Engineered Viruses as Genome Editing Devices

Xiaoyu Chen¹ and Manuel AFV Gonçalves¹

¹Leiden University Medical Center, Department of Molecular Cell Biology, Leiden, The Netherlands

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.

Received 8 July 2015; accepted 26 August 2015; advance online publication 6 October 2015. doi:10.1038/mt.2015.164

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.¹⁻³ 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 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.⁶ 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 AAAS1 (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 integrate-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

---

**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 \(\beta\)-sheet hairpin and two histidines in a \(\alpha\)-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. (c) RNA-guided nucleases (RGNs). RGNs are based on prokaryotic type II clustered, regularly interspaced, short palindromic repeat (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 adja-cent motif (NGG, in the case of *S. pyogenes*); RuvC and HNH, the two nuclease domains of the Cas9 nuclease protein.
amino acid substitutions at crucial positions within its catalytic pocket (i.e., D64, D116, and/or E152) (Figure 4). Importantly, these so-called class I integrase mutations are nonallelicotropic 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. 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. 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). 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, human epithelial stem cells, and iPSC lines. 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 and iPSCs from reprogrammed fibroblasts of Fanconi anemia patients. 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. 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. 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. 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 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.

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, it is possible that generating IDLVs containing 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. This makes the assembly of TALEN-encoding IDLVs dependent on substantial ORF optimization for minimizing the frequency and length of unstable repetitive tracts. 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. 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. These mechanisms involve the action of cellular histone deacetylases and have been shown to curtail DSB-induced mutagenesis after IDLV-mediated transfer of ZFN expression units. 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. These adverse genome-modifying events contribute to reduce the fidelity of the genome editing process as a whole.

**Adeno-associated viral vectors**

In contrast to lentiviral vectors, recombinant adeno-associated viral vectors (rAAVs) lack an integration machinery. This makes the assembly of TALEN-encoding IDLVs dependent on substantial ORF optimization for minimizing the frequency and length of unstable repetitive tracts. 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. 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. These mechanisms involve the action of cellular histone deacetylases and have been shown to curtail DSB-induced mutagenesis after IDLV-mediated transfer of ZFN expression units. 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. These adverse genome-modifying events contribute to reduce the fidelity of the genome editing process as a whole.

In contrast to lentiviral vectors, recombinant adeno-associated viral vectors (rAAVs) lack an integration machinery. This makes the assembly of TALEN-encoding IDLVs dependent on substantial ORF optimization for minimizing the frequency and length of unstable repetitive tracts. 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. 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. These mechanisms involve the action of cellular histone deacetylases and have been shown to curtail DSB-induced mutagenesis after IDLV-mediated transfer of ZFN expression units. 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. These adverse genome-modifying events contribute to reduce the fidelity of the genome editing process as a whole.
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. Despite the feasibility of this approach, including in vivo settings, the dominance of off-target insertions combined with the high dependency on large multiplicities of infection (>10⁴ total vector particles per cell) and cell selection schemes, has contributed to the initiation of research lines based on designer nuclease-assisted rAAV donor DNA targeting.

Like previous data had shown for HR substrates delivered in the context of standard plasmids, 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. 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 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 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, HEK 293 cells, HeLa cervix carcinoma cells, HT-1080 fibrosarcoma cells, and bona fide human embryonic stem cells (ESCs) as well as iPSCs. 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, 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. 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. 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 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. 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. 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.

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, Senis et al. 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 10⁶ 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

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 (VAl-VAlI) and intermediate gene products (IX and IIA2) 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.
Table 1 Overview of the main viral vector systems being repurposed as gene-editing tools

| Main characteristics | IDLV | rAAV | AdV |
|----------------------|------|------|-----|
| Parental virus family, Genus (prototype) | Retroviridae, Lentivirus (HIV-1) | Parvoviridae, Dependovirus (AAV-2) | Adenoviridae, Mastadenovirus (HAdV-5) |
| Particle structure (shape) | Enveloped phospholipid bilayer with trimeric spikes (spherical) | Nonenveloped protein capsid, fibreless (icosahedral) | Nonenveloped 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 replicative intermediates | Packaging of dsDNA from protein-primed replicative intermediates |
| Vector particle cell entry | Receptor-mediated vector envelop/cell plasmalemma fusion | | |
| Vector genome nuclear entry | Active ds cDNA import via a karyophilic preintegration complex | Remodeled or intact particle entry through the nuclear pore | Docking of remodeled 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 | Linear: ssDNA and dsDNA; circular: dsDNA single- and multicopy forms | Linear: dsDNA |
| Transduction potency (target cell replication status) | High (dividing and nondividing) | | |
| Tropism modification by pseudotyping | Straightforward | | |

**Note:** 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.

**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 **in vitro** implementation of rAAV/RGN tools in different **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.

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 retargeting 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, mesenchymal stromal cells, and muscle progenitor cells. Alternative AdV retargeting methods include capsid modifications by genetic fusion of fiber or pIX capsid proteins to heterologous ligands or by chemical binding of capsid components to targeting moieties. 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. 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 and of CCR5 and β-globin alleles in hematopoietic stem/progenitor cells. Moreover, homozygote-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.

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. 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. 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 ZFNs or TALEN dimers can be packaged intact in single vector particles. 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 as well as in CD34+ cells isolated from G-CSF-mobilized peripheral blood mononuclear cells. 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 mosaic character of many tumors. For instance, Zhang et al. 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%, respectively). Maddalò et al. 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 mutate the genes of murine cells, either by transfection or by adenovirus (AdV) delivery. The former approach involved the introduction of new DNA into the genome of the cells, while the latter method involved the use of AdVs to deliver the gene of interest. However, the use of AdVs has been limited by the high risk of adverse events associated with their use.

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 nucleases 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.

ACKNOWLEDGMENTS

We thank Ignazio Maggio, Josephine M. Janssen, Rob C. Hoeber (Department of Molecular Cell Biology, Leiden University Medical Center, the Netherlands), and Otto-Wilhelm Merten (Genethon, France) for their critical reading of the manuscript. X.C. is the recipient of a PhD research fellowship from the China Scholarship Council. Leiden University Joint Scholarship Programme. M.A.F.V. Gonçalves is coinventor in a patent application on the use of capped donor DNA constructs for targeted DSB-induced genome modifications. The funds for the research carried out in our laboratory include grants from the Dutch Prinses Beatrix Spierfonds (W.OR11-18) and the French AFM Téléthon (16621).

REFERENCES

1. Gaj, T., Gersbach, C.A., and Barbas, C.F. 3rd (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 31: 397–405.
2. Maggio, I. and Gonçalves, M.A. 2015. Gene editing at the crossroads of delivery, specificity, and fidelity. Trends Biotechnol. 33: 280–289.
3. Kim, H. and Kim, J.S. 2014. A guide to genome engineering with programmable nucleases. Nat Rev Genet 15: 321–334.
4. Kay, M.A., Glorioso, J.C. and Naldini, L. 2001. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat Med 7: 33–40.
5. Gonçalves, M.A. 2005. A concise peer into the background, initial thoughts and practices of human gene therapy. Bioseis 27: 506–517.
6. Skipper, K.A. and Mikkelsen, J.G. 2015. Delivering the goods for genome engineering and editing. Hum Gene Ther 26: 486–497.
7. Biasco, L., Baricordi, C. and Angrisani, A. (2012). Retroviral integrations in gene therapy trials. Mol Ther 20: 709–716.
8. Sterneckert, J.L., Reinhardt, P. and Schöler, H.R. (2014). Investigating human disease using stem cell models. Nat Rev Genet 15: 625–639.
9. Perez, E., Wang, J., Miller, J.C. and Hartley, Y. 2002. Viral vectors. In: HIV resistance in CD4+ T cells by genome editing using zinc-finger nucleases. Nat Biotechnol 26: 808–816.
10. Tebas, P., Stein, W.W., Tang, J., Frank, SQ., Wang, G. Lee, SK et al. (2014). Gene editing of CRISPR/Cas9 in autologous CD4 T cells of persons infected with HIV. N. Eng J Med 370: 901–910.
11. Sadelfaln, M., Papapetrou, EP. and Bushman, FD. (2012). Safe harbors for the integration of new DNA in the human genome. Nat Rev Cancer 12: 51–58.
12. Naldini, L. (2013). In vivo gene transfer and correction for cell-based therapies. Nat Rev Genet 12: 301–315.
13. Philipps, N. and Trasher, A. (2007). Use of nonintegrating lentiviral vectors for gene therapy. Hum Gene Ther 18: 483–489.
14. Waren, K. and Yáñez-Muñoz, B. (2009). Integration-deficient lentiviral vectors: a slow coming of age. Mol Ther 17: 1316–1332.
15. Cronin, J., Zhang, X.Y. and Resier, J. (2003). Altering the tropism of lentiviral vectors through pseudotyping. Curr Gene Ther 3: 387–398.
16. Lombardo, A., Genovese, P., Beaussier, CM., Colleoni, S., Lee, YL., Kim, KA et al. (2007). Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol 25: 1298–1306.
17. Benabdallah, BF., Duval, A., Rousseau, J., Chapdelaine, P., Holmes, MC., Haddad, E. et al. (2013). Targeted Gene Addition of Microdystrophin in Mice Skeletal Muscle via Human Myoblast Transplantation. Mol Ther Nucleic Acids 2: e68.
18. Holkers, M., Maggio, I., Heniques, SF., Janssen, JM., Cathomen, T., Gonçalves, MA et al. (2014). Adenoviral vector DNA for accurate genome editing with engineered nucleases. Nat Methods 11: 1031–1037.
19. Coluccio, A., Miselli, F., Lombardo, M., Marconi, A., Malageoli Tagliasuczci, G., Gonçalves, M.A. et al. (2013). Targeted gene addition in human epithelial stem cells by zinc-finger-nuclease-mediated homologous recombination. Mol Ther 21: 1695–1704.
20. Rio, P., Barioli, R., Lombardo, A., Quintana-Bustamante, O., Alvarez, L., Garate, Z et al. (2014). Targeted gene therapy and cell reprogramming in Fanconi anemia. EMBO Mol Med 6: 835–848.
21. Cass, EM and Jasin, M. (2010). Collaboration and competition between DNA double-strand break repair pathways. FIBS Lett 584: 3703–3708.
22. Doulatov, S., Nohta, S., Laurent, E. and Dick, J.E. (2012). Hematopoiesis: a human perspective. Cell Stem Cell 10: 120–136.
23. Genovese, P., Schirol, G., Escobar, G., Di Tomaso, T., Fimtro, C., Calabria, A. et al. (2014). Targeted genome editing in human repopulating haematopoietic stem cells. Nature 510: 235–240.
24. Hoban, MD., Cost, GJ., Mendel, MC., Romero, Z., Kaufman, ML, Joglekar, AV et al. (2015). Correction of the sickle cell disease mutation in human hematopoietic stem/ progenitor cells. Blood 125: 2597–2604.
25. Kumar, M., Keller, B., Makalou, N. and Sutton, RE. (2001). Systematic determination of the packaging limit of lentiviral vectors. Hum Gene Ther 12: 1893–1905.
26. Holkers, M., Maggio, I., Liu, J., Janssen, J.M., Miselli, F., Mussolino, C. et al. (2013). Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. Nucleic Acids Res 41: e63.
27. Yang, L., Guell, M., Byrne, S., Yang, J.L., De Los Angeles, A., Malli, P. et al. (2013). Optimization of scarless human stem cell genome editing. Nucleic Acids Res 41: 9049–9061.
28. Joglekar, AV., Hollis, RP., Kufniece, G., Sadanendra, S., Chan, R. and Kohan, DB. (2013). Integrate-defective lentiviral vectors as a delivery platform for targeted modification of adenovirus dexametase kinase. Mol Ther 21: 1705–1717.
29. Abaratagegui-Pontes, C., Crêneguy, A., Thirand, R., Fine, EJ., Thepenier, V., Fournier, le RL et al. (2014). Codon swapping of zinc-finger nucleases confer expression in primary cells and in vivo from a single lentiviral vector. Curr Gene Ther 14: 365–376.
31. Kantor, B, Ma, H, Webster-Cyriaque, J, Monahan, PE and Kafri, T (2009). Epigenetic activation of unintegrated HIV-1 genomes by gut-associated short chain fatty acids and implications for HIV infection. Proc Natl Acad Sci USA 106: 18786–18791.

32. Pelascini, LP, Pelascini, N, and Gonçalves, MA (2013). Histone deacetylase inhibition rescues gene knockout levels achieved with integrase-defective lentiviral vectors. Hum Gene Ther 24: 399–411.

33. McCarty, DM, Young, SM Jr and Samulski, RJ (2004). Integration of adeno-associated viruses delivered by integrase-defective lentiviral vectors. Nat Methods 1: 71–76.

34. Rouet, P, Smih, F and Jasin, M (1994). Expression of a site-specific endonuclease and its implications for HIV infection. Proc Natl Acad Sci USA 91: 7649–7657.

35. Saydaminova, K, Ye, X, Wang, H, Richter, M, Ho, M, Chen, H et al. (2015). Efficient genome engineering in hematopoietic stem cells with helper-dependent Ad5/35 vectors expressing site-specific endonucleases under microRNA regulation. Mol Ther Nucleic Acids 3: e216.

36. Thijink, J, Van der Heijst, J and Hoenen, RC (2005). The adenovirus capsid: major progress in minor proteins. J Gen Virol 86(Pt 6): 1581–1588.

37. Allikmets, R, Dirdjev, P, Roat, J and O’Riordain, M (2007). Evaluation of adeno-associated virus transfer vectors with synthetic polymers: a scientific review and technical guide. Mol Ther Nucleic Acids 4: 329–338.

38. Li, L, Krymskaya, L, Wang, J, Henley, J, Rao, A, Cao, LF et al. (2013). Genome editing of the HIV-1 co-receptor CCR5 in adult hematopoietic stem and progenitor cells using zinc finger nucleases. Mol Ther 21: 1299–1307.

39. McCarty, DM, Young, SM Jr and Samulski, RJ (2004). Integration of adeno-associated viruses delivered by integrase-defective lentiviral vectors. Nat Methods 1: 71–76.

40. Liu, GH, Suzuki, K, Qu, J, Sancho-Martinez, I, Yi, F, Li, M et al. (2011). Genome editing using Staphylococcus aureus Cas9. Nature 499: 158–162.

41. McCormy, DM, Young, SM Jr and Samulski, RJ (2004). Integration of adeno-associated viruses delivered by integrase-defective lentiviral vectors. Nat Methods 1: 71–76.