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REVIEW ARTICLE

Retargeting adenoviruses for therapeutic applications and vaccines

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Adenoviruses (Ads) are robust vectors for therapeutic applications and vaccines, but their use can be limited by differences in their *in vitro* and *in vivo* pharmacologies. This review emphasizes that there is not just one Ad, but a whole virome of diverse viruses that can be used as therapeutics. It discusses that true vector targeting involves not only retargeting viruses, but importantly also detargeting the viruses from off-target cells.

Keywords: Ad serotypes; detargeting; gain of function; liver; retargeting; sequestration; serotypes

Adenoviruses (Ads) have many features that make them useful as oncolytic viruses, as gene-based vaccines, or as gene therapy vectors. First and foremost, they can be produced at exceptionally high yields up to 10^{13} virus particles from 10^{9} cells. Ads are also stable nonenveloped viruses that can be lyophilized for long-term storage without a cold chain [1–5]. This is quite different from the diverse array of enveloped viral vectors that are inactivated by freeze-drying. This is important for storage, but also for deployment of global applications to regions where refrigeration is not always available.

Ad vectors mediate high transduction efficiency in dividing and nondividing cells. Ads do not actively integrate into the host genome reducing the risk of insertional oncogenesis [6], but this also limits their persistence in actively dividing cells [7,8]. On the other hand, Ad genomes can persist for years in nondividing cells provided that an immune response is not produced against Ad or the transgene product. For example, baboons injected once with helper-dependent Ad (HD-Ad) vectors have had persistent transgene expression for more than 7 years [9].

In many head-to-head *in vivo* comparisons, Ads mediate higher expression and more potent vaccine effects than most other vectors [10–15]. For example, when compared to DNA or vaccinia virus as an HIV vaccine in macaques, replication-defective Ad5 (RD-Ad5) vectors generated higher immune responses and better protection [12,13]. In an example from our laboratory, in gene therapy for propionic acidemia, a 10-fold lower gene dose of RD-Ad5 generated equal to or higher *PCCA* transgene expression than the popular adeno-associated virus 8 vector [10].

While Ads are arguably the most potent *in vivo* gene expression platform, they are also well known for their ability to provoke immune responses and for a tragic death in an early gene therapy trial for ornithine transcarbamylase deficiency [16]. This makes them highly sought as gene-based vaccines and oncolytic viruses,

Abbreviations

Ad, Adenovirus; BAP, biotin acceptor peptide; CAR, coxsackie and adenovirus receptor; CRAd, conditionally replicating adenovirus; DNA-TPC, Ad DNA/terminal protein complex; FIX, blood clotting factor IX; FX, blood clotting factor X; GMP, good manufacturing practice; HD-Ad, helper-dependent adenovirus; HVR, hypervariable region; IV, intravenous; LSEC, liver sinusoidal endothelial cell; RC-Ad, replication-competent adenovirus; RD-Ad, replication-defective adenovirus; RES, reticuloendothelial system; SC-Ad, single-cycle adenovirus.

but has restricted their use for gene therapy. This lack of use for gene therapy is largely political rather than scientific, since newer HD-Ad vectors and polymer shielding approaches largely mitigate most of their side effects [9,17–21].

This review discusses retargeting and detargeting Ads for therapeutic and vaccine applications. This retargeting can be imposed first by physically retargeting Ad particles to different receptors. For replication-competent vectors, a second layer of targeting can be applied postentry by controlling how Ads activate their genetic program in cell-specific ways. A third layer of control can be added by controlling transgene expression in cell or situation-specific fashions. We will delve into these technologies later in the article, but must first lay the foundations of how the genetically diverse adenovirus virome provides opportunities to start targeting efforts with viruses that are already tuned to different applications.

Adenovirus capsid proteins as platforms for physical particle targeting and off-target interactions

There are three major capsid proteins on Ads: fiber, penton base, and hexon (Fig. 1, reviewed in Ref. [22,23]). There are 36 monomers of fiber, 60 monomers of penton base, and 720 monomers of hexon on each Ad virion. There is good evidence that the fiber and penton base proteins of many Ad serotypes interact directly with cellular receptors, but there is little evidence showing that hexons directly target cellular receptors. One exception to this is binding of Ad hexons to scavenger receptors on macrophages, Kupffer cells, and endothelial cells [24,25]. While these interactions are usually destructive to Ads *in vivo* [24], ectopic expression of scavenger receptors on cells *in vitro* can lead to productive infection [26]. A more recent observation shows that certain human Ads can bind scavenger receptor MARCO (SR-A6) for productive infection [27].

There is also at least one minor protein, IX, that can also display targeting ligands. Beyond fiber, penton, IX, and hexon, all other viral proteins are hidden within the virion or are not packaged into virions. These four proteins can serve on scaffolds to display 36, 60, 240, or 720 copies of targeting ligands, respectively [28]. Low-affinity ligands like peptides from library selections may not work well if displayed in low copy fibers, but might work well if displayed on more capsomers to allow avidity interactions. High-affinity ligands should theoretically work on any

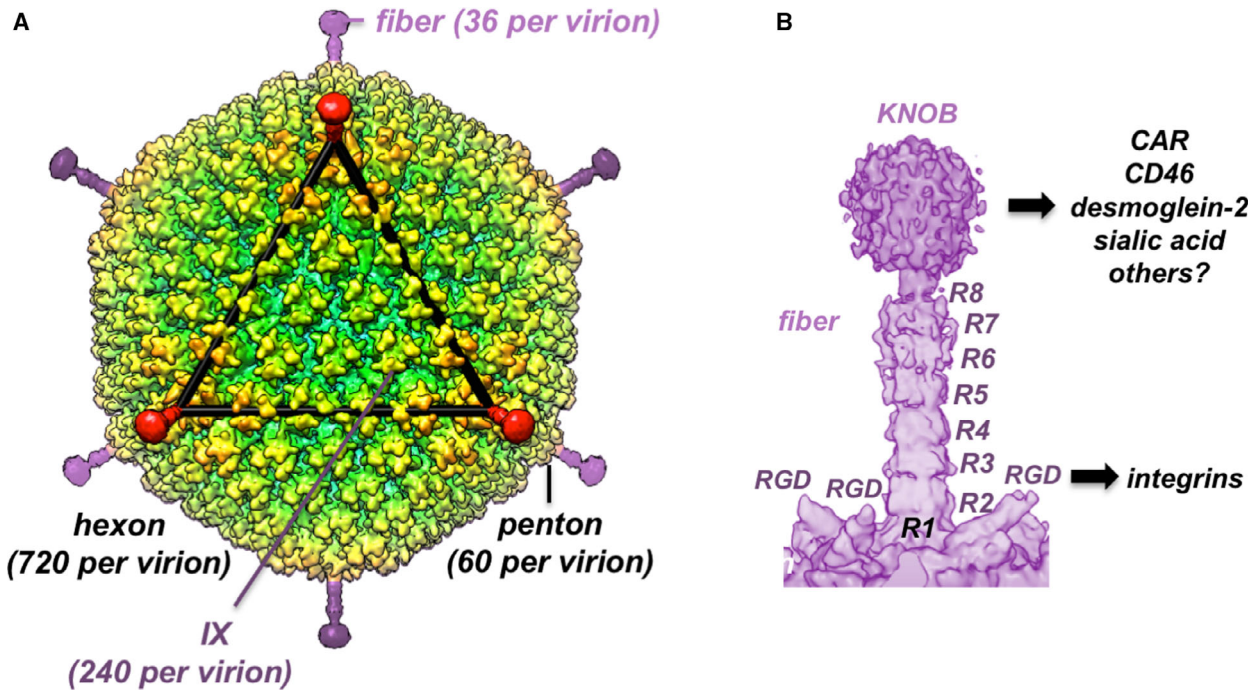

Fig. 1. Cryo-electron microscopic structures of Ad26. (A) Full virion structure. (B) Fiber and penton base. R indicated fiber shaft repeats. RGD indicates arginine–glycine–aspartic acid integrin binding motifs in the penton base. Knob indicates the receptor binding portion of the Ad26 fiber trimer. Receptors bound by these capsomers are shown on the right. Adapted from Ref. [33].

capsomer, but data using biotinylated vectors suggest that only fiber may be good for very high-affinity ligands (see below).

In vitro, Ad fiber proteins act as primary high-affinity attachment ligands for these viruses provided their receptors are expressed on target cells. Three fiber monomers trimerize to form a fiber at each vertex of the icosahedral capsid (Fig. 1B). These fibers form a 'knob' domain at their C terminus that is involved with most receptor interactions. Although fibers have this same basic structure, their shaft length, flexibility, and receptor binding vary considerably. The archetype adenovirus, human Ad serotype 5 (HAdV-5, hereafter referred to as Ad5) has a knob that binds to the coxsackie and adenovirus receptor (CAR). Most human Ads have only one fiber trimer, but three others express two different fibers [29], a long fiber and a short fiber. Fastidious gastrointestinal human Ad40 and 41 from species F were the examples for having novel dual fibers. In their cases, the long fibers bound CAR, but the short fiber did not appear to have overt receptor binding functions [30,31].

Most Ads have an RGD motif in their penton base that binds to integrins [32]. This RGD motif is displayed on loops with different lengths by the different viruses [33]. Ad5's fiber binds CAR with 10-fold higher affinity than its penton binds α_v integrins [32]. Because of this affinity difference, species C viruses have been shown to first engage CAR, and then bind integrins, which facilitate receptor-mediated endocytosis [34]. This is how it works *in vitro* in a cell culture dish and perhaps on mucosa, but this staged interaction is overwritten after an intravenous (IV) injection by other interactions with host factors in the blood.

Other Ads can bind to CAR, CD46, sialic acid, desmoglein-2, and perhaps other receptors (Fig. 1B, [23]). For many years, a non-CD46 additional receptor for species B viruses Ad3, Ad7, Ad11, and Ad14 was a mystery. This 'receptor X' was ultimately identified as desmoglein-2 by Lieber and colleagues [35]. More recent work with Ad3 shows that its fiber binds desmoglein-2 in an unusual 1 : 1 stoichiometry [36,37].

Species D Ad37 is the archetype for viruses using sialic acid as a receptor [38,39]. Ad37 and most Ads do not use simple sialic acid for binding. Species D human Ad37 is also the archetype virus for causing keratoconjunctivitis. Like Ad37, species D Ad8, Ad53, Ad54, Ad56, and Ad64 are also associated with this disease [40]. Recent comparison of these viruses' utilization of sialic acid on corneal cells *in vitro* demonstrated that Ad8, Ad53, Ad54, and Ad64 all use this receptor [40]. In contrast, Ad56 did not.

Sialic acid binding Ads can be quite specific for certain sialic acid structures. For example, Ad37 uses sialic acid only as presented in GD1a glycans [38,39]. The relatively new species D human Ad52 joins Ad40 and Ad41 in having two fibers: a short and long one. Like Ad40 and Ad41, Ad52 binds CAR, but also binds sialic acid [41]. More detailed examination of this interaction shows that the short fiber of Ad52 binds long chains of alpha-2,8-linked polysialic acid [29].

Human species D Ad26 is in rampant use as a gene-based vaccine and as an oncolytic virus [42–50]. There is ongoing debate on this virus' receptor utilization. Original *in vitro* data on artificial CAR- and CD46-modified cells indicated that Ad26 did not use CAR, but instead used CD46 for infection [42]. While Ad26 infection was increased on cells expressing CD46, this infection was half as efficient CD46-utilizing species B Ads [42]. Subsequent work by our laboratory on primary human B cells showed Ad26 used CD46 and integrin, but did not use sialic acid as evidenced by a lack of effect of neuraminidase on cells [46]. A more recent study reports that Ad26 does not use CD46 and instead uses αvβ3 integrin as its primary receptor [51]. Other work showed that Ad26 binds CAR and CD46 with 20 and 50 μM affinities, respectively [52]. A recent publication showed that the removal of cell surface sialic acid inhibits Ad26 infection [53]. This work also cocrystalized Ad26 knob with sialic acid, thus making the argument that sialic acid is 'the' Ad26 receptor [53]. Conversely, we find Ad26 knob binds CD46-D4 with 0.12 μM affinity and that it infects cells expressing CD46 in the presence or absence of sialic acid (unpublished observations). Regardless of whether Ad26 binds CD46 and/or sialic acid, its receptor binding is markedly weaker than the nM binding of most archetype viruses like Ad5, Ad35, and Ad11 for their receptors.

The affinities of these interactions vary. Ad5 fiber binds CAR with 15 nM affinity. Species B Ad11 binds CD46 with 13 nM affinity, whereas species B Ad21's affinity is 22-fold lower at 284 nM. Some species D Ads including Ad37 use sialic acid as a receptor, some with high selectivity for the GD1a glycan [38,39]. While Ad37 does bind sialic acid, the affinity of this interaction is only 19 μM. This is similar to the affinity of many other viruses for their receptors, but it is also 1000-fold lower than the affinity of Ad5 and Ad11 fibers for their cognate receptors [54,55].

Therefore, one might expect this low-affinity fiber to do well binding receptors in static conditions (e.g., on the eye, on mucosa, after an intramuscular injection), but struggle to interact in high shear conditions (e.g., after an IV injection). One might also expect that a

low-affinity fiber might rely more on secondary interactions of penton base with integrins on surfaces. This hypothesis might also play out in a similar fashion when exogenous low-affinity targeting peptides are added to Ad capsid proteins. Low-affinity ligands may perform well in the laboratory in a tissue culture dish or under static *in vivo* conditions, but may fail when challenged under high shear after IV injections.

Ad5 fiber has 21 or 22 β-spiral repeats in its shaft making this protein ~ 37 nm in length [23,56]). While one might think this long structure would prevent any access of penton base to integrins, this long fiber has a flexible joint near its base [57] allowing it to bend out of the way for binding to αv integrins in 'virus yoga' [57].

This flexibility can be appreciated by the inability to observe species C fibers in cryo-electron microscopic (cryo-EM) reconstructions [58,59]) because every fiber is in a different position on the virions and so their density gets averaged out. Most short-shafted fibers in species B and D viruses lack this flexible domain in their shafts, and so, these can be observed in cryo-EM (e.g., Ad26 fiber in Fig. 1B and Ref. [33,60]). This lack of flexibility may be compensated in some viruses by having more extended loops in penton base to display their RGD motif [33]. If these short-shafted fibers bind their cognate receptor with very low affinity (i.e., Ad37 for sialic acid), penton interactions may become more dominant than fiber interactions for cell binding [46]. Likewise, if target cells lack an Ad's cognate fiber receptor, the virus can use integrins as a fall back receptor.

To a certain degree, this can be observed based on the time allowed for the virus to infect cells *in vitro*. If the cell lacks CAR, for example, letting Ad5 infect it for only an hour yields little infection [61]. In contrast, if the virus is allowed to bind the same cell for 24 h, lower affinity integrin binding can occur allowing most of the cells to be infected. Similar effects can be observed when inserting cell-targeting peptides into Ad proteins. If the ligand has low affinity, it may appear to fail if given a short binding time, but may succeed under extended conditions.

Types of adenovirus vectors

Before discussing retargeting and detargeting, we will define the different types of Ad vectors onto which these approaches may be applied. Most gene-based adenovirus vectors in the literature are RD-Ad adenovirus serotype 5 (Ad5) vectors (Fig. 2). Ad5 is popular in part because commercial kits to make these viruses came out in the 1990s and no other serotype kits have been sold.

These RD-Ad vectors have their E1 gene deleted to prevent them from replicating their DNA and making progeny viruses. This prevents the vector from killing the cell that was just modified. This also avoids causing potentially dangerous and potentially lethal Ad infections from the vector itself. An RD-Ad infects a cell, delivers its one copy of a gene, and can express robust amounts of its transgene protein. They are safe, but do not replicate transgenes or amplify transgene expression.

Much that is known about adaptive T-cell responses against Ad vectors was learned with RD-Ad vectors. While the pivotal E1 gene is deleted, there can still be leaky expression from the remaining 17 or more viral ORFs and this leaky expression in transduced cells targets them for destruction by adaptive CD8 T cells [62].

Given this, HD-Ad vectors were developed that have all Ad ORFs deleted [63–65] (Fig. 2). No Ad proteins are produced, and this avoids immune cells killing the transduced cells [63–65]. This reduced immunogenicity allows HD-Ad to mediate sustained liver gene therapy for longer than 7 years in nonhuman primates [9]. Any residual HD-Ad toxicity can be blunted by genetic and chemical shielding approaches (reviewed in Ref. [20,26,66–68]). This makes HD-Ads viable platforms for gene therapy. However, bad public relations cloud the scientific merits of these improved Ad vectors. HD-Ads

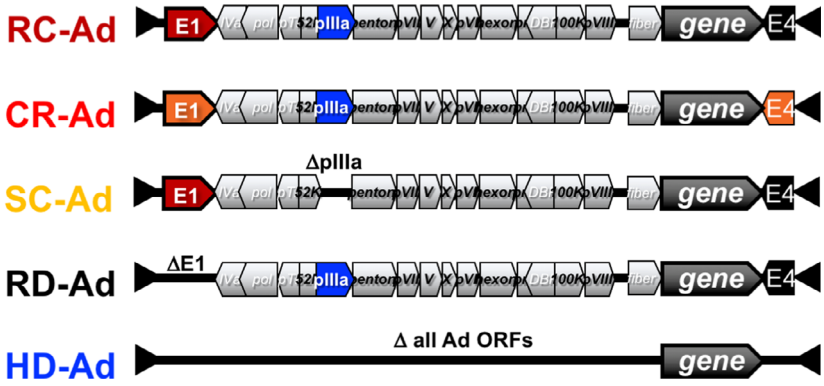

Fig. 2. Schematic of different types of adenovirus vectors. RC-Ad; SC-Ad; RD-Ad, E1-deleted Ad; HD-Ad.

are also replication-defective vectors that will not amplify transgenes. They avoid immune responses against encoded viral antigens. However, if the transgene protein itself is immunogenic, it will provoke T-cell responses that will delete these modified cells [69].

While RD-Ad and HD-Ad do not amplify transgenes, an E1 + replication-competent Ad (RC-Ad) vector could infect the same cell type and replicate the same transgene many thousands-fold [70–81] (Fig. 2). *In vitro,* this translates into 33- to 100-fold increases in protein production [82,83]. RC-Ad vectors are indeed more potent. However, fully RC-Ads run the real risk of causing adenovirus infections in humans. Indeed, when live RC-Ad vaccines are used in military service members, these wild viruses are delivered in enteric-coated capsules or tablets and given orally primarily to prevent them from causing Ad respiratory infections in nurses and vaccines [1]. More recent clinical studies of an RC-Ad4 influenza vaccine (clinical trial NCT01443936) showed that this replicating vaccine generates potent B-cell and antibody responses in humans after single intranasal (IN), tonsillar, or oral delivery [84]. However, this study also showed that 60% of the volunteers that received RC-Ad by the IN route came down with respiratory Ad infections (Mark Connors, NIH, personal communication).

To take advantage of transgene DNA replication by replicating Ads, but avoid the risk of adenovirus infections, we developed single-cycle Ad (SC-Ad) vectors (Fig. 2, [11,82,83,85] and reviewed in Ref. [86]). SC-Ads retain their E1 genes to allow them to replicate their genomes, but are deleted for their pIIIa gene to block the production of infectious progeny viruses. SC-Ads replicate their genomes and transgenes as effectively as RC-Ad (up to 10 000-fold) [82]. RC- and SC-Ad produce more transgene protein than RD-Ad vectors [82]. Like RC-Ads, SC-Ads also kill the first infected cell. However, they do not generate progeny viruses, so this initial cell death is limited to the first cells infected. SC-Ads generate more robust and more persistent immune responses than either RD-Ad or RC-Ads [83]. In head-to-head comparisons against standard RD-Ad vaccines, SC-Ad produces significantly higher antibodies and better protection against influenza virus [87]. SC-Ads have also shown potency as vaccines against Ebola virus and against *Clostridoides difficile* after single immunization [11] (W.E. Matchett, S.S. Anguiano-Zarate & M. A. Barry, unpublished results).

Conditionally replicating Ads (CRAds) are designed primarily for cancer applications with the goal of having Ads activate specifically in cancer cells while not activating in normal cells ([88–92], and reviewed in Ref. [93]). CRAds are engineered to activate in cancer cells by replacing promiscuous E1 or E4 promoters with cancer-specific promoters or by mutating the ability of E1A or E1B proteins to block their ability to interact with pivotal cellular proteins like pRB, p53, or p300 pathways.

While CRAds are a clever postentry strategy with demonstrated specificities, they are somewhat of an illusion when they are applied *in vivo*. It is true that the CRAd design can prevent the virus from activating in off-target cells and killing them directly. However, those cells will still die *in vivo*.

Any off-target cell that is infected by a CRAd *in vivo* will have leaky Ad ORF expression just like RD-Ad vectors [94]. The incoming capsid proteins and/or these leaky Ad ORF proteins will be detected by the immune system as a foreign invasion [94]. A CRAd-infected off-target cell may not die because of the direct cytotoxicity of the virus. However, it will still die, but in this case by execution by cytotoxic T lymphocytes. CRAd control may spare the host organism by reducing amplification of progeny viruses from off-target cells, but those off-target cells will still die and may provoke side effects.

This can be true for any Ad vector. If the immune system detects viral or transgene antigens, T cells will destroy that cell. HD-Ads can avoid this provided that their transgene protein is close enough to 'self' to escape detection. If an HD-Ad's transgene protein is foreign, these cells will also be destroyed by the immune system.

There is one final important note regarding testing of replicating Ads. Ad DNA replication and transgene amplification are highly species-dependent [95]. Human and nonhuman primate cells can amplify their genomes 3000- to 100 000-fold [82,83,96]. In contrast, most mouse lines do not allow any replication. *In vivo,* there can be as much as 13-fold DNA replication of Ad6 DNA in the liver after an IV injection [85]. In contrast, there is only threefold DNA replication of Ad DNA in the lungs after intranasal administration [85]. Syrian hamsters are thought to be a better model for human Ads [95]. This is true for species C Ads, but not for many other Ads [97]. In Syrian hamster HaK cells, one can observe 350-fold Ad6 DNA replication [83], but markedly less *in vivo*. Therefore, testing RC-Ad, SC-Ad, or CRAds in most small animals will underappreciate their potency and also their side effects.

Adenovirus serotypes as a diverse palette for physical particle targeting and postentry activation targeting

There are increasing numbers of Ad serotypes and genotypes that are being discovered nearly every day.

These serve as a genetically and functionally diverse palette of biologies on which to apply vector engineering and cell-targeting approaches (Fig. 3). Genetic diversity in human Ads can approach 40% at the whole genome level [98,99]. This genetic diversity translates into each virus having divergent protein surfaces that are able to evade each other's antibodies.

We and others have delved into the biologies of other human and nonhuman Ads in the quest for new functionalities or to evade anti-Ad5 immunity in patients and to have non-Ad5 genetic platforms for vector engineering [23,38,42,46–48,97,100–111]. They also allow one to avoid pre-existing immune responses against certain Ads (i.e., Ad5) and to vary the Ad serotype between treatments in a shell game called serotype-switching [100,112].

Adenovirus serotypes as a diverse palette for physical particle vector targeting

This diversity also translates into the evolution of viruses that naturally bind different receptors and natural differences in therapeutic potential based on this (Fig. 3). As discussed early, the fiber proteins of Ads bind CAR, sialic acid, CD46, desmoglein-2, and a few others (Fig. 3 and reviewed in Ref. [23]). Archetype Ad5 virus and its species C family members Ad1, Ad2, Ad6, and Ad57 bind CAR. Species B Ads like Ad21 and Ad35 bind CD46. Seminal work by Dmitry Shayakhmetov in Andre Lieber's laboratory generated some of the most potent retargeted Ads by given Ad5 CD46-binding fibers from species B Ads [101,113]. This approach has been stolen by many laboratories including ours. While this does retarget Ad5, it does not actually retarget Ads as a family of viruses as CD46 is already in the wheelhouse of human Ads.

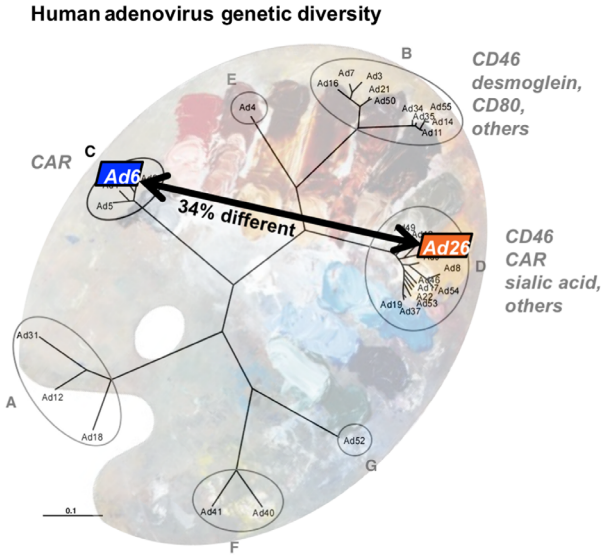

Fig. 3. Schematic of the human adenovirus virome palette for adenovirus targeting. Adapted from Ref. [99] and showing whole genome difference between species C Ad6 and species D Ad26 described in Ref. [106].

Different serotypes of Ad can also bind receptors indirectly by binding host proteins like vitamin K-dependent clotting factors [primarily blood clotting factor X (FX) and IX (FIX)], complement, natural antibodies, and other proteins that serve as 'bridges' to receptors [24,114,115]. These host-derived binding proteins and their effects on Ad tropism *in vivo* are discussed in detail in other articles in this collection.

The cell-binding proteins evolved by Ads and the host proteins that bind certain Ad serotypes can be modified by genetic or chemical engineering to physically retarget Ad particles to new receptors. These interactions can also be mutated or chemically blocked to detarget Ads from off-target tissue for therapy.

In many cases, wild Ads have been screened for utility prior to engineering. In another approach, Terri Hermiston's group bred multiple Ads together to encourage interspecies recombination to generate better oncolytic viruses [116]. One of these viruses known as ColoAd1 that is a chimera of two species B viruses, Ad11p and Ad3, was renamed Enadenotucirev and is in human clinical trials [117].

Adenovirus serotypes as A diverse palette for postentry vector activation targeting

Viral genetic diversity also translates into differences in the activation of different Ads after cell binding and entry has occurred. This is most relevant to Ads that retain E1 and activate DNA or full viral replication (i.e., SC-Ads, RC-Ads, and oncolytic Ads).

For example, we showed that species B, C, and D Ads infect primary human B-cell cancers to different degrees, but also activate DNA replication to different degrees [45]. CD46-binding Ad11 and Ad35 infected myeloma cells 100-fold more efficiently than species C Ad5 and 6 or species D Ad26 and Ad48. While one would predict that Ad11 and Ad35 would then dominate in genome and progeny virus replication, they never activated DNA replication in these cells. In contrast, the species C and D viruses activated and amplified their genomes in these primary cells. From this, different Ads have different entry and activation biologies that can be harnessed for postentry targeting.

We also directly compared the genetic activation programs of two of these genetically distant Ads: human species C Ad6 and species D Ad26 [106]. Ad6

and Ad26 differ by 34% at the whole genome DNA level (Fig. 4). Ad6 binds CAR, αv integrins, and FX. Ad26 binds CAR, CD46, sialic acid, and αv integrins, but not FX. Despite differences in receptor utilization, both infect human lung A549 cells. Both viruses initiate DNA replication within 12 h with identical kinetics and both begin killing cells within 72 h. Ad6-infected cells remain adherent until death. Ad26-infected cells detach from plates within 12 h, but remain viable in this detached state. Quantitative PCR and next-generation sequencing showed that both viruses activate their early genes at 6 h and transition to late gene activation by 12 h.

However, there are marked differences in how these viruses activate E1A and E1B genes and how E3A and E3B immune evasion mRNAs are activated (Fig. 4 and [106]). Differences in E1 activation could be related to differences in the sequences of their E1 promoters, but also perhaps due to differences in their ability to neutralize cellular proteins. For example, both viruses retain pRB binding motifs, but p300 and BS69 binding motifs are not conserved in Ad26. Variations in E3 mRNA expression translated into Ad6 being more effective at suppressing MHC I display on infected cells and evading extrinsic apoptosis signals than Ad26. These differences in E1 and E3 utilization likely underpin differences in the fundamental ability of these viruses to kill different cancers [45,46,97,118]. More differences are likely to be found in the diverse genetic palette of Ads. This provides a wide repertoire of genetic platforms on which to apply pre-entry and postentry vector targeting strategies.

Adenovirus pharmacology

Retargeting and detargeting of Ads are easy in cell culture; it is like shooting fish in a barrel. Ignoring the *in vivo* pharmacology of Ads is, however, a drastic mistake: You may develop the world's best targeted adenovirus, but if most of it is absorbed and destroyed by off-target cells and tissues, you will fail *in vivo*. Given this, we discuss important aspects of Ad *in vivo* pharmacology below before moving to retargeting and detargeting efforts.

Rapid blood protein and cell binding after intravenous injections

Other articles in this collection provide detailed review on these topics, so this will be a somewhat brief review with our opinions on these topics. More detailed reviews and our opinions on these topics can be found in Ref. [93,119]. When Ads are injected directly into the bloodstream, they can rapidly bind to blood proteins, platelets, red blood cells, and nucleated cells, and these fundamentally change the biodistribution of these viruses [120–126]. Because of this, Ads do not always perform as expected *in vivo* if these expectations are based on *in vitro* cell culture data.

Binding to blood clotting factors IX and X, natural antibodies, and complement can decide the fate of IV-injected Ads. Binding clotting factors can partially protect Ads from destruction in macrophages, particularly liver Kupffer cells. Species C human Ads can cloak themselves with FIX and FX and hide themselves to a certain degree from destruction by macrophages. Most other Ad serotypes do not and they can be drastically consumed and destroyed by macrophages. IX and X can also serve bridges to retarget Ads to heparan sulfate proteoglycans on cells. Binding to natural antibody IgMs can target Ads for covalent tagging by complement proteins to also target the viruses to macrophages for destruction. Uptake by macrophages and Kupffer cells and nonimmune cells can trigger potentially dangerous innate immune

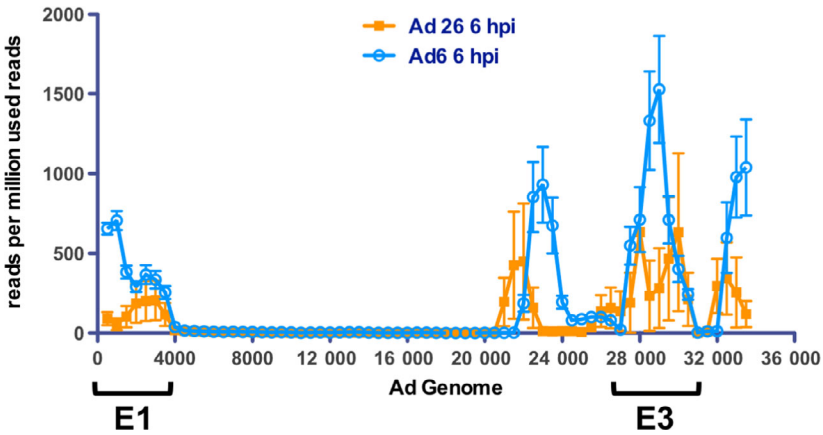

Fig. 4. mRNA activation after infection of human lung cells with species C Ad6 and species D Ad26. See the main text for further information. Adapted from Ref. [106].

responses and helps initiate adaptive immune responses against the virus and its transgene proteins.

Intravenously injected Ads can also bind to and activate platelets and endothelial cells [66,124,127,128]. Activation of these cells induces clotting and in extreme cases can lead to disseminated intravascular coagulation and death [129,130]. Platelet binding can also target Ads for degradation by macrophages [124].

Systemic distribution of adenoviruses after intravenous injection: interactions with organs and the reticuloendothelial system (RES)

An IV dose of Ad by most routes will usually encounter the heart and lungs before being distributed to the liver, spleen, and kidneys (reviewed in Ref. [93,119]). In mice, almost 98% of IV-injected Ad5 is found in the liver 30 min after injection [131]. At this same dose, only about 1% of injected Ad5 can be found in either the lungs or the kidney at this dose. If the dose is increased fourfold, Ad5 in the liver falls to 85% of injected dose and virus in the spleen and lung rises to 6% and 5% of injected dose, respectively. These are results in one strain of mice. No doubt there will be differences in relative distributions in humans. Different Ad serotypes may vary in their relative distributions, but the liver and spleen are likely to dominate all, since the RES cells housed in these tissues is evolved to absorb and neutralize particulate invaders.

The rapid distribution and sequestration of Ad after an IV injection can be easily appreciated by viewing movies of Ad5 labeled with near-infrared fluorophores distributing in mice [132]. In these, Ad5 can be seen entering the heart within 500 ms, flowing through distant arteries in 7 s, 'blushing' the skin and tissues within 11 s, and then accumulating in the liver within 3 min of the injection [132].

The liver as a dominant pharmacologic dead end for adenoviruses

Approximately 1.5 L of blood passes through the liver every minute in humans. After an IV injection and upstream absorption, Ads enter liver sinusoids where a large fraction of virions are absorbed by liver sinusoidal endothelial cells (LSECs) and Kupffer cells that line the sinusoids (reviewed in Ref. [119]). Kupffer cells are the resident macrophage of the liver. While they comprise only ~ 7% of liver cells, they may account for up to 90% of all of the macrophages in the body [133]. It has been estimated that liver Kupffer cells can sequester up to 98% of intravenously injected Ad5 vector in mice [134]. LSECs are also a major component of the RES, but their role in sequestration of Ads is underappreciated [135,136]. LSECs constitute ~ 25% of all liver cells [133]. LSECs and Kupffer cells work in concert to clear particles from the blood. Kupffer cells absorb particles up to 2 μm in diameter, and LSECs absorb particles below 230 nm [133,137]. Therefore, both cells can phagocytose or pinocytose ~ 100 nm Ads.

Viruses that evade LSECs and Kupffer cells enter the parenchyma of the liver through fenestrations in the sinusoid wall that are large enough to pass Ads. Once inside the liver, Ads can infect hepatocytes. If the goal is to transduce hepatocytes, this is great. If the goal is to reach more distant tissues or cancer cells, this is terrible, since more of the injected dose is depleted.

Beyond the liver

Beyond the liver, we know that a smaller, but significant fraction of Ad lands in the spleen, kidneys, and lungs after IV injection. As noted above, high doses of Ad5 in mice can result in 6% of the injected dose landing in the spleen and 5% in the lung. If you normalize viral genomes to organ weight, the spleen absorbs Ad5 as well as the liver kilogram for kilogram [138]. This specific activity representation is helpful for understanding adenoviral biology and immune responses against these viruses. However, viral genomes per organ weight or viral genomes per host genome underestimate the magnitude of the liver as a pharmacologic sink for IV-injected Ads, since it is considerably more massive than other tissues. Absorption in the spleen seems to be in large part due to uptake and sometimes transduction of macrophages. Absorption in the spleen can have drastic immunologic impacts as this organ can amplify innate and adaptive immune responses against the viruses or against their transgene products.

The kidney is another interesting organ for Ads. Its natural filtering functions prevent most entry into the organ Ads after IV injection. The glomerulus of the kidney actively excludes proteins above 50 kDa from entry into the organ (reviewed in Ref. [139,140]). In addition, slit diaphragms between podocytes in the glomerulus are only 10 nm. Therefore, on paper, 100 nm, 150 megadalton Ads have little likelihood of penetrating beyond the glomerulus deeper into the organ. In addition, there is only low-level infection of glomerular cells by most Ads [140]. Transduction of cells in the parenchyma of the kidney has been reported after IV injections of different Ad serotypes or retargeted Ads [141,142]. While this is reported, it is

unclear how these huge Ads break the glomerular barrier. Perhaps they overwhelm the glomerulus, form immune complexes, or perhaps enter by an unexpected route like retrograde through kidney lymphatics [143,144].

Adenoviral vector retargeting and detargeting

Discussions of early efforts to retarget and detarget Ads can be found in our previous reviews [22,93,118,119,145,146]. Activity in the Ad targeting and detargeting space since 2011 can be found in the following references [19,147–207]. General strategies are summarized in a schematic (Fig. 5).

Most early and recent work has been directed at retargeting Ads to new receptors rather than detargeting them from off-target receptors and cells. Much of this early work was championed by David Curiel's original group at the University of Alabama at Birmingham [208–214]. The reader should check out these seminal early works and follow subsequent work from Curiel and his 'progeny' scientists. Other seminal work on the basic biology of Ads including retargeting, swapping fibers, and *in vivo* sequestration can be found in publications by Andre Lieber and Dmitry Shayakhmetov [101,103,113,114,215–224].

Subsequent work aimed to detarget Ad from its cognate *in vitro* receptors. Many great retargeted Ads have been broken on the shores of the massive absorption of most of their injected doses by the RES. We believe that we must be effective at detargeting before we can be effective at retargeting.

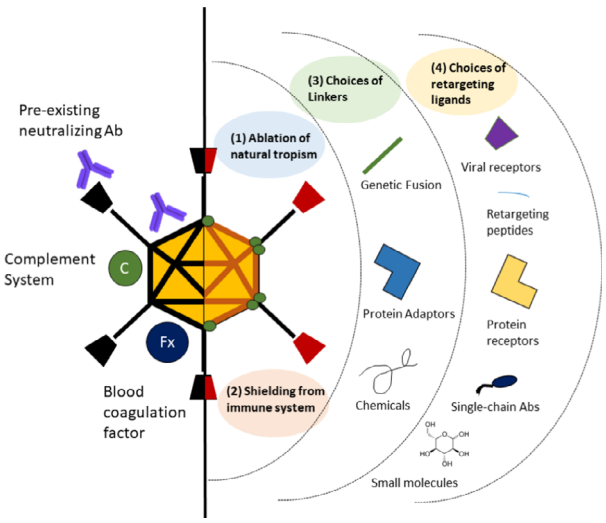

Fig. 5. Schematic of adenovirus retargeting and detargeting strategies. See the main text for further information.

Evading blood proteins and cells

After IV injection, the multivalent Velcro-like surface of Ad binds proteins and cells in the blood. Blocking these interactions is likely key to improving the ability of Ads to reach distant cells, particularly since many of these proteins target the viruses for destruction by the RES. A number of strategies can be used to detarget these cells (Fig. 5). The use of alternate Ad serotypes may avoid some of these interactions, since binding is receptor-mediated. Other approaches are to genetically delete viral ligands that bind CAR, CD46, integrin, and other interactions [225]. Another approach to evade interactions is to shield Ads with polymers like polyethylene glycol and poly-*N*-(2-hydroxypropyl) methacrylamide (HPMA) [20,66,123,131,134,226–242].

Shielding with these polymers prevents interactions of Ads with blood proteins, blood cells, endothelial cells, and Kupffer cells [20,66,123,189,243,244]. Polymer shielding can also reduce innate immune responses and liver damage after IV injection. Random covalent conjugation of these polymers has the down side that they can inhibit the ability of Ads to bind receptors and unpackage in cells. This problem can be avoided in part by targeting polymer modifications to specific sites on Ad by inserting cysteines into hexon and targeting conjugation to this amino acid with maleimide [26,235,236,245].

A novel new shielding approach was tested wherein Ads were 'cloaked' with silica (SiAd) [162]. This nanoparticle coating blocked the production of inflammatory cytokines and reduced production of neutralizing antibodies as well as increased virus infection after intratumoral injection.

Another innovative approach has been to insert albumin binding peptide into the hexon of Ad5 to shield it from neutralizing antibodies [246,247]. This approach allows the virus to cloak itself in albumin in the blood and may have utility to evade antibodies as well as other problematic factors in the blood.

Evading the reticuloendothelial system

In mice, 98% of Ad5 appears to be absorbed by liver Kupffer cells and LSECs at low dose after an IV injection [131]. If you increase the dose above the sequestration threshold, virus spills into liver hepatocytes [248]. This can increase liver transduction if this is your goal, but this also has immunologic side effects. One brute force way to avoid Kupffer cells and perhaps LSECs is to 'predose' the system by injecting other particles like gadolinium chloride, clodronate liposomes, or Ad5 itself to saturate and kill Kupffer

cells before injecting the therapeutic or reporter virus [134,217,249–252]. While predosing can be effective, uptake of Ad into Kupffer cells not only kills the virus, but also kills the Kupffer cells [253]. This creates a highly inflammatory milieu and results in dead Kupffer cell fragments lodging in the lung where they can provoke dangerous side effects.

Ad polymer shielding is an effective means to detarget Kupffer and LSECs [20,237,238]. Larger polymers that increase the diameter of Ad beyond the size of liver fenestrations also appeared to prevent entry into the parenchyma of the liver and uptake into hepatocytes.

Changing the serotype or the hypervariable regions (HVRs) of Ads can also hide the virus from Kupffer and other cells [68,96]. For example, giving Ad5 the HVRs from Ad6 block its uptake by scavenger receptors and macrophages, and increase transduction of hepatocytes [68]. Similarly, deleting the large, highly charged HVR1 of Ad5 reduces its binding to scavenger receptor MARCO (SR-A6) [27]. Conversely, giving an Ad HVRs that do not bind FX for shielding from IgM and complement can make uptake and side effects worse. For example, giving Ad5 the HVRs from Ad48 increased Kupffer cell uptake and inflammation against the virus [254].

Other approaches are to insert peptides or proteins into the HVRs of Ad [122,255–257]. Insertions into HVR5 appear able to block binding of FX to Ads. At the time, the expectation was that blocking this would prevent FX acting as a bridge to targeting heparan sulfate proteoglycans on cells like hepatocytes. *In vivo* data supported this paradigm, since insertion of RGD or a biotin acceptor peptide (BAP) into HVR5 markedly reduces hepatocyte transduction [122,255–257]. While this worked, the underlying hypothesis appears wrong as these FX binding insertions likely just block the ability of the virus to cloak itself in FX to avoid targeting to and destruction by Kupffer cells. If the viruses are more destroyed by Kupffer cells, hepatocyte transduction will also be reduced.

While Kupffer cells are a problem, depleting them by predosing does not reduce Ad genomes in the liver [252,253]. So, Kupffer cells are not the only story. To do better, Di Paolo *et al.* engineered Ad5 to target multiple liver cells 5 [225]. In this work, they showed that no single intervention by itself fully detargeted the virus from the liver. While one can detarget multiple interactions, in many cases, the virus actually needs these functions to be efficient. Therefore, detargeting can come at the cost of efficacy.

Adenoviral vector retargeting

Genetic insertions into Ad capsomers

Early work on inserting ligands relied on genetically adding known small peptides to the Ad5 fiber. First, proof of principle was adding a non-cell-targeting epitope tag to the C terminus of fiber [208]. Later work inserted a flag tag or an RGD motif into the nonconserved flexible loop between the H and I beta sheets of the Ad5 knob [211,212]. Many subsequent studies have inserted RGD into almost all capsomers of Ad. Most of these studies erroneously describe these as 'retargeted' vectors when in fact they are simply 'gain-of-function' vectors, since Ad already has its own RGD motif in its penton base. Placing RGD on fiber does not really retarget the virus. Rather, it just exposes the motif better for interactions with the same αv integrins that Ad already uses. More recent work has inserted more specific RGD peptides from foot-and-mouth disease virus to more specifically target αvβ6 integrins that are upregulated on cancer cells [258].

Other efforts have involved replacement of the trimeric fiber with heterologous trimeric proteins like bacteriophage fibritin [160,174,183,259–263] or reovirus sigma 1 protein [264–266]. Recent examples of retargeting by direct genetic introduction of ligands into Ad capsomers can be found in the following references: [19,152,154,158,164,166,173–175,183,185,187,191,192,204]. Other examples are discussed below in selected cases that provide guidance for future engineering efforts.

Choosing the best cell-targeting ligands for Ad genetic engineering

Incompatibility of secreted targeting ligands

Some of the best cell-targeting ligands are antibodies and other glycosylated proteins (Fig. 5). Unfortunately, these ligands are excreted through the secretory pathway where they are post-translationally modified with carbohydrates and disulfide bonds that are key to their targeting functions. This is unfortunate, because Ads are built in the reducing environment of nucleus where disulfides do not form and little glycosylation occurs. Therefore, one must usually engage in drastic ligand engineering to translate these excellent ligands from secretory tech to nuclear tech for direct genetic incorporation into Ad capsomer proteins [259]. Alternately, one can generate bridging molecules in which one end binds an Ad capsomer or tag and the other is the targeting ligand [267,268]. In contrast, chemical engineering approaches that chemically cross-link exogenous

ligands to Ads can bridge the divide between the secretory and nuclear world for targeting [229,233].

Peptide ligands and peptide-presenting phage libraries

What do you do if you do not have a targeting ligand already conveniently in hand? To quote Ghostbusters[tm]: 'Who you gonna call?'

This question actually served as part of the lead author's postdoctoral work. To find these needed ligands, we selected cell-binding and cell-internalizing peptides from peptide-presenting phage libraries [269]. Our goals were as follows: (a) to identify ligands without any prior knowledge of the biology or receptors of the target tissue; (b) to develop a technology that would identify ligands that bind directly to the cells of interest for direct transduction; and (c) to identify cell-binding ligands that would be compatible with genetic engineering into viral gene delivery vectors.

Peptides were attractive, since they are relatively small for genetic engineering into Ads, but also can be easily produced in good manufacturing practice (GMP) grade by chemical synthesis for targeting nonviral vectors or Ads by bioengineering approaches.

The possibility of identifying peptide ligands for vector targeting was suggested at the time by the early use of peptide-presenting phage libraries to select peptides against proteins *in vitro* in ELISA plates [270–272]. These peptide-presenting phage libraries had been developed by engineering filamentous bacteriophage to display random peptides by inserting semirandom DNA into their pIII receptor binding protein (analogous to Ad fiber) or their pVIII (analogous to the hexon). This peptide discovery technology was a uniquely powerful, since the actual ligand is physically attached to the DNA that encodes it. This allowed any good peptide sequence to be inferred by sequencing the DNA.

Proof of principle was demonstrated with peptide libraries build in filamentous phage and has been followed by other approaches like ribosomal display and yeast display [273–276]. Yeast display has the advantage over bacterial libraries of being able to generate ligands with some level of carbohydrate modifications. For example, ribosome display was used to generate bifunctional designed ankyrin repeat proteins that retarget Ad5 to Her-2 [273].

These library technologies used to need to be developed by individual laboratories. Phage peptide libraries have long been available commercially from companies like New England Biolabs, and you can see how this availability has generated most of the cell-targeting peptides that are in the adenovirus literature. Some peptide and single-chain antibody libraries can now be purchased from other vendors. This is a great expansion of availability, but is hindered by expense and sometimes stringent material transfer agreements.

The importance of ligand library size

More importantly, peptide libraries built in bacteria can have diversities of up to 10^{10} members. Consider that an average antibody recognizes six amino acids [270–272]. Therefore, if you want a targeting peptide that might be as good as an antibody, you may want to be able to screen 6-mer peptides. Consider that a peptide library must have at least 20 members in the library to cover one amino acid position with all possible 20 amino acids. To cover two amino acid positions, you need a library with 20^2 or 400 members. To cover four amino acid positions, you need 20^4 combinations or 1.6×10^5 library members. To cover six amino acid positions, you need a library of 6.4×10^7 library members. If you want to do better than the needed six amino acids of binding surface, a library covering all combinations of eight amino acids would need 2.56×10^{10} library members. At the time, we searched for peptide ligands, and even now, huge complexity libraries are still best generated in bacteria. Random PCR can generate more diversity, but they are not generally easy to apply in the context of a stable genetic platform for screening.

Selection of peptides against mammalian cells and their receptors

In the early 90s, peptide-presenting phage libraries had been used to select peptides against proteins on plates *in vitro* [270–272] and it had not been used to select peptides directly on mammalian cells. We demonstrated that you could do this by selecting 12 and 20-mer peptide libraries against mammalian cells [269]. As we pursued this work, other work described selecting peptides against purified cell surface receptors [277–280], against platelets [281,282], and notably selected the now famous RGD-4C peptide [279], which is the go-to ligand for Ad vector targeting. Subsequent work described selection peptide libraries *in vitro* and *in vivo* [229,283–288]. A few of these early investigators went the full distance and translated phage-selected peptides onto gene therapy vectors. Examples include translation of phage-selected peptides into adenovirus and AAV by genetic insertion [154,212,289–291], by chemical cross-linking to adenovirus [229,292], and by incorporation of targeting peptides into vector-specific antibodies [285,293].

Ligand context

Genetic insertion of foreign peptides into the viral capsomer proteins has been reviewed previously [22,146]. These methods can be unpredictable in terms of whether these peptide insertions would be compatible with capsomer folding and virion assembly and whether the peptide would retain its cell-binding functions when grafted into this foreign protein. For example, when we have had many disastrous experiences of having peptides that either fail to work in Ad or destroy things like fiber trimerization. We took one approach to circumvent these problems by engineering a bacteriophage library that displayed random peptides already in the context of the Ad fiber HI loop between fiber H and I beta sheets [290]. We showed that we could select muscle-binding peptides from these libraries and that at least one could be grafted back into the HI loop of Ad5 to yield functional retargeting of the virus [290].

We called these 'context-specific' phage libraries, but later realized that most nonenveloped viral proteins have an abundance of very similar beta sheets separated by flexible loops. Therefore, these are not so much 'context-specific', but viral capsomer 'compatible'. We proved this principle by grafting these HI loop-selected muscle-binding peptides into a similar beta sheet-loop-beta sheet structure of HVR5 in Ad5's hexon [291]. We showed that these HVR-modified viruses had increased infection of muscle cells *in vitro* and *in vivo*. Interestingly, we showed that only one of the two peptides inserted into HVR5 detargeted Ad5 from hepatocytes in the liver. This suggests variable effects of insertions into hexon on modulating interactions with FX and perhaps Kupffer cells.

In summary, abundant data demonstrate that viable cell-targeting ligands can be selected from bacterial phage libraries and can be used to retarget Ads by genetic or chemical engineering.

Direct adenovirus peptide libraries

More recently, peptide libraries have been created in which random peptides are cloned directly into the Ad capsid [294–296] without the pain and suffering of having to translate phage tech into Ad tech. This technique was modified to accommodate the insertion of peptides with known affinity for cellular targets. Lupold *et al*. designed an Ad peptide library that had a constant binding peptide insert flanked with random linker sequences [295]. Virions could then be selected with retained binding specificity [297].

Although these Ad libraries could theoretically contain up to 10^9 unique peptides as bacterial plasmids, there is a huge bottleneck in converting Ad plasmids to Ad viruses in mammalian cells. If you transfect 293 cells with 10 μg of DNA and get 20 plaques, your library is 20 members and you can only cover one amino acid position with all 20 amino acids. In practice, with concerted effort, these Ad libraries yielded sizes of up to 2×10^5 members [294,295] allowing coverage of all combinations of a four amino acid peptide.

The library size bottleneck was more recently addressed in part by work in the Yakamoto Lab [152,298]. In this approach, the transfection to plaque bottleneck with plasmids was circumvented in part. In the first iteration, full adenoviral genomes were generated by Cre-lox recombination between a fiber-modified plasmid library and Ad DNA/terminal protein complex (DNA-TPC) before transfection into mammalian cells [294]. In the second iteration, a fiber plasmid library and a fiberless Ad DNA-TPC were cotransfected into Cre-expressing 293 cells. This generated a library of ~ 10^4 members from ~ 10^6 cells and allowed selection of novel Ads with new functionalities [298].

This is a great step forward for Ad retargeting. However, total library size will still likely limit the affinity of any peptides selected from these libraries. If such a library is scaled up to cell factory scale with 10^9 cells, this may yield a library with 10^7 library members. This allows coverage of all combinations of 4-mer peptides, but not all combinations of 5-mers.

While RGD can be held up as a great cell-binding 3-mer peptide, it is an exception rather than a rule for binding. Bigger peptides are likely better for binding. When we directly competed 12-mer vs 20-mer phage libraries against each other, the bigger library always generated better cell-binding peptides (unpublished data). Even if one uses large complexity phage libraries, most peptides selected out of peptide libraries start with affinities in 10–100 μM level. Given this, it is standard practice to identify a lead peptide from a peptide and then generate a second mutant library based on the original peptide sequence and select this again to increase peptide affinity [269].

Therefore, direct peptide libraries in Ads are a great advance for the field. Efforts to increase library size and more importantly ligand affinity will help move these technologies forward.

Bioengineering to covalently attach targeting ligands to Ads

Another strategy to retarget Ad is to covalently attach targeting ligands to Ad capsid using mono- or

bifunctional cross-linkers (Fig. 5). In this approach, amine-reactive polymers bearing synthesized ligands can be cross-linked to Ad for shielding and retargeting. For example, polyethylene glycol-glucose and polyethylene glycol-galactose have been used to retarget Ad vaccines for intranasal immunization [226]. One end of a bifunctional polymer can be conjugated to lysines on the surface of the Ad, and the other end of the polymer can be cross-linked to targeting ligand. For example, small proteins like FGF-2 and EGF and phage-selected peptides have been cross-linked to Ads using bifunctional polyethylene glycol, HPMA, or other reagents [229,233,239,292,299]. Not only is this technology suitable for the display of ligands from the capsid, but native vector tropism can either be maintained or inactivated, depending on choice of amino acid targeted. This approach works best with ligands that have free single cysteines that are not tied up in disulfide bonds (i.e., FGF-2 and synthetic peptides with added cysteines). Complex disulfide-bearing ligands like antibodies can be used, but breaking these apart to liberate free cysteines can be tricky. Alternately, one can use things like Traut's reagent to convert amines on the ligand to cysteines for maleimide reaction. An improvement on this approach uses combined genetic and chemical engineering to target polyethylene glycols to cysteines inserted into specific sites in capsomer proteins [26,235,245,300]. Other approaches have utilized not only natural amino acids such as cysteine, but also unnatural amino acids to photo-cross-link ligands onto vectors [202,301].

Polymer coupling of ligands is an excellent way to screen peptide library-generated ligands before going to the trouble of genetically engineering them into capsomer proteins [292] and to shield or detarget Ad at the same time. It can also provide two layers of particle targeting: the first by the ligand and polymer and the second by the capsomers. In the context of a replicating oncolytic or vaccine vector, progeny virions coming from the first infection can have secondary targets now that they are no longer coated by polymers. More recent examples of chemical retargeting can be found in several studies [147,148,150,155,156,163,167–170,176,177,179–181,184,193–196,202,203,206].

Adenovirus targeting with adaptor proteins

Targeting Ads with high-affinity proteins like antibodies is hampered by their large size and improper folding of antibodies in the reducing environment of the nucleus where Ad is assembled. To circumvent this incompatibility, molecular adapters have been designed that bind Ad capsomers on one end and target receptors with the other end. Early adaptors bound Ad5 fiber by fusing the ectodomain of CAR to FGF-2 and EGF [302]. We used the GLA domain from FX to bind the hexons from species C Ad to fuse to single-chain antibodies targeting Her-2, EGFR, and the stem cell marker ABCG2 [268].

If CAR or FX adaptors are encoded by oncolytic Ads, the incoming Ad is not targeted by the adapter unless it is added to virus as an exogenous recombinant protein. This either weakens the adaptor technology or strengthens it by allowing two stages targeting. For the first stage, initial infection is mediated by whatever fiber or targeting ligands are displayed on the virus. For the second stage, the Ad is retargeted by the adaptor. Using CAR or FX may have different utilities. CAR binds 36 sites on Ad. FX adaptor binds up to 240 binding sites on Ad, so one might benefit by more avidity interactions to zipper up Ad to cellular receptors. CAR adaptor might block CAR binding during the second stage, which could be good or bad. FX adaptors preserve fiber functions while still providing a second level of retargeting. This may be useful to help oncolytic Ads spread in tumors since FX targeting is not affected by excess fiber production [268].

A major hurdle to this targeting method is that the adaptor molecules rely on noncovalent protein–protein interactions for their conjugation to the Ad capsid. Naturally occurring antibodies or CAR receptors could compete for Ad binding and displace the molecular adaptors from the capsid, abolishing the vector targeting activity. For example, we originally hoped that FX adaptors would not only retarget, but also detarget Ad from hepatocytes, but this did not work in practice [118], perhaps because the huge amounts of FX in the bloodstream competed the protein off the virus.

Other examples of Ads targeting with adaptor molecules can be found elsewhere [153,157,171,178,182,186,190,197,199,200].

Lessons learned from adaptor targeting with metabolically biotinylated adenoviruses

We developed a different adaptor system where BAPs were genetically fused to Ad fiber, protein IX, or inserted into hexon HVRs [28,61,303,304]. When BAP-modified viruses are produced in mammalian cells, the BAP tag is covalently biotinylated during vector production by the endogenous enzyme holocarboxylase synthetase [305] or by co-expression of bacterial BirA [61,145,305,306]. This allows biotinylated Ads to be bound to any biotinylated ligand using avidin as a bridge. Alternately, one can genetically or chemically

generate a single avidin targeting ligand for a two-component targeting complex. This system forms more stable complexes than other adaptors, because of the extreme affinity of avidin for biotin (10^{-15} M, which is about a quarter the strength of a carbon–carbon single covalent bond).

We showed that the system is adaptable, in that Ad-Fiber-BAP could be retargeted to new receptors using biotinylated peptides, proteins, carbohydrates, DNA, antibodies, and magnets [28,61,303]. There is also the advantage that you can buy many biotinylated ligands right off the shelf. The disadvantage would come in translating to clinic, since you would need to produce two or three GMP-rated components to target: GMP Ad-BAP + GMP avidin or streptavidin + GMP biotinylated ligand or GMP Ad-BAP + GMP avidin–ligand fusion protein.

While there are strengths and weaknesses in the Ad-BAP systems, they actually provide unique insights into (a) which capsomers work best for targeting; (b) how the biology of the ligand affects targeting; and (c) how the affinity of ligands might affect targeting on different capsomers.

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. While all of these capsomers could bind and display the same high-affinity targeting ligands, the fiber protein always worked for targeting, whereas IX and hexon did not [28]. When Ads were labeled with fluorophores and observed on cells by microscopy, it appeared that some of the failed vectors were actually trapped on their receptors and recycled to the cell surface rather than released from endosomes [28]. The one exception to this was transferrin, which could mediate intermediate levels of transduction when displayed on IX-BAP [304].

These results are most likely explained by differences in the biology of fiber, IX, and hexon proteins as well as the biology of ligand–receptor interactions during endosomal uptake and escape. Receptor binding is a critical first step in Ad infection, but virus release from the receptor after uptake is equally important. Ads normally accomplish this by shedding fiber and penton base in the endosome once their tasks are complete [34]. Following this, fibers and penton base are released from the virion [34,307]. In contrast, protein IX and hexon dissociate from virions 30 min or more after receptor binding, well after endosomolysis and cytosolic escape [308].

From this, we believe that fiber works with high-affinity ligands, because it naturally releases from the virion, so the virus can escape high-affinity interactions with receptors. Conversely, viruses bound to receptors with high-affinity ligands on IX and hexon on the icosahedron cannot be released from the same receptors because these capsomer proteins are not shed until they are in the cytoplasm or at the nuclear membrane. Unlike the high-affinity antibody ligands, transferrin works with the icosahedral proteins because this ligand is released from its receptor at endosomal pH [309].

If this model is correct, high-affinity ligands may have problems if directly inserted or coupled to IX or hexon, but may be functional if they can be designed to release from their receptors or the virion itself. This model also suggests that lower affinity ligands may not have these problems, since their kinetic off rates from receptors are likely high allowing for their spontaneous release after internalization. This is supported by observations of being able to insert low-affinity peptides into hexon and have them work for retargeting [255,291].

Combined targeting and detargeting

The vast majority of work in this space has been devoted to retargeting Ads. This works well *in vitro,* but frequently fails *in vivo* due to the many pharmacologic and host factors that were discussed above (reviewed in Ref. [119]). Early work used pharmacologic interventions to detarget Ads. This included 'predosing' discussed previously wherein a first injection of Ad or another particulate reagent like clodronate liposomes hours before therapeutic virus injection can destroy the Kupffer cells and allow the second virus to be effective. This effect can be garnered by two separate injections or by one very high-dose injection. The second pharmacologic approach was to use drugs like warfarin or snake toxins to knock out FX and blood factor binding to Ad to 'detarget' hepatocytes. In reality, this detargeting is really removing FX's shielding effects and is in actuality retargeting Ad for destruction in Kupffer cells and macrophages [24,310–313]. Predosing and blocking FX binding could yield improvements in treating tumors after IV injections [252], but the effects were not as strong as hoped likely because the viruses were still be absorbed by other cells like endothelial cells. In addition, destroying Kupffer cells can have profound consequences including death [253,314].

We described above retargeting Ad5 to muscle while detargeting it from the liver by inserting phage-derived peptides into the virus' hexon [291]. In this case, detargeting was largely sacrificial. The reduced liver transduction by the virus was likely mediated by blocking

FX binding to hexon and deprotecting the virus from complement and destruction in Kupffer cells. Better detargeting was achieved by Shayakhmetov and colleagues by blocking uptake into hepatocytes, Kupffer cells, and endothelial cells [225], but this lacked retargeting.

More recent efforts have combined targeting and detargeting in a vector called Ad5NULL-A20 that bears capsomer mutations that block binding to CAR, αvβ3/5 integrin, and FX, but that also includes peptide A20 that targets αvβ6 integrins [315]. After high-dose (10^{11} virus particle) IV injections, this virus provides remarkable 10^7-fold reductions in Ad genomes in the liver with other large reductions in uptake in other off-target organs. Given other work that shows that FX protects Ad5 from complement activation and Kupffer sequestration, one might expect that deleting FX binding would make the virus more susceptible to uptake and destruction in these cells [24,310–313]. This may occur even with Ad5NULL-A20, but the use of high-dose injections may have helped clear this block by destroying the Kupffer cells. Regardless, this is a significant step forward for detargeting and retargeting by genetic strategies.

Conclusions and Perspectives

As the diverse *in vivo* biology of the adenovirus virome has been better appreciated, it has become clearer that vector pharmacology relies only in part on evolved receptor binding ligands and can be significantly influenced by interactions with host proteins. We now understand that Ads encounter progressive viral distractions and sinks in the blood and in organs that can quantitatively deplete the vast majority of IV-injected Ad therapeutics. Retargeting efforts after IV injection that are pursued without considering detargeting are likely doomed to failure. Avoiding the blood and vasculature is a smart way to avoid these problems for those therapies that can be delivered by other routes. If this is not possible, selections of the right Ad serotypes combined with genetic or chemical modifications of the virus hold promise to bypass these viral sinks. Once detargeted, effective retargeting strategies can be applied to Ad vectors. Whether IV-administered Ads can penetrate into tissues from the blood is a separate question.

It is unclear to what degree genetic and chemical detargeting can shield the virus from blood protein binding and the host from this rapid toxicity. We speculate that chemical shielding may be better at reducing this immediate toxicity based on their general abilities to blunt binding and side effects. However, comprehensive genetic engineering of Ads that detarget certain proteins or cells may also succeed at blunting rapid and extended side effects after systemic Ad therapy.

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