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Molecular Therapy
Review

Genome Editing with mRNA Encoding ZFN, TALEN, and Cas9

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Genome-editing technologies based on programmable nucleases have significantly broadened our ability to make precise and direct changes in the genomic DNA of various species, including human cells. Delivery of programmable nucleases into the target tissue or cell is one of the pressing challenges in transforming the technology into medicine. *In vitro*-transcribed (IVT) mRNA-mediated delivery of nucleases has several advantages, such as transient expression with efficient *in vivo* and *in vitro* delivery, no genomic integration, a potentially low off-target rate, and high editing efficiency. This review focuses on key barriers related to IVT mRNA delivery, on developed modes of delivery, and on the application and future prospects of mRNA encoding nuclease-mediated genome editing in research and clinical trials.

Principles of Genome Editing

Genome-Editing Nucleases

The ability to efficiently and precisely edit genomic DNA sequences of cells from plants, animals, and humans has been a major focus of basic and biomedical research ever since the discovery of restriction enzymes. Traditionally, gene engineering has been achieved by homologous recombination (HR) through the incorporation of a homologous sequence-flanked transgene.[1] However, the extremely low efficiency of HR in many cell types and organisms limits its broad application.[1] Genome-editing nucleases can be engineered to recognize and cut specific DNA sequences in the genome, resulting in double-strand breaks (DSBs), which are efficiently repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR) in the presence of a donor DNA template (Table 1).[2,3] NHEJ is error prone, often resulting in small insertions or deletions (indels) at the nuclease cleavage site. Depending on the type and position, indels that shift the open reading frame can lead to nonfunctional protein and, thus, generate gene knockout. NHEJ can also be used to partially restore protein function by deleting a frameshift mutation containing exons.[4] In contrast to the error-prone NHEJ pathway, HDR-mediated genome editing enables precise modifications by incorporating the donor DNA with homologous sequences into the target loci.[4]

There are four groups of genome-editing nucleases based on their structures: meganucleases (MNs), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-associated nucleases.[5] MNs, also known as homing endonucleases, are endonucleases characterized by their large recognition site (14–40 bp).[6] The large recognition sites and low cytotoxicity to mammalian cells make MNs attractive tools for genome editing.[7] Existing engineering techniques include the creation of fusion protein from existing MN domains and engineering MN specificity via the direct alteration of protein residues in the DNA-binding domain. One of the most used starting scaffolds for the design of new artificial MNs is I-CreI, a member of the LAGLIDADG family, as the largest of five known families of MNs.[8,9] More recently, >100 MNs with different site specificities have been identified.[6,10–12] However, the number of naturally occurring MNs is still limited, and it is insufficient for addressing all potentially interesting loci.[13] Moreover, the complexity in re-engineering and low editing efficiency also limit the applications of MNs.[6,10–12]

ZFNs were created by fusing zinc-finger DNA-binding domains of zinc-finger proteins with the cleavage domain of FokI endonuclease.[14] The sequence specificity of ZFNs arises from the zinc-finger protein region that contains three to six Cys2-His2 fingers, each of which recognizes a triplet nucleotide code.[15] Two zinc-finger proteins bind opposite strands of DNA in the near space, allowing the fused FokI endonuclease to form a functional dimer that cleaves DNA in the target loci.[16] Similar to ZFNs, TALENs are engineered by fusing a DNA-binding domain derived from transcription activator-like effectors (TALEs) and a catalytic domain of FokI endonucleases.[17] The TALEs, originally identified from *xanthomonas* proteobacteria, encoded the DNA-binding domain that consists of monomers, each of which binds 1 nt in the target nucleotide sequence. Each monomer is conserved tandem repeats of 34 amino acid residues. The two residues located at positions 12 and 13 are highly variable and responsible for the recognition of a specific nucleotide.[18] Because the DNA-binding specificity of TALEs is easier to engineer than zinc-finger proteins, TALEs can be applied more widely to life sciences than ZFNs.[19,20]

CRISPR-Cas systems are essentially RNA-guided nucleases. Unlike the aforementioned nucleases that recognize the target sequence

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Table 1. Programmable Nucleases in Genome Editing

	Meganuclease	ZFN	TALEN	Cas9
Target sequence (bp)	14–40	9–18	14–20	~23
Number of target sites	limited	many	many	many
Enzyme engineering	difficult	difficult	easy	very easy
Size (kb)	~1	~1	~3	~3.5–4.5
Target recognition	protein-DNA	protein-DNA	protein-DNA	RNA-DNA base pairing and protein-DNA

through protein-DNA interaction, CRISPR-Cas nucleases target sequences through RNA and DNA base pairing[5] (Figure 1; Table 1). In addition to a target-specific CRISPR RNA (crRNA), a protospacer-adjacent motif (PAM) sequence is necessary for CRISPR-Cas systems to recognize target sequences.[21] In an updated classification system, CRISPR-Cas systems are divided into two classes and five types.[22] The class 1 system is defined by the presence of a multicomponent crRNA-effector complex, while the class 2 system is defined by the presence of a single-component crRNA-effector.[22] For example, the most widely used *Streptococcus pyogenes* CRISPR-Cas9 (SpCas9) system is a class 2 nuclease.[23] The SpCas9 system requires a simple PAM sequence (NGG) as well as a target-specific crRNA and a *trans*-activating CRISPR RNA (tracrRNA), which could be fused into a single-guide RNA (sgRNA) for efficient genome editing.[24] Recently, SpCas9 variants that can recognize a broad PAM sequence, including NG, GAA, and GAT, have been developed through protein evolution and structure-guided design.[25,26] CRISPR-Cpf1 (CRISPR from *prevotella* and *francisella* 1) is another widely used class 2 CRISPR system.[27] Different from Cas9 nucleases that require a PAM sequence downstream of the target sequence, dual RNAs (tracrRNA and crRNA), and a blunt-end DNA-cleavage site, Cpf1 nucleases require a PAM sequence upstream of the target sequence, crRNA only, and a staggered DNA-cleavage site.[27]

By changing the guide sequences of crRNA or sgRNA, it is very easy to target a new genomic sequence, making the CRISPR-Cas system a powerful genome-editing tool.[28] Because of the simplicity and high efficiency of the CRISPR-Cas9 system, Cas9 nuclease engineering has expanded to further applications. For example, Cas9 nickase, generated by inactivating one of the catalytic domains, nicks one strand of the double-stranded DNA, and it introduces double nicking when combined with paired guide RNAs. Paired nicking can improve genome-editing specificity of Cas9, and it is considered an alternative for genome-editing applications requiring high specificity.[29]

Catalytically inactive Cas9 (dead Cas9 [dCas9]), which loses its cutting activity due to the inactivation of both catalytic domains, can be engineered to form dimeric dCas9-Fok1 fusion protein with greater specificity than wide-type Cas9.[30,31] Fusion of dCas9 or Cas9 nickase to cytidine deaminase (also known as base editor [BE]) or adenosine deaminase (also known as adenine BE [ABE]) effectively mediates base conversion (C to T and G to A or A to G and T to C, respectively), without introducing DSBs.[32–34] To date, various apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC)3A BEs with better efficiency and specificity than APOBEC1 have been developed. An A3A-PBE BE, composed of Cas9 nickase and human APOBEC3A, converts C to T in rice, wheat, and potato with a 17-nt editing window independent of sequence context.[35] eA3A-BE3, which uses an engineered human APOBEC3A domain to preferentially deaminate cytidines in specific motifs, converts C to T with higher precision and reduced off-target mutation than BE3.[36] Moreover, human A3A-BE3 that can induce efficient C-to-T conversion in methylated regions and GpC context has been developed.[37] Recently, a Cpf1-based BE was developed as an alternative to a Cas9-based BE, expanding the application of BEs from recognizing G/C-rich PAM sequences to A/T-rich PAM sequences.[38] dCas9 has also been engineered to regulate transcriptional processes by fusing with transcription activators, such as VP64, VP48, and p65, to activate gene expression or with transcription repressor KRAB to suppress expression.[39–43] Moreover, dCas9 fused with acetyltransferase can be used to study epigenetic modifications at sites of interest.[44] In addition to Cas9 engineering, sgRNA can also be modified to enable transcriptional regulation by including a hairpin aptamer MS2 in the tetraloop and stem loop to recruit the corresponding regulators, such as MS2-VP64.[41]

Beyond editing genomic DNA, extensive research has been carried out to investigate RNA-editing platforms. RNA manipulation is usually reversible in time and is considered safer than DNA for therapeutic applications.[45] A CRISPR-Cas effector complex that consists of prokaryotic silencing (psi)RNAs from the CRISPR loci and Cas module repeat-associated mysterious proteins (Cmrs), namely, psiRNA-Cmr protein complexes, silenced invader RNAs by cleaving the target RNAs in a homology-dependent manner.[46] When the PAM sequence is presented separately *in trans* as PAM-presenting oligonucleotides (PAMmers), the CRISPR-Cas system could bind and cleave single-stranded RNA (ssRNA) targets.[47] Cas13a (also known as C2c2) is predicted to possess RNase activity and use a crRNA as a guide to cleave ssRNA carrying complementary protospacers.[48,49] Cas13a protein identified from *Leptotrichia wadei* (*Lwa*Cas13a) has been engineered for RNA targeting in mammalian and plant cells.[50] Recently, the type VI-D CRISPR-Cas system, which consists of a Cas13d effector and a WYL domain accessory, has been identified as an RNA-guided, RNA-targeting platform with minimal sequence and secondary structure requirements for targeting.[51,52] Notably, base editing of the RNA sequence is also feasible through fusing dCas13b to adenosine deaminases acting on RNA (ADAR).[53–56] Engineered RNA BEs can be used to convert adenosine to inosine.[53–56]

Modes of Nuclease Delivery

Nuclease delivery to the right tissues and cells at the right time is the key to successfully transforming the genome-editing technology

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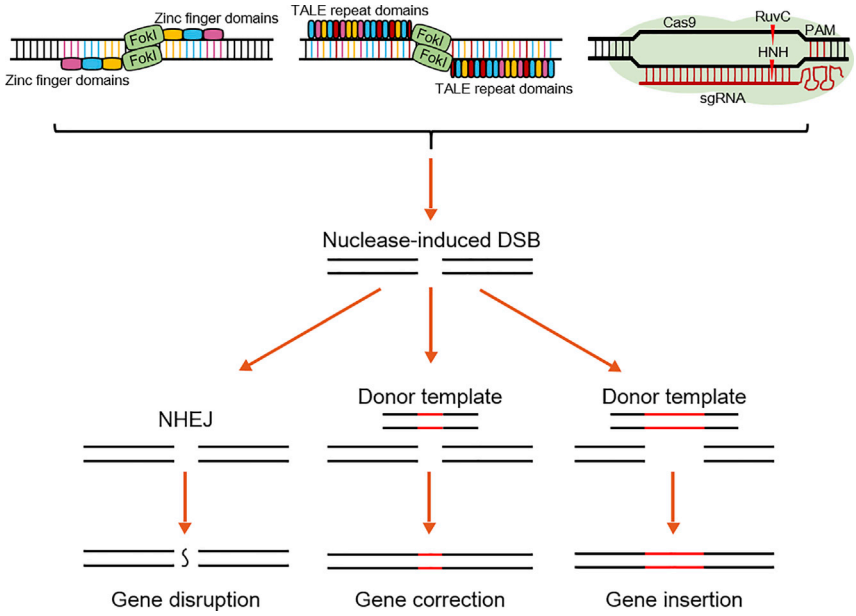

Figure 1. Genome Editing Mediated by Site-Specific Nucleases

The zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), or CRISPR-Cas genome-editing nucleases are able to quickly search the genome, specifically bind various DNA sequences of interest, and efficiently induce double-strand breaks (DSBs). The DSBs are then effectively repaired by non-homologous end joining (NHEJ), or potentially homology-directed repair (HDR) if provided a DNA donor template, resulting in gene disruption, gene correction, or gene insertion, respectively.

into medicine.[45] Given that nucleases can potentially be mutagenic and immunogenic, the ideal delivery system would permit transient nuclease activity. Currently, genome-editing nucleases can be delivered in the forms of DNA, mRNA, and protein, each of which has advantages and disadvantages (Table 2). Protein delivery of nucleases is the most straightforward, as it provides the best control over nuclease dosage without any signal amplification.[57] Protein delivery obviates the transcriptional and translational processes, such as promoter selection and codon optimization. It offers the most transient genome-editing duration with reduced off-target effects, and, thus, it is perfect for *ex vivo* cell therapy.[58] Cas9 ribonucleoprotein (RNP) delivery introduces genome editing almost immediately (approximately 3 h) after delivery and is degraded rapidly (approximately 24 h after delivery), while plasmid delivery takes about more than 8 h to start genome editing and has a duration of about several days.[58] However, protein delivery of nucleases has several limitations for *in vivo* therapy.

First, direct protein delivery of Cas9 might trigger a cellular and humoral immune response.[59] The nuclease protein might be proteasomally processed into peptides that are then presented by a major histocompatibility complex (MHC) on the cell surface to engage T cells when delivered through intravenous injection. A Cas9-specific T cell receptor has been identified in mice and humans.[60,61] The Cas9 protein exposure evokes Cas9-specific antibody production in animal models.[60,62] Since CRISPR-Cas is derived from bacteria, it is not surprising that 79% and 65% of the human population have preexisting antibodies against *Staphylococcus aureus* Cas9 (SaCas9) and spCas9, respectively.[63] Encapsulation of the Cas9 protein is one way to minimize protein degradation and immune response.[64] Delivery of Cas9 protein-sgRNA using lipoplexes achieved up to 80% and 20% of gene modification in cultured hair cells and mouse inner ear cells *in vivo* via local delivery, respectively.[64] Second, efficient systemic delivery of Cas9 protein is a challenge due to its charge and large size.[65] Different from the delivery of Cas9 in the mRNA or DNA format, delivery in the protein format is without signal amplification, requiring a sufficient amount of Cas9 protein delivered into cells. Third, the complicated protein purification process for large nucleases and the potential contamination of bacteria endotoxin during manufacture make protein delivery challenging.[65,66]

DNA delivery of programmable nucleases is a cost-effective method and is commonly used for basic research. Plasmid and virus are the most popular vectors to deliver nuclease, and they usually render more sustained expression than protein delivery.[67] However, there are several drawbacks of DNA delivery that limit its applications in therapeutics, the generation of animal models, and otherwise. First, the persistent nuclease expression, such as Cas9, may result in high off-target effects and genome instability.[66] Although various strategies have been developed to reduce off-target mutagenesis of Cas9, including the combination of a Cas9 nickase with paired guide RNA (gRNA),[29] truncation of guide RNA,[68] fusion of dCas9 with FokI nuclease,[30,31] fusion of Cas9 with a programmable DNA-binding domain,[69] structure-guided engineering of Cas9 nuclease,[70–72] and chemical modification of guide sequences,[73–75] transient expression of Cas9 is a key step to minimize off-target effects.[76] Second, the requirement of nuclear entry for DNA transcription may affect the delivery efficiency of the nuclease.[65,66] Third, non-viral delivery of DNA is associated with cellular toxicity *in vitro* and *in vivo*,[77–79] and viral DNA delivery could trigger immune responses and increase risks of random insertional mutagenesis by the viral vector itself.[80]

In vitro-transcribed (IVT) mRNA is another transient delivery system for nucleases. IVT mRNA minimizes the risk of genome insertion, and it bypasses the requirement of nuclear entry for transcription, resulting in efficient genome editing.[81] The short but robust expression of mRNA results in high activity and potentially low off-target effects.[82] In the case of genome editing, half-lives of both the nuclease mRNA and its protein products are critical determinants for editing efficiency.[83] As a result, the short half-life of mRNA may be a

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Table 2. Modes of Nuclease Delivery

	DNA	mRNA	Protein
Transcription in nuclear	yes	no	no
Translation in cytoplasm	yes	yes	no
End products function in nuclear	yes	yes	yes
Starting time after transfection (h)	>8	4–6	3
Stability	great	good (modified mRNA)	poor
Duration	1 week	several days	~24–48 h
Integration risk	yes	no	no
Off-target	high	low	low

limitation for its use in some cases.[83] To overcome this drawback, various modifications have been introduced into the structural elements of mRNA. The incorporation of anti-reverse m^7GpppG cap analog,[84] poly(A) tail, modified nucleotides,[85] 5′ and 3′ UTRs containing regulatory elements,[86] and synonymously frequent codons into mRNA[87] have exhibited enhanced stability and translational efficiency in IVT mRNA. As an alternative to linear mRNA, exogenous synthesized circular RNA (circRNA) has been developed for robust and stable protein expression.[88] In addition, protein expression kinetics also have contributed to the editing efficiency of mRNA encoding nucleases. When delivered, protein expression of mRNA starts almost transiently, peaks within several hours (~6–24 h), and persists for a few days.[89,90] The expression duration is long enough for genome editing. Like DNA, the exposure of mRNA in the cytoplasm may induce an immune response, which remains a key barrier for mRNA-based genome editing.[91,92] It has been shown that nucleotide modifications,[89,93] sequential engineering,[94] and high-performance liquid chromatography (HPLC)-purified mRNA resulted in lower immunogenicity and higher stability than unmodified nucleotides.[93,95] Therefore, mRNA delivery of nucleases holds great potential for therapeutic application.

Barriers for mRNA Delivery

Effective delivery of the mRNA encoding genome-editing nucleases and their corresponding partners (e.g., sgRNA for CRISPR and donor template DNA for targeted insertion) is the key and rate-limiting step for successful genome editing. The delivery of mRNA is usually mediated through non-viral synthetic materials and physical methods.[65] An ideal mRNA delivery system needs to overcome various delivery barriers to transport mRNA encoding nuclease to its destination (Figure 2). For IVT mRNA delivery *ex vivo* and *in vitro*, the delivery hurdles include the following: (1) cytotoxicity caused by manipulation and delivery vectors;[78,96,97] (2) cellular uptake;[98,99] (3) endosomal escape; (4) mRNA degradation triggered by lysosome and cytoplasmic nucleases;[99,100] (5) immune response mediated through the pattern-recognition receptors of the innate immune system, such as Toll-like receptors and retinoic acid-inducible gene I (RIG-I)-like receptors;[91] (6) low transfection efficiency; and (7) limited manufacture capacity of delivery formulations and low throughput of the delivery process.[65,66] Ideal non-viral synthetic materials should meet the following requirements: formation of stabilized complexes with the mRNA in order to protect them from degradation, efficient cellular uptake, endosomal escape, tissue specificity, and release of mRNA in the cytoplasm of the target cells.[101,102] For physical methods, including electroporation and microfluidic device-based transfection, effectiveness, harmlessness, and high throughput are desired.[103]

For *in vivo* delivery, mRNA encoding nuclease needs to travel toward target tissues and organs and then enter target cells. Additional hurdles exist both for systemic and local delivery *in vivo*: (1) rapid degradation of mRNA by nucleases in serum, tissue fluid, and mucus;[99,100] (2) nonspecific interactions with proteins and non-targeting cells, e.g., aggregation with red blood cells, taken up by macrophages and monocytes, or sequestered by serum proteins;[104] (3) extravasation from blood vessels to reach target tissues; and (4) clearance by the hepatic or renal system.[65]

Methods for mRNA Delivery

In general, approaches for mRNA delivery can be divided into two categories, physical methods and non-viral synthetic materials (Table 3).

Physical and Combinational Methods

Physical delivery methods, such as mechanical deformation (squeeze), electroporation, hydrodynamic injection, and microinjection, also known as carrier-free delivery, rely on transiently changing the plasticity of the plasma membranes to allow entry into the cell.[45,65,103]

For *ex vivo* cell therapies, electroporation or mechanical deformation is commonly used to deliver nucleic acids or proteins into the cells.[105–107] To introduce targeted integration of a GFP cassette into the adeno-associated virus integration site 1 (*AAVS1*) locus or a mutational hotspot of interleukin-2 receptor subunit gamma (*IL2RG*), CD34+ cells were transfected with mRNA encoding ZFNs and infected with integrase-defective lentivirus (IDLV) for donor DNA coding GFP.[108] An average of 5% GFP-positive cells were yielded, and most of the GFP-positive cells were with on-target insertion of GFP transgene.[108] By electroplating mRNA encoding ZFNs together with adeno-associated virus (AAV)6-mediated delivery of donor template DNA, approximately 17% and 26% targeted integration at the C-C chemokine receptor type-5 (*CCR5*) and *AAVS1* locus in human hematopoietic stem and progenitor cells (HSPCs) was achieved, respectively.[105] Another study using the optimized design of ZFN mRNA and donor constructs (gp91phox transgene) achieved rates of approximately 15% targeted integration at the *AAVS1* safe harbor locus in HSPCs from patients with X-linked chronic granulomatous disease (X-CGD).[106] Similarly, electroporation of TALEN mRNA targeting *CCR5* showed more than 50% on-target gene editing (disruption of *CCR5* gene) in primary T cells.[107] Electroporation of Cas9 mRNA or protein together with chemically modified sgRNAs enhanced genome-editing efficiency in human HSPCs and primary T cells compared to unmodified sgRNAs.[74] In addition, co-delivery of mRNA encoding megaTAL, a hybrid nuclease that consists of a

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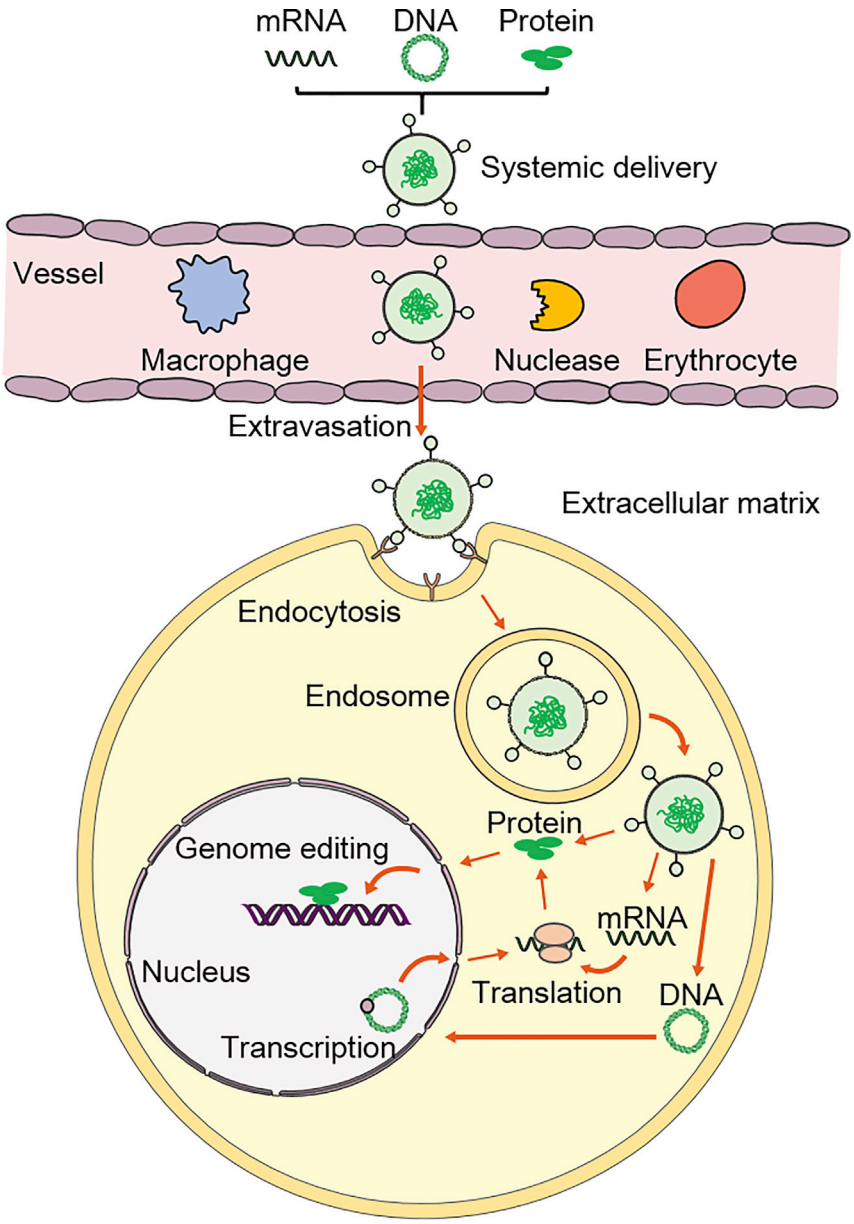

Figure 2. Barriers for Non-viral Delivery
Systemic delivery of genome-editing nucleases using non-viral vectors has to overcome various barriers.

TALE DNA-binding domain and an engineered homing endonuclease (HE), through electroporation with a donor template by AAV6 achieved 8%–60% rates of HDR at the *CCR5* locus in T cells.[109] An optimized mRNA electroporation protocol to handle a large amount of cells (>100 million) for clinical-scale manufacture was developed using the MaxCyte GT system.[110] The big downside of electroporation is the toxicity and cell death during the process. For example, 30%–50% T cells may die after electroporation.[111]

In addition to electroporation, the microfluidic-based membrane deformation method has been shown to deliver structurally diverse biomolecules, such as plasmid, protein, and small interfering RNA, into various types of cells, including various types of difficult-to-transfect primary cells.[112–114] A microfluidic platform for intracellular delivery named "squeeze" technology relies on the formation of transient membrane holes to facilitate the delivery of biomolecules into the target cells.[113] Considering its ability to deliver diverse loads into various types of cells with relatively low cellular toxicity, development of a suitable squeeze protocol for genome editing is valuable.[115]

Hydrodynamic injection causes transient deformation of the cell membrane to facilitate entry of nucleic acids into hepatocytes *in vivo*.[116] Hydrodynamic injection of a plasmid DNA expressing CRISPR-Cas9 and a single-stranded DNA donor resulted in the correction of a point mutation of fumarylacetoacetate hydrolase (*Fah*) in hepatocytes in a mouse model of hereditary tyrosinemia.[117] Microinjection of genome-editing nucleases into embryo or zygote has been widely used to obtain genetically modified animals.[118–120] Such methods allow one-step generation of animals carrying multiple mutations, making it straightforward to study functionally redundant genes and epistatic gene interactions *in vivo*.[121] Co-injection of Cas9 mRNA, sgRNAs, and DNA donors into zygotes created reporter and genetically modified mouse models.[122] More details are discussed in the next section.

Non-viral Synthetic Material-Mediated Delivery

A large number of nanomaterials, either newly developed or reformulated from plasmid DNA and small interfering RNA (siRNA) delivery systems, have been investigated or optimized for mRNA delivery. According to the nanomaterials used, nanoparticle-mediated delivery could be classified into lipid- and polymer-based vectors.[123]

Lipid-Based Delivery

Lipid materials have been extensively explored for small RNA (e.g., siRNA and microRNA) and plasmid delivery.[124,125] Similar delivery materials are transferred for mRNA delivery using optimized formulations.[126] DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) was the first cationic lipid used for IVT mRNA delivery into different cell lines.[127] DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane) is a derivative of DOTMA with

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Table 3. Non-viral Delivery of mRNA Encoding Nucleases

	Application	Efficiency	Toxicity	Other Characteristics
Microinjection	*ex vivo*	high	low	not applicable for large number of cells
Electroporation	*in vitro*, *ex vivo*	high	medium to high	not applicable for *in vivo* delivery
Squeeze	*in vitro*, *ex vivo*	high	medium	not applicable for *in vivo* delivery
Cationic lipids	*in vitro*, *in vivo*	high	medium to low	can be used in combination with systemic or local administration
Cationic polymers	*in vitro*, *in vivo*	high	medium to low	can be used in combination with systemic or local administration

higher delivery efficiency and lower synthetic cost.[128] Modification of DOTAP with carbonate apatite, inorganic additives, or fibronectin improves the cellular uptake of exogenous mRNA.[102] Commercially available lipids, such as lipofectamine, are widely used for IVT mRNA transfection *in vitro*.[129] Owing to electrostatic interaction, cationic lipids can spontaneously form lipoplexes with mRNA.[129]

Cationic or zwitterionic lipids are mixed with helper lipids, cholesterol, and polyethylene glycol (PEG) to form multicomponent lipid nanoparticles (LNPs).[130] 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), a common helper lipid, has been shown to improve intracellular delivery of LNPs by facilitating fusion and destabilization of endosomal membranes to enhance endosome escape.[130] PEG, the shield of the cationic liposomes, functions to reduce nonspecific interaction and extend blood circulation time.[131] Systemic delivery of LNP-encapsulated Cas9 mRNA in combination with AAV-mediated sgRNA and a repair template delivery resulted in correction of the *Fah*-point mutation in >6% of hepatocytes in adult animals.[126]

Zwitterionic amino lipids (ZALs) are lipid-like materials uniquely suited for the delivery of long RNAs.[132] Intravenous co-delivery of Cas9 mRNA and sgLoxP induced the expression of floxed tdTomato in the liver, kidneys, and lungs of engineered mice.[132] In addition, chemically modified and sequence-optimized sgRNAs have higher durability, lower toxicity, reduced off-target activity, and enhanced genome-editing activity.[73–75,133] By using chemically modified sgRNAs, LNP-mediated editing efficiency has been greatly improved. A single intravenous injection of LNP-encapsulated, chemically modified sgRNA and mRNA encoding Cas9 induced >80% genome editing of Proprotein convertase subtilisin/kexin type 9 (*Pcsk9*) in mouse liver.[75] *In vivo* activity of sgRNA was greatly improved through chemical modification independent of the target guide sequence.[133] Similarly, electroporation of unmodified sgRNA and Cas9 mRNA results in modest genome-editing efficiency, while chemically modified sgRNAs exhibited enhanced genome-editing efficiency when co-delivered with Cas9 mRNA or protein into human primary T cells and HSPCs.[64]

Cationic Polymer-Based Delivery

Cationic polymers are widely used for *in vivo* transfection of mRNA as an alternative to lipids.[134] There are a large number of naturally derived as well as synthetic polymers that render great structure diversity and modification.[135] Similar to lipids, cationic polymers can spontaneously form polyplexes with mRNA through electrostatic interaction.[136] Polyethylenimine (PEI) is one of the most studied cationic polymers with high transfection efficiency.[137,138] PEI can condense large nucleic acid molecules to form homogeneous spherical particles to facilitate cellular uptake.[139] One characteristic of PEI is that its protonation and charge density increase at low pH, which protects the nucleic acids from nuclease degradation and promotes endosomal escape at acidifying endosomes.[140,141] The most widely used PEI polymers are 22-kDa linear PEI (L-PEI) and 25-kDa branched PEI (B-PEI), both of which have moderate cytotoxicity.[142] Recently, an optimized PEI and its derivatives have been used in mRNA delivery.[143] Cyclodextrin-PEI has been synthesized for the intranasal delivery of mRNA vaccine.[143] Cyclodextrin-based nanoparticles partially surpass the nasal epithelial barrier with little damage to mucosal integrity.[143] 7C1, a low-molecular-weight PEI, is shown to effectively deliver siRNA into endothelial cells.[144,145] It will be interesting to test 7C1 for mRNA delivery.

Poly (D,L-lactide-co-glycolide) (PLGA) is another widely used polymeric vector for drug delivery *in vivo*.[146] Different from cationic polymers, the major limitation for PLGA-mediated mRNA delivery is the negative charge identical to nucleic acids, making packaging difficult.[146] Chitosan, a cationic polymer derived from cationic exoskeleton of crustaceans and cell walls of fungi, has been used to modify PLGA.[146,147] Chitosan-coated PLGA nanoparticle-encapsulated antisense oligonucleotides, DNA, or siRNA enable efficient *in vivo* delivery to various tissues in animal models.[146,147] Intratracheal delivery of chitosan-coated PLGA-encapsulated ZFN mRNA and AAV-mediated HDR templates achieved gene correction and an associated life-prolonging phenotype in a mouse model of surfactant protein B (SP-B) deficiency.[148] Recently, PLGA coated with a lipid-PEG shell was used with a cationic lipid-like compound G0-C14 to assemble stable and low toxic nanoparticles containing mRNA coding phosphatase and tensin homolog (PTEN), a tumor suppressor.[149] Systemic delivery of the *PTEN* mRNA-nanoparticles *in vivo* restored the expression of PTEN protein in prostate cancer cells and suppressed the tumor.[149]

Applications of IVT mRNA Encoding Genome-Editing Nucleases

For Research

IVT mRNA encoding genome-editing nucleases has been widely used in research and preclinical studies. Electroporation of ZFN, TALEN, and Cas9 mRNA has been used *ex vivo* to edit human

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T cells, HSPCs, and other cells (discussed above).[105,106,150] In addition to cell engineering, microinjection of mRNA encoding ZFN, TALEN, and CRISPR into one-cell-stage embryos or zygotes has been successful in various animal models, including zebrafish, mouse, and pig.[118–120,151] Injecting ZFN mRNA into one-cell-stage embryo results in robust gene knockout and enables the quick generation of genetically modified animal models.[118] In addition to site-specific nucleases, co-injection of transposase mRNA with a Tol2-transposon vector into the cytoplasm of fertilized cells efficiently generated transgenic mice, although such method introduced semi-random insertion.[152]

Similar to TALEN, microinjection of Cas9 mRNA and sgRNA into one-cell-stage embryos of zebrafish efficiently created mutants.[120] Recently, using CRISPR-Cas9 to generate genetically modified rodents became a standard service provided by transgenic core facilities in universities and commercial companies.[153,154] Microinjection of Cas9 mRNA and sgRNA with an optional DNA donor allows the one-step generation of mice or rats bearing multiple mutations[121,155], insertions, or conditional mutations[122] and gene knockouts.[156] For a proof of concept of potential therapeutic applications, such method can be applied to correct genetic mutations in genes in mouse zygotes as well as spermatogonial stem cells, leading to bear healthy animals.[157,158] Pigs bearing biallelic knockout have been generated in one step by an injection of Cas9 mRNA and sgRNA into zygotes.[159] Such a platform has also been applied to modify pigs and nonhuman primates.[159,160] For example, cynomolgus monkeys bearing two target gene (*Ppar-g* and *Rag1*) disruptions have been generated by injecting Cas9 mRNA and sgRNAs into one-cell-stage embryos.[160]

Genetically modified animals can be produced by mRNA coding a Cas9 BE.[161,162] Microinjection of ABE mRNA and a sgRNA into mouse embryos introduced a point mutation in the Tyrosinase (*Tyr*) gene and resulted in *Tyr*-mutant mice with an albino phenotype.[161] Microinjection of mRNA encoding BEs and sgRNAs targeting the Duchenne muscular dystrophy (*Dmd*) or *Tyr* genes into mouse zygotes generated mice with the desired point mutations.[162]

In Clinical Trials

Several clinical trials using mRNA encoding programmable nucleases are in progress. For HIV treatment, the deletion of *CCR5*, the major co-receptor required for HIV-1 infection, renders cells resistant to HIV-1 infection.[163] Allogeneic stem cell transplantation with donor HSPCs bearing naturally occurring homozygous deletion of *CCR5* has shown potential as a cure.[163] Thus, therapeutic genome editing on the *CCR5* genomic locus has been developed as an alternative for lifelong antiviral drug treatment of HIV-1 infections. Sangamo Therapeutics developed a project to utilize SB-728mR, mRNA of specialized ZFNs targeting the *CCR5* gene in human cells, for HIV treatment (SB-728mR-HSPC; ClinicalTrials.gov: NCT02500849).[164] To disrupt the *CCR5* gene expression, SB-728mR was delivered into HSPCs via electroporation.[164] Another trial (SB-728mR-T; ClinicalTrials.gov: NCT03617198) is to use SB-728mR to modify T cells, and the phase I trial has shown safe treatment.[165]

Intellia Therapeutics focuses on using LNPs to deliver CRISPR-Cas9 to the liver, aiming for treating transthyretin amyloidosis (ATTR amyloidosis), alpha-1 antitrypsin deficiency transthyretin amyloidosis (AATD), and primary hyperoxaluria Type 1 (PH1).[166] siRNA-based drugs for those diseases are also in the pipeline of Alnylam Pharmaceuticals.[167] An LNP-encapsulated siRNA drug named patisiran has been approved by the FDA for treating ATTR amyloidosis with polyneuropathy.[168] It is worth noting that, although CRISPR is likely used to target the same protein as RNAi therapy, CRISPR-based therapy is not a "me too" drug. First, the mechanism of action of CRISPR is very different from siRNA. Second, CRISPR holds the potential to finish the treating process via a one-time injection under certain conditions, such as editing in stem cells or progenitor cells, but not cell types with fast turnover features, while siRNA requires a lifetime of repeated dosing that increases the chance of drug-related adverse events. Third, CRISPR holds the potential to diminish the target while siRNA can only partially suppress target expression. Intellia Therapeutics reported up to 70% genome editing in the liver of mice using LNP-encapsulating chemically modified sgRNA and Cas9 mRNA.[133] More than a 97% decrease of serum transthyretin (TTR) levels was observed, and a decrease in serum TTR levels was persistent for more than 1 year in mice.[133]

Conclusions

After years of development, genome-editing therapeutics has become a clinical reality. The delivery technologies, in particular mRNA delivery, have significantly advanced, thanks to the discovery of new biomaterials and improved chemical modifications of mRNA. Currently, several clinical trials using ZFN mRNA targeting HIV are ongoing. Importantly, the breakthrough discovery of CRISPR-Cas genome-editing nucleases and their robust and high efficiency in precisely editing genome DNA has transformed many areas of research in biomedicine, and it has spurred RNA-based delivery to facilitate clinical translation. Three leading CRISPR biotech companies, namely, CRISPR Therapeutics, Intellia Therapeutics, and Editas Medicine, have programs already in clinical or preclinical stages and will likely follow their clinical route. The results of the upcoming clinical trials will help shape the knowledge of RNA delivery-based genome editing. Undoubtedly, the combination of IVT mRNA and genome editing, as two powerful platform technologies, is currently undergoing a significant expansion and will likely play essential roles in the biotech industry.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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