis of vesicular stomatitis virus (clathrin-mediated endocytosis) and SV40 (caveolae/lipid raft uptake) [Pelkmans *et al.,* 2005]. Uptake of adenovirus through clathrin coated endocytic vesicles activates and requires phosphatidylinositide-3OH kinase (PI3K) which can then activate downstream targets like protein kinase C (PKC) and the Rab5 GTPase [Li *et al.,* 1998c, Meier & Greber, 2004]. PKC activation can then lead to the localized remodeling of actin cytosketeton (Fig. **2**) [Li *et al.,* 1998b, Pietiainen *et al.,* 2005] and Rab5 is a marker and determinant of proper endosomal vesicular trafficking and maturation [Zerial & McBride, 2001]. Induction of the initial P13K activation signal is dependent on the physical interaction between the adenovirus capsid and cell surface integrin coreceptors [Li *et al.,* 1998c]. CryoEM reconstructions of the soluble extracellular domain of the $\alpha_v\beta_5$ integrin bound to the Ad virion has revealed a crown-like ring of integrin density docked above the pentons [Chiu *et al.,* 1999]. The pentameric spatial array of the penton base RGD motifs appears to be necessary for integrin clustering and initiation of both endocytic uptake and downstream signaling events. Integrin-mediated signaling events can differ between monovalent RGD ligand binding and integrin clustering via multivalent RGD ligands [Chen *et al.,* 1997, Stupack *et al.,* 1999]. The fact that the geometric arrangement of RGD motifs on the Ad capsid is strikingly similar to those on the unrelated foot-and-mouth disease virus emphasizes the importance of integrin clustering for efficient viral uptake [Acharya *et al.,* 1989].

The Ad5 fiber shaft has been shown to play an important role in coordinating the proper association between the Ad virion and the cell membrane-bound CAR and integrin receptors. Alterations of the shaft length, creating either shorter [Shayakhmetov & Lieber, 2000] or longer [Seki *et al.,* 2002] shafts resulted in decreased binding and infectivity. This effect was only seen for vectors with CAR-binding knobs, as those engineered with knobs from the CD46-utilizing subgroup B Ad35 were impervious to changes in shaft length [Shayakhmetov & Lieber, 2000]. Flexibility of the Ad5 shaft has also been shown to influence vector infectivity. Ad37 is a member of the subgroup D viruses and has short, straight shafts, lacking the flexible region present in the third pseudo-repeat of the Ad5 shaft [Chiu *et al.,* 2001]. Mutation of the flexible pseudo-repeat region of the Ad5 shaft to the corresponding sequence of the Ad37 shaft created a vector with long, straight fibers which led to a substantial decrease in infectivity [Wu *et al.,* 2003]. Apparently evolution has created fibers with the optimal length and flexibility for efficient interaction with both CAR and integrins [Chiu *et al.,* 1999, Wu *et al.,* 2003].

Receptor binding and uptake of Ad5 occurs relatively quickly (Fig. **2**), with the majority (80-85%) of the bound virions being taken up within 5-10 minutes on permissive cells [Greber *et al.,* 1993]. During uptake, the Ad5 virion undergoes a process of disassembly, beginning with the release of upwards of 90% of the fibers during the early stages of entry [Greber *et al.,* 1993, Nakano *et al.,* 2000]. Once the virion has been taken up, the natural process of endosomal acidification [Mellman, 1992] triggers a conformational change in the Ad5 capsid, ultimately resulting in endosomolysis and release of the virion into the cytoplasm. Recently, it has been shown that protein VI contains an N-terminal amphipathic alpha-helical domain that possesses the membrane lytic activity responsible for endosomal escape. The current working model suggests that the Ad capsid somehow senses the decrease in pH and responds by releasing various vertex components including the fiber, penton base, peripentonal hexons, protein IIIa, and protein VI. Upon release the N-terminal amphipathic alpha-helical domain of VI becomes free to disrupt the endosomal membrane [Wiethoff *et al.,* 2005]. The coordinated release of fibers and vertex components, necessary for endosomal escape, appears to result from the concerted action of several capsid components, as the protease-mutant *ts*1 which contains immature pIIIa, pVI, and pVIII [Hannan *et al.,* 1983] fails to undergo vertex disassembly and is consequently trapped in endosomes to be degraded in lysosomes [Wiethoff *et al.,* 2005]. Again the process of endosomal escape is believed to occur relatively rapidly, with the majority of virions reaching the cytoplasm

by 15-20 minutes post infection (Fig. **2**) [Greber *et al.*, 1993]. Interactions of the Ad penton with cellular integrins are also implicated in this vertex disassembly and endosomolysis event, as vectors deleted for the RGD motif in the penton base display not only delayed uptake kinetics but also delayed endosomal escape kinetics [Shayakhmetov *et al.*, 2005a]. This data suggests that the penton base/integrin interaction may be important for the pH-dependent conformational changes during residence in the acidifying endosomal compartment.

Once free in the cytoplasm, the partially dismantled capsid interacts with dynein (Fig. **2**), a cytoplasmic motor protein [Kelkar *et al.*, 2004]. The association of Ad with dynein enables the virions to translocate along microtubules towards the microtubule organizing center (MTOC), located near the host cell nucleus [Bailey *et al.*, 2003]. The cellular nuclear export factor CRM1 has recently been implicated in the release of Ad virions from the MTOC through an unknown mechanism, facilitating the transfer of Ad virions from the MTOC to the host cell nucleus [Strunze *et al.*, 2005]. Previous work has demonstrated specific interactions between the Ad5 capsid and cell nuclei using both *in vitro* binding assays as well as direct visualization with fluorescence microscopy [Wisnivesky *et al.*, 1999]. More detailed analysis has revealed that Ad specifically binds to the CAN/Nup214 protein of the nuclear pore complex (NPC) and this association, together with interactions between virions and histone H1, leads to further disassembly and eventual import of the adenoviral genome into the nucleus [Trotman *et al.*, 2001]. Overall, the entire process from initial cell surface binding to docking at the NPC is rapid and usually occurs within 30-60 minutes (Fig. **2**), depending on the cell type and morphology [Meier & Greber, 2004].

Interestingly, Ad5 vectors pseudotyped with fibers from group B Ad7 and Ad35 utilize an alternate intracellular trafficking pathway [Miyazawa *et al.*, 2001, Miyazawa *et al.*, 1999, Shayakhmetov *et al.*, 2003]. Uptake directed through these fibers leads to a longer residence within the endosomal compartment. Eventually some virions end up in perinuclear lysosomal compartments prior to cytoplasmic translocation and NPC docking while others are recycled back to the cell surface. This longer endosomal residence appears to be mediated solely through the fiber knob domains of these pseudotyped vectors [Shayakhmetov *et al.*, 2003]. It is not known whether the altered trafficking is caused by direct interactions between the subgroup B knobs and the CD46 receptor, or whether the knobs of subgroup B viruses somehow alter the fiber release and acid-dependent conformational changes of the capsid that are required for vertex release and protein VI-mediated vesicle lysis [Greber *et al.*, 1993, Wiethoff *et al.*, 2005].

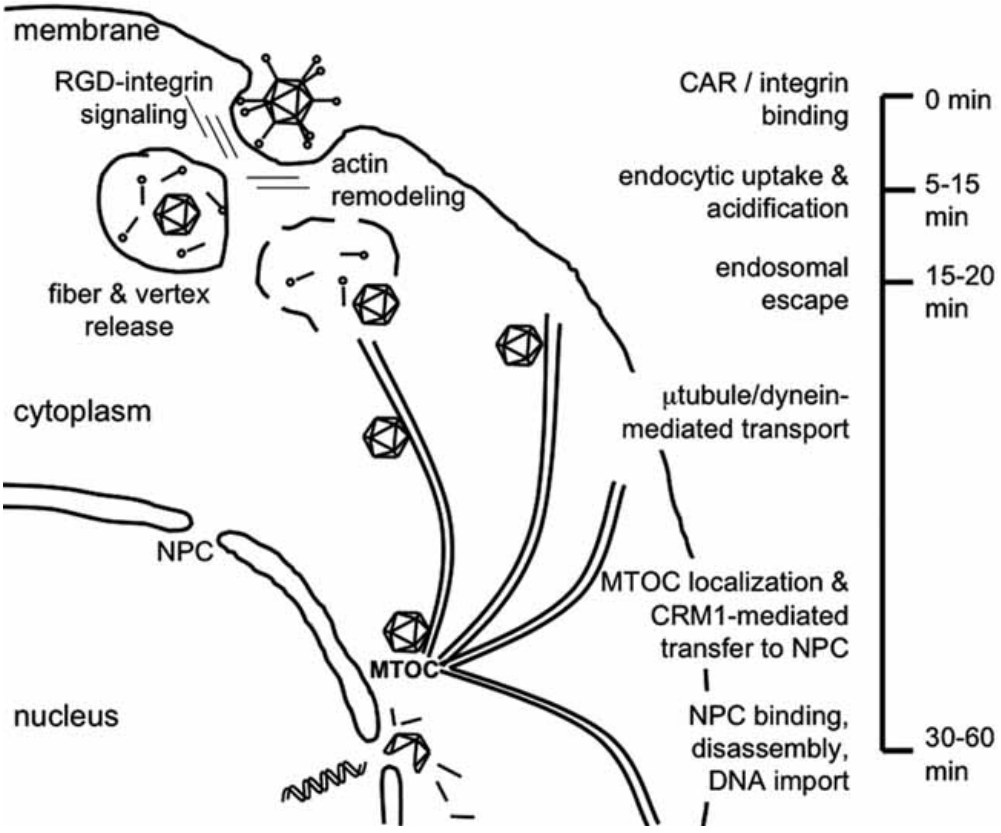

Fig. (2). Timeline of the Ad5 binding, entry, and trafficking pathway. Initial attachment is mediated through the fiber-CAR interaction. Subsequent binding of the penton base RGD motifs to integrins results in signaling cascades and endocytic uptake. Endosomal acidification is sensed by the incoming virion, resulting in fiber release, vertex disassembly, exposure of protein VI, and lysis of the endosomal membrane. Cytoplasmic virions can then interact with dynein for transport along microtubules to the microtubule organizing center (MTOC). Cellular export factor CRM1 then mediates transfer to the nuclear pore complex (NPC) where the virion binds, disassembles, and releases its genome into the host cell nucleus.

Although the mechanisms of *in vitro* attachment and uptake are relatively well understood, much less is known about the mechanisms governing *in vivo* Ad infection. Attempts to create detargeted Ad vectors by mutating critical CAR-interacting residues of the fiber knob [Bewley *et al.,* 1999, Kirby *et al.,* 2000], combined with deletion of the integrin-binding RGD motif of the penton base have led to vectors with greatly diminished *in vitro* transduction capabilities. Unfortunately these detargeting effects are not always recapitulated *in vivo* and many of these mutated vectors retain full infectivity upon systemic administration. [Alemany & Curiel, 2001, Mizuguchi *et al.,* 2002, Smith *et al.,* 2002]. Only now are we just beginning to understand and appreciate the complex nature of *in vivo* Ad-host interactions.

Recently, several blood factors have been identified that promote CAR-independent infection of murine hepatic tissues [Shayakhmetov *et al.,* 2005b]. Among these blood factors, factor IX (FIX) and complement C4-binding protein (C4BP) were shown to directly bind the fiber knob and act as molecular adaptors, redirecting uptake through alternate receptors including HSPGs and the low-density lipoprotein receptor-related protein (LRP). Another recent study demonstrates that in addition to FIX, other plasma zymogens including factor VII (FVII), factor X (FX) and protein C (PC) can augment Ad transduction both *in vitro* and *in vivo* [Parker *et al.,* 2006]. A liver perfusion technique was implemented to demonstrate that these blood factors are the predominant mode of CAR-binding ablated vector uptake in the murine liver. In the absence of blood (livers perfused with saline prior to vector administration), hepatocyte transduction by vectors with mutated knobs (CAR-binding ablated) was greatly diminished. Since the vectors used in this study contain the wild-type shaft sequence with the KKTK motif, these findings argue against the role of this putative HSPG-binding domain in cell binding and uptake. Importantly, FIX and C4BP augmented liver cell transduction of Ad5 (subgroup C) vectors with CAR-binding mutations as well as vectors pseudotyped with Ad35 (subgroup B) knobs, suggesting this *in vivo* pathway may be utilized by diverse Ad serotypes. FX has also been shown to augment transduction of Ad vectors pseudotyped with a variety of subgroup D fibers, again suggesting a conserved *in vivo* pathway of infection [Parker *et al.,* 2007]. Perhaps these blood factors bind to a region of the fiber knob conserved between different serotypes, a region distinct from the CAR-binding residues.

In addition to mediating CAR-independent liver transduction, these blood factors also contribute to Kupffer cell uptake and liver toxicity. Genetic insertion of a 12-residue peptide into the HI loop, combined with additional CAR-binding site mutations resulted in vectors with mutant knobs, diminished for blood factor binding. This mutant vector was had substantially decreased uptake by Kupffer cells and displayed near-baseline levels of plasma ALT (toxicity marker) upon systemic administration. The innate immune response against this mutant vector was blunted, with decreased levels of IL-6 and IFN-γ. Uptake and transduction of liver hepatocytes was also markedly diminished, supporting the potential of this or similar vectors as a "detargeted" platform upon which to introduce a targeted tropism [Shayakhmetov *et al.,* 2005b].

It is becoming ever apparent that the development of these "detargeted" vectors will not be an easy endeavor, as more host factors that modulate Ad infection are identified. For example hyaluronan, a non-sulfated glycosaminoglycan and primary component of extracellular matrix, has also been shown to increase transduction of Ad vectors with subgroup C and subgroup B fibers, *in vitro* and *in vivo*. Hyaluronan-based enhancement of transduction occurs through CD44, the receptor for hyaluronan, and may involve additional link proteins [Chaudhuri *et al.,* 2007]. Other studies have observed binding of complement protein C3 to the Ad capsid [Jiang *et al.,* 2004] in the presence of factor B and factor D, proteins involved in the antibody-independent alternative pathway of complement activation [Cichon *et al.,* 2001]. The precise roles of these complement interactions in vector transduction, clearance, cytokine induction, and activation of innate immunity still need to be elucidated.

Almost all the data on *in vivo* mechanisms of Ad transduction and host immune responses have been performed in mouse models. A very recent and important study highlights the differences between murine and human blood interactions with Ad5, and questions the relevance of studies performed in mouse models [Lyons *et al.,* 2006]. It has long been established that different Ad serotypes display characteristic erythrocyte agglutination activities, dependant on the red cell host species [Shenk, 2001]. Recent work suggests the interactions between Ad5 and human blood cells may severely interfere with systemic delivery of Ad5 vectors. Ad5 was shown to bind specifically to human erythrocytes and this sequestration inhibited transduction of permissive cells. Importantly, this effect was specific to human blood and was not observed with mouse blood. Remarkably, comparison of Ad5 vector levels in the blood from human clinical trial protocol samples to an experimental systemic injection in mice demonstrated that in humans the overwhelming majority of Ad5 vector is associated with blood cells. Only minute quantities (~1%) of the administered vector were present in the cell-free plasma fraction. In contrast, systemic administration in the mouse model revealed ~99.9% of the Ad5 vector to be present in the plasma fraction, unassociated with blood cells. In both cases the Ad5 vector was rapidly cleared from the circulation.

Ad vector interaction with platelets may have some involvement in this rapid clearance of vector from the blood. Adenovirus induces thrombocytopenia upon systemic administration in humans [Herman *et al.,* 1999]. Although this effect is not caused by Ad-induced platelet aggregation [Eggerman *et al.,* 2002], recent work has shown that CAR is present on human platelets and Ad can bind and activate platelets leading to subsequent activation of endothelial cells through a P-selectin and von Willebrand factor-dependent mechanism [Othman *et al.,* 2006]. This platelet binding and activation then leads to thrombocytopenia, with the clearance of platelets (and perhaps all platelet-bound Ad vector) from the bloodstream by tissue macrophages. Notably, this Ad-induced thrombocytopenia follows similar kinetics to Kupffer-cell mediated clearance of systemically administered Ad vector, suggesting the two processes may be connected [Alemany *et al.,* 2000, Othman *et al.,* 2006, Tao *et al.,* 2001].

Ad5 is a respiratory pathogen, and natural infections occur through epithelial tissues like the ciliated airway epithelia of the alveolae [Horwitz, 2001]. Interestingly, the primary attachment receptor CAR is a homophilic cell adhesion protein and has been localized to the tight junctions of polarized epithelium [Ashbourne Excoffon *et al.,* 2003, Cohen *et al.,* 2001, Honda *et al.,* 2000]. Sequestration of CAR at this inaccessible locale impairs Ad vector transduction of these monolayer tissues and disruption of these tight cell-cell contacts by transient EDTA treatment greatly enhances Ad vector transduction [Cohen *et al.,* 2001] by allowing the Ad vector access to cellular receptor. The fact that CAR naturally functions as a cell adhesion protein and resides within the inaccessible location of the tight junction raises questions regarding the *in vivo* relevance of the fiber-CAR interaction. As mentioned earlier, mutation of critical CAR-binding residues of the fiber knob that result in severe impairment of *in vitro* gene transfer may have little or no effect *in vivo*, again questioning the role of the fiber-CAR interaction during a natural infection. Interestingly, a CAR-independent uptake pathway in A549 cells has been shown to occur through interactions of the hexons with lung surfactant [Balakireva *et al.,* 2003]. The Ad fiber/CAR interaction has been implicated in viral egress and spread during infection [Walters *et al.,* 2002], and the large amounts of free unencapsidated fiber and penton that are produced and secreted during infection [Trotman *et al.,* 2003] appear to aid in the dissociation of cell-cell interactions and the spread of infection . Therefore the natural role of the Ad fiber-CAR interaction may be to promote viral escape and egress during infection [Walters *et al.,* 2002].

ADENOVIRAL VECTOR TARGETING

Systemic administration of Ad vectors results in rapid and efficient clearance of the vector by hepatic Kupffer cells, macrophage, and monocytes [Alemany *et al.,* 2000, Tao *et al.,* 2001]. Large amounts of vector can be administered to overcome this clearance mechanism, thereby allowing high levels of transduction of liver hepatocytes [Tao *et al.,* 2001]. Adenoviral vectors are immunogenic and the large amounts of vector needed to overcome liver clearance can lead to rapid and dangerous responses by the innate immune system, causing acute toxicity [Lozier *et al.,* 1999] and even death [Marshall, 1999]. Many efforts are underway to improve the efficacy of Ad vectors by altering their interactions with the body. The ablation of native tropism combined with the introduction of new targeting moieties should lead to vectors with improved safety and efficacy. This section will highlight the major advances towards achieving targeted adenoviral gene delivery.

Genetic Modification of the Capsid: Introducing New Tropism

Many of the therapeutically relevant target cells for gene therapy are refractory to Ad transduction due to low expression of primary receptors. Multiple strategies have been pursued to improve the efficacy of Ad vectors by targeting their transduction to specific cell and tissue types (Fig. **3**). Genetic manipulation of the adenoviral capsid is the most direct approach to modify vector tropism. Detailed structural data on the major capsid proteins [Rux & Burnett, 2000, Xia *et al.,* 1994, Zubieta *et al.,* 2005] have greatly facilitated the genetic incorporation of foreign peptides and proteins into exposed regions of the Ad capsid. Initial work demonstrated that short peptides and epitopes could successfully be grafted into the surface exposed HI loop [Dmitriev *et al.,* 1998, Krasnykh *et al.,* 1998] and C-terminus [Wickham *et al.,* 1996] of the fiber knob domain, the RGD-containing loop of penton base [Wickham *et al.,* 1997], the hypervariable region 5 (HVR5) loop of the hexon [Crompton *et al.,* 1994, Wu *et al.,* 2005], and the C-terminus of protein IX [Dmitriev *et al.,* 2002].

C-terminal fusions of poly-lysine residues [Wickham *et al.,* 1996] and HI loop insertions of RGD peptides [Dmitriev *et al.,* 1998] have created "gain of function" vectors with expanded tropism towards cells expressing HS-GAG's and integrins including cells normally refractive to Ad5 transduction like endothelial cells, skeletal and smooth muscle cells, and certain CAR-deficient cancer cells. Similarly, the insertion of RGD peptides into HVR5 of hexon [Vigne *et al.,* 1999] or the C-terminus of IX [Vellinga *et al.,* 2004] have resulted in vectors with broader tropism. Incorporation of mammalian cell-binding peptide ligands isolated by phage display [Barry *et al.,* 1996, Pasqualini & Ruoslahti, 1996] into the fiber knob can result in vectors with more specificity towards the target cells [Mailly *et al.,* 2006, Nicklin *et al.,* 2004, Work *et al.,* 2004]. Despite the numerous reports of successful modification of the fiber C-terminus and HI loop, there is no guarantee that any given peptide will be tolerated. Often insertion of foreign peptides into these locations can interfere with fiber folding and trimerization, preventing rescue of the modified vectors. Conversely, peptide ligands that are tolerated may lose their original ligand function once displayed within the structural context of the knob domain [Parrott *et al.,* 2003, Wickham *et al.,* 1997]. Towards this end, selection of peptide ligands from fiber knob or HI loop context-specific peptide display phage libraries provides a means for better translation of targeting ligand functionality from the context of the bacteriophage to the Ad vector [Fontana *et al.,* 2003, Ghosh & Barry, 2005].

Substantial progress in Ad capsid modification has been made since the initial studies with small peptides and several sites have been shown to tolerate the insertion of larger, more complex protein domains. The HI loop of the fiber knob can accept an 83 amino acid fragment of the RGD-containing loop of penton base [Belousova *et al.,* 2002], while the C-terminus has been fused to a 89 amino acid linker and biotin acceptor peptide (BAP) from *P. shermanii* [Parrott *et al.,* 2003]. Protein IX has garnered recent attention due to its ability to display large polypeptides and proteins on the surface of the Ad virion [Parks, 2005]. Fluorescent proteins like EGFP [Le *et al.,* 2004b, Meulenbrock *et al.,* 2004] and the mRFP1 and tdimer2 red fluorescent proteins [Le *et al.,* 2006] have successfully been displayed with negligible effects on virion thermostability and vector bioactivity. Even large reporter enzymes like luciferase, for bioluminescent imaging [Le *et al.,* 2004a] and HSV-TK for PET imaging [Li *et al.,* 2005] can be tethered to the virion surface through protein IX. Thus, the C-terminus of protein IX has been established as a flexible site for the incorporation of large targeting or reporter molecules. Indeed, labeling strategies based on the fusion of reporters to protein IX have

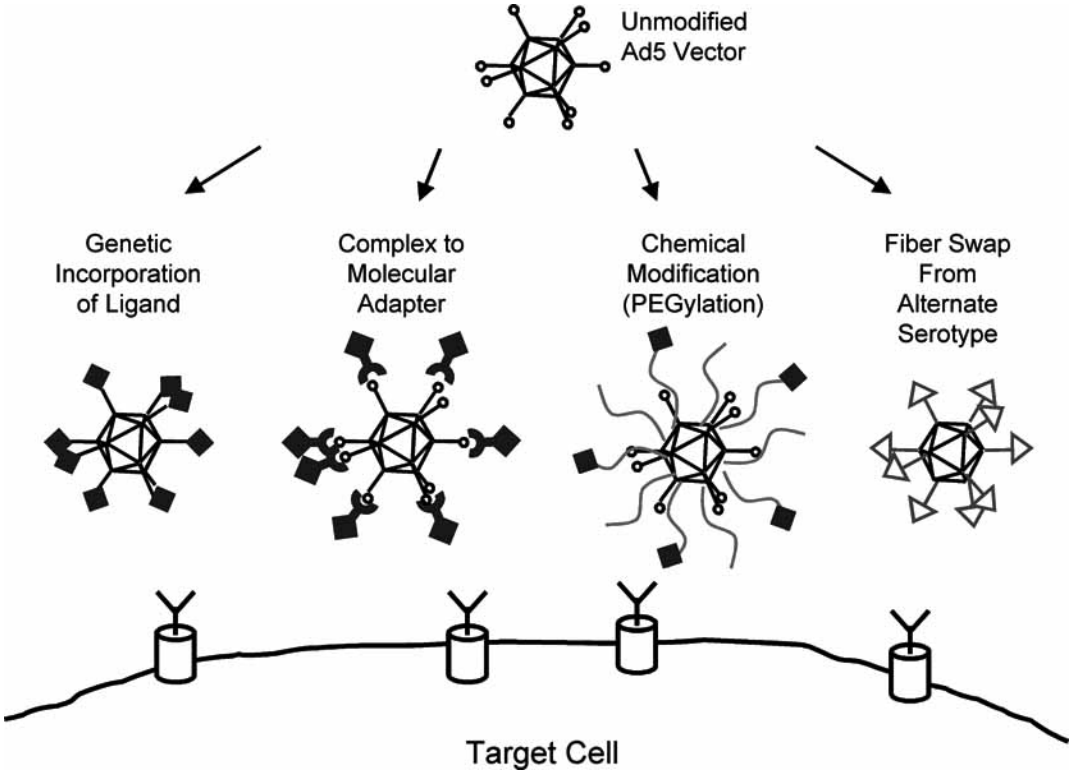

Fig. (3). General strategies towards redirecting Ad vector transduction to target cells through specific ligand-receptor interactions.

shown utility for non-invasive monitoring of viral replication and may become of great use in the study of oncolytic Ad vectors [Le *et al.*, 2006]. The hypervariable region 5 (HVR5) surface loop of hexon is another attractive site for possible ligand incorporation because of its sheer abundance (720 copies) in the Ad virion. With the exception of the 71 amino acid BAP from *P. shermanii* [Campos & Barry, 2004, Campos & Barry, 2006], this site has only been shown to accommodate small insertions like RGD, His_6, and other epitopes [Crompton *et al.*, 1994, Vigne *et al.*, 1999, Wu *et al.*, 2005].

Molecular Adaptors

One major limitation to direct genetic insertion of high affinity targeting ligands into the Ad capsid is a basic biological incompatibility. Not only does their size preclude the incorporation of larger, higher affinity ligands, but the nuclear life cycle of Ad also limits the repertoire of ligands to those that can fold properly in the reducing environment of the cytoplasm and cell nuclei. Naturally secreted ligands like cytokines, immunoglobulins, and growth factors require the oxidative environment of the golgi and ER compartments to fold correctly and form proper disulfide bonds [Magnusson *et al.*, 2002].

One targeting method to circumvent the problems of capsid-ligand incompatibility is the use of bi-functional fusion proteins (Fig. **3**). These molecular adaptor proteins consist of a capsid-binding domain genetically or chemically fused to a cell-binding ligand. These molecules can physically bridge the vector to the cell surface, thereby redirecting vector transduction to the target cell. Successful strategies have described anti-capsid antibody fragments or soluble CAR fragments fused to a diverse set of ligands including antibody fragments against a variety of cellular receptors, growth factors like the basic fibroblast growth factor (FGF-2) and epidermal growth factor (EGF), and even small molecules ligands like folate (reviewed in [Barnett *et al.*, 2002, Glasgow *et al.*, 2006]). Although this technology is versatile, the preparation of these adaptors, which are typically recombinant fusion proteins or chemically conjugated proteins, can vary from batch to batch. Also, the adaptor molecules rely on non-covalent protein-protein interactions for their conjugation to the Ad capsid, which are generally considered too weak to work *in vivo*. Naturally occurring antibodies or CAR receptors could compete for Ad binding and displace the molecular adaptors from the capsid, abolishing the vector targeting activity.

Chemical Modification of the Ad Capsid

Another general strategy to redirect Ad vector transduction is the direct chemical modification of the Ad capsid (Fig. **3**). Primary-amine reactive chemical groups can be used to covalently attach molecules to the surface of the Ad capsid. Direct attachment of ligands like FGF-2 and EGF as well as phage-selected peptides through bi-functional polyethylene glycol (PEG) or poly-N-(2-hydroxypropyl) methacrylamide (pHPMA) molecules has been shown to augment CAR-independent gene transfer [Fisher *et al.*, 2001, Lanciotti *et al.*, 2003, Menezes *et al.*, 2006, Romanczuk *et al.*, 1999]. Not only is this technology suitable for the display of ligands from the capsid, but native vector tropism can either be maintained or masked, depending on the specific reaction conditions. Given this approach normally requires the presence of free cysteines on the ligand for conjugation

to maleimide groups on PEG, it works best with ligands already having these displayed (i.e. FGF-2 and sythetic peptides with added cysteine). Conversely, conjugating more complex disulfide-bearing ligands like antibodies is technically much more challenging. Reaction to PEG has been shown to improve the *in vivo* pharmacokinetics of the vector by increasing vector persistence in the blood [Alemany *et al.*, 2000], preventing antibody neutralization [Croyle *et al.*, 2002, Fisher *et al.*, 2001], and decreasing the innate immune response against the vector [Mok *et al.*, 2005]. While PEG does ablate CAR-binding *in vitro*, to date its ability to substantially "detarget" Ad *in vivo* has not been realized. For example, saturation of Ad with ~15,000 5 kDa PEGs per virion does not change systemic transfection nor vector genome distribution in mice [Mok *et al.*, 2005]. Larger PEGs or polymer coating strategies may therefore be more productive for detargeting Ad.

Combinatorial Approaches

Combinatorial targeting methods in which versatile molecular adaptors are genetically incorporated into the Ad capsid have also been explored. The capacity of the Ad fiber to tolerate large insertions has enabled the fusion of various Fc-binding fragments from Staphylococcal protein A to both the C-terminus and HI loop of the fiber knob [Korokhov *et al.*, 2003, Volpers *et al.*, 2003]. This approach generates vectors that can then be complexed to cell-targeting antibodies through the specific interaction between protein A and the antibody Fc domain.

A similar molecular adaptor-based method was pioneered in our laboratory whereby the 71 amino acid biotin acceptor peptide (BAP) from Propionibacterium shermanii [Reddy *et al.*, 2000] was genetically fused to the C-termini of the fiber and protein IX [Campos *et al.*, 2004, Parrott *et al.*, 2003] as well as into the HVR5 loop of the hexon [Campos & Barry, 2004]. The BAP domain is a substrate for endogenous holocarboxylase synthetase (biotin protein ligase) in mammalian cells [Parrott & Barry, 2000] and the resulting Ad-BAP vectors are metabolically biotinylated during production in 293 cells. This versatile system permits attachment of any biotinylated ligand (protein, carbohydrate, or small molecule) to the Ad capsid through the extremely tight avidin-biotin interaction (up to femtomolar affinity) and provides a platform for ligand screening. In a recent study, we used the BAP system to directly compare targeting through the Ad fiber, protein IX and hexon capsomeres, using a variety of high-affinity ligands (antibodies, transferrin, and cholera toxin B subunit) on multiple cell types. Surprisingly, efficient Ad targeting was only observed when the fiber protein was used to anchor the targeting ligand to the capsid [Campos & Barry, 2006], a result also observed in a recent similar study by insertion of RGD motifs in the same capsomeres [Kurachi *et al.*, 2007].

Another combinatorial targeting method combines genetic and chemical modification of the Ad capsid, also providing a useful platform for ligand screening. Engineering of cysteine residues into the HI loop permits specific covalent coupling of diverse ligands through thiol chemistry [Kreppel *et al.*, 2005]. This method is also unique in that it permits simultaneous retargeting and detargeting through chemical modification. Detargeting can be achieved by PEGylation through amine-reactive conjugates *in vitro* while retargeting is facilitated through thiol chemistry at the introduced HI loop cysteine residues. While these versatile combinatorial systems may not be ideal for clinical use, they do enable the rapid screening of a wide variety of ligands for Ad vector targeting.

Fiber Swapping and Fiber Replacement

Another strategy for redirecting Ad5 vectors to alternate cell types exploits both the natural diversity and evolutionary conservation of the adenoviridae family. Previous work has shown that the Ad5 fiber can be swapped with fibers of other Ad serotypes thereby creating "pseudotyped" hybrid vectors [Havenga *et al.*, 2002]. The modular design of the fiber protein enables the short ~45 amino acid fiber tail of Ad5 to be swapped onto the shaft and knob of other fibers to create these pseudotyped vectors (Fig. **3**). The fiber-swapping of Ad5 fibers with those from subgroup B adenoviruses like Ad3, Ad35, or Ad11 results in vectors with CAR-independent transduction, mediated through the group B Ad receptors CD46, CD80/CD86, or "receptor X" [Gaggar *et al.*, 2003, Segerman *et al.*, 2003, Short *et al.*, 2004, Sirena *et al.*, 2004, Tuve *et al.*, 2006]. Although this strategy does introduce alternate tropism into the Ad5 capsid, it does not result in truly targeted vectors, as the natural cellular receptors for the group B adenoviruses are present on many cell types.

Fiber replacement is an even more innovative approach being pursued by several groups. It has been shown that the trimerization function of the fiber knob can be replaced by extrinsic trimerization motifs like the MoMuLV envelope glycoprotein trimerization domain [van Beusechem *et al.*, 2000], the neck region peptide (NRP) from human lung surfactant protein D [Magnusson *et al.*, 2001], and the foldon domain from the bacteriophage T4 fibritin protein [Krasnykh *et al.*, 2001]. The Ad fiber has also been replaced by the structurally similar reovirus σ1 protein [Chappell *et al.*, 2002], resulting in a chimeric vector that could transduce cells through the reovirus receptors JAM1 and sialic acid [Mercier *et al.*, 2004]. These fiber replacement strategies have proven useful as scaffolds for the display of various targeting ligands like small peptide ligands [Schagen *et al.*, 2006], *S. aureus* protein A domains for subsequent display of immunoglobulins [Henning *et al.*, 2005], human CD40L [Belousova *et al.*, 2003], and stabilized single chain antibodies, engineered to properly fold in the reducing environments of the cytoplasm and nucleus [Hedley *et al.*, 2006]. Other reports of "knobless" vectors whereby the knobs are proteolylically removed after vector production, thereby exposing cell targeting domains have also been described [Hong *et al.*, 2003].

One major caveat of these fiber replacement systems is the apparent inefficiency of chimeric fiber encapsidation relative to wild type fiber. A prerequisite for the development of a successful fiber psuedotype or chimera is trimerization of the fiber. Unfortunately, trimerization is necessary but not sufficient for encapsidation, and it has no predictive value on how well such a fiber trimer will encapsidate into the Ad penton base. Indeed, many chimeras for fiber-

replacement have encapsidation and stability defects [Henning *et al.*, 2006, Noureddini *et al.*, 2006]. While one might expect this for some of the most synthetic of chimeras, it is possible that there may be subtle structural and encapsidation phenotypes even when fusing fibers from two human Ads (e.g. Ad5/F35 can have particle to plaque ratios 5-fold higher than Ad5). Optimization of these fiber chimeras can restore the encapsidation efficiency to near wild-type levels, but it appears that different chimeras displaying different ligands will require separate optimization [Noureddini *et al.*, 2006]. The replacement of the Ad fiber with these foreign trimerization domains results in detargeted vectors, ablated for the natural cellular interactions necessary for transduction and these strategies may therefore provide the basis for truly targeted Ad vectors, deleted for natural host cell interactions and specifically targeted towards certain cell and tissue types.

Propagation of such vectors in standard 293 cells can be difficult since fiber replacement ablates the natural pathways for Ad infection. Cell lines engineered to express the wild type fiber protein [Von Seggern *et al.*, 2000] have been shown to complement the deletion of fiber and can support the production of these vectors. Effective "pseudo-receptor" systems have also been described that enable production of these fiber-deleted vectors [Krasnykh *et al.*, 2001, Roelvink *et al.*, 1999]. These systems are typically based on an epitope insertion in the Ad capsid binding to cell-surface single chain antibodies, acting as surrogate receptors. Adenovirus "stripping" is another elaborate way to avoid difficulties in vector production. These vectors have only a short fragment of the shaft fused to the NRP trimerization domain, a cell-binding ligand, a specific protease cleavage site, and the fiber knob domain. The vectors can propagate on unmodified 293 cells (via the knob domain) and after purification, treatment with a specific protease cleaves off the unwanted knob thereby exposing the cell targeting ligand [Hong *et al.*, 2003].

CONCLUSION

The basic biology of adenovirus, from its beautiful icosahedral architecture to its *in vivo* pharmacological behavior, is very complex. Substantial progress has been made towards our understanding of Ad vectors, but a more comprehensive knowledge and understanding of this biology must be achieved before the successful development of safer, more effective vectors, targeted to the site of disease. A detailed understanding of the structure/function relationships between Ad virions and host cells/proteins is necessary for the rational design of more effective vectors. More thorough investigations may enable genetic modification of the virions to improve their *in vivo* performance. Once the roles of specific capsid proteins are more defined, it may be possible to mutate capsid residues and domains involved in vector uptake and clearance by immune cells and activation of innate immunity without compromising critical functions like virion stability, target cell binding and uptake, intracellular trafficking, virion disassembly, and delivery of therapeutic transgenes to the nucleus. Ultimately, this rational engineering of the capsid, together with advances in Ad vector genome design, incorporating modified transposon elements and targeted integration capabilities, for the stable long term expression of therapeutic genes in human tissues [Izsvak & Ivics, 2004, Thyagarajan *et al.*, 2001, Yant *et al.*, 2002], may yield a new generation of Ad vectors for the safe and effective gene therapy of hereditary and acquired disease.

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REFERENCES

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D. & Brown, F. (1989). The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. *Nature*, **337**, 709-716.

Akalu, A., Liebermann, H., Bauer, U., Granzow, H. & Seidel, W. (1999). The subgenus-specific C-terminal region of protein IX is located on the surface of the adenovirus capsid. *J. Virol.*, **73**, 6182-6187.

Alemany, R. & Curiel, D. T. (2001). CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. *Gene. Ther.*, **8**, 1347-1353.

Alemany, R., Suzuki, K. & Curiel, D. T. (2000). Blood clearance rates of adenovirus type 5 in mice. *J. Gen. Virol.*, **81**, 2605-2609.

Amalfitano, A. (1999). Next-generation adenoviral vectors: new and improved. *Gene. Ther.*, **6**, 1643-1645.

Anderson, C. W. (1990). The proteinase polypeptide of adenovirus serotype 2. *Virology*, **177**, 259-272.

Ashbourne Excoffon, K. J., Moninger, T. & Zabner, J. (2003). The Coxsackie B virus and adenovirus receptor resides in a distinct membrane microdomain. *J. Virol.*, 4.

Athappilly, F. K., Murali, R., Rux, J. J., Cai, Z. & Burnett, R. M. (1994). The refined crystal structure of hexon, the major coat protein of adenovirus type., 2, at 2.9 Å resolution. *J. Mol. Biol.*, **242**, 430-455.

Bailey, C. J., Crystal, R. G. & Leopold, P. L. (2003). Association of adenovirus with the microtubule organizing center. *J. Virol.*, **77**, 13275-13287.

Balakireva, L., Schoehn, G., Thouvenin, E. & Chroboczek, J. (2003). Binding of adenovirus capsid to dipalmitoyl phosphatidylcholine provides a novel pathway for virus entry. *J. Virol.*, **77**, 4858-4866.

Baranowski, E., Ruiz-Jarabo, C. M. & Domingo, E. (2001). Evolution of cell recognition by viruses. *Science*, **292**, 1102-1105.

Barnett, B.G., Crews, C. J. & Douglas, J. T. (2002). Targeted adenoviral vectors. *Biochim. Biophys. Acta*, **1575**, 1-14.

Barry, M. A., Dower, W. J. & Johnston, S. A. (1996). Toward cell-targeting gene therapy vectors: Selection of cell-binding peptides from random peptide-presenting phage libraries. *Nat. Med.*, **2**, 299-305.

Bayo-Puxan, N., Cascallo, M., Gros, A., Huch, M., Fillat, C. & Alemany, R. (2006). Role of putative heparan sulfate glycosaminoglycan-binding site of the adenovirus type 5 fiber shaft on liver detargeting and knob-mediated targeting. *J. Gen. Virol.*, **87**, 2487-2495.

Belousova, N., Korokhov, N., Krendelshchikova, V., Simonenko, V., Mikheeva, G., Triozzi, P. L., Aldrich, W. A., Banerjee, P. T., Gillies, S. D., Curiel, D. T. & Krasnykh, V. (2003). Genetically targeted adenovirus vector directed to CD40-expressing cells. *J. Virol.*, **77**, 11367-11377.

Belousova, N., Krendelchtchikova, V., Curiel, D. T. & Krasnykh, V. (2002). Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein. *J. Virol.*, **76**, 8621-8631.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L. & Finberg, R. W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*, **275**, 1320-1323.

Bewley, M. C., Springer, K., Zhang, Y., Freimuth, P. & Flanagan, J. M. (1999). Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. *Science*, **286**, 1579-1583.

Brown, D. T., Westphal, M., Burlingham, B. T., Winterhoff, U. & Doerfler, W. (1975). Structure and composition of the adenovirus type 2 core. *J. Virol.*, **16**, 366-387.

Campos, S. K. & Barry, M. A. (2004). Rapid construction of capsid-modified adenoviral vectors through bacteriophage λ Red recombination. *Hum. Gene. Ther.*, **15**, 1125-1130.

Campos, S. K. & Barry, M. A. (2006). Comparison of adenovirus fiber, protein IX, and hexon capsomeres as scaffolds for vector purification and cell targeting. *Virology*, **349**, 453-462.

Campos, S. K., Parrott, M. B. & Barry, M. A. (2004). Avidin-based targeting and purification of a protein IX-modified, metabolically biotinylated adenoviral vector. *Mol. Ther*., **9**, 942-954.

Cepko, C. L. & Sharp, P. A. (1982). Assembly of adenovirus major capsid protein is mediated by a nonvirion protein. *Cell*, **31**, 407-415.

Chappell, J. D., Prota, A. E., Dermody, T. S. & Stehle, T. (2002). Crystal structure of reovirus attachment protein sigma1 reveals evolutionary relationship to adenovirus fiber. *EMBO J.*, **21**, 1-11.

Chatterjee, P. K., Vayda, M. E. & Flint, S. J. (1985). Interactions among the three adenovirus core proteins. *J. Virol*., **55**, 379-386.

Chaudhuri, S. R., Mallam, J. N., Chevez-Barrios, P., Wadhwa, L., Ng, P., Hurwitz, M. Y. & Hurwitz, R. L. (2007). Modulation of adenoviral transduction *in vitro* and *in vivo* by hyaluronan and its receptor CD44. *Mol. Ther*., In Press, doi:10.1038/sj.mt.6300044.

Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M. & Ingber, I. G. (1997). Geometric control of cell life and death. *Science*, **276**, 1425-1428.

Chiu, C. Y., Mathias, P., Nemerow, G. R. & Stewart, P. L. (1999). Structure of adenovirus complexed with its internalization receptor, avb5 integrin. *J. Virol*., **73**, 6758-6768.

Chiu, C. Y., Wu, E., Brown, S. L., Von Seggern, D. J., Nemerow, G. R. & Stewart, P. L. (2001). Structural analysis of a fiber-pseudotyped adenovirus with ocular tropism suggests differential modes of cell receptor interactions. *J. Virol*., **75**, 5375-5380.

Cichon, G., Boeckh-Herwig, S., Schmidt, H. H., Wehnes, E., Muller, T., Pring-Akerblom, P. & Burger, R. (2001). Complement activation by recombinant adenoviruses. *Gene Ther*., **8**, 1794-1800.

Cohen, C. J., Shieh, J. T. C., Pickles, R. J., Okegawa, T., Hsieh, J. T. & Bergelson, J. M. (2001). The Coxsackie and adenovirus receptor is a transmembrane component of the tight junction. *Proc. Natl. Acad. Sci. USA*, **98**, 15191-15196.

Colby, W. W. & Shenk, T. (1981). Adenovirus type 5 virions can be assembled *in vivo* in the absence of detectable polypeptide IX. *J. Virol*., **39**, 977-980.

Corden, J., Engelking, M. & Pearson, G. (1976). Chromatin-like organization of the adenovirus chromosome. *Proc. Natl. Acad. Sci. USA*, **73**, 401-404.

Cotter, M. J., Zaiss, A. K. & Muruve, D. A. (2005). Neutrophils interact with adenovirus vectors via Fc receptors and complement receptor 1. *J. Virol*., **79**, 14622-14631.

Crompton, J., Toogood, C. I. A., Wallis, N. & Hay, R. T. (1994). Expression of a foreign epitope on the surface of the adenovirus hexon. *J. Gen. Virol*., **75**, 133-139.

Croyle, M. A., Chirmule, N., Zhang, Y. & Wilson, J. M. (2002). PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. *Hum. Gene Ther.*, **13**, 1887-900.

Davison, A. J., Benko, M. & Barrach, B. (2003). Genetic content and evolution of adenoviruses. *J. Gen. Virol.*, **84**, 2895-908.

Dechecchi, M. C., Melotti, P., Bonizzato, A., Santacatterina, M., Chilosi, M. & Cabrini, G. (2001). Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. *J. Virol.*, **75**, 8772-8780.

Dmitriev, I., Krasnykh, V., Miller, C. R., Wang, M., Kashenteva, G., Mikheeva, N., Belousova, N. & Curiel, D. T. (1998). An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilitization of a coxsackievirus and adenovirus receptor-independent mechanism. *J. Virol*., **72**, 9706-9713.

Dmitriev, I. P., Kashentseva, E. A. & Curiel, D. T. (2002). Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX. *J. Virol*., **76**, 6893-6899.

Eggerman, T. L., Mondoro, T. H., Lozier, J. N. & Vostal, J. G. (2002). Adenoviral vectors do not induce, inhibit, or potentiate human platelet aggregation. *Hum. Gene Ther*., **13**, 125-128.

Everitt, E., Sundquist, B., Pettersson, U. & Philipson, L. (1973). Structural polypeptides of adenoviruses X. Isolation ond topography of low molecular weight antigens from the virion of adenovirus type 2. *Virology*, **52**, 130-147.

Fabry, C. M. S., Rosa-Calatrava, M., Conway, J. F., Zubieta, C., Cusack, S., Ruigrok, R. W. H. & Schoehn, G. (2005). A quasi-atomic model of human adenovirus type 5. *EMBO J*., **24**, 1645-1654.

Fisher, K. D., Stallwood, Y., Green, N. K., Ulbrich, K., Mautner, V. & Seymour, L. W. (2001). Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Ther*., **8**, 341-348.

Fontana, L., Nuzzo, M., Urbanelli, L. & Monaci, P. (2003). General strategy for broadening adenovirus tropism. *J. Virol*., **77**, 11094-11104.

Furcinitti, P. S., van Oostrum, J. & Burnett, R. M. (1989). Adenovirus polypeptide IX revealed as capsid cement by difference images from electron microscopy and crystallography. *EMBO J*., **8**, 3563-3570.

Gaggar, A., Shayakhmetov, D. M. & Lieber, A. (2003). CD46 is a cellular receptor for group B adenoviruses. *Nat. Med*., **9**, 1408-1412.

Ghosh, D. & Barry, M. A. (2005). Selection of muscle-binding peptides from context-specific peptide-presenting phage libraries for adenoviral vector targeting. *J. Virol*., **79**, 13667-13672.

Glasgow, J. N., Everts, M. & Curiel, D. T. (2006). Transductional targeting of adenovirus vectors for gene therapy. *Cancer Gene Ther*., **13**, 830-844.

Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from adenovirus type 5. *J. Gen. Virol*., **36**, 59-72.

Greber, U. F. (2002). Signalling in viral entry. *Cell Mol. Life Sci.*, **59**, 608-626.

Greber, U. F., Willetts, M., Webster, P. & Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. *Cell*, **75**, 477-486.

Hannan, C., Raptis, L. H., Dery, C. V. & Weber, J. (1983). Biological and structural studies with an adenovirus type 2 temperature-sensitive mutant defective for uncoating. *Intervirology*, **19**, 213-223.

Hartman, Z. C., Kiang, A., Everett, R. S., Serra, D., Yang, X. Y., Clay, T. M. & Amalfitano, A. (2007). Adenovirus infection triggers a rapid, MyD88-regulated transcriptome response critical to acute-phase and adaptive immune responses *in vivo*. *J. Virol*., **81**, 1796-1812.

Hassell, J. A. & Weber, J. (1978). Genetic analysis of adenovirus type 2. VIII. Physical locations of temperature-sensitive mutations. *J. Virol*., **28**, 671-678.

Havenga, M. J., Lemckert, A. A., Ophorst, O. J., van Meijer, M., Germeraad, W. T., Grimbergen, J., van Den Doel, M. A., van Deutekom, J., Janson, A. A., de Bruijn, J. D., Uytdehaag, F., Quax, P. H., Logtenberg, T., Mehtali, M. & Bout, A. (2002). Expoliting the natural diversity in adenovirus tropism for therapy and prevention of disease. *J. Virol*., **76**, 4612-4620.

Hedley, S. J., Auf der Maur, A., Hohn, S., Escher, D., Barberis, A., Glasgow, J. N., Douglas, J. T., Korokhov, N. & Curiel, D. T. (2006). An adenovirus vector with a chimeric fiber incorporating stabilized single chain antibody achieves targeted gene delivery. *Gene Ther*., **13**, 88-94.

Henning, P., Andersson, K. M., Frykholm, K., Ali, A., Magnusson, M. K., Nygren, P. A., Granio, O., Hong, S. S., Boulanger, P. & Lindholm, L. (2005). Tumor cell targeted gene delivery by adenovirus 5 vectors carrying knobless fibers with antibody-binding domains. *Gene Ther*., **12**, 211-224.

Henning, P., Lundgren, E., Carlsson, M., Frykholm, K., Johannisson, J., Magnusson, M. K., Tang, E., Franqueville, L., Hong, S. S., Lindholm, L. & Boulanger, P. (2006). Adenovirus type 5 fiber knob domain has a critical role in fiber protein synthesis and encapsidation. *J. Gen. Virol*., **87**, 3151-3160.

Herman, J. R., Adler, H. L., Aguilar-Cordova, E., Rojas-Thompson, T. C. & Scardino, P. T. (1999). *In situ* gene therapy for adenocarcinoma of the prostate: A phase I clinical trial. *Hum. Gene Ther*., **10**, 1239-1249.

Honda, T., Saitoh, H., Masuko, M., Katagiri-Abe, T., Tominaga, K., Kozakai, I., Kobayashi, K., Kumanishi, T., Watanabe, Y. G., Odani, S. & Kuwano, R. (2000). The Coxsackievirus-adenovirus receptor protein as a cell adhesion molecule in the developing mouse brain. *Mol. Brain Res*., **77**, 19-28.

Hong, S. S., Magnusson, M. K., Henning, P., Lindholm, L. & Boulanger, P. (2003). Adenovirus stripping: a versatile method to generate adenovirus vectors with new target specificity. *Mol. Ther*., **7**, 692-699.

Horne, R. W., Brenner, S., Walterson, A. P. & Wildly, P. (1959). The icosahedral form of an adenovirus. *J. Mol. Biol.*, **1**, 84-86.

Horwitz, M. S. (2001). Adenoviruses. In *Fields Virology*, Fourth Edition edn. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.

Howitt, J., Anderson, C. W. & Freimuth, P. (2003). Adenovirus interaction with its cellular receptor CAR. *Curr. Top. Microbiol. Immunol*., **272**, 331-364.

Iacobelli-Martinez, M. & Nemerow, G. R. (2007). Preferential activation of toll-like receptor nine by CD46-utilizing adenoviruses. *J. Virol*., **81**, 1305-1312.

Imelli, N., Meier, O., Boucke, K., Hemmi, S. & Greber, U. F. (2004). Cholesterol is required for endocytosis and endosomal escape of adenovirus type 2. *J. Virol.*, **78**, 3089-3098.

Izsvak, Z. & Ivics, Z. (2004). Sleeping beauty transposition: Biology and applications for molecular therapy. *Mol. Ther*., **9**, 147-156.

Jiang, H., Wang, Z., Serra, D., Frank, M. M. & Amalfitano, A. (2004). Recombinant adenovirus vectors activate the alternative complement pathway, leading to the binding of human complement protein C3 independent of anti-Ad antibodies. *Mol. Ther*., **10**, 1140-1142.

Kelkar, S. A., Pfister, K. K., Crystal, R. G. & Leopold, P. L. (2004). Cytoplasmic dynein mediates adenovirus binding to microtubules. *J. Virol*., **78**, 10122-10132.

Kiang, A., Hartman, Z. C., Everett, R. S., Serra, D., Jiang, H., Frank, M. M. & Amalfitano, A. (2006). Multiple innate inflammatory responses induced after systemic adenovirus vector delivery depend on a functional complement system. *Mol. Ther*., **14**, 588-598.

Kirby, I., Davison, E., Beavil, A. J., Soh, C., Wickham, T. J., Roelvink, P. W., Kovesdi, I., Sutton, B. J. & Santis, G. (2000). Identification of contact residues and definition of the CAR-binding site of adenovirus type 5 fiber protein. *J. Virol*., **74**, 2804-2813.

Korokhov, N., Mikheeva, G., Krendelshchikov, A., Belousova, N., Simonenko, V., Krendelshchikova, V., Pereboev, A., Kotov, A., Kotova, O., Triozzi, P. L., Aldrich, W. A., Douglas, J. T., Lo, K., Banerjee, P. T., Gillies, S. D., Curiel, D. T. & Krasnykh, V. (2003). Targeting of adenovirus via genetic modification of the viral capsid combined with a protein bridge. *J. Virol*., **77**, 12931-12940.

Krasnykh, V., Belousova, N., Korokhov, N., Mikheeva, G. & 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.

Krasnykh, V., Dmitriev, I., Mikheeva, G., Miller, C. R., Belousova, N. & Curiel, D. T. (1998). Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J. Virol*., **72**, 1844-1852.

Kreppel, F., Gackowski, J., Schmidt, E. & Kochanek, S. (2005). Combined genetic and chemical capsid modifications enable flexible and efficient de- and retargeting of adenovirus vectors. *Mol. Ther*., **12**, 107-117.

Kritz, A. B., Nicol, C. G., Dishart, K. L., Nelson, R., Holbeck, S., Von Seggern, D., Work, L. M., McVey, J. H., Nicklin, S. A. & Baker, A. H. (2007). Adenovirus 5 fibers mutated at the putative HSPG-binding site show restricted retargeting with targeted peptides in the HI loop. *Mol. Ther.*, In Press, doi:10.1038/sj.mt.6300094.

Kurachi, S., Koizumi, N., Sakurai, F., Kawabata, K., Sakurai, H., Nakagawa, S., Hayakawa, T. & Mizuguchi, H. (2007). Characterization of capsid-modified adenovirus vectors containing heterologous peptides in the fiber knob, protein IX, or hexon. *Gene Ther*., **14**, 266-274.

Lanciotti, J., Song, A., Doukas, J., Sosnowski, B., Pierce, G., Gregory, R., Wadsworth, S. C. & O'Riordan, C. (2003). Targeting adenoviral vectors using heterofunctional polyethylene glycol FGF2 conjugates. *Mol. Ther*., **8**, 99-107.

Le, L. P., Dmitriev, I. P., Davydova, J. G., Yamamoto, M. & Curiel, D. T. (2004a). Genetic labeling of adenovirus with luciferase on pIX for vector detection. *Mol. Ther*., **9**, 297-298.

Le, L. P., Everts, M., Dmitriev, I. P., Davydova, J. G., Yamamoto, M. & Curiel, D. T. (2004b). Fluorescently labeled adenovirus with pIX-EGFP for vector detection. *Mol. Imaging*, **3**, 105-116.

Le, L. P., Le, H. N., Dmietriev, I. P., Davydova, J. G., Gavrikova, T., Yamamoto, S., Curiel, D. T. & Yamamoto, M. (2006). Dynamic monitoring of oncolytic adenovirus *in vivo* by genetic capsid labeling. *J. Natl. Cancer Inst*., **98**, 203-214.

Li, E., Brown, S. L., Stupack, D., Puente, X. S., Cheresh, D. A. & Nemerow, G. R. (1998a). Integrin $\alpha_v\beta_1$ is an adenovirus coreceptor. *J. Virol*., **75**, 5405-5409.

Li, E., Stupack, D., Bokoch, G. M. & Nemerow, G. R. (1998b). Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. *J. Virol*., **72**, 8806-8812.

Li, E., Stupack, D., Klemke, R., Cheresh, D. A. & Nemerow, G. R. (1998c). Adenovirus endocytosis via av integrins requires phosphoinositide-3-OH kinase. *J. Virol*., **72**, 2055-2061.

Li, J., Le, L., Sibley, D. A., Mathis, J. M. & Curiel, D. T. (2005). Genetic incorporation of HSV-1 thymidine kinase into the adenovirus protein IX for functional display on the virion. *Virology*, **338**, 247-258.

Liu, Q. & Muruve, D. A. (2003). Molecular basis of the inflammatory response to adenovirus vectors. *Gene Ther*., **10**, 935-940.

Liu, Q., Zaiss, A. K., Colarusso, P., Patel, K., Haljan, G., Wickham, T. J. & Muruve, D. A. (2003). The role of capsid-endothelial interactions in the innate immune response to adenovirus vectors. *Hum. Gene Ther*., **14**, 627-643.

Lozier, J. N., Metzger, M. E., Donahue, R. E. & Morgan, R. A. (1999). Adenovirus-mediated expression of human coagulation factor IX in the rhesus maqaque is associated with dose-limiting toxicity. *Blood*, **94**, 3968-3975.

Lyons, M., Onion, D., Green, N. K., Aslan, K., Rajaratnam, R., Bazan-Peregrino, M., Phipps, S., Hale, S., Mautner, V., Seymour, L. W. & Fisher, K. D. (2006). Adenovirus type 5 interactions with human blood cells may compromise systemic delivery. *Mol. Ther*., **14**, 118-128.

Magnusson, M. K., Hong, S. S., Boulanger, P. & Lindholm, L. (2001). Genetic retargeting of adenovirus: Novel strategy employing "deknobbing" of the fiber. *J. Virol*., **75**, 7280-7289.

Magnusson, M. K., Hong, S. S., Henning, P., Boulanger, P. & Lindholm, L. (2002). Genetic retargeting of adenovirus vectors: Functionality of targeting ligands and their influence on virus viability. *J. Gene Med*., **4**, 356-370.

Mailly, L., Renaut, L., Rogee, S., Grellier, E., D'Halluin, J. C. & Colin, M. (2006). Improved gene delivery to B lymphocytes using a modified adenovirus vector targeting CD21. *Mol. Ther*., **14**, 293-304.

Mane, V. P., Toietta, G., McCormack, W. M., Conde, I., Clarke, C., Palmer, D., Finegold, M. J., Pastore, L., Ng, P., Lopez, J. & Lee, B. (2006). Modulation of TNF-alpha, a determinant of acute toxicity associated with systemic delivery of first-generation and helper-dependent adenoviral vectors. *Gene Ther*., **13**, 1272-1280.

Marsh, M. P., Campos, S. K., Baker, M. L., Chen, C. Y., Chiu, W. & Barry, M. A. (2006). Cryoelectron microscopy of protein IX-modified adenoviruses suggests a new position for the C-terminus of protein IX. *J. Virol*., **80**, 11881-11886.

Marshall, E. (1999). Gene therapy death prompts review of adenovirus vector. *Science*, **286**, 2244-2245.

Marttila, M., Persson, D., Gustafsson, D., Liszewski, M. K., Atkinson, J. P., Wadell, G. & Arnberg, N. (2005). CD46 is a cellular receptor for all species B adenoviruses except types 3 and 7. *J. Virol*., **79**, 14429-14436.

Matthews, D. A. & Russell, W. C. (1998). Adenovirus core protein V is delivered by the invading virus to the nucleus of the infected cell and later in infection is associated with nucleoli. *J. Gen. Virol*., **79**, 1671-1675.

McConnell, M. J. & Imperiale, M. J. (2004). Biology of adenovirus and its use as a vector for gene therapy. *Hum. Gene Ther*., **15**, 1022-1023.

Meier, O., Boucke, K., Vig Hammer, S., Keller, S., Stidwill, R. P., Hemmi, S. & Greber, U. F. (2002). Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J. Cell Biol*., **158**, 1119-1131.

Meier, O. & Greber, U. F. (2004). Adenovirus endocytosis. *J. Gene Med*., **6**, S152-163.

Mellman, I. (1992). The importance of being acid: The role of acidification in intracellular membrane traffic. *J. Exp. Biol*., **172**, 39-45.

Menezes, K. M., Mok, H. & Barry, M. A. (2006). Increased transduction of skeletal muscle cells by fibroblast growth factor-modified adenoviral vectors. *Hum. Gene Ther*., **17**, 314-320.

Mercier, G. T., Campbell, J. A., Chappell, J. D., Stehle, T., Dermody, T. S. & Barry, M. A. (2004). A chimeric adenovirus vector encoding reovirus attachment protein σ1 targets cells expressing junctional adhesion molecule 1. *Proc. Natl. Acad. Sci. USA*, **101**, 6188-6193.

Meulenbrock, R. A., Sargent, K. L., Lunde, J., Jasmin, B. J. & Parks, R. J. (2004). Use of adenovirus protein IX (pIX) to display large polypeptides on the virion--generation of fluorescent virus through the incorporation of pIX-GFP. *Mol. Ther*., **9**, 617-624.

Mitraki, A., Miller, S. & van Raaij, M. J. (2002). Review: conformation and folding of novel beta-structural elements in viral fiber proteins: the triple beta-spiral and triple beta-helix. *J. Struct. Biol*., **137**, 236-247.

Miyazawa, N., Crystal, R. G. & Leopold, P. L. (2001). Adenovirus serotype 7 retention in a late endosomal compartment prior to cytosol escape is modulated by fiber protein. *J. Virol*., **75**, 1387-1400.

Miyazawa, N., Leopold, P. L., Hackett, N. R., Ferris, B., Worgall, S., Falck-Pederson, E. & Crystal, R. G. (1999). Fiber swap between adenovirus subgroups B and C alters intracellular trafficking of adenovirus gene transfer vectors. *J. Virol*., **73**, 6056-6065.

Mizuguchi, H., Koizumi, N., Hosono, T., Ishii-Watabe, A., Uchida, E., Utoguchi, N., Watanabe, Y. & Hayakawa, T. (2002). CAR- or av integrin-binding ablated adenovirus vectors, but not fiber-modified vectors containing RGD peptide, do not change the systemic gene transfer properties in mice. *Gene Ther*., **9**, 769-776.

Mok, H., Palmer, D. J., Ng, P. & Barry, M. A. (2005). Evaluation of polyethylene glycol modification of first-generation and helper-dependent adenoviral vectors to reduce innate immune responses. *Mol. Ther*., **11**, 66-79.

Muruve, D. A. (2004). The innate immune response to adenovirus vectors. *Hum. Gene Ther*., **15**, 1157-1166.

Muruve, D. A., Cotter, M. J., Zaiss, A. K., White, L. R., Liu, Q., Chan, T., Clark, S. A., Ross, P. J., Meulenbrock, R. A., Maelandsmo, G. M. & Parks, R. J. (2004). Helper-dependent adenovirus vectors elicit intact innate but attenuated adaptive host immune responses *in vivo*. *J. Virol*., **78**, 5966-5972.

Nakano, M. Y., Boucke, K., Suomalainen, M., Stidwill, R. P. & Greber, U. F. (2000). The first step of adenovirus type 2 disassembly occurs at the cell surface, independently of endocytosis and escape to the cytosol. *J. Virol*., **74**, 7085-7095.

Newcomb, W. W., Boring, J. W. & Brown, J. C. (1984). Ion etching of human adenovirus 2: Structure of the core. *J. Virol*., **51**, 52-56.

Newcomb, W. W. & Brown, J. C. (1988). Use of Ar^+ plasma etching to localize structural proteins in viruses: studies with adenovirus 2. *Anal. Biochem*., **169**, 279-286.

Nicklin, S. A., White, S. J., Nicol, C. G., Von Seggern, D. J. & Baker, A. H. (2004). *In vitro* and *in vivo* characterisation of endothelial cell selective adenoviral vectors. *J. Gene Med*., **6**, 300-308.

Noureddini, S. C., Krendelshchikov, A., Simonenko, V., Hedley, S. J., Douglas, J. T., Curiel, D. T. & Korokhov, N. (2006). Generation and selection of targeted adenoviruses embodying optimized vector properties. *Virus Res*., **116**, 185-195.

Othman, M., Labelle, A., Mazetti, I., Elbatarny, H. S. & Lillicrap, D. (2006). Adenovirus-induced thrombocytopenia: the role of von Willebrand factor and P-selectin in mediating accelerated platelet clearance. *Blood*, In Press, doi:10.1182/blood.2006.06.032524.

Palmer, D. J. & Ng, P. (2005). Helper-dependent adenoviral vectors for gene therapy. *Hum. Gene Ther*., **1**, 1-16.

Parker, A. L., McVey, J. H., Doctor, J. H., Lopez-Franco, O., Waddington, S. N., Havenga, M. J., Nicklin, S. A. & Baker, A. H. (2007). Influence of coagulation factor zymogens on the infectivity of adenoviruses pseudotyped with fibers from subgroup D. *J. Virol.*, In Press, doi:10.1128/JVI.02786-06.

Parker, A. L., Waddington, S. N., Nichol, C. G., Shayakhmetov, D. M., Buckley, S. M., Denby, L., Kemball-Cook, G., Ni, S., Lieber, A., McVey, J. H., Nicklin, S. A. & Baker, A. H. (2006). Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes. *Blood*, **108**, 2554-2561.

Parks, R. J. (2005). Adenovirus protein IX: A new look at an old protein. *Mol. Ther*., **11**, 19-24.

Parrott, M. B., Adams, K. E., Mercier, G. T., Mok, H., Campos, S. K. & Barry, M. A. (2003). Metabolically Biotinylated Adenovirus for Cell-targeting, Ligand Screening, and Vector Purification. *Mol. Ther*., **8**, 689-702.

Parrott, M. B. & Barry, M. A. (2000). Metabolic biotinylation of recombinant proteins in mammalian cells and in mice. *Mol. Ther*., **1**, 96-104.

Pasqualini, R. & Ruoslahti, E. (1996). Organ targeting *in vivo* using phage display peptide libraries. *Nature*, **380**, 364-366.

Pelkmans, L., Fava, E., Grabner, H., Hannus, M., Habermann, B., Krausz, E. & Zerial, M. (2005). Genome-wide analysis of human kinases in clathrin- and calveolae/raft-mediated endocytosis. *Nature*, **436**, 78-86.

Pietiainen, V. M., Marjomaki, V., Heino, J. & Hyypia, T. (2005). Viral entry, lipid rafts and caveosomes. *Ann. Med.*, **37**, 394-403.

Prage, L., Pettersson, U., Hoglund, S., Lonberg-Holm, K. & Philipson, L. (1970). Structural proteins of adenoviruses. IV. Sequential degradation of the adenovirus type 2 virion. *Virology*, **42**, 341-358.

Rancourt, C., Keyvani-Amineh, H., Sircar, S., Labrecque, P. & Weber, J. (1995). Proline 137 is critical for adenovirus protease encapsidation and activation but not enzyme activity. *Virology*, **209**, 167-173.

Reddy, D. V., Shenoy, B. C., Carey, P. R. & Sönnichsen, F. D. (2000). High resolution structure of the 1.3S subunit of transcarboxylase from *Propionibacterium. shermanii*. *Biochemistry*, **39**, 2509-2516.

Roelvink, P. W., Lee, G. M., Einfeld, D. A., Kovesdi, I. & Wickham, T. J. (1999). Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science*, **286**, 1568-1571.

Romanczuk, H., Galer, C., Zabner, J., Barsomian, G., Wadsworth, S. C. & O'Riordan, C. (1999). Modification of an adenoviral vector with biologically selected peptides: A novel strategy for gene delivery to cells of choice. *Hum. Gene Ther.*, **10**, 2615-26.

Rowe, W. P., Huebner, R. J., Gillmore, L. K., Parrott, R. H. & Ward, T. G. (1953). Isolation of a cytopathic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. Soc. Exp. Biol. Med*., **84**, 570-573.

Roy-Chowdhury, J. & Horwitz, M. (2002). Evolution of adenoviruses as gene therapy vectors. *Mol. Ther*., **5**, 340-344.

Rux, J. J. & Burnett, R. M. (2000). Type-specific epitope locations revealed by X-ray crystallographic study of adenovirus type 5 hexon. *Mol. Ther*., **1**, 18-30.

Rux, J. J. & Burnett, R. M. (2004). Adenovirus Structure. *Hum. Gene Ther*., **15**, 1167-1176.

Rux, J. J., Kuser, P. R. & Burnett, R. M. (2003). Structural and phylogenetic analysis of adenovirus hexons by use of high-resolution x-ray crystallographic, molecular modeling, and sequence-based methods. *J. Virol*., **77**, 9553-9566.

Saban, S. D., Silvestry, M., Nemerow, G. R. & Stewart, P. L. (2006). Visualization of alpha-helices in a 6 angstrom resolution cryoelectron microscopy structure of adenovirus allows refinement of capsid protein assignments. *J. Virol*., **80**, 12049-12059.

Salone, B., Martina, Y., Piersanti, S., Cundari, E., Cherubini, G., Franqueville, L., Failla, C. M., Boulanger, P. & Saggio, I. (2003). Integrin $\alpha_3\beta_1$ is an alternate cellular receptor for adenovirus serotype 5. *J. Virol*., **77**, 13448-13454.

Schagen, F. H. E., Wensveen, F. M., Carette, J. E., Dermody, T. S., Gerritsen, W. R. & van Beusechem, V. W. (2006). Genetic targeting of adenovirus vectors using a reovirus sigma1-based attachment protein. *Mol. Ther*., **13**, 997-1005.

Scheres, S. H. W., Marabini, R., Lanzavecchia, S., Cantele, F., Rutten, T., Fuller, S. D., Carazo, J. M., Burnett, R. M. & San Martin, C. (2005). Classification of single-projection reconstructions for cryo-electron microscopy data of icosahedral viruses. *J. Struct. Biol*., **151**, 79-91.

Segerman, A., Atkinson, J. P., Marttila, M., Dennerquist, V., Wadell, G. & Arnberg, N. (2003). Adenovirus type 11 uses CD46 as a cellular receptor. *J. Virol*., **77**, 9183-9191.

Seki, T., Dmitriev, I. P., Kashentseva, E., Takayama, K., Rots, M., Suzuki, K. & Curiel, D. T. (2002). Artificial extension of the adenovirus fiber shaft inhibits infectivity in coxackievirus and adenovirus receptor-positive cells. *J. Virol*., **76**, 1100-1108.

Shayakhmetov, D. M., Eberly, A. M., Li, Z. & Lieber, A. (2005a). Deletion of RGD motifs affects the efficiency of both the internalization and the endosome escape of viral particles containing adenovirus serotype 5 or 35 fiber knobs. *J. Virol*., **79**, 1053-1061.

Shayakhmetov, D. M., Gaggar, A., Shaoheng, N., Li, Z. & Lieber, A. (2005b). Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J. Virol.*, **79**, 7478-7491.

Shayakhmetov, D. M., Li, Z. Y., Ni, S. & Lieber, A. (2005c). Interference with the IL-1-signaling pathway improves the toxicity profile of systemically applied adenovirus vectors. *J. Immunol*., **174**, 7310-7319.

Shayakhmetov, D. M., Li, Z. Y., Ternovoi, V., Gaggar, A., Gharwan, H. & Lieber, A. (2003). The interaction between the fiber knob domain and the cellular attachment receptor determines the intracellular trafficking route of adenoviruses. *J. Virol*., **77**, 3712-3723.

Shayakhmetov, D. M. & Lieber, A. (2000). Dependence of adenovirus infectivity on length of the fiber shaft domain. *J. Virol.*, **74**, 10274-10286.

Shenk, T. E. (2001). *Adenoviridae:* The Viruses and Their Replication. In *Fields. Virology*, Fourth Edition edn. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.

Short, J. J., Pereboev, A. V., Kawakami, Y., Vasu, C., Holterman, M. J. & Curiel, D. T. (2004). Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. *Virology*, **322**, 349-359.

Sirena, D., Lilenfeld, B., Eisenhut, M., Kalin, S., Boucke, K., Beerli, R. R., Vogt, L., Ruedl, C., Bachmann, M. F., Greber, U. F. & Hemmi, S. (2004). The human membrane cofactor CD46 is a receptor for species B adenovirus serotype 3. *J. Virol*., **78**, 4454-4462.

Smith, A. E. & Helenius, A. (2004). How viruses enter animal cells. *Science*, **304**, 237-242.

Smith, T. A. G., Idamakanti, N., Kylefjord, H., Rollence, M. L., King, L., Kaloss, M., Kaleko, M. & Stevenson, S. C. (2002). *In vivo* hepatic adenoviral gene delivery occurs independently of the Coxsackievirus-adenovirus receptor. *Mol. Ther*., **5**, 770-779.

Smith, T. A. G., Idamakanti, N., Rollence, M. L., Marshall-Neff, J., Kim, J., Mulgrew, K., Nemerow, G. R., Kaleko, M. & Stevenson, S. C. (2003). Adenovirus serotype 5 fiber shaft influences *in vivo* gene transfer in mice. *Hum. Gene Ther*., **14**, 777-787.

Stewart, P. L., Burnett, R. M., Cyrklaff, M. & Fuller, S. D. (1991). Image reconstruction reveals the complex molecular organization of adenovirus. *Cell*, **67**, 145-154.

Stewart, P. L., Chiu, C. Y., Huang, S., Muir, T., Zhao, Y., Chait, B., Mathias, P. & Nemerow, G. R. (1997). Cryo-EM visualization of an exposed RGD epitope on adenovirus that escapes antibody neutralization. *EMBO J*., **16**, 1189-1198.

Stewart, P. L., Fuller, S. D. & Burnett, R. M. (1993). Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO J*., **12**, 2589-2599.

Strunze, S., Trotman, L. C., Boucke, K. & Greber, U. F. (2005). Nuclear targeting of adenovirus type 2 requires CRM1-mediated muclear export. *Mol. Biol. Cell*., **16**, 2999-3009.

Stupack, D. G., Li, E., Silletti, S. A., Kehler, J. A., Geahlen, R. L., Hahn, K., Nemerow, G. R. & Cheresh, D. A. (1999). Matrix valency regulates integrin-mediated lymphoid adhesion via Syk kinase. *J. Cell Biol*., **144**, 777-788.

Sundquist, B., Everitt, E., Philipson, L. & Hoglund, S. (1973). Assembly of adenoviruses. *J. Virol*., **11**, 449-459.

Tao, N., Gao, G. P., Parr, M., Johnston, J., Baradet, T., Wilson, J. M., Barsoum, J. & Fawell, S. E. (2001). Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction of liver. *Mol. Ther*., **3**, 28-35.

Thyagarajan, B., Olivares, E. C., Hollis, R. P., Ginsburg, D. S. & Calos, M. P. (2001). Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. *Mol. Cell Biol*., **21**, 3926-3935.

Toietta, G., Mane, V. P., Norona, W. S., Finegold, M. J., Ng, P., McDonagh, A. F., Beaudet, A. L. & Lee, B. (2005). Lifelong elimination of hyperbilirubenemia in the Gunn rat with a single injection of helper-dependent adenoviral vector. *Proc. Natl. Acad. Sci. USA*, **102**, 3930-3935.

Trotman, L. C., Achermann, D. P., Keller, S., Straub, M. & Greber, U. F. (2003). Non-classical export of an adenovirus structural protein. *Traffic*, **4**, 390-402.

Trotman, L. C., Mosberger, N., Fornerod, M., Stidwill, R. P. & Greber, U. F. (2001). Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. *Nat. Cell Biol*., **3**, 1092-1100.

Tuve, S., Wang, H., Ware, C., Liu, W., Gaggar, A., Bernt, K., Shayakhmetov, D. M., Li, Z., Strauss, R., Stone, D. & Lieber, A. (2006). A new group B adenovirus receptor is expressed at high levels on human stem and tumor cells. *J. Virol*., **80**, 12109-12120.

Valentine, R. C. & Pereira, H. G. (1965). Antigens and the structure of adenovirus. *J. Mol. Biol*., **13**, 13-20.

van Beusechem, V. W., van Rijswijk, A. L., van Es, H. H., Haisma, H. J., Pinedo, H. M. & Gerritsen, W. R. (2000). Recombinant adenovirus vectors with knobless fibers for targeted gene transfer. *Gene Ther*., **7**, 1940-1946.

van Oostrum, J. & Burnett, R. M. (1985). Molecular composition of the adenovirus type 2 virion. *J. Virol*., **56**, 439-448.

van Raaij, M. J., Mitraki, A., Lavigne, G. & Cusack, S. (1999). A triple β-spiral in the adenovirus fibre shaft reveals a new structural motif for a fibrous protein. *Nature*, **401**, 935-938.

Vellinga, J., Rabelink, M. J., Cramer, S. J., van den Wollenberg, D. J., Van der Meulen, H., Leppard, K. N., Fallaux, F. J. & Hoeben, R. C. (2004). Spacers increase the accessibility of peptide ligands linked to the carboxyl terminus of adenovirus minor capsid protein IX. *J. Virol.*, **78**, 3470-3479.

Vigne, E., Mahfouz, I., Dedieu, J. F., Brie, A., Perricaudet, M. & Yeh, P. (1999). RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J. Virol*., **73**, 5156-5161.

Volpers, C. & Kochanek, S. (2004). Adenoviral vectors for gene transfer and therapy. *J. Gen. Virol*., **6**, S164-S171.

Volpers, C., Thirion, C., Biermann, V., Hussmann, S., Kewes, H., Dunant, P., von der Mark, H., Herrmann, A., Kochanek, S. & Lochmuller, H. (2003). Antibody-mediated targeting of an adenovirus vector modified to contain a synthetic IgG-binding domain in the capsid. *J. Virol*., **77**, 2093-2104.

Von Seggern, D. J., Huang, S., Fleck, S. K., Stevenson, S. C. & Nemerow, G. R. (2000). Adenovirus vector pseudotyping in fiber-expressing cell lines: Improved transduction of Epstein-Barr virus-transformed cells. *J. Virol*., **74**, 354-364.

Walters, R. W., Freimuth, P., Moninger, T., Ganske, I., Zabner, J. & Welsh, M. J. (2002). Adenovirus fiber disrupts CAR-mediated intercellular adhesion aloowing virus escape. *Cell*, **110**, 789-799.

Wickham, T. J., Mathias, P., Cheresh, D. A. & Nemerow, G. R. (1993). Integrins a_vb_3 and a_vb_5 promote adenovirus internalization but not adenovirus attachment. *Cell*, **73**, 309-319.

Wickham, T. J., Roelvink, P. W., Brough, D. E. & Kovesdi, I. (1996). Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. *Nat. Biotech.*, **14**.

Wickham, T. J., Segal, D. M., Roelvink, P. W., Carrion, M. E., Lizonova, A., Lee, G. M. & Kovesdi, I. (1997). Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J. Virol*., **70**, 6831-6838.

Wiethoff, C. M., Wodrich, H., Gerace, L. & Nemerow, G. R. (2005). Adenovirus protein VI mediates membrane disruption following capsid disassembly. *J. Virol*., **79**, 1992-2000.

Wisnivesky, J. P., Leopold, P. L. & Crystal, R. G. (1999). Specific binding of the adenovirus capsid to the nuclear envelope. *Hum. Gene Ther*., **10**, 2187-2195.

Wodrich, H., Guan, T., Cingolani, G., Von Seggern, D., Nemerow, G. & Gerace, L. (2003). Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. *EMBO J*., **22**, 6245-6255.

Work, L. M., Nicklin, S. A., Brain, N. J., Dishart, K. L., Von Seggern, D. J., Hallek, M., Buning, H. & Baker, A. H. (2004). Development of efficient viral vectors selective for vascular smooth muscle cells. *Mol. Ther*., **9**, 198-208.

Wu, E., Pache, L., Von Seggern, D. J., Mullen, T. M., Mikyas, Y., Stewart, P. L. & Nemerow, G. R. (2003). Flexibility of the adenovirus fiber is required for efficient receptor interaction. *J. Virol*., **77**, 7225-7235.

Wu, H., Han, T., Belousova, N., Krasnykh, V., Kashentseva, E., Dmitriev, I., Kataram, M., Mahasreshti, P. J. & Curiel, D. T. (2005). Identification of sites in adenovirus hexon for foreign peptide incorporation. *J. Virol*., **79**, 3382-3390.

Xia, D., Henry, L. J., Gerard, R. D. & Deisenhofer, J. (1994). Crystal structure of the receptor binding domain of adenovirus type 5 at 1.7A resolution. *Structure*, **2**, 1259-1270.

Yant, S. R., Ehrhardt, A., Mikkelsen, J. G., Meuse, L., Pham, T. & Kay, M. A. (2002). Transposition from a gutless adeno-transposon vector stabilizes transgene expression *in vivo*. *Nat. Biotechnol*., **20**, 999-1005.

Zerial, M. & McBride, H. (2001). Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol*., **2**, 107-117.

Zhang, Y., Chirmule, N., Gao, G., Qian, R., Croyle, M. A., Joshi, B., Tazelaar, J. & Wilson, J. M. (2001). Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophage. *Mol. Ther*., **3**, 697-707.

Zhu, J., Huang, X. & Yang, Y. (2007). Innate immune response to adenoviral vectors is mediated by both TLR-dependent and -independent pathways. *J. Virol.*, In Press, doi:10.1128/JVI.02192-06.

Zubieta, C., Schoehn, G., Chroboczek, J. & Cusack, S. (2005). The structure of the human adenovirus 2 penton. *Mol. Cell*, **17**, 121-135.

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