State-of-the-art gene-based therapies: the road ahead

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Abstract | Improvements in the gene transfer vectors used in therapeutic trials have led to substantial clinical successes in patients with serious genetic conditions, such as immunodeficiency syndromes, blindness and some cancer types. Several barriers need to be overcome before this type of therapy becomes a widely accepted treatment for a broad group of medical diseases. However, recent progress in the field is finally realizing some of the promises made more than 20 years ago, providing optimism for additional successes in the near future.

Zinc finger nucleases
Engineered DNA-binding proteins that produce double-strand breaks at specific sequences. They can be used to correct or induce mutations in genomic DNA.

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The idea of gene-based therapeutics has been around for some time, but it only received serious attention with the advent of recombinant DNA technology and the ability to transfer and express exogenous genes in mammalian cells. The first clinical trials were carried out in the late 1980s, and at that stage it was predicted that gene therapy would become a treatment for serious diseases in just a matter of years. However, during the ensuing two decades, numerous obstacles have tempered the enthusiasm for gene therapy. More recently, some important technical barriers have been overcome to the point where successful examples exist of treating specific diseases, as well as encouraging new preclinical trials that will expand on the number of treatable disease-targets.

This Review provides insights into the state-of-the-art accomplishments made with gene-based therapies and the major barriers that need to be overcome before they are more widely implemented by the medical community. The Review starts by describing the general approaches used — highlighting the growing application of therapies involving the transcription of non-coding RNA — and some of the practical considerations that are common to all gene transfer studies. The most clinically relevant vectors are discussed, providing examples of current successes (such as higher gene transfer efficiency and lower immunological responses) and the areas in which more effort is required to overcome technical barriers. The reader is referred to three other Reviews in this Focus issue that cover the use of viral vectors[1], silencing-based therapies[2] and cell-based therapies[3] in greater detail. I end with some thoughts on the prospects for the future applications of gene therapy in the era of personalized genomic medicine.

Although gene therapy has had a rocky course — which has been exacerbated in part owing to scepticism from some parts of the scientific community — it is now emerging as a promising discipline as a result of technical advances and the successful treatment of several devastating medical conditions.

The goals of gene therapy

Gene-based therapeutics is broadly defined as the introduction, using a vector, of nucleic acids into cells with the intention of altering gene expression to prevent, halt or reverse a pathological process. Here, I restrict the definition to include exogenous nucleic acids that provide a transcriptional template for the expression of a protein-coding or non-coding nucleic acid.

Gene therapy can be carried out by three routes — gene addition, gene correction/alteration and gene knockdown — that are sometimes used in combination. The vectors can be administered *in vivo* or *ex vivo* using autologous cells derived from an individual patient. Depending on the vector, the therapeutic DNA either integrates into host chromosomal DNA or exists as an episomal vector.

Gene addition and correction. Of the approaches mentioned above, gene addition is the most commonly attempted in current preclinical and clinical studies; it is used to provide therapeutic benefit or to supply a protein that is missing owing to genetic mutation.

The least common of the techniques, gene correction/alteration, has gained a lot of attention and has been covered in a recent Review[4]: in this approach, zinc finger nucleases and DNA recombination technologies are used to alter genomic sequences to correct a

mutation or create a mutation (for example, a mutation in C-C chemokine receptor type 5 (*CCR5*) can make cells resistant to HIV infection).

Gene knockdown. Newer tools for modulating single genes or complex gene networks have renewed enthusiasm in gene-based therapeutics owing to the elucidation of microRNA (miRNA)-mediated gene regulation circuits and the ability to virtually eliminate a gene product using RNAi. RNAi can be induced by expressing short hairpin RNAs (shRNAs) or by delivering short dsRNAs that have complete complementarity to a target mRNA. miRNAs generate similar small RNAs but downregulate genes by a non-cleavage-dependent degradation pathway and/or slowed translation of mRNAs. Compared to short RNAs that induce RNAi, miRNAs have limited mismatches to their mRNA target sequences, and although gene repression is less robust compared to RNAi, each individual miRNA sequence can fine-tune hundreds or even thousands of genes. miRNA sponges are a similar tool: they bind to specific miRNAs and reduce their effective concentration, and so restore or enhance protein production from the miRNA-targeted mRNAs.

Other intriguing new classes of nucleic-acid-based therapies are also amenable to gene therapy. These include non-coding RNAs that change or restore a particular protein function by altering splicing (for example, through exon skipping)[5], small RNAs that can upregulate gene expression[6] and RNA aptamers[7].

I concentrate on gene addition and miRNA/RNAi approaches because these are the most likely to provide clinical benefit in the near future. In terms of non-coding RNAs, I focus on small RNAs that are transcribed from transduced vector genomes, rather than RNAs that are delivered via macromolecular complexes. Ultimately, the disease and target organ will dictate whether transcription-based or delivered non-coding nucleic-acid-based therapies are more effective for a specific treatment. Many of the barriers associated with using vectors apply regardless of whether they express non-coding RNAs or proteins.

Dysregulation of miRNAs can cause or participate in serious medical diseases; gene replacement therapy that combines protein encoding and non-coding elements is likely to be the most useful in some complex human disorders that have a large polygenic origin (for example, cancer).

Technical barriers to successful gene therapy

The initial creation of gene transfer vectors made gene therapy conceptually simple. The various vectors' history, creation and use have been reviewed[8]. Over the past 10 years, the improvement in gene transfer into cells and/or tissues *in vivo* has resulted largely from advances in vector technologies that include refined vector systems, improved production methods, enhanced transduction rates and greater vector–host safety profiles. Nevertheless, not all of the problems and limitations have been solved, and in fact new, unanticipated barriers have emerged as clinical trials have proceeded[8]. The four main barriers are described below and in FIG. 1 (see also REF. 8).

Vector uptake, transport and uncoating. To be successful, a gene therapy vector that is administered into the body by localized means (for example, tissue injection) or systemic means (for example, intravenous infusion) must be taken up by the target tissue to allow for therapeutic levels of transgene expression. The distribution of the vector after administration is influenced by many parameters, including the vascular supply and endothelial barriers to a particular organ, vector size and interactions between the vector ligand and the host cell receptor. Depending on the vector, the target and the transgene product, the degree of inadvertent vector uptake by other tissues will vary and affect the tolerable levels of toxicity. Ultimately, optimal vectors would be cell-type-specific. Despite a great deal of effort, it has been technically challenging to design either non-viral vectors with specific ligands or viral vectors with re-engineered capsids or envelope proteins that would re-target the vectors to a specific cellular receptor; these designs have therefore not shown much success in clinical applications. The re-engineered vectors are too large, too unstable or are otherwise unable to progress from the cytoplasm to the nucleus. Viruses have evolved efficient mechanisms for entering the cell and the nucleus, but the major two barriers for non-viral vectors, assuming they make it through the cell membrane, are the inability to escape from the endosome and the inability to enter through the nuclear membrane.

Vector genome persistence. For some vector types, the active exogenous DNA molecule can exist as an episome, which will be lost during cell division. However, episomal vectors can persist for life in rodents and for many years in larger animals if they are delivered into relatively quiescent tissues, such as liver, brain, heart or muscle, perhaps because these cells turnover very slowly[8,9]. Vectors that integrate into the host chromosome are better candidates for targeting cells that have more rapid turnover (for example, haematopoietic cells), although they have the disadvantage of potentially activating or disrupting nearby genes by insertional mutagenesis.

Sustained transcriptional expression. Transgene expression — whether from integrated vector genomes of from persisting episomal genomes — can be extinguished by epigenetic modifications to the vector genome. In general, the duration of transgene expression should match the period of time required to treat the specific disease. In most cases, genetic diseases require lifelong expression, which can be achieved by vector re-administration or stable expression after single-vector infusion. By contrast, acquired disorders such as infection or cancer may require more limited periods of expression.

The host immune response. One of the major barriers to successful gene therapy is caused by activation of the host immune response, which can be directed against the transgene product and/or vector particles (BOX 1). A better understanding of the range of host immune responses is being used to inform innovative strategies for restricting vector delivery and transgene expression to the target cell type (or types).

RNAi
(RNA interference). The process by which the introduction or expression within cells of dsRNA leads to the sequence-specific cleavage and degradation of a target mRNA and therefore to gene suppression.

miRNA sponges
Exogenously delivered or expressed non-coding RNAs that bind and inhibit microRNA function in a sequence-specific manner.

RNA aptamers
Short RNAs selected from large libraries that, owing to their three-dimensional structure, bind to and activate or interfere with protein function and/or direct a macromolecular cargo (for example, small interfering RNAs) into cells via a targeted receptor.

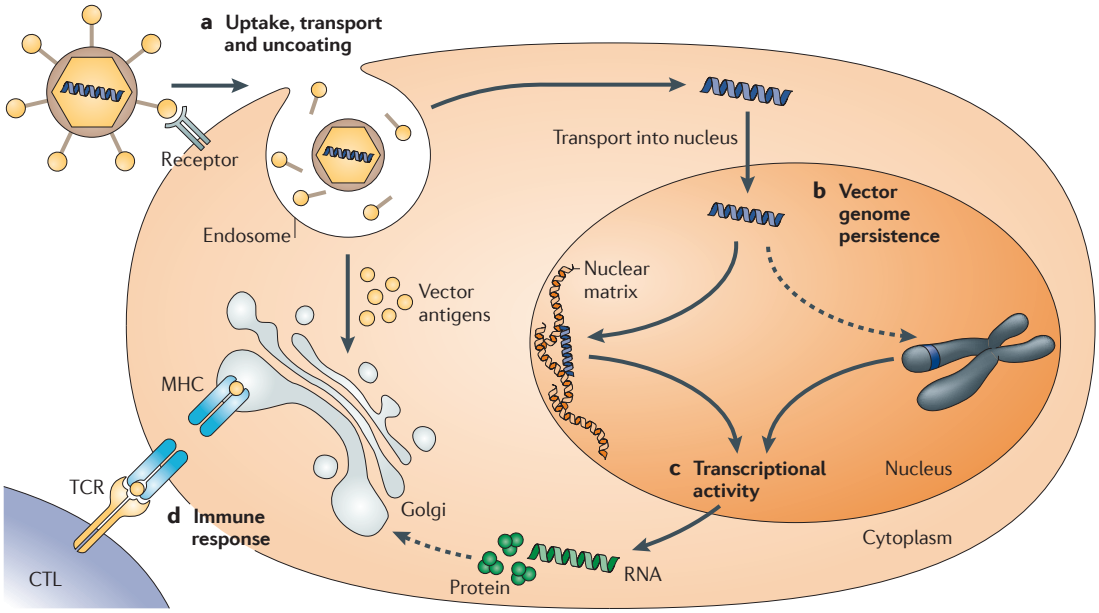

Figure 1 | **The four barriers to successful gene therapy. a** | Uptake, transport and uncoating. Vectors bind to a cellular membrane and are internalized by various processes. Most uptake steps involve a ligand–receptor interaction. Once internalized, most vectors enter the endosome and undergo a complex set of reactions that can result in their full or partial degradation. Viruses have evolved effective mechanisms for escaping from the endosome; for example, adenoviruses lyse the endosome. Transport to the nucleus is also required for successful therapy. **b** | Vector genome persistence. Once the vector reaches the nucleus, it can be further processed. Depending on the vector, the DNA can exist as an episomal molecule (and associate with the nuclear matrix) or it can be integrated (by covalent attachment) into the host chromosome. **c** | Transcriptional activity and transgene persistence are dependent on many factors, as described in the main text. **d** | The immune response can limit the viability of the transduced cells and/or the expression of the transgene product. CTL, cytotoxic T cell lymphocyte; MHC, major histocompatibilty complex; TCR, T cell receptor.

Possibly the biggest hurdle is the inability to predict both innate and antigen-dependent immune responses in humans, some of which cannot currently be replicated in animal models. Clinical success more generally is also being hampered by the inability to accurately correlate animal and human studies: it is currently not possible to know whether vector-based gene transfer efficacy in humans will reflect that seen in non-human species.

Retroviral vectors

Retroviral vectors are produced at relatively low titres, require proviral integration into the host chromosome for transduction, and most only infect dividing cells. These properties restrict most applications to *ex vivo* gene transfer approaches. Directly administering retroviral vectors into mammals, including humans, has generally been inefficient and subtherapeutic except under conditions that would not be amenable to clinical implementation[10]. One advantage of retroviral vectors is that they can be modified to infect non-rodent mammalian cells. Another advantage is that lentiviral vectors, most of which have been derived from HIV, can transduce cells that do not undergo cell division.

Mouse Moloney retroviruses and severe combined immune deficiency. Retroviral vectors derived from mouse Moloney retroviruses were the first vectors to be used in US Food and Drug Administration (FDA)-reviewed clinical trials. These viruses were chosen because their viral coding sequences (which are supplied in *trans*) can be easily replaced with therapeutic sequences and they can be pseudotyped with envelope proteins that allow vector entry into non-mouse cells[11,12]. The integration of these vectors into host DNA makes them desirable for treating genetic diseases that require permanent gene modification of cells. The permanency of gene correction will be influenced by the ability to transduce either the earliest progenitor cells or differentiated cells whose offspring are not dependent on progenitor cells. Permanency will also be influenced by any selective advantage or disadvantage conferred by the insertion of the transgene, and/or the degree of epigenetic silencing.

Because transduction of haematopoietic stem cells is inefficient, even *ex vivo*, most therapeutic applications involve conditions that can be cured by correcting only a small percentage of progenitor cells. The first successful cure came in 2000 when it was demonstrated that autologous transplantation of retrovirally transduced bone marrow cells expressing the common cytokine receptor interleukin-2 receptor, gamma (*IL2RG*) resulted in a functional immune system in children with X-linked severe combined immune deficiency (SCID)[13]. The success of the gene therapy was tempered by the fact that 5 out of the 20 patients who were successfully treated for this type of SCID developed leukaemia; this was due

Pseudotyping
The use of an unnatural or unmatched envelope or capsid protein to package a viral genome.

Severe combined immune deficiency
(SCID). A lethal disease caused by the lack of B cell and T cell immunity. The disease is caused by a deficiency of one of several genes. It is commonly referred to as the 'bubble boy' disease.

Box 1 | **Dealing with the immune response**

Immune responses can be divided into three types — innate, humoral and cell-mediated — and can be directed against the vector and/or the transgene product.

Pre-existing or primary humoral responses directed against the vector can limit vector efficacy or its re-administration, whereas a humoral response against the transgene product can neutralize the therapeutic protein. The cell-mediated response against the vector particle or transgene product can eliminate the transduced cells, whereas the innate response can cause local and/or systemic toxicity and enhance a secondary antigen-dependent immune response. Innate responses to directly delivered small RNAs have been observed, but not to the transgenes that express small, non-coding-RNA-based therapeutic agents.

Immunity against the vectors

As with most wild-type viral infections, vectors are subject to humoral immune responses that would probably limit any strategy that required re-administration. In addition, the conversion of human-derived viruses into vectors may limit their usefulness in patients who have been exposed to the wild-type virus before the administration of the vector. This brings into question the validity of using common human adenoviral serotypes (for example, serotype 5), for which most adults have detectable neutralizing antibodies. This is especially relevant in some cancer trials, in which multiple doses of vector are administered over a couple of weeks. There are over 40 human adenoviral serotypes, and many of these serotypes infrequently infect humans, making them potentially better candidates for gene therapy[107].

The same is true of other viral vectors. Most adults have natural humoral adeno-associated virus 2 (AAV2)-specific antibodies as a result of naturally occurring and innocuous infection[108,109]. However, there is discordance in the estimates of individuals with neutralizing AAV8-specific antibodies. Given that natural AAV8 infection is restricted to non-human primates, the neutralizing AAV8-specific antibodies that are observed might have been mounted to other human AAVs that contain AAV8-specific epitopes.

Non-viral vectors may induce humoral responses directed against a protein, carbohydrate and even lipid component, thus limiting the number of times that the vector might be delivered.

Immunity against the transgene

Humoral immunity directed against the transgene product can also be problematic. The probability of such a response will be influenced by the amount of gene product made in a specific tissue and whether that tissue is a natural site for synthesis. If the vector is inadvertently taken up by non-intended tissues, such as dendritic cells or robust antigen-presenting cells (APCs), there is a higher likelihood that an immune response will occur. The best solution would be to engineer vectors that specifically target their intended cell type. Until this can be accomplished, restricting expression of the transgene via a tissue- or cell-specific promoter is the only available means to limit expression to target cells.

Another clever strategy to avoid transgene expression in APCs is to take advantage of the differential expression of specific microRNAs (miRNAs) in different cells. By placing an miRNA target into the 3′ UTR of a transgene minigene, expression of the transgene can be mitigated in unintended cells because the mRNA target responds to a specific miRNA that is normally expressed in the unintended but not the intended cell target. Thus, the mRNA is degraded before translation in the APC[110,111]. In some animal models, such an approach has reduced humoral immune responses directed against the transgene product when delivered by lentiviral vector *in vivo*.

to the clonal expansion of haematopoietic-cell-derived cells containing an integration of the proviral retrovirus and the activation of the LIM domain only 2 (*LMO2*) proto-oncogene[14,15]. One patient who was treated for another immunodeficiency syndrome, Wiskott–Aldrich syndrome, also developed *LMO2*-dependent leukaemia[16].

The current prevailing hypothesis is that *LMO2* and the growth-promoting *IL2RG* transgene act synergistically to promote clonal expansion and the subsequent outgrowth of oncogenic cells. This implies that the risk of leukaemia is transgene-specific. Indeed, several children with clinically indistinguishable SCID caused by a deficiency in the adenosine deaminase (*ADA*) gene have been treated and none to date has developed leukaemia[15]; this is because, unlike *IL2RG*, the *ADA* gene does not have an independent growth-promoting activity. However, using a similar vector, non-malignant cellular clonal expansion has been observed in gene therapy trials for chronic granalomatous disease[17].

The safety of Moloney retroviruses remains a cause for concern. The integration profile for these vectors is widespread, but it is not entirely random[18–20]. Moreover, the retroviral long terminal repeat (LTR) can activate neighbouring genes. Moloney vectors with a self-inactivating LTR might be safer in this regard and are currently being tested in clinical trials for SCID[15]. Although Moloney vectors are still being used in clinical trials, the field is moving towards the use of lentiviral vectors.

Lentiviral vectors. Unlike their mouse-based vector counterparts, lentiviral vectors have the advantage of being able to transduce non-dividing cells, and of possessing an LTR that lacks a robust enhancer.

However, the true degree of safety of lentiviral integration is unknown. In a recent study, the transduction of haematopoietic progenitor cells with a lentiviral vector expressing a β-globin cDNA successfully reversed the transfusion dependency of one patient with β-thalassaemia-based anaemia[21]. In this patient, 10% of the erythroid cells contained the vector, but in 3% of cells the vector had integrated into the high mobility group AT-hook 2 (*HMGA2*) gene, resulting in dysregulation of this chromatin-modifying protein, which has been linked to cellular de-differentiation and metastasis of solid tumours[22]. However, at 33 months, this patient had no evidence of malignancy.

Conversely, no predominant clones containing the integrated lentiviral provirus were seen (after a 2 to 4 year follow-up) in transduced haematopoietic cells in patients who were treated for X-linked adrenoleukodystrophy (X-ALD)[23]. The progression of neurodegeneration was halted in two male infants (and more recently a third) who were treated with autologous $CD34^+$ haematopoietic cells transduced with a lentiviral vector expressing ATP-binding cassette, subfamily D, member 1 (*ABCD1*). This response was at least as good as that obtained with allogenic bone marrow transplantation.

It is still not clear whether the different integration site preferences of lentiviral vectors and Moloney retroviral vectors influence potential oncogenic risks[20,24]. Vectors that promote site-specific or selected integration are highly desirable and are discussed in a later section.

Cancer and HIV therapies. Of the ~70 active clinical gene therapy protocols (see the ClinicalTrials.gov website), most continue to be directed towards cancer. This is because of the obvious unmet needs of this condition as well as the interest of major pharmaceutical companies. Some of the different vectors are being used in cancer applications, and some examples are described in BOX 2.

X-linked adrenoleukodystrophy
An X-linked, neonatal, neuronal de-myelinating disorder.

RNA decoys
RNA molecules designed to bind and inhibit a biologically active RNA from binding to its target.

Epidermolysis bullosa
An inherited connective tissue disease resulting in mild to severe (fatal) skin blisters owing to a mutation in collagen or keratin genes.

HIV-based lentiviral vectors have also been used in an attempt to eliminate HIV in humans. One of the more recent attempts involved the construction of a vector expressing three types of non-coding RNAs (RNAi, ribozymes and RNA decoys) that were engineered to downregulate expression of *CCR5* (the HIV receptor) and replication of HIV[25]. The strategy in this clinical trial is to use this vector to transduce $CD34^+$ cells in HIV-positive patients who require an autologous bone marrow transplant for AIDS lymphoma. Even a low-dose infusion of gene-modified cells resulted in long-term (24 months to date) detection of the gene-modified cells and the expression of the encoded small RNAs[25]. Further trials with transplantation of a larger number of transduced cells will be required to establish the effectiveness of this approach.

Not all retroviral trials involve transducing haematopoietic cells. In an ongoing example of retrovirus-based treatment, human skin cells from an epidermolysis bullosa patient were implanted after *ex vivo* transduction of a corrective retroviral vector[26–28]. However, for successful clinical intervention, a means to genetically modify large areas of skin will be required. This may be achieved by multiple engraftments of skin patches, as used in individuals who are treated for severe burns.

Even though Moloney retroviral vectors are still in use, the number of trials using lentiviral vectors is increasing at a greater rate than the number of trials using other retroviral vectors. Two properties make lentiviral vectors more efficient at gene transfer than Moloney retroviral vectors in most experimental protocols. First, lentiviral vectors can transduce non-dividing cells, and second, they can easily be pseudotyped with the VSV-G (vesicular stomatitis virus G) envelope protein, allowing them to be concentrated to titres about three orders of magnitude higher than routine Moloney vectors. Foamy retroviral vectors are another group of vectors that are derived from a non-pathogenic human retrovirus, but these vectors have not yet gained widespread use[29]. It is likely that there will continue to be a niche for retroviral vectors in *ex vivo*-based therapies in the immediate future.

Adenoviral vectors

A stormy history. The 36 kb genome of adenoviral vectors provides ample space for inserting large sequences, and the vector can be purified to high titres relatively easily. The vector genome remains episomal and, in the absence of an immune response, stable therapeutic levels of plasma proteins (such as coagulation factors, α1 antitrypsin, erythropoietin and metabolic enzymes) have been achieved throughout the lifetime of a mouse when delivered into relatively quiescent tissues such as the liver, muscle or brain[8].

Although recombinant adenoviral vectors were the first to result in high levels of systemic gene transfer into many tissues in mammals, when delivered systemically they can induce severe toxicity at the dosage levels that are required for efficacy, especially in humans. Toxicity induced by adenoviral vectors stems from an immediate innate immune response and a secondary antigen-dependent response. As examples, pulmonary administration of the vector caused self-limiting toxicity in a patient with cystic fibrosis, and one patient with ornithine transcarbamylase deficiency died after intravenous infusion of a vector designed to treat this condition. The death was probably due to a systemic cytokine response to the vector[30–32]. However, even with this history, cumulatively these vectors have been the most frequently used in clinical trials, just surpassing retroviral vector trials[33].

Box 2 | **Cancer gene therapy**

Several approaches to cancer gene therapy have been tested in preclinical and clinical trials. These include: immunological strategies based on the expression of robust antigens and/or local cytokine production; the expression of anti-angiogenesis proteins or pro-drug suicide genes; the expression of tumour suppressor protein products, microRNAs (miRNAs) and/or miRNA 'sponges'; RNAi-mediated cleavage of oncogene-encoded mRNAs; and the stimulation of lytic replication in tumour cells, which leads to cellular lysis[112].

Below are some of the most interesting recent examples of cancer gene therapy.

Retroviral vectors

In melanoma, the expression of highly reactive T cell receptors directed against tumour antigens in autologous lymphocytes has led to an initial complete tumour regression in two patients[113]. Similarly, in neuroblastoma, clinical responses were observed using vectors to express tumour antigens[114]. In an ongoing lymphoma study, autologous cytotoxic T cell lymphocytes (CTLs) enriched against specific Epstein–Barr virus antigens (which are present in many of the lymphomas) have provided a complete remission in 50–60% of patients for at least 1.5 years. Some of the patients who were initially resistant to the CTLs and/or had relapsed have experienced partial or a full remission when their CTLs were transduced with a retroviral vector expressing a transforming growth factor-β (TGFβ) dominant-negative protein before transplantation. As a result, the immunosuppressive effects of the wild-type TGFβ were dampened, allowing for a more robust CTL response against the tumour cells[115].

$CD19^+$ leukaemias or lymphomas that are resistant to therapy are being treated with autologous T cells that are engineered to express a chimeric antigen receptor; this receptor targets CD19 that is attached either to the CD3 or CD3:4-1BB signalling domains. To date, 3 out of 3 patients have shown a clinical response (C. June, personal communication).

Although these early studies are encouraging, more patients and additional time are required to determine the overall effectiveness (the percentage of patients with a clinical response) as well as the persistence of the clinical response or remission in responders.

Adenoviral vectors

The localized tissue toxicity caused by adenoviral delivery may enhance anticancer strategies. There have been many reports related to adenoviral anticancer strategies. An adenoviral vector expressing the tumour suppressor gene or an oncolytic adenovirus modified to only replicate in specific tumour types were originally reported to be efficacious in clinical trials for head and neck cancer[116], especially when used in combination with radiation; however, there is much doubt as to the validity of these early claims, justifying the need for additional trials.

Adenoviral vectors expressing granulocyte-macrophage colony-stimulating factor (GMCSF) as a cancer vaccine have shown mixed results for the treatment of various tumours. More recently, a strategy that combines GMCSF production and a means to restrict vector replication to tumour cells has given some hints of efficacy in several different cancer trials[117,118].

AAV vectors

The properties of adeno-associated virus (AAV) vectors have made them less desirable for many of the anticancer therapies. However, the ability to use AAVs to express RNAi to knock down gain-of-function proto-oncogenes has potential as a therapeutic approach. One of the more interesting uses of AAVs as an anticancer therapy is based on the expression of miR-26a (a regulator of the cell cycle), which can inhibit hepatocellular carcinomas in mice[119].

The prototype adenoviral vector is derived from adenovirus type 5; because most adults have been exposed to and therefore have immunity to this particular serotype, its usefulness in human applications might be limited. Pseudotyping or changing serotypes is technically more difficult in adenoviruses compared to retroviruses and adeno-associated viral vectors[34].

Improvements to vector design. The first-generation recombinant vector retained many wild-type viral genes, which are expressed at low levels in transduced cells and can cause toxicity. The deletion of the viral master transcriptional E1 genes is insufficient to eliminate expression of the other early and late viral gene products. The amount of toxicity resulting from the particle and from leaky expression of the adenoviral proteins is not entirely clear and is influenced by the host species, the route and dose of administration, and the degree of vector purity.

Second- and third-generation adenoviral vectors had additional viral gene deletions; however, the least toxicity occurred when all of the viral coding sequences were removed by using a helper-dependent packaging system. The production of the helper-dependent vectors is more complex than that of most viral vectors developed for animal and human studies[30]. These vectors are not entirely devoid of toxicity and have not yet been administered systemically in humans. In rodents, adenoviral vectors result in large changes in endogenous gene expression profiles regardless of whether viral genes are deleted in the vector[35]. The final verdict has not yet been given on the human safety of helper-dependent adenoviral vectors.

Therapeutic applications. The high efficiency of transgene expression by adenoviral vectors has made them attractive as an *in vivo* oncology target (BOX 2) and the most frequently used vectors in clinical trials.

Adenoviral vectors may also offer an advantage for vaccine strategies against infectious diseases, as the innate immune responses mounted against the vector may enhance the antigenic response directed against the expressed antigen[36]. Adenoviral vaccines designed to protect against HIV infection were halted owing to concerns that the vaccine may actually enhance susceptibility to HIV infection[37,38].

Gene transfer vectors have also been used for *in vivo* cellular reprogramming for the treatment of diseases such as diabetes mellitus in animal models[39–42]. Specifically, injection of recombinant adenoviral vectors expressing one or several different β-cell transcriptional factors, systemically or into the pancreas of diabetic mice, results in correction of hyperglycaemia owing to the reprogramming of an undefined cell population into β-cells. Interestingly, ectopic expression of the pancreatic transcription factors alone is not enough for reprogramming — the adenoviral particle is a required secondary component[43] but the mechanism of this secondary requirement is not known. To attempt this therapeutic strategy in humans, a substitute for this secondary component or a means for isolated administration of an adenoviral vector will be required.

The value of adenoviral vectors in clinical gene therapy is still not clear, but there has been a resurgence of interest in using them when targeting cancer, and in clinical trials when localized administration of the vector is possible. In one interesting *ex vivo* application, an adenovirus expressing a zinc finger nuclease that is designed to inactivate the HIV *CCR5* gene is used in either autologous T lymphocytes or $CD34^+$ cells, and the cells are then transplanted back into the patients. The rationale for this strategy is that $CCR5^{-/-}$ cells have a selective advantage because they are resistant to HIV infection[44].

Adeno-associated viral vectors

The jury is still out. Adeno-associated viruses (AAVs) are small, single-stranded, nonpathogenic DNA viruses that require a helper virus for replication and completion of their life cycle. AAVs are simple viruses and are composed of two genes: *rep*, which is responsible for viral DNA replication, and *cap*, which packages the viral genome. The therapeutic expression cassette replaces *rep* and *cap*, leaving the viral inverted terminal repeats as the only viral sequences[45,46].

The vector is easily purified to high titres, can be pseudotyped and can transduce dividing and non-dividing cells, in which case it almost always remains episomal. One of the limitations of AAV-based therapeutics is that the insert size is restricted to just over 4 kb. There has been much excitement about the potential of this vector for use in clinical trials, but until more trials are completed it is difficult to know whether it will live up to expectations.

The prototype AAV2 vector, which is based on a human-derived virus, can be pseudotyped with one of various AAV capsids. Variations in the capsid are largely responsible for the specificity and transduction efficacy of the vector, although there is not always concordance between *in vitro* and *in vivo* transduction of even the same cell types. Hundreds of AAV isolates with variant capsids have now been found[47], but only a few have been incorporated into vectors and studied in depth in animals. Improved vectors have also been obtained by experimentally modifying the capsid sequence with site-specific mutations, by using hybrid capsids[48–50] and by constructing large libraries of randomly shuffled capsid DNA that are placed under selective pressure for certain desired properties[51–53] (FIG. 2).

Most (>99%) of proviral AAV genomes are episomal. Thus, <1% of AAV proviral transduction can be attributed to integration[54,55]. Integration preferentially occurs in regions of broken DNA[56], perhaps explaining the preferred integration in transcriptionally active genes[57]. There is doubt about whether integration can be pro-oncogenic. In one mouse model, hepatocellular carcinoma resulted from integration near an miRNA locus that is known to be involved in tumorigenesis[58]. The long-term risk of recombinant AAV therapy for tumorigenesis in humans is not known[59].

Clinical trials in eye and muscle. Recombinant AAV vectors have gained popularity for use in clinical trials. One of the most notable trials includes retinal infusion of an AAV2 vector containing the *RPE65* transgene in patients

Self-limiting toxicity
A toxicity reaction that resolves without intervention.

Ornithine transcarbamylase deficiency
Ornithine transcarbamylase is an enzyme in the urea cycle that is crucial for the conversion of ammonia to urea. Deficiency results in high blood ammonia levels, mental retardation and possible death.

Helper-dependent packaging system
A means of packaging adenoviral vectors that are devoid of all their genes. The pared-down adenoviral genome is expressed from a helper adenovirus that lacks a packaging signal; this permits the vector but not the helper virus to be packaged.

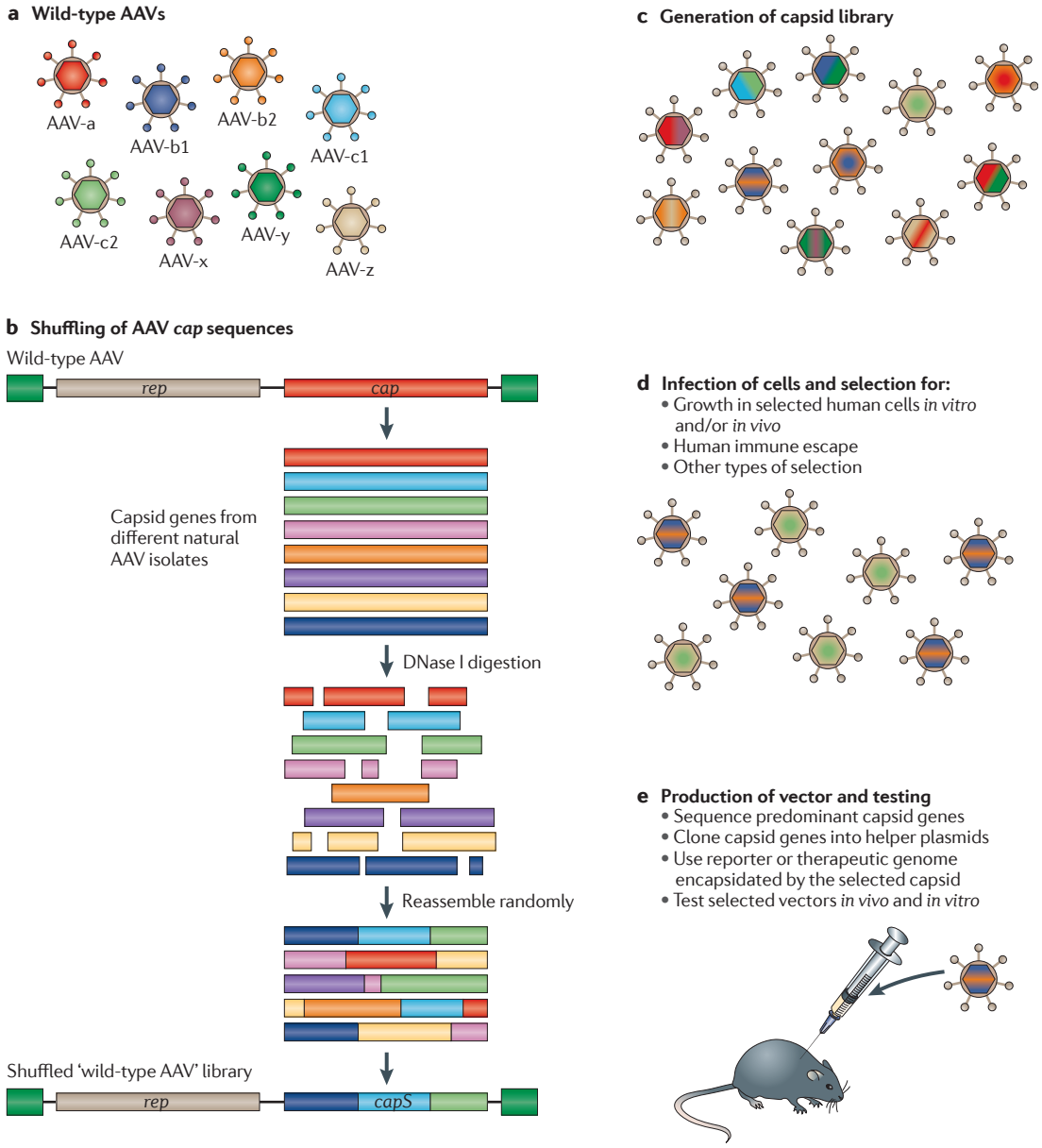

Figure 2 | **Adeno-associated virus capsid shuffling and directed evolution.** Although the capsid sequences can be easily modified, it is difficult to make predictions about how specific modifications in the amino acid sequence will affect the transduction parameters of the viral vector. **a** | Various capsid DNA sequences are derived from adeno-associated viruses (AAVs) with different transduction properties (hexagons of different colours). **b** | The capsid DNA sequences are randomly digested and then PCR ligated back into a 'wild-type' AAV plasmid (*capS*, shuffled *cap* gene). The AAV capsid library can contain between 10^6 and 10^7 unique sequences. **c** | The recombinant AAV wild-type viruses are expanded (with the addition of a replication helper virus, not shown) without any selection in cells. **d** | The AAV viral library is expanded under selective pressure, allowing viruses that survive the selection to be further propagated. With stronger selective pressure, the diversity of the capsid library is reduced and select clones are enriched. **e** | Selected capsid sequences that survive the selection are then cloned into a vector production system and used to pseudotype standard AAV vector genomes (containing a reporter or therapeutic expression cassette) and tested for transduction properties in cells, animals or humans.

Leber's congenital amaurosis
An inherited and incurable blindness disorder.

Ambulatory vision
The degree of vision that allows one to see enough to get around a room without bumping into objects.

with Leber's congenital amaurosis. In this early Phase I study, there was a marked improvement in measurable vision parameters as well as restoration of ambulatory vision in some patients[60,61]. Therapy is required before onset or with early disease onset because of the loss of the appropriate cell targets with disease progression. Interestingly, some forms of retinitis pigmentosa are the result of gain-of-function mutations, making AAV vectors expressing shRNAs against the mutant allele an attractive approach[62,63].

Recombinant AAV vectors efficiently transduce skeletal and cardiac muscle, and animal studies have

Box 3 | **Adeno-associated viruses and non-coding RNAs**

Exon skipping

The use of muscle-tropic adeno-associated virus (AAV) vectors expressing RNAs that promote exon skipping to restore an active dystrophin protein has gained much interest in recent studies[72]. An AAV vector expressing a modified U7 small nuclear RNA (snRNA) was able to block key splicing sites both in a transgenic mouse model of exon 51 Duchenne muscular dystrophy (DMD) and in patients' cells, and to restore functional human DMD expression[120].

Gene knockdown strategies

Short hairpin RNAs (shRNAs) can induce RNAi against various transgene products. shRNAs have been raised against the gene products of viral hepatitis viruses (for example, HBV and HCV) and of gain-of-function mutations such as those that cause neurodegenerative disorders, with Huntington's disease being a prime example[121,122]. It is not entirely clear whether total elimination of the huntingtin protein (mutant and wild-type allele) will be detrimental; strategies for knocking down specific alleles will be important in some applications.

This strategy has also been suggested for treating those patients with α1 antitrypsin deficiency who are homozygous for the PiZ allele[123]. Normally, α1 antitrypsin is made in the liver and secreted into the bloodstream, where its main role is to inhibit neutrophil elastase in the lung. There are two major forms of α1 antitrypsin deficiency: patients with Z-protein mutations that result in α1 antitrypsin being inefficiently secreted from the liver show severe liver disease, whereas other patients with null alleles or mutations that result in inactive α1 antitrypsin being excreted show early emphysema. In patients who are homozygous for PiZ, a vector can be made in which RNAi specifically targets the PiZ allele via expression of an shRNA, and the normal protein is expressed from a separate expression cassette contained within the same vector. This double knockdown/gene addition strategy would also be amenable for treating sickle cell disease (using retroviral or lentiviral vectors), as clinical success will probably require the removal of sickle globin and production of wild-type β-globin in erythrocytes.

Interestingly, overexpression of shRNAs in tissues can be lethal, presumably because these RNA molecules can interfere with the essential small-RNA metabolism of the host cell[124]. However, this is a dose-dependent event, and so lowering the amount of exogenous RNAi using a weaker promoter would still reduce the target RNAs by several orders of magnitude while eliminating the toxicity[125].

suggested they might be useful for treating muscular dystrophies, heart failure and myocardial infarction. Early clinical trials for all three groups of diseases are ongoing. For heart failure and myocardial infarction, a number of potential gene addition and knockdown strategies are possible. AAV delivery for gene addition or gene knockdown using RNAi strategies is in preclinical and clinical development. A Phase II trial using AAV1 encoding the *Serca2a* cDNA in dilated cardiomyopathic heart failure may establish the use of gene transfer for increasing cardiac output in these patients[64,65].

The skeletal muscle is a massive organ and has served as a gene factory, in that secreted proteins can be exogenously produced from this tissue. Clinical trials for lipoprotein lipase[66], α1 antitrypsin[67] and factor IX[68,69] have been carried out. For lipoprotein lipase and α1 antitrypsin, clinical trials are in their early stages, but sustained therapeutic levels of plasma factor IX were not achieved in humans. Muscles are also a target for AAV vectors in the treatment of muscular dystrophies, and initial studies have focused on Duchenne muscular dystrophy (DMD)[70–72]. However, the limit to the size of the vector payload and the doses required for systemic uptake by the skeletal, diaphragm and cardiac muscles has made treating DMD a challenge. Another approach for treating DMD includes exon skipping (BOX 3).

Image-guided vector placement
The use of imaging technologies, such as real-time magnetic resonance imaging, to pinpoint the delivery of a vector through a catheter.

Developments in central nervous system disorders and haemophilia. The transduction of AAVs into neurons after localized infusion into the brain is being developed for the treatment for neurological disorders, and Parkinson's disease has been the focus of several studies[73–76]. Three types of gene addition approaches, all of which are in clinical trials, are being attempted in Parkinson's disease: changing neurotransmitter production; producing dopamine-synthesis enzymes; and expressing growth factors to inhibit neurodegeneration of the substantia nigra. This last option would be the most physiological therapy as patients with Parkinson's disease slowly degenerate neurons in this portion of the brain; however, therapy by this route would depend on early detection of the disease. Although long-term transgene expression has been achieved in non-human primates (for >8 years) and even humans (for >5 years) after a single dose of the vector[77], long-term clinical benefit still needs to be established in larger, ongoing clinical trials. Moreover, it is likely that current and future clinical trials will be more efficacious because it is now possible to deliver the vector to the desired sites of the brain with extreme accuracy[78]. A combination of various strategies — the use of different AAV serotypes, image-guided vector placement[79], and anatomic blood–brain barrier containment of the vector — will greatly enhance the usefulness of this vector (as well as others) for treating neurological disorders. One important property related to AAV vectors is that vector-mediated transduction can occur after neuron body uptake and transport into specific neuronal pathways via axonal transport mechanisms[80]. Widespread, localized and cell-type (for example, glial versus neuronal) gene delivery is now possible.

Recombinant AAV vectors have also been administered systemically for liver-based treatment of factor IX deficiency (as occurs in haemophilia B). In mice and dogs, a single-dose of AAV2 vectors can successfully treat haemophilia B for many years[81,82], but similar success has not been achieved in humans. Therapeutic plasma levels of factor IX in a patient with haemophilia B lasted only a few months owing to a cellular immune response directed against the capsid peptides during degradation of the capsid in hepatocytes; this resulted in a transient, immune-based hepatitis and the loss of transduced hepatoctyes[83]. This response has not been observed in other mammalian species and is perhaps unique to humans because the AAV vector was derived from the human AAV2 virus — it is unclear whether a non-human AAV vector would give the same result. Even though it is a topic of great interest, it has not been possible to recapitulate this type of immune response in animal models. The diverse human polymorphic variations in genes affecting human immunity may make it difficult to predict which patients are most susceptible to these types of responses. This important issue may be resolved by a current haemophilia B clinical trial involving a vector packaged with a non-human primate-derived AAV8 capsid (which nonetheless shares 82% of its amino acids with the capsid of the human AAV2 virus)[84]. Transient administration of mild immunosuppressive agents provided at the same time as the vector

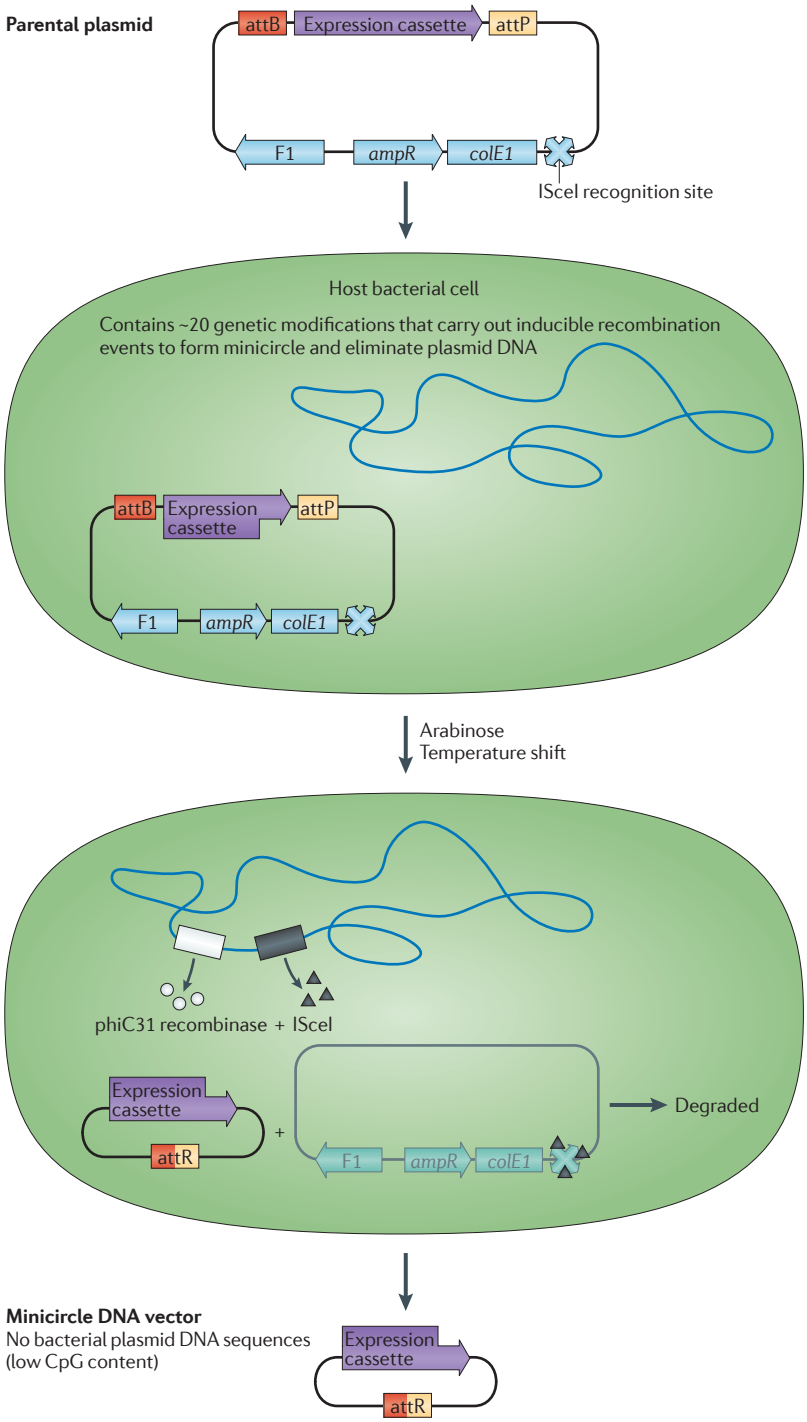

Figure 3 | **Robust minicircle production.** The parental plasmid containing the therapeutic expression cassette flanked by attB and attP sequences, and multiple ISceI endonuclease recognition sites in the bacterial plasmid backbone (only one ISceI recognition site is shown) is transformed into a genetically modified *Escherichia coli* bacterial strain for propagation. Under standard culture conditions, the plasmids are amplified in the same way as any routine plasmid DNA. At the end of routine culture, arabinose is added to the culture and the temperature is changed for several hours. The arabinose induces the expression of the host phiC31 recombinase and the ISceI homing endonuclease enzyme. The recombinase results in the formation of a minicircle and an unwanted bacterial plasmid DNA; the latter is degraded by ISceI-mediated cleavage. The remaining minicircle plasmid is purified by a standard plasmid-DNA purification procedure (such as column chromatography). *ampR* and *colE1* are selection markers that confer resistance to two antibiotics.

may be required because the capsid peptides will have a finite lifetime, after which the transduced hepatocytes will no longer be a target for reactive T cells.

Overall, although the AAV vector has some limitations, it shows great promise for a number of applications, and over the next few years the value of this vector in clinical gene therapy is likely to become clearer.

Non-viral vectors

These vectors can be divided into two basic components — the plasmid DNA molecule and the delivery constituent. Non-viral or DNA-based vectors offer several potential advantages: they do not contain viral contaminants and nor do they stimulate any pre-existing antigen-dependent immunity, and there is no size limit on the amount of DNA that they can deliver.

Although non-viral DNA delivery is efficient in many culture systems, which makes it feasible for *ex vivo* applications, there has been little success in achieving efficacious and safe *in vivo* gene transfer. An *in vivo* trial for cystic fibrosis is underway using a CFTR (cystic fibrosis transmembrane conductance regulator) expression cassette complexed in a liposome[85]. Overall, however, the use of non-viral vectors for *in vivo* gene transfer remains relatively sparse owing to limitations in vector delivery.

To create delivery platforms, DNA has been complexed to many different types of macromolecules, including those based on polylysine, polyethylenimine, polysaccharides (for example, cyclodextrin and chitosan) and/or other polycationic-lipid-based systems[86,87]. These are generally not small enough to traverse the vascular endothelial barriers and, even if they succeed, most are degraded in endosomal complexes. Moreover, the combination of lipid carriers with methylated CpG DNA motifs can cause severe toxicity *in vivo* by activating various cytokines[88]. Even if the DNA does make it to the nucleus[89], expression from the episomal plasmid does not persist for sufficiently long periods (unlike expression from proviral episomal genomes).

Delivery. DNA–macromolecular complexes can be delivered, albeit inefficiently, by various routes, including intravenous infusion or oral ingestion[90]. One method to overcome delivery into the liver has been hydrodynamic transfection. This consists of a high-pressure infusion of a large volume of naked DNA into the tail vein of a mouse, resulting in the transfection of up to 40% of hepatocytes by an unknown mechanism[91–93]. Modifications of the procedure for localized delivery into the hepatic vasculature are being studied in larger mammals and humans[94]. Similarly, muscle delivery of naked DNA can be achieved by isolation of the vasculature and pressured delivery into isolated skeletal muscle groups or cardiac muscle by infusion into the coronary arteries and/or direct injection into the myocardium[95]. Other delivery technologies, including electroporation and/or ultrasound-guided DNA uptake, have also been tried[96]. The relevance of these various physical delivery approaches for clinical therapies is still not clear, but at a minimum they have been invaluable for studying non-viral vector DNA-mediated transgene expression and disease correction in whole mammals.

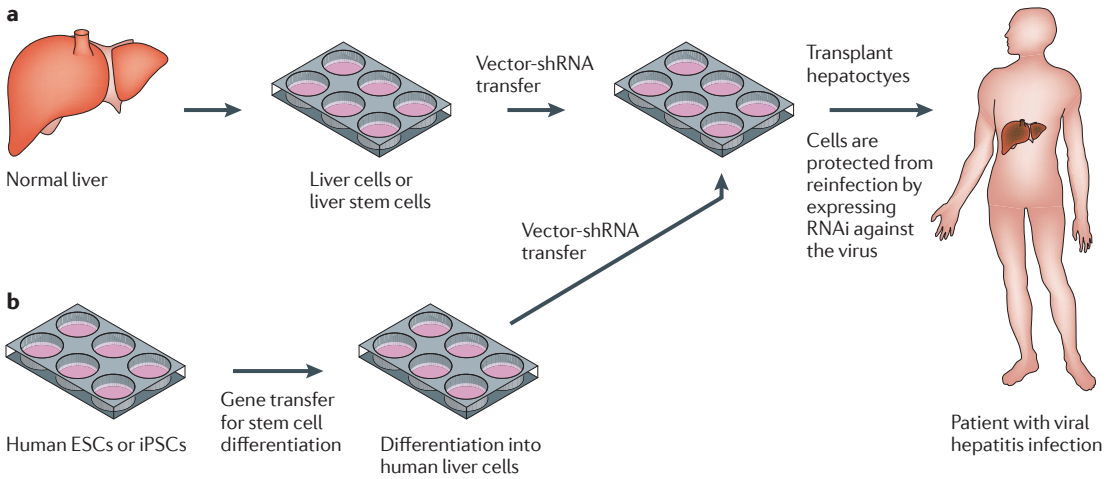

Figure 4 | **Combining stem cells and gene therapy: an example application.** Patients with chronic liver disease from viral hepatitis (for example, HCV or HBV) infection who require a liver transplant might be amenable to hepatocellular transplantation of mature hepatocytes (**a**), or hepatocytes-derived from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) (**b**). Not only might gene transfer be required to convert stem cells into hepatocytes (**b**) but, because the transplanted cells are likely to become reinfected by the hepatitis virus (as do whole liver organs after transplantation), gene transfer of a vector encoding RNAi (short hairpin RNAs (shRNAs)) directed against the virus would make the transplanted cells resistant or 'immune' to reinfection. The resistant cells can repopulate the liver over time and restore normal liver function.

Expression. Most of the DNA vectors that have been delivered are routine plasmids that have been cloned to contain a transgene expression cassette that is similar to the cassette in a viral vector. However, even with hydrodynamic infusion into the liver, transgene expression diminishes rapidly, falling by two to three orders of magnitude over several weeks. By contrast, minicircle DNAs give more persistent expression, generally at levels 100 to 1,000 times greater than the same expression cassette in a routine plasmid[97]. The difference in expression is not due to differences in loss of vector DNA but to loss of transgene-specific mRNA by mechanisms that are still not well understood[98]. Nevertheless, minicircle DNA vectors can now be easily produced[99] (FIG. 3), and in combination with other improvements in *cis*-DNA elements[100], they will probably replace routine plasmids in gene therapy trials when and if clinically relevant means of DNA delivery can be achieved.

Integration. Although plasmid DNAs are episomal in nature, I highlight two strategies to achieve transgene integration in target cells. The first is based on a variety of class II transposable elements — DNA transposons[101]. A transposase is transiently expressed, allowing a DNA sequence containing the therapeutic expression cassette — usually supplied on a second plasmid in *trans* and flanked by specific transposon DNA sequences — to integrate into the host chromosome. The first DNA transposon used for gene therapy studies in mammals was called *Sleeping Beauty*. Since this time, numerous different DNA transposons as well as hyperactive transposase alleles have been studied, and other transposons have been examined in preclinical gene transfer studies. DNA transposon integration is targeted into specific nucleotide sequences (for example, TA dinucleotides for *Sleeping Beauty* and TTAA for the insect-derived *piggyBac* transposon). *Harbinger-Dr3* is a more newly developed transposon-based vector, and recognizes the palindromic AAACACCWGGTCTTT sequence. Different strategies have been used to target integration to specific sites, including tethering a zinc-finger DNA-binding domain to transposases, but with limited success[102]. Moreover, there are studies in which the transposable elements are placed within vectors such as the helper-dependent adenoviral vector, resulting in integration of the therapeutic transgene[103].

A second means of achieving DNA integration into the host chromosome includes the use of bacteriophage recombinases, preferably those that do not allow for the reverse, excision reaction. Transient expression of the recombinase results in integration into pseudosites resembling the endogenous prokaryotic integration sequence[104,105]. In general, the recombinase-mediated integrations are more site-selective than the DNA transposons, but they are still quite promiscuous and can result in chromosomal translocations — something that is not observed with DNA transposons[106]. The safety of both DNA transposons and recombinases in gene therapy applications is not yet known.

Future developments. Non-viral vectors could replace many viral vector approaches if the problems associated with delivery efficacy can be overcome — perhaps by a novel nanoparticle chemical design.

The future of gene therapy

The successful implementation of gene therapy has been a lengthy and cumbersome process. Novel medical therapies are particularly vulnerable to delay because of the time required for safety testing. In retrospect, many

Minicircle DNAs
Expression cassettes that are devoid of the plasmid DNA backbone.

Sleeping Beauty
An ancient inactive transposon isolated from salmon. The transposase was reactivated by introducing various mutations.

of the early gene therapy clinical trials were carried out prematurely because they lacked enough scientific and preclinical evidence to support reasonable chances of success. After more than 20 years we are finally seeing some of the anticipated therapeutic benefits, as well as new and promising preclinical studies. Further successes — in addition to the treatment of immunodeficiency disorders and genetic blindness as proof-of-concept — will be needed to appease sceptics of gene therapy approaches. The immediate work required is obvious — to develop better vectors and gain a better understanding of interactions between the vector and the human host. Another important research effort will be to work in animal models to overcome the severe toxicity of the transfer vectors and/or their inefficient transduction into cells or tissues. Future efforts should therefore have two main aims — improving vector delivery and overcoming the host immune response.

In many ways, organ transplantation was really the first gene therapy. Bone marrow transplantation to treat SCID or liver transplants to treat life-threatening metabolic disorders are good examples. Moving full circle, the new interest in stem-cell-derived therapies in many scenarios represents a similar goal. As an aside, the American Society for Gene Therapy has recently changed its name to the American Society of Gene and Cell Therapy to underscore the exciting promise for cell-based therapies.

Gene transfer in combination with stem cell therapy is clearly a therapy of the future. We are likely to see the development of gene transfer vectors for the creation of induced pluripotent stem cells (iPSCs), for iPSC and embryonic stem cell (ESC) differentiation, and for modifying the stem cell or its differentiated derived cell to provide an additional phenotype. An example is shown in FIG. 4.

The range of phenotypes that will be targeted by gene therapeutic approaches is also bound to change. Not all genetic contributions to disease are heritable. As many biological traits and disease phenotypes are influenced by epigenetic parameters through histone, chromatin and DNA modifications, they may become important therapeutic targets.

We are now in the era of personalized genomic medicine, when the cost of sequencing a human genome is predicted to fall to only US$1,000 in the near future. This, in combination with our better understanding of genetic contributions to common medical conditions, makes it likely that genome sequencing at birth will replace the dried blood cards that are currently used for screening only a handful of serious medical conditions. As a result, infants with treatable predispositions detected by whole-genome sequencing would be offered an appropriate vector early in life in a similar way to the current practice of vaccine prophylaxis against serious infectious pathogens.

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