REVIEWS

Ex vivo gene transfer and correction for cell-based therapies

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Abstract | Cell-based therapies are fast-growing forms of personalized medicine that make use of the steady advances in stem cell manipulation and gene transfer technologies. In this Review, I highlight the latest developments and the crucial challenges for this field, with an emphasis on haematopoietic stem cell gene therapy, which is taken as a representative example given its advanced clinical translation. New technologies for gene correction and targeted integration promise to overcome some of the main hurdles that have long prevented progress in this field. As these approaches marry with our growing capacity for genetic reprogramming of mammalian cells, they may fulfil the promise of safe and effective therapies for currently untreatable diseases.

Transduction
The transfer of genetic material into a cell mediated by a viral vector.

Autologous
In transplantology, this refers to cells transplanted from an individual to that same individual (often after an *ex vivo* procedure has been performed).

Storage disorder
A disease caused by genetic deficiency of a lysosomal or peroxysomal enzyme, leading to intracellular accumulation of undegraded substrate, cellular pathology and malfunction of multiple organs.

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doi:10.1038/nrg2985
Published online
29 March 2011

Ex vivo cell therapies are based on the ability to isolate stem, progenitor or differentiated cells from a patient or a normal donor, expand them *ex vivo* — with or without genetic modification — and administer them to the patient to establish a transient or, more often, a stable graft of the infused cells and their progeny. This can be done to treat an inherited, infectious or neoplastic disease, to regenerate a tissue or to deliver a biotherapeutic molecule to a disease site. As compared to *in vivo* gene therapies[1,2], in *ex vivo* therapies there is no exposure of the patient to the gene transfer vector, and the target cells of transduction can be selected, expanded and/or differentiated, before or after gene transfer, to improve efficacy and safety.

The best developed and most successful cell therapy is haematopoietic stem cell (HSC) transplantation (HCT)[3] (TABLE 1). Thanks to the long-standing clinical experience with HCT, *ex vivo* gene transfer procedures have been readily integrated into standard HCT protocols and have achieved rapid clinical translation. Autologous HSC gene therapy represents an emerging therapeutic option for several monogenic diseases of the blood and the immune system as well as for storage disorders, and it may become a first-line treatment option for selected disease conditions. Another established cell and gene therapy application is adoptive immunotherapy, which exploits *ex vivo* expanded T cells, with or without genetic engineering to redirect their antigen specificity or to increase their safety profile, in order to harness the power of immune effector and regulatory cells for use against malignancies, infections and autoimmune diseases[4–7]. Finally, a range of other types of somatic stem cells — in some cases involving genetic engineering — are showing promise for therapeutic applications, including epidermal and limbal stem cells[8,9], neural stem/progenitor cells (NSPCs)[10,11], cardiac stem cells[12] and multipotent stromal cells (MSCs)[13,14].

Despite the promise of cell-based therapies, several important hurdles must be overcome before they achieve satisfactory efficacy, clinical feasibility and safety for broad application. Here, I highlight the crucial aspects and main challenges for *ex vivo* cell and gene therapy strategies, with a particular emphasis on HSC gene therapy, which has been chosen as the most representative example, given its advanced clinical development. However, most principles and issues discussed here similarly apply to other *ex vivo* cell and gene therapy applications.

This Review comes at an exciting time, when several new developments in the clinical arena have helped us to address long-debated questions in the field of gene therapy. Firstly, the long-term follow-up (up to 10 years after treatment) of the seminal clinical studies of HSC gene therapy for two types of severe combined immunodeficiency (SCID) has just been published[15–17]. These studies, which used early-generation retroviral vectors, now provide a comprehensive analysis of a sizeable number of patients, allowing a reliable assessment of long-term immune system reconstitution and the risk/benefit ratio of HSC gene therapy versus HCT. The verdict is favourable, with a clear long-term therapeutic benefit evident in most treated patients despite the occurrence of vector-related leukaemia in a few[18,19],

Table 1 | ***Ex vivo* HSC gene therapy in clinical trials**

Disease type	Rationale and target cells	Gene vector	Stage of development	Safety	Efficacy	Comments	Refs
Haematological							
ADA-SCID	Lymphoid reconstitution	γ-RV (MLV or SFFV LTR) expressing ADA	Phase I/II trials completed or ongoing	Long-term safety demonstrated	Long-term cure comparable to HCT	Registration for market underway; SIN LV trials planned	15,158, 159
X-linked SCID	Lymphoid reconstitution	γ-RV expressing IL-2R common gamma chain	Phase I/II trials completed	High rate of leukaemia triggered by insertional activation of *LMO2* oncogene	Long-term cure comparable to HCT	New trials with SIN γ-RV started	16–19
X-linked CGD	Myeloid reconstitution	γ-RV (SFFV or MFG LTR) expressing gp91$^{(phox)}$	Phase I/II trials recruitment closed, follow-up ongoing	Myeloid expansion triggered by insertion at oncogenic CIS and evolving to myelodysplasia in all patients treated with SFFV-LTR	Transient clinical benefit	New trials with SIN LV and myeloid-specific expression planned	22,160
WAS	Multi-lineage reconstitution	γ-RV (MPSV LTR) expressing WAS protein	Phase I/II trial recruitment closed, follow-up ongoing	One case of leukaemia reported by insertional activation of *LMO2* oncogene	Clear benefit at 2 year follow-up	-	20
WAS	Multi-lineage reconstitution	SIN LV expressing WAS from *WAS* gene promoter	Phase I/II trials recently started	-	-	-	*
β-thalassaemia	Erythroid reconstitution	SIN LV; large LCR expressing β-globin and cHS4 insulator	Phase I/II trial ongoing (one patient treated)	Clonal dominance possibly triggered by vector insertion in *HMGA2* gene	Transfusion independence	-	24
Fanconi's anaemia	Stem cell reconstitution	SIN LV expressing FANC-A protein from *PGK* promoter	Phase I/II trial approved	-	-	HSC gene transfer without stimulation to protect cells from DNA damage	161
Storage disorders							
ALD	Overexpression in tissue macrophages and microglia	SIN LV expressing the ATP-binding cassette transporter ABCD1 from *MND* promoter	Phase I/II trial ongoing	10–15% HSC marking	Clear therapeutic benefit comparable to HCT	-	23
MLD	Overexpression in tissue macrophages and microglia	SIN LV expressing ARSA from *PGK* promoter	Phase I/II trial recently started	-	-	-	*

All trials are using autologous haematopoietic stem/progenitor cells (HSPCs). ADA, adenosine deaminase; ALD, adrenoleukodystrophy; ARSA, arylsulphatase A; CGD, chronic granulomatous disease; cHS4 chicken hypersensitive site 4 insulator from β-like globin gene; CIS, common vector integration site; gp91$^{(phox)}$, cytochrome *b*-245, beta polypeptide of phagocyte oxidase; HCT, HSC transplantation; *HMGA2*, high mobility group AT-hook 2; HSC, haematopoietic stem cell; IL-2R, interleukin-2 receptor; LCR, locus control region from globin gene; *LMO2*, LIM domain only 2; LTR, long terminal repeat; LV, HIV-derived lentiviral vector; MLD, metachromatic leukodystrophy; MLV, Moloney leukaemia virus; MPSV, myeloproliferative sarcoma virus; *PGK*, phosphoglyceratokinase; γ-RV, γ-retroviral vector; SCID, severe combined immunodeficiency; SFFV, spleen focus-forming virus; SIN, self-inactivating; WAS, Wiskott–Aldrich syndrome. *These trials are currently ongoing at HSR-TIGET in Milan (A. Aiuti, A. Biffi, M. G. Roncarolo and L.N., unpublished observations). A similar trial for WAS is open in London and Paris, using the same lentiviral vector as the one in Milan.

and calls for further development of the therapy and testing of improved vectors to achieve better safety. However, more recent clinical trials for other types of immunodeficiency, carried out with retroviral vectors that have been modified to achieve more robust gene expression, have reported an alarming incidence of vector-induced haematological malignancy[20], although clinical benefits were initially observed in all patients after the treatment[21,22]. The rollercoaster-like reporting of, alternately, dramatic successes and severe adverse events in these clinical trials both testifies to the therapeutic potential of HSC gene therapy and casts doubt on the predictive value of conventional preclinical testing. Further studies have been prompted to allow us to better understand the mechanisms of vector insertional mutagenesis with a view to identifying novel vector configurations that may alleviate the risks. Here, I will discuss the outcomes of these studies and how they are providing confidence in promoting clinical testing of improved vectors.

Severe combined immunodeficiencies
A family of genetic disorders that affect T cell differentiation and B cell immunity, resulting in the absence of a functional immune system.

Box 1 | *In vivo* selection of transplanted cells

The potential for positive or negative selection of the administered cells in cell therapies may be an intrinsic feature of gene correction or may be engineered to improve efficacy or safety of the procedure.

In certain inherited disorders, a whole cell lineage may be lacking owing to developmental failure, as occurs for lymphoid cells in some types of severe combined immunodeficiency (SCID)[27]. In these settings, the functionally corrected cells have a selective advantage over their mutant counterparts, as only the former cells can expand, differentiate and occupy the empty tissue niches. This scenario enables lymphoid reconstitution after haematopoietic stem/progenitor cell (HSPC) gene therapy even if only a small fraction of the transplanted cells has been functionally corrected, in some cases even without the need to condition the patient for cell engraftment[15–17]. This outcome is in agreement with rare reports of revertant somatic mosaicism in lymphoid cells of patients with SCID consequent to a spontaneous genetic reversion in a progenitor cell[131]. Similarly, a key factor in the success of adenosine deaminase-deficient SCID (ADA-SCID) gene therapy was the withdrawal of enzyme replacement therapy after HSPC transplantation to favour selective survival of the gene-corrected cells[128]. *In vivo* selection of the transduced HSPCs has thus been a crucial factor ensuring the success of gene therapy trials using early-generation γ-retroviral vectors, despite inefficient haematopoietic stem cell gene transfer. However, the growth advantage that is conferred by the therapeutic gene may also increase the risk that clones harbouring a genotoxic vector insertion could expand vigorously and progress to malignancy[18,19].

An increase in the percentage of gene-modified cells following transplantation can also be obtained by engineering the transplanted cells for positive selection *in vivo*. This can be accomplished by introducing a transgene that confers resistance to a cytotoxic drug, for instance, the mutant methylguanine methyltransferase ($MGMT^{P140K}$). Administration of cytotoxic drugs (such as *N*,*N*′-bis(2-chloroethyl)-*N*-nitroso-urea or temozolomide) after the transplant selectively kills the non-transduced HSPCs, and the transduced ones expand progressively[132–134]. As we increase our understanding of the hierarchy and growth regulation of normal and malignant haematopoiesis, it may also become possible to design strategies that couple a transgene driving cell growth with the therapeutic gene. These approaches can improve the efficacy of cell therapy when the administered cell dose or the engraftment rate are limiting or can be used to bypass host conditioning requirements.

For a fail-safe cell therapy approach, the transplanted cells can be engineered *ex vivo* to express a conditional suicide gene, such as the thymidine kinase of human herpesvirus, so that they can be eliminated by drug treatment (ganciclovir) in case they give rise to serious adverse reactions *in vivo*, such as neoplastic outgrowth or severe graft versus host disease[44]. The reliability of this feature depends on ensuring stable expression of the conditional suicide gene in nearly all transduced administered cells.

Of major relevance to the testing of new vectors, two recent publications have reported the initial results of the first HSC gene therapy trials, which used late-generation lentiviral vectors for the treatment of the neurodegenerative storage disorder adrenoleukodystrophy[23] and the red blood cell disease β-thalassaemia[24]. Such data have long been awaited given the expectation that lentiviral vectors would provide a more efficient and safer gene shuttle into the genome as compared to earlier vector types. Both studies show a clear therapeutic benefit in the few patients treated so far, together with the achievement of substantial levels of HSC gene transfer. Whether these data support a broader application of lentiviral vectors in gene therapy will be addressed in this Review.

Finally, the engineering of transposons has provided alternative gene vectors[25]; this, combined with the development of powerful new technologies for highly efficient gene targeting and site-specific gene editing[26] raises the possibility of targeted rather than random integration, and gene correction rather than replacement. I discuss how these advances may offer radical new solutions to overcome the major hurdles that have long prevented progress of the field.

Applications of *ex vivo* therapy

Reconstituting dysfunctional cell lineages. For inherited diseases that are characterized by a defective or absent cell lineage, the lineage can be regenerated by functional progenitor cells, derived either from normal donors or from autologous cells that have been subjected to *ex vivo* gene transfer to correct the deficiency. An example is provided by SCIDs, in which a deficiency in any one of several genes blocks the development of mature lymphoid cells[27]. Transplantation of non-manipulated normal donor HSCs, which allows the generation of donor-derived functional haematopoietic cells of all lineages in the host, represents the standard of care or at least a valid therapeutic option for SCIDs, as well as many other diseases that affect the blood and immune systems[28,29]. Autologous HSC gene therapy, which replaces a functional copy of the defective gene in the transplanted haematopoietic stem/progenitor cells (HSPCs) and, similarly to HCT, provides a steady supply of functional progeny, may have several advantages over the allogenic procedure. First, it can be applied to every patient, whereas the requirement for a human leukocyte antigen (HLA)-compatible donor limits the number of patients that can benefit from allogenic transplant. Second, autologous HSC gene therapy reduces the morbidity and mortality of the transplant procedure, as there is no risk of graft versus host disease (GvHD) and consequently no need for post-transplant immunosuppression. There is also a reduced risk of graft rejection, which may allow the use of T-cell-sparing, reduced-intensity conditioning regimens before the transplant. The disadvantages are, first, the possibility that the *ex vivo* culture required for HSC gene transfer and/or a poor transduction rate may decrease the long-term reconstitution potential of the infused cells, and second, the risk of haematological malignancy, which can be triggered by vector insertion. If the gene-corrected cells have a selective growth advantage, gene therapy can be successful even when only a small fraction of the transplanted HSPCs has been transduced by the vector (BOX 1).

Augmenting therapeutic gene dosage. In some applications, HSC gene therapy may augment the therapeutic efficacy of allogenic HCT, thus providing a unique therapeutic opportunity. This is because the therapeutic gene dosage can be engineered to supra-normal levels in the transplanted cells by gene transfer. A positive correlation between gene dosage and the level of enzymatic activity reconstitution is emerging from studies of transplant recipients in lysosomal storage disorders (LSDs). In these studies, some patients receive transplants from a heterozygous-deficient donor, typically a parent or sibling whose cells, despite being normal from a functional standpoint, express only 50% of the wild-type level of enzyme. These recipients are compared

Allogenic
In transplantology, the use of cells or tissue from any human other than self or a monozygotic twin.

Graft versus host disease
A destructive attack on host tissues by immune cells that are derived from a transplant of allogenic haematopoietic cells.

with patients who have received transplants from a homozygous normal donor[30]. In agreement with the observations in these studies, a substantial advantage of gene therapy over normal donor HCT has been well established in several animal models of LSD. Such animal studies have shown that there is a dose–response relationship between vector load, enzyme reconstitution and prevention or correction of the disease phenotype[31,32]. The underlying mechanism of this response is multifaceted. An important target cell population in LSD is tissue scavenger cells of monocyte origin, such as macrophages and microglia, which accumulate most of the storage material and, in response, trigger local inflammatory and degenerative reactions. Expression of the exogenous functional enzyme in these blood-borne cells by transplantation of genetically modified HSCs restores their scavenging capacity and helps to clear the storage material[33]. Moreover, these cells, besides targeting the functional enzyme to their lysosomal compartment, also release it at least in part outside the cell, thus providing a local supply of functional enzyme that can be taken up by other tissue-resident cells, thereby cross-correcting their deficiency. If above-normal expression of the functional enzyme is achieved in these tissue-infiltrating haematopoietic cells by means of HSC gene transfer, more efficient enzyme delivery can be achieved. HSC gene therapy thus represents a powerful therapeutic strategy to establish widespread and sustained tissue sources of functional enzyme, even in poorly accessible tissues. Good examples are bones and the brain: macrophage and microglia populations in these tissues have been shown to undergo at least some replacement by blood-borne haematopoietic progenitors after conventional transplant procedures[34–39].

Instructing novel function and targeting gene therapy. *Ex vivo* gene therapy may also be used to confer a novel function to HSCs or their progeny, such as establishing drug resistance to allow administration of a high-dose antitumour chemotherapy regime[40] or establishing resistance to a pre-established infection with a virus, such as HIV, or other pathogen by expressing RNA-based agents (for example, ribozymes, RNA decoys, antisense RNA, RNA aptamers and small interfering RNA) and protein-based agents (for example, dominant-negative mutant viral proteins, fusion inhibitors and engineered nucleases that target the pathogen's genome)[41,162]. In some preclinical studies, HSPC gene therapy is being explored for the targeted delivery of a therapeutic transgene to a specific site. This is achieved by engineering selective expression of the transgene using lineage-specific promoters in the mature cell progeny that home to that site. Among the targeting cells are platelets, which have been used in the treatment of haemophilia that is resistant to replacement therapy[42]. Here, the platelets are engineered to express a clotting factor transgene within the clot to evade plasma inhibitors. Another example is tumour-homing macrophages, which can be manipulated to express an antitumour cytokine, such as interferon-α, locally within the tumour without systemic exposure[43].

Enhancing immune responses. In neoplastic diseases, allogenic adaptive immune cell types, such as T cells, can recognize and kill cancer cells. Unfortunately, recognition of healthy tissues by alloreactive lymphocytes can also result in detrimental GvHD. The transfer of a suicide gene in donor lymphocytes allows their antitumour potential to be exploited, while taming their toxicity[44]. In the autologous setting, lymphocytes with specificity directed against transformed or infected cells may be isolated from the patient's tissues and selectively expanded *ex vivo*[4,5]. Alternatively, they may be generated by transfer of a gene for a synthetic or chimeric antigen receptor that triggers the cell's response when it encounters transformed or infected cells[6,7,45]. These approaches may potentiate an underlying host response to a tumour or infection, or induce it *de novo*. Their potential efficacy comes from the possibility of harnessing the amazing biological power of immune effectors to induce antitumour or antiviral immunity or, in the case of autoimmune diseases, antigen-specific tolerance[46]. Their limitations are mostly due to the following challenges: modifying the output of complex immune networks *in vivo*[4]; overcoming the immune evasive and suppressive environment often found in tumours[47]; identifying suitable and, possibly, multiple tumour antigens that represent valid therapeutic targets[48]; achieving adequate *in vivo* persistence of the transferred cells[49,50]; and limiting toxicity, such as the potential detrimental interactions between the exogenous and pre-existing T cell receptor chains that compete for expression and may give rise to hybrid receptors with unpredictable auto-reactivity[51–53]. Where feasible, HSC engineering also represents an option to ensure a long-term *in vivo* supply of the modified immune cell progeny[54–56]. However, several additional hurdles limit this approach, such as the possibility that thymic selection might otherwise eliminate cells that carry the transgene.

Regenerating diseased tissues. Cell replacement also provides a route for the treatment of degenerative diseases that exhaust the local reservoir of differentiated cell types, such as demyelinating or neurodegenerative diseases, muscular dystrophies, chronic cardiomyopathies and arthropathies[10,11,57–59]. However, the capacity to regenerate mature and functionally integrated cells in the tissues has been limited, and most of the therapeutic benefit observed in preclinical trials of MSC or NSPC therapy has been ascribed to the protection of endogenous cells and the immune-modulatory activity of the administered cells. This is because the administered cells home preferentially to disease sites and locally exert an influence that favours tissue remodelling and angiogenesis rather than inflammation[13,59–63]. Indeed, MSCs are used in the clinic to treat GvHD following HCT[64]. Promotion of tissue repair and reperfusion is also the objective of cell therapies based on *in situ* administration of HSPCs or unfractionated bone marrow cells in ischaemic heart and limbs[65]. Because these last two approaches do not entail an *ex vivo* culture step, they are not discussed further here.

Suicide gene
A gene that encodes a protein that can convert a non-toxic pro-drug into a cytotoxic compound.

Thymic selection
The central tolerance that occurs during early T cell development in the thymus that causes cells with strong reactivity to self-antigens to undergo apoptosis and elimination.

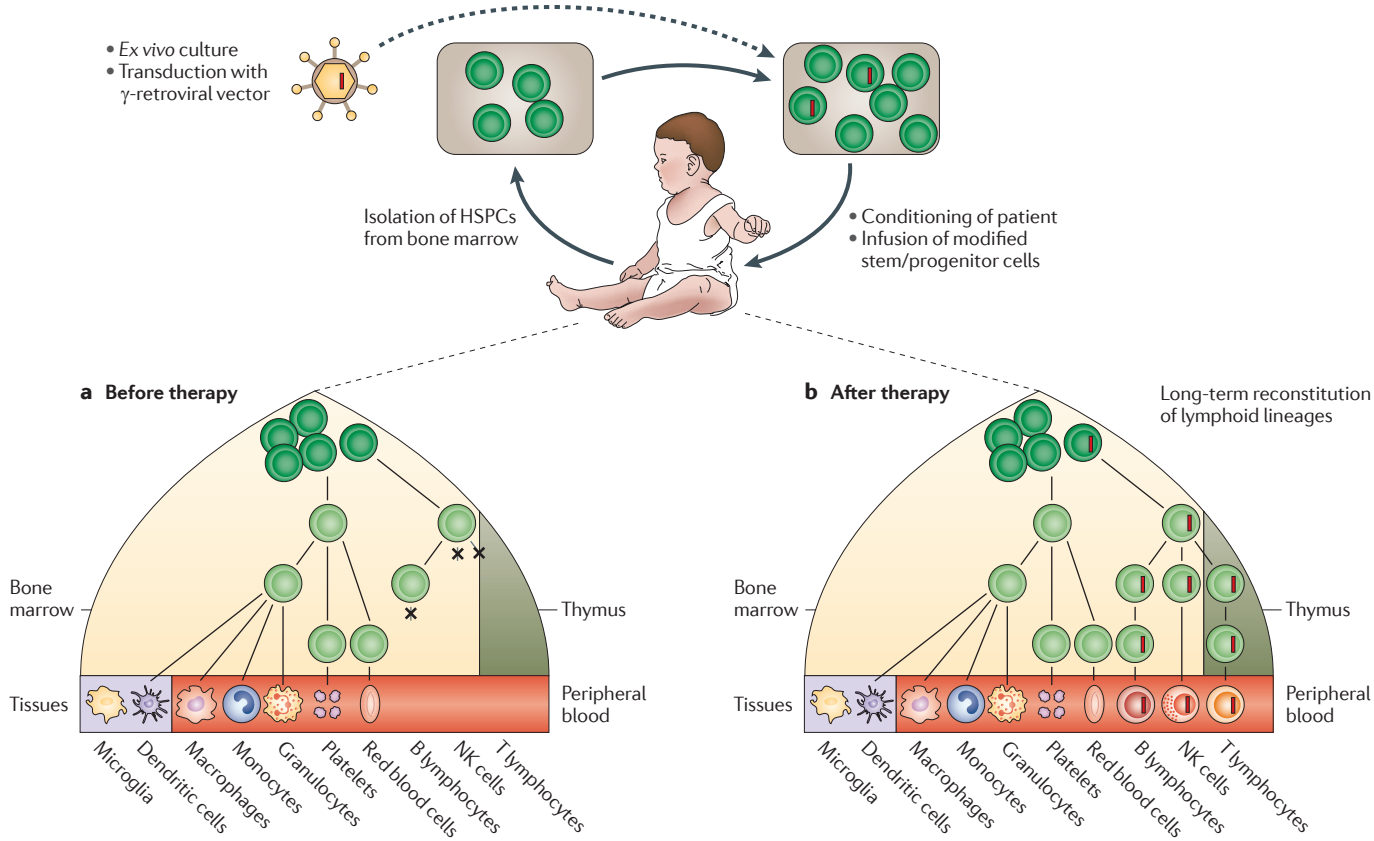

Figure 1 | **Outline of a typical protocol for *ex vivo* haematopoietic stem cell gene therapy.** Haematopoietic stem/progenitor cells (HSPCs) are isolated from the bone marrow (or mobilized peripheral blood) of a patient affected by a primary immunodeficiency — an inherited deficiency of a lymphocyte development gene that prevents formation of the lymphoid lineages. Following culture *ex vivo* in conditions that stimulate cell proliferation, the cells are exposed to a retroviral vector expressing a functional cDNA copy of the defective gene and then infused back into the patient after a few days. Infusion usually takes place following administration of a pharmacological conditioning regimen that eliminates the endogenous bone marrow progenitors and favours engraftment of the transplanted cells. The engrafted gene-corrected stem or progenitor cells generate functional progeny that reconstitute all lymphoid lineages and restore immune functions to the patient. If the gene-corrected cells have a selective growth advantage compared to the unmodified cells, full reconstitution of the immune cell compartments is obtained even from a few engrafted transduced progenitor cells, as depicted in the figure, and this may occur even without conditioning. If the engrafted progenitor cells have self-renewal capacity, they ensure long-term correction of the disease. If the engrafted cells are multipotent stem cells, they generate gene-marked cells in all haematopoietic lineages. NK cells, natural killer cells.

Ex vivo culture

The essential steps of *ex vivo* cell therapy are cell isolation and *in vitro* culture of the desired cell types, to allow their selection, expansion and/or differentiation, before or after genetic modification, if required (FIG. 1). When the aim of therapy is regeneration of one or more lineages of short-lived cells, as in haematological diseases, the administration of progenitor or stem cells that are capable of self-renewal is needed to ensure a long-term supply *in vivo*. When the aim is replacement of long-lived cells, as in neural or musculoskeletal diseases, the administration of committed progenitors that may undergo limited replication and complete maturation *in vivo* is often preferred to ensure that integration in the tissue architecture is favoured, as long as all the undifferentiated cells that may undergo hyperplastic or neoplastic growth *in vivo* are purged from the graft. A major concern with *ex vivo* culture is that it may irreversibly affect the phenotype, long-term viability, homing and repopulation capacity of the cells once they are transplanted *in vivo*. Terminal differentiation and exhaustion of the proliferative potential are feared consequences that may jeopardize therapy. These concerns strongly apply to HSC- and T-cell-based therapies[66–70]. An active field of study is the development of improved protocols that achieve efficient gene transfer in the absence of *ex vivo* stimulation and/or that actually expand populations of HSCs[71–74] or populations of T cells that can persist for long periods in culture[49,50]. In addition, the potential risk of accumulating mutations and karyotype abnormalities with prolonged *in vitro* growth must be considered.

Genetic modification of cells

The most common type of genetic modification of cells for *ex vivo* therapies is gene replacement or addition by a vector that integrates into the target cell genome. Whereas

gene replacement refers to the transfer of a functional copy (usually a cDNA) of a gene whose endogenous counterpart is defective, gene addition is a preferable term to describe the transfer of a selectable marker — to be used for cell purification, expansion or conditional elimination of the cells — and/or a novel function. In some applications, the nucleic acid that is introduced by the vector encodes an antisense or small interfering RNA in order to inhibit expression of an endogenous or exogenous gene[75] (for example, to establish cellular immunity to an infectious agent).

For therapies that exploit proliferating cells, such as stem or progenitor cells, integration of the vector, or at least its transgene expression cassette, into the target cell genome is required to ensure stability of the genetic modification and its transmission to the cell progeny. A possible alternative would be the transfer of a replicating episome that remains stably associated with the cell nucleus at each cell division, or even a whole minichromosome. Although some promising developments towards these aims have been reported[76], they have yet to reach widespread application. Transient expression may sometimes be used, for example to temporarily induce expression of molecules that enhance cell homing or engraftment.

The choice of vector. Currently, integrating vectors derived from retroviruses remain a preferred choice. In historical order and by the current stage of development, these vectors have been derived from γ-retroviruses, lentiviruses, foamy viruses and α-retroviruses. They integrate quasi-randomly throughout the genome, with a preference for certain genomic features that vary with each virus and probably reflect the mechanism used for tethering the integration complex to the chromatin and/or its differential accessibility[77]. Non-viral vectors that rely on transposition for stable integration into the genome have also been developed[78]. Here, gene transfer is obtained by transient transfection of a transposase, together with a donor plasmid carrying the gene of interest cloned between transposase binding sites[79]. The recent engineering of hyperactive transposases[80] provides a platform for *ex vivo* gene transfer with the benefit of simple manufacturing that is free from the potential risk of reconstituting a replication-competent virus. Site-specific recombinases[81], transposases and/or retroviral integrases fused with DNA recognition motifs[82] are also being developed to impart stronger preferences for integration at specific sites, thus offering the potential to better control the spectrum of genomic target sites. As these systems continue to be improved, they may eventually alleviate the concerns associated with insertional mutagenesis. Details on each vector design and performance can be found elsewhere; here, I focus on the essential features that dictate choice among the most advanced platforms.

Gene transfer efficiency. Efficient gene transfer is a paramount requisite, often representing the limiting factor for a positive outcome in therapeutic applications. As the first retroviral vectors developed, γ-retroviral vectors have had the longest-standing and broadest clinical use. But, for integration to occur, they require cells to enter division shortly after infection, thus imposing the need for prolonged culture under conditions of active proliferation. Furthermore, multiple cycles of infection are necessary to infect the majority of cells in a given culture, because only a fraction of the cells divides at a given time in non-synchronized cultures. This requirement may become a severe hurdle when *ex vivo* culture is detrimental to the maintenance of relevant stem cell properties and is accompanied by differentiation, as has long been the case with HSCs[66,67,83].

Lentiviral vectors were originally developed to overcome this hurdle[84]. Hybrid vectors exploit the capacity of HIV or other lentiviruses to reach the nucleus of non-dividing cells, while using the envelope of unrelated viruses to widen their tropism. Thus, these hybrid vectors allow proficient gene transfer in a wide variety of target cells. The advantages of lentiviral vectors have been shown in an extensive number of experimental and preclinical studies and, more recently, in clinical trials of T cell and HSC gene therapy[23,85]. Although lentiviral vectors offset the strict requirement for cell division that is imposed by γ-retroviral vectors, they still require T cells and HSPCs to be stimulated in order to progress into the late G1 phase for efficient transduction[86–90].

Because the culture conditions used for transduction of T cells and HSCs are essentially the same for γ-retroviral and lentiviral vectors, one may wonder what is gained by switching from the former to the latter. The answer is more efficient transduction with shorter culture times. HSC gene transfer protocols used in clinical trials have enabled detectable marking of long-term repopulating HSCs by γ-retroviral vectors. The extent of myeloid cell marking in patients with adenosine deaminase-deficient SCID (ADA-SCID) at long-term follow-up (which is a measure of HSC gene marking) was shown to vary from 0.1% to almost 10%, indicating that at least some HSCs had divided and were transduced *ex vivo*, without losing their long-term repopulation potential[15,91]. However, it is possible that these marking levels also reflect a growth advantage of the corrected cells, as similar levels have not been reported in other trials using the same vector platform. By contrast, the initial results of lentiviral vector-based HSC gene therapy trials show stable multilineage gene marking at around 15% in the three patients reported so far, with several common vector integration sites among the different lineages[23,24]. These results demonstrate a clear gain in HSC transduction efficiency in the absence of a selective advantage conferred by the therapeutic gene. Preliminary observations made in clinical trials for metachromatic leukodystrophy and Wiskott–Aldrich syndrome show that even higher levels of gene marking can be obtained by optimizing lentiviral vector manufacturing and transduction (A. Biffi, A. Aiuti and L.N., unpublished observations).

Insertional genotoxicity. Insertional genotoxicity is an equally important factor to consider when choosing a vector type and design for cell therapy. The mechanisms of insertional mutagenesis by integrating vectors

Episome
An extrachromosomal DNA element, such as a plasmid, in a cell nucleus.

Minichromosome
An extranumerary minimal chromosome that contains functional elements, such as telomeres and centromeres, and is transmitted in meiosis and mitosis.

Retroviruses
RNA-containing viruses that encode an RNA-dependent DNA polymerase, reverse transcriptase. Retroviruses replicate by reverse transcription and then integrate into the host genome. They comprise simple and complex retroviruses according to the genome organization. Simple retroviruses have oncogenic potential.

Lentiviruses
Retroviruses with a complex genome that usually causes delayed disease in their hosts. HIV is an example of a lentivirus.

Transposase
An enzyme that carries out the site-specific DNA recombination that is required for transposition.

Tropism
The spectrum of tissues and host species that a virus or viral vector can infect, owing to a restricted distribution of receptors or other essential cofactors of infection in certain tissues or species and not in others.

recapitulate those unravelled by early studies of retrovirus-induced oncogenesis and exploited in oncogene capture screenings[92,93]. A wealth of recent studies has compared different types of vector by transducing mouse or human HSPCs and monitoring them for rare cell transformation events. Monitoring is done either *in vivo* (by serial transplantation or using cells from tumour-prone mice to increase the sensitivity of detection for oncogene-activating insertions) or *in vitro* (by cell immortalization assays)[94–98]. These studies have yielded a better understanding of the key features underlying the relative genotoxic risk of different vector types and configurations[98–100] (FIG. 2).

The most dangerous insertions give rise to dominant gain-of-function mutations, such as the activation of proto-oncogenes flanking an insertion site, and are mediated either by enhancer and/or promoter elements in the vector or by aberrant splicing from the vector transcript[18–20]. Unfortunately, both events are strongly favoured by the genetic structure of retroviruses, which contain two sets of enhancers and promoters in their long terminal repeats (LTRs), and a strong splice donor site downstream of the 5′ LTR. This arrangement facilitates capture of flanking genes by splicing of transcripts that originate at the 5′ LTR or by transcriptional readthrough from the 3′ LTR, as well as their transcriptional activation from enhancers present in both LTRs[92,93,98]. These events are even more likely in γ-retroviruses, which have a bias for integration near promoters of active genes[101,102]. Thus, current approaches to improve vector safety call for: the use of self-inactivating (SIN) LTRs that delete transcriptional control elements from both LTRs during the transduction process[98,99]; the use of an internal promoter with weak-to-moderate activity to drive transgene expression[100]; the choice of vectors with an integration machinery that does not result in a bias towards insertion near promoters or active genes[103]; and, possibly, the incorporation of chromatin elements such as insulators that may shield the genes flanking the vector insertion site from the influence of the vector itself. However, the actual improvement in safety that is conferred by each of these changes remains to be fully established and validated in clinical trials. A brief discussion of the challenges that have been met in predicting the occurrence of adverse insertional events in clinical trials of gene and cell therapy is provided in BOX 2.

As vectors with improved safety profiles and transduction proficiencies are tested in clinical trials, we may become increasingly concerned about additional mechanisms of mutagenesis, which potentially could be associated with a lower risk or delayed onset of oncogenesis[24]. Such events may even be caused by SIN vectors, and may comprise loss-of-function mutations in tumour suppressor genes caused by intragenic vector insertions that would lead to premature termination or aberrant splicing of the endogenous transcript, or induce chromatin remodelling in the region flanking the vector insertion site. Studies are required to address the frequency of occurrence of these kinds of events, whether they may adversely affect safety, and how the vector design can be modified to abrogate them.

Concerning the risk of insertional mutagenesis, it is important to note that peripheral blood-derived T lymphocytes seem to be less susceptible to transformation by genotoxic events than are HSCs. Retroviral-mediated gene transfer to T cells has been tested in several clinical trials[4,6,7,44,45] and gene-modified cells have been detected in patients for more than 10 years[104,105] without any report of malignancy related to gene transfer.

Expression proficiency and regulation. The robustness of transgene expression varies with the vector type and design. It may depend on the integration site selection of each vector and the activity of genomic surveillance mechanisms. For instance, γ-retroviral vectors are known to be prone to silencing of expression by DNA methylation that specifically targets the LTR sequence, whereas SIN lentiviral vectors tend to be more robustly expressed[106]. Each integration site can influence the level of vector expression, probably through local epigenetic features and the underlying endogenous gene expression pattern. This accounts for the typical expression variegation that is observed in a bulk population of cells transduced with an average of one vector copy per genome. A fraction of integration sites may also be silenced from the time of insertion or become silenced after an initial phase of expression. Cell differentiation may result in substantial remodelling of chromatin, which could affect the stability and extent of expression of vectors that have become integrated in stem cells. DNA methylation, deposition of novel histone marks, spreading chromatin condensation and changes in nuclear sublocalization can all potentially affect the expression of an integrated transgene. As mentioned above, the integration site selection of several viral vectors favour expressed genes; although this feature may increase the chances of gene disruption, it may also favour the robustness and stability of expression in comparison with systems that target gene deserts or heterochromatic regions.

Expression of the exogenous gene may be driven ubiquitously in the transduced cells and their progeny or, conversely, targeted to a specific differentiation state or lineage. Ubiquitous expression is easier to engineer from a vector. It can be obtained by exploiting the original viral promoter or, more reliably, by inserting into the vector the constitutive promoter of a cellular housekeeping gene such as phosphoglycerate kinase, elongation factor 1α or ubiquitin. These promoters may also provide the best guarantee of stable expression. However, constitutive expression of a differentiation-stage-specific factor (such as a recombinase) in stem or progenitor cells and all their progeny might be risky or detrimental. In addition, certain applications strictly require switching on expression — and reaching very high expression levels — at only the proper stage of differentiation, such as in the case of globin transgenes used for HSC gene therapy of β-thalassaemia[107,108]. In these cases, transcriptional regulatory elements derived from the endogenous locus or from other genes with a similar expression pattern may be incorporated into the vector. It should be noted, however, that it is often difficult to achieve a stringent regulation of transgene

Serial transplantation
When donor-derived cells or tissue are used for another transplant after engraftment in a primary recipient.

Long terminal repeat
(LTR). A DNA sequence that is repeated at each end of an integrated retroviral DNA (provirus). A LTR contains regulatory sequences that are required to initiate transcription of the viral DNA into an RNA that is packaged into viral particles, retro-transcribed and integrated into the target cell DNA.

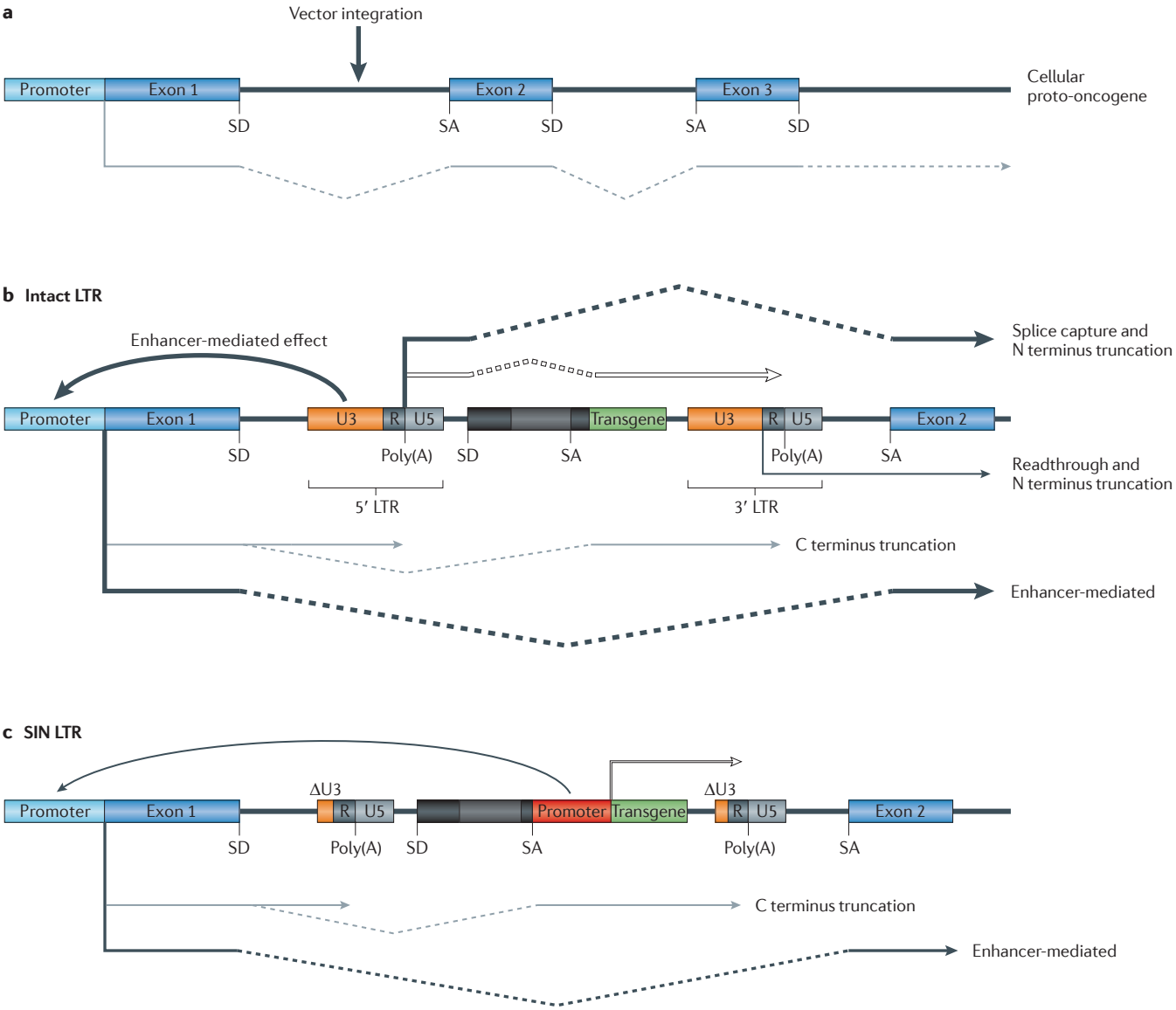

Figure 2 | **Mechanisms of vector insertional mutagenesis.** Certain genotoxic events could lead to activation of the transforming potential of a cellular proto-oncogene after integration of a retroviral vector within or near the proto-oncogene. **a** | A vector integration site is depicted in the first intron of a proto-oncogene. The grey arrow beneath the gene indicates the normal transcript, with the broken segments indicating intronic sequences that are removed by splicing. SD indicates a splice donor site and SA indicates a splice acceptor site. **b** | A conventional γ-retroviral vector is integrated at the site indicated in part **a**. The two long terminal repeats (LTRs) contain strong enhancers and promoter elements in the U3 region; the R and U5 regions are also indicated. The white arrow indicates the vector transcript, which encodes the transgene. This integration could lead to upregulated transcription of the proto-oncogene from its cellular promoter by enhancer-mediated effects. Alternatively, splice capture from the promoter in the vector 5′ LTR could give rise to a chimeric transcript encoding an N-terminally truncated form of the oncogene with constitutive activity and transforming potential. Another possible mechanism giving rise to an N-terminally truncated form of the oncogene is readthrough transcription originating from the vector 3′ LTR. This event is less likely when the vector encodes a transgene because of promoter interference (occlusion) between the upstream and downstream LTRs. Finally, truncation of the endogenous transcript might occur as a result of transcription termination at the polyadenylation (poly(A)) sites contained in the vector LTR, with or without aberrant splicing between the cellular and the vector splice sites. This transcript gives rise to a C-terminally truncated form of the oncogene that may have constitutive activity and transforming potential. **c** | All LTR-dependent events shown in part **a** are abrogated by the use of a vector with self-inactivating (SIN) LTRs. Here, the U3 transcriptional control elements are deleted (ΔU3) from both LTRs during the transduction process, and the vector expresses the therapeutic gene from an internal promoter. A residual concern is long-range transcriptional activation of the oncogene, which could be mediated by the enhancer elements of the internal promoter. However, this concern can be alleviated if an exogenous promoter with only moderate activity is used in the vector, resulting in lower proto-oncogene expression than shown in part **b**.

Box 2 | **Coping with insertional mutagenesis**

A persistent frustration of gene therapy investigators has been the inadequacy of preclinical models to predict the likelihood of insertional adverse events in clinical trials[135]. This is probably due to the limitations of mouse models, which can be transplanted with only a limited number of cells and then monitored for only their relatively short lifespan. For these reasons, rarely occurring transforming events cannot be distinguished from background oncogenesis and natural death with sufficient statistical power[136,137]. Similarly, no currently available mouse model for human cell engraftment provides the depth and time of analysis required to monitor low-incidence multi-step oncogenesis[138]. Large animal models, such as dogs and non-human primates, better recapitulate the dynamics of tissue or lineage reconstitution that are observed in clinical trials. Furthermore, they allow cell and vector doses to be escalated and they extend observation time to several years[139–141]. Such studies have provided the means to stringently validate some vector performance features and have yielded relevant insights into the biological function of certain transgenes. However, feasibility, time and cost issues prevent cell transplantation in large animal models as a means to screen for the occurrence of low-incidence oncogenesis in the routine preclinical testing of *ex vivo* gene therapy applications. Moreover, species-specific restriction factors may affect the cell permissiveness to gene transfer by certain vector types, thus limiting the power and sensitivity of the model[142].

A surrogate read-out for the occurrence of genotoxicity in transplantation studies can be provided by monitoring the clonal composition of the graft by taking advantage of vector insertions, which provide a unique genetic mark of each transduced cell[143–147]. If clones carrying vector integration within a common set of growth control genes repeatedly become dominant within the graft — and integration within these genes is not observed with similarly high frequency in the cells before transplant — this may indicate *in vivo* selection of gain-of-function insertional mutants before they progress to overt malignancy[20–22,98,148–154].

Although these findings still fall short of predicting the actual risk of oncogenesis for each individual subject or in a clinical trial, they can help in ranking the different vector types and design according to their genotoxic potential. In addition, these studies strongly support the ongoing clinical translation of safer vector designs in cell and gene therapies.

The inability to predict the oncogenetic risk of a cell therapy simply on the basis of the choice of vector and the total integration load in the transplanted cells arises from our lack of a comprehensive understanding of all the factors that determine whether a transplanted cell bearing a genotoxic insertion becomes established *in vivo*, and whether its outgrowth eventually progresses to malignancy. The cell type used, the fraction of progenitor or stem cells in the total infused population, the competition among transplanted cells for engraftment, the activity of the therapeutic gene and the underlying disease condition are all major variables that affect this outcome[155]. Furthermore, genomic constraints can limit the functional interaction between an inserted vector and the flanking genes, and only a small fraction of genotoxic insertions may trigger transformation in each specific cell type. The possibility that only a few insertions may cause transformation is indicated by the fact that all clinical cases of vector-related oncogenesis reported so far show a remarkable specificity for the type of oncogene targeted and the additional mutations that drive progression[18–22].

Overall, there is still no substitute for performing a clinical trial to assess the ultimate risk of a cell therapy procedure. Thus, the decision to start a trial is often based as much on science as on the ethically viable principle that the unpredictable risk of oncogenesis might be balanced by the potential expectation of a benefit that far outweighs the limitations or lack of alternative treatment options. As we increase patient follow-up in clinical trials using the improved vectors discussed in the main text and hopefully validate the preclinical prediction of better safety, it will become easier to broaden cell and gene therapy applications to more diseases.

expression solely by attempts to reconstitute a lineage-specific promoter in a gene transfer vector. This is due mainly to the challenge of identifying and reconstituting all genomic control elements within the compact genome and limited size of a vector. Moreover, if retroviral vectors are used, their infectious cycle entails transit through an RNA intermediate, which may eliminate all intronic sequences from the transferred genome, and its reverse transcription, which may further eliminate unstable and repeated sequences by recombination.

As mentioned above, integrating vectors are also prone to influences arising from their random integration site, and these effects can sometimes dysregulate the control exerted by the internal promoter. Finally, certain biological properties of stem cells, such as lineage priming, may also contribute to leaky expression from putative differentiation-specific promoters. A recently proposed strategy to improve the stringency of transgene expression regulation is to exploit the differential expression of microRNAs between stem or progenitor as compared to mature cells, or between distinct differentiation lineages[109,110]. By incorporating the target sequence for a microRNA in the 3′ UTR of a transgene, its expression is suppressed specifically in the cells that express that microRNA. By this approach, expression can be de-targeted from unwanted cell types[111]. Because this control is post-transcriptional, it can be combined with transcriptional control elements to obtain more specific transgene expression patterns[112]. Moreover, by optimizing the number, design and combination of microRNA target sequences that are incorporated in the vector, one can improve the robustness of suppression and alleviate concerns about inhibiting the regulation of the natural targets of the microRNA[113].

Gene correction and targeted integration

The recent development of designer endonucleases, such as zinc finger nucleases (ZFNs)[26,114,115], homing meganucleases[116] and transcription activator-like effector (TALE) nucleases[117,118], has brought the possibility

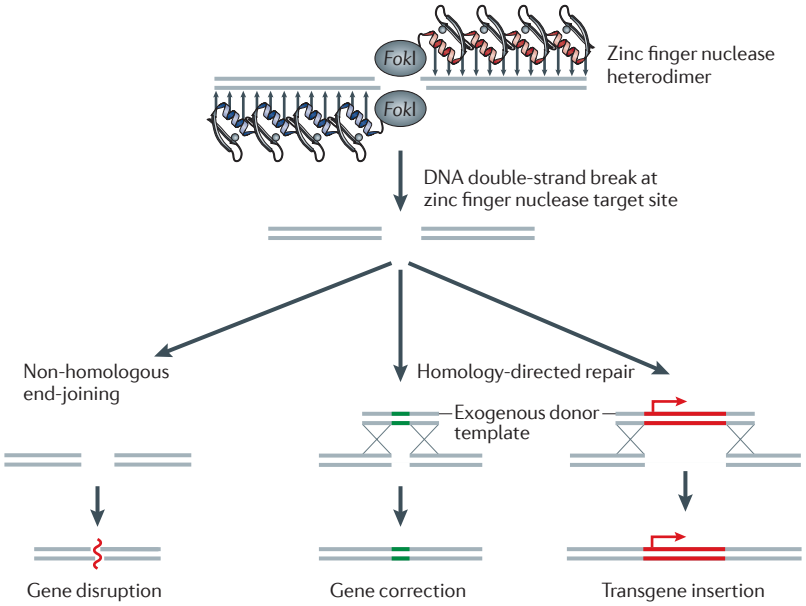

Figure 3 | **Gene targeting and gene editing using designer endonucleases.** A DNA double-strand break (DSB) is induced at a pre-selected sequence of the genome by an engineered endonuclease that is transiently expressed in a target cell. A zinc finger nuclease (ZFN) dimer is shown; each monomer consists of a nuclease domain from the *FokI* endonuclease and four zinc finger domains that recognize a specific 12 bp sequence. When two ZFN monomers bind on either side of a 5 bp or 6 bp spacer, an active nuclease complex is formed and DNA cleavage takes place in the spacer. Depending on the repair process that seals the break, the outcome can be gene disruption or reconstitution or editing of the original sequence. If the DSB is sealed by the error-prone process of non-homologous end-joining, which can occur at any time in the cell cycle, deletions and addition of non-templated bases are common. When essential coding or regulatory sequences are targeted, they can be stably disrupted, giving rise to a somatic knockout of a gene if both alleles are targeted. Alternatively, if the DSB is sealed during the S or G2 phase of the cell cycle, homology-driven repair is common because the two sister chromatids are in close proximity, providing a nearby homology donor and thus faithfully reconstituting the original sequence. If an exogenous donor template is provided that contains homology to the DNA sequences on each side of the DSB, the targeted sequence can be edited and novel sequences can be introduced at the site. This last mechanism allows the correction of endogenous mutations occurring at or near the site of the DSB or the introduction of novel sequences, such as a transgene expression cassette.

of gene targeting within the reach of cell and gene therapy[119]. These artificial proteins comprise a nuclease domain and a DNA binding domain whose sequence specificity can be engineered. They are used to target a DNA double-strand break (DSB) with high efficiency and specificity to a pre-selected and potentially unique sequence in the genome[26,114]. Depending on the repair process that seals the break[120], the outcome can be gene disruption, reconstitution or editing. If the DSB is sealed by the error-prone process of non-homologous end-joining, mutations and deletions can be introduced at the site and, when essential coding or regulatory sequences are targeted, they can be stably disrupted, giving rise to a somatic knockout if both alleles are targeted. Alternatively, if the break is sealed by homology-directed repair (HDR) and an exogenous template is provided, the targeted sequences can be edited and novel sequences introduced at the site (FIG. 3).

Gene disruption and gene editing. An example of the use of gene disruption in gene therapy is the production of T cells that are resistant to HIV infection. One way of doing this is through deletion of C-C chemokine receptor type 5 (*CCR5*), an essential viral co-receptor[121,122], as in a recently started clinical trial using ZFNs. Another approach is silencing expression of the endogenous receptor, followed by introduction into the T cells of a novel engineered receptor (L.N. and C. Bonini, unpublished observations). Gene editing allows correction of genetic mutations by restoring the wild-type sequence[123–126] or inserting a functional cDNA copy of the gene downstream of its own promoter[124]. The latter approach has the advantage that most disease-causing mutations affecting the locus, including deletions, can be treated with the same designer nuclease(s). Note that gene correction, as opposed to gene replacement, not only restores the function of the gene but also its endogenous expression control, coming close to the long-sought 'Holy Grail' of gene therapy.

Targeted integration. Another powerful application of gene editing is to target integration of a gene cassette into a genomic site that has been chosen for permissiveness to robust expression, tolerance to insertion without any detectable harmful consequence[124,127] and resistance to transcriptional perturbation of the flanking genes (A. Lombardo and L.N., unpublished observations). Such sites have been referred to as 'safe genomic harbours', although it remains difficult to define the preferred features of such sites and identify potential candidates. Furthermore, the predicted safety of an insertion must also take into account the specific design of the cassette that is being transferred. Once validated, such a combination of genomic acceptor site and transgene cassette would provide a platform for site-specific integration that would be widely applicable in cell therapy approaches.

Despite the great promise of these technologies, several challenges must be addressed to fully exploit their potential in gene and cell therapy. Delivery of the molecular machinery for homology-driven gene editing may be challenging when dealing with primary cell types and requires adapting vector platforms to induce a transient but robust spike of co-expression of nucleases and donor template DNA[124]. Permissiveness to HDR varies with the cell type and requires transit through the S–G2 phase of the cell cycle, whereas induction of DNA DSBs may induce apoptosis and growth arrest[120]. As the efficiency and specificity of designer nucleases continue to improve and make possible new therapeutic applications, we also need to thoroughly assess the potential impact of these nucleases on the genome through sensitive genome-wide assays for induced DSBs. This represents a challenge given the 'hit-and-run' nature of these events, which can escape detection upon repair (C. von Kalle, M. Schmidt and L.N., unpublished observations).

Overall, although site-specific gene editing requires *ex vivo* culture and selection, protocols for the rapid generation and selection of the cells that carry the desired targeted modification can now be accomplished and translated to clinical use — a quantum leap forward in comparison with standard gene targeting approaches.

In vivo cell administration

Conditioning of the patient before cell administration is a well-established procedure in HCT and has provided a crucial advance when applied to HSC gene therapy[128]. Irradiation or, more commonly, administration of chemotherapeutic drugs that are toxic to proliferating cells is used to empty bone marrow niches and/or induce homing and proliferation signals that favour engraftment and rapid clonogenic outgrowth of the transplanted HSPCs[3]. In addition, allogenic HCT requires T cell depletion to avoid immune-mediated clearance of the engrafted cells. Autologous HSC gene therapy may reduce the requirement for full myeloid and lymphoid ablation, thus sparing patients a prolonged window of myelosuppression and immunosuppression and substantially reducing the morbidity of transplantation, as well as abrogating the risk of graft versus host reaction. A possible concern, however, is that host HSCs that survive a reduced-intensity conditioning are more likely to harbour mutations that promote delayed secondary leukaemogenesis, given the mutagenic effect of some chemotherapeutic drugs.

Conditioning strategies are increasingly being adopted in other applications of cell-based therapy. For example, in T cell therapies a lymphoid-depletion regimen is being tested that is pre-administered to promote robust homeostatic expansion of the infused cells[129]. One may envision that the low grafting efficiency reported in preclinical studies of NSPC or MSC transplantation might be improved by the development of appropriate conditioning regimens that 'make space' in the relevant tissue niches and/or induce local microenvironments that favour tissue regeneration and angiogenesis, rather than inflammatory and immune reactions.

Box 3 | **Monitoring stem cell engraftment and activity**

In cell therapy, monitoring engraftment and clonogenic activity of the transplanted cells *in vivo* may be challenging. When the cells have been modified by gene transfer and their progeny can be conveniently sampled (as in the blood or bone marrow after haematopoietic stem cell (HSC) gene therapy), vector insertions can provide unique clonal markers to longitudinally track the survival and clonogenic activity of the engrafted cells[91,143–147,153].

Assessing gene transfer into human long-term HSCs has long been a challenge. *In vitro* assays have limited value, and repopulation of immunodeficient mice with human HSCs provides only a surrogate readout that is limited by short-term repopulation and the failure to reproduce all lineages[138,156]. Thus, clinical trials of HSC gene therapy provide a unique opportunity to validate these preclinical models as well as several long-held concepts about haematopoietic hierarchy and homeostasis. However, tracking vector insertion sites in the repopulated haematopoietic system of treated patients as clonal markers of the transduced HSPC, although representing the gold standard today, is still subject to technical challenges and biological confounding influences[23,144,153,157].

Technical challenges

Despite the remarkable advances in our capacity for high-throughput analysis and deep sequencing, several issues remain to be addressed, including:

- The occurrence of biases versus randomness in retrieval efficiency.
- The need to develop methods to identify and eliminate cross-contamination between sequencing data or biological material originating from different lineages, which may become intractable with increasing sensitivity of analysis and falsely indicate multi-lineage marking.
- The need to develop methods to estimate how exhaustive is the analysis, especially when highly polyclonal grafts are obtained and the likelihood of retrieval of the same integration site in a highly complex mixture may become small, hindering our ability to track individual clones.

The rapid pace at which genomic sequencing technologies and bioinformatics are evolving promises fast progress.

Confounding factors

Several biological confounding factors may influence the behaviour of gene-marked cells *in vivo*:

- The potential impact of the underlying disease on the stem cell niche, HSC frequency and engraftment.
- The function of the therapeutic gene, which may confer a selective growth advantage and drive expansion of the corrected cells.
- The conditioning regimen administered before cell infusion.
- Vector insertional effects on cell fitness; most importantly, the induction of gain-of-function mutations that drive clonal expansion.

The last concern raises a crucial issue: how neutral can gene marking ever be when it is obtained by quasi-random genome-wide vector insertion? This is still an outstanding question for the field, and I have discussed above how far we have come in understanding the features responsible for vector genotoxicity and in improving vector design to alleviate genotoxicity. If the ongoing trials with late-generation lentiviral vectors establish a relatively benign gene marking, they will also provide our first in-depth look at the dynamic behaviour of haematopoietic stem/progenitor cells (HSPCs) in humans after transplant. Indeed, these studies[23,24], as well as the clonal tracking of human HSPCs in severe combined immunodeficiency (SCID) mice[154], seem to be reporting a surprisingly higher frequency of long-term engrafting cells than would be expected from standard limiting dilution repopulation studies. Does this imply a non-neutral marking that affects cell survival or do we need to revisit established assumptions surrounding the dynamic of committed progenitors? As HSC gene therapy continues to progress in clinics, we can expect more intriguing findings and new insights into fundamental aspects of haematopoiesis.

Neoantigen
A newly encountered substance that the immune system can respond to by producing antibodies or immunoreactive T cells.

The recipient immune response remains a major hurdle to the safe deployment of any allogenic cell therapy, and sometimes even to autologous cell and gene therapy, when a transgene-encoded neoantigen triggers clearance of the transplanted cells. Emerging approaches to induce regulatory T cells *ex vivo* or directly *in vivo* to stimulate development of antigen-specific tolerance may provide a long-sought solution to this hurdle[46]. Approaches to longitudinally tracking the clonogenic activity of engrafted cells, by taking advantage of vector insertions as clonal markers, are discussed in BOX 3.

Conclusions

The long-term follow-up of patients with SCID who were treated with HSC gene therapy provides the most comprehensive and compelling assessment of the clinical impact of *ex vivo* gene- and cell-based therapy today. It shows that HSC gene therapy may compare favourably to allogenic HCT and, in selected conditions, become a first-line treatment and a registered medicine for the market. Although severe adverse events have been observed at an alarming frequency in HSC gene therapy trials conducted with early-generation vectors, their occurrence also seems to be influenced by the underlying disease condition and transgene function, thus tempering any move towards a general moratorium on the procedure.

In addition, a decade of working to understand the most relevant genotoxic features of vectors and insertional mutagenesis mechanisms has yielded several safety improvements in vector type and design that are now entering clinical trials for stringent validation. Among these improved vectors, late-generation SIN lentiviral vectors are already achieving substantial levels of HSC gene marking with a more benign insertion pattern as compared to earlier vectors. If established further through the treatment of more patients with a longer follow-up time, the therapeutic benefits that have been made possible by the increased transduction efficacy of these vectors, together with the apparently improved safety observed until now, will open the way to the treatment of a broader spectrum of diseases.

One of the key future challenges in translating this clinical progress into personalized medicines is to establish an efficient supply chain, which includes scaled-up vector manufacturing, distribution and cell transduction in a highly coordinated system that meets the regulatory requirements of the drug market. The involvement of the pharmaceutical industry, probably in close partnership with academia, will be crucial to addressing this area.

Crucial information is also eagerly awaited from new clinical trials using other somatic stem or progenitor cell types, such as NSPCs and MSCs. The recent development of powerful new technologies for highly efficient gene targeting and site-specific gene editing brings the possibility of somatic gene disruption, gene correction rather than gene replacement, and targeted rather than random integration within the reach of gene therapy, thus offering the potential to overcome the main hurdles preventing further progress of the field.

Finally, as we get ready for broader applications of *ex vivo* therapies, and especially as we explore how to exploit the new gene editing technologies, we need better sources of stem cells that can be more conveniently isolated and selected *in vitro* than most current somatic stem cell types. A fast-growing field of study has been using gene transfer to reprogramme somatic cells to pluripotency[130]. After safe and robust methods for generating these induced pluripotent stem cells and driving their differentiation towards different cell types are established, these cells may eventually provide new opportunities for cell-based therapies. Together with the new sophisticated gene transfer approaches described in this Review, they may provide the ultimate answer to the quest for safe and efficacious therapies for currently untreated or poorly treated diseases.

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Acknowledgements
I thank my colleagues at the Telethon San Raffaele Institute for Gene Therapy, The Division of Regenerative Medicine and Gene Therapy of the San Raffaele Institute, and all my past and current laboratory members for sharing in the excitement and challenges of making cell and gene therapy a clinical reality, as well as for critical discussion of this manuscript. The Naldini laboratory is supported by Telethon, the European Research Council, the European 7th Framework Program for Life Sciences, the Italian Association for Cancer Research, the Italian Ministries of Health and of Scientific Research, Sangamo Biosciences, the Cariplo Foundation and the Stop ALD Foundation. The author apologizes to all scientists whose research could not be properly discussed and cited in this Review owing to strict space limitations. For this reason, many primary and historical publications have not been cited, particularly in cases where topical reviews are available.

Competing interests statement
The author declares no competing financial interests.

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