REVIEW

Gene therapy: principles and applications to hematopoietic cells

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Ever since the development of technology allowing the transfer of new genes into eukaryotic cells, the hematopoietic system has been an obvious and desirable target for gene therapy. The last 10 years have witnessed an explosion of interest in this approach to treat human disease, both inherited and acquired, with the initiation of multiple clinical protocols. All gene therapy strategies have two essential technical requirements. These are: (1) the efficient introduction of the relevant genetic material into the target cell and (2) the expression of the transgene at therapeutic levels. Conceptual and technical hurdles involved with these requirements are still the objects of active research. To date, the most widely used and best understood vectors for gene transfer in hematopoietic cells are derived from retroviruses, although they suffer from several limitations. However, as gene transfer mechanisms become more efficient and long-term gene expression is enhanced, the variety of diseases that can be tackled by gene therapy will continue to expand. However, until the problem of delivery and subsequent expression is adequately resolved, gene therapy will not realize its full potential. The first part of this review gives an overview of the gene delivery technology available at present to transfer genetic sequences in human somatic cells. The relevance of the hematopoietic system to the development of gene therapy strategies as well as hematopoietic cell-based gene therapy is discussed in the second part. *Leukemia* (2001) 15, 523–544.

Keywords: gene therapy; hematopoietic cells; hematopoietic stem cells; T lymphocytes; viral gene transfer; recombinant viral vectors; retrovirus; adenovirus; adeno-associated virus (AAV); lentivirus; herpes simplex virus; nonviral gene transfer; lipofection; receptor-mediated gene transfer; particle-mediated gene transfer; DNA injection; electroporation; extrachromosomal replicating vectors

Gene delivery technology

Importantly, there is no such thing as an 'ideal' vehicle system for all gene therapy applications. In each gene therapy setting, the optimal vector system is determined by the nature of the disorder (inherited or acquired), the type of gene delivery (*in vivo* or *ex vivo*), the duration of transgene expression (long-term or short-term) and the target cell type. A variety of chemical, physical and biological gene transfer methods have been developed in order to deal with the 'delivery problem'. These techniques range from relatively simple forms of transfection such as electroporation, lipofection and gene gun to the exploitation of the natural infectious properties of recombinant viral vectors.

Recombinant viral vectors

Biological gene transfer methods make use of modified DNA or RNA viruses to infect the cell, thereby introducing and expressing its genome which contains the gene of interest (= 'transduction').¹ The most commonly used viral vectors are discussed below. In each case, recombinant viruses have had the genes encoding essential replicative and/or packaging proteins replaced by the gene of interest. Advantages and disadvantages of each recombinant viral vector are summarized in Table 1.

Retroviral vectors

After seminal work of the group of Rosenberg demonstrating the feasibility and safety of using retroviral gene transduction for human gene marking studies,² recombinant retroviruses have become widely used and well characterized vectors for preclinical and clinical gene therapy trials.^{3,4} It is the unique life cycle of the retrovirus that has made it an attractive vector for gene transfer.⁵ Retroviral transduction is usually performed in the presence of a poly-cation (eg polybrene or protamine sulphate) to reduce electro-static interference between the virus and its target cell. Retroviral vectors are packaged into infectious particles after introduction into a retroviral packaging cell line containing the necessary structural genes (*gag*, *pol* and *env*, coding respectively for structural proteins, reverse transcriptase/integrase and envelope protein) but lacking the packaging signal (Ψ, *psi*) required for encapsidation of vector RNA into virions (Figure 1). The recombinant vector containing the gene of interest is devoid of structural genes but has retained the long terminal repeats (LTR) required for viral integration and transcription and the packaging signal. This complementary process ensures that the producer cell line effectively generates replication-incompetent retroviral vectors that are capable of only one round of transduction (Figure 1).³

Advantages of retroviral gene transfer are high transfer efficiency and stable chromosomal integration. Disadvantages include the inability to infect nondividing cells,⁶ transcriptional repression,^{7,8} and potential insertional mutagenesis.⁹ Furthermore, the size of the gene insert is limited as retroviruses are only capable of packaging around 10 kb of genetic material.¹⁰ Noteworthy, with the latest generation of complementing packaging cell lines, the risk of producing replication-competent retroviruses (RCR) has practically been eliminated. By placing the *gag-pol* and *env* genes on to separate plasmids that could be introduced separately into the packaging cell line, the risk of RCR production has been virtually eliminated (Figure 1).¹¹ The problem of the LTR overlap that exists between the vector components has been solved

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Received 4 May 2000; accepted 2 January 2001

Table 1 Summary of viral gene transfer techniques

<i>Viral vector type</i>	<i>Advantages</i>	<i>Disadvantages</i>
Adenoviral vectors	Broad host range Transduce nondividing cells High titer No possibility of insertional mutagenesis High transduction efficiency	Possible (hepato)toxicity Transient expression Lack of tissue specificity Vector immunogenicity (1 fatal reaction described)
Adeno-associated viral vectors	Broad host range Transduce nondividing cells Chromosomal integration Stable expression Wild-type integrates at specific site	5 kb DNA insert Recombinant vectors lose specific integration Difficulty to purify vector (helper AdV contamination) Low titer Possible insertional mutagenesis
Herpes simplex viral vectors	Neurotropic No possibility of insertional mutagenesis Transduce nondividing cells Accept large DNA inserts (15 kb)	Lytic replication Transient expression Low titer
Retroviral vectors	Chromosomal integration Can be tissue-specific Stable expression Not immunogenic	Possible insertional mutagenesis Transduces dividing cells only Silencing of LTR promoter
Lentiviral vectors	Transduce nondividing cells Tissue-specific Stable expression High titer (10^{10} p.f.u./ml)	Possible insertional mutagenesis Ethical considerations

Figure 1 Retroviral gene transfer. A eukaryotic expression plasmid lacking the essential genes for replication (*gag*, *pol* and *env*) but containing a packaging signal is transfected into a complementing cell line. This cell line is stably transfected with the complementary retroviral genes, thereby providing the structural proteins and reverse transcriptase in *trans*, but lacking the packaging signal required for encapsidation of vector RNA into virions. This complementary system results in the packaging of replication-incompetent recombinant retroviruses. Retroviral vectors transduce target cells by interaction with specific receptors present on the cell surface. After entering the cell, the viral RNA is reverse transcribed, and the proviral DNA randomly integrates into the target cell genome, where the transgene is stably expressed.

through the use of heterologous promoters and polyadenylation signals in the retroviral helper constructs (Figure 1).¹²

The majority of retroviral vectors currently being used are derived from Moloney murine leukemia virus (MoMuLV). MoMuLV-based vectors bind to a target cell through an interaction with a receptor specific for the *env* protein encoded by the complementing cell line, which primarily determines the host range of the retroviral particle.¹³ For the ecotropic envelope protein, which allows transduction of murine cells, the receptor is a cationic amino acid transporter.¹⁴ In order to either expand or limit the viral host range, it is possible to incorporate a heterologous viral envelope protein in the vector particle, a process referred to as pseudotyping. MoMuLV vectors have been pseudotyped with the amphotropic envelope protein, allowing transduction of cells from a wide variety of species including human cells. The receptor target for amphotropic vectors is Pit-2 (formerly designated as Ram-1), a sodium-dependent phosphate transporter.¹³ It has been shown that the density of this receptor on cells correlates with the transduction efficiency of amphotropic retroviral vectors.¹⁵ In addition to using normal ecotropic and amphotropic envelope proteins, MoMuLV-based vectors are being pseudotyped with the gibbon ape leukemia virus (GALV) envelope protein which uses the phosphate transporter Pit-1 as target receptor. These GALV-pseudotyped MoMuLV vectors have been shown to be more efficient in transducing human hematopoietic cells than amphotropic vectors.^{16,17} Alternatively, MoMuLV-based vectors can be pseudotyped with the envelope G protein of the vesicular stomatitis virus (VSV-G) that interacts with ubiquitous membrane receptors such as phosphatidylserine. The vast distribution of these receptors appears to increase the vector host range from zebrafish to humans.¹⁸ Moreover, the stability of VSV-G allows pseudotyped particles to be concentrated by ultracentrifugation to titers (10^9 plaque-forming units (p.f.u.)/ml) that are higher than those of amphotropic vectors.¹⁸

In addition to using envelope proteins with different cell tropism, the *Env* protein has been modified by incorporation of ligands to alter its binding properties for targeted gene delivery.^{19,20} One method is by making fusion proteins of the *Env* protein and a single chain variable immunoglobulin fragment (scFv), the so-called 'chimeric envelopes', recognizing a target cell surface marker.^{21,22} After binding of the immunoglobulin component to its antigen, matrix metalloproteinases cleave off the antibody, thereby allowing *Env*-mediated transduction. These vectors efficiently transduce cells that express the corresponding antigen.

Further optimization of the basic MoMuLV vectors was performed in order to overcome transcriptional repression (= 'silencing') due to cytosine methylation or due to binding of inhibitory transcriptional factors to sequences in the viral primer binding site.^{7,8} This resulted in retroviral vectors with greatly improved function. For example, inclusion of the enhancer elements of the polycythemic strain of the spleen focus-forming virus (SFFVp) was shown to be superior to the classical MoMuLV enhancer configuration for transduction of hematopoietic cells.^{23–25} Also, intron-based retroviral vectors,²⁶ as well as inclusion of post-transcriptional elements such as the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE),^{27,28} have been used for increased transgene expression.

Most importantly, a recent trial using retroviral gene transfer in hematopoietic stem cells (HSC) to treat children with X-linked severe combined immunodeficiency (SCID) resulted in reconstitution of the patients' immune system with transduced

functional T lymphocytes. Patients did not receive additional therapies, are off-therapy and doing well. Although further follow-up is needed to assess long-term effects of gene transfer, this trial represents a major milestone in the field of gene therapy for a monogenetic disease.²⁹

Adenoviral vectors

In the fast growing area of vector systems, one of the most advanced vectors is the replication-deficient recombinant adenovirus (AdV). Although its natural tropism for respiratory epithelium has prompted its use in lung-related disorders (eg cystic fibrosis), it is remarkably efficient at transferring genes to a wide variety of cells *in vitro* and *in vivo* and at expressing exogenous genes at a high level.³⁰ AdV can transduce most cell types regardless of the cell cycle phase.³¹ After transduction, AdV expresses the transgene in replicating and nonreplicating cells in an episomal fashion, of which the duration of gene expression is transient. Therefore, AdV vectors are not likely to be used for stable transduction of HSC, notwithstanding recent reports on AdV transduction of CD34⁺CD38[−] hematopoietic progenitors.³² The widespread distribution of Cocksackie and adenovirus receptors binding the adenoviral pentons of the viral coat (each penton is composed of a penton base and a trimeric fiber) (Figure 2a) limits the use of AdV

Figure 2 Adenoviral vectors. (a) Structure of an adenovirus (AdV) particle. The protein coat comprises 240 hexons and 12 pentons forming 252 capsomeres in an icosahedral structure. Each penton is composed of a penton base and a fiber. The pentons interact with the Cocksackie and adenovirus receptors present on the target cell. The penton base of the viral coat contains a binding motif, arg-gly-aspartate (RGD). This RGD motif is recognized by integrins $\alpha_3\beta_1$ and $\alpha_5\beta_1$ that mediate internalization of the particle. (b) Structure of the recombinant AdV based on the AdV type 5 genome. First-generation AdV vectors are E1A-, partially E1B-, and E3-deleted, and carry the expression cassette in the position of the E1 deletion. Second-generation AdV vectors included a deletion of the E2 region, and in a later stage also of the E4 region. (c) Latest generation of AdV vectors are aimed at deleting all viral genes in order to circumvent immune response against the vector. These vectors are known as gutless or gutted AdV vectors.

for a targeted gene transfer. Therefore, it is mandatory to ablate the endogenous viral tropism and to introduce a new tropism by exchanging parts of the protein coat or by placing new molecules on the surface of the AdV vector. The penton base of the viral coat contains a binding motif, arg-gly-asp (RGD). This motif is recognized by integrins α_3 and α_5 that mediate internalization of the particle.³³ Consequently, replacement of the RGD motif by other peptide motifs can target the AdV vector to other integrin receptors.³⁴ In addition, the fiber protein can be modified by the introduction of chimeric fiber-fusion proteins or by placing specific monoclonal antibodies on top of the fiber protein.^{35,36} An alternative strategy to target AdV-mediated gene expression would be to use tissue-specific promoters.³⁷

A major disadvantage in adenoviral-based gene therapy strategies is the deleterious inflammatory responses against recombinant AdV. In a cystic fibrosis gene therapy trial, only transient expression (< 30 days) was observed after the first administration and no transgene expression could be observed after repetitive administration, due to neutralizing antibodies and cellular immune responses to viral components.³⁸ Very recently, administration of high-dose AdV vector to an 18-year old patient enrolled in an ornithin transcarbamylase gene therapy trial resulted in a fatal outcome due to a fulminant inflammatory immune response,^{39,40} thereby questioning the use of these vectors in future clinical trials for treatment of genetic diseases.

Although latest AdV producer cell lines prevent generation of replication-competent AdV,⁴¹ AdV pose a risk of infectious recombination between recombinant and wild-type AdV when injected into patients. To avoid the generation of replication-competent AdV, most recombinant adenoviral vectors carry deletions in their viral genome. Replication-incompetent recombinant AdV vectors of the first generation are E1A-, partially E1B- and E3-negative and carry the expression cassette with the gene insert at the position of the E1 deletion (Figure 2b). Subsequently, it became clear that further deletion of E2A resulted in an AdV vector with prolonged transgene persistence and a decreased inflammatory response. These E2A-negative constructs are called second-generation AdV vectors (Figure 2b).⁴² Other deletions, like E4, demonstrated a higher stability and are more efficient for use in gene therapy applications.⁴³ On the other hand, Ilan and colleagues⁴⁴ have demonstrated that the insertion of the E3 region into AdV inhibits host AdV-specific humoral and cellular immune responses. The deletion of different parts of the AdV genome aims to hide the AdV vector from the immune system. The ultimate AdV vector is one that is completely free of any adenoviral gene, also known as gutless or gutted AdV vectors (Figure 2c).⁴⁵ Gutless AdV vectors were shown to be significantly less cytotoxic and immunogenic and gave rise to prolonged transgene expression in rodent and primate models.^{46,47}

Due to the immunological obstacles to AdV-based long-term expression, the focus of the application of AdV vectors shifted from hereditary disorders to localized cancer gene therapy. In order to target as many tumor cells as possible, two strategies were developed. AdV vectors encoding 'suicide' genes such as pro-drug converting enzymes, eg herpes simplex virus thymidine kinase (HSV-TK), that have a 'bystander effect' for uninfected tumor cells, were constructed, thereby amplifying the effect of gene transfer.⁴⁸ Alternatively, oncolytic AdV were developed that are defective in replication in normal cells, but competent to selectively replicate in tumor cells. An AdV deletion mutant dl1520, also called ONYX-015, lacks expression of the E1B-55kDa gene. In wild-

type AdV, the early regulatory E1B-55kDa protein binds to and inactivates the host cell's p53 protein to promote its own replication.⁴⁹ Therefore, E1B-55 kDa gene-attenuated AdV are incapable of replication in normal cells harboring a wild-type p53.⁵⁰ In contrast, in tumor cells with a mutant p53, the E1B-55kDa gene is dispensable, thereby allowing ONYX-015 to replicate and cause tumor-specific cytolysis.^{51,52} Recently, Khuri *et al*⁵³ reported that ONYX-015 in combination with chemotherapy promotes tumor regression in patients with recurrent head and neck cancer.

Adeno-associated viral vectors

Adeno-associated virus (AAV) is a defective, nonpathogenic, single-stranded DNA parvovirus, which requires co-infection with a helper virus (usually AdV, herpesvirus or vaccinia virus), for productive replication.^{54,55} Without co-infection, wild-type AAV preferentially (> 70%) integrates into chromosome 19q13.3-qter and remains latent until it is rescued by a helper virus.⁵⁶ It has been reported that AAV requires both the inverted terminal repeats (ITR), present at the ends of the genome, and the nonstructural proteins, *rep78* and *rep68*, for site-specific integration to occur.⁵⁷ Although some reports have suggested recombinant constructs may partially maintain site-specific integration,⁵⁸ no AAV vectors have yet been manufactured that maintain this unique feature completely.^{59,60}

Some of the qualities of AAV vectors desirable for gene therapy include no proven pathogenicity in humans;⁹ broad host and tissue range;⁵⁴ stable integration into cellular DNA and stable expression of the transgene as shown in animal models;⁶¹ low risk of insertional mutagenesis, due to the weak activation of downstream genomic sequences or intrinsic promoters by the ITR;⁹ lack of viral genes preventing immune responses against the vector and allowing multiple administrations;⁶² and transduction of both proliferating and nonproliferating cells, including post-mitotic neurons and muscle cells.^{63,64}

A major disadvantage is that, when both open reading frames – *Rep* genes coding for regulatory proteins and *Cap* genes coding for capsid proteins – are deleted, recombinant AAV can only accommodate about 4.7 kb of DNA insert, thereby limiting the potential cDNA that can be employed as therapeutic agent. Another disadvantage of this delivery system is the low titer of AAV particles obtained from producer cell lines. Large-scale production is still cumbersome and should be improved for future clinical gene therapy applications.⁶⁵ In most packaging protocols, it is still necessary to perform cotransfection of the transgene and helper plasmid (containing *Rep* and *Cap*) and subsequent infection with helper virus, ie adenovirus (Figure 3). Production of efficient packaging cell lines, stably expressing *Rep*, is hampered by the propensity of *Rep* proteins to suppress cell growth.⁶⁶ One interesting approach to overcome these obstacles is the use of adenovirus-polylysine-DNA complexes, which allow the simultaneous delivery of recombinant AAV plasmid, AAV complementation plasmid and adenovirus.⁶⁷ Another major advance would be the generation of helper virus-free packaging cell lines through transfection of packaging cells with adenovirus genes necessary for AAV gene expression, DNA replication and packaging.⁶⁸

Data regarding the utility of AAV vectors for transduction of HSC have been controversial in the literature. The majority of researchers who have investigated AAV transduction of

Figure 3 Generation of adeno-associated virus (AAV) vectors. A packaging cell line (a human 293 cell line) is transfected with a plasmid containing the transgene flanked by AAV inverted terminal repeats (ITR) and with a helper plasmid providing the AAV regulatory (*rep*) and structural genes (*cap*) flanked by adenovirus (AdV) ITR in order to prevent recombination events leading to wild-type AAV. Co-infection with AdV is required for productive replication and generation of recombinant AAV particles. The resulting AAV vector particles can be separated from the AdV particles by density gradient centrifugation or by heat inactivation of the thermolabile adenovirus.

HSC observed a relatively low gene transfer efficiency,^{69–71} while others reported efficient and stable transduction of CD34⁺CD38[−] cells in long-term cultures.⁷² This controversy originates, at least in part, from differences in the integration/transduction process which is highly dependent on AAV second-strand DNA synthesis. AAV particles carry a single-stranded DNA genome which, upon transduction in cells, needs to be converted to double-strand DNA, in order to allow integration into the host genome. This conversion is brought about by second-strand DNA synthesis, the rate-limiting step for efficient AAV transduction.^{73,74} While this crucial step of AAV-mediated gene transfer does not seem to occur consistently in HSC, it readily occurs in muscle, liver and brain, making these cell types attractive targets for AAV-mediated gene therapy.^{75,76} Based on successful gene therapy trials in canine and murine models of hemophilia B,^{77,78} intramuscular injection of an AAV-based vector expressing human factor IX recently resulted in clinically beneficial effects in three hemophilia B patients.⁷⁹

An important characteristic and consideration for the use of AAV as a vector system, is its natural tropism. The primary AAV type 2 (AAV-2) receptor was recently shown to be membrane-associated heparan sulphate proteoglycan (HSPG), mediating both AAV attachment to and transduction of target cells.⁸⁰ This finding provides an explanation for the broad host range of AAV. Nevertheless, although AAV uses the ubiquitously expressed HSPG as an attachment receptor, the transduction efficiency of AAV vectors varies greatly between different cells and between tissues *in vitro* and *in vivo*, suggesting co-receptor involvement. Human fibroblast growth factor receptor 1 was identified as a co-receptor for successful viral entry into the host cell.⁸¹ Also, $\alpha_5\beta_1$ integrin – the primary receptor for AdV – was identified as a co-receptor for

AAV-2, facilitating virus internalization. Identification of HSPG as a viral receptor, as well as characterization of co-receptors should facilitate development of new reagents for virus purification and provide critical information for *in vivo* distribution of AAV vectors.⁸²

Lentiviral vectors

Recombinant lentiviral vectors based on human immunodeficiency virus type (HIV)-1,^{83,84} HIV-2,⁸⁵ feline immunodeficiency virus (FIV)⁸⁶ and simian immunodeficiency virus (SIV)⁸⁷ have been investigated as vector systems. Although lentiviruses, such as HIV or SIV are not yet being used extensively as vectors, there are certain features of these viruses that render them appropriate for gene therapy applications. Unlike other retroviruses, HIV can infect noncycling quiescent cells. The ability to infect nondividing cells is due to the preintegration complex traversing the intact nuclear envelope.⁸⁸ Recent observations, however, show that in some cases cell-cycle activation is required for transduction, even though cellular mitosis is not an absolute requirement for integration.⁸⁹

Improved packaging systems have been developed. The viral titer from these producer cell lines reached up to 10¹⁰ p.f.u./ml, and the produced virions were replication-incompetent. The cell tropism of HIV-based vectors can be broadened by pseudotyping with VSV-G.⁹⁰ As a result, these vectors can transduce virtually every cell type, including CD34⁺CD38[−] human hematopoietic cells.^{91,92} Also, pseudotyped HIV vectors are capable of transducing nondividing neuronal cells *in vivo*.⁹³ In order to improve the safety features of lentiviral vectors, so-called third-generation lentiviral pack-

aging systems have been constructed, which are devoid of all accessory HIV genes not essential for transduction (except *gag*, *pol* and *env*) in order to prevent viral recombination.^{94,95} Self-inactivating (SIN) lentiviral vectors have been developed to further increase biosafety.^{96,97} In these vector constructs, the 3' LTR contains a 133 bp deletion, including the TATA box and binding sites for transcription factors Sp1 and NF-kappaB. The deletion is transferred to the 5' LTR after reverse transcription and integration in the transduced cells, resulting in elimination of expression from the HIV-1 LTR and allowing expression from an internal promoter. The inactivation design significantly improves the biosafety of HIV-derived vectors, as it reduces the risk for replication-competent lentiviruses, and hampers recombination with wild-type HIV in an infected host. Therefore, these SIN vectors may provide a safer alternative for future gene therapy trials.⁹⁸ As an extra safety measure, the HSV-TK suicide gene could be incorporated, allowing HIV-transduced cells to be selectively destroyed by administration of ganciclovir. However, due to safety and ethical considerations, it may take several years before HIV-based vectors enter clinical trials.

The HIV elements allowing CD4-specific gene transfer have been exploited in other viral-based systems. Pseudotyping of MoMuLV with a truncated HIV-1 envelope glycoprotein resulted in CD4⁺ cell-targeted gene transfer.⁹⁹ Also, MoMuLV vectors have been modified with *cis*- and *trans*-acting genetic sequences of lentiviruses in order to make vectors capable of infecting nondividing cells. However, the initial HIV-MoMuLV chimeras have not led to cell division-independent transduction.¹⁰⁰

Herpes simplex virus vectors

Herpes simplex virus (HSV) is a neurotropic vector.¹⁰¹ It has therefore been developed for therapeutic gene delivery in the potential treatment of neurodegenerative disorders such as Parkinson's disease.¹⁰² Neurons have been transduced with a recombinant HSV vector containing the tyrosine hydroxylase gene. This resulted in increased production of the neurotransmitter, dopamine. Alzheimer's disease, epilepsy, Lesh-Nyan syndrome and multiple sclerosis may become targets for similar gene therapy techniques.¹⁰³ Strategies for treating brain tumors with HSV-based vectors are also being developed.¹⁰⁴ HSV may remain latent or cause cell lysis following cell transduction. As this feature is detrimental for gene therapy strategies, it will be necessary to develop HSV vectors in which the genes responsible for lytic replication are removed.¹⁰⁵ The construction of such a vector has proved to be difficult due to the size of the vector (± 150 kb) and the number of genes it harbors (~ 70).

Nonviral gene delivery

The process of introducing DNA or RNA by nonviral means into a target cell and consequently altering its genotype and phenotype is known as 'transfection'. Nonviral gene transfer formulations, although not reaching the efficiency of viral vectors, have already been introduced in clinical, *in vivo* gene therapy trials. Nonviral systems are attractive for the following reasons: (1) they may be very flexible with respect to size of DNA that has to be transported; (2) they are cost-effective and easily allow large-scale production of plasmid DNA; (3) they implicate less laborious safety testing as compared to recom-

binant vectors; and (4) theoretically there is no restriction on DNA insert length (although transfection efficiency decreases with increasing DNA length). However, nonviral gene delivery techniques suffer from a number of disadvantages as well: (1) low gene transfer efficiency *in vivo*; (2) transient expression due to lack of integration into the host genome; (3) immunostimulatory properties of plasmid DNA; (4) *in vivo* toxicity due to accumulation of lipid components and rapid serum clearance *in vivo*, if lipid technology is applied.

Nonviral strategies include physical methods, such as direct injection, particle-mediated bombardment ('gene gun') and electroporation, which actually force the DNA or RNA into the cells. Other methods involve active cellular internalization of mono- or polycationic lipids or polymers, suitably complexed with DNA or RNA.

Advantages and disadvantages of the nonviral methods described below are summarized in Table 2.

Lipofection

The use of a positively charged lipid to deliver nucleic acids to mammalian cells was first reported in 1987.¹⁰⁶ Cationic lipids consist of a pair of fatty acids possessing a hydrophobic tail and a hydrophilic head group. Cationic liposomes (= membranous spherical vesicles enclosing an aqueous volume) can be formed from double-chain cationic lipid, which form liposomes spontaneously; however, most positively charged liposomes produced for nucleic acid transfer are cationic amphiphiles, such as DMRIE-C and Lipofectamine, consisting of a nonbilayer-forming cationic lipid (DMRIE and DOSPA, respectively) and a neutral helper lipid (cholesterol and DOPE, respectively).

Because of the rapid speed with which this technology has developed, the terms associated with it have at times been confusing. In an attempt to simplify at least some of the aspects of this evolving terminology, Felgner *et al*¹⁰⁷ described a consensus nomenclature. Accordingly, 'lipoplex' refers to any cationic lipid-nucleic acid complex, and 'polyplex' refers to any polymer-nucleic acid complex (Figure 4). Consequently, 'lipofection' should be used for nucleic acid delivery by cationic lipids, eg commercially available mono- and polycationic lipids, and 'polyfection' for nucleic acid delivery mediated by cationic polymers, eg polyethylenimine, dendrimers, poly-L-lysine.

The principal reasons for this type of gene transfer have been the ease of application of this methodology to a wide range of cell types; the high efficiency of nucleic acid transfer in comparison to methods such as calcium phosphate or diethylaminoethyl-dextran-mediated transfection; and the potential application of these systems to human gene therapy.

The use of positively charged lipid-based macromolecules to deliver genes exploit the anionic character of DNA, RNA and oligonucleotides, that carry a negative charge due to the phosphate groups that are a part of these molecules. The electrostatic interaction between the negatively charged nucleic acids and the positively charged macromolecules induces a range of structural changes resulting in condensation or compaction of the nucleic acid and physical association ('encapsulation') of the nucleic acids with the lipid (Figure 4a).¹⁰⁸ Little is known about the mechanism of this complex formation although several models have been proposed.^{108,109} This interaction generates a complex that is more amenable to cellular uptake through fusion with the plasma membrane. The exact mechanism of membrane interaction and subsequent cell entry is unknown.

Table 2 Summary of nonviral gene transfer methods

Method of transfection	Advantages	Disadvantages
Lipoplexes	Flexible formulations Accept large nucleic acids	Transient expression Low transfection efficiency <i>In vivo</i> instability
Receptor-mediated gene transfer	High transfection efficiency Tissue-specific expression	Lysosomal degradation Transient expression
Electroporation	Simple High transfection efficiency <i>In vivo</i> applications	Transient expression Relatively toxic
Direct DNA injection	Simple <i>In vivo</i> gene transfer into specific target cells (skin, muscle)	Low transfection efficiency
Particle bombardment	High transfection efficiency	Transient expression
Extrachromosomal replicating vectors	Stable, long-term expression Nonintegrating Large DNA insert possible Physiologically regulated gene expression	Difficult to construct

Figure 4 Assembly and nomenclature of synthetic nonviral vectors. (a) The electrostatic interaction between the negatively charged nucleic acid and the positively charged polycationic DNA carrier induces a range of structural changes resulting in condensation or compaction of the nucleic acid. Physical association ('encapsulation') of the nucleic acids with a polycationic lipid results in a DNA–lipid complex, designated as lipoplex. (b) Receptor-mediated gene transfer involves the covalent or noncovalent binding of a cell-specific ligand to a polycationic polymer. This ligand conjugate is further complexed with DNA to generate a cell-specific DNA–polymer complex, designated as polyplex. Hence, transfection using lipoplexes or polyplexes is designated as lipofection or polyfection, respectively.

The initial interaction between the lipoplex and the cell membrane is electrostatic as a result of the excess positive charge associated with the lipoplex and the net negative charge of the cell surface. Endocytosis, phagocytosis, pinocytosis, and direct fusion with the cell membrane may all play

a role in lipoplex cell entry, depending on the cell type.¹¹⁰ Following cell entry, the lipoplex is surrounded by an endosome and either degraded by lysosomal action or released into the cytoplasm.¹¹¹ The DNA or lipoplex then migrates to the nucleus, where gene expression will take place. DNA prob-

ably needs to be released from the lipoplexes prior to entry to the nucleus, because micro-injection (see below) of lipoplexes directly into the nucleus results in poor transgene expression as compared to naked DNA injection.¹¹¹ This nuclear entry step is likely to be the major bottleneck of lipofection, leading to relatively low gene expression efficiency.¹¹² Addition of the SV40 nuclear localization signal peptide covalently attached to the plasmid DNA by peptide-nucleic acid (PNA) technology could overcome this limitation.¹¹³ Mixing DNA with a nonhistone, chromosomal high mobility group-1 protein also increases the nuclear translocation and expression of the gene.¹¹⁴ Alternatively, in an attempt to mimic viral transduction, AdV- and AAV-based plasmids have also been conjugated to liposomes in order to increase transfection efficiencies.^{115–117}

Cationic lipid- and polymer-mediated nucleic acid transfer have been used mostly for the transfection of plasmid DNA in applications in which transient gene expression is sufficient or required, but nucleic acids in all forms, ranging from small oligonucleotides to artificial chromosomes, can be transferred using these systems.^{118,119}

The need to optimize the formulation of a lipoplex cannot be overstated. A significant number of biochemical, biophysical and cellular variables can influence the final level of transgene expression by any given lipoplex by several orders of magnitude. The most critical, and thus most studied, variables are: (1) the DNA:lipid ratio which determines the net positive charge of the lipoplex; (2) initial concentration of both components which influences the interaction and the degree of condensation; (3) the total amount of nucleic acid and lipid since high concentrations of DNA and lipid can be cytotoxic while low DNA amounts result in reduced transgene expression; (4) the composition of the diluent in which the lipoplex is formed because pH, chelating agents, proteins and salts can interfere with the complex formation process.

As with other chemical transfer methods, a major disadvantage of lipoplexes is their low efficiency of gene transfer both *in vitro* and *in vivo*, as well as *in vivo* toxicity due to lipid accumulation. Moreover, *in vivo* application of lipoplexes is hampered by their *in vivo* instability and fairly rapid serum clearance, although recent data provide evidence for improved formulation with a slower serum clearance and higher *in vivo* stability.^{120,121} Currently, of all 425 gene therapy trials, 75 (18%) protocols use lipoplex technology for the treatment of cystic fibrosis, arthritis, cancer, cardiovascular disease and neurological disorders. Recent advances in liposome-mediated gene delivery have improved the efficiency of DNA gene transfer by using receptor-mediated techniques described below.

Receptor-mediated gene transfer

Receptor-mediated techniques employ polycation complexes, or polyplexes (see above), to transfer genes into target cells.¹²² Apart from viruses, many other compounds are internalized inside cells by receptor-mediated endocytosis. Cells use this process for the uptake of proteins such as asialoglycoproteins, transferrin, insulin, epidermal growth factor and small vitamins like folic acid. In order to target DNA to a given cell population, a suitable receptor ligand has to be attached covalently or non-covalently by conjugation to a polycationic DNA carrier (Figure 4b). A commonly used ligand is transferrin, since transferrin receptor is expressed ubiquitously. In several, but not all studies, a correlation was found between

receptor density and transfection efficiency.¹²³ Transferrin-mediated gene transfer into T cell lines is not efficient, although these cell do express the transferrin receptor. However, targeting polyplexes to the CD3–T cell receptor complex results in vigorous internalization and subsequent high transgene expression under appropriate conditions.¹²⁴

However, as well as choosing the proper ligand, packaging of the DNA by a proper carrier and (active or passive) delivery across the cell membrane are crucial for successful transfection.¹²⁵ Most of the described receptor-mediated systems contain the cell-binding ligand conjugated to the polycationic polymer, poly-L-lysine (PLL), to provide a DNA-binding domain. Neutralization and concomitant condensation of the DNA facilitates the endocytic uptake and survival of DNA on its way to the nucleus.¹²⁶

PLL cannot be considered as a biologically inert molecule as it enhances pinocytosis and phagocytosis, and activates membrane phospholipases thereby affecting membrane permeability. Furthermore, it activates the complement system and may induce an immune response upon repeated injection. A series of other DNA-binding compounds have been used in receptor-mediated gene delivery. Of all tested PLL alternatives, low molecular weight polyethylenimine (PEI) showed favorable characteristics for *in vitro* and *in vivo* applications, as PEI seems to lack toxicity *in vivo*. Furthermore, among all known cationic polymers, PEI has the highest protonation level resulting in maximum positive charge and improved gene transfer efficiency, eg in mammalian CNS.¹²⁷

Although polyplexes of DNA and ligand-conjugated cationic polymers can be efficiently delivered to cells, the accumulation in internal vesicles may greatly reduce the efficiency of gene transfer. Addition of lysosomotropic agents like chloroquine or glycerol to the transfection medium may increase transfection efficiency, probably by interfering with lysosomal degradation and enhancing the release of DNA into the cytoplasm. However, the use of chloroquine or glycerol is limited because only some cell types respond to this treatment. Moreover, chloroquine/glycerol shock treatment is often accompanied by cell toxicity.¹²⁸

Recently, several functional domains involved in viral entry and nuclear trafficking have been incorporated into DNA, since trafficking, nuclear entry and maintenance of the transfected DNA in the cell are additional steps where nonviral systems may benefit from including viral elements. The coupling of replication-defective AdV to polyplexes has been shown to facilitate lysosomal disruption and to consequently augment the levels of receptor-mediated gene transfer 100- to 1000-fold in cell lines expressing ligand and AdV receptor.^{122,129} In a next step to mimic viral entry and trafficking, ligand-bearing DNA polyplexes were provided with endosomal destabilizing viral peptides derived from influenza virus, rhinovirus, Sendai virus and adenovirus,^{130,131} whether combined or not combined with a chloroquine shock.¹³² Using PEI-based conjugates, high-level gene expression was observed in the absence of additional endosome-destabilizing agents.

Fusogenic viral liposomes, or 'viriosomes', include the hemagglutinating virus of Japan (HVJ).¹³³ HVJ is nonpathogenic to humans and its envelope protein allows fusion with the target cell membrane. This viriosome facilitates DNA delivery directly into the nucleus, bypassing the lysosomal degradation pathway. HVJ has been used to transfect a variety of tissues *in vivo* with greater efficiency than lipoplex or naked DNA. However, low-level immunogenicity of HVJ was reported,

and, since it is a viral hybrid, safety issues should be addressed.¹³³

A nonlipid, water-soluble polycationic amine has recently been reported to achieve high-efficiency gene transfer *in vivo* when compared to the amphiphile Lipofectamine. The transfer efficiency was comparable to a recombinant adenoviral vector. In preliminary experiments the novel polyplex was also shown to have no toxic effects in mice, nor was any inhibition by serum observed.¹³⁴

Direct DNA injection

This gene delivery technique is perhaps the most conceptually simple, and therefore appealing, approach to gene delivery. One drawback, however, is that micro-injection can be achieved at only one cell at a time, which limits its *ex vivo* use to applications in which individual cell manipulation is desired and possible, such as producing transgenic organisms. Although relatively efficient, the method is also rather slow and laborious and therefore not appropriate for *ex vivo* transfection of large numbers of cells.

In vivo transfection of mammalian cells has been achieved through direct injection of naked DNA.¹³⁵ Striated muscle was initially considered to be the only tissue capable of efficiently taking up and expressing free plasmid DNA in aqueous solution. PCR and Southern blot analysis revealed that the injected plasmid persisted episodically, presumably due to the low turn-over of myocytes. However, this gene delivery technique is relatively inefficient as demonstrated by the limited number of cells expressing the transgene. *In vivo* transfection of muscle was extensively studied by gene therapists to achieve long-term expression of recombinant proteins, eg α -1-antitrypsin,¹³⁶ and could provide a valid alternative for myoblast-mediated gene therapy.¹³⁷ Importantly, Isner and colleagues demonstrated proof of principle that direct injection of naked plasmid DNA encoding vascular endothelial growth factor can be applied for the treatment of patients with critical limb ischemia and myocardial disease.^{138,139} To date, as for particle-mediated gene delivery (see below), direct DNA injection into muscle and skin has been extensively used to express viral antigens *in vivo* to achieve antiviral immune responses, and has been referred to as DNA immunization.^{140,141} Boyer *et al*¹⁴² recently demonstrated that an HIV-specific immune response could be mounted in chimpanzees following inoculation with the *env*, *rev*, and *gag-pol* genes.¹⁴² Interestingly, transfection efficiency of the target tissue was not necessarily correlated with the efficiency of immunization. Indeed, one of the striking results of earlier studies examining different target cell types was that intradermal DNA injection can elicit potent immune responses to the encoded antigen despite the relatively low efficiency of DNA uptake and expression in the skin, as compared to muscle.¹⁴³ Recent evidence suggests involvement of antigen-capturing bone marrow-derived antigen-presenting cells – epidermal Langerhans cells – capable of initiating primary immune responses following injection of naked DNA,¹⁴⁴ as well as the presence of immunostimulatory sequences, also known as unmethylated CpG motifs, found in plasmid DNA preparations.¹⁴⁵ CpG motifs were found to be necessary for effective intradermal DNA immunization,^{146,147} and are believed to mediate their effect through activation of epidermal Langerhans cells.¹⁴⁸

Particle-mediated gene delivery

Also known as the particle bombardment or ‘gene gun’ method, this gene delivery technology is a physical means of transferring exogenous DNA into target cells.¹⁴⁹ Although initially developed to achieve genetic transformation of various crop plants, particle-mediated gene transfer (PMT) has found its applications for transferring materials including DNA, RNA, proteins, peptides, and pharmacological compounds into a wide variety of tissue and cell types.¹⁵⁰ The technique involves coating microparticles (usually 1- to 3- μ m inert gold beads) with plasmid DNA and projecting them on to a target tissue by an electrical discharge or gas pulse device. The physical force involved in this bombardment allows the coated particles to penetrate the cell membrane. The DNA construct is then gradually released from the microparticles and expressed within the cell. In most cases, the transferred genes are present as episomes within the host cell nucleus; however, it has been demonstrated that stable integration in human lymphocytes¹⁵¹ and human CD34⁺ hematopoietic progenitor cells¹⁵² can be achieved *in vitro*. The advantages of PMT include: (1) the delivery of large amounts of DNA fragments to specific target tissues, (2) the ability to deliver large DNA fragments (eg cosmids or artificial chromosomes), and (3) the lack of cytopathic responses.¹⁵³ PMT has been successfully applied to a range of somatic tissues *in vivo*. Clearly, the most accessible somatic tissue *in vivo* is the skin which is an excellent target for immunization. Consequently, PMT is being explored as a method for DNA immunization.¹⁵⁴ Since plasmid DNA is directly delivered to the cytoplasm, in contrast to injection where DNA is most probably taken up by cells from extracellular spaces, PMT to the epidermis of mice requires up to 5000 times less DNA than intramuscularly or intradermally injected DNA.¹⁵⁵ However, the major hurdle of this new technology is the apparent lack of long-term gene expression *in vivo* and the need for repeated injections to achieve long-term immunization, in contrast to direct plasmid DNA injections.¹⁴⁰

Only two clinical gene therapy protocols currently employ PMT, both being *ex vivo* approaches. One trial involves PMT-mediated transfection of autologous T lymphocytes with a plasmid coding for a negative transdominant Rev protein (RevM10) and reinfusion into HIV-positive individuals.¹⁵⁶ In the second trial, patients with melanoma or sarcoma are being immunized with autologous tumor cells transfected by PMT with the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene.¹⁵⁷

Electroporation

Electroporation or electrotransfection is a well-established, physical technique for *in vitro* and *ex vivo* gene delivery into a variety of mammalian cell types, both primary cells and established cell lines.^{158,159} Advantages of electroporation as a gene transfer method include millisecond transfection speeds, the lack of size constraints on the transgene and minimal need for complex DNA manipulation, high reproducibility and ease of performance.¹⁶⁰ Moreover, cotransfection of unlinked multiple genes can be easily performed by electroporation.¹⁶¹

In this technique, cells are exposed to a high-voltage electrical discharge in the presence of exogenous ‘naked’ nucleic acids. The consequent formation of hydrophilic pores in the cell membrane due to the applied electric field (hence the

name 'electroporation') allows uptake of nucleic acids.^{162,163} The need to optimize gene transfer efficiency by electroporation is somewhat less complex than the optimization of a lipoplex formulation as only two important electrical parameters determine, at least to a great extent, the degree of physical gene transfer.^{164,165} The most important parameter is the electric field strength E determined by the formula $E = V/d$, where V is the applied voltage and d is the distance between the electrodes of the cuvette (usually 0.4 cm). The higher the voltage, the more and the larger pores will be formed, although cell toxicity will increase with increasing voltage. For eukaryotic cells, low voltage pulses in the range of 150–350 V should be considered.^{166,167} The second crucial parameter is the pulse time (t) determined by the formula $t = R \times C$, where R is the total circuit resistance and C the capacitance between the electrodes. The total circuit resistance depends on the internal shunt resistance (generally + in order to allow the entire electrical pulse to pass through the cuvette) and on the resistance of the cuvette determined by the nature and the volume of the electroporation medium as well as by the number of cells. The higher the pulse time, the longer the pores will remain open. Pulse times of 10–40 ms have been found to be optimal for mammalian cells. Generally, t varies by varying C , since the parameters influencing R are kept constant by the electroporation device.¹⁶⁸

Although electroporation generally only achieves transient transgene expression, stable integration of the genetic material into the host genome of a small proportion of the cell population does occur.¹⁶⁹ As electroporation is a physical transfection method, it does not require cell type-specific receptors, nor does it depend on actively dividing cells for efficient gene transfer.¹⁷⁰ Aside from its ability to introduce DNA into cells, electroporation also facilitates transport of all types of biomolecules (RNA, antisense oligonucleotides, proteins, drugs). Currently, one gene therapy trial uses electroporation as a means of *ex vivo* gene transfer, involving reinfusion of IL-2 gene-transfected natural killer cells in patients with metastatic renal cell carcinoma, colorectal carcinoma and lymphoma.¹⁷¹

Although *in vivo* electroporation is currently an unfamiliar method of gene transfer, accounting for only about 1% of total studies related to *in vivo* gene transfer and gene therapy, it could become extensively used for experimental and therapeutic purposes in the near future, particularly for immunization purposes.¹⁷² Like other nonviral methods, *in vivo* electroporation has a variety of advantages over viral vectors including: (1) any type of cells and of tissues could in theory become a target; (2) handling is easy and quickly done within a matter of seconds; (3) repeated administration of DNA is possible; and (4) there is no restriction on DNA insert size.

Gene transfer efficiency of *in vivo* electroporation was found to be equivalent to or even superior to that of *in vivo* lipofection, gene gun and direct DNA injection methods.¹⁷³ Although gene expression is transient and foreign genes are likely to be present in an episomal form when transferred by *in vivo* electroporation, foreign gene products could be detected for more than 1 month depending on tissues and DNA constructs used.¹⁷⁴ Electroporation was recently combined with direct DNA injection *in vivo* to obtain high-efficiency gene transfer into a specific tissue. This 'electro-gene therapy' involved injection of a mammalian expression plasmid containing the *LacZ* gene into the internal carotid artery of rats whose brain tumors had been subjected to an electrical pulse just prior to injection of the plasmid.¹⁷⁵ In addition to avoiding the potential disadvantages of viral

transduction systems, this study demonstrated that transgene expression could be expressed in particular target organs while avoiding toxic effects. This new technology using *in vivo* electroporation as a nonviral gene transfer method is likely to be developed further in the future.

Extrachromosomal replicating vectors

Although integrating vectors (eg MoMuLV) can maintain stable gene expression, there are potential risks involved using these vectors, such as insertional mutagenesis and generation of replication-competent retroviruses (see Retroviral vectors). Furthermore, current viral vectors which do not integrate cannot replicate extrachromosomally and therefore have a limited lifetime in proliferating cells. Vectors that can replicate extrachromosomally include mammalian artificial chromosomes (MAC), Epstein–Barr virus (EBV)-based vectors and human *ori* vectors.¹⁷⁶ MAC contain the minimum chromosomal components required for DNA replication and segregation, namely, the origin of replication, telomeres and centromeres. For gene therapy, MAC would also need to contain sequences to drive physiologically regulated expression of the therapeutic gene.¹⁷⁷ The first complete construction of a human artificial chromosome was recently described.¹⁷⁸ Although this is a significant step forward in the production of a MAC-based vector, many problems still exist. Currently, the largest size of DNA that can be transferred to a target tissue is around a few hundred kilobases. The human MAC described ranged from 6 to 10 megabases, which is an impractical size for delivery into cells. For MAC to become a reality in gene therapy, gene delivery systems will have to accommodate larger DNA fragments or the size of the MAC will have to be reduced. Although MAC will at present not integrate into the natural genome, there is still the possibility of recombination with cellular chromosomal sequences and potential oncogenesis due to the introduced genetic instability. No artificial chromosomes have yet been developed for clinical gene therapy trials.

EBV-based vectors have also been used for gene therapy as extra-chromosomally replicating vectors. It is the EBV origin of replication (*ori*) and Epstein–Barr nuclear antigen (EBNA)-1 gene that confer the unique ability of these vectors to be maintained as stable, self-replicating plasmids.¹⁷⁹ Although EBV-based vectors are retained in the nucleus, they have a random segregation pattern and they do gradually disappear from rapidly dividing cells after a few months. It has been demonstrated that EBV vectors can accommodate up to 160 kb of DNA and are B cell specific.¹⁸⁰ These cells are attractive targets for hematopoietic diseases such as immunoglobulin deficiency and agammaglobulinemia. Furthermore, the EBNA-1 protein required for replication and retention of the vector has been shown to overcome immune surveillance, allowing efficient *in vivo* persistence.^{181,182} Nevertheless, the EBNA-1 protein has some oncogenic potential,^{183,184} a problem that has to be addressed in further detail before the initiation of clinical trials.

Hematopoietic cell-based gene therapy

The hematopoietic system

Most mature blood cells are short-lived and must be replaced continuously throughout adult life. The turnover of hemato-

ietic cells in humans may be estimated to be close to 1 trillion cells per day.¹⁸⁵ This remarkable cell renewal process in the hematopoietic system, called hematopoiesis, is supported by hematopoietic stem cells (HSC) located mainly in the bone marrow. Hematopoiesis is a tightly regulated process of cell proliferation and differentiation of HSC resulting in the production of terminally differentiated blood cell types (T and B lymphocytes, natural killer cells, monocytes, dendritic cells, granulocytes, eosinophils, basophils, megakaryocytes, and erythrocytes) and proceeds by the proliferation of four successively larger classes of cells: HSC, committed progenitor cells, bone marrow precursors and some blood cells (eg lymphocytes).¹⁸⁶

HSC have been defined as pluripotent cells (able to give rise to all hematopoietic lineages through differentiation) with the ability to self-renew, that is to self-replicate without differentiation. In the adult, HSC reside primarily in the bone marrow.^{185,186} HSC generate progenitor cells committed irreversibly to one of the various hematopoietic lineages. These committed progenitor cells can therefore be discriminated from the HSC by the loss of self-renewal potential and by their ability to generate morphologically distinctive progeny. Hematopoietic progenitor cells can be revealed in test systems that permit *in vivo* or *in vitro* differentiation into recognizable cell types, thus uncovering their identity.¹⁸⁷ Proliferation and differentiation of HSC are driven by hematopoietic growth factors, some of which have pleomorphic actions, acting at more than one stage of lineage development as well as stimulating production of more than one cell type. *Ex vivo* generation of maximal numbers of cells from HSC requires synergistic combinations of growth factors, particularly those that act at the initial stages of lineage development.¹⁸⁸

Targeting HSC using CD34 as a marker

One of the differences between mature and immature hematopoietic cells is the expression of a sialomucin, CD34. At least a part of human and mouse HSC populations and most hematopoietic progenitor cells express the CD34 antigen.^{189,190} In humans, the CD34 antigen is expressed on 1 to 3% of low-density bone marrow mononuclear cells, and on about 0.1% of normal peripheral blood mononuclear cells.¹⁹¹ CD34⁺ cell-enriched bone marrow was shown to restore hematopoiesis in lethally irradiated baboons, demonstrating that the CD34⁺ population comprises the HSC with the capacity to reconstitute multi-lineage hematopoiesis.¹⁹² As immature cells undergo differentiation, the level of expression of this antigen diminishes progressively, and CD34 expression is rapidly lost beyond the progenitor cell compartment. This stage-specific, rather than lineage-specific expression implicates a potential regulatory function for CD34 in developmental hematopoiesis. Several lines of evidence indicate that it may play a role in adhesive interactions with stromal cells or with the extracellular matrix. Outside the blood system, CD34 is found on vascular endothelial cells, supporting the hypothesis of involvement in cellular interactions or adhesion.¹⁹³ The endothelial glycoform of CD34 can function as a ligand for L-selectin.¹⁹⁴ Moreover, CD34 is encoded by a gene located on chromosome 1q, as are other genes encoding a family of leukocyte vascular endothelial adhesion molecules.¹⁹⁵

CD34⁺ cells represent a phenotypically heterogeneous population of cells, most of which are already committed to a particular lineage, for example pro-B cells co-expressing CD19, T cell precursors CD5 and CD7, and myeloid progenitors CD33

and CD13.¹⁹⁰ Hematopoietic cells not yet committed to any lineage do not express any of these surface markers and are designated CD34⁺Lin⁻ (lineage-negative) cells. However, these CD34⁺Lin⁻ cells may still express activation antigens such as CD38. CD38 expression occurs regardless of the particular lineage to which the cell commits. Recent *in vivo* studies using sublethally irradiated NOD-SCID mice indicate that human SCID-repopulating cells (SRC) are comprised of the CD34⁺CD38⁻Lin⁻ population.¹⁹⁶ The CD34⁺CD38⁻Lin⁻ population is believed to contain the HSC.^{196–199}

To date, clinical-grade devices have been developed for CD34⁺ cell isolation for use in bone marrow transplantation or gene therapy protocols. However, recent data suggest the existence of some human HSC expressing low or undetectable CD34.^{200,201} Moreover, Kato and co-workers showed generation of CD34⁺ cells from CD34⁻ hematopoietic cells, suggesting that CD34 acts as an activation marker on HSC.²⁰² This new information implies reconsideration of CD34 as a marker of HSC and may mandate new isolation strategies to perform alternative enrichment strategies for HSC.²⁰³

Hematopoietic stem cell biology: implications for gene therapy

It has been shown that, *in vivo*, only a minor proportion of HSC are cycling, and relatively small numbers of cycling HSC contribute to the process of steady-state hematopoiesis.^{204,205} Thus, the process of hematopoiesis is sustained by the maintenance of a quiescent HSC pool, which undergoes cell division relatively infrequently, unless stimulated by hematopoietic growth factors in (patho)physiological conditions. As retroviral vectors transduce only cells undergoing active mitosis,⁶ HSC were found to be relatively refractory to retroviral-mediated gene transfer. Using the human-NOD/SCID mouse model, human SRC were unfrequently transduced with retroviral vectors, distinguishing them from most colony-forming cells and long-term culture-initiating cells (LTC-IC).²⁰⁶ This observation is consistent with the low level of gene marking seen in human gene therapy trials. Therefore, gene therapy trials were initially focused on gene transfer in T lymphocytes because efficiency of retroviral gene transfer was higher in lymphocytes, as these cells can be readily stimulated for cell division.²⁰⁷

Prestimulation of HSC with IL-3 and IL-6 together with c-kit ligand (KL) resulted in beneficial effects on stem cell viability and transduction efficiency.^{208–211} However, *in vitro* stimulation of HSC with these cytokines is associated with cellular differentiation and irreversible lineage commitment.^{212,213} Therefore, optimization of the cytokine cocktail, especially with the addition of thrombopoietin (TPO) and Flt-3/Flk-2 ligand (FL) may favor HSC cycling with preservation of stem cell properties including self-renewal and *in vivo* engraftment capacity.^{214–219} HSC can also be induced to proliferate *in vivo* after infusion of cytokines such as granulocyte colony-stimulating factor (G-CSF) and KL. This *in vivo* priming was shown to have a beneficial effect on transduction of HSC.²²⁰ Another approach to induce HSC cycling without loss of *in vivo* engraftment, was recently reported by Nolte and co-workers. In order to overcome cellular quiescence, HSC were exposed to antisense oligonucleotides directed against p27 (Kip-1), in combination with neutralizing antibodies to transforming growth factor (TGF)- β .²²¹ These data showed a significant increase in retroviral transduction efficiency and successful engraftment of NOD-SCID mice. These data sup-

port the hypothesis that it is feasible to manipulate the cell cycle of HSC to induce self-renewal expansion for retroviral transduction without loss of pluripotency.

The recently discovered 'plasticity' of stem cells to differentiate into cells of seemingly unrelated lineages (eg HSC generating muscle or liver cells, neuronal or muscle-derived stem cells producing hematopoietic cells) has re-invigorated the field of hematopoietic cell-based gene therapy.^{222,223} Indeed, nonhematopoietic stem cells, in contrast to HSC, may be capable of extensive proliferation *in vitro* without concomitant differentiation, potentially facilitating gene therapy for hematopoietic disorders.²²⁴

Hematopoietic cell-based gene therapy

Ever since the development of technology enabling the transfer of new genes into eukaryotic cells, hematopoietic cells, in particular HSC and T lymphocytes, have been an obvious and desirable target for gene therapy, as they can be readily removed from the body, manipulated *ex vivo*, and reintroduced. The pluripotent nature of HSC makes these cells ideal targets for introducing genes into multiple hematopoietic lineages, thereby providing a potentially therapeutic tool with applications in transplantations, genetic disorders, cancer and AIDS.²²⁵ An overview of gene therapy strategies using hematopoietic cells is summarized in Table 3.

First, basic requirements such as integration of the transgene and stable expression in the target HSC to obtain a long-term effect, must be met. In addition, transduction efficiency must be high enough in order to allow correction of the disease phenotype.

Hematopoietic stem cell-based gene therapy

Improved retroviral vectors: Currently, retroviral vectors are most commonly used for gene delivery into HSC.²²⁶ However, retroviral transduction of HSC presents a number of technical problems: (1) HSC comprised, at least partly, of the CD34⁺CD38⁻ fraction, are rare cells (less than 0.05% of total adult bone marrow mononuclear cells) and require high-performance cell separation devices for purification; (2) they reside in a quiescent state and require growth factor stimulation prior to retroviral transduction; and (3) there is a poor transduction efficiency of human HSC with amphotropic retroviral vectors as compared to murine HSC where transduc-

tion is more efficient.¹⁵ Therefore, considerable efforts have been made to refine retroviral gene transfer into HSC.

GALV-pseudotyped retroviral vectors have been shown to be more efficient than amphotropic vectors at transduction of human HSC.²²⁷ In both a nonhuman primate and canine competitive repopulation assay, Kiem *et al*^{16,228} reported a significantly increased gene marking of peripheral blood leukocytes with GALV-pseudotyped vectors as compared to amphotropic vectors. As with amphotropic envelope receptor, transduction efficiency using GALV-pseudotyped vectors correlates with the levels of GALV receptor mRNA in CD34⁺ cells.²²⁹

Furthermore, design of optimized transduction modalities have led to increased transduction efficiency. To this end, the use of chymotryptic fragments of the extracellular matrix protein fibronectin have been used instead of a stromal layer to support HSC in suspension culture and to enhance contact between cells and viral particles.²³⁰ The use of the recombinant fibronectin fragment CH-296 in combination with IL-6, KL, FL and TPO has led to superior retroviral transduction of baboon marrow repopulating cells.²³¹ Moreover, adhesion to fibronectin maintains long-term engraftment during *ex vivo* culture and transduction of human HSC in a immune-deficient beige/nude/xid human (bnx/hu) xenograft model, while in HSC cultured in suspension under the same conditions, transduction was undetectable and long-term multilineage regenerative capacity was severely diminished.²³²

Modification of the basic MoMuLV vectors in order to optimize transcription resulted in greatly improved retroviral vectors for use in hematopoietic cell-based gene therapy.²³⁻²⁵ To this end, the so-called FMEV vectors combining the LTR of Friend mink (FM) cell focus-forming viruses with the 5 untranslated leader region of the murine embryonic stem cells virus (MESV) were developed to achieve high transgene expression in hematopoietic progenitor and stem cells.^{233,234}

Lentiviral and other vectors: HIV-based vectors have the great advantage of stably transducing noncycling cells, making them promising vectors for gene delivery into HSC. Indeed, the ability of lentiviral vectors to transduce cells without cytokine prestimulation is advantageous because efficient transduction can be achieved within 1 day, thereby circumventing prolonged *in vitro* culture and concomitant loss of pluripotency. Recently, efficient transduction of quiescent CD34⁺CD38⁻ cells using lentiviral vectors has been reported,^{91,235} and HIV-transduced HSC gave rise to long-term reconstitution in recipient NOD-SCID mice *in vivo*.^{92,236}

Recombinant AdV are non-integrating vectors and therefore not likely to be used for stable transduction of HSC. Nevertheless, AdV may be used to transiently express receptor genes for retroviral vectors.²³⁷ Alternatively, AdV vectors can be exploited for tumor purging of stem cell transplants in cancer patients as they deliver genes into tumor cells more efficiently than into HSC. Using this strategy, suicide genes could be selectively delivered to tumor cells contaminating autologous stem cell transplants.^{238,239}

Also, nonviral gene delivery systems capable of transducing CD34⁺ cells *in vitro* are of value, including particle-mediated gene transfer,²⁴⁰ electrotransfection,¹⁶⁶ and lipid-based vectors.^{241,242} However, although these methods could lead to transient high-level expression in CD34⁺ cells, integration is unlikely to occur in HSC and more studies are required to address the potential utility of these methods in hematopoietic stem cell-based gene therapy.

Table 3 Targets for gene therapy using hematopoietic cells

<i>Single-gene disorders:</i> Severe combined immunodeficiency (SCID), adenosine deaminase (ADA) deficiency, Gaucher's disease, Hurler's syndrome, Fanconi's anemia, chronic granulomatous disease
<i>Transplantation and cancer:</i> Gene marking, suicide genes, chemotherapy resistance genes
<i>Immunotherapy:</i> DNA/RNA vaccines, cytokine enhancement of lymphocyte effector function, chimeric T cell receptors
<i>Acquired immunodeficiency syndrome:</i> Catalytic ribozymes, antisense RNA, dominant repressors of HIV genes, RNA decoys

Identification of transduced HSC: The development of vectors with reporter genes that can be detected by flow cytometry is an encouraging strategy which may facilitate the design of more efficient gene therapy protocols in which transduced HSC can be preselected by fluorescence-activated cell sorting (FACS), thereby improving identification and tracking of engrafted HSC.²⁴³ These markers include murine CD2, murine CD4, a truncated version of the human nerve growth factor receptor and the green fluorescent protein (GFP) from the jellyfish *Aequoria victoria*.^{244,245}

Stem cell division, homing, and subsequent lineage development can only be studied definitively by marking of pluripotent HSC, followed by tracking and clonal analysis of the progeny in a long-term transplantation system. Kohn and co-workers have developed a bnx/hu xenograft model, in which transduced human hematopoietic cells can be individually tracked into different lineages over the course of 1 year post-transplantation.^{246,247} The tracking is accomplished by single cell cloning of individual T lymphoid and myeloid progenitors recovered from the marrow of the mice, and clonal integration analysis by the sensitive technique of single-colony inverse PCR. The recovered cellular DNA segments that flanked proviral integrants are then sequenced to confirm their descent from a transduced common HSC.

Animal models: Various immunodeficient mouse models such as the NOD-SCID or the bnx/hu xenograft models have been exploited to establish transduction modalities, efficiency, safety and bone marrow-repopulating capacity of transduced human HSC.^{218,248} Recently, studies in nonhuman primate transplantation models have documented potentially therapeutic levels of gene transfer in a large animal/nonhuman primate model (Figure 5). In previous reports, transduction efficiency was in general lower than 1% of gene-marked cells in the blood of transplanted monkeys, using classical

MoMuLV vectors and basic transduction protocols.^{249,250} However, recently with the latest improvements in vectorology and transduction protocols as described above, gene marking efficiencies of 1–10% in both myeloid and lymphoid lineages have consistently been obtained after retroviral gene delivery into HSC from monkeys (Figure 5).^{231,251,252} Detectable levels of gene marking in HSC were also achieved using lentiviral vectors in a rhesus model, demonstrating the potential and safety of these vectors for future gene therapy trials.²⁵³

Preclinical animal models have been established to test the therapeutic efficacy of HSC gene therapy. Bunting *et al*²⁵⁴ demonstrated induction of specific immunity through transfer of the Jak3 cDNA into hematopoietic repopulating cells by an optimized retroviral vector in a Jak3-deficient SCID mouse model. Long-term correction of phagocyte NADPH oxidase activity in a murine X-linked chronic granulomatous disease model was obtained following transduction with gp61phox-expressing MESV-based retroviral vectors.²⁵⁵ Murine models of lysosomal storage disorders, such as mucopolysaccharidosis VII, demonstrated that HSC gene therapy results in long-term expression of low levels of beta-glucuronidase which partially corrects the disease by reducing lysosomal storage in liver and spleen.²⁵⁶

Clinical trials: The majority of human HSC gene therapy trials are basically gene marking studies in cancer patients undergoing bone marrow transplantation. These trials were aimed at determining the source of tumor relapse following transplantation of autologous bone marrow that had been genetically marked with a neomycin resistance (*neo^r*) gene. Importantly, these gene-marking trials have yielded data which showed the long-term persistence of the transduced gene in a small proportion of CD34⁺ hematopoietic progenitor cells.^{257,258}

Several clinical trials have been performed targeting

Figure 5 HSC gene transfer in primate nonhuman repopulation assay. HSC are mobilized in the peripheral blood of monkeys using granulocyte colony-stimulating factor (G-CSF) and c-kit ligand (KL) for a defined period. HSC are purified using CD34⁺ cell isolation devices and stimulated *in vitro* with an optimized hematopoietic growth factor cocktail consisting of Flt-3/Flk-2 ligand (FL), thrombopoietin (TPO) and KL, followed by gene marking with retroviral vectors in the presence of recombinant fibronectin fragment CH296 and the aforementioned cytokines. Afterwards, HSC are re-infused into lethally irradiated monkeys to test whether transduced HSC are capable of repopulating the bone marrow to give rise to all blood cell lineages. Transgene expression kinetics, as well as integration events, in differentiated blood leukocytes are then analyzed to assess gene marking efficiency.

adenosine deaminase (ADA) deficiency, X-linked SCID, Gaucher's disease, Hurler's syndrome, Fanconi's anemia, chronic granulomatous disease, autologous bone marrow transplantation and HIV-1 infection (Table 3).²⁵⁹ So far, all clinical HSC gene therapy trials have used MoMuLV-based retroviral vectors. Only low levels of circulating gene-transduced leukocytes were observed and there was no sustained expression of the transgene, nor proven clinical benefit.^{4,260}

Nevertheless, some major milestones have already been achieved. Four years after transplantation of transduced autologous cord blood CD34⁺ cells in three neonates suffering from ADA deficiency, the frequency of T lymphocytes with a normal ADA gene have accumulated to 1–10%, whereas the frequencies of other hematopoietic and lymphoid cells containing the gene remain at 0.01–0.1%.^{261,262} Most importantly, Cavazzana-Calvo *et al*²⁹ recently reported the first gene therapy trial as mono-therapy with proven clinical benefit in two SCID-X1 patients through gene transfer of the γ -common chain of the IL-2-receptor into the CD34⁺ cells. The researchers made use of an optimized retroviral transduction protocol, also used in recent nonhuman primate models (ie KL+FL+TPO in combination with recombinant human fibronectin fragment CH-296).

HSC gene therapy might also be beneficial for treatment of HIV-1 disease as an alternative approach for T cell-based HIV gene therapy.²⁶³ By transducing CD34⁺ progenitors, expression of the transgene in the HSC pool could not only protect CD4⁺ T cells but also CD4⁺ monocytes/macrophages or dendritic cells from HIV infection, and may also result in more persistent expression of the transferred gene.²⁶⁴

T cell-based gene therapy

As lymphocytes can be readily purified, manipulated and expanded *ex vivo*, gene transfer in T cells has in some ways been less problematic than in HSC. Therefore, many groups have initially focused on gene transfer into T lymphocytes, resulting in the very first gene transfer into humans in 1990.² This trial involved retroviral marking with a neomycin resistance (*neo^r*) gene of tumor-infiltrating T lymphocytes (TIL) in patients with advanced melanoma. Later, ADA deficiency was the first genetic disorder to be targeted by gene therapy using retroviral gene transfer into autologous T lymphocytes, demonstrating the safety, but ultimately not the efficacy, of this gene therapy approach.²⁰⁷

Gene therapy has also been applied to improve the safety and/or the efficacy in adoptive immunotherapy protocols. The development of retroviral transduction systems for stable expression of a foreign gene into T lymphocytes allows the introduction of a marker gene (eg *neo^r*), as a means to analyze *in vivo* persistence and distribution of adoptively transferred T cells, such as TIL,² EBV-specific CTL,²⁶⁵ and allogeneic donor-derived T lymphocytes.²⁶⁶

TIL have also been a major focus for the delivery of immune-enhancing cytokines, since they have the capacity to home to tumor sites, thereby avoiding the potential toxicity of systemically administered cytokines.^{267,268} A major hurdle of adoptive therapy with TIL alone is the failure of this subset to produce IL-2, which may be necessary for *in vivo* proliferation and persistence. This obstacle could be circumvented by genetically modifying TIL with chimeric receptors containing the extracellular domains of the GM-CSF receptor and the transmembrane and cytoplasmic domain of the IL-2 receptor.²⁶⁹ Thus, the production of GM-CSF by such modified CD8⁺ CTL,

which normally occurs upon antigen recognition, could potentially provide an endogenous IL-2 receptor signal to the CTL and improve *in vivo* survival and proliferation. Moreover, these chimeric receptors could provide a means of establishing the fate and distribution of re-infused genetically modified T cells.

Genetically re-engineering the tumor tropism of TIL was recently demonstrated using chimeric receptors composed of scFv serving as extracellular recognition elements joined to lymphocyte triggering molecules, eg the Fc receptor (so-called 'T bodies').^{270,271} These receptors with specificity for tumor-associated antigens can redirect the specificity of T cells in an MHC-independent manner and are successfully used to target T cells to tumor cells.²⁷² It is anticipated that genetically modified TIL with such enhanced functions will be clinically evaluated in the future and may provide recipients with more effective responses than those achieved with unmodified TIL therapy.

As primary targets of HIV-1, T lymphocytes are obvious candidates for HIV-1 gene therapy. At present, various genetic approaches are being developed to target HIV-1 replication: (1) expression of ribozymes that catalytically cleave and inactivate HIV-1 mRNA molecules;²⁷³ (2) antisense RNAs that inhibit reverse transcription, processing, and translation of HIV-1 RNA molecules;^{274,275} (3) mutant HIV-1 structural or regulatory genes with dominant repressor activity, eg RevM10;²⁷⁶ (4) RNA decoys to inhibit HIV-1 transcription, processing and packaging;²⁷⁷ and (5) intracellular immunization through expression of intracellular antibodies ('intrabodies'),²⁷⁸ that bind viral elements.

In the context of allogeneic bone marrow transplantation, the incorporation of an HSV-TK suicide gene into donor T cells renders them susceptible to ablation by administration of ganciclovir or acyclovir and can be used as a safety measure in gene therapy protocols allowing elimination of transduced cells if undesirable side-effects develop.²⁷⁹ Suicide gene-based strategies have been explored to promote a graft-versus-leukemia effect while minimizing graft-versus-host disease (GVHD) and have already entered the clinical phase.^{280–282} Despite the positive results of the pilot clinical trial, the induction of a strong immune response against the *neo^r* gene encoded by the retroviral vectors was observed. As an alternative, new generation retroviral vectors lacking the *neo^r* gene but carrying a modified form of the low-affinity nerve growth factor receptor (LNGFR) cDNA as a selectable cell surface marker have been developed.²⁸³

Conclusion

Recent advances in aspects of vectorology and the biology of hematopoietic cells have paved the way for improved gene transfer strategies into hematopoietic cells, in particular into HSC, of which efficiency can now be assessed in primate repopulation studies. The applications of novel vector systems such as lentiviral vectors has only just begun to be evaluated and holds much promise for future HSC gene therapy. Also, the field of nonviral vectors is the subject of future research as these systems involve minimal risks, and may ultimately be more cost-effective than their viral counterparts. However, low gene transfer efficiency and lack of integration of nonviral vectors may require inclusion of (viral) components needed for cell entry and/or nuclear transport.

To date, four early-stage gene therapy trials have documented clinical successes including complete cure of SCID

patients in one HSC gene therapy trial. Thus, the field of gene therapy seems to be turning the corner after 10 years of being criticized for too much hype and too few successes. The challenge to correct hematopoietic disorders and immune deficiency diseases using gene-modified HSC is finally becoming a reality.

Acknowledgements

This work was supported by grant Nos 3.0109.96, G.0313.01 and G.0157.99 of the Fund for Scientific Research – Flanders, Belgium (FWO), by grants of the Scientific Committee of the FB Verzekeringen (formerly ASLK)-financed Cancer Research, and by a grant of the Belgian Federation against Cancer. VVT is a research assistant of the FWO. We thank Dr Thierry VandenDriessche (Department of Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology (VIB), Katholieke Universiteit Leuven, Belgium) for critical reading of the manuscript.

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