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Adenoviral Vectors

A Promising Tool for Gene Therapy

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Abstract

Gene therapy is a promising tool for treatment of the human diseases that cannot be cured by rational therapies, and its primary success depends on suitable vectors to deliver therapeutic genes. Adenoviruses (Ads) are among the most commonly used vectors for gene therapy, second only to retroviruses. During the last decade, remarkable progress has been made in the development of Ad vectors and in the understanding of the toxicity related to the Ad vector system. Ad vector has certain advantages such as high transduction efficiency for different quiescent and dividing cell types and high levels of short-term expression to provide therapeutic benefits. However, researchers are facing the challenges associated with tissue-specific targeting of vectors and the vector-mediated immunogenicity. This review mainly focuses on the studies that have employed methods to improve Ad vectors and reduce viral toxicity for different applications. These methods include minimization or elimination of viral genes, retargeting of vector to the tissue of interest, and generation of immunocompromised recombinant vectors that lead to safer use of Ad vector systems that improve persistence of transgene expression. Moreover, the therapeutic applications of Ad vectors for liver-targeted gene therapy, suicide gene therapy, delivery of small interfering RNA, and production of recombinant vaccine under regulated conditions used in clinical trials are discussed.

Index Entries: Adenovirus vector; inverted terminal repeat; small interfering RNA; Coxsackie and adenovirus receptor.

Introduction

Extensive research on adenoviral structure, the mechanism of DNA replication, and transcription maps has stimulated a growing interest in adenovirus (Ad) as a potential gene therapy vector *(1,2)*. Several advantages

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Table 1
Advantages and Disadvantages of Ad Vectors

Advantages	Disadvantages
Ability to infect both dividing and quiescent cells	Long-term correction not allowed
Stability of recombinant vectors	Humoral and cellular immune response from high vector doses
Large insert capacity	
Relatively safe	
Nononcogenic	
Can be produced at high titers	
No integration, which prevents germ line gene transfer	
Transgenes expressed episomally, unlike retrovirus-based vectors, which are dependent on integration (and cell division) for transgene expression	

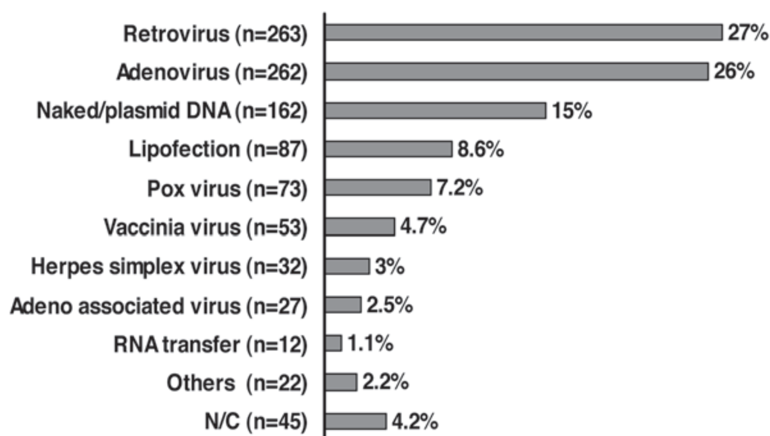

Fig. 1. Vectors that are currently used in gene therapy clinical trials. *n* is the number of individuals in gene therapy clinical trials (www.wiley.co.uk/genmed/clinical). N/C, numerical control.

make Ad suitable for gene therapy applications (Table 1). Consequently, the recombinant Ad is commonly used in gene therapy compared to other viral systems (Fig. 1). The most commonly used recombinant Ads are generated from human Ads of serotypes 2 and 5 (Ad2 and Ad5 of subgroup C) *(3)*. They are nonenveloped particles of 60–90 nm in diameter possessing icosahedral symmetry, and their capsids are made of 252 capsomers comprising 240 hexons and 12 pentons at vertices of icosahedron *(4)*. A thin trimeric glycoprotein fiber protrudes from the center of each penton, terminating in a bulbous knob. The initial interaction of Ads with the cells occurs

through binding of the distal knob domain of the fiber to a host cell-surface molecule Coxsackie and adenovirus receptor (CAR), which mediates internalization of Ad *(5,6)*. In addition to the fiber-CAR interaction, the adenoviral penton base interacts with the α_v integrin family of cell-surface heterodimers via the RGD (Arg-Gly-Asp) motif *(7)*. The α_v integrins act as coreceptors and assist in viral endocytosis into clathrin-coated vesicles *(8)*. The heparan sulfate glycosaminoglycan receptor identified on different cell surfaces is also involved in binding with the capsids of Ad2 and Ad5 *(9,10)*. On entry into the cells, virus core uncoats from endocytotic vesicles and migrates to the nucleus, where DNA replication and transcription occurs to produce progeny virions that ultimately come out by lysing the host cells (Fig. 2). Tissue distribution of Ad depends on the widespread distribution of CAR *(11)*, which is expressed on many cell types, including the basolateral surface of epithelial cells, endothelial cells, myoblasts, heart muscle cells, and hepatocytes. The recombinant Ads can transfer genes into both dividing and quiescent cells with high efficiency and localize preferentially to the liver after iv administration in rodents *(12)*. However, Ads are highly immunogenic *(13)*. They activate the innate immune system of the host by expressing pathogen-associated molecular patterns, which on binding to pathogen recognition receptors on host cells produce proinflammatory cytokines and initiate differentiation of immature dendritic cells (DCs) into antigen-presenting cells (APCs). Systemic administration of high doses of recombinant Ads into mice triggers the release of interleukin (IL)-6, IL-12, and tumor necrosis factor-α and accumulation of transduced macrophages and DCs in lymphatic tissues *(14,15)*.

Problems associated with the Ad-mediated immunogenicity can be overcome by generating vectors that are completely devoid of viral-coding genes or by coexpressing the hybrid immunomodulator protein (CTLA4-Ig) *(16)*. Adaptive immune responses could be directed to both early and late antigens of Ad. Therefore, antibodies generated owing to adenoviral infections against the surface loops of the viral hexon or the penton base or the fiber could neutralize Ad.

One potential advantage of Ad vectors is their ability to package large fragments of homologous sequences. Moreover, Ad vectors have a low frequency of nonhomologous chromosomal integration (10^{-5}–10^{-3} integrins per infected cell), suggesting that they may be useful for gene targeting. Ad vectors may be used as a powerful gene-targeting tool if advances can be made, given their ability to infect many types of cells efficiently, both in vitro and in vivo. Viral retargeting for tissue-specific gene delivery can be achieved by chemical, genetic, or immunologic modifications of virus.

Generation of Recombinant Ad Vectors

The 35-kb double-stranded DNA (dsDNA) linear Ad genome consisting of a 103-bp inverted terminal repeat (ITR) on each end is functionally divided into two major overlapping regions, termed early (E) and late (L)

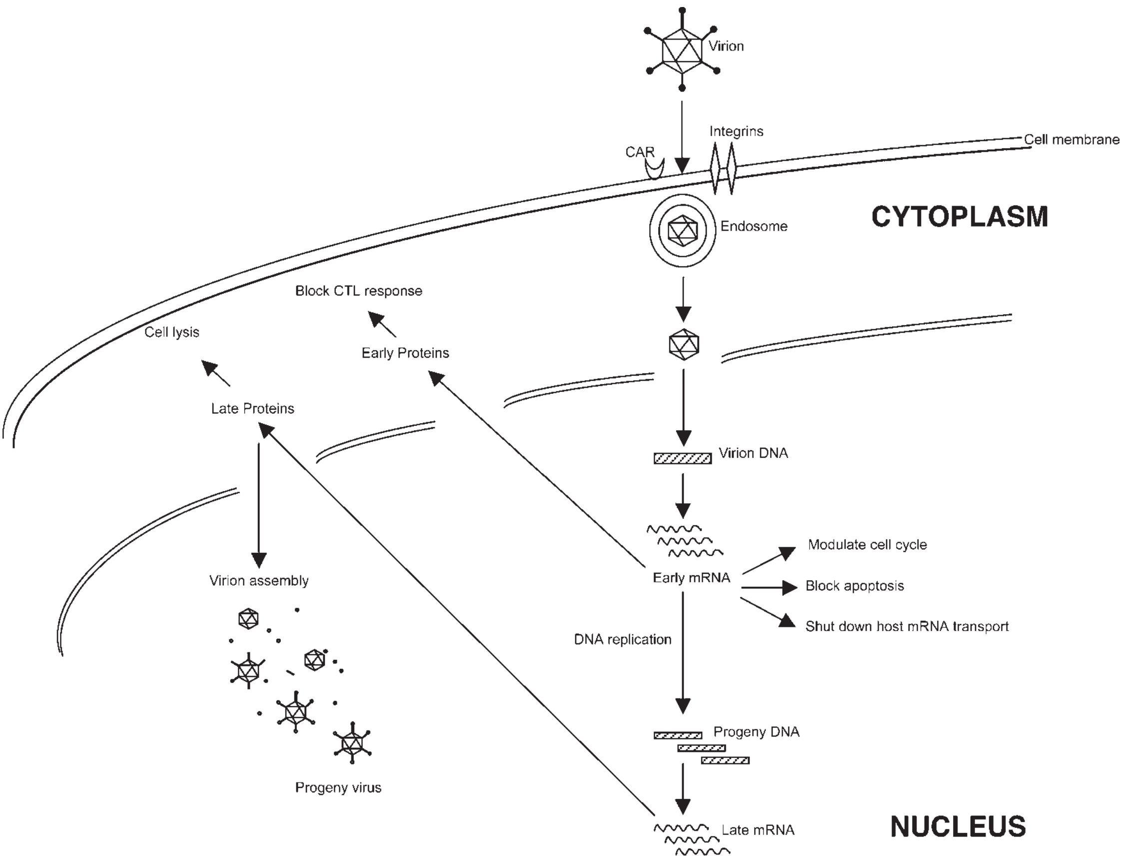

Fig. 2. Schematic representation of life cycle of Ad. Ad subgroup C attaches to the host cell surface by CAR and integrin receptors. Virus then internalizes via clathrin-mediated endocytosis. Next, viral DNA replication and transcription occurs in the nucleus. Finally, encapsidated progeny virions are released by lysis of host cells. CTL, cytotoxic T-lymphocyte.

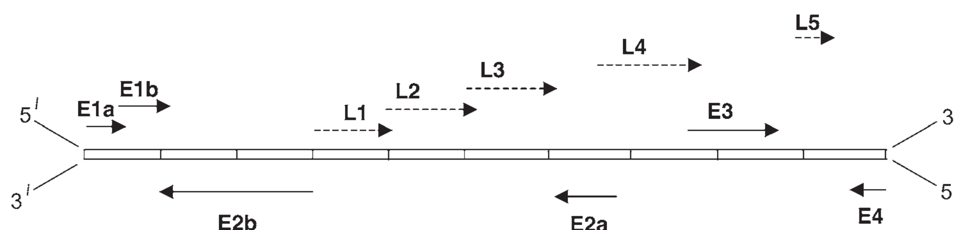

Fig. 3. Schematic diagram of Ad5 genome. Each box unit corresponds to 360 bp of Ad5 genome. The E and L regions indicate early and late Ad transcripts, respectively.

Table 2
Early Genes and Their Functions

Genes	Function
E1	Required for activation of transcription of early genes
E2	Required for virus DNA replication
E3	Required for modulation and evasion of host's immune response, prevention of untimely cell death through apoptosis, and efficient cell lysis once new particle assembly is complete
E4	Involved in virus RNA metabolism and transport, preferential downregulation of host-cell protein synthesis, and enhancement of virus DNA replication

regions. A packaging site (ψ) located between the left ITR and the early region-1 (E1) is essential for localization of replicated virus DNA into the preassembled capsids *(17,18)* (Fig. 3). Table 2 provides the functions of early Ad genes (E). The E1 region encodes transcription factors that are required for the expression of Ad genes, and its disruption markedly inhibits the expression of the viral proteins. According to the conventional method, the E1-deleted Ad vectors are constructed by in vivo homologous recombination disrupting the E1 gene by insertion of transgene. In this method, a large plasmid containing E1-deleted adenoviral genome and a shuttle vector containing the left-handed ITR, the E1a enhancer, the packaging signal, the gene of interest cloned under the control of cytomegalovirus (CMV) promoter, and the SV40 poly (A) signal flanked by 3' sequences of the E1 are cotransfected into human embryonic kidney cell line (HEK 293). The recombinant Ad is produced by in vivo homologous recombination between these two plasmids, and the necessary transcription units are supplied *in trans* by HEK 293 cell lines *(19–21)*. In the modified method, the transgene carrying shuttle plasmid is directly ligated to the linearized adenoviral genome backbone in vitro. The recombinant plasmid produced by this cloning method can be easily propagated in *Escherichia coli*, and subsequently the bacterial sequences can be removed. Finally, the virus can be produced rapidly by transfecting the packaging cell lines with the recombinant viral backbone (Fig. 4). The in vitro molecular ligation

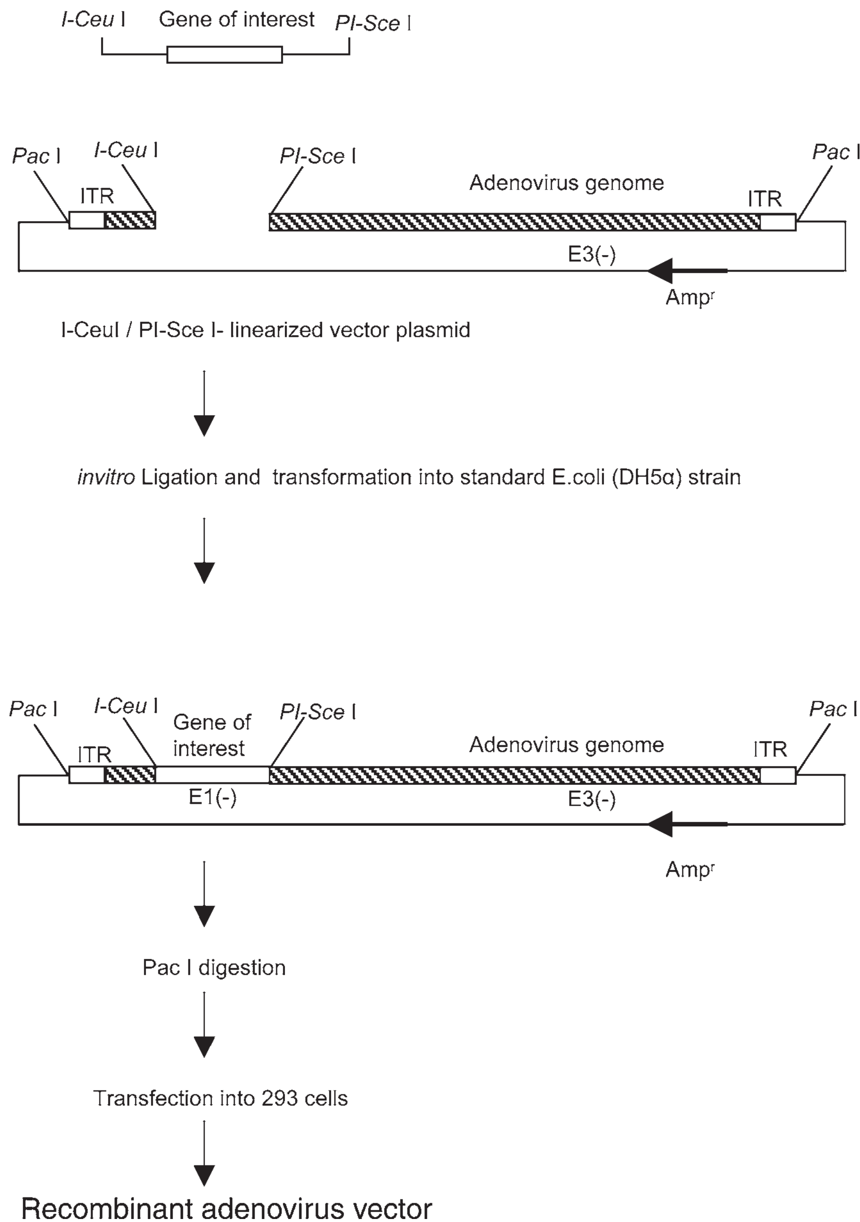

Fig. 4. Construction of recombinant Ad vector by in vitro ligation method.

technique is a rapid method to generate most of the E1-deleted recombinant Ad vectors and is now available as commercial kits *(22)*.

Choice of Ad Vectors

Ad vectors are selected on the basis of their expression level, size of the target genes, and characteristics of the target tissues. The first-generation

viral vectors are relatively easy to generate and produce high levels of foreign gene products, but the duration of gene expression is short, whereas the second-generation viral vectors exhibit long-term expression, which can be regulated. The replication-defective E1-deleted Ad vector that is currently being used as vaccine carrier sustains antigen presentation with reduced ability to induce cell death. The E3 gene deletion accommodates a 3.5-kb foreign DNA sequence in viral genome, but the virus is replication competent so the virus is less predictable than replication-defective vectors. Deletion of both E1 and E3 gene can accommodate up to 7.5 kb of foreign DNA and is commonly used in gene therapy *(23)*. The E4 gene deletion further reduces induction of vector-specific immune responses and minimizes outgrowth of replication-competent virus in packaging cell lines *(24)*. The viral yield reduces by 30- to 40-fold in E1 and E2A gene deletion compared to E2A containing vector. The E1, E2A, E3, and E4 viral genes are deleted to generate "gutless" vectors with more available space for transgene insertion. However, the production of these "gutless" vectors is labor intensive and requires several serial passages in the presence of helper virus to provide the structural proteins *(25–27)*. A new-generation ΔAd.IR vector has been developed in which the transgene cassette is flanked on both sides by the duplicated inverted repeats (IRs), Ad packing signals, and Ad ITRs *(28)*. The ΔAd.IR vectors are formed by homologous recombination between the inverted repeats during DNA replication, and the necessary functions for ΔAd.IR replication and packaging are provided by the full-length genome (termed FL-Ad.IR) amplified in the same cells (Fig. 5). The ΔAd.IR vector is not cytotoxic because it does not express any viral protein and its production involves only a single round of large-scale infection. Moreover, the ΔAd.IR vector can be applied at very high multiplicity of infection (MOI) to increase the transduction rate. However, the production of ΔAd.IR vector is less compared with full-length genome FL-Ad.IR (the ratio of FL-Ad.IR to ΔAd.IR particles is 5:1 to 10:1), the vector also contains 0.1% of first-generation virus contaminant, and the separation of ΔAd.IR vector from empty or defective particles is complicated.

Tissue-specific Ad vector can be generated either by using a tissue-specific transcriptional unit for the target gene or by modifying the tropism of the recombinant Ad vector *(29)*. Immunogenicity can be overcome by using helper-dependent, high-capacity, "gutless" Ad vector (HC-Ad), which is devoid of all viral coding sequences. This vector has large cloning capacity (approx 28–32 kb) and provides long-term gene expression. The HC-Ad vectors could transfer larger inserts (>20 kb) into target brain cells, even in the presence of antiadenoviral immune responses, and these HC-Ad vectors are generated by transfection of producer cells with HC-Ad DNA, followed by infection with helper virus *(30)*. Contamination by helper virus has been shown to be further reduced by excision of the packaging signal using Cre recombinase expressed in 293 cells or by yeast FLP recombinase *(31)*.

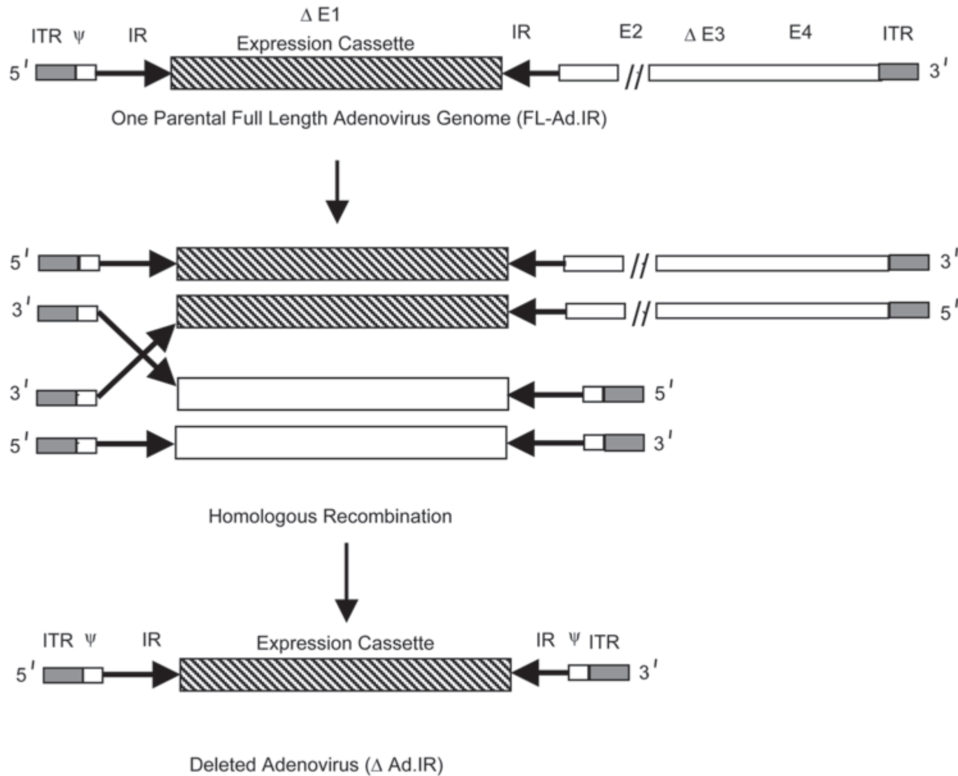

Fig. 5. Generation of ΔAd.IR vector devoid of all viral genes that contain transgene expression cassette. The parental full-length vector (FL-Ad.IR) provides the homologous elements including IRs. The ΔAd.IR vector consisting of the transgene cassette is generated by homologous recombination process; ψ is the Ad packaging signal.

Adenoviral Retargeting

Recombinant Ads are currently being used for gene delivery to a wide variety of cells. One major obstacle for the use of adenovirus is the lack of high-affinity viral receptors on the surface of certain cell types. Retargeting of Ad to those cell types can be achieved by altering the viral structures that mimic CAR and heparan sulfate-assisted viral entry to the cell types. Viral structures can be altered by either chemical or genetic modifications. Ligands for cell-surface receptors can be linked to capsid proteins of Ad vectors via biotin-avidin bridges or via polymers such as polyethylene glycol (PEG) and poly [*N*-(2-hydroxypropyl) methacrylamide] *(32)*. The attachment between ligand and viral surface should be covalent to minimize the dissociation following in vivo gene delivery. The efficacy of chemical modifications depends on the moiety used for targeting, the method of attachment onto the viral surface, and the targeted cell types. PEGylation is the most common method for chemical modifications of Ad.

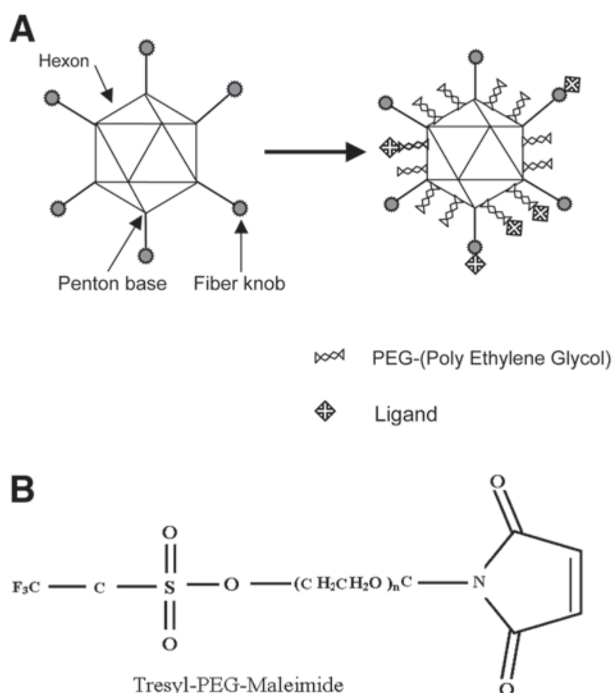

Fig. 6. **(A)** PEGylation of Ad. Icosahedral Ad capsid is composed of three structural proteins: hexon, fiber, and penton base. Ad vector is modified by covalent attachment of PEG ligand. **(B)** Chemical structure of TMPEG. The tresyl group reacts with Lys amino acid on capsid protein, and the maleimide group reacts to the thiol group of the ligand.

PEGylation

PEG is an immunologically inert water-soluble polymer that can be covalently attached to proteins. Immunogenicity owing to PEGylated coupled moieties can be overcome by biologic optimization of the method using tresyl-MPEG-maleimide (TMPEG) *(33)*. The optimized PEGylation method has been used to attach both monofunctional and heterofunctional PEG molecules to capsid proteins without compromising the integrity of the virion (Fig. 6A,B). Modification of Ad with monofunctional PEG has been shown to protect the virus from neutralizing antibodies, both in vitro and in vivo *(34,35)*. The protein fibroblast growth factor-2 (FGF2) coupled to the surface of an Ad vector via PEGylation could enhance gene transfer to the cells that expressed the FGF receptor. Ad vectors modified by this FGF2 protein have been shown to increase efficiency of transduction of the ovarian cancer cell lines *(36)*. Moreover, both Th1 and Th2 responses could be markedly suppressed following systemic delivery of the modified vector. The cystic fibrosis transmembrane conductance regulator gene delivery by the modified Ad vector has resulted in correction of defective

Cl^- transport in well-differentiated epithelial cultures from human cystic fibrosis donors *(37)*. Efficient gene transfer with minimal toxicity has been reported in mouse skin fibroblasts by 20% PEG6000-treated Ad vector *(38)*. Retargeting of Ad to activated endothelial cells can also be achieved by coupling of an αv integrin-specific RGD peptide or E-selectin-specific antibody to the functional group of PEG molecule *(39)*. These findings indicate that the strategy of using heterofunctional PEG to adapt Ad vectors for targeted gene delivery can be safe, convenient, nonimmunogenic, and nontoxic.

Genetic Modifications

Targeted Ad vectors can be generated by modifications of the fiber, penton base, and hexon capsid proteins. CAR-independent gene transfer can be achieved by substituting fiber genes from the Ad2 or Ad5 backbone with genes encoding fiber proteins from alternate Ad serotypes. The Ad2-based vector pseudotyped with Ad 17 fiber enhances viral infectivity to well-differentiated ciliated human airway epithelia and fetal rat central nervous system cells. An Ad5 vector with pseudotyped Ad35 fiber gene increases transduction rate for $CD34^+$ cells and DCs. Moreover, Ad5-based vector containing the Ad16 fiber shaft and knob domains has shown an 8- and a 64-fold increase in gene transfer to endothelial and smooth muscle cells (SMCs) compared with Ad5 alone. Replacement of the Ad5 knob with the subgroup B Ad3 knob resulted in an Ad5/3 chimeric fiber that confers improved gene delivery to human fibroblasts and head-and-neck cancer cells. Pseudotyping Ad5 fiber with other serotypes is advantageous because the number of neutralizing antifiber antibodies to Ad serotypes in the patient serum is less than that of the most commonly occurring Ad5. The tropism of Ad vectors can be altered by genetic engineering of the fiber knob. Replacing a large portion of the C terminus of Ad5 fiber with the σ1 protein of reovirus type 3 produces stable recombinant vectors, which use the reovirus receptor junctional adhesion molecule 1 for viral entry, showing high transduction of DCs *(40)*. Ad5 vectors pseudotyped with fibers from Ads of subgroups B, C, D, and F generate vectors with reduced tropism for myoblasts and endothelial cells (fibers from subgroup D) and increased tropism for DCs (fibers from subgroup B) in vitro and in vivo without affecting transgene product-specific antibody responses *(41)*. Moreover, insertion of RGD sequences into the fiber knob increases transduction efficiency in DCs *(42)*. Ad vector targeting can also be achieved by direct genetic modifications of capsid proteins. Vectors with ablated tropism through loss of CAR and integrin binding can be used to evaluate the targeting potential of peptide ligands incorporated in Ad capsids. Ad vectors are transduced poorly on nonepithelial cells such as endothelial cells, fibroblasts, and SMCs owing to low-level expression of the CAR receptor. Ad vectors can be generated with stretches of positively charged basic amino acids such as lysine moieties inserted genetically in fiber knob or,

alternatively, a polylysine peptide can be conjugated to an Ad/PEG vector to achieve the binding to heparin/heparan sulfate moieties *(43)*. The modified Ad by incorporation of six histidine residues (His tag) at fiber knobs (H1 loop) can be used to infect human glioma cells that express a single-chain antibody for the His tag *(44)*. Similarly, hemagglutinin peptide from influenza virus inserted into fiber or the penton base can be used to infect cells expressing the single-chain antibody ligand *(45)*. Therefore, Ad vectors with altered tropism can infect a wide variety of cell types.

Applications of Ad Vectors

Ad-Mediated Liver-Targeted Gene Therapy

Liver-directed gene therapy is being actively pursued and developed as a method for treating various liver diseases. It is effective on both inherited disorders and acquired conditions, such as infectious and neoplastic diseases, cirrhosis of the liver, and immune rejection of transplants. Recombinant Ads can infect nondividing cells with high efficiency and are rapidly concentrated in the liver after systemic administration; hence, they are used extensively in liver-directed gene therapy *(46,47)*. Ad vector–mediated gene expression could replace missing gene products that cause inherited diseases. Specific genes could be overexpressed by Ad for therapeutic purposes, such as the overexpression of metalloproteases for the treatment of cirrhosis. In certain situations, such as CN1 or low-density lipoprotein (LDL) receptor deficiency (familial hypercholesterolemia), the missing gene product was expressed by Ad vector in liver for appropriate metabolic effect *(48)*. The catalytic subunit of the apolipoprotein B mRNA editing enzyme (APOBEC-1), which is normally expressed in the intestinal epithelial cells, could be expressed ectopically in the liver by Ad vector to switch the hepatic apolipoprotein production from apo B100 to apo B48, thereby reducing the production of LDLs *(49)*. Expression of PDX, a homeobox protein that is responsible for pancreatic differentiation in liver, has shown secretion of insulin from hepatocytes *(50)*. In other cases, nucleic acids may be used to inhibit the expression of toxic proteins, such as viral proteins or mutant α_1-antitrypsin *(51)*. In an attempt to develop new therapeutic agents for human hepatocellular carcinoma, the high-affinity and high-stability antibodies for targeting tumor-specific antigens, Ad vectors expressing monoclonal antibody (MAb) AF-20 raised against a human hepatoma cell line and high-affinity single-chain MAb fragments (scFv) have been generated *(52)*. Loss of p53 function is one of the most frequent genetic alterations in liver cancers. Both replication-incompetent (rAd.p53, or SCH58500) and replication-selective (dl1520, or Onyx-015) Ads are being developed for the treatment of p53-deficient cancers *(53,54)*. Table 3 provides some of the inherited and acquired disorders targeted for liver-directed gene therapy.

Table 3
Liver Disorders That Are Current Targets for Gene Therapy

Inherited liver disorders	Acquired diseases
Disorders causing damage to liver architecture	Infectious diseases, such as hepatitis B and C and malaria (prophylaxis)
α_1-Antitrypsin deficiency	Liver tumors: hepatomas, cholangiocarcinomas, metastatic tumors
Wilson disease	Cirrhosis of the liver
Tyrosinemia	Extrahepatic tumors (inhibition of neovascularization)
Progressive familial intrahepatic cholestasis	Allograft or xenograft rejection
Glycogen storage diseases, such as von Gierke disease and Pompe disease	
Disorders not affecting liver architecture	
Crigler-Najjar syndrome type I	
Mucopolysaccharidosis VII	
Ornithine transcarbamylase deficiency	
Phenylketonuria	
Familial hypercholesterolemia and other lipid metabolic disorders	
Maple syrup urine disease	
Hemophilia A and B	
Oxalosis	

Immunologic Modifications of Ad for Liver-Targeted Gene Therapy

Cellular and humoral immune responses to adenoviral proteins limit the clinical application of adenovectors. In humans, antibodies often exist from previous infections by adenoviruses, and naive individuals readily develop humoral and cell-mediated immunity against the viral antigens after the initial injection of the recombinant Ad. Neutralizing antibodies block gene transfer by the recombinant vectors. Moreover, Ad-specific cytotoxic lymphocytes attack the host cells, resulting in liver damage and rapid loss of the transgene after secondary gene transfer *(55,56)*. The helper-dependent adenovectors, in which all viral genes are deleted, may also retain immunogenicity, because of the viral proteins provided *in trans* by the packaging cells. Although these vectors express transgenes for a longer duration than the first-generation Ad vectors, secondary or tertiary administration fails to transfer the transgene (Table 4).

Development of immune response requires presentation of antigenic peptides by APCs. Following docking of the APCs with uncommitted T-cells, APCs and T-cells costimulate each other via the B7-CD28 and CD40-CD40 ligand interactions. Inhibition of B7-CD28 costimulation prevents

Table 4
Advantages and Limitations of Ad-Mediated Liver-Targeted Gene Therapy

Integration and persistence	In vivo gene transfer efficiency to liver	Liver specificity	Immunologic issues
Episomal. Transgene is expressed for several months in the absence of host immune response.	Efficiently infects both dividing and nondividing cells. Very high efficiency for liver on systemic administration.	Liver is targeted in rodents and mice but not in humans.	Evokes both antibody and cell-mediated immune response. Deletion of viral genes reduces primary immunogenicity but does not permit repeated injection. Host tolerization or coexpression of immunomodulatory genes permits repeated gene transfer.

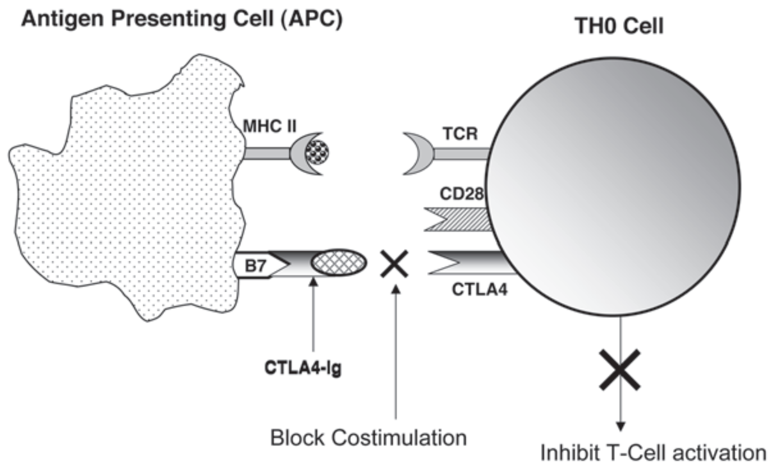

Fig. 7. Ad vector–mediated expression of CTLA4-Ig. The soluble CTLA4-Ig protein blocks the costimulation pathway. The TCR (T-Cell receptor) recognizes the peptide-MHC complex on antigen presenting cell (APC).

effective immune response. CTLA4-Ig, a soluble inhibitory protein, inhibits this costimulation. Injecting CTLA4-Ig alone at the time of administration of Ads does not prevent antibody formation. However, coexpression of CTLA4-Ig with the target transgene permits multiple administration of the recombinant Ad *(16)* (Fig. 7). Host tolerance toward adenoviral proteins can be induced by injecting recombinant Ads in newborn rats, inoculating adenoviral proteins into the thymus of young adult rats, or orally administering small doses of adenoviral proteins.

Suicide Gene Therapy/Prodrug Therapy in Cancer

Cancer is a disease of high incidence for which conventional treatments are not necessarily effective. Suicide gene therapy, also known as prodrug therapy, in which the transgene products metabolize the nontoxic prodrug to toxic drug, resulting in cell death, could be an alternative strategy for cancer therapy *(57)*. Treatment efficiency can be enhanced by the bystander effect in which the death of transduced cells affects neighboring nontransduced cells. The addition of ganciclovir (GCV) to human oral squamous cell carcinoma cell lines transduced with Ad vector containing herpes simplex virus thymidine kinase gene (HSVtk) led to apoptosis *(58)*. In another study, Ad vector–mediated cytosine deaminase::uracil phosphoribosyltransferase (CD::UPRT) expression in the presence of 5-fluorocytosine has shown high antitumor activity in human glioblastomas *(59)*. The replication-activated Ad vectors express a secreted form of β-glucuronidase and a CD/UPRT, which activate the prodrugs 9-aminocamptothecin glucuronide to 9-aminocamptothecin and 5-fluorocytosine to 5-fluorouracil and further to 5-fluoro-UMP, respectively. The activated prodrugs 9-aminocamptothecin and 5-fluoro-UMP are highly cytotoxic and

kill cancer cells. Tissue- or tumor-specific gene delivery is crucial for achieving successful results in suicide gene therapy. In this context, Ad vectors combining the Cre-loxP CD gene system with prostate-specific antigen (PSA) promoter/enhancer to drive Cre recombinase activity were found to inhibit the growth of PSA producing prostate cancer cells in vivo *(60)*. The RGD fiber-mutant Ad vector (Ad-RGD)–mediated HSVtk/GCV system is an attractive approach for melanoma treatment *(61)*. The use of either melanoma-specific tyrosinase (Tyr) promoter or tumor-specific telomerase reverse transcriptase (TERT) promoter instead of universal CMV promoter in HSVtk/GCV treatment exhibits high transgene expression specificity for melanoma cells. Recently, a conditionally replicative Ad expressing UPRT has been constructed, which replicates only in cancer cell–expressing transgene *(62)*.

Small Interfering RNA and Ad Vectors

RNA interference is a process in which dsRNA induces homology-dependent degradation of cognate mRNA mediated by 21- to 23-nucleotide (nt) small interfering RNA (siRNA) *(63)*. These siRNA molecules can be chemically synthesized and transfected into cells or directly expressed intracellularly from a plasmid by the function of cellular RNA polymerase III. Successful application of siRNA in functional genomics and proteomics, cancer gene therapy, and virus protection depends on the efficient delivery of siRNA into the target cells that are refractory to transfection by DNA or RNA. In this context, Ad vectors serve as useful tools for siRNA delivery because of high virus titer, a broad spectrum of cell-type infectivity, and independence of active cell division. Ad vectors expressing siRNA molecules targeted against p53 or VprBP/KIAA0800, a cellular protein that interacts with the human immunodeficiency virus (HIV) auxiliary protein Vpr, have shown specific reduction in the target protein and the corresponding mRNA level *(64)*. Reports have shown that the Ad vectors expressing siRNA (Adv-siSurv) targeted against survivin, an antiapoptotic molecule widely overexpressed in malignancies but not detected in terminally differentiated adult tissues, successfully exerted a gene knockdown effect and induced apoptosis in HeLa, U251, and MCF-7 cells. Moreover, intratumoral injection of Adv-siSurv could significantly suppress tumor growth in a xenograft model using U251 glioma cells *(65)*. A two-vector Ad system for efficient and controlled expression of hairpin siRNA (shRNA) by Tet operator has been reported for transcriptional coactivator p300 regulation, in which the tetracycline repressor protein (TetR) encoded in a second Ad represses shRNA expression, but the addition of tetracycline abolished TetR binding and allowed shRNA transcription, leading to targeted reduction of mRNA and protein level *(66)*. Regulated adenoviral shRNA vectors have the advantages of being able to infect a wide array of replicating and nonreplicating cells, allowing controlled gene silencing. Another study showed that expression levels of hypoxia-inducible factor 1α (HIF-1α) could be significantly attenuated by the use of siRNA in combina-

tion with Ad-mediated gene transfer. Downregulation of the HIF-1α protein could enhance hypoxia-mediated tumor cell apoptosis in vitro *(67)*. Recombinant Ads drive siRNA expression efficiently in islet β-cells and β-cell-derived cell lines. Transduction with a virus expressing siRNA(s) targeted against GLUT2 and glucokinase reduced mRNA and protein levels significantly in β-cell lines *(68)*. Therefore, recombinant Ad siRNA vectors are useful for suppression of specific genes in pancreatic islets and β-cell lines. The Ad-based siRNA gene transfer approach could be a potentially effective adjuvant strategy for cancer treatment.

Gene Transfer to Stem Cells by Adenovectors

Stem cells are quiescent, self-renewing cells that can differentiate into different lineages. Differentiations of stem cells depend on the expression of specific transcription factors. The delivery of transcription factors to the stem cells by conventional DNA/RNA transfection method is very inefficient. Recent studies have shown that Ad vectors could be used as an effective tool for gene transfer to stem cells. Ad vector–mediated expression of the Oct-3/4 transcription factor, a critical regulator of embryonic stem (ES) cell differentiation in neurospheres consisting of neural stem cells and neural progenitor cells, has influenced cell fate *(69)*. Downregulation of Oct-3/4 could induce neuronal differentiation while its prolonged expression prevents neuronal differentiation. Rapid genetic manipulation of ES cells to obtain multiple gene targeting by Ad-Cre-mediated gene recombination and to facilitate efficient loxP-neo-loxP removal has been reported. Mesenchymal stem cells transduced with an Ad vector carrying the human interferon-β (IFN-β) gene have been used to target delivery of IFN-β to tumors, which inhibited tumor cell growth *(70)*. Angiogenic mitogens Bv8 and endocrine-gland-derived vascular endothelial growth factor delivered by Ad vectors promote the survival of hematopoietic cells and enhance progenitor mobilization, where Bv8 induces differentiation of the granulocytic and monocytic lineages *(71)*. Until recently, the Ad vector was not considered an efficient gene transfer vector for the cells of hematopoietic origin, because these cells lacked receptors for Ad vectors and Ad infection has inherent limitations, such as short-term expression and a non-integrating nature, whereas hematopoietic stem cells (HSCs) require long-term expression. However, mouse bone marrow highly enriched for HSC can be transduced with Ad5 at a low MOI. The transduced HSCs have shown normal in vitro myeloid differentiation potential, retained long-term in vivo repopulating activity, and contributed to all blood cell lineages. Ad5 transduction of mouse HSCs was dependent on CAR, because the use of anti-CAR blocking antibody greatly reduced transduction *(72)*. Strategies including the use of Ad vector possessing synthetic polymer coatings, genetically modified capsid proteins, or antibody-redirected fibers could redirect and retarget Ad vectors to transfer genes into HSCs. A soluble fusion protein that bridges adenoviral fibers and the c-Kit recep-

tor to alter Ad5 tropism to immature hematopoietic cells has been developed *(73)*, in which the CAR-stem cell factor (SCF) fusion protein consisted of the extracellular domains of CAR and SCF. Hematopoietic cell lines can be infected with an Ad5 vector expressing the enhanced green fluorescent protein gene (Ad5-eGFP) in the presence of CAR-SCF. New chimeric Ad5/F35 vectors that have been engineered to substitute the shorter-shafted fiber protein from Ad35 efficiently infect the committed hematopoietic cells with low toxicity *(74)*. Additionally, new hybrid Ad vectors that are engineered with both modified capsid proteins and novel *cis*-acting integration sequences could efficiently deliver and integrate Ad vector–delivered genes into HSCs *(75)*.

Ads as Recombinant Vaccines

Ads can be engineered to express a number of heterologous proteins in vitro. Studies in animal models have shown that recombinant Ad vectors are efficient in inducing protective immunity against various pathogens. The inherent immunogenicity of these vectors is a desirable feature for vaccine development. Ad vectors are being developed as therapeutic vaccine delivery systems for many infectious agents including HIV type 1 and for biodefense against potential bioterror pathogens *(76,77)*. The tropism of Ads for mucosal epithelium makes them ideal vectors for the development of recombinant Ad-HIV vaccines. Replication-defective Ad-simian immunodeficiency virus (SIV) gag vaccines elicited cellular responses that could control iv infection with an HIV/SIV chimeric immunodeficiency virus, while replication-competent Ad-SIV env/rev/gag/nef vaccines have induced cellular and humoral responses and protected rhesus monkeys from a mucosal challenge with pathogenic SIV *(78)*. Currently, several Ad-HIV vaccine candidates are being tested in clinical and preclinical trials. Administration of Ad vectors expressing herpes simplex virus glycoprotein B through the intranasal route could give greater protection than ip inoculation *(79)*. Ad vector–mediated cellular immune response also plays a pivotal role in the clearance of hepatitis C virus (HCV) infection. Replication-deficient recombinant Ad expressing HCV NS3 protein (RAdNS3) could protect a recombinant vaccinia virus–mediated HCV-polyprotein (vHCV1-3011) expression and is useful for the induction of prophylactic or therapeutic anti-HCV immunity *(80)*. The effective APCs and DCs, which elicit both primary and secondary T-cell responses critical for antitumor immunity and elimination of intracellular pathogens, can be pulsed ex vivo with antigens for using cell-based vaccines against tumors. Ads that accommodate large expression cassettes encoding antigens could be extensively used to pulse DCs ex vivo by delivering genes encoding immunomodulatory molecules and tumor antigens to DCs for effective induction of DC maturation *(81)*. The viral capsid proteins of Ad vectors can be modified for effective gene delivery to DCs to circumvent the paucity of Ad receptor on DCs. A new class of nonreplicative Ad vector influenza vac-

cines has been developed without the prerequisite of growing influenza virus, which could be administrated as a nasal spray or skin patches and are found to be well tolerated by human volunteers *(82)*. The nasal vaccine, which offers a less invasive means of delivery, is a potential candidate for further human testing of needleless vaccines and a promising alternative to current vaccines.

Conclusion

The potential of Ad vectors as promising tools for gene therapy can be explored by the development of new-generation vectors with refined specificity and improved efficacy. This review elucidated construction of new-generation Ad vectors and different methods of Ad vector retargeting and their applications. The problems associated with Ad vector specificity and host immune response against these vectors can be further solved with greater understanding of viral immunoregulatory genes and their cellular targets. In addition to an important role in human gene therapy, Ad vectors have great potential as effective tools in genomic studies. New advances in vector technology will significantly improve the efficiency of Ad production, especially for new generations of Ad vectors. Efforts are currently under way to streamline the large-scale manufacturing of replication-deficient Ad vectors for commercial manufacturing under Good Manufacturing Practice conditions. The design to generate safe vectors and tailor producer cells will have immense importance for the production of high-titer vectors. Advances in cell culture engineering, cell metabolism, bioreactor design and operation, and downstream processing will have a positive impact on the large-scale production of Ad vectors. Procedures for the separation, purification, and formulation of vector preparations are also being developed. Novel gene expression using tissue-specific and inducible systems in specific cells could enhance the potential applications of Ad in different stages of diseases. Candidate genes can be manipulated using activated/dominant-negative mutants or antisense/ribozyme in the Ad vector system to validate their function both in vitro and in vivo. By combining the latest versions of the Ad 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. Recombinant Ads should be considered an important vehicle to explore the vast unknowns of human genome science.

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