
Gene Delivery Technologies for Efficient Genome Editing: Applications in Gene Therapy

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Abstract

Specific nucleases (SNs), including ZFNs, TALENs, and CRISPR (clustered regularly interspaced palindromic repeats), are powerful tools for genome editing (GE). These tools have achieved efficient gene repair and gene disruption of human primary cells. However, their efficiency and safety must be improved before translation into clinic. In particular, one of the main hurdles of GE technology is the delivery of the different components into the nucleus of target cells. Successful gene editing must be able to deliver the SNs and/or the donor DNA into a large number of target cells in order to have a therapeutic benefit. In addition, the delivery must be nontoxic and the SNs must be innocuous to the target cells. In this chapter, we will summarize the different ways to deliver SNs and donor DNA.

Keywords: gene edition, gene therapy, delivery, viral vectors, specific nucleases

1. Introduction

Although genome editing (GE) technologies have been used for more than 30 years, the efficiency and specificity was too low to be used in gene therapy (GT). However, the development of specific nucleases (SNs) that can enhance homologous directed recombination (HDR) up to 10,000 times and allows the generation of specific mutations have open new possibilities for the use of GE for GT applications. SNs can create specific DSBs at target locations in the genome. These DSBs must be repaired by the cell's endogenous mechanisms by either HDR, a high-

fidelity repair process, or by nonhomologous end joining (NHEJ), an error prone process that results in insertions or deletions (indels) at the cleavage site. This repair mechanism is used as a platform for GE. Depending on the mechanism used by the cells to repair the DSBs, we can repair, insert, or delete DNA fragments in the genome of the target cells. There are four families of SNs being used: meganucleases (MNs) [1], zinc finger nucleases (ZFNs) [2], transcription activator-like effectors nucleases (TALENs) [3], and the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR Associated 9 (Cas9) (CRISPR/Cas9) system [4] also named RNA-guided nucleases (RGNs). MNs, ZFNs, and TALENs used the principles of DNA-protein recognition to target specific locus. However, the difficulties of protein design, synthesis, and validation blocked the widespread adoption of these engineered nucleases for routine use in many laboratories. The field is experiencing a new phase thanks to the development of the RGNs [4]. This two-component system can achieve specific cleavage in any target DNA location guided by a small RNA molecule named gRNA [5].

However, in spite of the great advances in SNs design, the translation of GE technologies into clinic still requires several refinements both in terms of specificity and efficiency [6]. The efficiency of a particular GE strategy largely relies on the efficiency of delivery of the SNs and/or the donor DNA. In this chapter, we will discuss the different tools available for the delivery of the different components required for GE.

2. Viral-based vectors

GE using SNs and/or a donor DNA requires vector systems that efficiently deliver both components into the target cells. This is a relative easy task for cells that are growing in the laboratories but is a much difficult task when we target primary human cells. In general, viral-based vectors are more efficient than non-viral for most primary human cells. Since transient expression of GE components is preferred over stable expression, we will focus on episomal (non-integrative) viral-based vectors.

2.1. Adenoassociated virus (AAV)-based vectors

AAVs have been used for the delivery of ZFNs; however, the limited capacity of the AAV vectors makes difficult the use of these vectors for the delivery of Cas9 and TALEN. The first report showing the efficacy of AAV vectors to deliver ZFNs was published in 2012 [7, 8]. Soon later, the group of Katherine High showed that systemic AAV-ZFNs and AAV-corrective donor template enables production of high levels of human factor IX in a murine model of hemophilia B [9]. In another study, Weber et al. [10] reported that the administration of AAV-ZFNs-targeting HBV polymerase achieved an inhibition of HBV replication. In spite of their big size, several groups have attempted to deliver *S. pyogenes* Cas9 and its gRNA using AAV vectors [11]. However, oversized AAV vectors render inconsistent results [12]. As an alternative, different groups deliver the gRNA and the Cas9 in separate AAVs with very promising

results [13]. Indeed, these two-AAV vectors systems have achieved correction of dystrophin expression in a mouse model of Duchenne muscular dystrophy (DMD) [14] and correction a metabolic liver disease [15].

Recently, the development of a new RGN based in the smaller Cas9 from *S. aureus* [16] opened the possibility of generating AAV vectors harboring both Cas9 and gRNA expression cassettes. Taking the advantage of this smaller Cas9, several groups have developed "All-In-One" AAV-RGN systems obtaining very promising results in animal models of DMD [17, 18]. In these experiments, systemic delivery of AAV-Cas9-gRNA to DMM mice resulted in the expression of the dystrophin gene with improvements of muscle biochemistry and enhancement of muscle force.

Beside the potential of AAV as a gene delivery tools, it has been reported that these vectors are able to enhance up to 1000-fold the HDR rate in mammalian cells [19]. In fact, the delivery of corrective donor DNA (without SNs) with AAV has achieved correction of mucopolysaccharidosis [20] and hereditary tyrosinemia [21] in mouse models. Of course, scientists have taken advantage of this property and have combined SNs with delivery of Donor DNA by AAV vectors. Wang and colleagues [22], combined electroporation of ZFN mRNA (see below) with donor delivery by AAV serotype 6, achieving efficient GE in HSPCs (up to 50% of CCR5 specific insertion) and T cells. Anguela et al. [9] and Sharma et al. [23] showed that systemic delivery of AAV-ZFNs and AAV-Donor to adult mice can achieve high levels of human factor VIII and IX in murine models of hemophilia A and B. Recently Yang et al. [15] corrected a metabolic liver disease using a similar strategy. Using AAV to deliver ZFNs and donor DNA, Sharma et al. [23] reported a general strategy for liver-directed protein replacement therapies that allows site-specific integration of therapeutic transgenes within the albumin gene. The authors achieved long-term expression of human factors VIII and IX as well as lysosomal enzymes in different animal models of hemophilia, Fabry and Gaucher disease, and Hurler and Hunter syndrome. An additional property of AAV that make these viruses an ideal tool for delivery of Donor DNA is its high specificity for the target locus [24]

2.2. Adenovirus (AdV)-based vectors

AdVs are highly attractive for viral delivery of SNs and in particular for TALENs due to their high cargo capacity, their ability to transduce dividing and nondividing cells and their transient expression. In addition, similarly to AAV, AdVs started to be used as a tool to deliver large donor DNA for homology-directed gene-targeting experiments (without the use of SNs). This approach has been used in order to correct the HPRT in mouse ES cells [25] or LMNA gene in pluripotent stem cells [26]. Of course, the appearance of SNs prompts scientists to use AdV not only for delivery of the donor DNA but also for the delivery of SNs. In this context, one of the major successes was achieved by the expression of ZFNs targeting the CCR5 locus into CD4+ T cells [27–29]. In fact, AdV delivery of ZFNs was the first GE strategy been approved for their use in clinical trials. The strategy aimed to knock down CCR5 expression (a co-receptor for HIV-1 entry into cells) from T cells derived from HIV-1 patients [29]. On the other hand and taking into account the large cargo capacity of AdVs in comparison with AAV, the AdV have been used not only for the delivery of TALENs [30] and Cas9/gRNA [31, 32], but also as

a source of donor DNA templates for homology-directed gene editing after site-specific chromosomal DSB formation by ZFNs, TALENs, and RGNs. Interestingly, it was found that protein-capped adenovirus genomes favored a more specific GE by HDR templates compared to un-capped linear templates. However, the strong immune response elicited by these viruses may limit their potential in clinical settings [33]

2.3. Lentiviral vectors (LVs) and integration-deficient lentiviral vectors (IDLVs)

LVs have been successfully used for efficient transduction of the most cell types, including hard to transfect primary differentiated cells (such as neurons, T cells, or macrophages) as well as multipotent (MSCs, HSCs) and pluripotent stem cells (hESCs and iPSCs). Due to the high efficiency of LVs, scientists have used this platform also for the transient delivery of transgenes by mutating the integrase protein and develop IDLVs [34]. The development of IDLVs opened the possibility of using these systems for GE in therapeutic settings. IDLVs have been used to deliver cDNAs expressing ZFNs, TALENs, and RGNs. However, only ZFNs genes have been delivered with high efficiency using these systems [24, 35–37]. In one of the first demonstration of IDLVs efficacy for GE, Lombardo et al. [35] showed that IDLV delivery of ZFNs can achieve high levels of gene addition (over 50%) in several primary human cell lines, including hematopoietic stem cells. IDLVs have also been very successful for GE of T cells [38, 39].

Delivery of RGNs and TALENs by IDLVs has been more challenging due to the larger size of Cas9 and the high recombination rates of TALENs [40]. Some authors have developed LVs with mutated reverse transcriptase to deliver mRNA avoiding recombination. Using this system, the authors showed efficient CCR5 and TCR gene suppression in different cell lines [41].

IDLVs have also been adapted for the delivery of SNs proteins instead of delivery of cDNA, providing efficient targeted gene disruption in several primary cells [42]. By co-packaging ZFNs or TALENs proteins and donor RNA in lentiviral particles, the authors achieved homology-directed DNA insertion and gene correction.

The ability to deliver circular DNAs into the nuclei of target cells, including quiescent cells, make IDLVs a very interesting tool to deliver donor DNAs [35–37]. Compared with AAV and AdV, IDLVs have the advantage of enhanced efficiency in some target cells, such as hematopoietic stem cells (HSCs), a very interesting target population for GT strategies. Using IDLVs to deliver Donor DNA, Genovese et al. [43] managed to restore up to 6% of CD34+ cells from a SCID-X1 patient. The main advantage of using IDLVs for delivery of the Donor DNA is the high efficacy; however, quite often (5–20%) the Donor DNA integrates outside that target locus. These off-target integrations can have undesired side effect and is something that need to be monitored in detailed.

3. Non-viral-based vectors

As we have discussed in the previous section, the different viral vectors have different applications in GE, each one with its own limitation. In this section, we will discuss the best

non-viral gene transfer technologies that have been developed for *ex vivo* and *in vivo* delivery of GE tools, in particular for the delivery of SNs. These systems have often been combined with viral-based methods for the delivery of donor DNAs.

3.1. Nucleofection

Nucleofector™ Technology-Lonza, also named nucleofection, is a electroporation-based system that allows high transfection efficiencies with high cell viability in most cell types including hHSCs, dendritic cells, and iPS. Nucleofection of SNs in the form of DNA, RNA, and proteins has been a successful approach for GE of primary human cells. This technique has been used to achieve therapeutic benefits by NHEJ and by HDR (often combined with viral-based methods for delivery of the donor DNA).

Examples of mRNA nucleofection for NHEJ-based GT can be found in the clinical trial for Duchenne muscular dystrophy patients. The protocol resulted in a deletion of a defective sequence, partially restoring the expression of dystrophin [44]. In a similar strategy, Poirot et al. [45] nucleofected TALEN mRNA into T cells allowing highly efficient gene disruption of alphabeta T-cell receptor (TCR) and CD52 (a protein targeted by alemtuzumab). These cells did not mediate graft versus host reactions and were resistant to alemtuzumab, increasing the safety and efficacy of CAR T cells immunotherapies [45]. The CCR5 T cell receptor (see above) was also targeted efficiently by nucleofection of TALEN mRNAs [46]. Recently, a clinical scale protocol for gene disruption of the PD-1 gene in tumor-infiltrating lymphocytes (TILs) has been developed [47]. In this protocol, nucleofection of T cells with ZFNs mRNA resulted in 80% reduction in PD-1 surface expression in TILs.

The first experiments showing HDR in primary human cells used nucleofection of plasmids to deliver both SNs and Donor DNA [48]. The authors showed specific modification (inclusion of new restriction enzymes) of the IL2Rgamma gene in over 10% of primary human T cells. However, efficiencies in other target cells remained very low for therapeutic applications. Different groups have shown that combining mRNA nucleofection with delivery of Donor DNA by IDLVs [43] or AAV [22] rendered better results due to the ability of the viral vectors to improved efficiency of HDR.

3.2. Liposomes and cationic polymers

Liposomes and cationic polymers (i.e., polyethylenimine-PEI) allow delivery of large DNA fragments due to the interactions between the cationic charge of the particles and the anionic charge of the cell membranes. Cationic liposomes have shown good efficacies for transfection of DNA expressing ZFNs [27, 48, 49], TALEN [50] and Cas9/gRNAs [50, 51].

In a GT application, HPV-targeted TALEN plasmids were used for *in vivo* delivery using TurboFect1 [50], a proprietary cationic polymer (ThermoFisher Scientific). Direct applications of the TurboFect1-Cas9-gRNA complex into the cervix of transgenic mice displaying HPV infection and cervical cancer reduced viral loads and tumor size [50]. Other reports have showed efficient *ex vivo* and *in vivo* delivery of Cas9 protein complexes with gRNA using liposomes [53, 54].

Some groups have combined the delivery of the CRISPR/Cas as a protein but fusion to a cationic liposome reagent. Zuri et al. [54] hypothesized that proteins that are highly anionic could be delivered by the same electrostatics-driven complexation used by the cationic liposomal reagents. They showed that the Cas9 nuclease protein with the polyanionic single guide RNA could be delivered efficiently and functional into mammalian cells using this cationic lipid formulations and at the same time is able to create indels in a efficient way; approximately 10 folds compared with the plasmid transfection [54]

3.3. SNs proteins and cell-penetrating peptides (CPPs)

It was soon observed that ZFNs have an intrinsic cell-penetrating activity due to the positive charge of the zinc finger domains [55]. In fact, direct delivery of ZFNs proteins achieved up to 24% gene disruption of CCR5 in HEK and HDF cells and up to 8% in human T cells [55]. However, unlike ZFNs, TALEN are incapable of penetrating cellular membranes [56] and therefore, cell-penetrating peptides (CPPs) are required to promote cellular uptake. CPPs, also known as protein transduction domains or membrane translocation sequences, are short cationic or amphipathic peptides of 5–40 amino acids that can traverse mammalian plasma membranes [57]. Ru et al. fused TALENs with HIV-1 TAT protein (a CPPs) and showed 3 and 5% of CCR5 gene disruption in HeLa and hiPSC cells, respectively. TALENs have also been conjugated with poly-Arg9 peptides (R9CPP) [58]. The conjugated R9-TALEN proteins were able to knockout CCR5 and BMPR1A genes in HeLa and HEK293 cells.

The delivery of the CRIPR/Cas9 system by its fusion with CPPs has also been used in different cells types, such as fibroblast and pluripotent stem cells. Delivery of the Cas9 and gRNA conjugated with m9R and 9R CPPs, respectively, resulted in mutation frequencies ranging from 2.3 to 16% in several human cells including embryonic stem cells [59].

3.4. Nanoparticles

Nanoparticles are particles between 1 and 100 nanometers in size generated by different strategies such as attrition, pyrolysis, or hydrothermal synthesis. The group of Marie E. Egan developed a new method for surface-modifying PLGA nanoparticles with cell-penetrating peptides [60] and then combined to develop PLGA/PBAE/MPG nanoparticles, achieving modification of 5% of the cells in the nasal epithelium and more than 1% in the lung. Using this system, the authors deliver PNAs and donor DNA molecules to correct the F508del *CFTR* mutation achieving *in vitro* and *in vivo* gene correction an order of magnitude higher than previously achieved [61].

Other strategies combined triplex-forming peptide nucleic acids (PNAs), synthetic oligonucleotide analogs that are resistant to degradation with nanoparticles [62]. PNAs have also the advantage that can induce DNA repair upon sequence-specific triplex formation at targeted genomic sites. The direct delivery of PNAs and Donor DNA by nanoparticles can mediate GE of human cells at frequencies of 0.05% in HSCs [63]. Other authors used biodegradable poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) encapsulating PNAs and donor DNAs to

disrupt CCR5, achieving up to 1% gene disruption in HSCs and conferring HIV-1 resistance to an humanized mice model [64].

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