# 24 Gene Therapy

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## INTRODUCTION

The human body is composed of a variety of proteins. Almost all human diseases are the results of improper production or function of proteins. Traditional small molecule drugs usually interact with proteins such as enzymes, hormones, and transcriptional factors to exert their therapeutic potential. However, many severe and deliberating diseases (e.g., diabetes, hemophilia, cystic fibrosis) and several chronic diseases (e.g., hypertension, ischemic heart disease, asthma, Parkinson's disease, motor neuron disease, multiple sclerosis) remain inadequately treated by the conventional pharmaceutical approaches.

Gene therapy is the use of nucleic acids as a pharmaceutical agent to treat disease (Fig. 24.1). It derives its name from the idea that DNA can be used to supplement or alter genes within an individual's cells as a therapy to treat disease. Unlike small molecule drugs or protein drugs which are usually formulated in capsule or tablet forms, therapeutic nucleic acids are packaged within a specialized vector to get inside cells within the body. Gene therapy usually targets one or more defected genes without affecting the normal gene at the site of diseases. A gene therapy target can be an abnormal oncogene whose product has the potential to cause a tumor or a defect gene whose product is critical to maintain normal physiological functions.

The potential use of nucleic acids as therapeutics has attracted great attention to treat severe and debilitating genetic diseases. Compared with small molecule drugs, gene therapy per se will not induce drug resistance even after repeated treatments since the targets of gene medicine are not certain receptors but the genes encoding them. Moreover, this technique could be a permanent treatment to give someone who is born with a genetic disease a chance to live a normal life once and for all. However, there are certain limitations to gene therapy as well. The major disadvantage of gene therapy is that gene medicines are not easy to be formulated into conventional dosage forms and delivered for a routine use. Therefore, the clinical applications of gene therapy can so far only be conducted in hospitals with well-trained specialists. The cost of gene therapy is so far also much higher compared with traditional medicines.

The first approved gene therapy case in the United States took place on September 14, 1990, at the National Institute of Health (NIH). The patient was a 4-year-old girl with severe combined immunodeficiency (SCID) disease caused by a defect adenosine deaminase (ADA) gene. In the therapy procedure, the medical group extracted some of the girl's T lymphocytes, exposed them to a genetically engineered retrovirus that had lost its virulence but carried the normal ADA gene, and transfused them back to the girl's bloodstream. The treatment was successful (Blaese et al. 1995). Ten years after treatment, lymphocytes from the patient continued to express the recombinant transgene, indicating that the effects of gene transfer can be long lasting (Muul et al. 2003).

In this chapter, we will discuss the current state of gene therapy and common approaches to gene transfer. The biology and utility of gene transfer systems, recent advances in cell-based gene delivery systems, the diseases currently subjected to gene therapy, and the regulation of gene products will be discussed and reviewed.

## VECTORS FOR GENE TRANSFER

### ■ Basic Components of Plasmid (cf.Chap. 1)

Gene therapy can be classified into viral gene therapy and nonviral gene therapy, both of which rely on the successful construction of a gene expression plasmid.

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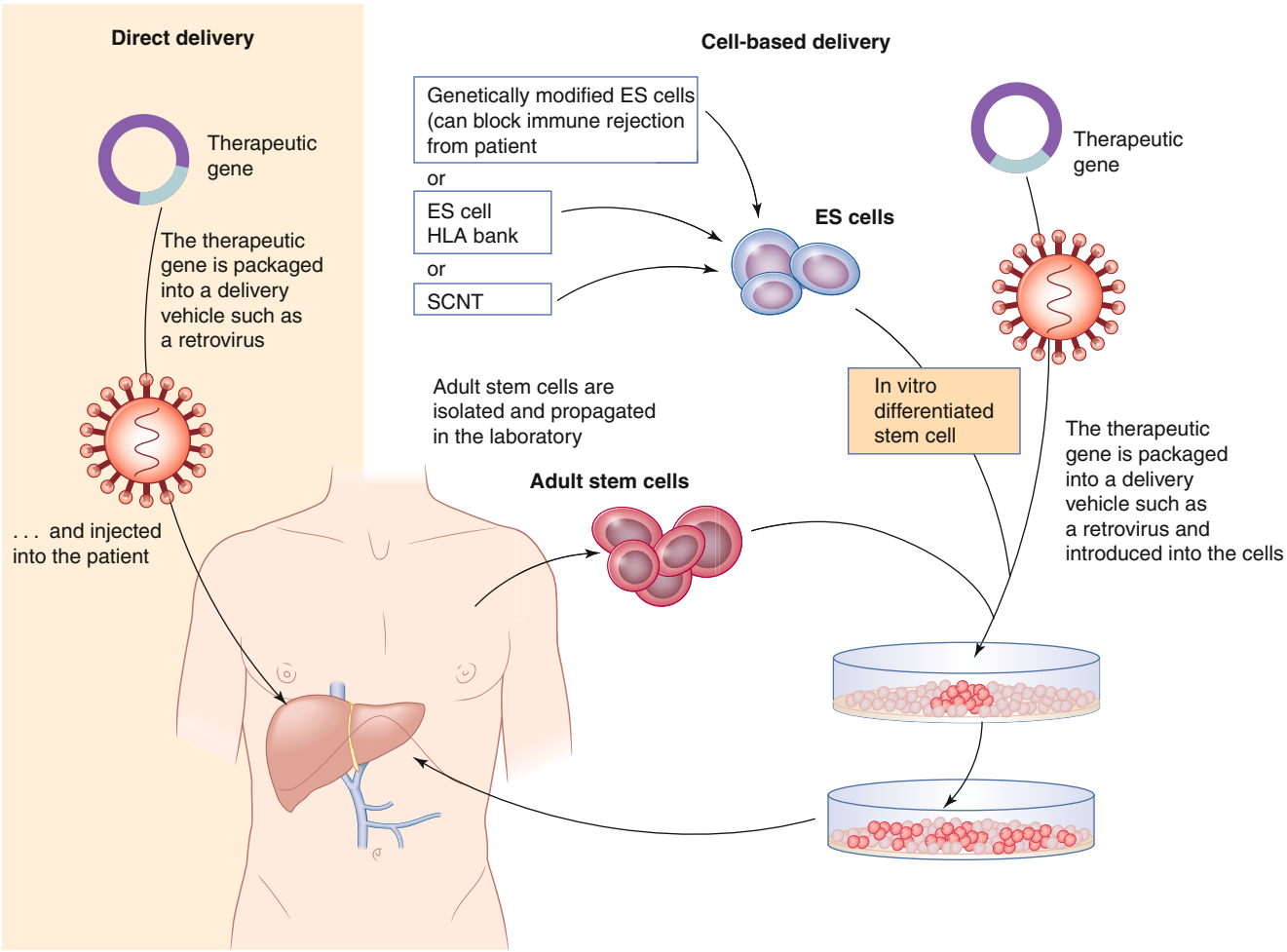


**Figure 24.1** ■ Methods of administration of gene therapy vectors. In vivo gene transfer involves direct administration of the vector in the tissue of interest. Ex vivo gene transfer requires collection of cellular targets from the patient. The cells are treated in culture with the vector. Cells expressing the therapeutic transgene are harvested and given back to the patient (From (Zwaka 2006) with permission to reprint (also cf. Chap. 25)).

A plasmid is a circular, double-strand DNA molecule which contains a complementary DNA (cDNA) sequence coding for the therapeutic gene and several other genetic elements including bacterial elements, transcription regulatory elements (TRE), multiple cloning sites (MCS), untranslated regions (UTR), introns, polyadenylation (polyA) sequences, and fusion tags, all of which have great impact on the functioning of the final genetic products. After constructing the plasmid, certain screening methods are needed to validate the construct. For example, DNA sequencing, polymerase chain reaction (PCR), and Southern blot are useful to validate the structure of the construct. Western blot and enzyme-linked immunosorbent assay (ELISA) are useful to confirm the function of the construct (see also Chaps. 1 and 2).

### *Bacterial Elements*

Plasmids have two features that are important for their propagation in bacteria. One is the bacterial origin of replication (Ori), which is a specific DNA sequence that binds to factors that regulate replication of plasmid and in turn control the number of copies of plasmid per bacterium. The second required element is a selectable marker, usually a gene that confers resistance to an antibiotic. The marker helps in the selection of bacteria that have the gene expression plasmid of interest. *Escherichia coli* (E. coli) is a commonly used bacterium for propagating plasmids. It has the property to transfer DNA either by bacterial conjugation, transduction, or transformation. The extensive knowledge about E. coli's physiology and genetics accounts for its preferential use as a host for gene expression. Human insulin was the first product to be produced using recombinant DNA technology from E. coli.

### *Transcription Regulatory Elements (TRE)*

Gene-expressing plasmids contain transcription regulatory elements (TRE) to control transcription. Various TREs (promoters, enhancers, operators, silencers,

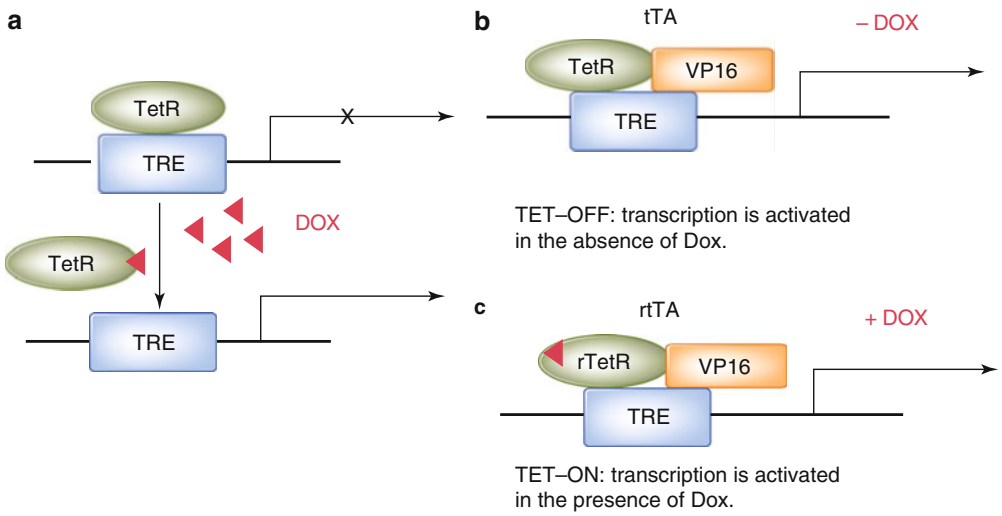


**Figure 24.2** ■ Tetracycline (*Tet*)-based reversible inducible gene expression systems. (**a**) Unmodified Tet system. In the absence of doxycycline (*Dox*), tetracycline repressor (*TetR*) binds to Tet response element (*TRE*) and inhibits expression through steric hindrance; after administration of Dox, Dox binds to TetR, removes TetR from TRE, and activates expression. (**b**) Modified Tet-off system. In the Tet-off system, the chimeric tetracycline transactivator protein (*tTA*) consists of the TetR domain fused to the VP16 transactivating domain of herpes simplex virus. tTA binds to TRE to activate gene expression. (**c**) Modified Tet-On system. TetR is mutated to rTetR. Therefore tTA is mutated to rtTA, which must have Dox present in order to bind to TRE and activate gene expression, *r* repressor.

insulators, etc.) interact with the molecular machinery (general transcription factors, activators, co-activators, and repressors) to control the patterns of gene expression.

A promoter is a DNA sequence that enables a gene to be transcribed. The promoter is recognized by RNA polymerase and transcriptional factors. Any mutation in this region will prevent the binding of RNA polymerase and the subsequent transcription and translation. The proper choice of promoter governs the strength and duration of transgene expression. Cytomegalovirus (CMV), Rous sarcoma virus (RSV), and Simian virus 40 (SV40) are some of the strongest known viral promoters. The potency of a promoter can be cell and tissue specific. Overwhelming evidence suggests that the CMV promoter is surprisingly silenced in both embryonic stem cells (ES) and other adult stem cells, making such promoter unsuitable for the new evolving stem-cell-based gene therapy (Qin et al. 2010; McGinley et al. 2011). Other promoters such as EF-1α, chicken β-actin promoter coupled with CMV early enhancer (CAGG), and SV40 are more efficient to drive the transgene expression in stem cells (Qin et al. 2010; McGinley et al. 2011). The distance between the promoter and the transgene cassette also has great impact on gene expression. Several reports have suggested that an insertion between the CMV promoter and transgene cassette surprisingly increases the transgene expression (Li and Mahato 2009).

An enhancer is a short DNA sequence that can bind transcription factors or activators to enhance transcription levels of genes in a gene cluster. While most enhancers are usually close to the promoters and genes, certain enhancers control gene expression from a far distance or even from different chromosomes (Spilianakis et al. 2005). Enhancer-promoter interaction plays a major role to drive gene expression. Enhancers do not directly act on the promoter region, but elicit their effects once they are bound by activators or other transcription factors. These proteins recruit the RNA polymerase and the general transcription factors and stabilize the transcription initiation complex. Different enhancer-promoter combinations have been widely explored to improve the gene transfer efficiency in a variety of tissues and species (Hagstrom et al. 2000).

Other TREs include insulators, operators, and silencers. Insulators are mainly genetic boundary elements to block the enhancer-promoter interaction or more rarely barriers against condensed chromatin proteins. Operators and silencers are usually short DNA sequence close to the promoter with binding affinity to a set of proteins named repressors and inducers. Based on these interactions, an inducible or repressible system can be constructed to either increase or decrease transcription depending on the requirements. For example, the tetracycline-repressor-regulated gene expression system is a popular inducible system in constructing transcription regulatory plasmids (Fig. 24.2a). Based on this system, more advanced Tet-On and Tet-Off systems are constructed for reversible control of the transgene expression (Fig. 24.2b, c).

### *Multiple Cloning Site (MCS)*

A multiple cloning site (MCS), also known as a polylinker, is a short DNA segment which contains many restriction endonuclease recognition sites. Restriction sites within an MCS are typically unique and occur only once within a given plasmid. Within each MCS, there are usually up to 20 restriction sites which can be identified and easily cleaved with commonly used restriction endonucleases. MCS allows the insertion of single cDNA or multiple cDNA depending on the requirement of the therapeutic genes. Generally, the choice of the restriction site for cDNA cloning has no impact on the ultimate transgene expression. However, the choice of the cloning site might occasionally lead to a change in the secondary structure of mRNA and a subsequent translation inhibition. It might call for reengineering MCS for function and convenience (Crook et al. 2011).

### *Untranslated Regions (UTR)*

To express a therapeutic protein, an mRNA must be generated from a cDNA template which is inserted in the MCS and transported into the cytoplasm to be translated. In molecular genetics, untranslated regions (UTR) refer to two sections on each side of a coding sequence on a strand of mRNA. The 5′ UTR is the region of the mRNA transcript that is located between the capsite and the initiation codon. The 5′ UTR contains regulatory elements controlling gene expression. Such elements include the binding sites for proteins to stabilize the mRNA structure, the riboswitches to regulate mRNA's own translation activities, the binding sequence to stabilize or inhibit the translation-initiation complex, and introns to control mRNA splicing and export. The 3′ UTR is the region of the mRNA transcript following the termination codon. 3′ UTR plays an important role in mRNA stability. It contains binding sites for proteins which may affect the mRNA's stability or location in the cell; a polyadenylation tail which is important for the nuclear export, translation, and stability of mRNA; and binding sites for miRNAs which are part of the endogenous gene-silencing machinery.

### *Introns*

The protein coding region in the eukaryotic gene is often interrupted by the stretches of noncoding DNA called introns. In any eukaryotic cells, introns are co-transcribed with protein-encoding exons into a premature mRNA and removed by the mRNA splicing. There is no intron in the cDNA sequence. However, extensive studies have shown that transcripts from the intronless gene are degraded rapidly and that at least one intron should be included in the transcription unit for the optimal transgene expression (Ryu and Mertz 1989). Introns are frequently inserted into the 5′ UTR of the transcript unit (Huang and Gorman 1990).

### *Polyadenylation (polyA) Sequence*

The polyadenylation (polyA) sequence is important for the nuclear export, translation, and stability of mRNA. At the end of transcription, the 3′ segment of the newly made RNA is first cleaved off by a set of proteins. These proteins then synthesize the polyA tail at the RNA's 3′ end. The polyA signal is a recognition site consisting of AAUAAA hexamer positioned 10–30 nucleotides upstream of the 5′ end and a GU- or U-rich element located maximally 30 nucleotides downstream of the 3′end (Mahato et al. 1999). The most important function of polyA sequence is to prevent the mRNA from enzymatic degradation.

### *Fusion Tag*

Fusion tag is a protein or a peptide located either on the C- or N-terminal of the target protein to exert one or several functions such as improving expression, solubility, detection, purification, or localization. For example, fusion of the N-terminus of the target protein to the C-terminus of a highly expressed fusion partner results in high-level expression of the target protein. Maltose binding protein (MBP) is frequently used to increase the solubility of recombinant proteins expressed in E. coli systems (Bedouelle and Duplay 1988). Fluorescent protein tags, such as green fluorescent protein (GFP), provide information about the intracellular location of the transgene expression. Fusion tags like glutathione S-transferase (GST) and MBP made the isolation of recombinant proteins easy using affinity chromatography with specific resins. Other fusion tags such as several peptide sequences of the human c-myc protein were used to increase the nuclear translocation of the target protein to exert physiological functions (Dang and Lee 1988).

## VIRAL VECTORS

All viruses hold the inherited advantage to bind to their hosts and introduce their genetic material into the host cell with high efficiency. To construct a viral vector, the genes responsible for the viral replication and pathogenicity are first removed and replaced with a transgene cassette. Then the recombinant viral genome is inserted into a shuttle plasmid and transduced into a packaging cell line which contains the genes responsible for the viral replication to generate the recombinant viral vectors (Fig. 24.3). The vector construct contains the terminal sequences (ITRs or LTRs), the packaging signal (ψ), and the transgene cassette. The packaging signal (ψ) regulates the essential process of

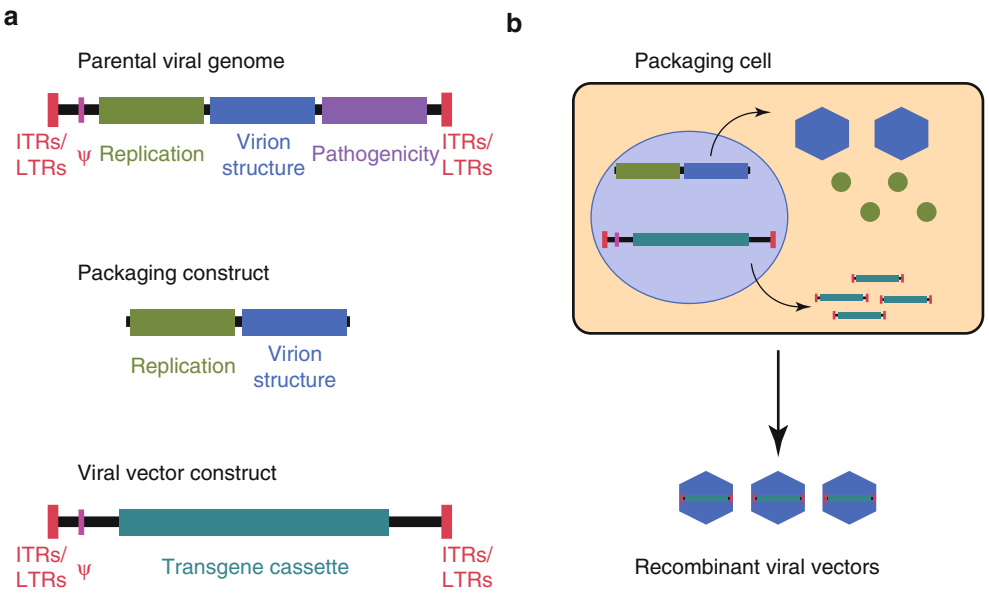


**Figure 24.3** ■ General overview of constructing and maintaining a viral vector. (**a**) Schematic overview of a viral genome, the packaging construct and the vector construct. The viral genome containing genes involved in replication, production of the virion structure, and pathogenicity of the virus is flanked by the terminal sequences (ITRs or LTRs) and the packaging signal (ψ). The packaging construct contains only genes that encode replication and structural proteins. The vector construct contains the terminal sequences (ITRs or LTRs), the packaging signal (ψ), and the transgene cassette. (**b**) The packaging and vector constructs are introduced into the packaging cell by transfection, by infection with helper virus, or by generating stable cell lines. Replication-related proteins and viral particles are expressed by the packaging construct. The vector constructs are replicated and encapsidated into virus particles to generate the recombinant viral vector.

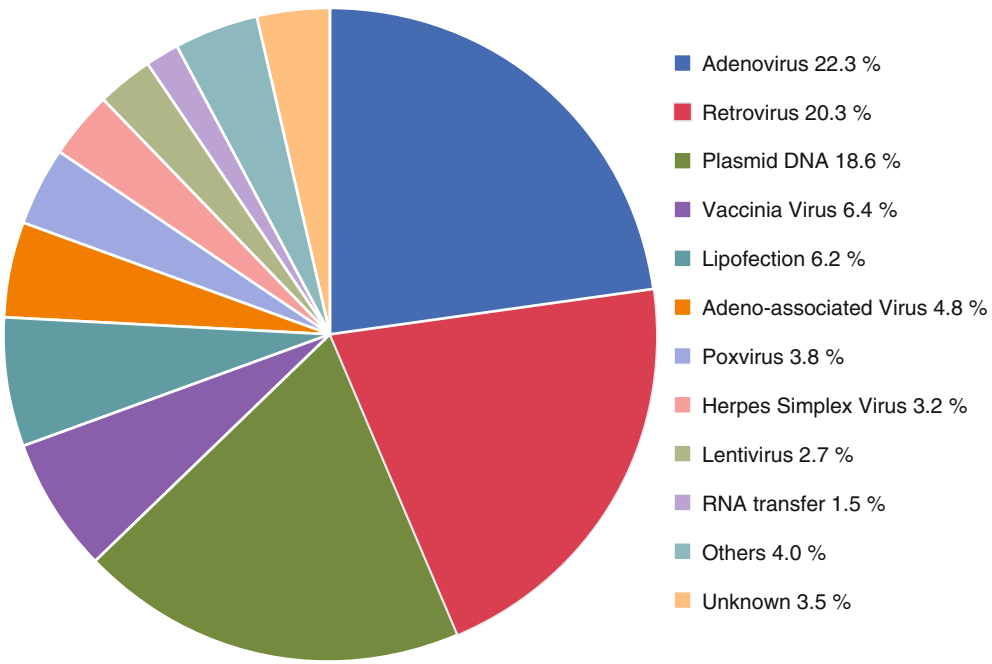


**Figure 24.4** ■ Vectors of gene therapy clinical trials (Wiley 2012). Others indicate the clinical trials in which vector used was not reported.

packaging the genetic materials into the viral capsid during replication. Viral vectors typically hold high transduction efficiency and do not need additional carriers for effective gene delivery. To date, approximately 70 % of all gene therapy clinical trials employ viral vectors (Fig. 24.4). Retrovirus, lentivirus, adenovirus, and adeno-associated virus (AAV) are the most extensively studied and used viral vectors for human gene therapy. Their characteristics have been listed and compared in Table 24.1.

| | Retrovirus | Lentivirus | Adenovirus | Adeno-associated virus |
|---|---|---|---|---|
| Genetic material | RNA | RNA | dsDNA | ssDNA |
| Genome size | 7–11 kb | 8 kb | 26–45 kb | 4.7 kb |
| Cloning capacity | 8 kb | 8 kb | 7[b]–35[c] kb | < 5 kb |
| Genome forms | Integrated | Integrated | Episomal | Stable/episomal |
| Diameter | 100–145 nm | 80–120 nm | 80–100 nm | 20–22 nm |
| Tropism | Dividing cells only | Broad, dividing & nondividing cells | Broad, dividing & nondividing cells | Broad, not suitable for hematopoietic cells |
| Virus Protein Expression | No | Yes/no | Yes b/no c | No |
| Transgene expression | Slow, constitutive | Slow, constitutive | Rapid, transient | Moderate, constitutive, transient |
| Delivery method | Ex vivo | Ex vivo | Ex/in vivo | Ex/in vivo |
| Typical yield (viral particle/ml) | $<10^8$ | $<10^7$ | $<10^{14}$ | $<10^{13}$ |
| Preexisting immunity | Unlikely | Perhaps, post-entry | Yes | Yes |
| Immunogenicity | Low | Low | High | Moderate |
| Potential pathogenicity | Low | High | Low | None |
| Safety | Insertional mutagenesis | Insertional mutagenesis | Potent inflammatory response | None to date but long term not clear |
| Physical stability | Poor | Poor | Fair | High |

[a]Information compiled from references (Edelstein et al. 2004; Weber and Fussenegger 2006)

[b]First-generation, replication-defective adenovirus

[c]Helper-dependent adenovirus

**Table 24.1** ■ Characteristics of viral vectors for gene transfer[a].

## ■ Retrovirus

### *Biology*

Retroviruses are enveloped RNA viruses containing two copies of a single-stranded RNA genome (Fig. 24.5). Retroviruses are 80–100 nm in diameter and have a genome of about 7–10 kb, composed of group-specific antigen (gag) gene codes for core and structural proteins of the virus; polymerase (pol) gene codes for reverse transcriptase, protease, and integrase; and envelope (env) gene codes for the retroviral coat proteins. The long terminal repeats (LTRs) control the expression of viral genes, hence act as enhancer-promoter. The final element of the genome, the packaging signal (ψ), helps in differentiating the viral RNA from the host RNA (Verma 1990).

After viral binding and introducing the viral RNA into the host cell, reverse transcriptase converts the viral RNA to linear double-stranded DNA that integrates into the host genome with the help of the viral integrase. The integrated construct, the provirus, will later undergo transcription and translation as cellular genes do to produce viral genomic RNA and mRNA encoding viral proteins. Virus particles then assemble in the cytoplasm and bud from the host cell to infect other cells.

### *Suitability of Retroviruses as Vectors for Gene Transfer*

To generate replication-deficient retroviral vectors, the sequences encoding the virion proteins (gag, pol, and env) responsible for the viral replication and pathogenicity are replaced by transgenes. The transgenes can be controlled by the native LTRs or exogenous enhancer-promoter sequences which can be engineered into the genome along with the transgene. The chimeric genome is then introduced into packaging cell lines, mostly HEK293 cells to produce the retroviral vectors.

Retroviral vectors have several features for gene transfer applications (Table 24.1). They can accommodate transgene cassettes of 8 kb. They are capable of integrating into the host genome. Therefore retroviruses can produce stable, long-term transgene expression in dividing cells with low immunogenic potential. Retroviruses can also be used to direct the transdifferentiation of stem cells or reprogram the differentiated somatic cells to have stem-cell-like properties (see later). Such features made retroviruses a valuable tool in a new emerging area named "stem-cell-based gene

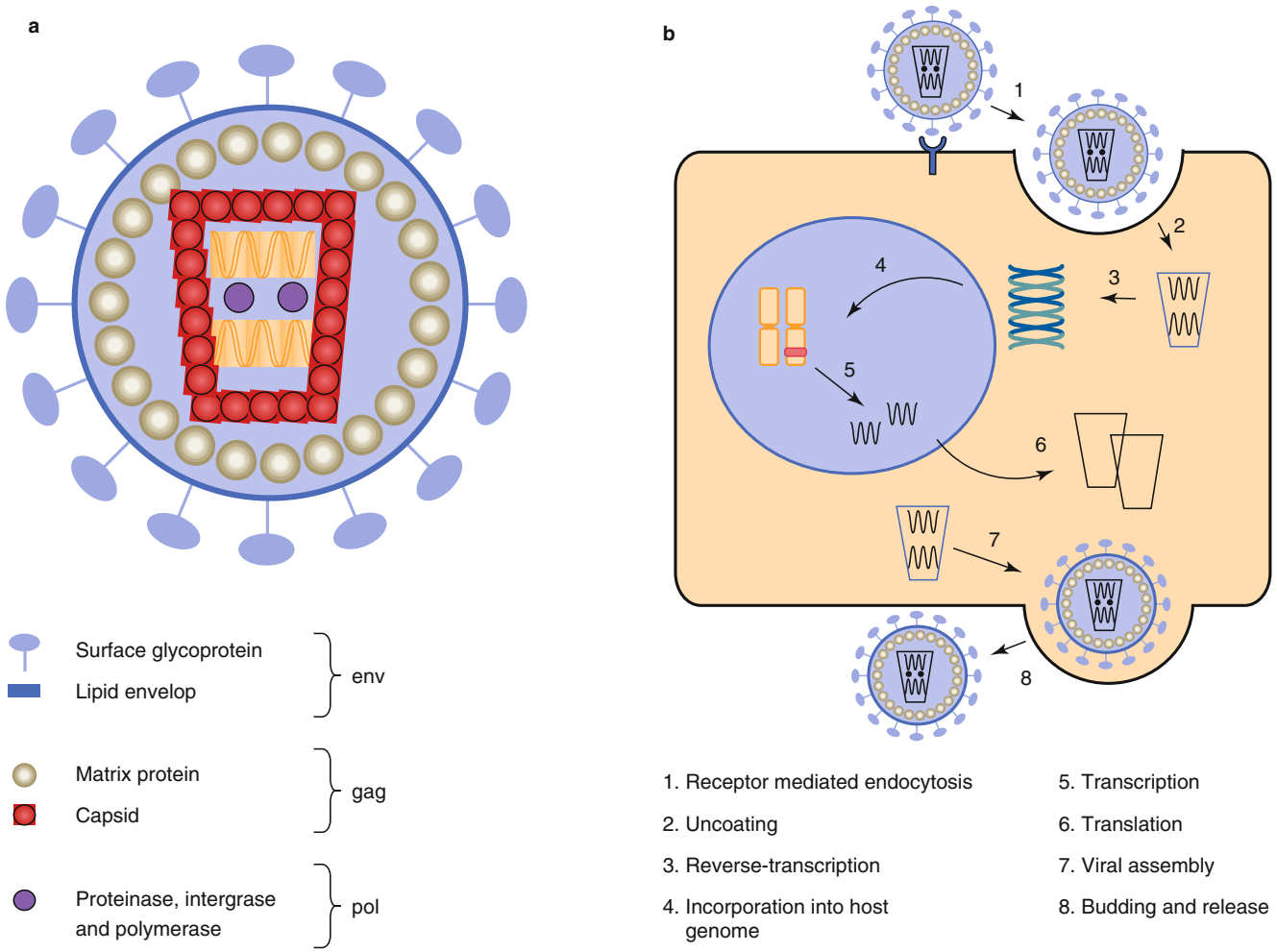


**Figure 24.5** ■ The retrovirus. (**a**) Cross section of a retrovirus. (**b**) The retrovirus replication cycle. Retroviruses enter cells by receptor-mediated endocytosis. In the endosome, the lipid envelope and capsid matrix proteins are degraded. The viral RNA is reverse-transcribed into double-stranded DNA which is then shuttled to the nucleus. In the nucleus, the double-stranded viral DNA is inserted into genomic DNA as a provirus. RNA polymerase in the cell copies the viral RNA in the nucleus. These molecules are shuttled out of the nucleus and serve as templates for making additional copies of viral RNA and mRNA that is translated into viral proteins that form the envelope. New virus particles are assembled in the cytoplasm and bud from the cell membrane.

therapy" in the past two decades. However, there are several disadvantages of these vectors. Retroviruses cannot transduce nondividing cells, which are often targets for many gene transfer applications. In addition, current methods of virus production generate preparations in which the virus titer is very low ($1 \times 10^5$–$1 \times 10^7$ active virus particles/mL), making its clinical use difficult. Retroviruses are also inactivated by complement systems and rapidly removed from the systemic circulation in response to cellular proteins incorporated in the viral envelope during the budding process.

The major limitation of retrovirus-based gene therapy is that the retrovirus randomly inserts the genetic material into the host genome. If genetic material happens to be inserted in the middle of a gene of the host cell, this gene will be disrupted (insertional mutagenesis). If the gene happens to be one regulating cell division, uncontrolled cell division (i.e., insertional oncogenesis) can occur. Fortunately, this problem has begun to be addressed by custom-designed zinc finger nucleases (ZFNs) [1] or by genetic manipulation of the LTRs of the viral genome (Montini et al. 2009).

### *Clinical Use of Retrovirus*

Approximately 20 % of the currently active clinical trials employ retroviral vectors for gene transfer (Fig. 24.4). The Moloney murine leukemia virus (MoMLV), one of the most thoroughly characterized retroviruses, was the first viral vector to be used in the clinic for treating ADA deficiency caused by SCID, an inherited disease in which the buildup of deoxyadenosine caused by ADA deficiency prohibits the expansion of lymphocytes (Blackburn and

Kellems 2005). MoMLV-expressing recombinant ADA was used to transduce autologous T lymphocytes isolated from the patient ex vivo. Sustained engraftment of cells has been documented 10 years after the last infusion, and no severe adverse effects from this therapy have been reported (Muul et al. 2003).

Another successful clinical trial employing retroviruses was for treating a rare form of X-linked severe combined immunodeficiency (X-SCID) (Cavazzana-Calvo et al. 2000). MoMLV expressing the γc-interleukin receptor was used to transduce hematopoietic stem cells (HSCs) isolated from the patient ex vivo. Then the genetically modified HSCs were transfused back to the patient to reconstitute the immune system. More than twenty patients have been treated worldwide, with a high rate of immune system reconstitution observed. However, a leukemia syndrome was reported in several patients enrolled in the trial (Hacein-Bey-Abina et al. 2003). As a result, the United States Food and Drug Administration (FDA), the Gene Therapy Advisory Committee (GTAC), and Committee of Safety of Medicine in the United Kingdom have declared that this approach should not be first-line therapy for X-SCID, but should be considered in the absence of other therapeutic options.

## ■ Lentivirus

### *Biology*

Lentiviruses are unique retroviruses being able to replicate in both dividing and nondividing cells. The biology of lentiviruses is quite similar to retroviruses. Apart from the genes gag, pol, and env, lentivirus has six accessory genes such as tat, rev, vpr, vpu, nef, and vif, which regulate the synthesis and processing of viral RNA and other replicative functions.

Human immunodeficiency virus (HIV) is the best known lentivirus. HIV virus has been genetically manipulated to generate viral vectors for efficient gene transfer into human helper T cells and macrophages. Apart from the genes gag, pol, and env, the accessory genes of the lentiviral genome can also be removed to incorporate more genetic materials without affecting the production efficiency of the virus. HEK293 cells were the most frequently used packaging cell lines for lentivirus generation.

### *Suitability of Lentiviruses as Vectors for Gene Transfer*

The significance of lentiviral vectors lies in the fact that they can efficiently transduce nondividing cells or terminally differentiated cells such as neurons, macrophages, hematopoietic stem cells, muscle, and liver cells as well as other cell types for which traditional retrovirus-based gene therapy methods cannot be used. Previous studies have shown that when injected into the rodent brain, liver, muscle, or pancreatic islet cells, lentivirus promoted a sustained gene expression for over 6 months (Miyoshi et al. 1997). Lentiviruses do not elicit significant immune responses and thus can be ideal for in vivo gene expression. Magnetic nanoparticles have been employed for targeted delivery of lentiviral vectors to the endothelial cells even in perfused blood vessels (Hofmann et al. 2009).

Lentiviruses only have limited integrating potential and consequently induce less risk of insertional mutagenesis. However, the generation of replication-competent lentiviruses (RCL) during the production phase or after introduction into target cells is still a primary concern for the clinical use of lentiviruses. Development of self-inactivating vectors that contain deletions within the 3′ LTR, eliminating transcription of the packaging signal to prevent virus assembly, has significantly improved the safety profile of lentiviruses (Zufferey et al. 1998). Another choice is to develop non-integrating lentiviral vectors by point mutations into the catalytic site, chromosome binding site, and viral DNA binding site of the viral integrase (Apolonia et al. 2007).

### *Clinical Use of Lentiviral Vectors*

Because of the perceived risks associated with the use of lentiviruses, clinical trials with these vectors were not initiated until 2001, most of which are for treating HIV infection (MacGregor 2001). In these studies, peripheral blood mononuclear cells (PBMCs) were obtained from the patient. After selective depletion of CD8 T cells, the remaining cells, CD4 T cells were enriched, transduced with the lentiviral vector VRX496, and expanded in culture. The VRX496-transduced cells were then infused back into the patient. The VRX496 vector contains an antisense sequence targeted to the HIV env gene. Expression of the antisense env from an HIV vector transcript would target wild-type HIV env RNA and destroy it and hence, decrease the productive HIV replication from CD4 T cells. The clinical goal for this treatment approach is to decrease HIV viral loads and promote CD4 T cell survival in vivo. Results from this trial showed that although no serious treatment-related adverse events have occurred, no statistically significant anti-HIV effects could be observed in a pilot trial (Manilla et al. 2005).

## ■ Adenovirus

### *Biology*

Adenoviruses are non-enveloped (without an outer lipid bilayer), icosahedral, lytic DNA viruses composed of a nucleocapsid and a linear double-stranded genome (Fig. 24.6a). Adenoviruses are capable of infecting both dividing and nondividing cells. Fifty-seven serotypes of adenoviruses have been identified

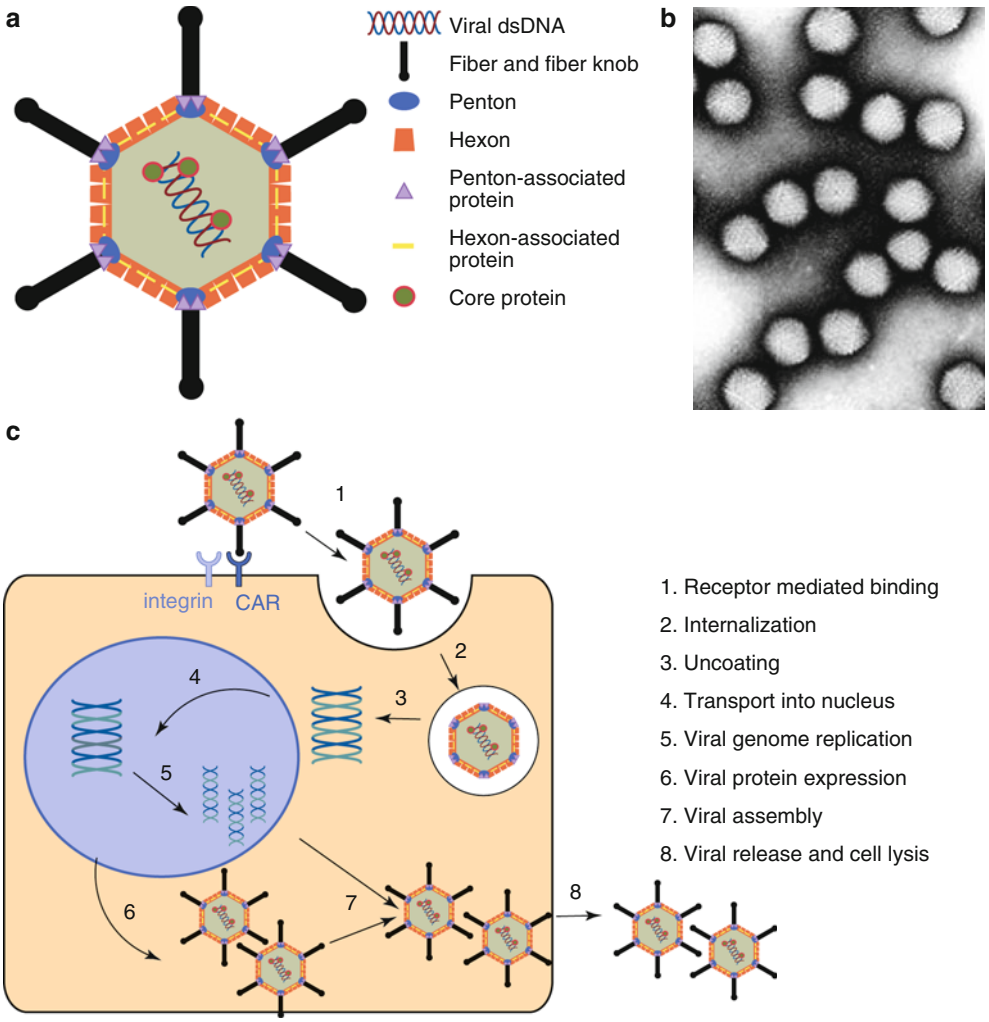


**Figure 24.6** ■ The adenovirus. (**a**) Cross section of an adenovirus particle. The virus consists of a double-stranded DNA genome encased in a protein capsid. The capsid is primarily made up of hexon proteins. Penton proteins are positioned at each of the vertices of the icosahedral capsid and serve as the base for each fiber protein. Hexon-associated and penton-associated proteins are the glue that holds these proteins together within and across the facets of the capsid. Core proteins bind to penton proteins and serve as a bridge between the virus core and the capsid. (**b**) Electron micrograph of intact adenovirus serotype 5 particles. (**c**) The adenovirus replication cycle. Adenovirus infection begins with the attachment of fiber proteins to cellular receptors such as coxsackie and adenovirus receptors (*CAR*) and integrins. Through receptor-mediated endocytosis, the virus enters the cytoplasm. In the endosome, capsid proteins are degraded and viral DNA is released into the cytoplasm and transported to the nucleus for replication. After assembly into new viral particles in the cytoplasm, the host cell is lysed and viruses are released. In the case of gene therapy, recombinant replication-defective adenoviruses are used to transduce targeted cells. The inserted transgenes are transcribed to mRNA in the nucleus. Messenger RNA is then transported out of the nucleus and into the cytoplasm where it is translated to therapeutic proteins.

to date. They are grouped into 7 subgroups (A-G) based on genome size, composition, hemagglutinating properties, and oncogenicity. The adenoviruses serotypes 2 and 5 are the most extensively studied and the first to be used as vectors for gene therapy. The adenoviral genome is linear, non-segmented dsDNA, between 26 and 45 kb, composed of six early (E1a, E1b, E2a, E2b, E3, and E4) and five late (L1, L2, L3, L4, L5) genes. The early genes encode proteins necessary for the viral replication, while the late genes encode proteins to assemble into viral particles.

Adenovirus infection begins with binding of the fiber knob on the surface of the viral capsid to the coxsackievirus and adenovirus receptor (CAR) and major histocompatibility complex (MHC) class I (Fig. 24.6c). After initial binding, the penton base interacts with integrin on the cell surface to initiate a series of cell signaling processes allowing internalization via receptor-mediated endocytosis. (Nemerow and Stewart 1999; Medina-Kauwe 2003). Adenovirus particles enter the nucleus as early as 30 min after initial cellular contact. Viral DNA replication and particle assembly in the

nucleus starts 8 h after infection and culminates in the release of $10^4$–$10^5$ mature virus particles per cell, 30–40 h post-infection by cell lysis (Majhen and Ambriovic-Ristov 2006).

### *Suitability of Adenoviruses as Vectors for Gene Transfer*

To construct an adenoviral vector for gene therapy, the E1 region and the E3 region of the viral genome were often removed to prevent viral replication and accommodate transgene cassettes. Adenoviruses have a big genome capable to accommodate large transgene cassettes. The adenoviral genome is also easy to be manipulated to generate a vector with multiple deletions and inserts without affecting its transduction efficiency. Recently, adenoviruses with both E1 and E3 inserts to simultaneously express two therapeutic genes have been reported (Panakanti and Mahato 2009). Moreover, adenoviruses with E1, E3, and E4 deletions and even "gutless" adenovirus (adenovirus without viral coding regions) have been constructed to drive transgene expression (Armentano et al. 1995; Chen et al. 1997).

Other favorable characteristics of adenoviruses include that the biology of the virus is well understood, that recombinant virus can be generated with high titer and purity, that transgene expression from adenoviruses is rapid and robust, and that adenoviruses can infect a wide range of dividing and nondividing cells. Adenoviruses do not integrate into the host genome. While this minimizes the risk of insertional mutagenesis, gene expression is transient making adenoviruses unsuitable for long-term correction of genetic defects.

A significant drawback to the use of recombinant adenoviruses is the ability of the virus to elicit a strong immune response including T lymphocyte-mediated "cellular immunity" and antibody-producing "humoral immunity." The cellular response results in the killing of adenovirus transfected cells by T lymphocytes, whereas the humoral response results in the production of antibodies to adenovirus, resulting in the clearance of the adenoviral vectors from the bloodstream. Both actions bring an end to the transgene expression (Dai et al. 1995). Moreover, the preexisting adenovirus serotype 5 immunity in human populations has been shown to significantly reduce the efficacy of these vectors in both preclinical studies and clinical trial (Ertl 2005).

The immunogenicity of recombinant adenovirus also raises intensive safety concern of its clinical applications. The massive immune responses caused by administration of adenovirus could lead to multiple organ failure and brain death. In 1999, a patient died 4 days after an injection with an adenoviral vector, which is the first death of a participant in a clinical trial for gene therapy (Stolberg 1999). Another patient experienced a severe immune response syndrome characterized by multiple organ failure and sepsis and died soon after an adenoviral injection in 2003 (Raper et al. 2003). Preclinical studies also confirmed that the immune response generated by adenoviral vectors must be suppressed before a therapeutic effect can be expected. The transgene expression from adenovirus-transduced cells lasted for about 5–10 days, partially due to the clearance of the transduced cells by the host immune system (Lochmuller et al. 1996). Adenoviruses show extended duration of expression when given to nude mice (mice with an "inhibited" immune system) or when an immunosuppressant is administered (Dai et al. 1995).

A significant effort has been put forth to address the issue of the adenovirus-induced systemic immune response. Adenoviruses with more deletions in the early genes and even "gutless" helper-dependent adenoviruses have been constructed to reduce the inflammatory response and accommodate more transgene cassettes (Chen et al. 1997). Other strategies involved the incorporation of an arginine-glycine-aspartic acid (RGD) sequence and tissue-specific ligands to the surface of the viral particles to decrease the systemic immune responses and increase the gene transfer efficiency (Stewart et al. 1997; Wu et al. 2011). However, none of these strategies provided the full elimination of the immune response. Coadministration of immunosuppressive agents such as cyclophosphamide, FK506, and cyclosporin A extended the duration of transgene expression, but did not prevent development of neutralizing antibodies (Xu et al. 2005; Lochmuller et al. 1996). "Stealth" adenoviruses coated with polyethylene glycol (PEG) or other polymers were also designed to reduce the immunogenicity, increase the blood circulation time, and prolong the transgene expression (Chillon et al. 1998). However, masking adenovirus with PEG or other polymers significantly decreased gene transfer efficiency of adenoviruses.

Generation of replication-competent adenovirus (RCA) is another problem of using adenovirus in the human body. Although the early genes responsible for viral replication and pathogenicity are already removed in the vector construction process, RCA can still be generated by homologous recombination if there is some overlap between sequences in the virus genome and packaging cell. The RCA mixed in the clinically used adenoviral products could be extremely dangerous for the patients. Several groups have observed the production of RCA from HEK293 cells owing to sequence overlap (Louis et al. 1997). Some new packaging cell lines with less overlap have been reported to overcome such problem (Fallaux et al. 1998; 1999).

### *Clinical Use of Adenoviral Vectors*

Today, approximately 23 % of all gene therapy clinical trials involve recombinant adenoviruses, making them the most widely used vector for gene transfer (Fig. 24.4). The safety concern regarding the immunogenicity of adenovirus is the major hurdle for its clinical application. Adenoviral gene therapy faced a major setback in 1999, when a patient died 4 days after injection with an adenoviral vector carrying a corrected gene to test the safety of the procedure (Stolberg 1999). Gendicine, an adenoviral p53-based gene therapy was approved by the Chinese food and drug regulators in 2003 for treatment of head and neck cancer. Advexin, a similar gene therapy approach from Introgen, was turned down by the US Food and Drug Administration (FDA) in 2008. Moreover, despite over 300 clinical trials that have shown it to be well tolerated and efficient in gene transfer, the clinical efficacy of this vector has yet to be proven (Shirakawa 2009).

However, certain breakthroughs in adenovirus-based gene therapy have been made. With the help of the tissue-specific targeted delivery strategies, the new generation of adenoviral vectors is less likely to induce severe systemic immunity. For example, the aerosol administration of a recombinant adenovirus expressing the cystic fibrosis transmembrane conductance regulator (CFTR) to cystic fibrosis patients demonstrated the safety and the proof of concept of adenovirus-based gene therapy (Bellon et al. 1997). In another phase I/II trial, using the gene-directed enzyme-prodrug therapy concept ("suicide gene therapy," see Figs. 24.12 and 24.13 and Disease Targets for Gene Therapy), the intratumoral administration of adenovirus encoding a suicide gene (thymidine kinase, TK) and of intravenous ganciclovir increased the median survival time of patients with malignant glioma from 37.7 to 62.4 weeks without adverse effects (Immonen et al. 2004). Cerepro, a drug composed of thymidine kinase (TK) encoding recombinant adenoviruses, has been granted orphan drug status by the European Committee for Orphan Medicinal Products and by the Office of Orphan Products Development, FDA (see also under: Disease Targets for Gene Therapy).

## ■ Adeno-Associated Virus (AAV)

### *Biology*

The AAV genome is a 4.7 kb linear, single-stranded DNA molecule composed of two open reading frames (ORF), rep, cap, and two inverted terminal repeats (ITRs) that define the start and end of the viral genome and packaging sequence. The rep genes encode proteins responsible for viral replication, while the cap genes encode structural capsid proteins. ITRs are required for genome replication, packaging, and integration.

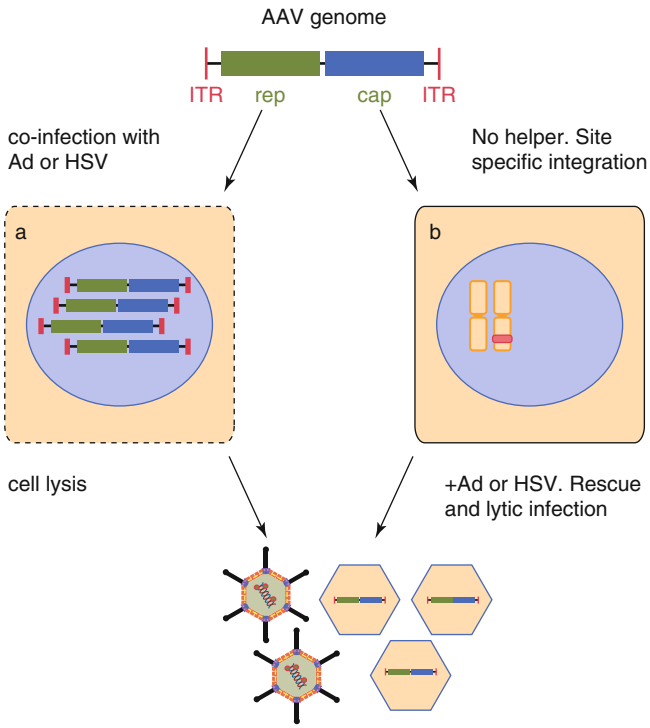


**Figure 24.7** ■ Life cycle of adeno-associated virus (*AAV*). AAV can enter cells through receptor-mediated endocytosis. Once in the nucleus, the virus can follow one of two distinct and interchangeable pathways. (**a**) In the presence of helper virus (adenovirus or herpes simplex virus), AAV enters a lytic phase. The AAV genome undergoes DNA replication resulting in amplification of the genome and production of progeny virions. The newly formed AAV viral particles along with helper viruses are released from the cell by helper-induced lysis. (**b**) In the absence of helper virus, it enters a latent phase. During this phase, part of the AAV vectors integrates into host genomic DNA. While the majority of the AAV vector persists in an extrachromosomal latent state without integrating into the host genome. The latent AAV genome cannot undergo replication and production of progeny virions in the absence of helper virus. However, the transgenes carried in the AAV genome are transferred to the host cell and co-express using the host gene expression machinery *ITRs* inverted terminal repeats, *rep* replication.

The icosahedral AAV capsid is 25 nm in diameter. AAV is deficient in replication, and there are no packaging cells which can express all the replication-related proteins of the AAV. Therefore AAV requires coinfection with a helper virus, such as an adenovirus or a herpes simplex virus to replicate (Fig. 24.7). Eleven distinct AAV serotypes have been identified, and over 100 AAV variants have been found in human and nonhuman primate tissues in 2006 (Wu et al. 2006; Mori et al. 2004). The biology of AAV serotype 2 (AAV2) has been the most extensively studied, and this serotype is most often used as a vector for gene transfer.

### *Suitability of Adeno-Associated Viruses for Gene Transfer*

Recombinant AAV vectors have rapidly gained popularity for gene therapy applications within the last decade, due to their lack of pathogenicity and ability to establish long-term gene expression (Table 24.1). The viral genome is simple, making it easy to manipulate. The virus is resistant to physical and chemical challenges during purification and long-term storage (Wright et al. 2003; Croyle et al. 2001). The ability of the virus to integrate in the human chromosome was an initial concern, but eventually it turned out that AAV only integrates into a fixed location of human chromosome and the integration frequency of recombinant AAV is quite low (Surosky et al. 1997).

The AAV vectors are produced by replacing the rep and cap genes with the transgene. Only one out of 100–1,000 viral particles is infectious. Apart from the production of AAV vectors being laborious, these vectors also have the drawback of limited packaging capacity (4.7 kb) for the transgene. Large genes are therefore not suitable for use in a standard AAV vector. To overcome the limited coding capacity, the ITRs of two AAV genomes can anneal to form a head-to-tail structure through trans-splicing between two genomes, almost doubling the capacity of the vector (Yan et al. 2000).

Since recombinant AAV vectors do not contain any viral open reading frames (ORFs), they induce only limited immune responses in humans. Intravenous administration of AAV vectors in mice causes transient production of pro-inflammatory cytokines and limited infiltration of neutrophils, in contrast to an innate response lasting 24 h or longer induced by aggressive viruses (Zaiss et al. 2002). However, despite the limited innate immunity elicited by AAV vectors, the humoral immunity elicited by AAV is still a common event. Up to 80 % of individuals are thought to be positive for AAV2 antibodies in the human population (Erles et al. 1999). The associated neutralizing activity limits the usefulness of the most commonly used serotype AAV2 in certain applications.

### *Clinical Use of Adeno-Associated Virus Vectors*

To date, 86 clinical trials employing recombinant AAV vectors have been initiated worldwide (Fig. 24.4). The first clinical use of recombinant AAV was to transfer the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to the respiratory epithelium for treating cystic fibrosis (Flotte et al. 1996). This is the first trial to suggest that gene therapy could treat cystic fibrosis in a positive manner. Other phase I and phase II trials have shown that AAV-mediated gene transfer is safe and effective for treating Leber's congenital amaurosis (High and Aubourg 2011; Simonelli et al. 2010), hemophilia (Nathwani et al. 2011), lipoprotein lipase deficiency (Rip et al. 2005), and Parkinson's disease (LeWitt et al. 2011). There are currently four trials using AAV2 vectors in phase III testing for metastatic hormone-resistant prostate cancer (Simons and Sacks 2006).

## NONVIRAL VECTORS

The inherent problems with recombinant viruses such as immunogenicity, a.o. reflected in the generation of neutralizing antibodies, and insertional mutagenesis have called for the design of efficient, nonviral vectors for human gene therapy. Nonviral vectors are significantly less immunogenic and are not likely to induce insertional mutagenesis and unwanted homologous recombination after uptake by the cells. They are also relatively easy to be manipulated, produced, and purified in a large scale compared with their viral counterparts. Nonviral gene therapy includes local administration of naked plasmid or using specialized carriers to deliver plasmids to this area. However, their clinical utility is still hampered by the low transfection efficiency, which stems from nonspecific uptake of the vector by epithelial barriers and extracellular matrix and poor delivery into the therapeutic target (Fig. 24.8). The intracellular gene-silencing machinery also prevents the long-term transgene expression. New emerging delivery systems and vector-constructing technologies try to address these issues (cf. Chap. 4)

### ■ Delivery Methods for Nonviral Gene Transfer

#### *Physical Methods for Gene Transfer*

Physical methods involve transfer of naked plasmid by direct disruption of (target) cell membranes. Chemical methods increase the plasmid uptake by the targeted cells using lipid-, peptide-, or polymer-based carriers.

The earliest techniques to deliver recombinant DNA to cellular targets include microinjection, particle bombardment, and electroporation (Table 24.2). Microinjection, direct injection of DNA or RNA into the cytoplasm or nucleus of a single cell, is the simplest and most effective method for physical delivery of genetic material to cells. This transfects 100 % of the treated cells and minimizes waste of plasmid DNA. But, it requires highly specialized equipment and skills. Moreover, microinjection is not suitable for in vivo gene transfer or in vitro gene transfer into tissues or organs composed of a large amount of cells. Particle bombardment, or gene gun treatment, starts with coating tungsten or gold particles with plasmid DNA. The coated particles are loaded into a gene gun barrel, accelerated with gas pressure and shot into targeted cells or tissues in a petri dish. Particle bombardment can be used to introduce a variety of DNA vaccines into desirable cells in vitro. However, particle bombardment has a low penetration capacity, making it unsuitable for in vivo gene delivery apart from easily

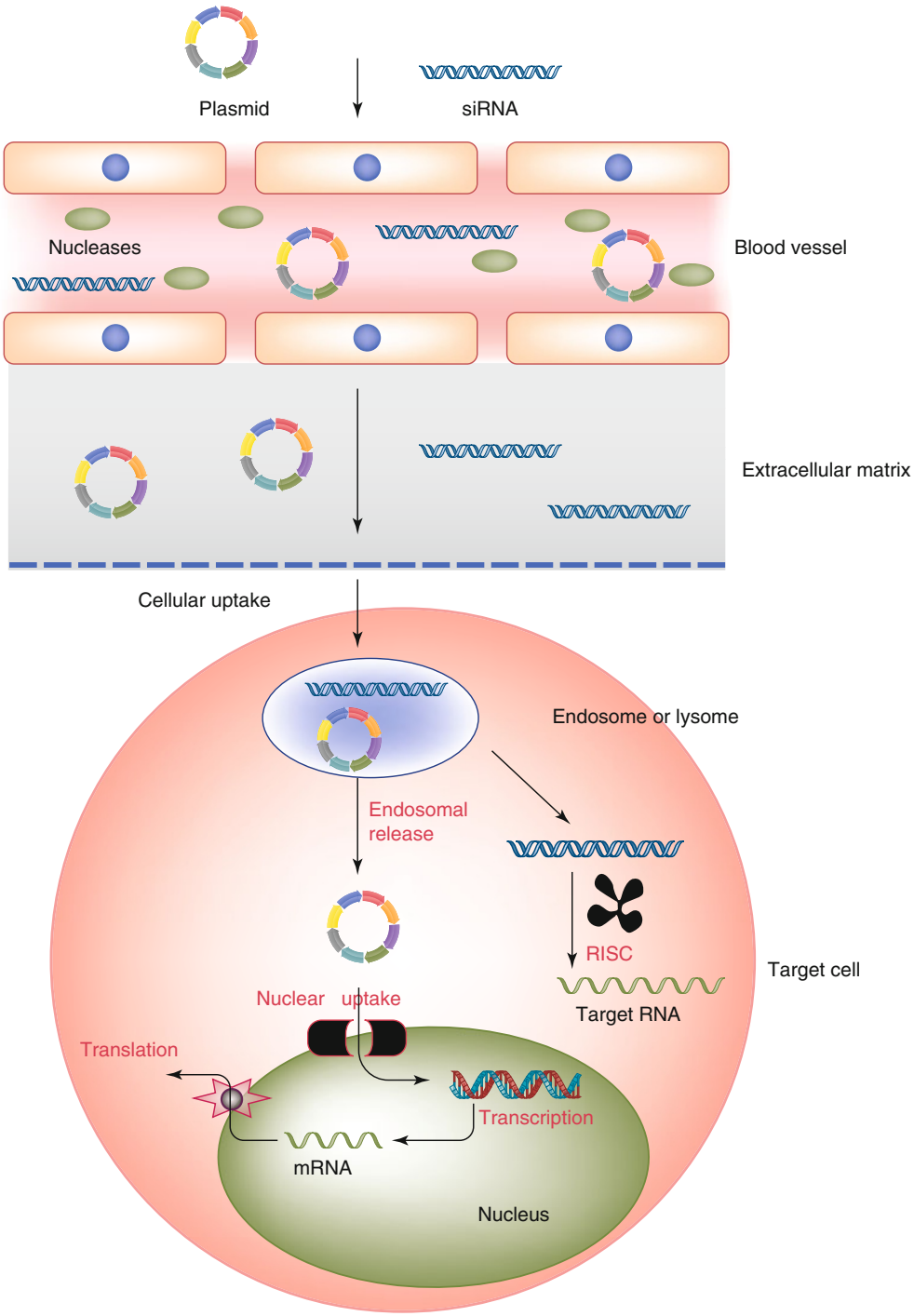


**Figure 24.8** ■ Barriers for nonviral gene delivery. Following systemic administration, gene medicine (plasmid or siRNA) comes in contact with blood nucleases. Then they may traverse the blood vessel barrier and the extracellular matrix compartment prior to crossing the plasma membrane barrier. Upon entering the cell via receptor-mediated endocytosis, they are trapped in endosomes and need to be released in the cytosol. Endosomal escape is a major rate-limiting step in gene delivery (From (Singh et al. 2011) with permission to reprint).

accessible tissue, e.g., the skin. Electroporation is used to generate temporary pores in the plasma membrane to transfer plasmid DNA to the cells by an externally applied high-voltage electrical field. Electroporation increases the gene transfer efficiency by 100–1000 folds compared to naked DNA solutions and has met with great success in laboratory practices and clinical trials (Wells 2004). For example, it is frequently used to produce transgenic animals, a powerful tool in preclinical studies (see Chap. 8). Electroporation-mediated gene transfer also demonstrated safety and efficacy in clinical trials to treat melanoma, prostate cancer, and HIV infection (Daud et al. 2008; Vasan et al. 2011). Other physical methods for gene transfer include sonoporation, laser irradiation, magnetofection, and hydroporation. However, because most of the physical methods induce stress and disruption of cellular structure and function, physical methods are less widely studied compared with chemical methods (see below) and are generally restricted to in vitro gene transfer of cultured cells or embryonic stem cells (Table 24.2).

| | Advantages | Disadvantages |
|---|---|---|
| Naked DNA | No special skills needed Easy to produce | Low transduction efficiency Transient gene expression |
| *Physical methods* | | |
| Microinjection | Up to 100 % transduction efficiency (nuclear injection) | Requires highly specialized skills for delivery<br>Limited to ex vivo delivery |
| Gene gun | Easy to perform<br>Effective immunization with low amount of DNA | Poor tissue penetration |
| Electroporation | High transduction efficiency | Transient gene expression<br>Toxicity, tissue damage<br>Highly Invasive |
| Sonoporation | Method well tolerated for other applications | Transient gene expression<br>Toxicity not yet established |
| Laser irradiation | Can achieve 100 % transduction efficiency | Special skills and expensive equipment necessary |
| Magnetofection | Safety of method established in the clinic | Poor efficiency with naked DNA |
| *Chemical methods* | | |
| Liposomes | Easy to produce | Protein and tissue binding Transient gene expression |
| Micelles | Easy to produce and manipulate | Unstable<br>Protein and tissue binding |
| Cationic polymers | High DNA loading<br>Easy to produce and manipulate | Transient gene expression Toxicity |
| Dendrimers | High DNA loading<br>High transduction efficiency | Extremely toxic |
| Solid lipid nanoparticles | Low toxicity<br>Controlled release and targeting | NA |

**Table 24.2** ■ Summary of nonviral methods used for gene transfer.

### *Cationic Lipids*

Since the invention of lipofectamine in 1987, numerous cationic lipids have been synthesized and tested for gene delivery. Most cationic lipids are composed of three parts: (i) a hydrophobic lipid anchor group; (ii) a linker group, such as an ester, amide, or carbamate; and (iii) a positively charged head group, which interacts with the negatively charged plasmid DNA, leading to its condensation and aggregate (nanometer/micrometer range) formation (Fig. 24.9) (Mahato et al. 1997). 2, 3-dioleyloxypropyl-1-trimethyl ammonium bromide (DOTMA) and 3-β[N(NV,NV-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) are two commonly used cationic lipids with different structures. Cationic lipids are usually mixed with a neutral co-lipid such as dioleoylphosphatidylethanolamine (DOPE) at a certain molar ratio to reduce the toxicity and enhance gene delivery. PEGylation of polyplexes is frequently used to reduce the plasma binding and increase the circulation time of cationic liposomes. Targeting strategies for these colloidal (pegylated) lipoplexes have been discussed in Chap. 4.

Lipoplexes are taken up by cells through the endosomal route (Fig. 24.8). For endosomal escape and transport to and through the nuclear membrane, additional functional elements may be attached: for endosomal escape (pH sensitive fusiogenic peptides), for transport in the cytoplasm, and nuclear membrane passage (a nuclear translocation peptide).

### *Peptides*

Just like cationic lipids, cationic peptides condense DNA in a similar manner and can be used as gene delivery carriers. Poly (L-lysine) (PLL), a polydisperse, synthetic repeat of the amino acid lysine, was one of the first cationic peptides to deliver genes. However, increase in length of PLL leads to increasing cytotoxicity. Besides, PLL shows limited transfection efficiency and needs the addition of endoosmolytic agents such as fusogenic peptides (see above) to facilitate plasmid

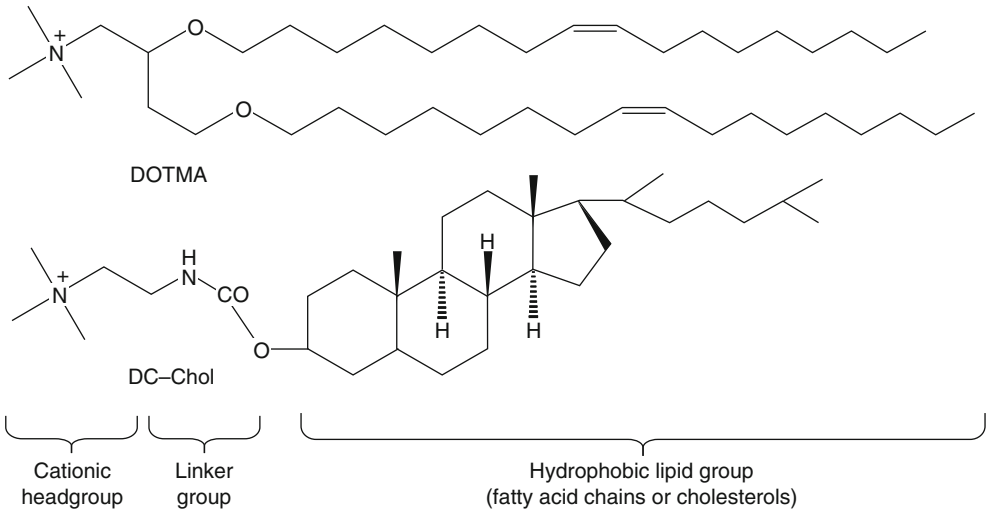


**Figure 24.9** ■ Basic components of cationic lipids N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and 3-β [N(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) (From (Mahato et al. 1997) with permission to reprint).

release into the cytoplasm. Due to these issues, many researchers have turned to the development of PLL-containing "active" peptides and have met with some success (McKenzie et al. 1999). Such peptides offer many advantages over PLL, such as lower toxicity, precise control of synthesis, and homogeneity of peptide length (Martin and Rice 2007).

Peptide-based gene delivery systems have the potential to overcome extracellular and intracellular barriers of gene delivery using a single peptide sequence. However, they suffer from nonspecific plasma protein binding and uptake by the reticuloendothelial system. Here again, PEGylation was demonstrated to be an effective strategy to increase the blood circulation time of the complex (Mannisto et al. 2002). Another unique challenge for peptide-based gene delivery systems is cytosolic proteasomes, which degrade unneeded or damaged proteins by proteolysis. Proteasomes destabilize and degrade the DNA/peptide condensates and prevent effective gene transfer. The involvement of proteasomes in the gene delivery process was first identified in AAV-mediated gene transfer and further confirmed in peptide-based, nonviral gene transfer strategies. In both cases, gene transfer efficiency was significantly higher in the presence of a proteasome inhibitor (Duan et al. 2000; Kim et al. 2005). Coadministration of proteasome inhibitors is the most effective strategy to address this issue.

### *Polymers*

Synthetic and naturally occurring cationic polymers constitute another category of gene carriers. Polyethyleneimine (PEI) has been the most widely used cationic polymer for gene delivery in the last two decades. Boussif et al. first reported that PEI condensed with oligonucleotides and plasmids forms colloid particles (1–1,000 nm) that are a highly efficient delivery system, both in vitro and in vivo (Boussif et al. 1995). However, PEI, especially PEI with a high molecular weight (>25 kD), is extremely cytotoxic. PEI induces the disruption of the cell membrane leading to immediate necrotic cell death and disruption of the mitochondrial membrane after internalization leading to apoptosis. PEI binds to blood components, extracellular matrix, and untargeted cells after intravenous injection. Chemical modification of PEI was proposed to overcome these problems. For example, cholesteryl chloroformate readily formed micelles (10–100 nm) in aqueous solution when conjugated to branched PEI (Wang et al. 2002). This new lipopolymer showed decreased toxicity and optimal gene transfer efficiency.

A simple mixing of plasmid DNA and PEG-b-PLL polymer resulted in the spontaneous formation of polyion complex (PIC) micelles characterized by a small particle size, excellent colloidal stability, and optimal gene transferring ability in serum-containing media (Itaka et al. 2003). However, it should be noted that micelles are in a thermodynamically unstable state. The micelle structure may disintegrate upon the drastic dilution following intravenous injection and lead to inefficient gene transfer. To address this issue, cross-linked micelles were prepared using thiol-modified PEG-b-PLL through the formation of disulfide bonds in the core area (Miyata et al. 2004). These cross-linked micelles are more stable in the blood during circulation and the disulfide bonds are assumed to be cleavable in the cytoplasm (Miyata et al. 2005).

## ■ Clinical Use of Nonviral Vectors

It is not possible, with current nonviral technologies, to match the high transduction efficiencies and high levels of expression reported with certain viral methods in vivo. Nevertheless, nonviral gene therapies may pro-

vide a means for achieving short-term expression of therapeutic gene products in certain tissues with a high degree of safety. Principal approval specifications and recommended assays for assessing the final plasmid DNA preparation purity, safety, and potency for gene therapy and DNA vaccines applications are listed in Table 24.3. There are currently 333 clinical trials using plasmid DNA to treat a number of diseases (Fig. 24.4). Many of these trials are still in phase I testing so far. Collectively, these clinical studies provided "proof-of-principle" for nonviral gene therapy but also highlighted the need for development of formulations with enhanced transfection efficiency and therapeutic efficacy. It should also be mentioned that the majority of these trials were uncontrolled, open label, phase I designs primarily investigating safety and feasibility. The possibility of strong placebo effects cannot be overlooked in these trials. The efficacy results from these studies should be interpreted with caution and can only be assessed by conducting further phase II/III trials.

## STEM-CELL-BASED GENE THERAPY

Stem-cell-based gene therapy emerges from recent progress in both cell therapy and gene therapy. Cell therapy describes the process of introducing new cells into a tissue to treat a disease. Recent advances in cell therapy, especially evolving basic insights in stem cell behavior, fostered breakthroughs in regenerative medicine, which is the process of replacing or regenerating human cells, tissues, or organs to restore or establish normal function. Stem-cell-based gene therapy is a multistep process, starting with the isolation of stem cells from the patients. This step is followed by ex vivo expansion of the stem cells and transduction with gene transfer vectors. Finally, the transfected stem cells are infused back into the patient to treat a specific disease.

## STEM CELL THERAPY (cf. CHAP. 25)

Stem cells exist in all multicellular organisms and share two characteristic properties. They have prolonged or unlimited self-renewal capacity and the potential to differentiate into a variety of specialized cell types. The earliest stem cells in human life are embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst and capable to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. Besides the ESCs, which can only be isolated from early embryos, there are other types of stem cells in the mature tissues of all aged mammals, the adult stem cells. Adult stem cells have unlimited self-renewal capacity and a more restricted differentiation potential. They multiply by cell division to replenish dying cells and regenerate damaged tissues. The most famous adult stem cells are bone marrow hematopoietic stem cells (HSCs), which give rise to all the blood cell types and lymphoid lineages. Bone marrow also contains a population of adult stem cells named mesenchymal stem cells (MSCs).

Among all types of stem cells, MSCs have attracted special attentions because of their wide applicability in regenerative medicine. Direct injection of highly pluripotent ESCs into ectopic organs often gives rise to teratoma, a benign tumor containing derivatives of all three germ layers (Nussbaum et al. 2007). MSCs are less potent to induce teratoma or other malignant transformations as they only have restricted differentiation potential (Rubio et al. 2005). Compared with other adult stem cells such as HSCs, mammary stem cells, or neural

| Impurity | Recommended assay | Approval specification |
|---|---|---|
| Proteins | BCA protein assay | < 3 μg/mg pDNA |
| RNA | Analytical HPLC | <0.2 μg/mg pDNA |
| gDNA | Real-time PCR | <0.2 μg/mg pDNA |
| Endotoxins | LAL assay | < 10 E.U./mg pDNA |
| Plasmid isoforms (linear, relaxed, denatured) | Analytical HPLC or capillary gel electrophoresis | <3 % |
| Bacterial and fungal | Method outlined in 21 CFR 610.12 | No growth |
| Biological activity and identity | Restriction endonucleases | Coherent fragments with the plasmid restriction map |
| | Agarose gel electrophoresis | Expected migration from size and supercoiling |
| | Transformation efficiency | Comparable with plasmid standards |

Information compiled from references (Manthorpe et al. 2005; U.S. Department of Health and Human Services 1998)

**Table 24.3** ■ Principal approval specifications and recommended assays for assessing the final plasmid DNA preparation purity, safety, and potency for gene therapy and DNA vaccines applications.

stem cells, MSCs have a well-characterized trophic effect and immunomodulatory property, making them good candidates in treating degenerative diseases. As a trophic mediator, MSC produces bioactive factors which inhibit apoptosis, promote angiogenesis, and stimulate mitosis and differentiation into tissue-specific reparative cells. For example, intravenous transplantation of MSCs was successful in treating systemic diseases such as graft-versus-host disease (GVHD) and osteogenesis imperfecta in humans (Le Blanc et al. 2004). Wakitani et al. also reported several successful clinical cases treating cartilage defects with MSCs (Wakitani et al. 2007). Primary MSCs or genetically modified MSCs have also been employed in regenerating hematocytes, tendon, bone marrow, muscle, and other connective tissues (Phinney and Prockop 2007).

There are two major directions of stem-cell-based gene therapy: (1) stem cells were used as gene delivery vehicles to express therapeutic genes in the target sites and (2) stem cells were reprogrammed or transdifferentiated by genetic modification to replenish the defect cells or tissues (regenerative medicine).

## STEM CELLS AS GENE DELIVERY VEHICLES

The advances in gene therapy in the recent two decades have had a great impact on how stem cells could be used to treat certain diseases. Since the first successful gene therapy case in which an ADA gene was inserted into autologous T lymphocytes to treat ADA deficiency-induced SCID, several groups had the ambitious goal to permanently correct ADA deficiency by inserting the ADA gene into hematopoietic progenitor cells from bone marrow and umbilical cord blood (Bordignon et al. 1995; Kohn et al. 1995). Although the overall outcome was disappointing, transgene expression in hematopoietic progenitor cells did provide a selective survival, growth, and differentiation advantage to the lymphocyte descendants.

Stem-cell-based gene therapy also showed promising results in the X-SCID clinical trial in 1999. The protocol consisted of the isolation of CD34-enriched bone marrow progenitor cells that were harvested from the iliac crest of the patients and the ex vivo transduction with retrovirus encoding the common cytokine-receptor γ-chain (γc). In four out of five patients, the infusion of transduced CD34+ cells led to the generation of functional peripheral-transduced T cells similar to those of age-matched controls within 6–12 weeks (Cavazzana-Calvo et al. 2000). Traditional gene therapy (as discussed before) focuses on the introduction of genetic material in mature cells to treat a hereditary, genetic disease, while the stem-cell-based gene therapy may represent a permanent treatment for these genetic diseases.

Recent preclinical studies also demonstrated the promising future of using genetically modified stem cells as therapeutic agents. For example, MSCs were widely reported to be competent trophic mediator for islet transplantation. However, MSCs alone are insufficient to support a rapid and functional revascularization of islet grafts. Genetically modified MSCs not only reversed the incompetence of primary MSCs but also provided MSCs with new functions to target various diseases (Dzau et al. 2005).

## STEM CELLS AS REGENERATIVE MEDICINE (cf. CHAP. 25)

Stem cells can be reprogrammed or transdifferentiated by genetic modification to replenish defect cells or tissues. These features make genetically modified stem cells a powerful tool in regenerative medicine. Viral vectors efficiently transduce stem cells and direct their differentiation. Peng and coworkers first demonstrated that muscle-derived stem cells genetically engineered with retrovirus to express human bone morphogenetic protein-4 (BMP4) and VEGF promoted bone formation and bone healing in a mouse model (Peng et al. 2002). This finding was further supported by the finding of Tsuda and coworkers that MSCs genetically engineered with adenovirus to overexpress bone morphogenetic protein-2 (BMP2) enhanced ectopic bone formation in rats (Tsuda et al. 2003). From these studies one can conclude that viable human bone grafts can be engineered under laboratory conditions by using human MSCs and a "biomimetic" scaffold-bioreactor system (Grayson et al. 2010).

Other preclinical studies showed that stem cells can readily transdifferentiate into different types of cells through genetic modification using viral or nonviral vectors. For example, genetically modified induced pluripotent stem cells (iPSCs) and MSCs represent a major source of artificial β-cells and islets for treating diabetes. Karnieli et al. and Li et al. both reported the reversal of hyperglycemia in streptozotocin-induced diabetic mice after transplantation of insulin-producing cells originated from genetically modified Pdx-1 expressing MSCs (Karnieli et al. 2007). In another report, Li et al. demonstrated the in vitro formation of islet-like structures using genetically modified MSCs (Li et al. 2008).

Viral vectors were the most popular tools to direct the differentiation of stem cells in regenerative medicine. However, because of the risks of insertional mutagenesis and generation of replication-competent viruses, nonviral vectors were also studied in stem-cell-based gene therapy. Corsi et al., described a way to transfect MSCs using chitosan-DNA nanoparticles

(Corsi et al. 2003). Kaji et al., introduced a "smart" nonviral transgene expression system to efficiently reprogram somatic cells to iPSCs and shut down the transgene expression once the reprogramming was done (Kaji et al. 2009). Nevertheless, the application of nonviral vectors is still limited because stem cells are typically hard to be transfected by nonviral vectors.

Sources for stem cells are another problem for stem-cell-based gene therapy. iPSCs induced from somatic cells offer potential alternatives to the ESCs and other adult stem cells whose supply is limited nowadays. However, teratoma formation and immunogenicity remain an unsolved issue hampering the wide application of iPSCs (Zhao et al. 2011).

It should be noted that most stem-cell-based gene therapies, especially the ones that use retroviruses or lentiviruses as gene delivery vectors, do not provide a mechanism to shut down the therapeutic gene expression when further expression is unnecessary or to clear the proliferative stem cells when the damaged tissue is fully healed. An inducible system can be added to the vector structures to achieve temporal and spatial control of the transgene expression (Fig. 24.2). Self-inactivating retroviruses were also constructed for safe and efficient in vivo gene delivery (Miyoshi et al. 1998). To clear the excess of stem cells when the damaged tissue is fully healed, Schuldiner et al. reported that human embryonic stem cells genetically engineered to express a "suicide" gene could be eliminated in vivo by administration of the FDA-approved drug ganciclovir (Schuldiner et al. 2003) (cf. Fig. 24.12).

## DISEASE TARGETS FOR GENE THERAPY

There are currently 1786 active gene therapy clinical trials worldwide (Wiley 2012). Approximately 65 % of these trials are for cancer treatment. Treatment of monogenetic diseases, cardiovascular diseases, and infectious diseases each take ~10 % of the number of active clinical trials. Treatment of neurological diseases, which has expanded very fast in the last 5 years, is the goal of 2 % of active clinical trials (Fig. 24.10). Currently, gene therapy trials are primarily performed in the United States (64 % of all trials), the United Kingdom (11 %), and Germany (4.5 %). The geographical distribution of gene therapy clinical trials is summarized in Fig. 24.11. General indications for all gene therapy trials in the clinic are summarized in Table 24.4.

## CANCER GENE THERAPY

The majority of today's gene therapy clinical trials are devoted to treat cancer. There are two potential benefits of using gene therapy to treat cancer: (a) gene-based treatments can attack existing cancer at the molecular level, eliminating the need for drugs, radiation, or surgery and (b) identifying cancer susceptibility genes in individuals or families may have significant impact in preventing the disease before it occurs.

Strategies to achieve these goals include (a) correction of genetic mutations contributing to the malignant phenotype by replacing missing genes or altered defect genes with healthy genes, (b) enhancement of a patient's immune response to cancer (immunotherapy), (c) insertion of genes into cancer cells to make

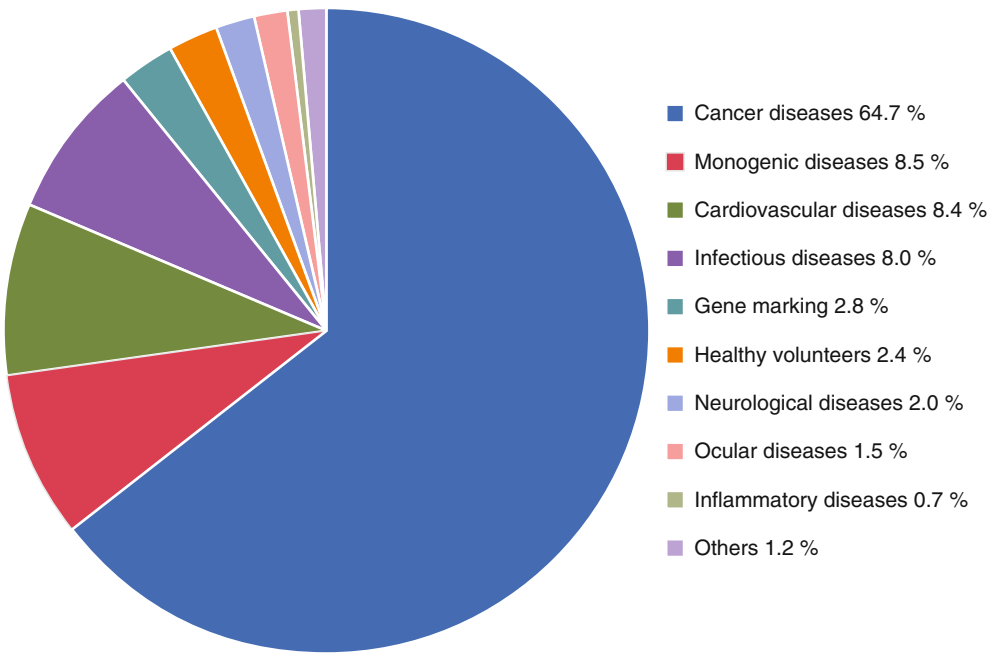


**Figure 24.10** Disease targets of gene therapy clinical trials (Wiley 2012). Other diseases include inflammatory bowel disease, rheumatoid arthritis, chronic renal disease, carpal tunnel syndrome, Alzheimer's disease, diabetic neuropathy, Parkinson's disease, erectile dysfunction, retinitis pigmentosa, and glaucoma.

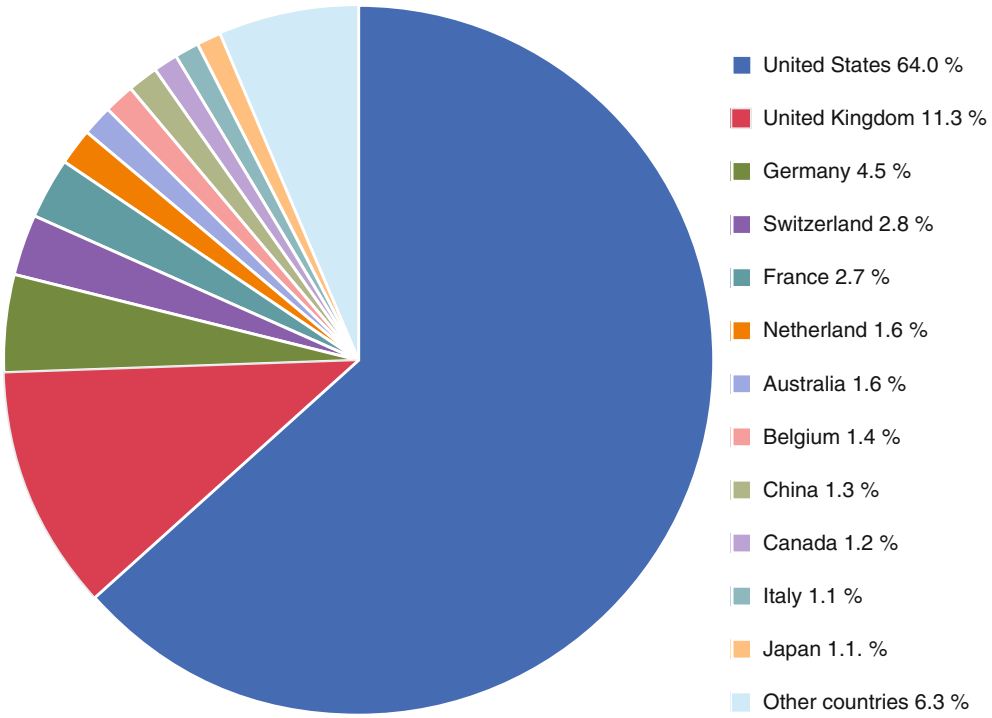


**Figure 24.11** ■ International status of gene therapy clinical trials (Wiley 2012). Other countries include Austria, Czech Republic, Denmark, Egypt, Finland, Ireland, Israel, Mexico, New Zealand, Norway, Poland, Romania, Russia, Singapore, South Korea, Spain, Sweden, and Taiwan. Trials held in each of these countries represent less than 1 % of all clinical trials held worldwide.

them more sensitive to conventional chemotherapy and radiotherapy or other treatments, (d) introduction of "suicide genes" into a patient's cancer cells that can enzymatically activate a prodrug in these cells to the destroy them, and (e) direct tumor killing through oncolytic viruses.

### ■ Correction of Genetic Mutations

In this approach, gene therapy is used to correct genetic mutations contributing to the malignant phenotype by replacing missing genes or removing defect genes. Understanding cancer at the molecular level is the platform for gene correction in cancer therapy. The inactivation or activation of certain genes may contribute to tumor growth. Although the complex process of tumor development and growth limits the utility of this strategy, approximately 12 % of cancer gene therapy clinical trials involve overexpression of tumor-suppressor genes such as p53, MDA-7, and ARF (Majhen and Ambriovic-Ristov 2006). Mutations in the p53 gene are most commonly seen in a wide spectrum of tumors (Roth and Cristiano 1997). Efficient delivery and expression of the wild-type p53 tumor-suppressor gene prevents the growth of human cancer cells in culture, causes regression of established human tumors in nude mice, or sensitizes the existing tumors to the therapeutic effect of conventional chemotherapy and radiotherapy (Roth and Cristiano 1997). The results from clinical trials indicated that the therapeutic effect of gendicine, the first gene therapy product, was promising in patients with head and neck squamous cancers (Peng 2005). However, the results were only validated in China. Efficient delivery of tumor-suppressor genes deep within tumors is difficult, and restriction of gene expression in malignant tissue is challenging. Gene silencing by this approach has also limited success, especially when a prolonged silencing effect is required. Despite these reservations, prostate, lung, and pancreatic tumors have been successfully treated in the clinic with a variety of genes and transfer methods.

### ■ Immunotherapy

In this approach, gene therapy is used to stimulate the body's natural ability to attack cancer cells. In one study, autologous peripheral blood lymphocytes genetically engineered with retroviral vectors encoding the melanoma specific T cell receptor (TCR) were administered to patients with metastatic melanoma. The genetically engineered T lymphocytes then recognize the antigens on the surface of the tumor cells through TCRs and kill the tumor cells. Cancer regressions were first reported by Morgan et al. and further confirmed by Johnson et al. (Morgan et al. 2006; Johnson et al. 2009). In other clinical studies, expression of either pro-inflammatory cytokines (IL-2, IL-4, and IL-12), co-stimulatory molecules (HLA-B7 LFA-3), or tumor-specific antigens (mucin-1 and CEA) to stimulate antitumor immune responses has also been tested (Majhen and Ambriovic-Ristov 2006).

### ■ Tumors Sensitization

In this approach, genes are inserted into cancer cells to make them more sensitive to conventional chemotherapy and radiotherapy or other treatments. We

| Cancer | Other diseases | Cardiovascular disease |
|---|---|---|
| Gynecological | Inflammatory bowel disease | Peripheral vascular disease |
| Breast, ovary, cervix | Rheumatoid arthritis | Intermittent claudication |
| Nervous system | Chronic renal disease | Critical limb ischemia |
| Glioblastoma, leptomeningeal carcinomatosis, glioma, astrocytoma, neuroblastoma | Fractures | Myocardial ischemia |
| Gastrointestinal | Erectile dysfunction | Coronary artery stenosis |
| Colon, colorectal, liver metastases, post-hepatitis liver cancer, pancreas | Anemia of end stage renal disease | Stable and unstable angina |
| Genitourinary | Parotid salivary hypofunction | Venous ulcers |
| Prostate, renal | Type I diabetes | Vascular complications of diabetes |
| Skin | Detrusor overactivity | Pulmonary hypertension |
| Melanoma | Graft-versus-host disease | Heart failure |
| Head and neck | | |
| Nasopharyngeal carcinoma | **Monogenic disorders** | **Infectious disease** |
| Lung | Cystic fibrosis | HIV/AIDS |
| Adenocarcinoma, small cell, non-small cell | Severe combined immunodeficiency (SCID) | Tetanus |
| Mesothelioma | Alpha-1 antitrypsin deficiency | Epstein-Barr virus |
| Hematological | Hemophilia A and B | Cytomegalovirus infection |
| Leukemia, lymphoma, multiple myeloma | Hurler syndrome | Adenovirus infection |
| Sarcoma | Hunter syndrome | Japanese encephalitis |
| Germ cell | Huntington's chorea | Hepatitis C |
| | Duchenne muscular dystrophy | Hepatitis B |
| **Neurological diseases** | Becker muscular dystrophy | Influenza |
| Alzheimer's disease | Canavan disease | |
| Carpal tunnel syndrome | Chronic granulomatous disease (CGD) | |
| Cubital tunnel syndrome | Familial hypercholesterolemia | |
| Diabetic neuropathy | Gaucher disease | |
| Epilepsy | Fanconi's anemia | |
| Multiple sclerosis | Purine nucleoside phosphorylase deficiency | |
| Myasthenia gravis | Ornithine transcarbamylase deficiency | |
| Parkinson's disease | Leukocyte adherence deficiency | |
| Peripheral neuropathy | Gyrate atrophy | |
| | Fabry disease | |
| **Ocular diseases** | Familial amyotrophic lateral sclerosis | |
| Age-related macular degeneration | Junctional epidermolysis bullosa | |
| Diabetic macular edema | Wiskott-Aldrich syndrome | |
| Glaucoma | Lipoprotein lipase deficiency | |
| Retinitis pigmentosa | Late infantile neuronal ceroid lipofuscinosis | |
| Superficial corneal opacity | RPE65 mutation (retinal disease) | |
| | Mucopolysaccharidosis | |

Information obtained from reference (Wiley 2012)

**Table 24.4** ■ Conditions for which human gene transfer trials have been approved.

previously mentioned that transgene expression of p53 sensitized the tumors to the therapeutic effect of conventional chemotherapy and radiotherapy (Lesoon-Wood et al. 1995; Chen et al. 1996). In other studies, the RNAi mechanism (see Chap. 23) was used to overcome multidrug resistance (MDR) in cancer cells. MDR, which typically represents overexpression of P-glycoprotein, a drug efflux transporter on cancer cell membranes, is a frequent impediment to successful chemotherapy. Synthetic siRNA- or vector-mediated MDR1 gene silencing were widely reported to be successful to reduce the chemoresistance of certain types of cancers (Huang et al. 2008).

### ■ Gene-Directed Enzyme-Prodrug Therapy

In this approach, gene therapy aims to maximize the effect of a toxic drug and minimize its systemic effects by generating the drug in situ within the tumor. In the first step of this procedure, the gene for an exogenous enzyme is delivered and expressed in the tumor cells. Subsequently, a prodrug is administered and converted to the active drug (toxic metabolites) by the foreign enzyme expressed inside or on the surface of tumor cells (Fig. 24.12). The suicide gene is usually of viral or prokaryotic origin with no human homolog. However, this is not an absolute requirement provided the prodrug is not activated to any significant degree by the native cellular enzyme. In preclinical studies, Chen et al. first reported a successful combination of a suicide gene/prodrug system and immunotherapy to treat hepatic metastases in mice (Chen et al. 1996). Uckert et al. further improved the system to a double suicide gene system as a safety mechanism for the elimination of tumor cells in a reliable fashion (Uckert et al. 1998). There are several variants of gene-directed enzyme-prodrug therapy. The herpes simplex virus-thymidine kinase (HSV-tk)/ganciclovir system, the cytosine deaminase/5-fluorocytosine system, the nitroreductase/CB1954 system, and the carboxypeptidase G2/CMDA system are the most "popular" systems (Fig. 24.13). Cerepro, a gene medicine developed by Ark Therapeutics Group PLC, has been granted orphan drug status by the European Medicines Agency and the FDA. Cerepro is an adenovirus containing a herpes simplex type-1 thymidine kinase transgene for treating malignant glioma together with ganciclovir. In a phase II clinical trial with Cerepro, the mean survival time of patients increased to 15 months as compared to 7.4 months in patients treated with retroviral therapy or 8.3 months with a noneffective adenovirus (Sandmair et al. 2000).

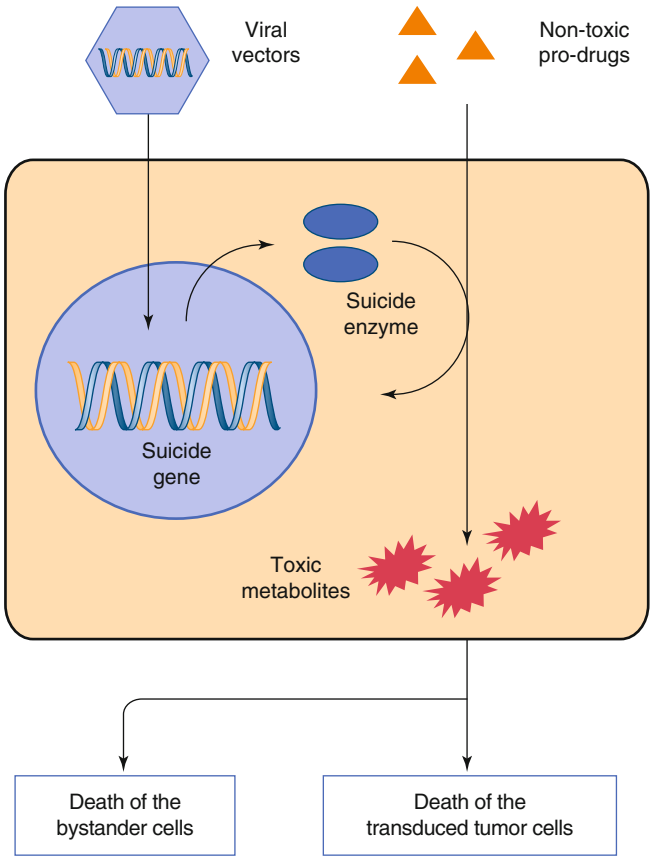


**Figure 24.12** ■ A schematic illustration of gene-directed enzyme-prodrug therapy. The suicide gene for an exogenous enzyme is delivered and expressed in the tumor cells. Then a prodrug is administered and converted to the active drug by the foreign enzyme expressed inside or on the surface of tumor cells

### ■ Oncolytic Viruses (Virotherapy)

In this approach, oncolytic viruses were directly introduced into tumors to induce cell death through viral replication, expression of cytotoxic proteins, and cell lysis. Vaccinia virus, herpes simplex type-I (HSV), reovirus, Newcastle disease virus, poliovirus, and adenovirus are often selected for this application because they naturally target cancers and contain genomes that can be easily manipulated (Aghi and Martuza 2005). Despite some clinical success, significant safety precautions must be taken, making clinical trials with these viruses extremely expensive and cumbersome (Markert et al. 2000; Varghese and Rabkin 2002). The clearance of the virus by cellular immunity and preexisting neutralizing antibodies in the majority of the population also negatively affects the efficacy of virotherapy. In 2006, Oncorine, a drug made of conditionally replicative adenoviruses by Sunway Biotech, Shanghai, China, gained marketing approval in China for treating head and neck cancer. This adenovirus contains a deletion in the E1B 55 K region and only replicates in p53-deficient cancer cells. The company has claimed significant benefits of using Oncorine in clinical trials of lung cancer, liver cancer, pancreatic cancer, and malignant effusion (Xia et al. 2004).

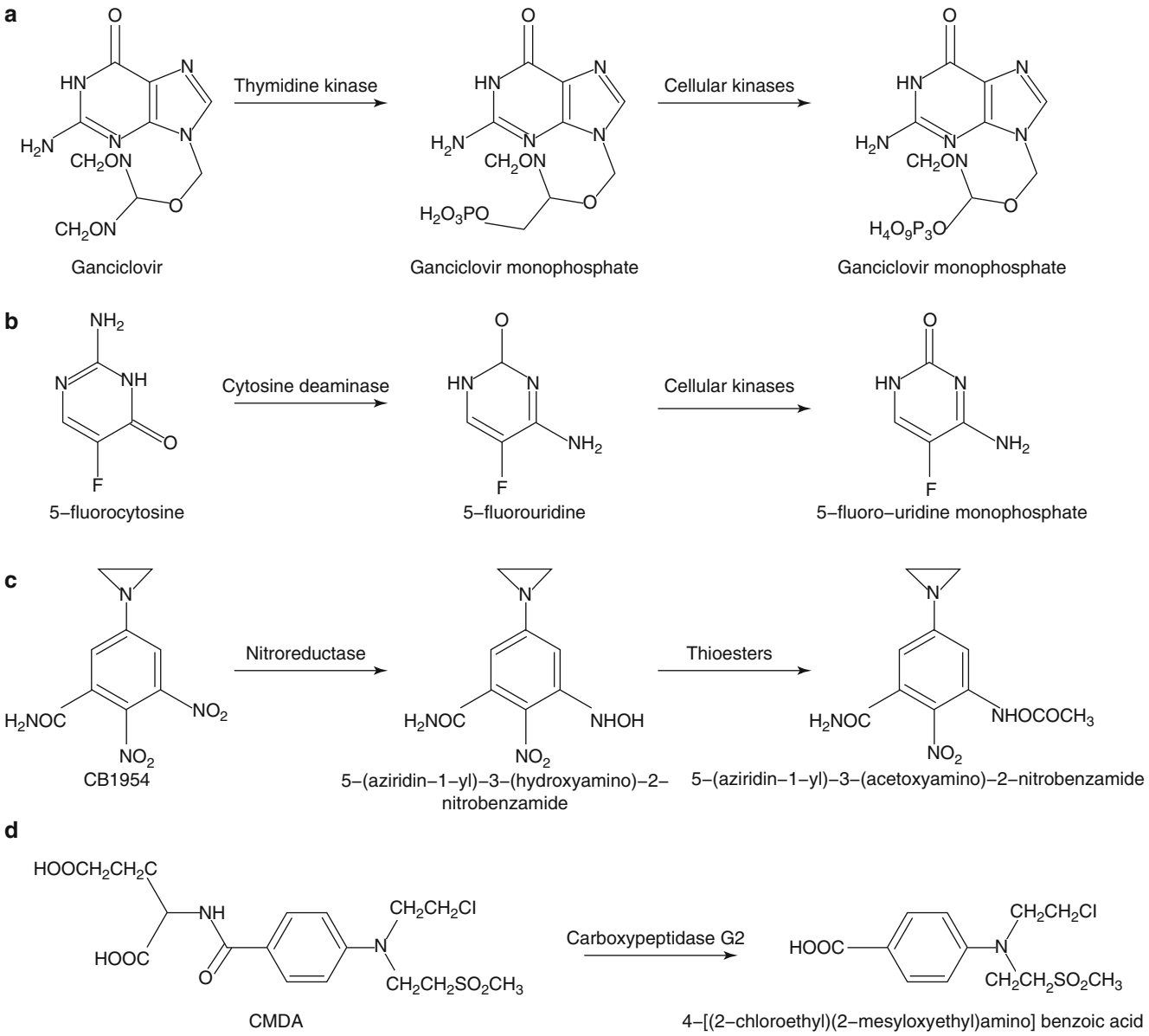


**Figure 24.13** ■ Models of gene-directed enzyme-prodrug therapy. (**a**) The herpes simplex virus-thymidine kinase (HSV-tk) system. A vector expressing the gene for HSV-tk enters a cellular target. This enzyme is expressed and phosphorylates the drug ganciclovir (GCV). This is subsequently converted to the di- and triphosphate forms by guanylate kinase and other cellular kinases. The triphosphate is incorporated into cellular DNA during cell division causing single strand breaks. (**b**) The cytosine deaminase/5-fluorocytosine system. A vector expressing cytosine deaminase (CD) enters a cellular target. Overexpression of CD activates 5-fluorocytosine (5-FC) to 5-fluorouridine (5-FU). 5-FU is converted to mono-, di-, and triphosphate forms by cellular kinases. All of these compounds are cytotoxic. (**c**). The nitroreductase/CB1954 system. A vector expressing E. coli nitroreductase (NTR) enters a cellular target. Expression of NTR allows the cell to convert the compound CB1954 to a potent DNA-cross-linking agent. (**d**) The carboxypeptidase G2/CMDA system. The bacterial enzyme carboxypeptidase G2 (CPG2) is able to cleave the glutamic acid moiety from the prodrug releasing the DNA-cross-linking mustard drug 4-[(2-chloroethyl-2-mesyloxyethyl) amino] benzoic acid without further catalytic requirements.

## ■ Nonviral Gene Therapy

In the last two decades, gene therapy has been widely used in clinical trials for cancer treatment and the results were quite encouraging. Most of the clinical trials employed viral vector-based gene therapeutics, probably because of the high transfection efficiency of this strategy. Despite the fact that nonviral-based gene therapeutics are relatively safer and less tumorigenic, extensive work is still needed to further optimize this strategy (increase transgene expression, reduce plasma protein binding, escape reticuloendothelial system (RES) and endosome, etc.) to make it clinically

acceptable. Chapter 4 deals with new insights and strategies to improve targeting efficiency, and the artificial virus approach (for endosomal escape and nuclear membrane passage) describes means to enhance intracellular transport and delivery (Mastrobattista et al. 2004).

## MONOGENETIC DISEASES

The greatest successes of gene therapy to date have been achieved in treating monogenetic diseases, which is the second largest disease group treated by gene therapy, comprising 8.5 % of all the active gene therapy clinical trials. The ultimate therapeutic goal of gene therapy for monogenetic disorders is to permanently replace a defect gene with a good copy to restore normal function and permanently reverse disease processes. To date, clinical trials have not met this objective. Of the 151 active clinical gene therapy trials for monogenetic diseases, approximately one third targeted cystic fibrosis (CF), the most common inherited genetic disease in Europe and the United States (Wiley 2012). Up until now, the severe combined immunodeficiency syndromes, comprising 20 % of trials for inherited disorders, is the only group of diseases in which gene therapy has shown a lasting, clinically meaningful therapeutic benefit. Other monogenetic diseases currently in clinical trials are listed in Table 24.4.

Issues which have prevented successful gene transfer for monogenetic diseases to date include (a) lack of suitable gene delivery technologies, (b) unfavorable interactions between the host and gene transfer vector, (c) complex biology and pathology of monogenetic diseases and target organs, and (d) lack of relevant measures to assess the clinical efficacy of gene transfer. The greatest challenges that remain in treating monogenetic disease are to induce gene expression sufficiently to correct the clinical phenotype without precipitation of host immune responses and minimizing the risk of insertional mutagenesis for integrating vectors in dividing cellular targets. Improvements in vector technology and advancements in the understanding of cellular processes will vastly improve methods for correction of genetic disease.

## CARDIOVASCULAR DISEASES

Cardiovascular diseases are the third largest group of diseases actively treated by gene therapy clinical trials. The current understanding of molecular mechanisms of cardiovascular diseases has uncovered a large number of genes that could serve as potential targets for molecular therapies. For example, overexpression of genes involved in vasodilation such as endothelial nitric oxide synthase (eNOS) and heme oxygenase-1 (HO-1) or inhibition of molecules involved in vasoconstriction (angiotensin converting enzyme (ACE), angiotensinogen (AGT)) have reduced blood pressure in animal models of hypertension (Melo et al. 2006). Most clinical trials for cardiovascular diseases are designed for treating coronary and peripheral ischemia. Overexpression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) have been effective in myocardial and peripheral ischemia in preclinical studies (Springer 2006). Despite the lack of significant benefit in several earlier clinical trials, VEGF gene therapy did show an excellent safety profile and improvement of symptoms in patients following adenoviruses or plasmid intramyocardial administration in both pilot studies and long-term follow-ups (Stewart et al. 2006; Reilly et al. 2005). However, limited success was experienced in using gene therapy to treat cardiovascular diseases compared to other areas. Larger, double-blind, randomized, controlled trials are needed to minimize the potential bias for placebo effects suspected to occur in some trials. Stringent criteria for patient selection are needed as many with cardiovascular disease often have underlying conditions that may influence the results. Endpoints for assessing efficacy and measures to assess potential short- and long-term complications must also be standardized among research groups. The efficacy of gene therapy for cardiovascular disease will most likely be enhanced by strategies that incorporate multiple gene targets with cell-based approaches.

## INFECTIOUS DISEASES

One hundred and forty-two clinical trials for treating infectious diseases have been initiated, comprising 8 % of the total number of gene therapy clinical trials (Wiley 2012). Gene transfer for acquired immunodeficiency syndrome (AIDS) is the main application in this category. Many gene therapy trials for AIDS involve ex vivo transfer of genetic material to autologous T cells using self-inactivating or conditionally replicating viral vectors to improve the immune system of the patients (Levine et al. 2006; Manilla et al. 2005). Other trials employed overexpression of HIV inhibitors such as RevM10 to increase $CD4^+$ T cell survival in HIV-infected individuals (Morgan et al. 2005; Ranga et al. 1998).

The most important achievement in the gene therapy studies to treat infectious diseases is the development of DNA vaccination, a technique to protect the host from diseases by producing an immunological response through injecting genetically engineered viral DNA (see Chap. 22). Although DNA vaccination was

first proposed and studied for HIV, it has achieved little success in the last 10 years (MacGregor et al. 1998). Clinical studies using DNA vaccine for other infectious diseases caused by hepatitis B virus (HBV), influenza virus, and Ebola virus were also reported (Tacket et al. 1999). In 2010, researchers from the USA and France reported the first HIV DNA vaccine which can induce a long-lasting HIV-specific immune response in nonhuman primates, a discovery that could prove significant in the development of HIV vaccines (Arrode-Bruses et al. 2010). Currently, PENNVAX™, a DNA vaccine product for HIV developed by Inovio Pharmaceuticals, is in phase I clinical studies.

## NEUROLOGICAL DISEASES

Significant progress has been made in gene therapy for neurological diseases in the last 5 years. The two most common neurological diseases targeted by gene therapy are Alzheimer's disease and Parkinson's disease. In 2005, a phase I trial of ex vivo nerve growth factor (NGF) gene delivery in eight individuals with mild Alzheimer disease was performed at the University of California in San Diego. Briefly, autologous fibroblasts obtained from small-skin biopsies in each individual were genetically modified to produce and secrete human NGF using retroviral vectors and reimplanted into the forebrain. The results indicated improvement in the rate of cognitive decline, significant increases in cortical 18-fluorodeoxyglucose concentrations (PET imaging), and robust nerve growth responses to NGF (Tuszynski et al. 2005). In 2007, the first gene therapy clinical trial was conducted at the New York Presbyterian Hospital. Briefly, serial doses of adeno-associated virus (AAV) encoding glutamic acid decarboxylase (GAD) were infused into the subthalamic nucleus of patients with Parkinson's disease. The results indicated that AAV-GAD gene therapy is safe and well tolerated by patients with advanced Parkinson's disease. Although this open label, nonrandomized phase I study did not include a sham group and was not designed to assess the effectiveness of gene therapy, the preliminary data were encouraging, showing substantial improvements in Unified Parkinson's Disease Rating Scale (UPDRS), beginning at 3 months after surgery and continuing until the end of the trial (12 months after surgery) (Kaplitt et al. 2007).

## REGULATORY ISSUES OF GENE THERAPY PRODUCTS

Any studies involving humans must be reviewed with great care. Gene therapy presents unique safety and infection control issues, which make it necessary for scientists to take special precautions with gene therapy. In the USA two organizations within the United States Department of Health and Human Services (DHHS), the Office for Human Research Protections (OHRP) and the Food and Drug Administration (FDA), have specific authority described in the Code of Federal Regulations (CFR). The OHRP mandates any gene therapy clinical trial involving human subjects to be reviewed, approved, and monitored by the Institutional Review Board (IRB) at each investigative site. The FDA's Center for Biologics Evaluation and Research (CBER) oversees human gene therapy clinical trials conducted by the manufacturers. Any gene therapy product must be tested extensively to meet the FDA requirements for safety and efficacy before approval for marketing. All gene therapy clinical trial protocols must be conducted under Investigational New Drug (IND) applications. Regulations pertaining to this process appear in Title 21 of the Code of Federal Regulations (CFR), Part 312. Another DHHS organization, the National Institutes of Health (NIH) oversees the gene therapy studies and clinical trials conducted by federally funded investigators. The NIH monitors scientific progress in human genetics research through the Office of Biotechnology Activities (OBA). Inside OBA, the Recombinant DNA Advisory Committee (RAC) was established in 1974 in response to public concerns regarding the safety of manipulating genetic material through the use of recombinant DNA techniques. Any human gene transfer research receiving NIH funding must be registered with OBA and reviewed by the RAC. Another responsibility of RAC is to cooperate with the Institutional Biosafety Committees (IBC) to oversee recombinant DNA research at each investigative site. Figure 24.14 shows the interactions of these different regulatory agencies in the development process of a gene product. Other countries also have a number of committees that must approve gene therapy protocols and address other scientific and ethical concerns associated with clinical trials.

## CONCLUDING REMARKS

Within the last 20 years, the field of gene therapy has come a long way from bench to bedside. Many vectors developed for gene transfer have now been tested in the clinic. Three products (Gendicine and Oncorine in China, Cerepro in Europe) have been given marketing approval and several others are in late phases of testing. Although the biology of gene transfer vectors is well understood, several barriers must be overcome for turning genes into therapeutics. The immune responses and the insertional mutagenesis of viral vectors and the lack of transgene efficiency of nonviral vectors are the most significant barriers of gene therapy.

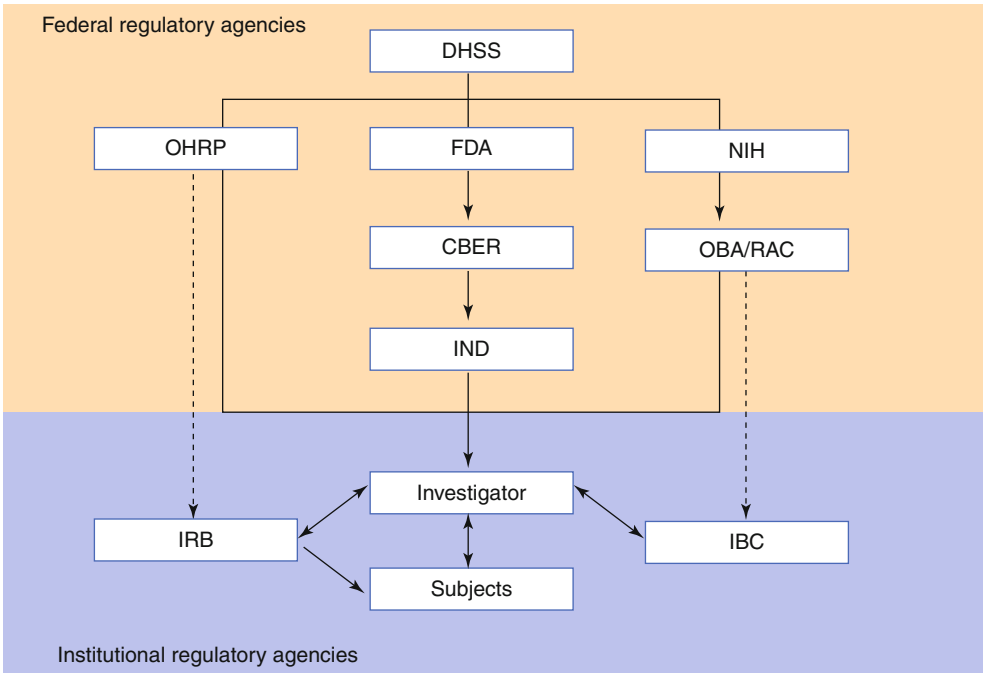


**Figure 24.14** ■ The interactions of the regulatory agencies involved in the implementation of a gene therapy protocol (From Mendell and Miller (2004). with permission to reprint).

Targeted delivery of gene expression systems and spatial and temporal control of transgene expression in target tissues based on the severity and the process of the disease are also critical to the success of many gene therapy applications. Although clinical trials have shown short-term safety and efficacy, long-term surveillance over a period of decades is lacking, and the safety and efficacy of genetic medicines have so far only been validated in limited patient populations. Other factors such as the use of concurrent medications and concurrent medical conditions, objective assessment of improvements and endpoints, and the assessment of placebo effects need to be standardized to get reliable and reproducible results among different research groups. In addition, cost-effectiveness analyses have to be considered as the production of gene therapy vectors itself is costly and requires specialized equipment and personnel. In the future, the further development of genetic medicines that can be widely used will heavily rely on collaborations between academic institutions and commercial partners from the pharmaceutical and biotechnology industries.

## SELF-ASSESSMENT QUESTIONS AND ANSWERS

### ■ Questions

1. What was the disease target for the first gene therapy clinical trial? What vector was selected for gene transfer?
2. Identify and describe five transcription regulatory elements (TRE) discussed in the chapter.
3. Several clinical trials involve gene transfer for treating malignant glioma. One approach involves the use of a recombinant retrovirus expressing the HSV-tk transgene. Another involves the use of a recombinant adenovirus expressing the p53 transgene.
   (A) Which of the five current strategies to treat cancer by viral gene therapy does each of these trials employ? Describe the principle behind each strategy.
   (B) List 2 advantages and 2 disadvantages associated with the vector used in each of these trials.
   (C) Outline potential drawbacks to the use of each of these strategies for cancer therapy.
   (D) What other approaches could have been selected to prevent the growth and spread of malignant tissue? Explain the principle behind each.
4. What is the purpose of the packaging cell line during the production of recombinant viral vectors for gene transfer? What is the risk associated with using packaging cell lines for vector production?
5. Provide two examples of how gene therapy is used to modulate the immune system to fight infection.
6. Describe one clinical trial for retrovirus-based gene therapy and adenovirus-based gene therapy, and identify the most significant adverse effects that have been reported for each trial.
7. Identify the three marketed gene therapy products in the world and describe the mechanism of actions of each product.

## ■ Answers

1. The first gene therapy clinical trial was initiated in 1990 for treating adenosine deaminase (ADA) deficiency. In this trial, patients with ADA deficiency were given peripheral blood lymphocytes treated with a retroviral vector expressing the ADA transgene.
2. Promoter is a DNA sequence that enables a gene to be transcribed. The promoter is recognized by RNA polymerase and transcriptional factors. Enhancer is a short DNA sequence that can bind transcription factors or activators to enhance transcription levels of a gene from a distance. Insulators are mainly genetic boundary elements to block the enhancer-promoter interaction or rarely as a barrier against condensed chromatin proteins. Operators and silencers are usually short DNA sequence close to the promoter with binding affinity to a set of proteins named repressors and inducers.
3. (A) Retrovirus trial

   Gene-directed enzyme-prodrug therapy. Cells transduced by the virus express an enzyme capable of converting a prodrug (in this case ganciclovir) to a cytotoxic metabolite. This conversion cannot occur in cells that do not express the transgene, limiting the cytotoxic effect to transduced cells and their neighbors through the bystander effect.

   Adenovirus trial

   Correction of genetic mutations that contribute to a malignant phenotype. Cells transduced by the virus express a gene such as p53 that is necessary for controlled cell division and development. This prevents the uncontrolled growth and division associated with malignant disease.

   (B) Retrovirus

   Advantages – (i) Retroviruses can infect dividing cells which are the therapeutic target in this trial. Despite this fact, transduction efficiency of this vector in vivo has been low. (ii) Retroviruses are capable of inducing long-term gene expression which should be sufficient for effective removal of malignant tissue.

   Disadvantages – (i) Retroviruses have the potential for inducing insertional mutagenesis in normal, healthy cells. (ii) Transgene expression is sometimes limited by the host immune response to cellular components acquired by the virus during large scale production.

   Adenovirus

   Advantages – (i) Adenoviruses can infect dividing cells which are the therapeutic target in this trial. (ii) Adenoviruses can induce high levels of transgene expression in short periods of time. (iii) Adenoviruses do not have the risk of insertional mutagenesis. (iv) It is relatively easy to produce large amounts of recombinant adenovirus sufficient for clinical use.

   Disadvantages – (i) Transgene expression is transient, making readministration necessary for continued effect. (ii) Adenoviral vectors are capable of inducing a potent immune response. This not only limits the success of gene transfer after a second dose of virus but also is associated with severe toxicity at certain doses. (iii) Preexisting immunity to adenovirus serotype 5 is common in the general population. This may also limit gene transfer.

   (C) Drawbacks to gene-directed enzyme-prodrug therapy.

   (i) Efficacy relies on efficient transgene expression and drug bioavailability. (ii) The therapeutic effect may spread to healthy cells through the bystander effect.

   Drawbacks to gene correction therapy.

   (i) Gene correction may stop tumor growth but not eliminate it. (ii) Expression is not limited to malignant tissue.

   (D) Other approaches for cancer gene therapy

   (i) Immunotherapy. A vector expressing pro-inflammatory cytokines, co-stimulatory molecules, or tumor-specific antigens is injected directly into the tumor mass. This facilitates the formation of an antitumor immune response that targets and destroys malignant cells.

   (ii) Virotherapy. A replication-competent virus that naturally targets cancers is directly injected in the tumor mass. The virus can induce cell death during replication in malignant tissue by producing cytotoxic proteins and subsequent cell lysis.
4. (i) The primary purpose of the packaging cell line is to provide genetic elements that support virus replication and assembly. These have been eliminated from the vector to prevent it from causing disease in the patient. (ii) The recombinant virus can incorporate elements for replication into its genome through homologous recombination during the production process. The potential for generation of replication-competent virus (RCV) in this manner does exist for each vector but can vary due to specific features of a given packaging cell line.
5. (i) Gene transfer into autologous immunocytes to increase the immune system of a patient. (ii)

Overexpression of protein inhibitors that interfere with virus infection and replication. (iii) Overexpression of known antigenic epitopes of the pathogen by DNA vaccination to stimulate an immune response.

6. (i) One trial employed aerosol administration of a recombinant adenovirus expressing cystic fibrosis transmembrane conductance regulator (CFTR) to treat cystic fibrosis (CF). Another trial employed a recombinant retrovirus expressing recombinant adenosine deaminase (ADA) to transduce autologous T lymphocytes isolated from patients for treating ADA deficiency-induced severe combined immunodeficiency (ADA-SCID). (ii) CF trial. Massive immune response to the recombinant viral vector.

   ADA-SCID trial. Lymphoproliferative leukemia caused by insertional mutagenesis.

7. Gendicine is a recombinant adenoviral vector which expresses p53 tumor suppressor and is used to treat patients with head and neck squamous cancers.

   Oncorine is a recombinant adenoviral vector which contains a deletion in the E1B 55 K region and only replicates in p53 deficient cancer cells. Oncorine kills tumor cells through viral replication, expression of cytotoxic proteins, and cell lysis.

   Cerepro is a recombinant adenoviral vector encoding herpes simplex type-1 thymidine kinase (TK). Cerepro is used for treating malignant glioma together with ganciclovir through gene-directed enzyme-prodrug therapy.

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