Adenovirus technology for gene manipulation and functional studies

Yibin Wang and Shuang Huang

Recombinant adenoviral vectors are highly efficient at gene transfer in a broad spectrum of cell types and species, and have been used, both *in vitro* and *in vivo*, to achieve gain or loss of function in functional studies. In recent years, there have been several significant advances in adenovirus technologies, including new generations of vectors, improved production systems and sophisticated methods of delivery. In this review, recent progress and innovative applications are discussed to demonstrate the potential of the recombinant adenoviral vector as an effective tool in functional genomics.

Databases, bioinformatics and biological studies constitute the three crucial components of functional genomics[1]. With the sequencing of the entire human genome nearing completion, it has become imperative that more efficient approaches to uncover, confirm and evaluate the physiological functions of genes need to be developed. At the level of molecular genetics, there are two main routes to determine the function of genes with known sequences: one is through loss of function, the other is through gain-of-function. Often, gene function must be studied in the context of the entire organism or in a particular cell type at a specific stage of development and disease. Therefore, an ideal strategy for functional studies should encompass both effectiveness and versatility. Recent advances in gene therapy research have provided more effective tools to achieve genetic manipulation through somatic gene transfer. In particular, the recombinant adenovirus has become a widely used delivery vector for both gene therapy and functional studies[2,3]. In this review, the advantages and limitations of adenoviral vectors will be discussed in the light of recent advances, illustrating their potential in human genome research.

Background to recombinant adenoviruses

The adenoviral genome

Almost all recombinant adenoviral vectors are derived from the human serotype 2 or type 5 of the group C adenovirus. The genome of both serotypes consists of 36 kb double-stranded linear DNA with inverted terminal repeat sequences at each end. Based on the expression profile, the adenoviral genome can be divided into three categories:

- The early genes (E), which encode five early transcripts, including E1A, E1B, E2, E3 and E4
- The delayed early genes, which encode transcripts IX and IVa2
- The single major late unit (L), which generates five families of late transcripts through a posttranscriptional process.

The early gene products are mostly involved in adenoviral gene transcription, DNA replication, host immune suppression and inhibition of host cell apoptosis. The late gene transcripts encode proteins that are required for virus assembly[4]. Among the early genes, E1A is the first viral gene to be expressed after infection, and is the essential

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transcriptional activator for subsequent adenoviral gene expression. E1A gene products are also involved in viral replication by inducing G1–S transition in the host cell[5,6].

Recombinant adenovirus generation and production

The prototype recombinant adenovirus was generated using a two-plasmid system[7], consisting of 'shuttle' and 'helper' plasmids. The shuttle plasmid contains a section of the 5′ genome in which E1A and E1B genes have been replaced by a transgene that is driven by its own regulatory system. The helper DNA is either a plasmid that contains a complete but unpackageable viral genome (e.g. pJM17) or adenoviral genomic DNA being truncated by a restriction enzyme. Following co-transfection of these two DNAs, homologous recombination occurs to generate a recombinant adenovirus in which the transgene is incorporated into the viral genome, replacing the E1A and E1B genes (Fig. 1). Recombinant adenoviruses cannot replicate as efficiently as wild-type viruses, but can replicate efficiently in permissive host cells, such as 293 cells (these are human embryonic kidney cells, transformed by sheared adenoviral genomic DNA), in which E1A proteins are provided *in trans*. A large quantity of recombinant adenovirus can be produced in 293 cells or in other E1A-producing cell lines, and purified by an established method such as CsCl gradient ultracentrifugation[8]. A viral solution with a titre of 10^{12} particles ml^{-1} can be routinely prepared. The entire process of generating and preparing stocks of a particular viral vector usually takes 2–4 weeks.

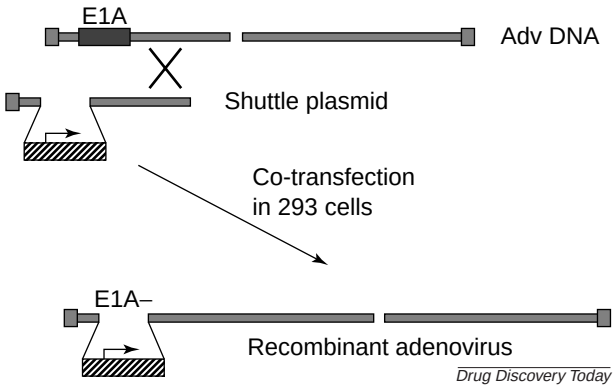

Figure 1. *Generation of a prototype recombinant adenoviral (Adv) vector. The shuttle plasmid contains the 5′ portion of the adenoviral genome in which the transgene expression unit (hatched bar with arrow) is inserted in place of the E1A region (filled bar). Homologous recombination (represented by the large cross) between the co-transfected shuttle plasmid DNA and the viral DNA (pJM7 or pBHG) in 293 cells will result in the incorporation of the transgene (hatched bar) into the adenoviral genome (bottom) at the place where the E1A was deleted.*

Adenoviral infection

Adenoviral infection is initiated by the high-affinity binding of the fibre protein knob domain to cell surface receptors, such as the coxsackie adenovirus receptor (CAR)[9] or MHC-I α2-domain[10]. Subsequent internalization of virus particle requires a further interaction between the penton base protein of the viral capsid and α_v integrins on the host cells[11]. The process of internalization and release of the virus into the cytosol takes only 15 min to complete. Transgene expression can be detected within 18 h of infection, reaching a maximal level 48–72 h after infection[12,13]. The susceptibility of different cell types to adenoviral infection is often dictated by the availability of the receptors[14,15]. Therefore, the specific multiplicity of infection (MOI) that is used to achieve optimal infection varies between different cell types. For example, primary ventricular myocytes or skeletal muscle cells from neonates require a low MOI of ~50–100 to obtain nearly 100% infection. By contrast, mature skeletal muscle cells and undifferentiated myoblast C2C12 cells require a much higher MOI to achieve the same level of infection[13,16]. This difference can be attributed largely to differing levels of receptor expression in a variety of host cells. The route of viral vector infection in intact animals can also affect the transgene expression patterns, and the accessibility of a given tissue to the injected virus is an important factor[17]. For example, systemic delivery of adenoviral vectors often results in very low levels of infection in adult cardiac muscle cells, skeletal muscle fibres and brain tissues, whereas these cells or tissues can be more efficiently infected in culture conditions or by direct intramuscular or intracerebral injection[17–20]. Therefore, consideration of target cell receptor density and choice of delivery technique are essential to achieving a satisfactory level of transgene expression for *in vivo* studies.

Limitations of first-generation recombinant adenoviral vectors

Several factors limit the application of the adenovirus as a gene delivery vector for functional studies. Firstly, the inserted transgene cannot exceed the packaging capacity of the virus, limiting the cDNA size to 4–5 kb (Ref. 21). Secondly, E1A-deficient adenoviruses can still cause significant cytotoxicity in infected cells and immunological responses in the infected animal, which limit the duration and level of target gene expression and complicate the functional

analysis[22,23]. Thirdly, the generation of recombinant adenoviruses through homologous recombination in 293 cells is a low-efficiency and technically demanding process.

Innovative adenovirus technologies

New generations of adenoviral vectors

In the past few years, there have been substantial efforts to improve adenoviral vector systems, with particular emphasis on enhancing transgene expression whilst accommodating larger transgenes and reducing the cytotoxic or immunological effects in the host organism. Viral vectors with both E1A and E3 or E4 deletions have been developed in which the capacity of the foreign gene can reach 7 kb (Refs 24,25). A second-generation vector with both E1 and E2 deletions/mutations was developed that had prolonged transgene expression and reduced cytotoxic effects *in vivo*[26–28]. By using a helper virus system, the generation of so-called 'gutless' adenoviral vectors, in which most of the viral genome is replaced by exogenous DNA, has recently been accomplished[29–31]. The gutless vector, the most advanced vector system to date, has a capacity of up to 35 kb of foreign gene and has eliminated the production of viral gene products in infected cells. As a result, the gutless adenovirus has significantly reduced the host immune response *in vivo*[32], and is capable of achieving long-term, regulated expression of multiple target genes in one vector[33,34].

New methods of generating recombinant adenoviruses

To overcome the low efficiency of homologous recombination in recombinant adenovirus production, several improved systems have been developed. One such system uses the highly efficient DNA recombination, mediated by the Cre/loxP system, to facilitate recombinant adenoviral generation in a Cre-expressing 293 cell line[35]. Two other recent reports have demonstrated the use of powerful bacterial genetics to select the recombinant vectors[36,37]. In such systems, the recombination events between the shuttle plasmid and the adenovirus helper plasmid occur in a bacterial system. The resulting recombinants can be identified using genetic markers, such as antibiotic resistance, much more efficiently than when co-transfecting in mammalian cells. These new methods of recombinant adenovirus production now make it possible to generate a large quantity of adenoviral vectors efficiently and predictably.

New adenoviral vectors for regulated expression

A broad spectrum of infectivity and ubiquitous expression of the transgene are common features of adenoviral vectors. However, regulated expression and targeted infection are also desirable for many functional studies. To achieve regulated expression, different transcription systems have been incorporated into adenoviral vectors. Instead of employing strong viral promoter/enhancer sequences, such as cytomegalovirus (CMV), rous sarcoma virus (RSV) and herpes simplex virus (HSV-tk), tissue-specific promoters are used to drive the transgene expression[38–40]. A common problem with the use of tissue-specific promoters is a loss of specificity when they are incorporated into the adenoviral genome. It is possible that negative and positive regulatory elements residing in the viral genome might be capable of influencing the specificity of the incorporated cellular promoters[41]. Indeed, by eliminating the viral genome (in gutless vectors) or by using insulating sequences, the function of tissue-specific and inducible promoters in adenoviral vectors can be preserved[32,42,43]. For temporal regulation, a tetracycline-regulation cassette or hormone-regulated system can be incorporated into an adenoviral vector[42,44]. Furthermore, a gene-switch system, based on Cre/loxP-mediated DNA recombination, has been successfully incorporated into adenoviral vectors and has been used in combination with loxP-engineered transgenic animals to achieve targeted gene activation/inactivation *in vivo*[18,45–47]. These vectors can enable quantitative and regulated expression of the transgene for precise functional studies both *in vitro* and *in vivo*.

New adenoviral vectors for targeted infection

The broad expression of the primary adenovirus receptors enables the adenoviral vector to efficiently transduce several different cells and tissues. However, the lack of tissue or cell specificity, coupled with the inability of adenoviral vectors to transduce certain tissues, has led to efforts to redirect adenoviral tropism.

Viral tropism can be altered by several mechanisms. One approach involves switching the Ad5 fibre or fibre knob region with another adenovirus serotype. For example, replacing the Ad5 fibre knob with Ad3 fibre knob enables the chimeric vector to transduce Epstein–Barr virus (EBV)-immortalized human B lymphocytes more efficiently than conventional Ad5 vectors[48]. Alternatively, the transduction spectrum can be altered by inserting a peptide ligand sequence into the fibre protein[49]. Furthermore, a single-chain antibody with both fibre-neutralizing activity and a ligand peptide fused to its C-terminal was used to generate an antibody–adenovirus complex termed 'adenobody'. This adenobody could direct the adenovirus to the targeting cells in a receptor-dependent manner[50,51]. It is likely that future advances in adenoviral targeting will

generate improved viral vectors that can transduce targeted cells with greater efficiency.

Application of recombinant adenoviral vectors in functional studies

Advantages of using adenoviruses in functional studies

The use of adenoviral vectors in functional studies has several advantages over other commonly used gene delivery vectors (Table 1). One of the most desirable features is its high infection efficiency in a broad range of cell types, including both actively replicating cells and terminally differentiated quiescent cells[3]. Thus, a single adenoviral vector can be used in both *in vitro* and *in vivo* model systems to study cellular and physiological responses in many species. In some instances, *in vivo* transgene expression by adenoviral vectors can produce results that are similar to those using traditional transgenic approaches, but at only a small fraction of the cost and time. For example, adenovirus-mediated overexpression of phospholamban in the heart resulted in suppression of global contractility[20], similar to that reported when using a transgenic approach[52].

Strategies for using adenovirus in functional studies

Three elements need to be considered in the design of adenovirus-based functional studies (Fig. 2).

Choice of vector. Adenoviral vectors are selected on the basis of their expression pattern and level, the size of the target genes and the characteristics of the targeted tissue. First-generation vectors usually produce high levels of foreign gene products but have a short duration of expression *in vivo* with significant cytotoxicity from viral gene products. They are relatively easy to generate and amplify with established procedures. Second-generation and gutless vectors can deliver long-term and, if necessary, regulated expression. However, the generation and production of these new vectors require special cell lines and helper viruses, involving more demanding experimental procedures. If the expression of the transgene is to be targeted to a particular cell type, a tissue-specific adenoviral vector can be generated either using a tissue-specific transcriptional unit for the target gene, or by modifying the tropism of the recombinant adenoviral vector, as described earlier.

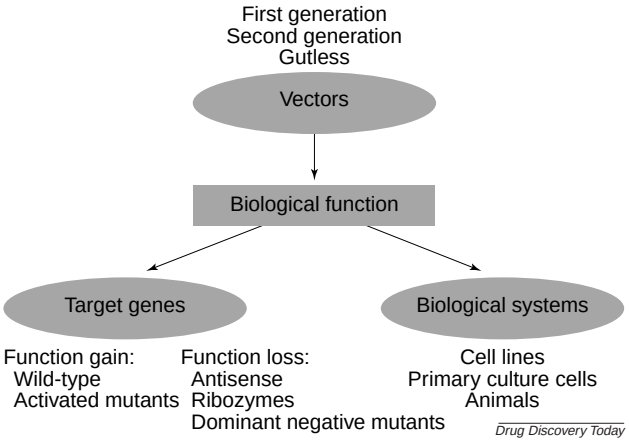

Figure 2. *Strategies for using recombinant adenoviral vectors in functional studies.*

Choice of target genes. Recombinant adenoviral vectors can be used to acheive gain-of-function by ectopic or overexpression of a wild-type gene product or an

Table 1. Comparison of gene transfer vectors in functional studies[a]

Studies	Vectors					
	Non-viral	**Retrovirus**	**Lentivirus**	**HSV**	**AAV**	**Adenovirus**
Host tropism	Broad	Restricted	Broad (pseudotyped)	Restricted in neurons	Broad	Broad
Construction system	Difficult	Established	Difficult	Difficult	Established	Established
Transduction efficiency	Very low	Low	Low	Moderate	Moderate–high	Very high
Capacity for transgene	Unlimited	<4–5 kb	~9 kb	Large	<4–5 kb	5–37 kb
Yield (titre)	High	Low (10^5–10^7)	Low (10^2–10^6)	High (10^{10})	Low (10^8–10^9)	High (10^{11}–10^{12})
Host-cell replication	Not required	Required	Not required	Not required	Not required	Not required
Regulated expression	Available	Possible	Possible	Difficult	Available	Available
Duration of expression	Days	Long term	Long term	Days	Long term	Weeks–months
Cytotoxic response	Low	Low	Low	High	Low	High/low

[a] Non-viral vectors are based on the ligand-mediated DNA–protein complex method. The retrovirus represents amphotrophic murine retrovirus vectors. Lentivirus is derived from the HIV-1 virus VSV-G pseudotyped vectors[55]. HSV represents herpes simplex virus type 1 vector with the transgene in the LAT region[56]. AAV represents adeno-associated virus[57]. Adv includes first and second generation, and gutless adenoviral vectors.

activated mutant protein[13]. Alternatively, they can be used to express antisense molecules, ribozymes or dominant negative mutant molecules to achieve loss of function[13,53].

Choice of targeted biological system. Adenoviral vectors can be used in cultured cells to assess the biochemical and cellular functions of a target gene. They can also be applied *in vivo* to elucidate physiological functions of living animals. Furthermore, adenoviral vectors can be used in combination with other genetically manipulated biological systems to activate or inactivate specific genes. For example, adenoviral vectors expressing the *Cre* gene can be used in transgenic mice in which loxP sequences have been incorporated into the genome (floxed alleles) to achieve targeted gene activation or inactivation[18,45–47].

Delivery methods of adenoviral vectors in functional studies

In tissue-cultured cells, adenoviral infection can be easily performed with minimum effort. The infection is reproducible and can therefore be used routinely with large quantities of samples. This method is particularly attractive for studies of primary culture cells, such as neuronal cells and muscle cells, for which cell lines are not available and conventional transfection methods produce low yields[13].

The application of recombinant adenoviruses for functional studies *in vivo* requires optimization of delivery techniques, tailored for the target organ. For cardiac myocytes, intrachamber injection, intramyocardial injection and intracoronary injection have been reported. Intrachamber injection can achieve global infection in the heart of neonatal mice because the cardiac wall is still thin and accessible to the virus solution; however, the same protocol used in older mice produces little infection[54]. Intramyocardial injection resulted in restricted expression along the needle track. Of all the cardiac injection methods, intracoronary delivery, although more technically challenging (particularly in small rodents), has given the best results in terms of global infection and functional alteration[19]. Improved surgical techniques have made it possible to transiently close the aorta during adenovirus administration, thus forcing the viral solution into the coronary circulation and thereby enhancing the infection efficiency in adult myocytes[20]. In general, virus delivery methods must be designed to maximize exposure of the targeted organ to the viral solution.

Summary

In addition to an important role in human gene therapy, adenoviral vectors have great potential as effective tools in genomic studies. New advances in vector technology will significantly improve the efficiency of adenovirus production, especially for new generations of adenoviral vectors. Novel genes can be expressed using tissue-specific or inducible adenoviral vectors in specific cells and tissues to uncover their potential functions during specific stages of development and disease. Candidate genes can be manipulated, using activated/dominant negative mutants or antisense/ribozyme, to validate their function both *in vitro* and *in vivo*. By combining the latest versions of the vectors with the improved delivery techniques, disease models can be established in a broad range of species based on somatic gene transfer rather than traditional transgenic approaches. In summary, recombinant adenoviruses should be considered as an important vehicle to explore the vast unknowns of human genome science.

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Collaborations…

ChemRx (San Diego, CA, USA), a wholly-owned subsidiary of Discovery Partners International, has signed a research agreement with **Pharmacia and Upjohn** (Peapack, NJ, USA). In this agreement, ChemRx will develop multiple classes of compounds on a fee-for-service basis for screening in a wide range of Pharmacia and Upjohn's assays. These compounds will be developed using the combinatorial chemistry tools developed by ChemRx's sister company, IRORI, to convert synthesis methods into chemical libraries for lead discovery and optimization. The compounds will be supplied on an exclusive basis with no milestone or royalty obligations.

Sequenom (San Diego, CA, USA) has initiated a beta-site agreement with **Genzyme Genetics** (Hamburg, Germany) for the development of mutation detection assays. This project will use the MassArray™ technology from Sequenom, which utilizes MALDI–TOF (matrix-assisted laser desorption ionization with time-of-flight) mass spectrometry, SpectroCHIP arrays, proprietary solid-phase enzymatic reactions and customized software to validate and score genetic markers to genotype DNA mutations associated with genetic disease states. Katherine W. Klinger, Senior Vice President of R&D, Genetics and Genomics at Genzyme said 'Genzyme has focused on the development, evaluation and use of state-of-the-art genetic testing platforms that provide accurate and reliable clinical test results with high information content. Sequenom's development of the MassArray analytical system offers us the possibility of being part of the next generation of clinical tests for molecular genetics.'

The **Cystic Fibrosis Foundation** (Bethesda, MD, USA) is providing funding from the Foundation's Therapeutics Development Program for a collaboration with **Aurora Biosciences** (San Diego, CA, USA) to identify potential therapies to treat the defect identified as the primary cause of cystic fibrosis. This gene defect causes cells to produce damaged ion channels, inhibiting the efflux of chloride ions and interfering with the exchange of sodium ions. Under the terms of the agreement, Aurora will develop screening assays for this defect using their dual fluorophore voltage sensor system and VIPR™ high-throughput kinetic plate reader, and then conduct high-throughput screening using their compound library. The company will then try to prioritize any lead compounds identified through this process. The collaboration might then be expanded for further development.