Determinants of genome editing outcomes: the impact of target and donor DNA structures

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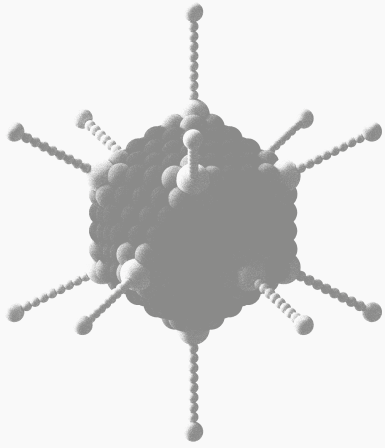
Chapter 6

Engineered **Viruses** as **Genome Editing** Devices.



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Abstract

Genome editing based on sequence-specific designer nucleases, also known as programmable nucleases, seeks to modify in a targeted and precise manner the genetic information content of living cells. Delivering into cells designer nucleases alone or together with donor DNA templates, which serve as surrogate homologous recombination (HR) substrates, can result in gene knockouts or gene knock-ins, respectively. As engineered replication-defective viruses, viral vectors are having an increasingly important role as delivery vehicles for donor DNA templates and designer nucleases, namely, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated Cas9 (CRISPR/Cas9) nucleases, also known as RNA-guided nucleases (RGNs). We review this dual role played by engineered viral particles on genome editing while focusing on their main scaffolds, consisting of lentiviruses, adeno-associated viruses, and adenoviruses. In addition, the coverage of the growing body of research on the repurposing of viral vectors as delivery systems for genome editing tools is complemented with information regarding their main characteristics, pros, and cons. Finally, this information is framed by a concise description of the chief principles, tools, and applications of the genome editing field as a whole.

Introduction

Genome editing based on sequence-specific designer nucleases, also known as, programmable nucleases (**Figure 1**) is opening a vast array of scientific and technological possibilities. Its broad range of action stems from granting researchers the means to modify, in a targeted and precise manner, the genetic make-up of cells from an increasing number of higher eukaryotes, including those of humans and other mammals.[1,2,3] In general, this is achieved by inducing double-stranded DNA breaks (DSBs) at predefined chromosomal sequences after designer nuclease delivery into target cells. The delivery of designer nucleases alone (**Figure 2**) or together with so-called donor DNA (**Figure 3**) can result in different targeted genome modification outcomes, each of which resulting from the repair of site-specific DSBs by nonhomologous end-joining (**Figure 2**) or homologous recombination (HR) (**Figure 3**), respectively.

Therefore, a crucial aspect pertaining to the application of genome editing strategies is that of introducing into target cells designer nucleases (**Figure 2**) and, whenever new genetic information needs to be added, surrogate HR substrates in the form of exogenous donor DNA templates (**Figure 3**). Viral vectors are particularly suitable options to introducing genome editing reagents into target cells because, while being replication-defective, they retain the efficient cell entry mechanisms evolved by their wild-type counterparts.[2,4,5] Indeed, as engineered replication-deficient viruses,

viral vectors have been extensively used in academia and industry to deliver foreign genetic payloads into virtually any cell type of interest. Moreover, besides nucleic acids they are also starting to be adapted for the direct transduction of recombinant proteins into target cells, including designer nucleases. In these cases, designer nucleases are fused to structural components of vector particles (for a recent review, see ref. [6]).

The on-going adaptation of viral vectors to genome editing settings builds upon

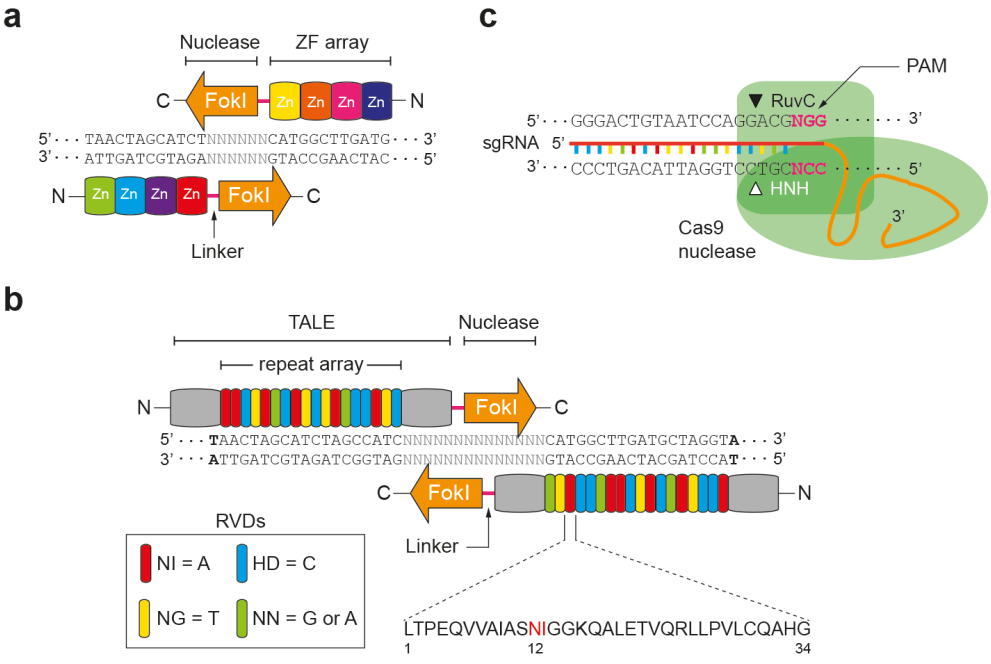

Figure 1

Diagrams of the three principal designer nuclease platforms. (**a**) Zinc-finger nucleases (ZFNs). ZFNs are based on artificial zinc-finger motifs in which two cysteine residues in a β-sheet hairpin and two histidines in an α-helix are tetrahedrally coordinated by a zinc ion. ZF, zinc-finger motif dictating the interaction with a specific nucleotide triplet; FokI, nuclease domain of the type IIS restriction enzyme FokI. (**b**) Transcription activator-like effector nucleases (TALENs). TALENs are based on type III secretory systems of phytopathogenic bacteria (e.g., *Xanthomonas sp.*). TALE, transcription activator-like effector comprising a central DNA-binding domain consisting of an highly conserved 33–34 residue-long repetitive motif; FokI, nuclease domain of the type IIS restriction enzyme FokI; RVDs, repeat variable di-residues located at positions 12 and 13 of each TALE repeat governing the interaction with a particular nucleotide. (**c**) RNA-guided nucleases (RGNs). RGNs are based on prokaryotic type II clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated (Cas) systems (e.g., Cas9 from *Streptococcus pyogenes*). sgRNA, chimeric single-guide RNA consisting of a fusion between a sequence-tailored CRISPR RNA (crRNA) and a scaffolding trans-activating crRNA (tracrRNA). PAM, protospacer adjacent motif (NGG, in the case of *S. pyogenes*); RuvC and HNH, the two nuclease domains of the Cas9 nuclease protein.

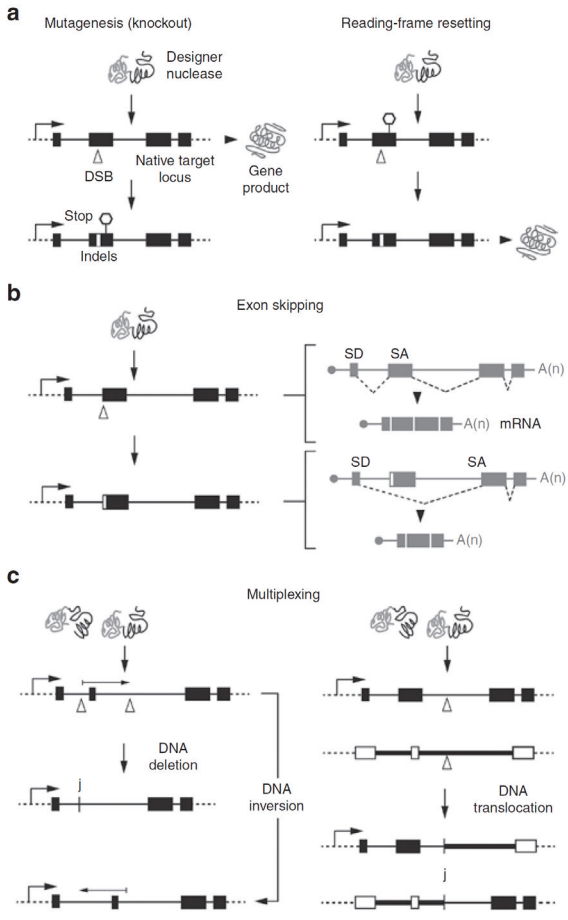

Figure 2

Genome-editing approaches based on nonhomologous end-joining (NHEJ)–mediated repair of chromosomal DSBs using designer nucleases. NHEJ DNA repair mechanisms acting at site-specifically generated DSBs can result in different genome editing outcomes. (**a**) NHEJ-mediated introduction of small insertions and deletions (indels) at the target site often leads to DNA sequence frame shifting, which in turn, can yield gene-specific knockouts whenever protein or RNA coding sequences are targeted (left panel). Conversely, targeted DSB-induced frame shifting can restore the proper reading frame usage (right panel). Of note, targeted mutagenesis resulting from the activity of designer nucleases might also generate protein variants whose mode of action involve dominant negative, or positive, effects (not drawn). (**b**) DNA-level exon skipping can be achieved by targeting genomic sequences corresponding to key splice acceptor elements. (**c**) Coordinated DSB formation by designer nuclease pairs (multiplexing) can yield specific deletions or inversions if the target sites are located in a particular chromosome (left panel) or translocations if they are present in different chromosomes (right panel). *Solid boxes* and *horizontal lines*, exons and introns, respectively; DSB, double-stranded DNA break (*open vertical arrowheads*); *Broken arrows*, *cis*-acting gene regulatory elements including promoters/enhancers. Indels, small insertions and deletions (*open vertical bars*); j, chromosomal DNA junctions formed by non-homologous recombination events triggered by designer nuclease multiplexing. SD and SA, splice donor and splice acceptor, respectively.

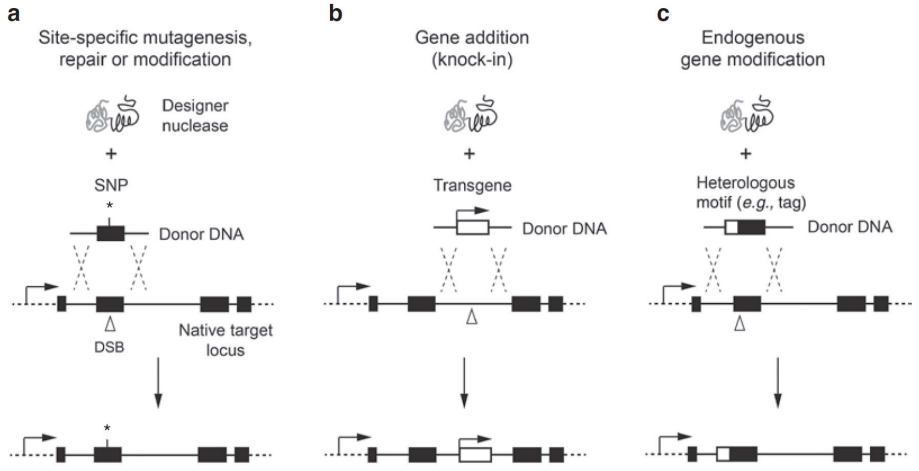

Figure 3

Genome-editing approaches based on HR-mediated repair of chromosomal double-stranded DNA breaks (DSBs) using donor DNA templates and designer nucleases. Robust homology-directed gene targeting, or knock-in, is achievable by combining designer nuclease-induced DSB formation with the delivery of donor DNA templates whose sequences share identity to regions flanking the targeted chromosomal lesion. These genome-editing procedures, based on the activation and recruitment of the homologous recombination (HR) DNA repair machinery, are particularly useful for the precise modification of predefined chromosomal target sequences of choice. Indeed, these DNA-modifying approaches offer the possibility to (**a**) repair, mutate or modify endogenous genes in a site-specific manner, (**b**) introduce entire recombinant transcriptional units (transgenes) into predefined positions in the genome, such as at so-called "safe harbours", and (**c**) endow endogenous proteins with new domains or tag endogenous genes, such as for tracing their expression patterns or isolating their encoded products. *Asterisks*, nucleotide change, such as, addition or deletion of a single nucleotide polymorphism (SNP) or mutation; *Large and small open boxes*, recombinant transcriptional unit (transgene) and heterologous motif (e.g., a tag), respectively. For a description of the other symbols and abbreviations see the legend of **Figure 2**.

a vast amount of knowledge gained from their development for "classical" gene therapy or gene replacement approaches in which the delivered foreign nucleic acids remain mostly in an episomal state or integrate randomly or semi-randomly throughout the target cell's genome.[2,4,5]

Clearly, inserting instead transgenes, or any exogenous DNA for that matter, into specific genomic sequences reduces the chance for various problematic events sometimes emergent whenever using systems that lead to the uncontrolled chromosomal integration of foreign nucleic acids (e.g., retroviral vectors and transposons/transposases). These unwarranted outcomes include positional-effect variegation, transgene silencing and, in some cases, insertional mutagenesis caused by transcriptional deregulation or physical disruption of endogenous target-cell genes.[7] The more defined genome modification outcomes resulting from the aforementioned designer nuclease-assisted genome editing strategies, are having a clear impact in

many fields. For instance, in functional genomics by helping deciphering the role of *cis*- and *trans*-acting nucleotide sequences, in transgenesis by speeding-up animal model generation via direct zygote engineering and in disease modeling by mimicking the origins of certain cancers through the deliberate induction of specific mutations or oncogenic rearrangements. Likewise related to disease modeling, and besides its potential role in future cell therapies, the integration of genome editing with induced pluripotent stem cell (iPSC) technologies is already helping in establishing genotype–phenotype relationships underlying not only monogenic but also polygenic or complex illnesses.[8]

In addition, genome editing strategies are being investigated for developing new treatment modalities aiming at tackling infectious diseases and advancing gene- and cell-based therapies. A first example of the former already exists in the shape of clinical studies testing whether designer nuclease-induced knockout of the HIV-1 coreceptor gene *CCR5* confers therapeutic benefit to acquired immune deficiency syndrome patients.[9,10] In parallel, the investigation of many other candidate gene therapies based on designer nuclease-induced gene knockout and gene knock-in approaches proceeds at the experimental and preclinical levels. These "genome surgery" research lines include deploying designer nucleases for disrupting alleles linked to dominant disorders and triggering homology-directed DNA targeting for repairing or complementing defective genes. The former entails the direct *in situ* correction of endogenous loci; the latter encompasses the targeted insertion of therapeutic DNA at ectopic "safe harbour" loci such as the *AAVS1* (19q13.42). Transgene insertion at such loci results in much higher probabilities for stable and homogeneous expression levels while lessening the chances for the deregulation of target-cell endogenous genes.[11]

In view of the many common goals and substantial overlap between "classical" gene therapy and therapeutic gene-editing research, the co-option of viral vector technologies for the latter purpose is logical and multifaceted in that they are being investigated for delivering not only designer nucleases but also donor DNA templates. Related to this, different types of viral vectors are, in some cases, combined in individual gene-editing transduction protocols. Here we review the roles that the main classes of viral vectors are having on improving the performance of and expanding the scope for genome-editing technologies.

Viral Vectors as Gene-Editing Tools

Lentiviral vectors

Conventional lentiviral vectors based on HIV-1 establish permanent genetic modification of target cells owing to the fact that their integrase-dependent mechanisms ensure semirandom chromosomal insertion of the transported foreign nucleic acids.[12] In "classical" gene therapy settings, these mechanisms are a crucial feature for achieving stable complementation of genetic defects in proliferating target cells and effector progenies.[13] In the context of genome editing approaches, however, the lentiviral DNA insertion mechanisms should best be disabled in order to ensure that the resulting episomal vector templates are available as substrates for HR or for transient designer nuclease expression. As previously mentioned, the short-term presence of designer nucleases in target cells is important for reducing the chances that deleterious effects caused by off-target activity arise. Therefore, by using trans-complementing packaging constructs harboring specific point-mutations in the HIV-1 *pol* region, researchers can assemble lentiviral particles whose integrase moiety contains disabling amino acid substitutions at crucial positions within its catalytic pocket (i.e., D64, D116, and/or E152) (**Figure 4**).[12,14,15] Importantly, these so-called class I integrase mutations are nonpleiotropic in that they interfere specifically with proviral establishment and not with any other of the viral transduction steps, such as, receptor binding, uncoating and nuclear import of the reverse-transcribed linear double-stranded vector genomes. Hence, integrase-defective lentiviral vectors (IDLVs), made with the aid of such packaging constructs serve as valuable vehicles for delivering nucleic acid templates for gene targeting and/or transient designer nuclease gene expression. Of note, similarly to their integration-proficient counterparts, the tropism of IDLV particles are normally altered by endowing them with envelop proteins derived from viruses whose cell surface receptors are different from those engaged by HIV-1. Accordingly, such pseudotyping manoeuvres permit narrowing or expanding the range of cell types transduced by vector particles.[16] For instance, to confer broad host range and high physical particle stability to lentiviral vectors, the vesicular stomatitis virus glycoprotein-G (VSV-G) is often selected as the heterologous envelop moiety (**Figure 4**).

IDLVs were the first viral vectors to be tested in the context of designer nuclease-assisted genome editing experiments.[17] These experiments, involving ZFN technology and various human target cell types (e.g., K562 erythromyeloblastoid leukemia cells, lymphoblastoid cells, and embryonic stem cells), provided an initial proof-of-concept for using IDLVs in designer nuclease-induced gene addition and gene repair studies. The former and latter experiments comprised, respectively, inserting recombinant DNA at specific genomic sequences (i.e., *CCR5*) and correcting *IL2RG* mutations underpinning X-linked severe combined immunodeficiency (X-SCID).

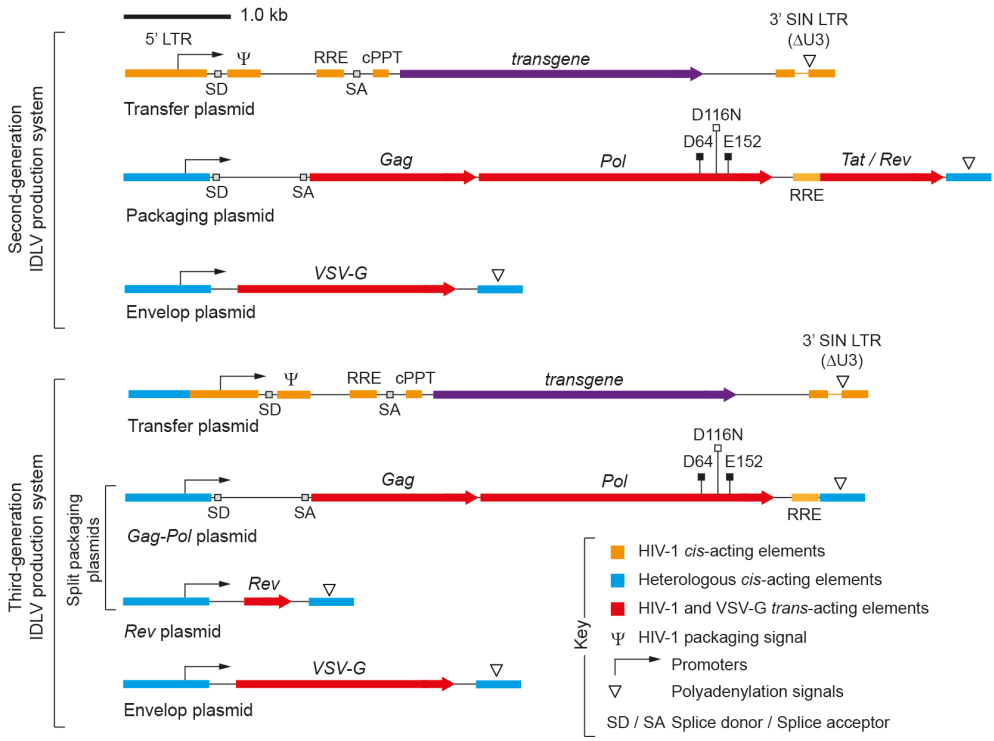

Figure 4

Schematics of the main HIV-1-based vector systems. HIV-1-based vectors are assembled by cotransfecting producer cells (e.g., HEK293T cells) with transfer, envelop, and packaging constructs with the resulting particles being collected and purified after budding from producer cells. Transfer plasmids harbor foreign nucleic acid sequences flanked by HIV-1 5′ and 3′ long terminal repeats (LTRs). To confer HIV-1 Tat independence to vector genome expression, third-generation transfer plasmids have hybrid 5′ LTRs composed of HIV-1 and heterologous transcriptional elements (e.g., *cytomegalovirus* and *Rous sarcoma virus*). Self-inactivating (SIN) vectors are deleted from specific LTR enhancers (ΔU3) to abrogate unwarranted transcriptional activity. Additional HIV-1 *cis*-acting elements include the packaging signal (Ψ), for vector genome encapsidation, the Rev-responsive element (RRE), for nuclear export of unspliced and singly spliced transcripts, and the central polypurine tract (cPPT), for transduction enhancement. The *in trans*-acting envelope plasmids typically encode the pseudotyping VSV-G moiety to confer a pantropic host range to vector particles. The also *in trans*-acting packaging constructs drive expression of HIV-1 *Gag* and *Pol* alone (third-generation) or together with *Tat* and *Rev* (second-generation). Owing to the *Tat* removal and the splitting of Rev from *Gag-Pol* templates, the former systems display a superior biosafety profile. The proteolytic processing of precursor *Pol* sequences yields mature reverse transcriptase and integrase (IN) molecules. Crucially, integrase-defective lentiviral vectors are assembled by using packaging constructs encoding IN moieties with substitutions of one or more amino acids of the DDE triad; D64, D116, and E152. These so-called class I mutations (e.g., D116N) abrogate specifically proviral establishment.

These data revealed that IDLV genomes can serve as efficient HR substrates yielding, in some cell types, homology-directed DNA targeting frequencies exceeding 10% of the total target cell population with the majority of cells harboring mono-allelic insertions. These initial data has been followed-up by various other studies in which IDLV transfer of donor DNA templates resulted in the addition of reporter and therapeutically relevant transgenes into "safe harbour" loci in a diverse set of target cells, including human myocytes,[18,19] human epithelial stem cells,[20] and iPSC lines.[21] Examples of these experiments are the site-specific chromosomal insertion of *microdystrophin* and *FANCA* transgenes into the "safe harbour" *CCR5* locus in, respectively, human muscle progenitor cells[18] and iPSCs from reprogrammed fibroblasts of Fanconi anemia patients.[21] It is worth mentioning however that, in common with any other HR-based genome editing approaches, the recruitment of IDLV donor DNA for gene addition or for gene repair purposes is limited in non-dividing or quiescent cells due to the fact that HR occurs preferentially during the G2/S phase of the cell cycle, when endogenous repairing templates are available.[22] Hence, the cellular DNA of quiescent primary cells, of which many display a high therapeutic relevance, is particularly difficult to edit through HR. An outstanding example of such cells is provided by primitive CD34+ human hematopoietic stem cells (HSCs). HSCs are defined as cells capable of long-term multilineage repopulation of the hematopoietic compartment in conditioned immune-deficient mice.[23] Of note, only genome modification at the HSC level is expected to ensure life-long correction of genetic disorders affecting components of the hematopoietic system. Aiming at improving HR-based genome editing of these cells, Genovese et al.[24] have developed a protocol in which donor DNA and ZFN delivery into HSCs is carried out by IDLV transduction and synthetic mRNA electroporation, respectively. Crucially, this transduction/electroporation protocol is combined with exposing target cells not only to cytokines but also to 16,16-dimethyl-prostaglandin E2 (dmPGE2) mixed with the aryl-hydrocarbon receptor protein antagonist, StemRegenin 1 (SR1). The rationale for including dmPGE2 and SR1 was to interfere with the loss of stem cell properties resulting from HSC exposure to extended *ex vivo* culture conditions and cell cycle-activating cytokines. By using these methods, the authors report that homology-directed gene targeting frequencies at *AAVS1* and *IL2RG* in *bona fide* HSCs are increased, as stringently demonstrated by serial transplantation of human CD34+ cells from primary to secondary NSG (NOD-SCID-*Il2rg*$^{-/-}$) mice. In a subsequent study, Hoban et al.[25] have also tested an *ex vivo* protocol based on the transfer of ZFN-encoding mRNA and IDLV donor templates into bone marrow-derived CD34+ cells for correcting the A-to-T transversion in *β-globin* alleles causing sickle cell anemia.

Hitherto, the delivery of designer nucleases by IDLVs remains mostly restricted to ZFNs. Considering that the yields of functional lentiviral vector particles decrease sharply with increasing transgene size,[26] it is possible that generating IDLVs contain-

ing the 4.1-kb *Cas9* open reading frame (ORF) from *Streptococcus pyogenes* results in IDLV preparations with low functional particle titers. In addition, experimental results indicate that the genetic instability resulting from frequent reverse transcriptase template switching events within TALE repeats in lentiviral vector genomes leads to defective particles.[27] This makes the assembly of TALEN-encoding IDLVs dependent on substantial ORF optimization for minimizing the frequency and length of unstable repetitive tracts.[28] Of note, the same principle of sequence identity reduction has permitted to package and deliver transcriptional units encoding two ZFN monomers in single IDLV particles.[29,30] This two-in-one approach is especially useful at low transduction rates since it ensures that each transduced cell is exposed to both members of a working ZFN pair at the proper 1:1 stoichiometry.

An issue pertaining to the optimal use of IDLVs as designer nuclease expression platforms is that of the susceptibility of their genomes to epigenetic silencing mechanisms in transduced cells.[31,32,33] These mechanisms involve the action of cellular histone deacetylases and have been shown to curtail DSB-induced targeted mutagenesis after IDLV-mediated transfer of ZFN expression units.[34] Finally, another issue regards the susceptibility of free-ended double-stranded IDLV genomes to "illegitimate" recombination processes such as nonhomologous end-joining. As a result, IDLV templates can become "captured" at off-target or spontaneous DSBs and form undesirable DNA structures such as concatemers and non-HR-derived junctions involving target or off-target sequences.[17,19] These adverse genome-modifying events contribute to reduce the fidelity of the genome editing process as a whole.[2]

Adeno-associated viral vectors

In contrast to lentiviral vectors, recombinant adeno-associated viral vectors (rAAVs) lack an integration machinery (**Figure 5**).[35,36] As a result, once in target cell nuclei, their genomes remain mostly in an episomal status with only a small fraction of them becoming incorporated in the cellular DNA (0.1–0.5 integrations per infectious unit)[37] presumably upon nonhomologous end-joining–mediated repair of sporadic chromosomal DSBs.[36] These vectors entered the scene of homology-directed gene targeting during the late 1990's, after the demonstration that viral particle transduction of single-stranded rAAV donor DNA yields more than 1,000-fold higher frequencies of gene repair (up to 1% of the total target-cell population) when compared to those achieved by transfecting conventional donor plasmids.[38] Despite the feasibility of this approach, including in *in vivo* settings,[39] the dominance of off-target insertions combined with the high dependency on large multiplicities of infection (>10^4 total vector particles per cell) and cell selection schemes,[40,41] has contributed to the initiation of research lines based on designer nuclease-assisted rAAV donor DNA targeting.

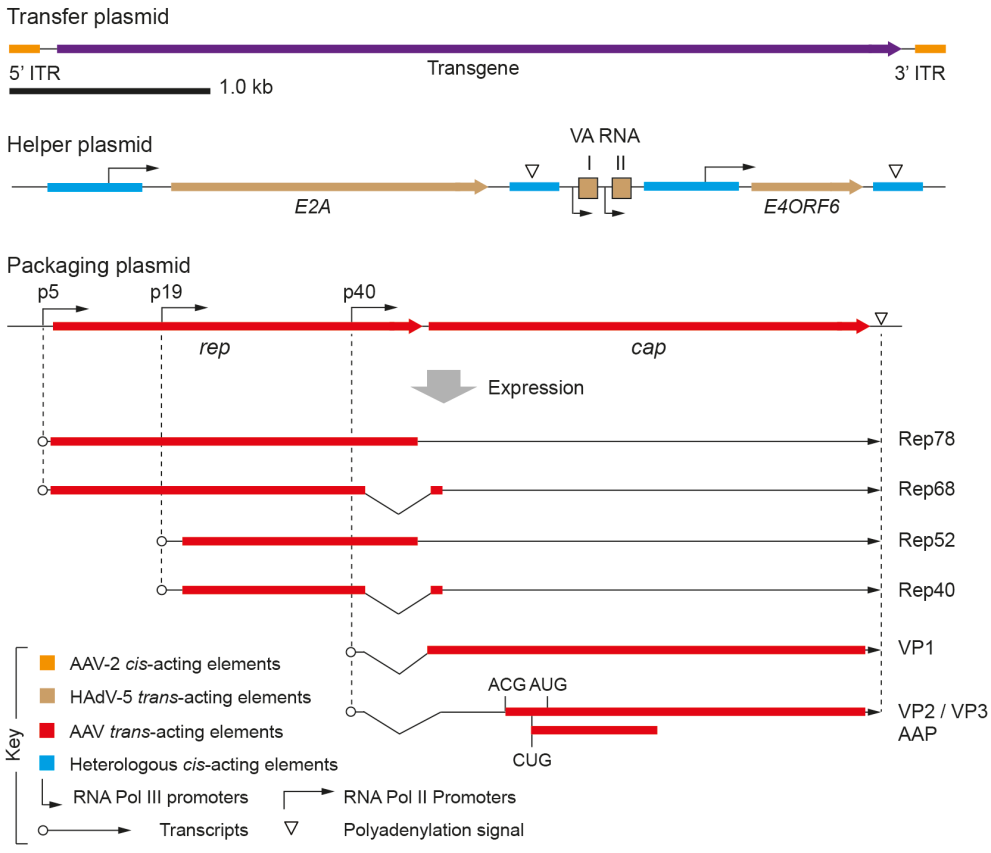

Figure 5

Schematics of the main recombinant adeno-associated viral vector (rAAV) production system. Recombinant AAV vectors are typically assembled by co-transfecting producer cells (e.g., HEK293T cells) with transfer, packaging and helper constructs with the resulting particles being purified after producer cell lysis. Transfer plasmids harbor foreign nucleic acid DNA flanked by 145 bp-long palindromic AAV-2 inverted terminal repeats (ITRs) whose primary sequence and T-shaped secondary structure form the origins of replication. The *in trans*-acting packaging plasmids contain the three AAV ORFs, *rep*, *cap*, and *AAP*. Transcription of *rep* from two different promoters and splicing of each of the resulting transcripts yields four proteins. Rep78 and Rep68 participate in DNA replication; Rep52 and Rep40 are necessary for DNA packaging into preformed empty capsids. The *cap* ORF is transcribed from a single promoter with alternative splicing resulting in two mRNA templates for the synthesis of three viral capsomers (VP1, VP2, and VP3). VP2 and VP3 share the same mRNA template with the former being translated from a "weak" start codon (ACG) located upstream of that for the latter product (AUG). Moreover, another "weak" start codon (CUG) present within the VP2-VP3 mRNA marks the beginning of a third reading frame, which codes for the assembly-activating protein (AAP). Productive wild-type AAV infections depend on the presence of an unrelated virus for providing AAV helper gene functions (*e.g.*, HAdV-5 *E1A-E1B*, *E2A*, *VAI-VAII*, and *E4ORF6*). In the context of rAAV production, these functions are supplied by transfecting E1A-E1B-expressing cells (e.g., HEK293) with a helper construct containing *E2A*, *VAI-VAII* and *E4ORF6*. Often, the packaging and helper functions are combined in a single plasmid.

Like previous data had shown for HR substrates delivered in the context of standard plasmids,[42,43] experiments based on inducing DSBs at chromosomally integrated reporter genes by the homing endonuclease I-SceI, provided a proof-of-concept for combining sequence-specific nucleases with rAAV donor DNA in gene-targeting settings. Indeed, these initial studies revealed that rAAV-based gene targeting can be enhanced by approximately 100-fold if a DSB is generated at a predefined target locus.[44,45] In this realm, and similarly to IDLVs, rAAVs have been mostly used so far for delivering donor DNA templates and ZFNs. Of note, when compared to those of *Cas9* and *TALEN*, *ZFN* ORFs are the smallest (i.e., ~1.2 kb per monomer versus ~4.1 kb and ~3 kb for *S. pyogenes Cas9* and *TALEN* ORFs, respectively). This permits the flexible construction and packaging of transcriptional units encoding one or even two ZFNs in single rAAV particles[46] whose effective maximum capacity is only ~4.5 kb (**Figure 5**). Clearly, in addition to TALEN and Cas9 nuclease delivery, the low packaging capacity of rAAV also introduces some limitations on the designing of HR substrates for the purpose of site-specific addition of whole transcriptional units. In any case, the combination of ZFN and rAAV technologies has clearly proven its potential for not only targeted gene disruption and deletion[46] but also for gene repair strategies. In what the latter genome editing approaches are concerned, these experiments involved the targeting of both reporter and endogenous loci after the delivery of ZFNs and gene correcting templates into a diverse panel of human cell types. These different cell types included, U2OS osteosarcoma cells,[47,48] HEK 293 cells,[46] HeLa cervix carcinoma cells,[48] HT-1080 fibrosarcoma cells,[48] and *bona fide* human embryonic stem cells (ESCs) as well as iPSCs.[49] Noticeably, due to the very diverse range of tools, experimental models and conditions, the gene-targeting frequencies in both absolute and relative terms (i.e., targeted versus random insertion events), varied substantially. As an example, Asuri et al.[49] compared ZFN-induced gene repair levels after transducing ESCs with a HR template packaged either in natural or variant AAV capsids. The latter capsid type, isolated by sequential cycles of biopanning of libraries of cap-mutant viruses on target cells, confers high-level rAAV transduction of hard-to-transfect ESCs and iPSCs. The authors showed, by using a highly quantitative readout system based on the rescue of defective reporter gene expression, that the transfer of corrective donor DNA by the molecularly evolved rAAV variant (R459G) yielded significantly higher (~10-fold) ZFN-induced gene repair levels in ESCs (~1.3% of the total target cell population) when compared to those resulting from using a prototypic, serotype 2-based, rAAV. Importantly, the proportion of random rAAV DNA chromosomal insertions was not augmented by the presence of active ZFNs in the transduced cells. Collectively, this and the above-mentioned studies established that site-specific DSB formation serves as a potent trigger for homology-directed gene targeting of donor DNA delivered in the context of single-stranded rAAV genomes.

Owing to a favorable set of characteristics, rAAVs are particularly suited for testing genome-editing strategies *in vivo*. These characteristics include low immunogenicity in immunocompetent animal models and amenability to tissue tropism modification methodologies based on engineered capsids generated by rational or directed evolution approaches.[50] Moreover, reminiscent of the above-described tropism engineering strategies involving enveloped lentiviral vectors; nonenveloped rAAVs can also be pseudotyped. In this case, rAAV genomes consisting of foreign DNA flanked by prototypic AAV serotype 2 inverted terminal repeats, are packaged within the capsids of other natural AAV isolates such as those of serotypes 1, 5, 6, 8, or 9.[50] These novel capsid-modified rAAVs are powerful gene delivery tools in that they can bypass pre-existing immunity associated with the presence of neutralizing antibodies against particular rAAV serotype(s) and can overcome transductional blocks linked to the absence of viral receptor(s) on the surface of specific cell types or tissues. In addition to the previously mentioned work in which a molecularly evolved rAAV was used,[49] another case in point is provided by the body-wide transduction of murine tissues by rAAV2/6 vectors, that is, AAV serotype 2-derived rAAV genomes pseudotyped by packaging in AAV serotype 6 capsids.[51] Moreover, it has been shown that rAAV2/8 particles achieve frequencies of murine liver cell transduction that are 10- to 100-fold higher than those obtained by using vectors based on other serotypes.[52] Importantly, these experiments equally revealed that the rAAV2/8 gene delivery activity was not hindered in animals preimmunized by exposure to other AAV serotypes.

The relevance and utility of rAAVs in *in vivo* settings is also underscored by the fact that a first proof-of-principle for designer nuclease-induced genome editing *in vivo* involved the use of these vectors in a murine model of hemophilia B, a blood coagulation disorder caused by factor IX deficiency.[53] In particular, rAAV2/8 particles containing a corrective cDNA spanning exons 2 through 8 of human *F9* were administered to new-born hemophilia B mice together with rAAV2/8 particles encoding donor-matched ZFNs targeting intron-1 of a defective human *F9* transgenic allele. Gene targeting was detected and meaningful in that it resulted in 3–7% of normal levels of circulating factor IX that led to the improvement of the disease phenotype as measured by clot-formation kinetic assays. Of note, molecular analysis of genomic DNA from treated mice revealed that therapeutic construct insertions at the intended target site occurred through both homologous and non-HR.[53] The latter, vector genome capture events, were likely caused by end-to-end nonhomologous end-joining of broken chromosome and AAV inverted terminal repeat sequences. A subsequent study extended these findings of AAV/ZFN-mediated *in vivo* therapeutic genome editing to adult hemophilia B mice.[54]

The *in vitro* and *in vivo* transfer of RGN components by rAAVs, has also been initiated. After constructing and validating shortened expression units encoding Cas9 and

sgRNAs, Senís et al.[55] were able to demonstrate delivery of Cas9 alone or together with a sgRNA by single vector particles built on chimeric AAV-DJ capsids. The latter "all-in-one" rAAV construction achieved approximately 8% indel formation at a target miRNA locus in HEK 293T cells when applied at a multiplicity of infection of 10^6 particles per cell. However, in mouse livers, RGN-induced indel formation at the conserved miRNA target locus by different rAAV constructs was invariably below 1% at 2 weeks postadministration. These *in vivo* results have been complemented by other animal model experiments in which rAAV-mediated delivery of RGN components served as a direct, transgenesis-free, approach for studying gene function in the mammalian brain.[56] These initial studies together with the advent of shorter Cas9 variants *bode well* for the implementation of rAAV/RGN tools in different *in vitro* and *in vivo* systems. Indeed, Ran et al.[57] have recently used a comparative genomic analysis to isolate and characterize a *Staphylococcus aureus* Cas9 protein whose relatively small size permits flexible rAAV design, including copackaging of both RGN components within single vector particles. The delivery of these tools into the livers of C57BL/6 mice by rAAV2/8 particles led 1 week after intravenous administration to approximately 5 and 40% indel formation at *Apob* and *Pcsk9* sequences, respectively.[57]

Adenoviral vectors

The sizable packaging capacity of adenoviral vectors (AdVs) combined with their high-titers and efficiency in transducing dividing and nondividing cells, makes them a broadly applicable option for *in vitro* and *in vivo* delivery of designer nucleases and donor DNA templates (**Figure 6**). Similarly to rAAVs, AdVs started to be deployed in the context of homology-directed gene targeting experiments that did not involve designer nuclease-induced DSB formation. In these experiments, helper-dependent AdVs, also known as "gutless" AdVs, were chosen owing to their lack of viral genes, permitting the use of high multiplicities of infection, and high capacity, allowing for large donor DNA packaging and delivery. Indeed, Ohbayashi et al.[58] utilized helper-dependent AdVs with 18.6 kb homology arms to correct a mutation in *HPRT* through HR without the involvement of artificial DSB formation in mouse ES cells. With the emergence of iPSCs, helper-dependent AdVs were also shown to be useful for correcting disease-related mutations in these pluripotent stem cells. In particular, they were used to repair several mutations in *LMNA* alleles associated with laminopathies, thus expanding the application of this gene delivery system to human disease modeling and targeted gene repair.[59] A follow-up study by Aizawa et al.[60], demonstrated that regardless of the transcriptional status of the target gene, helper-dependent AdVs can mediate both gene knock-ins and gene knockouts by HR with high fidelity in both iPSCs and ESCs of human origin. Of note, however, the absolute gene targeting levels achieved by helper-dependent AdVs are rather low requiring as a result the use of drug-based selection pressure for isolating the

desired targeted clones.

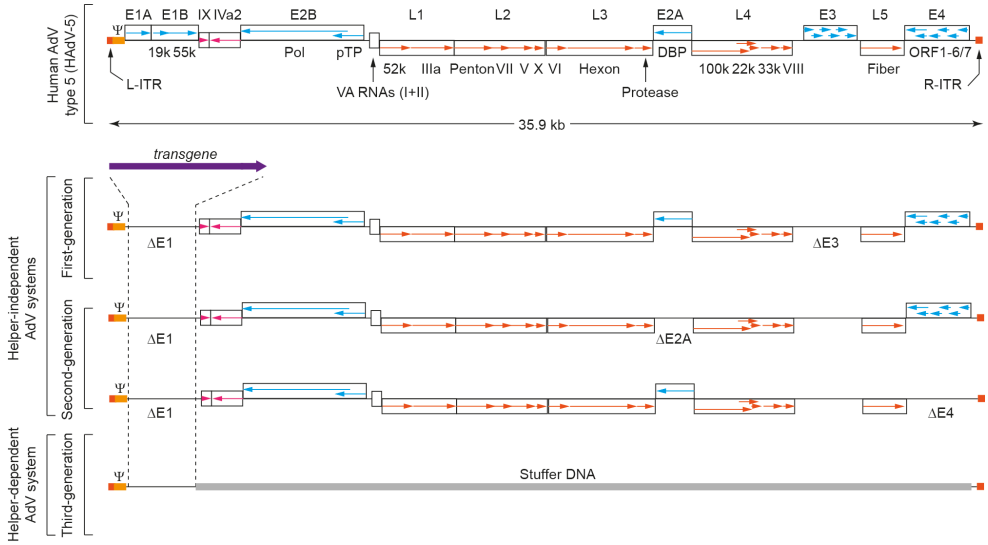

Figure 6

Schematics of the principal adenoviral vector (AdV) systems. The genome structures of the main AdV classes are drawn in relation to that of the prototypic HAdV-5 from species C. The 103 bp-long "left" and "right" inverted terminal repeats (L-ITR and R-ITR, respectively) contain the origins of replication, with the viral DNA packaging signal (Psi) being located adjacent to the L-ITR. The early (E) and late (L) regions are expressed before and after the onset of viral DNA replication, respectively. The former regions (i.e., *E1A-E2A*, *E2A-E2B*, *E3*, and *E4*) encode proteins involved in gene regulation (viral and host) and viral DNA replication; the later encode gene products primarily responsible for virion maturation and assembly (L1-L5). Expression units corresponding to small RNAs (VAI-VAII) and intermediate gene products (IX and IVa2) are also shown. First-generation AdVs lack E1A-E1B or E1A-E1B plus E3. Since E3 is dispensable during *in vitro* replication, all these vectors can be produced in packaging cell lines expressing exclusively the E1 functions (e.g., HEK293 or PER.C6). Second-generation AdVs have deletions in additional early regions (e.g., E2A and/or E4) being, as a result, produced in their respective complementing cell lines. Third-generation AdVs (also known as "gutless" or high-capacity) lack all viral DNA sequences except for the *cis*-acting ITRs and packaging signal. These vectors are produced in E1-complementing cells in the presence of a first-generation helper AdV which furnishes *in trans* all the viral gene products necessary for the replication and assembly of "gutless" AdV particles. The helper has its packaging elements framed by target sites for a site-specific recombinase (e.g., Cre or FLP) so that in recombinase-expressing producer cells is rendered packaging-defective in a selective manner.

Similarly to lentiviral and adeno-associated viral vector systems, AdVs are equally amenable to tropism modification and Good Manufacturing Practice methodologies. The former strategies include exchanging the apical fiber motifs of prototypic species C serotypes, which interact with the Coxsackie B virus and adenovirus receptor (CAR), with those of other natural serotypes (e.g., species B adenoviruses), which interact with other primary receptors. This "fiber swapping" genetic retar-

geting strategy allows by-passing the absence of CAR on the surface of human cells with scientific and therapeutic value such as hematopoietic stem/progenitor cells,[61,62] mesenchymal stromal cells,[63,64,65] and muscle progenitor cells.[65,66] Alternative AdV retargeting methods include capsid modifications by genetic fusion of fiber or pIX capsid proteins to heterologous ligands[67] or by chemical binding of capsid components to targeting moieties.[68] In this regard, it is noteworthy mentioning that the first testing of a therapeutic approach based on genome editing entails ZFN-mediated *CCR5* knockout in CD4+ T-cells from acquired immune deficiency syndrome patients after their *ex vivo* transduction with fiber-modified AdV particles.[9,10] Examples of other genome-editing studies based on the integration of AdV and ZFN technologies include the targeted mutagenesis of endogenous T-cell receptor genes in lymphocytes[69] and of *CCR5* and *β-globin* alleles in hematopoietic stem/progenitor cells.[70,71] Moreover, homology-directed gene targeting induced after AdV-mediated delivery of ZFNs, is equally being pursued in various cell types such as myoblasts, epithelial stem cells, and keratinocytes.[18,20]

Highlighting their versatility, AdV systems have in addition to ZFNs been validated for delivering TALENs and RGN complexes into human somatic cells regardless of their transformation status.[27,72] Concerning the former research it was found that, in striking contrast to lentiviral vector systems, the direct repeat arrays coding for the DNA-binding domains of TALENs are stable during AdV production in complementing packaging cell lines.[27,73] Importantly, the resulting vector preparations led to dose-dependent and high-level (up to 67%) targeted DSB formation in exposed cells (e.g., muscle progenitor cells and mesenchymal stromal cells). The genetic stability of AdVs is also underscored by the fact that transcriptional units encoding ZFN[9,74] or TALEN dimers can be packaged intact in single vector particles.[71,75] Due to the sizable length of *TALEN* ORFs (~3.0 kb per monomer), the latter studies deployed the high-capacity "gutless" AdV platform (**Figure 6**). In addition to the aforementioned muscle progenitor cells and mesenchymal stromal cells, the combination of AdV and TALEN technologies has served for inducing site-specific DSB formation in iPSCs[75] as well as in CD34+ cells isolated from G-CSF-mobilized peripheral blood mononuclear cells.[71]

Recently, various research groups started exploiting the efficient transduction of particular murine tissues by AdVs for studying genetic lesions underlying the emergence of specific cancers and, subsequently, modeling their progression *in vivo*. Such approaches based on the direct induction of targeted genomic changes *in vivo* (e.g., mutations, inversions, and translocations) are more expeditious than those based on transgenic mice and mimic more accurately the stochastic mosaicism characteristic of many tumors. For instance, Zhang et al.[76] succeeded in inducing higher rates of *Apc* mutations in the murine liver after tail vein injection of TALEN-encoding AdVs than those achieved after plasmid hydrodynamic injections (33 versus 7–19%, re-

spectively). Maddalo et al.[77] have in turn deployed RGN-encoding AdVs for inducing an approximately 11 Mb chromosomal inversion involving the *Alk* and *Eml4* loci to model the development of non–small-cell lung cancer *in vivo*.

Besides cancer modeling, other experiments sought to mutagenize *Cebpα*[78] and *Pcsk9* (ref. [79]) in murine livers after the administration of AdVs encoding RGN complexes. The former gene is a transcriptional factor involved in the activation of metabolic target genes; the latter is associated with low-density lipoprotein cholesterol levels, with its loss-of-function correlating with reduced risk of coronary heart disease development. Collectively, these experiments strengthened the view that, together with rAAVs, AdVs serve as a valuable platform for introducing designer nucleases *in vivo*. However, with the expansion and finer follow-up of *in vivo* genome editing procedures, one can expect encountering the immunological hurdles identified previously in countless gene transfer studies in animals. These hurdles include the activation of innate and adaptive immune responses against viral particle components and foreign antigens derived from transgenic and, in the case of helper-independent AdVs, viral ORFs.[80-82] Moreover, the long-term presence of designer nucleases in target tissues adds yet another hurdle that needs to be tackled by, for instance, incorporating regulatory devices for minimizing the risks of chromosomal mutations and/or rearrangements.

In addition to the introduction of designer nucleases into target cells, AdVs are also being exploited as a source of donor DNA templates for homology-directed gene editing after site-specific chromosomal DSB formation by ZFNs,[20,74] TALENs,[19,75] and RGNs.[19,75] In this regard, it has been shown that combining designer nucleases (i.e., TALENs and RGNs) and AdV-mediated donor DNA transfer induces homology-directed gene targeting that is more specific and accurate than that resulting from delivering donor DNA templates through conventional nonviral vectors or IDLVs.[19] The finding of precise genome editing resulting from designer nuclease-induced AdV donor DNA targeting ("Ad.iting", in short) could be attributed to the capping of linear AdV DNA by the 5′ covalently-attached viral terminal protein which, presumably, reduces non-HR events. The resulting targeted, single-copy, donor DNA integrants lead to uniform transgene expression in gene-modified cell populations.[19]

A synopsis of the main characteristics of the viral vector systems being repurposed as gene-editing devices is presented in **Table 1**, whereas their principal pros and cons are summarized in **Table 2**. On the basis of this review and on the information gathered in **Table 1** and **Table 2**, there is no evidence for an "ideal" one-fits-all combination of gene delivery and gene-editing tools. Instead, one can put forward the view that a specific arrangement(s) of these tools is best suited to achieve a particular goal.

In conclusion, viral vectors can serve a dual role in genome engineering efforts by delivering into virtually any human cell type, templates for not only designer nuclease expression but also for targeted chromosomal integration of foreign DNA. These features, combined with their well-established production systems and regulatory history build-up, are expected to foster and expand their application in genome editing settings, including in the realm of translational research.

Table 1. Overview of the main viral vector systems being repurposed as gene editing tools.

Main characteristics	IDLV	rAAV	AdV
Parental virus family, Genus (Prototypic element)	*Retroviridae*, Lentivirus (HIV-1)	*Parvoviridae*, Dependovirus (AAV-2)	*Adenoviridae*, Mastadenovirus (HAdV-5)
Particle structure (Shape)	Enveloped Phospholipid bilayer with trimeric spikes (spherical)	Non-enveloped Protein capsid, fibreless (icosahedral)	Non-enveloped Protein capsid with 12 trimeric fibres (icosahedral)
Vector particle size	~120 nm	~20 nm	~90-100 nm
Vector genome structure	HIV *cis*-acting LTRs and packaging signal flanking foreign DNA	AAV *cis*-acting ITRs flanking foreign DNA	HAdV *cis*-acting ITRs and packaging signal flanking foreign DNA and vector backbone
Typical vector assembly schemes in producer cell lines	Transfection of vector DNA, in *trans* complementing (*Gag*, *Pol*, *Rev*) and pseudotyping (*VSV-G*) constructs	Transfection of vector DNA, in *trans* complementing (*rep*, *cap*) and helper (HAdV genes) constructs	Transfection of vector DNA and propagation of assembled particles in complementing cells
Vector genome polymerases	Particle-associated Reverse transcriptase	Cellular DNA polymerases	Virus-encoded DNA polymerase
Packaged genome structure, polarity (Topology)	2× ssRNA, + strand (linear, free-ended)	1× ssDNA, + or - strand (linear, hairpin-capped)	1× dsDNA, ± strands (linear, protein-capped)
Vector particle assembly processes	Packaging of full-length vector genome transcripts	Packaging of ssDNA from hairpin-primed dsDNA intermediates	Packaging of dsDNA from protein-primed dsDNA intermediates
Vector particle cell entry	Receptor-mediated vector envelop/cell plasmalemma fusion	Receptor-mediated endocytosis via clathrin-coated pits	
Vector genome nuclear entry	Active ds cDNA import via a karyophilic pre-integration complex	Remodelled or intact particle entry through the nuclear pore	Docking of remodelled capsids at the nuclear pore, DNA entry
Prevalent genome status in transduced cell nuclei		Episomal	
Prevalent genome topologies in target cell nuclei	Linear: ds cDNA; circular: ds cDNA 1-LTR and 2-LTR forms	Linear: ssDNA and dsDNA; circular: dsDNA single- and multi-copy forms	Linear: dsDNA
Transduction potency (Target cell replication status)		High (dividing and non-dividing)	
Tropism modifications, including pseudotyping		Straightforward	

AAV-2, human adeno-associated virus type 2; cDNA, complementary DNA; ds, double-stranded; HIV-1, human immunodeficiency virus type 1; HAdV-5, human adenovirus type 5; IDLV, integrase-defective lentiviral vector; ITRs, inverted terminal repeats; LTRs, long terminal repeats; rAAV, recombinant adeno-associated viral vector; ss, single-stranded; *vsv-g*, vesicular stomatitis virus glycoprotein-G gene.

Table 2. Overview of the main pros and cons of IDLV, rAAV and AdV systems.

Parameters	IDLV	rAAV	AdV
Production timelines (research-level batch)	< 2 weeks	< 2 weeks	> 1 month
Vector production up-scaling[a]	+	++	+++
Functional vector particle yields	+	++	+++
Vector particle stability	++	+++	++
Viral genes[b]	Absent	Absent	Present or absent
Effective packaging capacity	< 7 kb	~ 4.5 kb	5-37 kb[c]
Compatibility between packaging capacity and nuclease ORF size[d]	++	+	++ or +++
Genetic stability (mutations, rearrangements and deletions)[e]	+++	+	+
Transgene expression kinetics[f]	++	+	+++
Transgene expression levels	+	++	+++
Susceptibility to transgene silencing	++	+	+
Background chromosomal insertion[g]	++	++	+
In vivo Immunogenicity[h]	+	++	+++
In vivo vector neutralizing antibodies[i]	+	+++	+++

[a]Typically, lentiviral vector production involves transient transfection methods.Virtually all of the AdV and some of the rAAV production platforms encompass instead infection of producer cell cultures with rescued viral vector particles. In these cases, the viral gene-deleted vector genomes are complemented *in trans* by either another viral vector and/or by transgenes stably integrated in the producer cell's DNA.[80,83]

[b]Lentiviral vectors, rAAVs, and helper-dependent, "gutless", AdVs can be made without any of their parental virus coding sequences. The latter system requires, however, a coinfecting helper AdV modified in its packaging signal region to provide *in trans* all the replicative and structural elements needed for vector DNA amplification and packaging, respectively.[80]

[c]The AdV packaging capacity is contingent upon the particular system and varies from a lowest value of ~5 kb for E1-deleted, helper-independent, AdVs to the highest value of ~37 kb for "gutless", helper-dependent, AdVs.[80]

[d]The approximate *ZFN* and *TALEN* ORF lengths are 1.2 and 3.0 kb per monomer, respectively. The combined length of the *S. pyogenes Cas9* ORF plus a typical gRNA expression unit is about 4.4 kb.

[e]The error rates of the HIV-1 reverse transcriptase exceeds those of viral dsDNA polymerases by several orders of magnitude.[84] This contributes to make lentiviral vector genomes mutation-prone.

[f]The transgene expression kinetics of rAAVs is particularly slow due to rate-limiting transduction steps, e.g., vector genome uncoating, nuclear entry and ssDNA to dsDNA conversation for transcriptionally active template generation.[35]

[g]IDLV and rAAV genomes are often "captured" at chromosomal DSBs, presumably due to the action of the nonhomologous end-joining pathway. This contributes to their relatively high levels of basal chromosomal DNA integration.[36,85]

[h]Helper-independent AdVs are particularly immunogenic in part because of adaptive immune responses triggered by "leaky" viral gene expression at high vector doses.[80] rAAVs are immunogenic in part because of capsidspecific T-cell activation at high vector doses.[82]

[i]AdVs and rAAVs based on prototypic serotypes 5 and 2, respectively, are particularly affected by pre-existing neutralizing antibodies due to the fact that a high fraction of the population has been exposed to these, or related, viral serotypes.[81,86]

AdV, adenoviral vector; DSB, double-stranded DNA breaks; IDLV, integrase-defective lentiviral vector ; rAAV, recombinant adeno-associated viral vector.

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