# PROGRESS AND PROBLEMS WITH THE USE OF VIRAL VECTORS FOR GENE THERAPY

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Gene therapy has a history of controversy. Encouraging results are starting to emerge from the clinic, but questions are still being asked about the safety of this new molecular medicine. With the development of a leukaemia-like syndrome in two of the small number of patients that have been cured of a disease by gene therapy, it is timely to contemplate how far this technology has come, and how far it still has to go.

TERMINAL REPEAT
A short non-coding DNA sequence found at each end of the viral genome, which contains elements required for the replication and packaging of the viral DNA.

CAPSID
A protein shell that encapsulates the viral genetic material.

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The science of gene therapy has a turbulent history. Initially perceived as a revolutionary new technology with the promise to cure almost any disease — provided that we understood its genetic or molecular basis — enthusiasm rapidly waned as clinical trial after clinical trial failed to show efficacy[1]. The stumbling block seemed to be the vehicles that were used to deliver the therapeutic genes to the target tissue; early recombinant viral vectors were inefficient, failed to persist in host cells and transgene expression was typically short-lived. Then, in 1999, an adverse patient reaction to an adenovirus vector during a clinical safety trial led to the realization that the failure to understand the biology of vector interactions with the human immune system could have fatal consequences (BOX 1). The year 2000 brought the first gene-therapy success in which three children were cured of a fatal immunodeficiency disorder, but this therapy has subsequently caused a leukaemia-like disease in 2 of the 11 patients who have been treated (BOX 1). Such severe blows have overshadowed the substantial progress that has been made in the development of gene-transfer technologies over recent years. The message we have extracted from a history of anticipation and disappointment is that the future success of gene therapy will be founded on a thorough understanding of vector biology and pharmacology. Over the past few years, intense efforts have been concentrated on understanding the molecular basis of how viruses and viral vectors interact with the host. Our findings have allowed us to develop vectors with improved efficiency, specificity and safety, and some clinical successes have recently been achieved. This article highlights some of the advances in the development of viral vectors, as well as discussing the substantial challenges that remain before gene therapy can truly fulfil all of its promises.

## From pathogen to medicine

Viruses are highly evolved biological machines that efficiently gain access to host cells and exploit the cellular machinery to facilitate their replication. Ideal virus-based vectors for most gene-therapy applications harness the viral infection pathway but avoid the subsequent expression of viral genes that leads to replication and toxicity. This is achieved by deleting all, or some, of the coding regions from the viral genome, but leaving intact those sequences (usually the TERMINAL REPEAT sequences) that are required *in cis* for functions such as packaging the vector genome into the virus CAPSID or the integration of vector DNA into the host chromatin. The expression cassette of choice is then cloned into the viral backbone in place of those sequences that were deleted. The deleted genes encoding proteins that are involved in replication or capsid/envelope proteins are included in a separate packaging construct to provide helper functions *in trans*. The packaging cells into which the vector genome and packaging construct are co-transfected then produce the recombinant vector particles (FIG. 1).

DISSEMINATED INTRAVASCULAR COAGULATION
Inappropriate blood clotting.

TRANSDUCTION
The introduction of genetic material into a cell using a viral vector.

TITRE
A measure of vector concentration that is usually expressed as the number of transducing units, or the number of particles per millilitre.

After production in a packaging cell line, the recombinant vector particles are purified and quantified (TITRED). Purification strategies have traditionally relied on the separation of vector particles from cellular components by density gradient centrifugation (usually a caesium chloride gradient); however, this process is laborious, difficult to scale up for industrial purposes and can sometimes damage the vector particles and reduce the infectious titre of the vector stock. Advances in column-chromatographic methods for the purification of several classes of vector have alleviated these concerns[2,3] and most of the main classes of vector that are described here are now able to be grown and purified to the high titres required for administration to humans.

## The main groups of viral vectors

Gene therapy was first conceived as a treatment for hereditary single-gene defects[4]. Today, acquired diseases such as cancer[5], cardiovascular disease[6], neurodegenerative disorders[7] and infectious disease[8] are the subject of most gene-therapy research (FIG. 2). Given the diversity of disease targets that are potentially amenable to gene transfer, it has become clear that there can be no single vector that is suitable for all applications. Perhaps the only characteristics that are required by all vectors are the abilities to be reproducibly and stably propagated and purified to high titres, to mediate targeted delivery (that is, to deliver the transgene specifically to the tissue or organ of interest without widespread vector dissemination

### Box 1 | Adverse events in gene therapy

**1999: adenovirus vector causes patient death**

In September 1999, 18-year-old Jesse Gelsinger took part in a gene-therapy clinical trial at the University of Pennsylvania in Philadelphia. Gelsinger suffered from a partial deficiency of ornithine transcarbamylase (OTC), a liver enzyme that is required for the safe removal of excessive nitrogen from amino acids and proteins. OTC deficiency leads to an accumulation of ammonia in the bloodstream, which, in turn, causes an elevation of ammonium ions in the brain, leading to encephalopathy, brain damage and coma. The University of Pennsylvania trial was designed to test the safety of using a second-generation E1- and E4-deleted adenovirus vector to deliver the gene for OTC to the liver. The therapy would eventually be intended for babies suffering from severe and fatal OTC deficiency, but ethical concerns over whether parents would be able to give informed consent for their sick children meant that the safety trial was conducted on 18 relatively fit adult volunteers who had only a mild form of the disease that was controlled by diet and drugs. Gelsinger received the highest dose of vector in the trial ($3.8 \times 10^{13}$ particles). A female patient who received a similar dose ($3.6 \times 10^{13}$ particles) experienced no unexpected side effects, but 4 hours after Gelsinger's treatment, he developed a high fever. By the morning after his treatment, he was displaying symptoms of liver injury and DISSEMINATED INTRAVASCULAR COAGULATION. Within four days of treatment, Gelsinger died from multiorgan failure.

Jesse Gelsinger's death was directly attributable to the administration of the adenovirus vector. An autopsy showed that, although the vector had been infused directly into the liver through the hepatic artery, substantial amounts of the vector had disseminated into the circulation and had accumulated in the spleen, lymph nodes and bone marrow. The systemic delivery of the vector triggered a massive inflammatory response that led to disseminated intravascular coagulation, acute respiratory distress and multiorgan failure[40,88]. Subsequent studies in monkeys have indicated that the adenovirus capsid proteins, rather than the genetic cargo, might elicit an early inflammatory cytokine cascade[89]. Exactly why Gelsinger suffered such severe side effects, whereas a second patient tolerated a similar dose of the vector, remains unclear. However, it has been indicated that previous exposure to a wild-type virus infection might have sensitized his immune system to the vector[90].

**2002–2003: retrovirus vector induces a lymphoproliferative disorder**

In April 2000, a paper was published in the journal *Science* that marked the highest point in the turbulent history of gene therapy. In a paper entitled "Gene therapy of human severe combined immunodeficiency (SCID)-XI disease", Maria Cavazzana-Calvo, Alain Fischer and colleagues at the Necker Hospital for Sick Children in Paris reported the first definitive cure of a disease by gene therapy[64]. Three young children suffering from the fatal X-linked SCID-XI syndrome had developed functional immune systems after the reinfusion of haematopoietic stem cells that were TRANSDUCED *ex vivo* with an MLV vector that carried the gene encoding the γ-c chain cytokine receptor. Without the γ-c chain receptor, developing lymphocytes are unable to respond to cytokine signals and mature into functional T cells and natural-killer (NK) cells. Since the publication of this report, several more patients have been treated with the same gene therapy with apparent success, but elation recently became anxiety after the development of a leukaemia-like disorder in two of Fischer's patients. The cancerous T cells in both patients are thought to be derived from single transduced cells in which the retrovirus genome had inserted near, or in, the LIM domain only 2 (*LMO2*) oncogene, activating LMO2 expression[65,91,92]. A similar insertion into the *LMO2* region has recently been identified in a third child in the SCID-XI study, although this child has not developed leukaemia (limited peer-reviewed data on this subject are available at present).

In the SCID-XI trial, haematopoietic stem cells that were genetically reconstituted with the γ–c chain cytokine receptor needed to undergo many cell divisions to generate a repopulating functional T-cell repertoire. Although there was already a strong selection for genetically modified cells to proliferate, the activation of *LMO2* gene expression probably boosted the ability of these clones to proliferate to the point of malignancy. Therefore, it seems probable that the cancer in the two SCID-XI patients was a consequence of a particular combination of vector, transgene and disease target (in which the proliferation of single transduced cells was a therapeutic end point in young children with compromised immune systems). New recommendations from the United States Food and Drug Administration (FDA) Biological Response Modifiers Advisory Committee (BRMAC) state that this form of therapy should not be the first line of treatment for SCID-XI, but it can be considered in the absence of other options such as matched bone-marrow transplant[93].

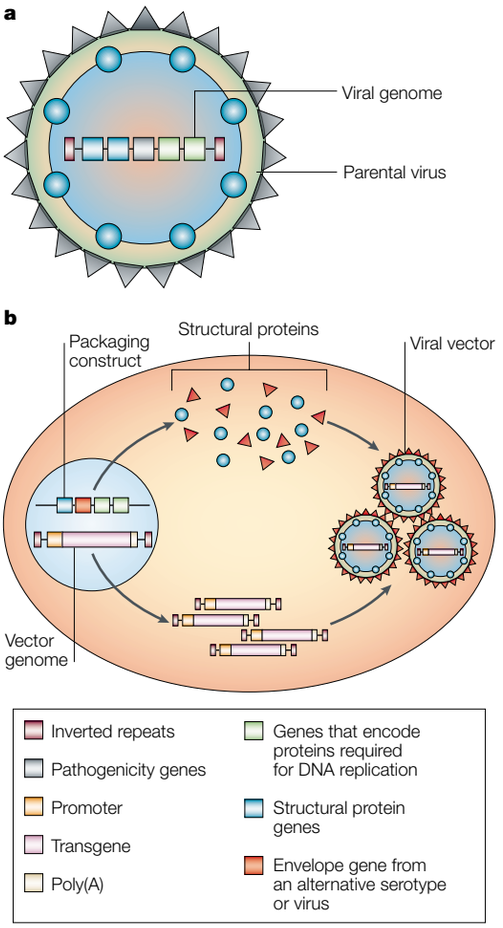


Figure 1 | **Converting a virus into a vector. a** | Schematic diagram of a generic viral vector. **b** | A packaging (helper) construct, containing viral genes derived from the parental virus that encode structural proteins and proteins that are required for vector genome replication, is introduced into a packaging cell line along with a construct that contains the vector genome. The helper DNA can be delivered as a plasmid or helper virus, or it can be stably integrated into the chromatin of the packaging cell. Pathogenicity functions and the sequences that are required for encapsidation are eliminated from the helper construct so that it cannot be packaged into a viral particle. The vector genome contains the transgenic expression cassette and is flanked by inverted terminal repeats and *cis*-acting sequences that are required for genome encapsidation. Some vector genomes retain viral genes that are relatively inactive, as a result of the elimination of viral early genes that are required for their transcription. Viral structural proteins and proteins that are required for replication of the vector DNA are expressed from the packaging construct and the replicated vector genomes are packaged into virus particles.

EPISOME
A stable DNA molecule that persists in the nucleus without integrating into the cellular genome.

TROPISM
The range of cell types or tissues in which a virus can sustain a productive infection.

elsewhere) and to mediate gene delivery and transgene expression without inducing harmful side effects.

The number of different viruses that are under development as gene-therapy vectors is steadily increasing, but there are, at present, five main classes of clinically applicable viral vector that are derived from oncoretroviruses, lentiviruses, adenoviruses, adeno-associated viruses (AAVs) and herpes simplex-1 viruses (HSV-1s) (reviewed in REF. 9). Each of these classes of vector is characterized by a set of different properties that make it suitable for some applications and unsuitable for others.

## Vector applications

The five main classes of viral vector can be categorized in two groups according to whether their genomes integrate into host cellular chromatin (oncoretroviruses and lentiviruses) or persist in the cell nucleus predominantly as extrachromosomal EPISOMES (AAVs, adenoviruses and herpes viruses). This distinction is one important determinant of the suitability of each vector for particular applications; non-integrating vectors can, under certain circumstances, mediate persistent transgene expression in non-proliferating cells, but integrating vectors are, at present, the tools of choice if stable genetic alteration needs to be maintained in dividing cells. Integration is not, however, a guarantee of stable transcription as transgene expression from integrated vector genomes can be gradually silenced over time[10].

Oncoretrovirus vectors were the first class of viral vector to be developed and have, so far, been the most widely used in clinical trials. They have traditionally been the vectors of choice for the *ex vivo* transduction of repopulating haematopoietic stem cells. A limitation to the usefulness of C-type retrovirus vectors is that they can only gain access to the cell nucleus if the nuclear membrane breaks down; therefore, they can only transduce dividing cells. Recently, a nuclear localization signal was engineered in the matrix protein of an avian C-type retrovirus — spleen necrosis virus (SNV) — to enable an SNV vector to transduce non-proliferating cells[11]. However, most work has focused on the development of lentivirus vectors, which can naturally penetrate an intact nuclear membrane and transduce non-dividing cells (BOX 2; TABLE 1). This characteristic enables lentivirus vectors to transduce haematopoietic stem cells *ex vivo* without first inducing them to proliferate with cytokine stimulation. As such, lentivirus vectors might supersede the C-type retrovirus vectors for most *ex vivo* haematopoietic gene therapy. Lentivirus vectors will probably be important vector systems in the future treatment of a wide range of diseases besides haematopoietic disorders. They have proven to be effective tools for gene delivery to the central nervous system (CNS), generating long-term gene expression in the absence of inflammation[12]. Therapeutic efficacy has been shown in animal models of mucopolysaccharidosis type VII (REF. 13), metachromatic leukodystrophy[14] and Parkinson disease[15]. Lentiviral transduction of muscle and liver has also been shown in animals, but, interestingly, studies in the liver have indicated that not all non-dividing cells are equally susceptible to transduction by lentivirus vectors; some cell types (such as the hepatocyte) might require cell cycling for efficient gene transfer *in vivo*[16].

Vector TROPISM, the duration of transgene expression and vector immunogenicity are other factors that influence the suitability of a vector for specific therapeutic applications. The potent immunogenicity and consequent short-lived transgene expression of early-generation adenovirus vectors are undesirable

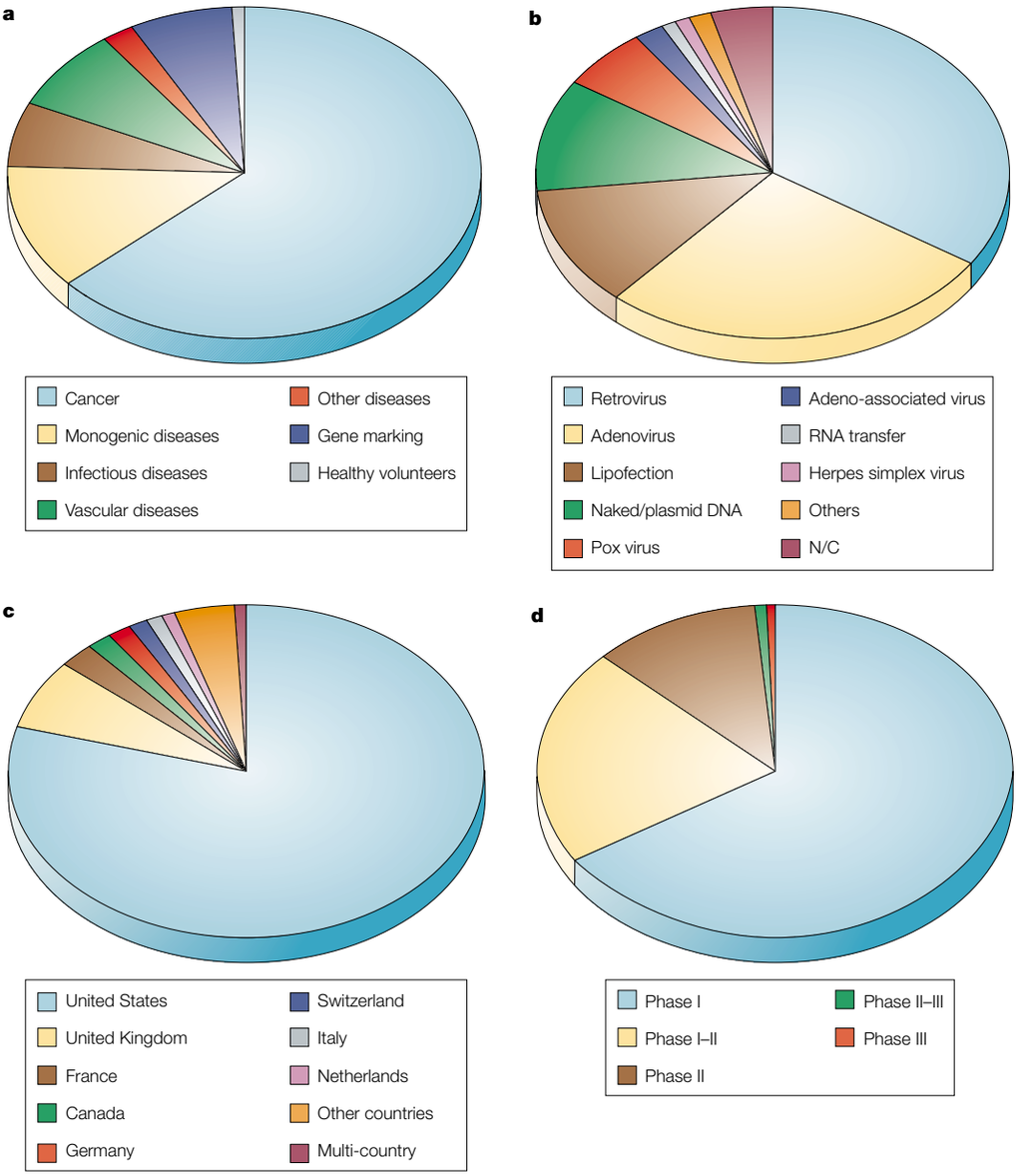


Figure 2 | **A survey of gene transfer clinical trials.** The Journal of Gene Medicine Clinical Trial Database (Wiley Database) contains information on 636 completed, ongoing or pending human gene-transfer clinical trials worldwide. **a** | Most gene-therapy clinical trials are designed to treat cancer. **b** | Retrovirus vectors and adenovirus vectors have, so far, been the most commonly used vectors in gene-transfer trials. Non-viral gene transfer has been assessed in roughly one-quarter of all trials. **c** | Most gene transfer clinical trials are conducted in the United States. **d** | Most gene-transfer trials are designed to assess only the safety of a particular gene-therapy approach (PHASE I). Few gene therapies are being assessed in PHASE II or PHASE III efficacy trials. The Wiley Database represents, at present, the best available compendium of information on gene-therapy trials that have been conducted worldwide. Here, we reproduce some of the information from this database to convey a general impression of the nature of different gene-transfer trials. However, analysis of more up-to-date protocol listings obtained from national registries shows that the database is not wholly accurate. For example, the Office of Biotechnology Activities (OBA) lists, at present, a total of 558 human gene-transfer protocols that are completed, ongoing or pending in the United States (358 for cancer, 57 for monogenic disorders, 38 for infectious diseases, 41 for gene marking, 5 for non therapeutic gene transfer and 59 for other diseases/disorders; last updated on 28 February 2003), compared with a total of 505 US trials that are listed in the Wiley Database. Similarly, the Gene Transfer Advisory Committee (GTAC) in the United Kingdom registered 83 gene-therapy research trials between 1993–2002 (64 for cancer, 7 for monogenic disorders, 4 for infectious disease and 8 for other diseases; last updated on 3 October 2002), compared with just 43 UK trials listed in the Wiley Database. The creation of a reliable international registry that contains information on all gene-transfer clinical trials would greatly benefit the gene-therapy community. Such a registry should provide detailed information on aspects of each trial protocol, including the dose of vector administered, the target tissue, the route of vector administration, the numbers of patients enrolled and the length of their observation period. Panels **a**–**d** are reproduced with permission from the Journal of Gene Medicine Clinical Trial Database. N/C, not classified.

PHASE I TRIAL
The first stage in a clinical trial, which is designed to assess only the safety and dosage levels of a new treatment and usually involves only a few patients.

PHASE II TRIAL
The assessment of efficacy, usually on a small scale.

PHASE III TRIAL
The assessment of efficacy and side-effects, which generally involves hundreds of patients from different clinics nationwide or worldwide.

properties for many gene-therapy applications; however, immunogenic adenovirus vectors will probably find niches in the treatment of vascular and coronary artery disease in which transient transgene expression is advantageous, and for cancer in which cellular toxicity and immunogenicity might enhance antitumour effects[6,17,18]. Adenovirus vectors are, arguably, the most efficient class of vector in terms of delivering their genetic cargo to the cell nucleus[19], and direct injection of adenovirus vectors can efficiently transduce most tissues. Recent improvements that reduce the immunogenicity of adenovirus vectors (BOX 2; TABLE 1) have enhanced their prospects for long-term gene transfer in a wide range of different tissues.

Box 2 | **Engineering the main groups of viral vectors**

**Oncoretroviruses**

The earliest gene-therapy vectors were based on the simple Moloney murine leukaemia virus (MLV) — a C-type oncoretrovirus. PSEUDOTYPING was first developed for MLV and is now widely applied to other vector systems. Generally, the MLV envelope glycoprotein is pseudotyped with the G protein of vesicular stomatis virus (VSV-G); this modification confers an extremely broad host range and markedly stabilizes the vector particles, allowing the vector stocks to be concentrated to high titres.

**Lentiviruses**

Lentiviruses are members of the retrovirus family. Lentivirus vectors are often pseudotyped with VSV-G, and have been derived from human immunodeficiency virus (HIV) and other non-human lentiviruses. Vectors that are based on HIV retain <5% of the parental genome, and <25% of the genome is incorporated into packaging constructs, which minimizes the possibility of the generation of revertant replication-competent HIV. Biosafety has been further increased by the development of self-inactivating (SIN) vectors that contain deletions of the regulatory elements in the downstream long-terminal-repeat sequence (LTR), eliminating the transcription of the packaging signal that is required for vector mobilization[94].

Reverse transcription of the retroviral RNA genome occurs in the cytoplasm. Unlike C-type retroviruses, the lentiviral cDNA complexed with other viral factors — known as the pre-initiation complex (PIC) — is able to translocate across the nuclear membrane and transduce non-dividing cells. A structural feature of the viral cDNA — a DNA 'flap' — seems to contribute to efficient nuclear import. This flap is dependent on the integrity of a central polypurine tract (cPPT) that is located in the viral polymerase gene, so most HIV-1-derived vectors now retain this cPPT sequence[95–97]. A recent study has, however, indicated that the presence of a valine residue at position 165 in the viral integrase — a PIC component — is more important for the nuclear import of viral nucleic acids than the cPPT[98].

**Adenoviruses**

Adenovirus vectors have been extensively engineered to reduce their potent immunogenicity. First-generation adenovirus vectors were deleted for only one or two viral EARLY GENES (E1 and E3). Cells that were transduced with these vectors expressed other adenoviral genes at low levels, inducing strong cytotoxic T-cell responses that rapidly eliminated transgene expression. Second- and third-generation vectors that contain additional deletions in other early genes (E2 and/or E4) have shown reduced toxicity in animal models[99–101], but the development of helper-dependent adenoviruses (HD-Ads) that are deleted for all viral genes has been the most important advance to decrease immunogenicity, prolong transgene expression and improve the prospects of adenovirus vectors for long-term gene therapy[32].

**Adeno-associated viruses (AAV)**

Most rAAV vectors have been derived from AAV2, but, so far, a total of eight distinct AAV serotypes have been identified that infect different cell types with different efficiencies[47–49]. Pseudotyping the rAAV2 vector genome with capsids from alternative serotypes to achieve more efficient gene transfer in tissues refractive to rAAV2 transduction, is becoming common practice. An important barrier to efficient transduction with rAAV2 vectors is conversion of the single-stranded DNA genome into a double strand. Recently, this obstacle has been overcome by the development of double-stranded vectors that exploit a hairpin intermediate of the AAV replication cycle. These vectors mediate 10 to 100-fold higher levels of transgene expression *in vitro* and *in vivo*, although as they can only package 2.4 kb of double-stranded DNA their usefulness for therapeutic gene transfer will be limited[102]. The limited packaging capacity has been addressed by exploiting *in vivo* concatemerization of rAAV genomes (episomal rAAV genomes persist in a variety of molecular forms, including circular monomers, linear monomers and linear concatemers[103,104]). By splitting an expression cassette across two vectors, a functional cassette can be reconstituted after concatemerization in the cell nucleus[105–107].

**Herpes simplex virus-1 (HSV-1)**

Replication defective HSV-1 vectors are produced by deleting all, or a combination, of the five immediate-early genes (ICP0, ICP4, ICP22, ICP27 and ICP47), which are required for lytic infection and expression of all other viral proteins. Unfortunately, the ICP0 gene product is both cytotoxic and required for high level and sustained transgene expression. As such, the production of non-toxic quintuple immediate-early (IE) mutant vectors is a trade-off against efficient and persistent transgene expression[108,109]. An HSV-1 protein that is activated during latency has recently been shown to complement mutations in ICP0 and overcome the repression of transgene expression that occurs in the absence of ICP0 (REF. 110). Substitution of this protein in place of ICP0 might facilitate efficient transgene expression without cytotoxicity in non-neuronal cells. Long-term expression can be achieved in the nervous system by using one of the HSV-1 neuron-specific latency-activated promoters (LAP) to drive transgene expression[111].

PSEUDOTYPING
The alteration of the vector tropism by substitution of the virus receptor-binding proteins with those from other virus strains.

EARLY GENES
The first viral genes that are expressed after infection. Early-gene expression does not require *de novo* viral protein synthesis. Early-gene products activate viral DNA replication and the expression of viral structural proteins.

Table 1 | **The main groups of viral vectors**

| Vector | Genetic material | Packaging capacity | Tropism | Inflammatory potential | Vector genome forms | Main limitations | Main advantages |
|---|---|---|---|---|---|---|---|
| ***Enveloped*** | | | | | | | |
| Retrovirus | RNA | 8 kb | Dividing cells only | Low | Integrated | Only transduces dividing cells; integration might induce oncogenesis in some applications | Persistent gene transfer in dividing cells |
| Lentivirus | RNA | 8 kb | Broad | Low | Integrated | Integration might induce oncogenesis in some applications | Persistent gene transfer in most tissues |
| HSV-1 | dsDNA | 40 kb* 150 kb‡ | Strong for neurons | High | Episomal | Inflammatory; transient transgene expression in cells other than neurons | Large packaging capacity; strong tropism for neurons |
| ***Non-enveloped*** | | | | | | | |
| AAV | ssDNA | <5 kb | Broad, with the possible exception of haematopoietic cells | Low | Episomal (>90%) Integrated (<10%) | Small packaging capacity | Non-inflammatory; non-pathogenic |
| Adenovirus | dsDNA | 8 kb* 30 kb§ | Broad | High | Episomal | Capsid mediates a potent inflammatory response | Extremely efficient transduction of most tissues |

*Replication defective. ‡Amplicon. §Helper dependent. AAV, adeno-associated viral vector; dsDNA, double-stranded DNA; HSV-1, herpes simplex virus-1; ssDNA, single-stranded DNA.

Recombinant AAV vectors (rAAVs) are one of the most promising vector systems for safe long-term gene transfer and expression in non-proliferating tissues. AAV is unique among viruses that are being developed for gene therapy in that the wild-type virus has never been shown to cause human disease. The small size and simplicity of the vector particle makes it possible to administer high doses of vector systemically without eliciting acute inflammatory responses or toxic side effects. The successful gene transfer and expression of human coagulation factor IX was recently shown in haemophilia B patients after muscle-directed gene transfer of an AAV2 vector[20]. A further clincal trial to treat the same disease through liver-directed gene transfer is ongoing, as are other trials for cystic fibrosis, muscular dystrophy and several CNS disorders.

The space available in the vector genome for the incorporation of exogenous DNA is another criterion that influences the choice of vector for specific therapeutic applications. HSV-1 is the largest and most complex of all the viruses that are being developed for gene therapy, and one important feature of this vector is its capacity to carry large fragments of foreign DNA. Replication-defective HSV-1 vectors can carry up to 40 kb of foreign DNA, facilitating the delivery of several separate expression cassettes, or large single genes[21]. The carrying capacity of HSV-1 vectors is further expanded in amplicon vectors (bacterial plasmids that contain the HSV-1 origin of replication and the HSV-1 packaging signal that are packaged into infectious HSV-1 virions). Recently, the full carrying potential of HSV-1 was realized by amplicon-mediated delivery and expression of the complete genomic human hypoxanthine phosphoribosyltransferase locus (115 kb) to cultured cells[22]. Wild-type HSV-1 is a neurotropic virus that can establish lifelong persistence in sensory neurons. This natural tropism has made neuropathological disorders one of the most promising applications of replication-defective HSV-1 vectors[23].

### Hybrid vectors

In the quest for better vectors, many researchers are attempting to combine the best features of different viruses in hybrid vectors. One of the most interesting hybrids couples the site-specific integration machinery of wild-type AAV with the efficient internalization and nuclear targeting properties of adenovirus. AAV is a helper-dependent parvovirus; in the presence of adenovirus or herpes virus infection it undergoes a productive replication cycle, but in the absence of helper functions the virus genome integrates into a specific site on chromosome 19 (19.13.3-qtr, also called AAVS1). Integration of the AAV genome into AAVS1 requires expression of the AAV Rep protein. As conventional rAAV vectors are deleted for all viral genes, including *rep*, they are not able to specifically integrate into AAVS1, but this potentially useful feature of the parental wild-type virus has been harnessed in hybrid vectors. Site-specific integration of AAV inverted terminal repeat (ITR)-flanked transgenes has been shown in cell culture from adenovirus vectors and from herpes-virus amplicon vectors that express the AAV Rep68/78 proteins[24,25].

Although hybrid vectors that contain AAV Rep could be useful for *ex vivo* transduction, their use for *in vivo* gene transfer might be limited as a result of the intracellular toxicity of the Rep proteins. Our laboratory has used a transposon approach to achieve integration from an adenovirus vector: we constructed a gene-deleted

Box 3 | **Selectively replicating viruses for cancer gene therapy**

The potential of gene-deleted viruses for tumour-specific replication was first shown with a herpes simplex virus-1 (HSV-1) vector that was deleted for thymidine kinase (*dl*sptk)[112]. Tk is essential for HSV-1 replication and endogenous levels are elevated in tumour cells. The *dl*sptk mutant was able to replicate in, and destroy, malignant glioma in an animal model, but the *tk* mutation rendered the virus insensitive to antiherpetic drugs, and biosafety concerns prompted the development of HSV-1 vectors that were deleted in alternative genes[113]. One such vector, which contains mutations in the *γ-34.5* gene (encoding the ICP34.5 product, which inhibits apoptosis by infected cells) and the gene encoding HSV-1 ribonuclease reductase (required for HSV-1 replication and elevated in tumour cells) is, at present, in Phase I/Phase II trials for recurrent malignant glioma.

Conditionally replicating adenoviruses (CRADs) also show great promise for tumour gene therapy. The first CRAD — *dl*1520, also known as ONYX-015 — incorporated a deletion in the *E1B-55kD* gene[114]. E1B-55kD binds and inactivates p53 during adenovirus replication and it was expected that *dl*1520 would only be able to replicate in p53-deficient tumour cells. The *dl*1520 mutant has proven to be tumour-selective in patients with advanced head and neck cancer[115], but controversy has raged over its mode of action. Contrary to expectations, *dl*1520 replication does not seem to be solely determined by the absence of p53, and other cellular factors involved in the p53 and RB pathways probably contribute to its tumour selectivity[116–119]. The *dl*1520 strain has shown antitumoral efficacy in clinical trials if used in combination with chemotherapy and is now in Phase II trials[29], but it replicates poorly in comparison with wild-type adenovirus and has not shown significant therapeutic benefit when used alone. As such, a repertoire of alternative adenoviruses with increased antitumoral potency has been developed and several strains are in early-phase trials for various types of cancer[17,28].

adenovirus vector that carried an hFIX transposon flanked by *Flp* motifs. Systemic delivery of this vector with a second gene-deleted vector that expressed the *Flp* and *Sleeping Beauty* recombinases resulted in the generation of transposon circles and the random integration of the *hFIX* gene in mouse liver. Therapeutic levels of hFIX were maintained for more than 6 months in the presence of extensive liver proliferation[26].

## Viral vectors for cancer gene therapy

According to the Journal of Gene Medicine Database, in March 2003, 63.4% of all gene-therapy clinical trials were for cancer. A number of different approaches to cancer gene therapy are being investigated, which mainly use replication-defective viral vectors to deliver anti-angiogenic factors, tumour-suppressor genes, prodrug-activating genes (such as HSV-1 thymidine kinase) and immunostimulatory genes[27]. An alternative approach to cancer gene therapy has been to harness the inherent ability of viruses to replicate and lyse cells[28]. Viruses have evolved to maximize their chances of replication by inducing changes in cellular metabolism that mimic changes that are acquired by transformed cells (for example, p53 inactivation). Approaches to achieve safe tumour-specific replication have generally involved the deletion of viral genes that are necessary for replication in normal cells, which creates a mutant virus that can only replicate in tumour cells in which the missing function is supplied.

A number of these selectively replicating viruses have been developed (BOX 3). Most oncolytic viruses have been engineered from adenovirus or HSV, although inherently tumour-selective viruses, such as Newcastle disease virus, reovirus and autonomous parvoviruses, are also being tested in trials[28].

Data from animal models and clinical trials have indicated that some of these oncolytic viruses will be effective tools for the treatment of solid tumours[29] (BOX 2; TABLE 1). The treatment of metastases will be a more substantial challenge; nevertheless, 'virotherapy' will probably become a viable alternative treatment for some types of cancer in the next few years.

## Hazards and hurdles

***The immune response.*** The 'Achilles heel' of gene therapy is that many of the immunological defence systems that are used to tackle wild-type infections are activated against the vectors and/or new transgene products that might be recognized as foreign. Adenovirus vectors are the most immunogenic of all the viral vector groups, and the largest hurdle that has faced gene therapists using adenovirus vectors is overcoming this immunogenicity. Adenovirus vectors induce multiple components of the immune response: cytotoxic T-lymphocyte (CTL) responses can be elicited against viral gene products or 'foreign' transgene products that are expressed by transduced cells, and the capsid itself — in the absence of viral gene expression — induces humoral virus-neutralizing antibody responses and potent cytokine-mediated inflammatory responses. Incoming adenovirus capsid components can also enter the major histocompatability complex (MHC) class I processing pathway and target transduced cells for recognition by pre-existing CTLs[30,31].

Progress has been made in reducing T-cell responses against viral gene products that are expressed by transduced cells, by engineering 'gutted' or 'helper-dependent' (HD) vectors that are stripped of all viral genes[32] (BOX 2; TABLE 1). This advance has improved the prospects of adenovirus vectors for long-term gene transfer and HD adenoviruses (HD-Ads) have facilitated life-long phenotypic correction in mouse models with negligible toxicity[33–35]. Elimination of all viral genes from the adenoviral vector genome has reduced vector-mediated cytokine responses after the systemic administration of HD vectors[33,36], but studies in the rat brain caution that these highly disabled vectors still retain the potential to induce a capsid-mediated inflammatory response[37]. The

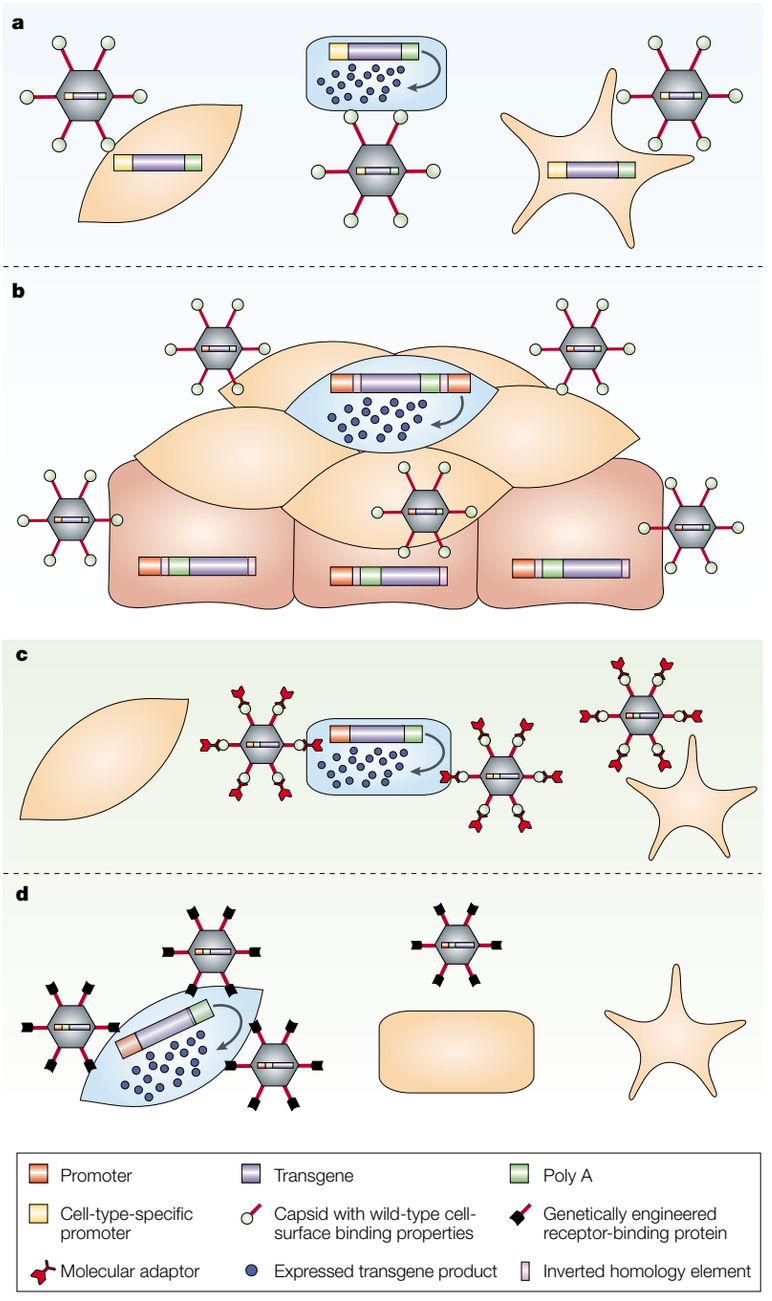


Figure 3 | **Strategies to achieve targeted gene expression from adenovirus vectors.** **a** | Transcriptional targeting is generally achieved by placing the transgene under the control of a cell-type-specific promoter. The viral particles infect many different cell types, but the transgene is not expressed in cell types that do not actively express the transcription factors that are necessary to drive expression from the cell-type-specific promoter. **b** | A new approach for achieving tumour-specific transcriptional targeting from a conditionally replicating adenovirus vector was recently proposed by Lieber and colleagues[120]. Their approach exploited homologous recombination between inverted homology elements to bring a promoter sequence into conjunction with a reporter gene — a process that was dependent on adenoviral genome replication that occurred specifically in tumour cells. **c** | Transductional targeting can be achieved by redirecting the vector capsid to new cellular receptors using molecular adaptors (usually bi-specific antibodies) that are conjugated to the capsid structure, or by genetically altering receptor-binding proteins in the virus capsid so that they recognize and bind to alternative receptors (**d**). Combining transductional targeting with transcriptional targeting can further increase the efficacy and specificity of viral vector-mediated transduction[53].

inappropriate activation of inflammatory responses can be highly dangerous; a massive systemic inflammatory response that was induced by an adenovirus vector led to fever, disseminated intravascular coagulation, multiorgan failure and the eventual death of a patient during a 1999 trial for ornithine transcarbamylase (OTC) deficiency (BOX 1).

As with all drug-induced toxicities, the degree to which viral vectors induce harmful immune-mediated and inflammatory responses and other toxic side effects is a question of vector dose. Studies in the immunoprivileged rodent brain (in which innate immune responses can be studied in isolation from adaptive cellular immune responses) have shown that inflammatory responses to the adenovirus capsid increase linearly with an escalation in vector dose[38], but the situation could be more complicated in other organs, particularly if the vector particles become disseminated into the circulation. Dose-escalation studies have shown that the relationship between adenovirus vector dose and direct cellular toxicity (as distinct from immune-mediated toxicity) is characterized by a 'threshold effect'; cellular toxicity occurs over a narrow dose range and often no symptoms are observed until a slightly higher vector dose is administered, which induces severe cellular injury[38,39].

The ability to accurately predict vector-related side effects at a particular dose is confounded in human studies by the degree of variability between immune responses in different individuals. The disastrous OTC trial of 1999 has made it clear that different patients have markedly different inflammatory and immune responses to the same dose of adenovirus vector (BOX 1). It is not yet clear how to predict which patients will mount severe inflammatory reactions, but in the light of the OTC trial, a National Institutes of Health (NIH) report recommended that "all research participants enrolled in gene-transfer clinical trials should be monitored for several types of acute toxicities before and after vector administration" and that monitoring "should routinely include a research participant's immune status, cytokine profile, and predisposing or underlying conditions that might elevate an individual's sensitivity to a particular vector"[40].

Other vector systems are less inflammatory and immunogenic than adenovirus vectors. Lentivirus vectors and AAV vectors in particular do not seem to induce inflammatory or immune responses against viral proteins, but T-cell responses can still be elicited against the expressed transgene product, particularly if the vectors transduce cells that are robust for antigen presentation, including DENDRITIC CELLS. The route of vector administration might affect the degree to which dendritic cells are transduced; route of administration has a profound effect on the development of T-cell responses to transgenes that are expressed from AAV vectors[41]. Pre-existing humoral immunity to the parental wild-type viruses is another obstacle that affects all classes of viral vector. Circulating virus-neutralizing antibodies can preclude efficient transduction with the viral vector. Humoral immune responses against adenovirus and

AAV vectors have been addressed by switching capsid SEROTYPES, but antibody responses towards secreted therapeutic proteins remain a theoretical problem for the long-term therapy of certain disorders[42,43].

***Specificity of transgene delivery.*** Natural infections with wild-type viruses are restricted to those tissues that are accessible through the route of transmission, but recombinant vectors are not subject to the same physical limitations. For example, adenoviruses and AAVs do not naturally infect the CNS, yet both these vectors efficiently infect neurons if they are injected into the brain. From certain perspectives, the promiscuity of viral vectors is more of a liability than a benefit, as the systemic delivery of vector generally leads to unwanted vector uptake by many different cell types in multiple organs. Even the local delivery of vector can lead to leakage and dissemination to other tissues. Transgene expression can be restricted to particular cell types and even switched on and off using tissue-specific and/or regulatable promoters (FIG. 3a) (reviewed in REFS 44,45), but dissemination of the vector particle itself can have harmful consequences; lack of adenovirus vector specificity was directly linked to the induction of the massive systemic immune response that caused the death of Jesse Gelsinger in 1999 (BOX 1; REF. 40).

As discussed in the previous section, the severity and risk of eliciting harmful immune responses and other toxic side effects is intimately connected with vector dose. Increasing the efficiency with which viral vectors infect specific cell populations will increase the safety of gene therapy by allowing lower viral loads to be administered. Modifying the vector capsid to achieve TRANSDUCTIONAL TARGETING (FIG. 3b) is therefore an important focus of vector development, to address the significant problem of nonspecific and/or inefficient uptake. Transductional targeting is a particular focus for cancer gene-therapy research, as tumour cells often downregulate the expression of the cellular receptors that are normally used by the virus for infection.

The simplest form of transductional retargeting, which requires little prior knowledge of specific virus–receptor interactions, is pseudotyping. Pseudotyping has been well established for retroviruses, but has similarly been used to create a chimeric adenovirus vector comprising the adenovirus type-35 fibre protein incorporated in a type-5 capsid[46]. AAV vector genomes flanked by AAV2 inverted terminal repeats (ITRs) have also been successfully cross-packaged in the capsids of alternative serotypes, creating a portfolio of pseudotypes with different specificities[47–49]. The limitation of pseudotyping for achieving transductional retargeting is that tropism is determined by the pre-existing specificities of the parental viruses.

A second approach to targeting vector capsids to distinct cell populations has been to conjugate capsids with molecular adaptors (usually bi-specific antibodies) with particular receptor-binding properties. This approach has been used to enhance the transduction of various cultured cell types using adenovirus[50], retrovirus[51] and AAV vectors[52], and has shown some limited efficacy with adenovirus and retrovirus vectors in animal models[53,54]. However, it remains to be seen whether conjugated vector production will be feasible for clinical trials. A third approach is to genetically engineer the capsid genes in such a way that normal receptor binding is abolished and/or a small peptide ligand for alternative receptor binding is incorporated into the capsid structure. This genetic approach to transductional retargeting has been successful at redirecting adenovirus vector tropism in cell culture, but few studies have evaluated the performance of these vectors *in vivo*. In one study, mutation of the fibre gene to replace the coxsackie-adenovirus receptor (CAR) binding motif with an integrin-binding motif increased gene transfer into CAR-deficient cell lines[55], whereas in another study, the ablation of CAR binding alone was sufficient to redirect adenovirus tropism in the brain[56].

Adenovirus vectors are relatively easily re-routed through alternative internalization pathways, but the genetic retargeting of other vectors is more difficult. The internalization of herpes viruses into cells is complex and is mediated by many different viral glycoproteins. Similarly, retrovirus receptor binding exposes FUSOGENIC domains in the viral envelope and, therefore, it is difficult to modify retrovirus binding without negatively affecting internalization[57]. The AAV capsid has been successfully genetically engineered but, in general, AAV engineering has been more 'hit-and-miss', as the AAV capsid does not easily accommodate heterologous peptides and modified vector particles are often unstable or defective at a step subsequent to internalization[58,59]. The recent determination of the crystal structure of the AAV2 capsid might facilitate this type of approach in the future[60].

Two novel approaches to genetic targeting allow nature to design and select functional and stable targeted viruses. The first approach used DNA family shuffling to genetically recombine envelope genes from six different strains of MLV, producing a library of $1 \times 10^6$ MLV variants containing chimeric envelope proteins. Screening by infection of Chinese hamster ovary (CHOK1) cells (which are normally refractive to MLV infection) yielded an infectious clone with altered tropism for CHOK1 cells[61]. In the second approach, a library of rAAV clones with randomized peptide insertions at a tolerant site in the viral capsid protein VP3 was generated, and infectious viruses with increased tropism for the B-cell lymphocytic leukaemia cell line Mec-1 or the human megakaryocytic cell line M07e were isolated[62]. Provided that a relevant selection system exists, vector libraries that are produced by DNA shuffling or randomized insertion can be screened for many different properties, and these powerful technologies will probably be used extensively in the future to overcome the present limitations of vector engineering.

The large body of data from many different studies has shown that it is possible to target vectors to new cell types, but it is difficult to prevent nonspecific uptake by other cell populations. Nevertheless, increasing the efficiency of transduction of the appropriate cell type through a combination of transductional targeting and the use of optimal promoters should reduce the

DENDRITIC CELLS
A subset of antigen-presenting cells, which are particularly active in stimulating T cells.

SEROTYPE
Related members of the same virus species that are distinguishable by serological methods.

TRANSDUCTIONAL TARGETING
The direction of vector-mediated transgene expression to particular cell types by the alteration of vector tropism.

FUSOGENIC
Facilitating fusion of the viral envelope with the cellular plasma membrane.

potential for toxic side effects by allowing lower doses of virus to be administered.

***Insertional mutagenesis.*** Integrating viral vectors, which are mostly derived from retroviruses, have been used for more than 10 years in clinical trials in attempts to obtain stable gene transfer in proliferating cells such as haematopoietic cells. The prevailing dogma held that retroviral vector genomes integrated randomly into host chromatin and the risk of disrupting a cellular sequence connected with malignancy was predicted to be in the region of 1 in 10 million insertions[63]. Even though more than 10 million cells are typically modified with retroviral vectors during *ex vivo* gene transfer, the risk of inducing cancer was considered to be negligible, as oncogenesis usually requires multiple genetic lesions. This viewpoint was reinforced by the fact that vector-induced cancer had never been observed in any of the hundreds of patients that were treated with retroviral vectors in many different gene-therapy trials.

Recent evidence from a number of separate studies has challenged our perceptions about the risks of using integrating retrovirus vectors for certain types of gene therapy[64,65]. One study showed that the transplantation and expansion of a clone of retrovirally transduced bone-marrow cells had induced leukaemia in mice[66]. The transgene used in this study might itself have had growth-promoting properties, but the development of cancer in this case is thought to have been a particular consequence of cooperation between the transgene product and the fact that the retroviral integration event had disrupted the gene encoding a transcription factor that has been implicated in the pathogenesis of acute myeloid leukaemia[67]. The findings in this study were ominously echoed when it was discovered that 2 of the 11 patients treated during the successful severe combined immunodeficiency (SCID)-XI trial[64] had developed a leukaemia-like disorder, which was apparently caused by retroviral vector genome integration in, or near, the oncogene *LMO2* (BOX 1)[65]. A third study has heightened these concerns by showing that retroviral integration is not as random as was previously thought; the analysis of hundreds of human immunodeficiency virus (HIV) integration sites in cell culture showed that HIV is more likely to integrate into transcriptionally active genes than into non-coding regions of chromatin[68].

In the SCID-XI case, there was clearly selection for transduced cells to proliferate, which might have favoured the development of malignancy (BOX 1). The question that gene-therapy researchers must now address is whether similar risks exist for other applications of gene therapy using integrating vectors. Clonal expansion (required in *ex vivo* haematopoietic gene-therapy applications) seems to be a risk factor that contributes to cellular transformation and it is improbable that integrating vectors would induce cancer in non-dividing tissues in individuals with functional immune systems, in which cell proliferation was not a therapeutic end point. Nevertheless, much interest will probably focus on making existing integrating vectors safer (for example, by engineering SUICIDE GENES into the vector backbone to provide a self-destruct mechanism in case of oncogenesis) and on developing new vector systems that are capable of mediating integration into specific predetermined sites. Recently, the site-specific integration machinery of bacteriophage ΦC31 has been exploited in non-viral delivery approaches to achieve the targeted integration of transgenes in mice and human cells[69–71]. Incorporating the ΦC31 integrase system into a viral vector is an obvious next step.

Initially hailed as the safest of gene-therapy vectors, rAAV vector integration has also received a share of scrutiny. A recent study has shown that rAAV genomes share with retroviral genomes a predilection for integrating into genes rather than non-coding regions of the host chromatin (H. Nakai and M.A.K., manuscript in preparation). As the frequency of rAAV integration *in vivo* is low (<10% of persistent vector genomes are integrated in the liver[72]) and most applications of rAAV vectors target non-proliferating cells, the risks that are associated with rAAV integration will be much lower than those for retroviral vector applications. Nevertheless, unlike retrovirus integration, the analysis of rAAV integration sites has shown that the integrated rAAV genomes are frequently associated with chromosomal rearrangements and deletions of large segments of chromosomal DNA[73]. Integration of rAAV genomes into the host chromosomes is thought to occur through NON-HOMOLOGOUS END-JOINING (NHEJ) of rAAV free-ends with broken chromosomes. A current topic of study is whether the rAAV free DNA ends induce chromosomal damage, or whether they are simply fused by NHEJ to pre-existing chromosomal breaks. Two pieces of evidence from cell culture and mouse studies indicate the latter: genotoxic agents that induce double-strand breaks increase rAAV integration in cell culture[74,75], and increasing rAAV vector dose above a threshold level does not increase the number of integrated genomes in mice[76].

## Perspectives and future directions

The focus of the past few years on developing better vectors is beginning to translate into some encouraging preliminary results in the clinic[20,29]. Great advances have been made in the following areas: the creation of new systems for the efficient production of gene-deleted less-immunogenic vectors; improvement of the efficiency of the *ex vivo* transduction of haematopoietic cells; improvement of the specificity and efficiency of *in vivo* transgene expression through the optimization of tissue-specific and inducible promoters; expansion of the repertoire of vector tropisms and the evasion of pre-existing immune responses through the development of alternative viral serotypes; the identification of new virus species for vector development (for example, Epstein–Barr virus[77], foamy viruses[78], SV-40 (REF. 79), α-viruses[80] and negative-strand RNA viruses[81]) and the identification of disease targets that can be realistically tackled given the present limitations of viral vectors.

Many hurdles remain to be overcome. Important concerns have emerged over the safety of present integrating vector systems that are based on retrovirus vectors. It remains to be seen whether integrating vectors

SUICIDE GENE
A gene that encodes a protein that can convert a non-toxic prodrug into a cytotoxic compound.

NON-HOMOLOGOUS END-JOINING
(NHEJ). One of two cellular DNA-repair pathways that are involved in the repair of double-strand breaks.

that are based on transposases or other integrases will show the same predilection for inserting into active genes as do retrovirus-based systems. The wealth of genetic information that has become available from the sequencing of the mouse and human genomes will be fundamental to addressing these issues. To deal with concerns over the potential risk of an integration event disrupting an oncogene, much interest will probably focus on developing alternative vector systems that are able to achieve targeted integration into a single predefined benign site in the genome. Particular promise lies with incorporating sequence-specific integrases, such as the bacteriophage ΦC31, into viral vectors. In fact, ΦC31 integrase targets multiple sequence-specific sites in the genome. It is estimated that there are between 100 and 1,000 pseudo-attP sites into which the ΦC31 integrase can mediate genome insertion, probably with different efficiencies[82]. It might be possible to restrict integration into a smaller subpopulation of these sites by directed evolution or directed mutagenesis of the integrase protein[83].

As more work is needed to develop site-specific integrating vectors, more work is also needed to improve the ability of vectors to home in on and infect specific target-cell populations. Understanding how to predict the response of individual patients to inflammatory vectors also remains a substantial challenge. Preclinical studies in large-animal models are important for evaluating vector performance and efficacy, but because human immune responses are more variable than those observed in animal models, it is difficult to make complete predictions on the basis of preclinical trials. One of the most important areas for gene-transfer research will be to dissect and understand these vector–host interactions. Monitoring pre-existing immunity to parental wild-type viruses will probably be an important component of patient evaluation in future clinical trials. Rigorous and uniformly recognized standards for measuring vector potency and concentration also need to be introduced to allow meaningful data comparison across different clinical studies.

Several gene-therapy applications seem promising in early-phase clinical trials, including the treatment of haemophilia B using rAAV, the treatment of certain types of cancer using conditionally replicating oncolytic viruses and the treatment of vascular and coronary artery disease using viral vectors that express angiogenic factors. The next few years will probably bring another gene-therapy success in one or more of these areas, but the future might also see new disease targets becoming amenable to gene therapy through the fusion of viral vector-mediated gene transfer with new technologies such as RNA interference (RNAi). RNAi technology has already been incorporated into adenovirus[84], lentivirus[85] and retrovirus[86] vectors and used to knockdown gene expression in cell culture and in experimental animals. This powerful tool to achieve gene silencing will probably be evaluated in many viral vector systems, and be used to develop therapies for a range of diseases, including dominantly-inherited genetic disorders, infectious disease and cancer.

At the present time, viral vectors are the best available vehicles for efficient gene transfer into most tissues. Non-viral gene delivery is potentially safer than viral-mediated delivery, but — with the exception of a few promising applications, such as vaccines[87] — non-viral systems are, at present, limited by their inefficiency. As we continue to unravel and understand the biological mechanisms that underlie virus entry into cells, transport of viral particles to the cell nucleus and the persistence of viral DNA, we will be able to apply this knowledge to the development of more efficient non-viral vectors that will ultimately rival virus-based systems. In the long term, gene-therapy vectors will probably be very different from those that are in use today. Vectors will be tailor-made for each specific therapeutic application, possibly even for each individual patient, and might combine non-viral delivery technology with properties from different viruses, some of which have yet to be exploited or even discovered. Whatever the gene-therapy vectors of the future look like, they must all achieve a specific set of functions: they must target specific populations of cells in a target tissue, preferably after delivery by a non-invasive route, and they must express therapeutic levels of transgene expression in a safe and regulated manner for the appropriate length of time.

There is still a tremendous amount of work to be done in gene-therapy research. We have encountered many obstacles so far, and will probably encounter more, but these obstacles are not insurmountable. By continuing to identify and address potential hurdles and by maintaining a strong focus on improving vectors, gene therapy will surely improve the outcome of a range of diseases.


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