Title: **Non-integrating Gene Therapy Vectors**

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Synopsis:

Gene delivery vectors that do not rely on host cell genome integration offer a number of advantages for gene transfer, chiefly the avoidance of insertional mutagenesis and position effect variegation. However, unless engineered for replication and segregation, non-integrating vectors will dilute progressively in proliferating cells, and are not exempt of epigenetic effects. Here we provide an overview of the main non-integrating viral (adenoviral, adeno-associated viral, integration-deficient retro/lentiviral, poxviral) and non-viral (plasmid vectors, artificial chromosomes) vectors used for pre-clinical and clinical cell and gene therapy applications. We place particular emphasis on their utilisation for hematologic disease indications.

Keywords: Non-integrating, Gene Therapy, Vector, Adenovirus, Adeno-associated virus, IDLV, Poxvirus, Plasmid, Genome Editing

Key points:

- Host cell genome integration of first-generation gene therapy vectors may result in various effects on cellular genes (knockout, over-expression, altered splicing), variegated levels of transgene expression or transcriptional silencing, as well as clonal expansion and oncogenic transformation.

- Non-integrating gene therapy vectors can be viral and non-viral. Viral vectors can be non-integrating like their parental organisms (adenovirus, herpesvirus, poxvirus, Sendai) or engineered to minimise integration (adeno-associated virus, retro/lentivirus).
- Non-integrating vectors can provide stable transgene expression in quiescent cells and transient or stable expression in proliferating cells.
- Variants of non-integrating vectors carrying suitable payloads (transposons, site-specific recombination cassettes, genome editing cassettes) are suitable platforms for genetic modification of the cellular genome by transposition, site-directed integration and genome editing.
- Successful clinical trials have already been reported using adenoviral vectors (genome editing of *CCR5* for AIDS), herpesvirus vectors (cancer) and adeno-associated virus vectors (hemophilia).

Non-integrating Gene Therapy Vectors

Brief historical overview of gene therapy

The concept of gene therapy arose during the 1960s and early 1970s. Rogers carried out the first genetic modification of a virus (Tobacco mosaic virus, TMV), and proposed in 1970 that "good DNA" could be used to replace defective DNA in people afflicted by genetic disorders[1]. In 1972, Friedmann and Roblin assessed the requirements and risks and called for a moratorium[2]. An unsuccessful early attempt at gene therapy was reported in the scientific literature in 1975[3]. The first patient with some degree of long-term transgene persistence from a gene therapy clinical trial was in adenosine deaminase severe combined immunodeficiency (ADA-SCID), following an autologous transplant of T-cells treated *ex vivo* with an integration-proficient retroviral vector, initiated in 1990 and reported in 1995[4]. The first clear success was published by the group of Fischer in 2000, describing the treatment of X-linked severe combined immunodeficiency (SCID-X1) patients with autologous hematopoietic stem/progenitor cells (HSPC) genetically modified with similar retroviral technology[5]. These efforts with integrative retroviral vectors led to the approval of *Strimvelis™* in Europe for the treatment of patients with ADA-SCID for whom no suitable human leukocyte antigen (HLA)-matched stem cell donor is available.

Despite the initial success with integrating vectors, non-integrating viral vectors were the first approved products for cell and gene therapies in China and Europe. Onyx-015©[6] (originally named Ad2/5 dl1520), an experimental oncolytic adenoviral (Ad) vector trialled as a possible treatment for head and neck cancer, was the first gene therapy product licensed, in China in 2006, under the name *H101©*[7]. An adeno-associated viral (AAV) vector, Glybera®

(uniQure/Chiesi)[8], was subsequently approved by EMA in 2012 as the first gene therapy in Europe, for lipoprotein lipase deficiency. An oncolytic herpes simplex virus-1 (HSV-1; IMLYGIC®, Amgen)[9], was approved in 2015 for the treatment of advanced melanoma by both FDA and EMA.

Non-integrating vectors

Optimal vectors for gene delivery should exhibit high payload capacity, cell tropism for specific target cell types, high transduction efficiency, little or no geno- and cytotoxicity, and should elicit minimal or no immune response. Non integrating vectors specifically share reduced risk of genotoxicity, offering a safer profile *in vivo* and *in vitro*, and expression can be retained for long periods in post-mitotic tissues. However, unless they have been specifically genetically engineered for replication and segregation, non-integrating vectors will dilute progressively in proliferating cells. If stable expression in dividing cells is required, repeated administration of non-integrating vectors is an option, provided that an immune response can be avoided or managed.

Here we provide an overview of the main non-integrating viral vectors: Ad, AAV, integration-deficient lentiviral vectors (IDLVs), poxviral and others. Non-viral vectors (plasmid, artificial chromosomes) will also be discussed. We illustrate the structure of the main non-integrating viral vectors (**Fig. 1**), and summarise the use of non-integrating vectors in clinical trials (**Table 1**). Different vector systems provide a variety of advantageous properties and challenges (**Table 2**). Traditionally, viral vectors are considered more efficient while non-viral methods have advantages in terms of large-scale production and low immunogenicity.

Non-Integrating Viral Vectors

Adenovirus vectors

Adenovirus vectors key features:

- Efficient delivery to dividing & non-dividing cells.
- Retained as non-integrated nuclear linear episomes.
- High but transient expression.
- High immunogenicity.
- ~8-30kb capacity.

Adenoviruses are a family of DNA viruses with an icosahedral, 70-100nm in diameter, non-enveloped capsid engulfing a double-stranded (ds) DNA genome. These viruses can infect quiescent and dividing cells and replicate in the cell nucleus. Ad vectors were used early in clinical trials of cystic fibrosis, in various cancer types and in more recent clinical trials of peripheral vascular and coronary artery disease[10]. Serotypes 2 and 5 are the most extensively characterised human Ad serotypes from a range of >50 Ad subdivisions/clades, with a typical Ad5 vector genome of ~36kb encoding genes that are expressed before (Early, E) and after (Late, L) viral replication. Early transcription units encode proteins required for viral transactivation and host-virus interactions. >30 novel non-human primate (NHP) adenoviruses from chimpanzees, bonobos and gorillas and various other species such as canine, equine etc have been isolated and characterised.

Conventional Ad vectors were constructed by substituting the E1 region of the adenovirus genome with the transgene cassette of interest [E1-] (**Fig. 1A,B**). Thus, first generation Ad vectors [E1-] had a carrying capacity of <8kB. However, other viral genes are expressed and

Ad capsid proteins appear to activate innate host immune responses that within 2 weeks can result in the loss of Ad transduced cells[11]. Different combinations of early region Ad deletions have been tested providing the Ad vector with modified properties and allowing for enhanced duration of transgene expression. Subsequent generations of adenoviral vectors were characterised by deletions of E1 and E2 and /or E4 genes, although toxicity from an E1/E4 deleted Ad vector lead to the first reported fatality in the field of gene therapy[12]. Ad vectors with multiple backbone deletions also reduce the risk of generation of replication competent adenoviral (RCA) particles. Latest generations of 'gutless' helper-dependent (HD) Ad vectors are devoid of most viral sequences, minimally retaining only the viral ITRs, and the packaging recognition signal. They can accommodate up to 28-32kb foreign exogenous DNA sequences and have been used in various pre-clinical animal studies with apparent stable expression and low levels of toxicity[13]. Hence, HD Ad vectors appear to have significant advantages over 1st generation Ad vectors. However, it should be noted that production of HD Ad is considerably more challenging.

Neutralising Abs and pre-existing immunity (discussed also in the AAV vector section of this review) represent a significant barrier to repeated vector administration, a strategy of potential importance with episomally maintained vectors. Low-level expression of viral vector genes in such settings almost always results in the generation of immune responses directed against Ad-transduced cells and ultimately in loss of transgene expression. Latest-generation HD-Ad vectors represent a significant advantage, but it is possible that they may still potentiate cytotoxic T-cell responses even in the absence of *de novo* viral gene expression. However, repeated administration using HD-Ad vectors of different serotypes has been achieved[14].

Future directions and hematopoietic application

Current Ad vectors are primarily derived from common serotypes 2 and 5. There are now efforts, however, to exploit other human/nonhuman adenoviral serotypes or mosaic/chimeric/hybrid[15] adenoviruses to avoid administration problems associated with pre-existing immunity and transduction longevity issues. Approaches targeting uncontrolled blood thrombogenesis by systemically over-expressing prothrombin via Ad vector-mediated gene transfer have been explored[16]. Efficient genome editing in HSPC with helper-dependent Ad5/35 vectors expressing site-specific endonucleases, under microRNA regulation, can be a useful tool for therapeutic purposes[17].

Adeno-associated virus vectors

Adeno-associated virus vectors key features:

- Efficient delivery to dividing and non-dividing cells.
- ~4.5 kb cloning capacity.
- Capable of sustained expression as episomal concatemers in post-mitotic tissues.
- Relatively low immunogenicity.

AAV is a human defective parvovirus whose 4.7 kb single-stranded (ss) genome is flanked by two inverted terminal repeats (ITRs) and comprises of two genes, *rep* and *cap*. *rep* encodes for non-structural (replication) proteins, and is also important for site-specific integration into *AAVS1*[18] (**Fig. 1C,D**). *cap* encodes for structural (encapsidation) proteins, and the accessory assembly-activating protein (AAP), essential for serotype-specific assembly. In recombinant AAV vectors the viral backbone segment including the *rep* and *cap* genes is

removed and supplied in *trans*. The ITRs are the most prominent genomic characteristic of the virus; they consist of a 125 nucleotide-long palindromic hairpin structure and a 20 nucleotide stretch, designated as the D-sequence, which remains single-stranded. The ITRs contain recognition signals for replication, packaging into functional virions and integration. Efficient replication and lytic growth of AAV depends on co-infection by a helper virus. Ads, HSV type I/II, pseudorabies virus and cytomegalovirus can provide complete helper functions for AAV replication.

Wild-type (wt)-AAV2 preferentially integrates into the *AAVS1* site, in the long arm of human chromosome 19 (19q13.3-qter), in a process mediated by rep binding[18]. In addition, more than 20 alternative integration sites have been identified[19]. The absence of obvious pathogenic effects of AAV integration into *AAVS1* has led to the development of this site as a safe-harbour locus for multiple applications. However, recombinant AAV vectors fail to integrate in the absence of rep protein and instead essentially become non-integrating vectors, able to impart long-term episomal persistence in post-mitotic tissues like muscle. Mostly they are maintained as large head-to-tail circularized multimeric concatemer structures, with a common structural element including a complete ITR flanked by two D-region elements. The generation of such concatemers may involve recombination and a possible rolling circle-type DNA replication mechanism[20].

More than 170 (7.2%) of human trials have utilized AAV vectors (clinical studies available as of August 2016; **Table 1**). The low immunological response to AAV-mediated gene transfer was initially attributed to limited transduction of antigen-presenting dendritic cells, although further data demonstrated the ability of AAV to transduce immature dendritic cell populations[21] and provoke humoral immune responses. At least 12 naturally isolated AAV

serotypes have been identified to date. In addition, a wide range of mosaic/hybrid and novel capsids generated by *de novo* shuffling approaches are rapidly expanding the current AAV toolbox[22]. This is important, as different serotypes display varying tropism, and because dose-escalation of recombinant AAV-2 in clinical trials may have caused acute inflammatory responses to viral coat proteins similar to the ones encountered in Ad trials. The use and combination of various AAV serotypes provides flexibility, low-immunogenicity, possible differential antigenic display and lack of antibody cross-reactivity. A detailed understanding of the tropism of AAV serotypes is playing a key role in transduction studies and clinical trials[23].

Future directions and hematopoietic application

The field of AAV gene therapy has progressed rapidly over the past decade, with the advent of novel capsid serotypes, organ-specific promoters and an increasing understanding of the immune response to AAV administration[24]. Recently, utilising non-biased haploid/knockout genetic screening approaches, AAVR has been identified as a universal host receptor for AAV-2 infections[25]. New AAV isolates, particularly AAVHSCs, represent a new class of genetic vectors that may be particularly suited for the manipulation of HSPC[26].

In a clinical setting, as discussed extensively elsewhere in this issue, peripheral-vein infusion of scAAV2/8-LP1-hFIXco (codon-optimised factor IX gene targeted to the liver) resulted in FIX transgene expression at levels sufficient to improve the bleeding haemophilia B phenotype, with few side effects[27]. Following on from these studies, there are now three ongoing trials (with more under way) of AAV-mediated gene transfer in haemophilia B, all aiming to express the factor IX gene from the liver[28]. Additionally, AAV liver expression of the hyperactive variant FIX-Padua prevented and eradicated FIX inhibitor without increasing

uncontrolled thrombogenesis in hemophilia B dogs and mice, supporting the potential translation of gene-based strategies using FIX-Padua at lower vector doses[29]. Recently, preliminary data from infused participants in an ongoing Phase 1/2 clinical trial of FIX-Padua-containing AAV vector (SPK-9001) for hemophilia B have been released. Following a single administration of 5 x 10^{11} vector genomes (vg)/kg, all participants experienced consistent and sustained (~30% in average) increases in factor IX activity[30]. Developments of relevance to other forms of hemophilia are also in the pipeline. Liver-directed gene therapy with AAV-FVIII in two outbred dogs with severe hemophilia A resulted in sustained expression of 1-2% of normal FVIII levels and prevented 90% of expected bleeding episodes[31]. Engineering of factor VIII to reduce its size and facilitate its delivery with AAV vectors has recently shown success in interim results from a clinical trial. B-domain deleted FVIII construct was administered in an AAV5 vector (BMN 270) to subjects with severe hemophilia A. High-dose (6 x 10^{13} vg/kg) subjects required no further FVIII prophylactic post-BMN 270 infusion[32].

Codon optimization of human FVII (hFVIIcoop) improved AAV transgene expression by 37-fold compared with the wild-type hFVII cDNA, whereas in the same study, in adult macaques, a single peripheral vein injection of 2 x 10^{11} vg/kg of the hFVIIcoop AAV vector resulted in therapeutic levels of hFVII expression[33]. Regarding other diseases, AAV has also been used in a murine model of thrombotic thrombocytopenic purpura (TTP), which is caused by severe deficiency of plasma ADAMTS13 activity. Current treatment of hereditary TTP is through plasma infusions. AAV8-hAAT-mdtcs (expressing MDTCS, a C-terminus truncated variant of ADAMTS13, driven by the liver-specific alpha-1 anti-trypsin promoter) at doses greater than 2.6 x 10^{11} vg/kg body weight (which were required to achieve therapeutic levels of ADAMTS13 plasma/antigen activity and were comparable with those reported for haemophilia B studies) resulted in sustained expression of plasma ADAMTS13 activity at

therapeutic levels[34]. Separately, AAV is also a very promising tool for genome editing, both for delivery of chimeric nucleases and repair template in culture and *in vivo*[35].

Integration-deficient lentiviral vectors (IDLVs)

Integration-deficient lentiviral vectors key features:

- Generated through the use of mutations in the integrase gene.
- ~7.5 kb capacity for foreign DNA.
- Efficient delivery to dividing and non-dividing cells.
- Transient expression in proliferating cells and sustained expression in post-mitotic tissues.
- Relatively low immunogenicity.

Retroviruses are single-stranded RNA viruses whose genome is reverse transcribed into double-stranded DNA and integrated into the infected cell genome. Genomic integration leads to stable maintenance and potentially sustained expression. These features are kept in retroviral vectors, which makes them particularly appreciated when stable, long-term expression is sought. The lentivirus genus of retroviruses includes species with a more complex genome, which in addition to genes *gag, pol* and *env* features a variable array of accessory genes[36]. Lentiviruses offer additional tropism as they are able to infect quiescent as well as dividing cells, the former not being suitable targets for classical gamma retroviruses. Lentiviruses infecting various mammals have been converted into lentiviral vectors, but the most used are those based on HIV-1[37] (**Fig. 1E,F**). Vectors based upon equine infectious anaemia virus (EIAV) are also highly engineered and have been developed for

commercial use[38]. Both HIV-1 and EIAV vectors have been used successfully for transduction of the central nervous system (CNS)[37,38].

Several strategies have been implemented to improve the bio-safety of lentiviral vectors. These vectors do not encode any viral product, as the viral proteins are provided in *trans* from several packaging plasmids to split the original viral genome. Accessory genes, often responsible for pathogenic features, have been progressively removed from the production system[39]. Vectors have also been made self-inactivating (SIN) by deleting the transcriptional promoter/enhancer from the 3' LTR in the transfer plasmid; this deletion is copied onto the 5' end of the vector during the reverse transcription cycle, abolishing expression from the viral LTR. SIN vectors are therefore dependent on an internal promoter to provide transgenic expression[40].

The theoretical risk from insertional mutagenesis mediated by retroviral vectors unfortunately first materialised in SCID-X1 clinical trials, where *ex vivo* transduction of HSPC with retroviral vectors led to clinical success but also some cases of leukaemia[41]. Lentiviral vectors display a safer integration pattern than gamma-retroviral vectors, and the SIN configuration also contributes to increasing bio-safety[42]. Additionally, high-efficiency lentiviral transduction can be achieved with IDLVs, produced through the use of integrase mutations that specifically prevent proviral integration, resulting in the generation of increased levels of circular vector episomes[43] (**Fig. 2A**). Lacking replication signals, lentiviral episomes mediate transient transduction in dividing cells and stable expression in quiescent cells. We and others have shown efficient and sustained transgene expression *in vivo* in rodent ocular, cerebral and spinal cord tissues[44-46], and substantial rescue of clinically relevant rodent models of retinal degeneration[46] and Parkinson disease[47]. It is also possible

to use retroviral vectors for so-called retrovirus particle-mediated mRNA transfer (RMT), whereby vector mutants unable to start reverse transcription are instead transiently translated[48], and lentiviral vectors for protein delivery[49].

Future directions and hematopoietic application

IDLVs have been shown to transiently transduce HSPC[50]. More recently, other applications for IDLVs have emerged (**Fig. 2B-D**). They can be used as platforms to deliver cassettes and zinc-finger nuclease genes for gene editing by homology-dependent repair in hematopoietic cells and others[51], for transposition[52] and for site-specific recombination[53]. Using the latter, IDLVs can yield site-specific recombination of a selectable donor cassette at the 'safe-harbour' *AAVS1* locus previously edited by zinc-finger nuclease to contain an acceptor site[54]. Dendritic cells are of particular interest and have been successfully targeted for transgenic expression with IDLVs[55]. An IDLV-based platform (ID-VP02) that targets and delivers antigen-encoding nucleic acids to human dendritic cells has also been recently developed. It was constructed by incorporating a novel genetic variant of Sindbis virus envelope glycoprotein with posttranslational carbohydrate modifications in combination with Vpx, a SIVmac viral accessory protein[56]. Replicating IDLVs would be of considerable importance, and attempts to develop them have included the incorporation of the simian virus 40 (SV40) promoter/origin of replication in cells expressing SV40 large T-antigen[57]. A version of this vector incorporating HSV thymidine kinase allows suicide gene therapy using ganciclovir[58]. We have shown that replicating IDLVs can be established at high frequency by inducing transient cell-cycle arrest at the time of vector transduction, with no requirement for replication sequences[59].

Poxviral vectors, including vaccinia

Poxviral vectors key features:

- Large-capacity dsDNA viruses (>25 kb of foreign DNA).
- Provide transient expression of immunologically relevant proteins.
- Ability to activate appropriate innate immune mediators upon vaccination.
- Relatively high-level of biological safety but potential for adverse events.

Poxviruses have played an important role in the development of virology, immunology and vaccinology. Deliberate inoculation of cowpox virus to humans by Jenner in 1796 demonstrated protection against the antigenically related smallpox virus (variola)[60]. Poxviruses are members of the family Poxviridae. They are dsDNA viruses about 200-400nm in length with a genome of about 190kb, which is flanked by ~10kb ITRs, and exist in two forms: an intracellular naked virion (INV) and an extracellular enveloped virion (EEV). Transcription and DNA replication occur in the cytoplasm, where the progeny DNA is generated by the synthesis and resolution of large concatemeric molecules[60]. Recombinant forms of the virus (with vaccinia as the prototype vector) are the vectors of choice for transient expression of immunologically relevant proteins[61] and thus serve as an alternative approach to the development of vaccines against a variety of infectious agents (reviewed by Moss[62]). Recombinant poxviruses have the transgene of interest commonly inserted by homologous recombination and driven by a poxviral promoter rather than a constitutive viral or mammalian promoter, since they are cytoplasmatic viruses and encode their own RNA polymerase. Classical experiments showed that vaccinia can carry at least 25 kb of foreign DNA[63]. Modified Vaccinia virus Ankara (MVA) is licensed as third-generation vaccine against smallpox. Recombinant MVAs (rMVAs) can be used for protein production and as vaccines against infectious diseases, cancer and other pathologies[64].

Future directions and hematopoietic application

Airway epithelial cells are the initial replication site of vaccinia virus, before spreading to secondary sites of infection, mainly the draining lymph nodes, spleen, gastrointestinal tract, and reproductive organs[65]. Stimulation of NK cell subsets during the antiviral response occurs through receptors apparently directed at viral products[66]. Understanding of the importance of NK cells in viral infections is improving, and poxviruses have been instrumental to improve this knowledge. After smallpox was eradicated, vaccinia has been used for the development of a variety of therapeutics: recombinant vaccines, immunotherapies, oncolytic therapies and others[67].

Other non-integrating viral vector systems

No single viral vector is optimal for all potential gene therapy applications. The availability of many vector systems differing in cloning capacity, stability, tropism, immunogenicity and other properties provides options to target the tissue and strategy of interest. In addition to those reviewed above, a prominent place is also due to herpesvirus vectors, and particularly those based on HSV-1 for the purposes of gene transfer for cancer and neurodegenerative disease[68]. Sendai virus, an RNA virus with no risk of genomic integration that can infect a wide range of cell types including HSPC, has recently attracted significant interest. Sendai vectors have been used for delivery of CRISPR/Cas9 for efficient gene editing[69], and for the generation of human induced pluripotent stem cells (iPSCs)[70].

Non-viral vectors

Non-viral vectors key features:

- Potentially unlimited packaging capacity.
- Inexpensive to manufacture at GMP.
- Can be endowed with replication and segregation capabilities for stable expression in proliferating cells.
- Relatively inefficient delivery.
- Relatively low immunogenicity.

Non-viral vectors for gene delivery were amongst the earliest to be developed, starting with plasmids: Wilson *et al*. cloned the β-globin cDNA into a bacterial plasmid in 1978[71], and Green *et al*. successfully transfected the plasmid into cells by calcium phosphate co-precipitation[72]. A distinctive feature of non-viral gene delivery is the ability to carry and deliver a broad range of nucleic acids. For gene addition studies, plasmids have been the most commonly used. However, more recently next-generation DNA molecules, including minicircle DNA[73], mini-intronic plasmids[74] and closed-ended linear duplex (CELiD) DNA[75], have been developed that show both enhanced transgene expression and persistence compared to conventional plasmids. In addition to DNA, mRNA, siRNA and gRNA can also be delivered using non-viral vectors to provide short-term transgene expression, gene suppression and CRISPR/Cas genome editing, respectively. In the case of mRNA and siRNA, as they only need to enter the cytoplasm to function, nuclear entry is removed as a significant barrier to function. This can be particularly advantageous in non-dividing cells. The transient expression obtained with mRNA delivery is also useful for gene editing applications where expression and function of nucleases and transposons are needed for

only a short period of time and where constitutive long-term expression could induce off-target mutagenesis[76].

For large transgenes, or whole genes, human artificial chromosomes (HAC) are an option that can be utilised with non-viral vectors. HACs can carry entire genes, including their introns and be maintained in dividing cells as the artificial chromosomes are replicated and segregated with the host chromosomes. Yeast artificial chromosomes (YACs) with 200- to 800-kilobase inserts of human DNA and first approaches to human mapping were described in the late 1980s[77]. Since these early days, applications of HACs have included development of transgenic mice with megabase-sized transgenes; stable maintenance in human embryonic stem cells; combination of human alpha satelite and single copy DNAs; and development of therapeutic human artificial chromosomes in preclinical models for Duchenne muscular dystrophy, caused by defects in the largest gene, *DMD*[78]. There is little consensus as to what constitutes a true 'artificial' *versus* an 'engineered' human chromosome, as recently reviewed[79]. However, the low efficiency of chromosome transfer and the relatively complex engineering of artificial chromosomes present significant challenges that have limited their utility as a therapeutic.

A number of non-viral methods for nucleic acid delivery have been developed, which can be classified as physical or chemical. Physical methods include the use of ultrasound[80] or electrical currents[81] to temporarily increase the permeability of target cells (sonoporation and electroporation, respectively), direct injection of DNA into single cells[82], ballistic propulsion of DNA-coated particles[83] and hydrodynamic gene delivery involving the rapid injection of a large volume of DNA solution (8-10% of body weight)[84]. Gene delivery by physical methods is fairly simple but offers no protection from nucleases for the nucleic acid.

In contrast, chemical carriers typically encapsulate nucleic acids thereby protecting the payload from nucleases. Chemical gene delivery vectors usually employ a cationic species to condense the anionic nucleic acids and in the process form nanoparticles for delivery. Cationic liposomes have been extensively studied and are among the most widely used non-viral vectors[85]. Later, addition of cationic polymers (producing so-called lipopolyplex) was shown to enhance gene delivery[86] (**Fig. 3**). Mechanistically, the liposome likely provides the mechanism for endosomal escape whilst the polymer enables efficient condensation and packaging of the nucleic acid therefore forming small, stable, discrete and homogenous nanoparticles. Further attempts at improving non-viral formulations have been made with the addition of components to improve bioavailability *in vivo* through shielding of complexes using polyethylene glycols, to enhance cell-specific targeting using targeting moieties, to aid endosomal escape using fusogenic lipids or pH sensitive polymers, and to improve nuclear entry using nuclear targeting sequences or nuclear localisation signal-containing peptides.

Future directions and hematopoietic application

The application of non-viral vectors to the hematopoietic system has been limited, but recent technologies are promising. T-cells can be genetically engineered to express a chimeric antigen receptor (CAR) using electroporation for sleeping beauty (SB) transposition, where SB was delivered as an mRNA or minicircle and the SB donor (consisting of the CAR sequences, flanked by SB inverted repeats) was provided as a minicircle[87]. Of importance, the integration pattern observed was considered safer than that present in CAR T-cells obtained with integrating lentiviral vector. Given the ease of scaling up production of mRNA and mini-circles and the close-to-random integration profile of SB transposition, there may be significant advantages to using non-viral methods for generating functional CAR T-cells.

Novel electroporation technologies like nucleofection have also been reported to mediate efficient delivery of DNA, mRNA and siRNA to various hematopoietic cell lineages[88].

Concluding remarks

HSPC have great therapeutic potential because of their ability to self-renew, differentiate and accomplish corrective reconstitution of the hematopoietic system in patients with various hematologic disorders. Specialised blood lineages are of relevance to specific strategies, like dendritic cells for immunisation or engineered CAR T-cells for lymphoma and leukaemia treatment. Gene addition therapies targeted to HSPC and using integrating retroviral vectors-have shown clear clinical benefits and potential in multiple diseases, among them immune deficiencies, storage disorders and hemoglobinopathies. However, the potential for insertional mutagenesis remains a risk. Gene editing technologies are also undergoing massive expansion and optimised, transient delivery is of clear benefit. The development of non-integrating nucleic acid delivery methods that combine low or no genotoxicity and high efficiency in hematopoietic cells is therefore highly desirable. We have described several such methods using Ad, AAV, IDLV, poxviral and non-viral vector technologies and are optimistic regarding the potential of non-integrating vectors in gene and stem cell therapy-based regenerative medicine.

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FIGURE LEGENDS

Figure 1. Diagrammatic representation of the basic genome structure of commonly used viruses and derived non-integrating vector constructs for gene therapy applications. Adenoviral (1st generation and helper-dependent), AAV and lentiviral vector structures are illustrated. Diagrams are not to scale. **A & B**: Adenovirus and Ad-based gene transfer vectors. The capacity of [E1-] Ad vectors is further increased by deletion of E3. HD Ad vectors contain the expression cassette of interest and ~500 bp of viral sequences required for DNA replication and packaging sequences. The 'stuffer' fragments are important for vector stability. **C & D**: wt AAV and recombinant AAV vector with expression transgene cassette flanked by ITRs; **E &F**: Lentiviral provirus and gene transfer vectors. Integrating lentiviral vectors (LVs) and IDLVs are produced similarly but for the latter the packaging plasmid encodes a mutated version of the integrase gene. ITR, inverted terminal repeat; Ψ, encapsidation sequence; pA, polyadenylation signal; LTR, long terminal repeat; SD, splice donor; SA, splice acceptor; cPPT, central polypurine tract; RRE, rev response element.

Figure 2. Generation and uses of integration-deficient lentiviral vectors. **A**: Following reverse transcription of the lentiviral genome, double-stranded DNA present in the cytoplasm is translocated into the nucleus. In the absence of catalytically active integrase, proviral integration is minimized and increased levels of transcription-proficient viral episomes are generated by non-homologous end-joining mediated self-ligation and homologous recombination of dLTR sequences. dLTR: 5'-deleted self-inactivating LTR. **B**: Genome editing using IDLV-mediated template delivery. In the presence of an engineered chimeric nuclease able to cut the target locus (thin black arrow), efficient homology-dependent repair can take

place, repairing a mutant gene. The mutation is represented as an orange signpost. **C**: IDLV-mediated transposon delivery. Co-introduction of a transposase like *sleeping beauty* or *piggybac* and the corresponding transposon allows efficient transposition into the target cell genome. TA: di-nucleotide targeted by *sleeping beauty* and flanking the inserted transposon. Inverted orange arrows: transposon *cis*-acting sequences. **D**: IDLV-mediated site-specific integration. An IDLV containing a site-specific recombination cassette can be integrated at the target genomic site in the presence of the relevant integrase. Examples are flp-mediated recombination at *FRT* sites and cre-mediated recombination at *loxP* sites. The recombination site is represented as a black arrow. Note that in **B-D** genes encoding engineering nuclease, transposase or site-specific recombinase can also be delivered with IDLVs.

Figure 3. Diagrammatic representation of barriers to non-viral transfection. Red arrows show limiting steps for lipoplex and polyplex technologies, potentially overcome by the use of lipopolyplex complexes.

Vector	Gene Therapy Clinical Trials Worldwide	
	Number	%
Adeno-associated virus	173	7.2
Adenovirus	505	21.0
Adenovirus + Modified vaccinia Ankara virus (MVA)	11	0.5
Adenovirus + *Retrovirus	3	0.1
Adenovirus + Sendai virus	1	0.0
Adenovirus + Vaccinia virus	8	0.3
Alphavirus (VEE) Replicon Vaccine	1	0.0
Antisense oligonucleotide	6	0.2
Bifidobacterium longum	1	0.0
Escherichia coli	2	0.1
Flavivirus	8	0.3
Gene gun	5	0.2
Herpes simplex virus	89	3.7
Lactococcus lactis	6	0.2
*Lentivirus	144	6.0
Lipofection	115	4.8
Listeria monocytogenes	22	0.9
Measles virus	10	0.4
Modified Vaccinia Ankara virus (MVA)	7	0.3
mRNA Electroporation	5	0.2
Naked/Plasmid DNA	414	17.2
Naked/Plasmid DNA + Adenovirus	4	0.2
Naked/Plasmid DNA + Modified Vaccinia Ankara virus (MVA)	2	0.1
Naked/Plasmid DNA + RNA transfer	1	0.0
Naked/Plasmid DNA + Vaccinia virus	3	0.1
Naked/Plasmid DNA + Vesicular stomatitis virus	3	0.1
Newcastle disease virus	1	0.0
Non-viral	2	0.1
Poliovirus	3	0.1
Poxvirus	70	2.9
Poxvirus + Vaccinia virus	36	1.5
*Retrovirus	449	18.6
RNA transfer	43	1.8
RNA virus	5	0.2
Saccharomyces cerevisiae	9	0.4
Salmonella typhimurium	4	0.2
Self-adjuvanting RNA	1	0.0
Semliki forest virus	2	0.1
Sendai virus	4	0.2
Shigella dysenteriae	1	0.0
*Simian Immunodeficiency Virus (SIVagm)	1	0.0
Simian virus 40	1	0.0
siRNA	5	0.2
*Sleeping Beauty transposon	10	0.4
Streptococcus mutans	1	0.0
Vaccinia virus	125	5.2
Venezuelan equine encephalitis virus replicon	3	0.1
Vesicular stomatitis virus	3	0.1
Vibrio cholerae	1	0.0
Unknown	80	3.3
Total	2409	100%

Table 1. Clinical trials using Non-Integrating Viral and Non-Viral vectors. To date at least 71.5% (1722 trials of a total 2409 registered) of the clinical trials reported have used non-integrating viral and/or non-viral vectors. Blue: non-integrating vectors; red: integrating vector or mixture (* indicates integrating vector); black, unknown. Adapted from the Journal of Gene Medicine, Gene Therapy Clinical Trials Worldwide, Wiley Database, (August 2016). http://www.abedia.com/wiley/vectors.php

Vector	Carrying Capacity (kb)	Features	Advantages	Disadvantages	Initial applications
Adenoviruses	~8-30	Nuclear Episome	Efficient delivery to dividing and non-dividing cells High, but transient expression	High Immunogenicity	Cancer Therapeutics Vaccination
AAV	~4.5	Episomal concatemers	Efficient delivery to dividing and non-dividing cells Relatively low immunogenicity	Possible long-term persistence of capsids *in vivo* Potential for encapsidation of prokaryotic sequences	Efficient and persistent *in vivo* delivery to post-mitotic tissues Delivery of gene editing components
IDLV	~7.5	Mutations of integrase gene in packaging plasmid	Efficient delivery to dividing and non-dividing cells Transient expression in proliferating cells and sustained expression in post-mitotic tissues Relatively low immunogenicity	Low expression in proliferating cells	Vaccination Delivery of gene editing templates
Poxvirus	>25	Poxviral RNA pol-based	Large capacity Delivery of substantial cassettes of heterologous antigens Activates innate immune mediators	Potential for adverse events, particularly in immunocompromised patients	Transient expression of immunologically relevant proteins Vaccination
Non-Viral	Potentially Unlimited	Chemical formulation	Inexpensive to manufacture Can achieve stable expression with replication and segregation	Relatively inefficient delivery	Gene delivery to muscle

			capacity Relatively low immunogenicity		

Table 2. Properties of Non-Integrating Gene Therapy Vectors.

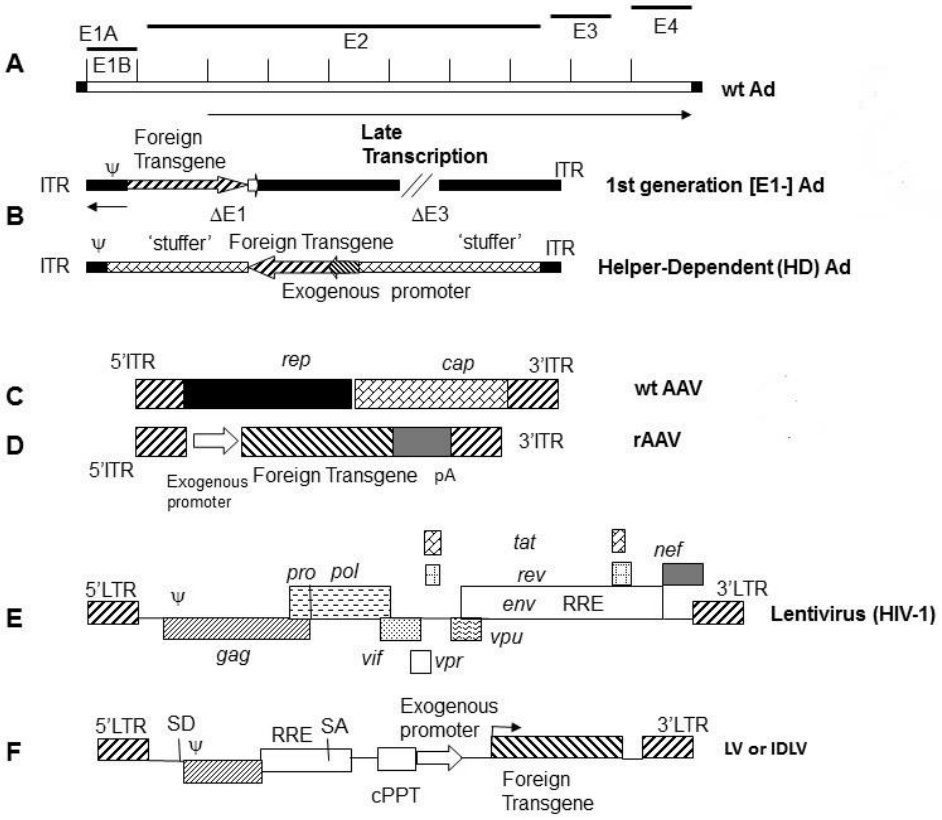
A
E1A
E1B
E2
E3
E4
wt Ad
Late
Transcription
B
Foreign
Transgene
ψ
ITR
ITR
1st generation [E1-] Ad
ΔE1
ΔE3
ψ
'stuffer'
Foreign Transgene
'stuffer'
ITR
ITR
Helper-Dependent (HD) Ad
Exogenous promoter
C
5'ITR
rep
cap
3'ITR
wt AAV
D
5'ITR
3'ITR
rAAV
Exogenous promoter
Foreign Transgene
pA
E
tat
nef
pro
pol
rev
5'LTR
ψ
env
RRE
3'LTR
Lentivirus (HIV-1)
gag
vif
vpr
vpu
F
5'LTR
SD
ψ
RRE
SA
Exogenous promoter
3'LTR
LV or IDLV
cPPT
Foreign Transgene

Figure 1

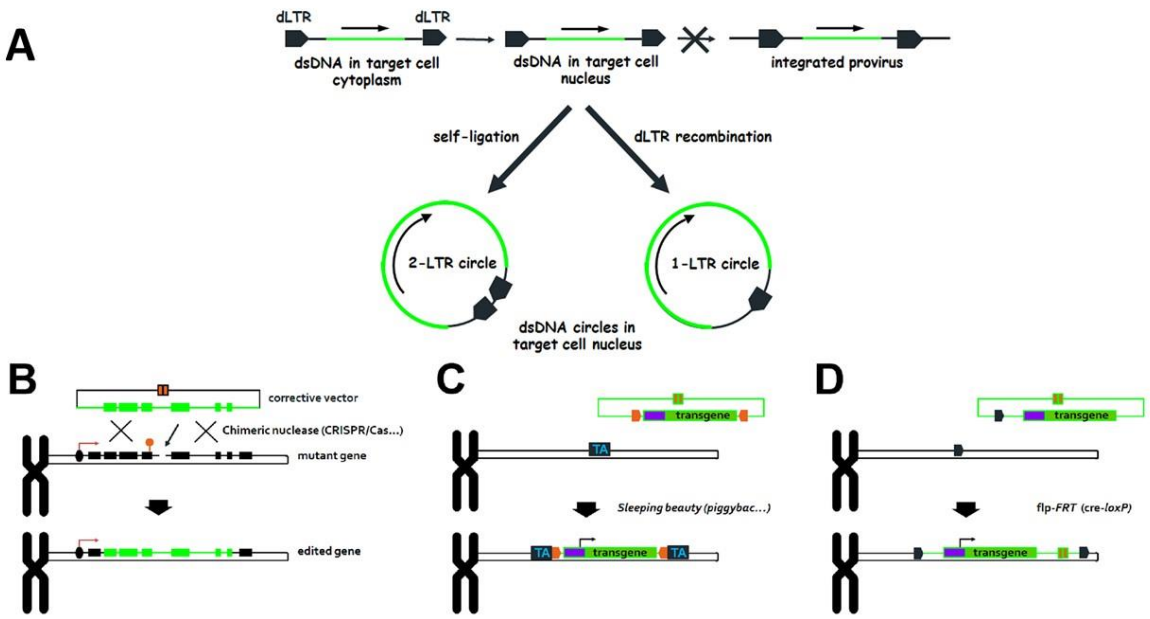
A
dLTR
dLTR
dsDNA in target cell cytoplasm
dsDNA in target cell nucleus
integrated provirus
self-ligation
dLTR recombination
2-LTR circle
1-LTR circle
dsDNA circles in target cell nucleus
B
corrective vector
Chimeric nuclease (CRISPR/Cas...)
mutant gene
edited gene
C
transgene
TA
Sleeping beauty (piggybac...)
TA
transgene
TA
D
transgene
flp-FRT (cre-loxP)
transgene

Figure 2

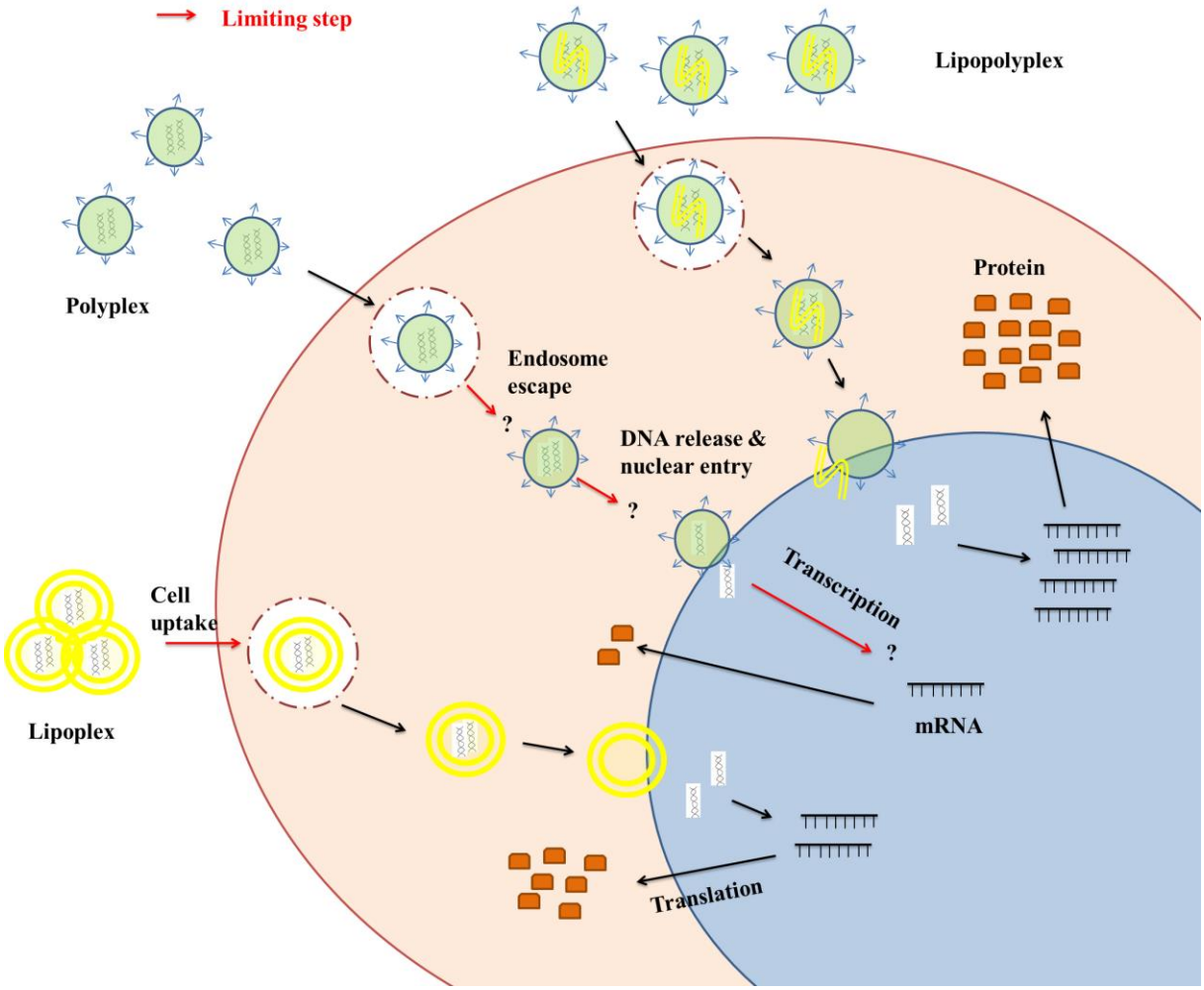
Limiting step
Lipopolyplex
Polyplex
Protein
Endosome
escape
?
DNA release &
nuclear entry
?
Transcription
?
Cell
uptake
Lipoplex
mRNA
Translation

Figure 3