



REVIEW ARTICLE

Adenovirus-mediated gene delivery: Potential applications for gene and cell-based therapies in the new era of personalized medicine



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Abstract With rapid advances in understanding molecular pathogenesis of human diseases in the era of genome sciences and systems biology, it is anticipated that increasing numbers of therapeutic genes or targets will become available for targeted therapies. Despite numerous setbacks, efficacious gene and/or cell-based therapies still hold the great promise to revolutionize the clinical management of human diseases. It is widely recognized that poor gene delivery is the limiting factor for most *in vivo* gene therapies. There has been a long-lasting interest in using viral vectors, especially adenoviral vectors, to deliver therapeutic genes for the past two decades. Among all currently available viral vectors, adenovirus is the most efficient gene delivery system in a broad range of cell and tissue types. The applications of adenoviral vectors in gene delivery have greatly increased in number and efficiency since their initial development. In fact, among over 2000 gene therapy clinical trials approved worldwide since 1989, a significant portion of the trials have utilized adenoviral vectors. This review aims to provide a comprehensive overview on the characteristics of adenoviral vectors, including adenoviral biology, approaches to engineering adenoviral vectors, and their applications in clinical and preclinical studies with an emphasis in the areas of cancer treatment, vaccination and regenerative medicine. Current challenges and future directions regarding the use of adenoviral vectors are also discussed. It is expected that the continued improvements in adenoviral vectors should provide great opportunities for cell and gene therapies to live up to its enormous potential in personalized medicine.

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Introduction

By using directed gene transfer to treat human disease, gene therapy may hold the potential to revolutionize medicine – in part because this approach is capable of treating the root cause of a disease, not merely its symptoms.¹ Despite numerous setbacks in the past decades, gene therapy remains as a field that is constantly growing and developing as scientists look for new strategies to treat some of the most difficult health issues. There has been a long-lasting interest in using viral vectors, especially adenoviral vectors, for gene therapy in the past two decades.^{1–3} It is well recognized that poor gene delivery is the limiting factor for most *in vivo* gene therapies while the only exceptions are where therapeutic viruses can be injected directly into the target site or where they can be introduced into target cells *ex vivo*.

Adenovirus has received tremendous attention as an effective gene delivery vector and was in fact the first DNA virus to enter rigorous therapeutic development, largely because of its well-defined biology, its genetic stability, its high gene transduction efficiency and its ease of large-scale production.^{2–4} Adenovirus (Ad) is a non-enveloped, linear double-stranded DNA virus with 57 identified human Ad serotypes.^{2,5} Ad serotypes differ in tropism and are further divided into six subgroups, A–G. Differences in viral capsids delineate tropisms among serotypes. The viral capsid is comprised of capsid proteins, core proteins, and cement proteins. These diverse serotypes can give rise to a vast range of therapeutic candidate viruses. Thus, it is not surprising that adenovirus continues to occupy the center stage in gene therapy arena.^{2–4}

Compared with other viral gene delivery systems, adenoviral vectors offer significant advantages.² First, adenovirus is the most effective means of delivering genes

in vivo as most human cells express the primary adenovirus receptor and the secondary integrin receptors. Thus are easily infected with adenovirus vectors and consequently yield high levels of the transgene expression.² Second, the development of gutless adenoviral vectors allows us to circumvent anti-adenoviral vector immunity. Third, despite the concern over safety of their use, there has been extensive experience with adenovirus vectors in many different clinical applications, and the safest dosing and routes of administration are now well established.² In fact, adenovirus vectors are the most common vector used in clinical trials worldwide and account for >20% of all gene therapy trials (see below). Fourth, adenovirus vectors offer a versatile platform for developing strategies to modify viral capsids in order to enhance therapeutic properties and improve targeting specificity of the virus. Interestingly, some of the inherited shortcomings of adenovirus, such as immunity evoked against the adenovirus capsid and low-level expression of adenovirus genes, may now prove beneficial for the development of anticancer immunotherapies, where inducing immunity against the cancer or directly killing the cancer cell is the goal. Furthermore, the combined immunity against the adenovirus together with the short time of expression is ideal for using the adenovirus as a platform for developing vaccines.²

The past two decades have witnessed many advances in the adenovirus vector system, ranging from its deployment as a vector for transgene delivery and supplementation for vaccination to its use as an oncolytic agent. Together with adeno-associated viruses and lenti/retrovirus vectors, the adenovirus now represents one of the three major viral vector categories in the gene therapy “tool box”. In this review, we will focus on the basic features of adenovirus, namely by the comparison of adenovirus to other viral and non-viral vectors, the common methods to produce

adenovirus, the current clinical and preclinical use of adenovirus as gene transfer tools, as well as the existing challenges and opportunities in using adenovirus vectors in gene and cell-based medicine.

Commonly-used methods for gene delivery

A variety of other gene delivery techniques, largely subdivided between viral and non-viral methodologies, have come into use over the last few decades (Table 1). In addition to adenovirus (AdV), lentivirus (LV), herpes simplex virus (HSV), adeno-associated virus (AAV), and baculovirus have also been studied for use in gene therapies.^{1,3} Within the non-viral subclass, techniques utilizing naked DNA injection, electroporation, gene gun, sonoporation, magnetofection, and lipoplexes have also been developed for gene delivery.^{6–9}

While viral vector techniques make use of the natural aptitude of viruses to efficiently deliver genes directly into target cells, these applications do not come without potential for adverse side effects (Table 1). Namely, the use of viruses often present risk for marked immunogenic response, insertional mutagenesis, and cytotoxicity. Furthermore, the transgenic capacities of most viral vectors are largely limited in size.^{10,11} On the other hand, though non-viral systems do not exhibit immunotoxic concerns nor significantly constrain transgene size, they are generally far less efficient in delivering transgenes to a target cell.^{12,13}

Despite great strides in gene delivery innovation over the last 20 years, no single class of gene delivery system has been developed without falling prey to one of these major limitations or potential adverse side effects.^{14–17} As such, adjunct techniques, like those of episomal vectors and

Table 1 Characteristics of the commonly-used viral vectors.

Viral system	Adenovirus (Ad5)	AAV	Retrovirus	Lentivirus	HSV-1	Baculovirus
Genome material	dsDNA	ssDNA	RNA	RNA	dsDNA	dsDNA
Genome size	36 kb	8.5 kb	7–11 kb	8 kb	150 kb	80–180 kb
Enveloped	No	No	Yes	Yes	Yes	Yes
Biosafety level	BSL-2	BSL-1	BSL-1/2	BSL-2/3	BSL-2	BSL-1
Insert size	8–36 kb	5 kb	8 kb	9 kb	30–40 kb	No limit known
Max titer (particles/mL)	1×10^{13}	1×10^{11}	1×10^9	1×10^9	1×10^9	2×10^8
Tropism	Broad, low for blood cells	Broad, low for blood cells	Broad (pan or psuedo-typed)	Broad (pan or psuedo-typed)	Neurons	Some mammalian cells
Infectivity	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells
Transgene expression	Transient	Transient or stable	Stable	Stable	Transient	Transient or stable
Vector genome form	Episomal	Episomal (>90%), site-specific integration (<10%)	Integrated	Integrated	Episomal	Episomal or integrated
Inflammatory potential	High (low for HC-AdVs)	Low	Low	Low	High	High
Advantages	High titers; extremely efficient transduction of most cell types and tissues	Safe transgene delivery; non-inflammatory; non-pathogenic	Persistent gene transfer in dividing cells	Persistent gene transfer in most tissues	Large packaging capacity; strong tropism for neuronal cells	Large cargo sizes; high level of gene expression
Drawbacks	Capsid mediates a potent inflammatory response (eliminated in HC-AdVs)	Small packaging capacity; requiring helper AdV for replication and difficult to produce pure viral stocks	Only transduces dividing cells; integration might induce oncogenesis in some applications	Integration might induce oncogenesis in some applications	Inflammatory; no expression during latent infection; transient gene expression in non-neuronal cells	Limited mammalian host range

transposon-based vectors, may be applied in combination with larger gene delivery systems to overcome such obstacles. These complementary techniques may be tailored in combination to enhance stable gene expression and efficient gene transfer; or alternatively, used stand-alone to mitigate side effects and improve the overall safety profile of a system.

Non-viral episomal vectors for safe and persistent gene delivery

The use of episomal vectors provides yet another system for gene delivery with its own set of benefits and limitations. Since these vectors remain extrachromosomal without direct integration into the host genome, they spare host cells the potential side effects of insertional mutagenesis.^{18–21} And though the immunogenicity of these systems can be greatly decreased by excluding viral integration, non-viral episomal vectors are generally inefficient transfection agents – much like stand-alone transposon-based systems.²¹ Transgenes delivered via episomal plasmids classically exhibit transient rates of expression; similar to AdVs and AAV vectors.¹⁵ However, recent advances have provided options to circumvent this major drawback by constructing episomal vectors that incorporate genetic material from viruses like the BK virus (BKV), Epstein–Barr Virus (EBV), Bovine Papilloma Virus-1 (BPV-1), and Simian Virus 40 (SV40).^{21,22} These viruses, and consequently their derivative episomal constructs, contain trans-activating factors that can be used to uncouple transgene replication from the regulatory mechanisms of the host cell to establish an autonomous ‘episomal replicon’.^{21,23,24}

One of the most studied episomal vector systems integrates the SV40 large T-antigen, an oncoprotein and replicative helicase, to confer amplified and sustained transgene expression.^{25–27} Similarly, the BKV T antigen has also been used in studies as a trans-activating factor to induce rapid transgene amplification.²⁸ Yet the broad range of interaction exhibited by these trans-acting factors, particularly their inhibition of the p53 tumor-suppressor protein, make them too volatile for application in human gene therapy.^{21,28}

Non-viral DNA transposons for efficient long-term and footprint-less gene delivery

DNA transposons represent a powerful emerging tool for gene delivery that is unencumbered by many of the toxic and undesirable side effects that burden viral vector modalities.^{29–32} Furthermore, most transposon systems are non-immunogenic and are able to deliver transgene cargos between 9 and 10 kb in size for Sleeping Beauty or up to 100 kb for piggyBac transposon.³¹ Transposon-based systems utilize transposase enzymes to mediate a ‘cut-and-paste’ transfer of transgenes from vector to host genome.³³ The transposon vector, carrying a transgene cassette between unique flanking DNA sequences, is commonly delivered to the cell in the form of a circular DNA plasmid.^{31,34} Cellular uptake of plasmid molecules by non-viral techniques are far less efficient than that of viral vector techniques; which marks one of the largest limitations faced by

stand-alone transposon-based delivery systems.^{31,35–38} However, once inside the cell, transposon systems enable direct integration of transgenes into the host genome with significant reliability.^{31,36,38} We demonstrated that the piggyBac transposon-mediated gene expression is more efficient than that of retroviral vector transduced cells.³⁹

The benefits conferred by direct genomic integration toward the stability and longevity of transgene expression have been described above. Transposons used in mammalian hosts are considered non-autonomous systems. Here, the transposase enzyme must be supplied by a secondary source, since functional transposase enzymes are not inherent to vertebrates.^{36,40} Such a non-autonomous system prevents the transposon from independently continuing to excise and re-integrate itself in a haphazard fashion, largely diminishing the risk of oncogenic mutagenesis.^{36,37,40} Given their relatively simple design, composed almost entirely of DNA, the production costs of transposons are considerably lower than those of viral vector systems.^{29,31,38}

Lentiviral vectors for stable and long-term gene transfer

The lentivirus is unique among retroviruses given its ability to transduce non-replicating cells in addition to replicating cells.^{9,41} As a retrovirus, however, lentiviral vectors contain RNA gene products that must undergo processing by reverse transcriptase enzymes to produce double-stranded DNA proviral transgenes. Most lentiviruses are generally apathogenic in humans and therefore exhibit low levels of immunogenicity.⁴² The lentiviral vector capacity, at 8 kb, is small like that of AAV vectors. Though dissimilar to AAV vectors and AdVs, lentiviral vectors ultimately integrate transgenes into the host genome through the activity of retroviral integrase enzymes.^{43,44} This integration of transgenes into the host genome presents a double-edged sword when considering the efficiency and safety of lentiviral vectors in comparison to AdV and AAV vectors.³⁵

While genomic integration provides the benefits of stable transgene expression in addition to continued expression among daughter cells, the risk of inactivating tumor-suppressor genes, activating oncogenes, or otherwise altering various other vital regulatory genes (a phenomenon termed “insertional mutagenesis”) depends on the site of integration and may potentially lead to cancer.^{44–52} The precise integration site of transgenes delivered by lentiviral vectors, though non-random, is more or less unpredictable.^{53–55} As such, concerns regarding the development of cancer by insertional mutagenesis certainly exist when considering the use of lentiviral vectors in gene therapy.

Lentiviruses have been found to demonstrate strong tropism for neural stem cells and have been studied for their utility in delivering genes within CNS tissues *ex vivo*.^{43,44} Accordingly, numerous groups have applied lentiviral vectors within animal models to study potential therapies for Parkinson Disease, Alzheimer Disease, and Huntington’s Disease.^{56–61} Various methods for modifying and pseudotyping lentiviral vectors to enhance their versatility and specificity among certain cell types,

including hematopoietic precursors, neurons, lymphoid cells, and macrophages, have also been reported.^{1,62–65} Alternative studies seeking to enhance transgene expression in a cell-specific manner have incorporated tissue- and cell-specific promoters within transgene constructs and coupled their delivery to a diversity of viral vectors (including AdV, lentivirus, AAV, and HSV).

Adenovirus-associated virus (AAV) for safe and small cargo gene delivery

Adeno-associated virus (AAV) is a small, helper-dependent, single-stranded DNA virus capable of transducing both dividing and non-dividing cells by delivering a predominantly episomal transgene product.^{66–71} In comparison to AdVs, AAV vectors provide a safer option for transduction given their naturally diminished pathogenicity and immunogenicity in humans.^{9,72} AAV vectors have been found to display the potential for host genome integration. However, in these rare instances, they will reliably integrate in a site-specific manner to the q arm of human chromosome 19 – where expression remains silent unless the AAV lytic cycle is induced by a helper virus.^{1,62,73,74} Such site-specificity further bolsters the safety profile of AAV vectors.

One considerable disadvantage of AAV vectors is their small transgene capacity of approximately 4.8 kb, which severely restricts the breadth of therapeutic genes that may be delivered via AAV vector.^{9,35,62,75} AAVs have been studied in the treatment of Hemophilia B, Leber's Congenital Amaurosis, Alpha-1 Antitrypsin Deficiency, and Cystic Fibrosis.^{72,76–78} Surprisingly, some AAV vectors, particularly vectors derived from the AAV9 serotype, have been found to cross the blood–brain barrier and proved capable of transducing cells of the CNS.^{79–83} Such breakthroughs hold great clinical promise in developing novel therapeutic options for diseases of the brain and spinal cord.

Herpes simplex virus (HSV) vectors for neural tissue-specific and large cargo gene delivery

The Herpes Simplex Virus (HSV) is a double-stranded DNA virus capable of delivering up to 50 kbp of transgenic DNA when used as a vector; making HSV one of the largest viral vectors under routine study.^{62,84–86} Similar to the adenovirus, pre-existing immunity to HSV infection is prevalent within the general population; however, HSV vectors are largely able to evade inactivation by host immune response.^{68,69,84,85,87} HSV vector genomes also remain episomal like those of AdVs. As a result, they are expectably burdened by the same limitations of transient expression faced by AdVs as discussed above.^{62,88} If an HSV vector co-infects a cell with an underlying latent HSV infection, the opportunity for genetic recombination between the vector and wild-type genomes also presents a considerable safety concern.^{89,90}

One of the most striking characteristics of HSV vectors is their natural tropism for neuronal cells, which contributes to their great therapeutic potential in delivering genes in the selective treatment of nervous system disorders.^{91,92} A recent study by Goss et al has evaluated HSV vectors in targeting sensory neurons to express pain reliever genes in patients

with chronic pain disorders.⁹³ Though early HSV vectors were limited to transduction of non-dividing cells, modifications have been recently developed to produce replication-conditional HSV vectors that demonstrate significant toxicity within proliferating tumors.⁹⁴ The neurotropism of HSV vectors in combination with these oncolytic properties has proved valuable in targeting tumors and cancerous cells of neuronal origin.^{92,95–99} Studies have demonstrated the successful use of HSV vectors against gliomas, glioblastomas, melanoma and other solid tumors.^{88,91,100,101}

Adenoviral vectors as one of the most efficient gene delivery systems

One of the most salient features of the adenovirus is its prevalence among healthy individuals. As such, a broad pre-existing immunity against Adenovirus is common in the general population.¹⁰² Such immunity largely hinders the utility of AdVs derived from the most prevalent serotypes of adenovirus.⁶² Therefore, investigators have focused on using the rare serotypes 2 and 5 to construct AdVs for gene therapy.^{35,42,103} Of additional concern, the proteins of all AdVs exhibit high immunogenicity. As a result, repeat administrations of AdVs within a single patient are severely limited to prevent risk anaphylactic shock or death.^{62,104–108} Interestingly, the strong immunogenicity of AdVs makes them ideal candidates for applications in oncolysis and vaccination.^{35,109,110} AdVs can be used to trigger antitumoral immunity within the tumor microenvironment,^{110,111} and have been studied in the development of vaccines against HIV and influenza, among other pandemic diseases.^{62,103,112}

Adenoviral vectors are able to transduce both replicating and quiescent cell populations, making them a valuable tool in delivering transgenes *in vivo* and within mature tissues.⁶² In contrast to lentiviral vectors, AdVs are able to deliver larger transgenes up to 8 kbp in size; however, their DNA does not integrate into the host genome, but rather, resides episomally in the host nucleus.^{113,114} Such episomal transduction precludes the risks of insertional mutagenesis, without direct integration into the host genome although transgene expression is transient, is vulnerable to cell silencing mechanisms, and is destined for dilution among daughter cells should cell division ensue.⁶²

Nonetheless, it is clear that no single vector system outlined above presents a ubiquitously ideal method for achieving gene delivery across all applications. However, a thorough review of the advantages and limitations characteristic of each modality, with an eye for integrating techniques, enables investigators to devise a highly modulatory strategy to approach the task at hand. By studying these vectors within novel and innovative applications, we may realize the full therapeutic promise these techniques hold for patients. Here, we only focus on the characteristics of adenoviral vector-mediated gene transfer.

The characteristics of adenovirus

The adenoviral genomes are linear, non-segmented double-stranded DNA with sizes ranging from 26 kb to 45 kb in

length, depending on the serotype. The genome of the commonly-used human adenovirus type 5 (Ad5) is approximately 36 kb (Fig. 1).³ The DNA is flanked on both ends by hair-pin-like, inverted terminal repeats (ITR), which serve a variety of purposes. One of the roles of ITRs is to act as a self-primer to promote primase-independent DNA synthesis, making them important elements in DNA multiplication. Another function of the ITRs is to facilitate integration into the host genome (Fig. 1). In addition to ITRs, another genetic element of the adenovirus is the packaging signal, which is located on the left arm of the genome and is required for proper viral transcript packaging. Viral transcripts are classified as either early or late. The four early transcriptional units, E1, E2, E3, and E4, are responsible for expressing non-structural proteins (Fig. 1). These proteins have regulatory functions and are involved in viral DNA replication. The late proteins encode for structural components of the Ad virion (Fig. 2A and B).

The adenovirus virion has unique viral structure: 90–100 nm in size, non-enveloped, icosahedral particle containing a nucleocapsid (Fig. 2A and B). The viral capsid consists of 252 proteins, involving three distinct types: fiber, penton and hexon based proteins. There are 240 hexon proteins and 12 penton proteins (Fig. 2A and B). Both fiber and penton base proteins are key in receptor binding and internalization, while hexon composes most of the viral capsid. The “spikes” or fibers are associated with each penton base protein of the capsid and facilitates attachment to host cell receptors, including the coxsackie-Ad receptor (CAR) (Fig. 3). After endocytosis, the Ad capsid escapes from the endosome and reaches the nuclear pores

via microtubule-mediated translocation (Fig. 3). In nature, wild-type AdVs do not integrate their genomes into host cell chromosomes. With a few exceptions, they replicate as linear, extra-chromosomal DNA elements in the nucleus. After DNA replication, the Ad genomes are packaged into the newly assembled capsids in the nucleus of the infected cell. The release of Ad progeny requires the lysis of the infected cell.

The evolution of three generations of adenoviral vectors

The use of adenoviral vectors (AdVs, mostly derived from human AdV5) has become more efficient with time and experience. To establish safety in use, the first improvements were made by eliminating essential viral-replication genes (Fig. 4). First generation AdVs are those stripped of regulatory genes E1a and E1b – the first transcriptional regulatory factors to be produced during the viral life cycle. The depletion of this gene resulted in replication-deficient AdV with an initial transgene cloning capacity of 5.2 kb.⁵ Looking to improve, researchers wanted to increase cloning capacity and continue to decrease capacity for viral replication. Thus, second generation AdVs describe vectors with deletion of other non-structural genes (E2/E3/E4) in addition to the original E1 gene absent in first generation vectors (Fig. 4). While the second generation of vectors demonstrated increased cloning capacity and reduced cytotoxicity, they still triggered immune responses *in vivo* resulting in the reduced yields of transduced cells.⁵ Born

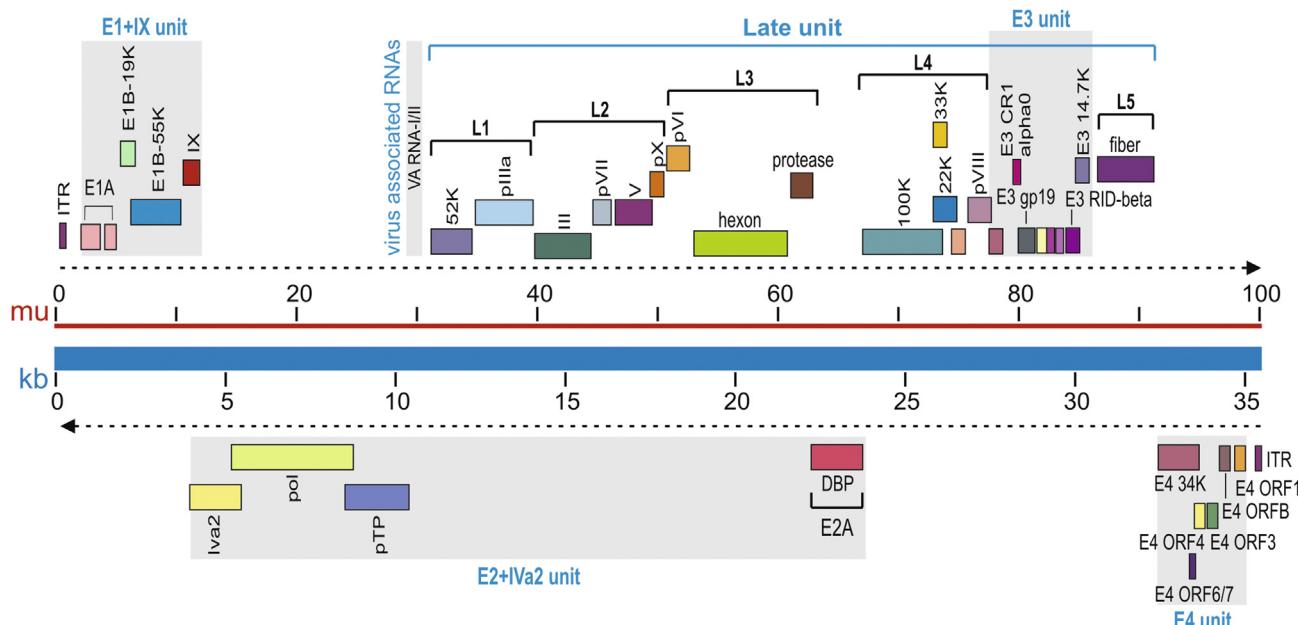


Figure 1 The genome structure and major transcript units of human Ad5. The Ad5 genome is composed of 36 kb linear double-stranded DNA, also shown in map units (mu). The Ad5 genes are temporally transcribed as early units (E1, E2, E3 and E4 units) or late unit (L1 to L5) in both directions. The top part of the listed genes are transcribed from left to right, while the lower part of the genes are transcribed from right to left, as indicated by the dotted arrows. E1 gene products are involved in the replication of the virus. The E2 region is sub-divided into E2A and E2B. These proteins provide the machinery for viral DNA replication and the ensuing transcription of late genes, which mostly encode structural proteins for virus packaging. Most of the E3 proteins are involved in modulating the immune response of infected cells and are not essential for viral production *in vitro*.

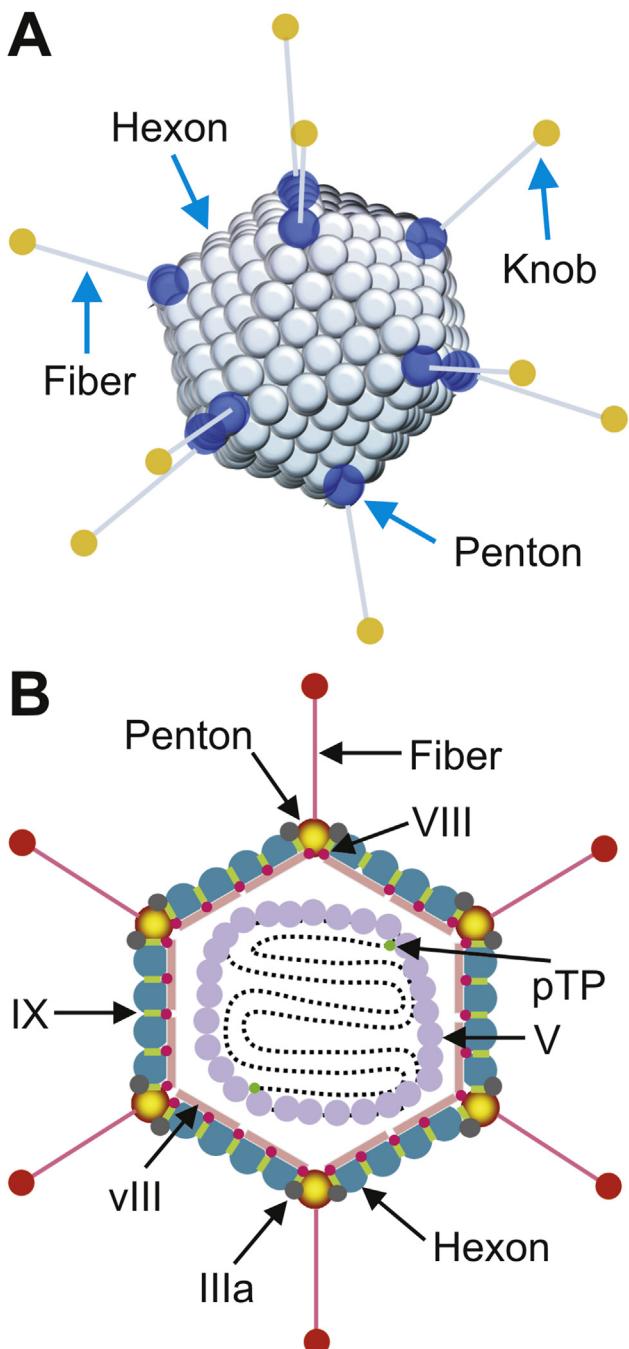


Figure 2 The structure of adenovirus. (A) Adenovirus is a large, non-enveloped virus presenting icosahedral symmetry. The hexon, penton base, and knobbed fiber, are the most important capsid proteins for gene delivery. (B) Hexon is the major protein forming the 20 triangular faces of the viral capsid. The 240 hexon capsomeres in the capsid are trimers, each interacting with six other trimers. The 12 vertices are formed by the penton capsomere, a complex of five copies of the penton base, and three copies of fiber. Each penton capsomere interacts with five hexon capsomeres, one from each of the five faces that converge at the vertex. The knobbed fiber protrudes from the fiber base. In addition, the 5' termini of adenovirus genome bind covalently to the precursor terminal protein (pTP). The viral genome DNA is wrapped in a histone-like protein and contains the inverted terminal repeats (ITRs), which act as origins of replication.

from this struggle to develop less immunogenic vectors, a third generation of AdVs emerged, termed high-capacity adenoviral vectors (HC-AdVs), also known as gutless AdVs or helper-dependent AdVs (HD-AdVs) (Fig. 4). The HC-AdV is stripped of all viral coding sequences, resulting in a vector with only 5' and 3' ITRs in addition to a packaging signal, thus providing a larger capacity for transgenic cloning sequences (36 kb). Because of this structure, they are often referred to as "gutless adenoviral vectors." Furthermore, the structure of the HC-AdV minimizes cytotoxicity, thus enabling prolonged expression of therapeutic genes, rendering the HC-AdV the most promising AdV to use for gene therapy to date.

High-capacity adenoviral vectors (HC-AdVs) are beneficial because they lack the viral elements that can cause an immune response in the host. HC-AdV's are deemed "helper-dependent" because while these vectors lack viral genetic components, they also lack the necessary packaging components. A complementary virus, or helper virus (HV), is used to provide the necessary proteins *in trans* for the packaging of an Ad-based vector.¹¹⁵ The HV is not packaged along with the desired HC-AdV because it has its packaging sequences flanked by *loxP* recognition sites, which is sufficiently excised by Cre recombinase so that the helper virus DNA remains unpackaged. While the packaging ability of the HV is stunted, the HV is replicated at normal levels and can thus express all of the functions necessary *in trans* for replication and packaging of a vector genome containing the appropriate *cis*-acting elements.

Optimization of the delivery system is essential for its efficiency as a gene therapy tool because HC-AdV is often needed in large quantities. Large-scale production of HC-Ad via HVs can be optimized through modification of HV with "stuffer" sequences to enhance growth characteristics. Such stuffer sequences help to optimize HC-AdV replication, increase the total production of HC-AdVs, and reduce the incidence of HV contamination.^{116,117}

The HC-Ad system relies on minimal HV contamination. Another way to minimize contamination is by alternative methods of selection against the HV. The traditional method is through a Cre/*loxP* system, but Ng described a FLP/FRT system that was shown to be equivalent with respect to HC-AdV amplification efficiency and HV contamination levels.¹¹⁸ This provides scientists with a second recombinase system for HC-AdV production, which will enhance the utility and flexibility of HC-AdVs. The Cre/*loxP* system requires final, density-based separation of HC-AdV and residual HV by ultracentrifugation to reduce contamination levels.¹¹⁹ This step hinders the optimization of large-scale production. FLP recombinase has shown to mediate maximum levels of excision (about 100% versus 80% for Cre) and, without any purification, showed the same levels of residual contamination as Cre after ultracentrifugation, rendering the FLP/FRT system as a promising strategy in optimizing HC-Ad production.

Approaches to the efficient productions of recombinant adenoviral vectors

Although the use of recombinant adenoviruses has been widespread, the initial generation of a given adenoviral

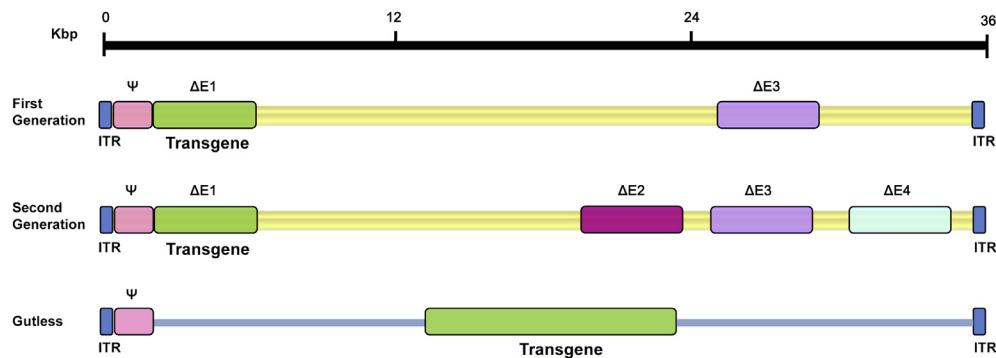


Figure 3 Adenovirus-mediated gene delivery to mammalian cells. The initial step of adenovirus infection is gaining access to the host cell, and the 12 spikes of the capsid are adhesion receptors, which recognize and bind to specific glycoprotein receptors, such as CAR on the target cell membrane, with the irreversible binding of cell-surface integrins to the penton at the base of the spike. This leads to endocytosis, and the cell-surface membrane invaginates, forming a pit. This pit invaginates and pinches off as a vesicle in the cytoplasm, coated by clathrin and containing the virus. The vesicles are sent to endosome. When the endosome becomes more acidic, the virus is uncoated as the outer capsid disassembles, revealing the viral DNA-protein core. The shed spikes have a toxic function and breach the endosome membrane, allowing the viral core to escape from the endosome into the cytosol of the host cell. Subsequently, the transgenes or nucleic acids are expressed in the target cells.

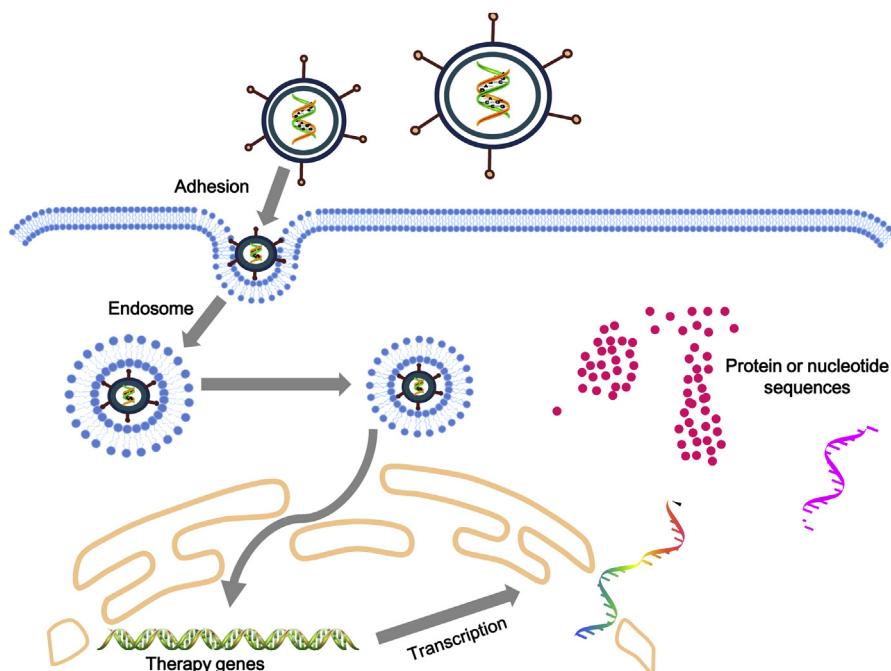


Figure 4 Three generations of adenoviral vectors. The first generation vectors are deficient in the E1 and E3 regions and have a maximum capacity of 8.2 kb for introducing of a therapeutic gene. The second generation vectors are deficient in E2 and E4, in addition to the deletion of the E1 and E3 regions. Gutless Ad vectors, also known as helper-dependent adenoviruses (HD-Ad), or high-capacity adenoviruses (HC-Ad), can be created to prevent the problem of Ad-created cellular immune response. HD-AdVs are helper-dependent because they depend on a helper adenovirus to be able to produce. HD-AdVs are of high capacity because they allow insertions of up to 36 Kb. Gutless Ad maintain only the 5' and 3' ITRs and the packaging signal (Ψ), an essential for final assembly of the virion.

vector (mostly the first generation AdVs) remains a technically challenging and time-consuming process.^{1,3,120,121} In the past decades, numerous efforts were devoted to the development of techniques for rapid and efficient generation of recombinant AdVs.^{1,3,121–125}

The classical method for generating E1-deleted AdVs utilizes homologous recombination between two DNA vectors, one carrying sequences mapping to the very left end

of the adenovirus genome and the gene of interest, and the other carrying sequences that slightly overlap the 3'-most viral sequences on the first molecule and continue to the right end of the adenovirus genome.^{123,124} This recombination takes place inside E1-expressing cells such as HEK-293 cells, generating the desired recombinant viral DNA (Fig. 5A). While functional, this approach is rarely used now because its AdV generation process is extremely inefficient.

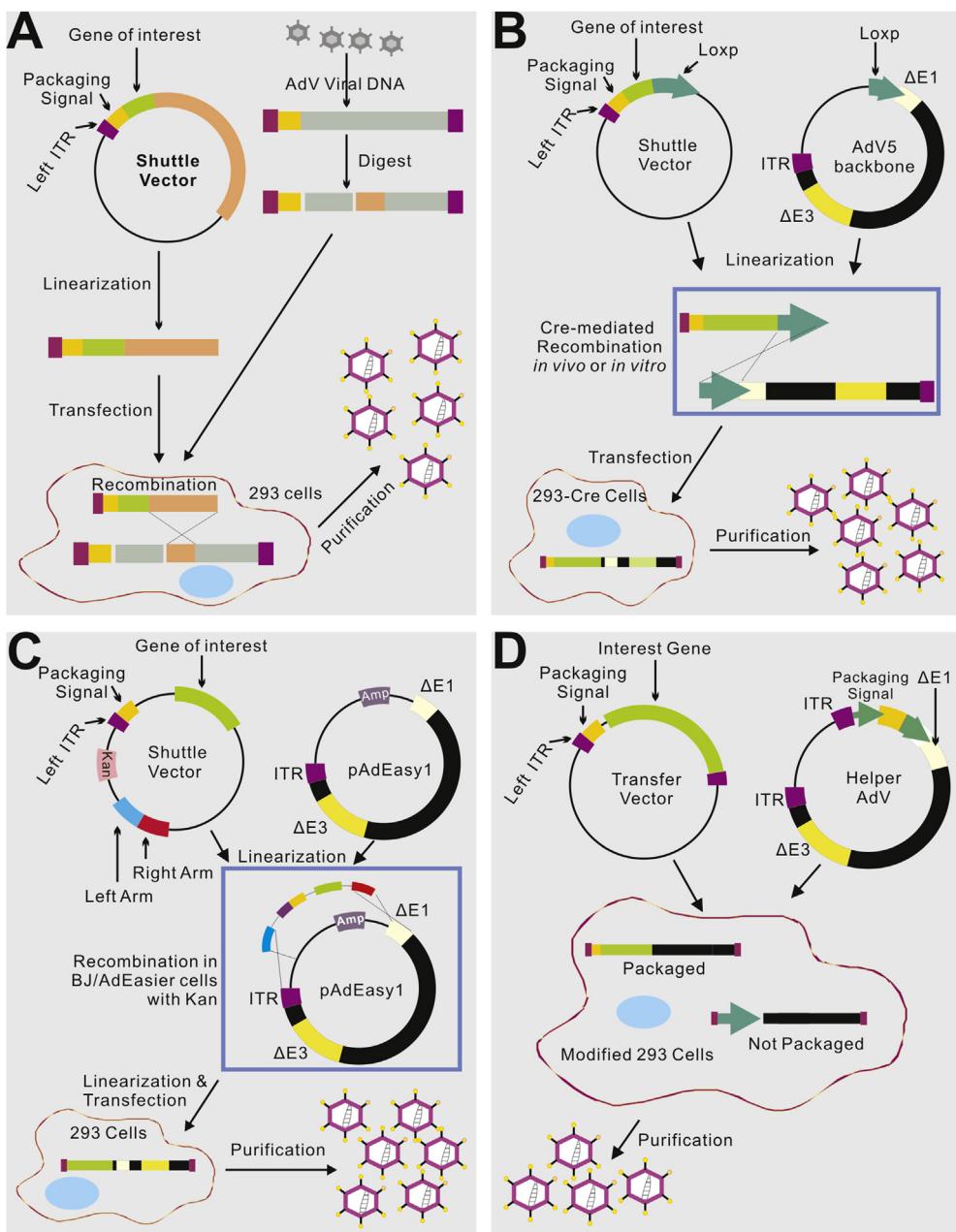


Figure 5 Four commonly-used methods to generate and produce adenovirus vectors for gene delivery. (A) The traditional method – recombination in HEK-293 cells. The gene of interest (GOI) is first cloned into a shuttle vector, which contains 5'-ITR, packaging signal and homologous regions to adenoviral genome. Adenoviruses are generated in HEK-293 cells through recombination between shuttle vector and adenoviral backbone vector, which is unable to produce virus by its self. (B) Cre/LoxP-mediated recombination. The GOI is cloned into a shuttle vector that contains LoxP site(s). Cre recombinase-mediated recombination occurs with a LoxP-containing adenoviral backbone vector *in vitro* or 293-Cre cells, leading to the generation of adenoviruses. (C) The AdEasy system. The GOI is subcloned into a shuttle vector that contains 5'-ITR and packaging signal, as well as a kanamycin-containing bacterial replication unit flanked with homologous arms. Recombinant adenoviral plasmids are generated through homologous recombination between the linearized shuttle vector and ampicillin-resistant adenoviral backbone vector, such as pAdEasy1, in the bacterial strain BJ5183 cells under kanamycin selection. The resultant adenoviral plasmids are linearized and used for adenovirus production in HEK-293 cells. (D) The use of helper adenovirus for the production of HC-AdVs (or HD-AdVs, or Gutless AdVs). The GOI is cloned into a transfer vector that contains both ITRs and packaging signal only. Adenoviruses are generated with a helper adenovirus, which will not be packaged due to the deletion of packaging signal in the modified HEK-293 cells, usually through Cre/LoxP or FLP/FRT excision system.

An early alternative to this method involved an *in vitro* direct ligation of the transgene-containing fragment to the linear E1/E3-deleted adenoviral genome DNA through a restriction enzyme site, such as *Cla* site.¹²⁶ However, due

to the large size of Ad genome, unique and useful restriction sites are limited, making the construction of Ad vectors relatively labor intensive. Nonetheless, the direct ligation approach was significantly improved when additional

unique restriction sites, such as I-CeuI, Swal and PI-SceI were engineered and added to the E1 deletion region.¹²⁷ However, this ligation approach is not commonly used because the efficiency is low and the recombinant virus often requires purification of contaminating wild-type and transgene-null viruses.

To overcome the low efficiency of homologous recombination in HEK-293 cells, the Cre-lox site-specific recombination system was later developed to generate AdVs (Fig. 5B).^{128,129} This system requires three components (1) a recombinant adenovirus that contains two *loxP* sites flanking the packaging signal; (2) a shuttle vector which contains the left ITR, the packaging signal, the expression cassette and a *loxP* site; and (3) a 293-Cre cell line which expresses the Cre recombinase (Fig. 5B). When the shuttle vector carrying the gene of interest and the viral DNA are co-transfected into the 293-Cre cells, an intramolecular recombination occurs between the two *loxP* sites present in the viral DNA to generate an adenovirus genome which is able to replicate and express viral genes, but is unable to be packaged. A second recombination occurs between the *loxP* site of this product and that on the shuttle vector, generating the desired recombinant virus (Fig. 5B). A similar method using FLIP/FRT system was later developed.¹¹⁸ The major disadvantage of this method is the presence of the parental AdV in the preparation, which is difficult to completely remove from the viral preparations even after multiple passages in 293-Cre cells. Thus, this method requires a careful verification of the identity of the recombinant virus.

One of the most popular methods to overcome the inefficiency of homologous recombination in HEK-293 cells is to take advantage of the highly efficient homologous recombination in microorganisms, such as yeast and bacterial cells.^{121–125,130–132} As a result, AdV generation has been drastically facilitated by the techniques exploiting the efficient homologous recombination in bacterial cells. Chartier et al developed a system in which the large adenovirus plasmid is linearized in the E1 or near the E3 region. This DNA molecule is introduced into a *RecA*⁺ *E. coli* strain together with a DNA fragment containing the gene of interest flanked by E1 or E3 sequences. Recombination between both DNA molecules generates a plasmid containing the expression cassette in the desired region.¹³¹ Alternatively, a co-integrate plasmid is first obtained by recombination between a large adenovirus plasmid and a small shuttle vector that contains the gene of interest and is selected for in the presence of two antibiotics. The final recombinant plasmid is generated via a second homologous recombination event that excises it from the co-integrated *B. subtilis* *sacB* sequences, which encodes a protein that kills the bacterium in the presence of sucrose.¹³²

We further improved the bacterial recombination system and developed the highly simplified and more user-friendly AdEasy system (Fig. 5C).^{121,122,125} Our system was designed to introduce expression cassettes specifically into the E1 region and thus facilitate the recovery of the recombinant *E. coli* clones. Specifically, the large adenovirus plasmid is introduced into *E. coli* BJ5183 cells as a supercoiled DNA in order to increase transformation efficiency, and a kanamycin-resistance gene, provided by the shuttle vector containing the expression cassette, is used to select the

recombinant clones. The recombinant plasmid DNA is purified, and the viral chromosome is released by restriction digestion and transfected into the appropriate cell line.^{122,125} In the AdEasy system, the backbone vector, containing most of the adenoviral genome, is used in supercoiled form, obviating the need to enzymatically manipulate it. The recombination is performed in *E. coli* rather than in mammalian cells, as the process takes advantage of the highly efficient homologous recombination machinery present in bacteria. We also engineered multiple vectors to allow inclusion of up to 10 kb of transgene sequences and/or multiple transgenes to be produced from the same virus. These modifications resulted in highly efficient viral production systems that can often obviate the need for plaque purification and can significantly decrease the time required to generate usable viruses. In fact, the AdEasy has been one of the most commonly-used AdV making systems worldwide.^{122,125}

While the generation of HC-AdVs or helper-dependent AdVs is more straightforward, the technical challenge is to remove contaminating helper AdVs from the final adenoviral preparations and to obtain relatively pure HC-AdVs. One of the most efficient methods for producing HC-AdV is the use of Cre/loxP system (Fig. 5D).¹¹⁵ The HC-AdV genome contains the adenoviral ITRs and ϕ packaging signal and a noncoding eukaryotic "stuffer" DNA fragment that is needed to bring the vector genome size within the size range (27.7 kb–37 kb) for efficient packaging into virions.¹¹⁵ On the other hand, the helper virus (HV) is E1-deleted and bears a packaging signal flanked by *loxP* sites. Following infection of 293-Cre cells, this allows the packaging signal to be excised from the HV genome, rendering the HV genome unpackageable but still able to undergo DNA replication and thus complement the replication and encapsidation of the HC-AdV genome *in trans*.¹¹⁵ Alternative HC-AdV production systems based on FLP/FRT site-specific recombination system have also been developed and similar outcomes were obtained.^{118,133} While packaging signal excision was relatively efficient in either the Cre/LoxP or FLP/FRT system, it was not complete, leading to HV contamination, albeit at low levels.¹²⁹ Further studies showed significant reduction in HV contamination was achieved using producer cells that expressed much higher levels of Cre.¹²⁹ Because the genome size of the HC-AdV (~30 kb) and the HV (~36 kb) are engineered to be different, they can be physically separated by CsCl ultracentrifugation which provided an additional method to purify the HC-AdV from residual HV. Nonetheless, removing residual HV remains the top priority regarding the production and use of HC-AdVs.

It is noteworthy that, while critical to develop techniques for efficient AdV production, the improvement of AdV infection efficiency and packaging efficiency should also significantly benefit the AdV production process. We found that adenovirus-mediated gene transfer in mesenchymal stem cells can be significantly enhanced by the cationic polymer polybrene.¹³⁴ Furthermore, we have demonstrated that overexpression of Ad5 precursor terminal protein (pTP) accelerates recombinant adenovirus packaging and amplification in HEK-293 packaging cells.¹³⁵ More recently, we have engineered the RAPA cell line for rapid adenovirus production and amplification.¹³⁶ By

assessing human Ad5 genes (E1A, E1B19K/55K, pTP, DBP, and DNA Pol) and host factor OCT1 for their contributions to adenovirus production, we demonstrated that over-expression of both E1A and pTP led to a significant increase in adenovirus amplification, whereas other transgene combinations did not significantly affect adenovirus amplification. When E1A and pTP were stably expressed in 293T cells, the resultant RAPA line showed high efficiency in adenovirus amplification and production.¹³⁶ Furthermore, adenovirus production efficiency in RAPA cells was dependent on the amount of transfected DNA. Under optimal transfection conditions high-titer adenoviruses were obtained within 5 days of transfection.¹³⁶ Therefore, a combination of using the AdEasy system and the RAPA cell line should significantly expedite AdV production.

Adenovirus-mediated gene therapy for monogenic diseases

The first *in vivo* application of AdVs for genetic therapy in humans was reported in 1992 with the successful transfer and expression of alpha-1 antitrypsin (A1AT) cDNA within hepatocytes of a patient with alpha-1 antitrypsin deficiency.¹³⁷ Simultaneously, A1AT gene transfer methods were under development in native lung tissues using an E1⁻E3⁻ serotype 5 adenoviral vector.² Soon thereafter, with the identification and characterization of the CFTR gene underlying cystic fibrosis, and the known tropism of the adenovirus for respiratory epithelium, studies investigating the transduction of CFTR cDNA via AdV in humans were soon underway.¹³⁸ With each subsequent administration, investigators came to recognize that a response by the patients' humoral and cellular immunity to adenoviral vectors was responsible for this transient, 1–2 week expression of AdV transgenes, while also leading to adverse immunogenic events in other patients.^{139,140} The death of one patient under experimental treatment for ornithine-transcarbamylase deficiency using an AdV vector in 1999,¹⁰⁷ followed by the development of leukemia in two patients with X-linked severe combined immunodeficiency in 2002, safety concerns surrounding the application of viral vectors in human subjects lead to a sharp decline in studies over the next decade.^{52,73} Much of the research focus in that time then shifted toward developing less immunogenic viral vectors. Hence, the utility of AAV and lentiviral vectors emerged.¹⁴¹

Despite these initial setbacks, AdVs have returned to the spotlight of human gene therapy in recent years. Adenoviral vectors now represent the most commonly studied viral vectors among clinical trials currently underway worldwide (Table 2).¹⁴² The potent immunogenic properties that once undermined the use of AdVs in sustained treatment of genetic deficiencies have since been leveraged to purposefully evoke host immunity, thereby highlighting the adenovirus as an ideal vaccine carrier and antitumoral agent.

Adenoviral vectors as anticancer agents

There are three main therapeutic categories to which AdVs are applied as anticancer therapies. In the first,

Table 2 The registered gene therapy clinical trials.

Vector system	Number of registered trials	%
Adenovirus	505	21
Adeno-associated virus	173	7.2
Retrovirus	449	18.6
Naked/Plasmid DNA	414	17.2
Lentivirus	144	6
Vaccinia Virus	125	5.2
Herpes Simplex Virus	89	3.7
Poxvirus	70	2.9
Other	440	18
Total (as of March 2017)	2409	

Note: Vectors used in clinical gene therapy trials registered with the international gene therapy clinical trial registry up to March 2017.

Source: Gene Therapy Clinical Trials Worldwide (provided by the Journal of Gene Medicine); <http://www.wiley.co.uk/genmed/clinical/>.

replication-defective (RD) AdVs are used for their immunogenic properties to deliver immune-related genes/epitopes directly to tumor cells in order to attract and induce a local antitumoral immune response.^{143–150} In the second category of strategies, replication-competent (RC) AdVs (also called conditionally replicative Ad, CRAds; or oncolytic AdVs) may be used to preferentially replicate within cancer cells and achieve oncolysis by executing the natural lytic life cycle of the virus in cancer cells (Fig. 6).^{2,151} Lastly, either RD or RC AdVs may be used to deliver and/or overexpress tumor-suppressor genes or cytotoxic/suicide genes within cancerous cells to directly induce an intrinsic cytotoxic cascade, cause cell cycle arrest, or trigger apoptosis.^{151,152}

Two RD adenoviral vector systems, Advexin and Gendicine, have produced considerable data among a number of clinical trials. Advexin is an E1⁻E3⁻ Ad5 vector expressing p53 from a Cytomegalovirus (CMV) promoter, while Gendicine is very similar except that it expresses p53 from a Rous Sarcoma Virus promoter.^{151,152} In numerous studies, including phase I and II trials, Advexin was judged to be safe within the settings of colorectal cancer, hepatocellular carcinoma (HCC), non-small cell lung cancer (NSCLC), prostate cancer, breast cancer, ovarian cancer, bladder cancer, glioma, and squamous cell carcinoma of the head and neck (HNSCC).^{153–156} In a phase III trial comparing Advexin vs. Advexin + Methotrexate in the treatment of recurrent HNSCC, this vector system proved to be well tolerated with evidence of antitumoral activity.¹⁵⁷ In October of 2003, the State Food and Drug Administration of China approved Gendicine for its use in combination with chemotherapy to treat HNSCC, making Gendicine the first commercially available gene therapy vector in the world.^{152,158–161} More than 7000 patients have been treated with Gendicine while post-market studies have reported extremely low rates of vector shedding and a mild side effect profile, even after intravascular administration.^{158,159,162} Gendicine has since been further studied in 16 controlled clinical trials in China for the treatment of advanced and unresectable cancers, including HCC, NSCLC,

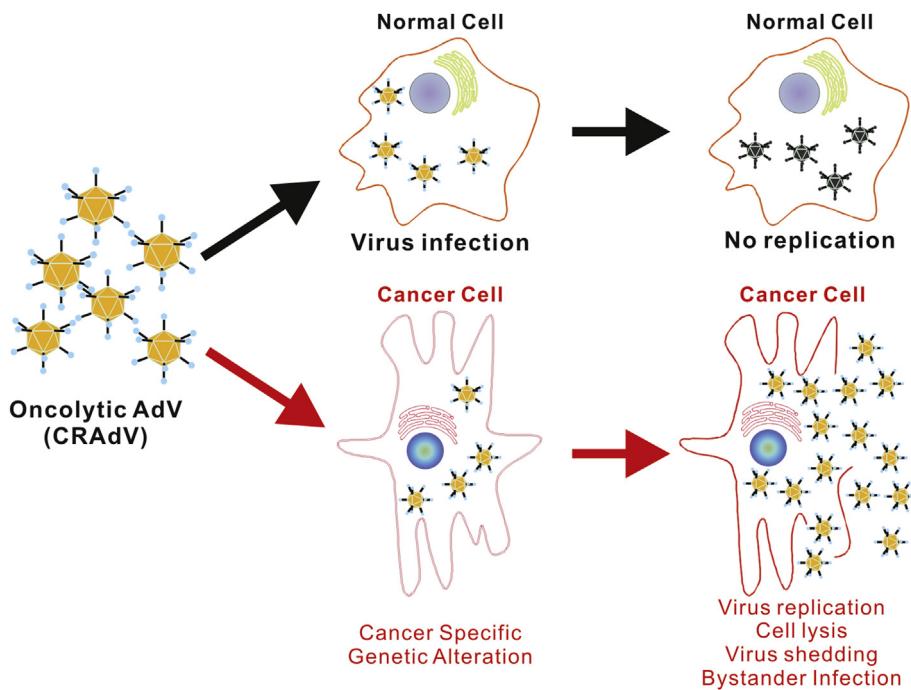


Figure 6 The anticancer effect of oncolytic adenoviruses (CRAdVs). The oncolytic adenoviruses infect both normal and cancer cells with similar efficiency. In cancer cells cancer-specific genetic changes activate the expression of Ad5 E1 genes, which initiates viral replication cascade, leading to virus production and cancer cell lysis. The packaged viruses are released and infect neighboring cancer cells to achieve bystander killing effect. However, normal cells lack the cancer-specific activation of viral replication, and thus no Ad5 viruses are reproduced. As the result, normal cells are spared by oncolytic adenoviruses.

malignant glioma, and epithelial ovarian carcinoma.^{64–79} Even among patients who failed conventional chemotherapy/radiation, Gendicine still displays synergistic effects in combination with these therapies while overall demonstrating better response and survival rates than those of control groups.^{65–83}

The advantages of RC AdVs in targeted cancer therapy become evident given that cancer cells are generally more conducive to Ad replication than quiescent or non-cancerous cells.¹⁶³ Furthermore, additional cancer-targeting modifications have been developed to support efficient oncolytic replication of AdVs in cancer cells while limiting their replication in healthy cells.^{151,164} In this way, many conditionally-replicative AdVs have been developed to target high-replicating cell populations or cell populations with specific oncogenic mutations.¹⁶⁵ In 1996, ONYX-015 became the first replication-selective AdV to enter clinical trials.¹⁶⁶ With a partial E1B gene deficiency that inhibits the replication of ONYX-015 in healthy cells expressing the p53 tumor suppressor, this oncolytic AdV exclusively replicates and amplifies in p53-deficient cancer cells.^{73,167,168} In phase I and II trials, ONYX-015 has demonstrated safety and tolerance when administered via intratumoral, intravascular, or intraperitoneal injection in patients with various advanced cancers.^{166,169} Furthermore, available results have since shown ONYX-015 is most effective in conjunction with conventional chemotherapy.^{168,170} In November of 2005, the replication-selective AdV, Oncorine (or "H101"), was approved in China for the treatment of cancer.^{151,161} Similarly, this oncolytic AdV also possesses a 55 kb E1B deletion, and has

demonstrated significantly higher response rates in combination with traditional chemotherapy when compared to chemotherapy alone.¹⁷¹

In light of such promising findings, numerous other clinical trials are currently underway to evaluate newer generations of oncolytic AdVs.^{73,151,172–174} These newer generations incorporate suicide genes (such as HSV thymidine kinase)¹⁷⁵ or cytotoxic pro-drug converting properties under the control of tissue or tumor-specific promoters (like the promoter of the prostate antigen PSA)^{176–178} to further enhance the efficacy, potency, and specificity of these vector systems.^{179,180} Additionally, advances in our understanding of adenoviral capsid antigens and immunogenic properties will undoubtedly reveal a broadened versatility of these vectors in vaccination efforts. Though current AdV systems have achieved some notable clinical successes, many challenges still largely impede the widespread use of AdVs in human gene therapy. Improvements in clinical efficacy and safety are needed to bring these innovations from the bench to the bedside, and thus require continued studies and trials despite their notorious debut to the clinical arena.

Adenoviral vectors for vaccine development

Adenoviral vectors have been incorporated into the development of vaccines against tuberculosis,¹⁸¹ HIV,^{182–184} Ebola,^{185–190} malaria,¹⁹¹ and influenza.^{192,193} Different from most traditional vaccines, immunization techniques using recombinant viral vectors trigger both a robust

cytotoxic T-cell response in addition to a humoral response.^{62,194,195} This cytotoxic T-cell response is far more efficient in destroying virus-infected cells, intracellular pathogens, and cancerous cells — while also expanding protective immunity against strains of similar pathogens that express homologous epitopes.¹⁹⁶

In the early 2000s, Merck began manufacturing a mixed-valent vaccine product composed of three Ad5-based vectors expressing several HIV-1 proteins.¹⁸⁴ This product was evaluated in the notable STEP study, which began enrolling volunteer subjects from around the world in 2004.^{184,197} Early findings suggested the vaccine was well tolerated, even upon repeat intramuscular injections, and was able to induce T-cell responses to HIV antigens.^{182,183,193} However, the study was prematurely stopped in 2007 when subjects in the treatment group revealed higher rates of HIV infection than those in the placebo group.¹⁵¹ Though multiple theories have surfaced to explain the increased trend for HIV acquisition in this treatment arm (particularly among men-who-have-sex-with-men, men who were uncircumcised, and/or those with pre-existing Ad5 neutralizing antibodies prior to vaccination),^{103,184} none such theories have undergone direct investigation, and so this phenomenon remains to be completely understood.^{103,153}

Adenoviral vaccine regimens based upon sequential exposure to HIV antigens and/or different serotypes (techniques suggested to induce more profound T-cell responses) then became the focus of investigation in subsequent years.^{151,198} An NIH-sponsored phase II clinical trial (called the "HVTN trial") began in 2009 and exposed subjects first to a DNA-based primer vaccine expressing HIV proteins to then be followed by a booster Ad5 vector vaccine expressing the same proteins. Yet again, this trial was brought to an early end in 2013 when the vaccine failed to reduce the rate of HIV infection or attenuate disease contracted by subjects during the trial.¹⁹⁹ Most recently, such "prime-boost" strategies continue to be employed among techniques that utilize heterologous vectors and/or heterologous inserts with the aim of creating improved coverage against HIV.^{200,201} In comparison to homologous pairing strategies, studies by Walsh et al have shown that vaccination strategies using heterologous vector pairs (particularly Ad5/Ad35 vectors) and inserts (particularly EnvA/EnvB inserts) improve T-cell response rates and induce significantly higher numbers of total T-cell epitopes, all with well accepted tolerance.²⁰⁰ Similarly, Baden et al found that both humoral and cellular immune responses to HIV-1 can be augmented with either homologous or heterologous (vector and insert) adenoviral booster vaccines using novel vector serotypes, Ad26 and Ad35, without adverse events and despite pre-existing vector immunity.²⁰¹

The charge to design an Ebola vaccine became of paramount international priority when the WHO declared an epidemic in 2014. Accordingly, a surge of research and collaboration efforts have accelerated vaccine development in recent years, leading to nearly a dozen candidate vaccines now in various stages of clinical trials. Among three of the key candidate vaccines designated by the WHO, two are derived from adenoviral vectors, namely, ChAd3-ZEBOV¹ from GlaxoSmithKline and Ad26-ZEBOV/MVA-BN-Filo² from Johnson & Johnson.¹⁸⁵ In a phase I trial, the ChAd3-ZEBOV vaccine was safe and well-tolerated.²⁰²

At low doses, found tolerable in a small cohort of macaques,²⁰³ a single test dose of this same vaccine was shown to induce antibody and T-cell responses in humans detectable as far out as 6 months post-inoculation.¹⁸⁷ Since February of 2015, the ChAd3-ZEBOV vaccine has been tested in thousands of healthy volunteers in Liberia while proceeding into phase II and phase III efficacy trials.^{185,187} With similar findings, the Ad26-ZEBOV/MVA-BN-Filo vaccine advanced from phase I trials in the UK in 2013 prior to the West African outbreak, with subsequent phase II and III studies relocated to West Africa upon the outbreak in 2014.^{189,204,205} And though this vaccine has demonstrated rapid and durable protection against Ebola in macaques, conclusive evidence supporting the efficacy of this vaccine in human subjects has not yet been reported.^{206–209} Lastly, another Ebola vaccine candidate using an Ad-C5 vector has been tested in phase 1 trials among healthy Chinese adults, showing safe and immunogenic findings, though with insufficient clinical efficacy in individuals with pre-existing immunity to the adenovirus.^{190,210–212}

Adenovirus-mediated gene delivery in regenerative medicine

The use of adenoviral vectors for genetic engineering has opened new areas of investigation in most areas of medicine. From basic science laboratories to multi-center clinical trials, this promising field has captured the attention of the scientific community. The number of clinical trials alone has skyrocketed in the last two decades. The breadth of these trials will be discussed in the next section to show the diverse nature of potential clinical applications. These clinical trials have been possible due to the efforts in basic and translational studies across many disciplines. The volume of preclinical studies that have paved the way for these clinical trials is tremendous. It is beyond the scope of this review to highlight, even superficially, the many areas explored by these studies as they touch nearly every medical discipline. Instead, we will focus on several pre-clinical advances in the field of bone tissue engineering that have led to clinical trials in this area. We highlight this limited area to demonstrate how cumulative efforts, at the basic and translational levels, have made clinical use possible. A look at advances in genetic engineering across other disciplines shows a similar path from benchtop to bedside. In this way, a look at the evolution of bone tissue engineering advancements is loosely representative of how genetic engineering has evolved in other areas of medicine.

Successful bone tissue engineering requires four key components: osteoinduction, osteoblast differentiation, osteoconduction, and mechanical stimulation.^{213–217} In general, preclinical developments in bone tissue engineering have centered on augmenting growth factors necessary for the first three of these elements. The majority of these studies have utilized adenoviral vectors largely because transient expression is more advantageous than constitutive expression in bone tissue engineering.^{213,218} This approach utilizes adenoviral vectors containing genes that code for essential osteoinductive growth factors.^{219,220} Efforts aim to induce osteogenic differentiation, facilitate ossification, and encourage integration

with surrounding bone tissue. Osteoblastic differentiation can be induced by a variety of osteoinductive growth factors both *in vivo* and *in vitro*. PDGFs (Platelet Derived Growth Factors), FGFs (Fibroblast Growth Factors), IGFs (Insulin-Like Growth Factors), EGF, Wnts, retinoic acids, NOTCH, and BMPs (Bone Morphogenetic Proteins) are some of the many factors that have displayed osteogenic activity in studies.^{220–226} At least 15 BMPs have been identified in humans and mice.^{219,220,227} They display a spectrum of osteogenic activity, with BMP-2, BMP-6, BMP-7, and BMP-9 among the most osteogenic.^{213,219,220,228–230} With their potent osteogenic action on the most important steps in the bone formation cycle from osteoblast to osteocyte, many of these BMPs were an early focus for researchers.^{231–233}

Early bone regeneration studies focused on rhBMPs (recombinant human BMPs.) However, difficulties with stability, cost, and efficacy limited the more widespread adoption of this strategy for meeting bone engineering goals.²¹³ Investigators were able to largely overcome these difficulties by implementing a gene therapy approach by transducing BMP with an array of vectors, most commonly adenoviral vectors. In 1999, Lieberman et al compared rhBMP-2 and BMP-2 transduced via adenoviral vector gene transfer of bone marrow cells in their ability to heal skeletal defects in a rat model. The results strongly favored the gene transfer group, which demonstrated increased quality and quantity of bone formation.²³⁴ Other studies followed that supported a gene therapy approach to improving bone engineering techniques via BMP.²³⁵

After establishing the merits of utilizing gene therapy to study bone engineering, the techniques were refined over the following years to accumulate a large body of work in which many factors in the bone formation cascade were studied for the potency of their osteogenic effect.^{219,228–230} Other key aspects were also studied including mesenchymal stem cell source, injection sites, skeletal defect sites, and direct *in vivo* vs. *ex vivo* approaches. Many of these efforts have been instrumental in advancing bone engineering to humans via clinical trials and in some cases FDA approval for clinical use. BMP-2 has been used in clinical trials examining its use in spinal fusion and in the repair of tibial fractures.^{236,237} BMP-7 has been used in clinical trials for the management of tibial nonunion fractures and spinal fusion.^{238,239} This work, among others, led to FDA approval for recombinant BMP-7 for use in nonunion of long bones and in anterior spinal fusions. Other clinical uses for BMPs are likely on the horizon. While not currently in clinical use, BMP-9 has demonstrated more robust osteoinduction than any other BMPs in several pre-clinical animal models.^{228,240,241}

AdV-mediated gene delivery has also been used in numerous preclinical studies involved other aspects of regenerative medicine and tissue engineering. We demonstrated that AdV-mediated expression of Sox9 significantly improved the healing of intervertebral degenerative discs in a rabbit model.²⁴² Adenoviral vectors were shown to deliver transgenes effectively into the flexor tendon of rabbit injury model.²⁴³ Furthermore, we showed that adenovirus-mediated gene transfer of BMP14 expedited tendon-healing and increased tendon tensile strength in a rat model of Achilles tendon injury.²⁴⁴ In this animal model, no adverse immunological response to the adenoviral

vector was detected in the host tissue, and the local production of BMP-14 did not induce unwelcome bone or cartilage formation within the healing tendon.²⁴⁴

Challenges and future directions

The use of AdVs in gene therapy comes with its challenges. Some of the major obstacles surrounding the use of viral vectors include reactions with the immune system, viral longevity, vector packaging capacity, and contamination with HV. As previously discussed, the removal of viral components and the emergence of HC-AdV have significantly reduced immune reaction among other benefits. In addition to preventing tissue damage and inflammation, the immune reduction also helps to prolong the lifespan of the virus. One major challenge relating to the prolonged expression of HC-AdV is due to previous immunization against Ad. To troubleshoot this issue, HC-AdV longevity can be optimized by encoding novel tetracycline-dependent (TetOn)-regulatory elements.²⁴⁵ With expression tightly regulated by doxycycline, the HC-Ad-TetOn vectors demonstrated better regulation and effectiveness than constitutively active HC-AdV in the presence of an immune response.²⁴⁶ The lack of viral protein genes also provides increased packaging capacity, as previously discussed. However, even the presence of viral packaging machinery can impose challenges, namely those related to the issue of having too little unique enzyme sites for transgene insertion. To overcome this issue, Shi and colleagues demonstrated efficacy of HD-AdVs that have a unique transgene insertion site, while maintaining tissue-specificity.²⁴⁷

Another issue surrounding the use of AdV for gene therapy is contamination with helper virus (HV). Previously discussed strategies for reducing contamination have proven to be successful, but contamination levels can still be too high to use the AdV for human purposes. To combat this issue, one group has constructed a HV (AdTetCre) in which the packaging signal is flanked by *loxP* sites that can be excised by a chimeric MerCreMer recombinase encoded in the same viral genome. The MerCreMer expression was under control using a TetOn system²⁴⁸ and was able to produce titers with significant numbers of infectious units and with <0.1% HV contamination. Self-inactivating HVs based on virally encoded recombinases are promising tools for reducing contamination in the production of HC-AdV.

Additional challenges include cellular barriers and viral targeting and transport. Promising research has demonstrated that expression levels of AdV receptors do not correlate with their transduction efficiency or biological function.²⁴⁹ Further research related to the challenges faced by HC-AdV should be conducted to improve transduction yields and overall efficacy.

In summary, the use of AdVs for gene therapy and gene delivery tools has produced promising results among numerous breakthrough studies for human applications. More importantly, AdVs may be used in many emerging fields of biomedical research. For example, the HC-AdVs can be used to effectively deliver engineered CRISPR/Cas9 systems to targeted cells and/or tissues for efficient genome editing,^{250,251} which cannot be effectively accomplished by other viral systems. Furthermore, the HC-AdVs

can be used to deliver anti-PD1/PD-L1 immune checkpoint therapeutics for cancer treatment.^{252,253} While the use of AdV in gene therapy has shown substantial promise and increasing potential, future work will need to be done regarding the current pitfalls of using the vector. Although great strides have been made, improvements regarding reactions with the immune system, viral longevity, vector packaging capacity, and contamination with HV will need to be made to continue making breakthroughs. Nevertheless, the evolution of the adenoviral vector as a tool for transfer of genetic material has revolutionized the way physicians and scientists can approach treating even the most debilitating diseases.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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