Drug Discovery: Recent Progress and the Future

Review

Advances in CRISPR/Cas9 Technology for in Vivo Translation

Yağız Anıl Çiçek, David C. Luther, Jessica A. Kretzmann, and Vincent M. Rotello

Department of Chemistry, Middle East Technical University (METU); Ankara 06800, Turkey:
Department of Chemistry, University of Massachusetts; 710 North Pleasant St., Amherst, MA 01003, U.S.A.: and School of Molecular Sciences, The University of Western Australia; 35 Stirling Hwy., Crawley, WA 6009, Australia.

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The CRISPR/Cas9 system has been extensively used for the targeted manipulation of genes in the mammalian genome and has been applied as a therapeutic for genetic diseases such as sickle cell disease, muscular dystrophy, and various cancers.15–21) Although CRISPR/Cas9 has already begun to revolutionize genome editing technology, there are still barriers to overcome prior to the clinical translation of this system. These obstacles largely arise from issues associated with the effective in vivo delivery of the CRISPR/Cas9 machinery. This topical review will discuss recent and landmark advances to in vivo delivery methods used for the CRISPR/Cas9 system, as well as current barriers to widespread translation of this technology.

2. CURRENT STRATEGIES IN CRISPR/CAS9 IN VIVO DELIVERY

Therapeutic translatability of the CRISPR/Cas9 system requires that the various components of the system be delivered intracellularly, and with the desired localization in vivo. The CRISPR/Cas9 machinery can be introduced in multiple forms,
namely as Cas9-encoded plasmid DNA (pDNA) or mRNA delivery, or direct delivery of the Cas9 protein. These different delivery formats each take a unique approach for introduction of the CRISPR machinery into the cell, ultimately allowing for editing of the genomic DNA by the Cas9 RNP, as shown in Fig. 1.

The different therapeutics approaches for CRISPR/Cas9 each have advantages and disadvantages associated, as summarized in Table 1. For example, a major limitation to both pDNA and protein delivery is the relatively large size of each, which makes it difficult to encapsulate them within a virus, or form a protective complex with a non-viral carrier. In contrast, mRNA is small, and can be packaged and protected efficiently for delivery. Additionally, both mRNA and protein allow for faster gene editing activity (fewer intracellular checkpoints, illustrated in Fig. 1), and more direct control over the dosage delivered to each cell, while pDNA expression (and therefore final dosage) depends on the design of the plasmid (promoters and enhancers present) as well as the cell type and activity. However this flexibility in design can also serve as an advantage to using plasmids, as plasmids can be designed with selective promoters and enhancers to be used as targeting mechanisms for tissue- or cell-specific editing, preventing editing in off-target tissue. Another major advantage to pDNA is its relatively high stability, compared to mRNA in particular. However, pDNA delivery of CRISPR/Cas9 is associated with more off-target editing, which may lead to potentially harmful unintended mutations to the host genome. These factors are important to consider when deciding on a therapeutic strategy for delivery of the CRISPR/Cas9 machinery, but can also be partially mitigated depending on the type of delivery vehicle chosen.

There are several prominent delivery methods that we will discuss for in vivo delivery of the CRISPR machinery. These strategies can be broadly considered as either viral, physical, or chemical, as demonstrated in Fig. 2.

### 3. VIRAL DELIVERY METHODS

Viruses have naturally evolved to deliver their own genetic cargo to infect their hosts, and so make an ideal choice for the delivery of CRISPR/Cas9-encoded genetic material, as illustrated in Fig. 2. Viruses can be utilized to deliver CRISPR/Cas9 in the form of pDNA or RNA, which is either incorporated into the host genome or expressed extrachromosomally depending on the viral method used.

Viral delivery is currently the most effective method for delivery in vivo. However, this approach has challenges related to toxicity, initial and adaptive immune responses, and limited packaging capacity. Notably, viral methods also entail significant expense, which inherently limits their applicability to widespread treatments and therapeutics for those with low socioeconomic status, who often suffer disproportionately high rates of diseases such as human immune deficiency virus infection (HIV) and sickle cell anemia. Traditionally, attempts at viral delivery have involved the use of lentiviruses, which result in patients suffering from unwanted insertional mutagenesis due to the incorporation of viral DNA into the host genome at a position which could collateralely induce oncogenesis. More recently, complications arising from lentiviral delivery, have shifted the focus of these studies to the use of adenoviral (AV) and adeno-associated viral (AAV) delivery.

AV and AAV strategies avoid some of the complications of lentiviral delivery and have shown notable therapeutic efficacy. AVs can be used to transduce both dividing and non-dividing cells, and because they express extrachromosomally are considered safer than lentiviruses, and have been used to encapsulate and deliver the CRISPR/Cas9 machinery. A study done by Xu et al. used intramuscular injection to administer AVs to excise a 23-kb genomic region by NHEJ using co-delivery of 2 sgRNAs, removing a mutant exon linked to Duchenne muscular dystrophy (DMD) in a murine model. Dystrophin expression in the transduced muscles was restored to approximately 50% of that in wt (wild type) muscles.

AAVs are also among the most widely used viral vectors due to their broad tropism and relatively low immunogenicity. Due to their limited packaging capacity of ca. 4.7kb however, AAVs can only package the CRISPR/Cas9 machinery and a single sgRNA, and so for gene corrections and insertions AAVs require dual-delivery or modified methods to encapsulate Cas9-encoded DNA, sgRNA and template DNA. A study by Kemaladewi et al. administered AAVs loaded with pDNA via intramuscular injection in a congeni-

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**Table 1. A Generalized Overview of Select Benefits to Each Method of Non-viral Delivery**

<table>
<thead>
<tr>
<th>Method</th>
<th>mRNA</th>
<th>pDNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>4500 nt</td>
<td>9.2 kb*</td>
<td>160 kDa</td>
</tr>
<tr>
<td>Control over dosage</td>
<td>Moderate</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Avoids integration</td>
<td>Yes</td>
<td>Potential to integrate</td>
<td>Yes</td>
</tr>
<tr>
<td>Relative stability</td>
<td>Low</td>
<td>High</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

*Cas9 pDNA size is representative of full vector, spCas9 itself is 4.5 kilobase (kb) in size.
tal muscular dystrophy type 1A (MDC1A) mouse model to achieve NHEJ to correct a pathogenic splice-site mutation that causes the exclusion of exon 2 from laminin-2 subunit alpha gene (Lama2) (mutations in this gene results in MDC1A) mRNA and the disruption of Lama2 protein. The authors report the inclusion of exon 2 in the Lama2 transcript and restoration of full-length Lama2 protein. Treated mice displayed substantial improvement in muscle histopathology and function without signs of paralysis. In recent years, further studies using CRISPR/Cas9-encoded AAVs for the treatment of DMD demonstrated editing of the dystrophin mutation in dystrophic mice with single and dual AAV vector delivery. Cas9 expression was restricted to skeletal and cardiac muscle by use of a muscle-specific regulatory cassette with promoter regions for murine muscle creatine kinase and α-myosin heavy-chain genes, to reduce the risk of off-target events in non-muscle cells and to minimize elicitation of an immune response. The muscle-restricted Cas9 expression results showed treated muscles express dystrophin in up to 70% of myogenic area. Moreover, systemic delivery of the vectors results in expression of dystrophin in both skeletal and cardiac muscles.

Combinations of viral and non-viral delivery methods have also been explored for CRISPR/Cas9 delivery. In 2014, Yin et al. utilized lipid nanoparticle-mediated delivery of Cas9 mRNA with AAVs encoding sgRNA and an HDR template via tail vein injection to induce repair of the disease-linked gene Fumarylacetoacetate hydroxylase (Fah) in a mouse model of human hereditary tyrosinemia. Results showed somewhat low efficiency of correction (6%) but rescued disease symptoms including weight loss and liver damage, as shown in Fig. 3.

4. NON-VIRAL DELIVERY METHODS

Significant progress has been made into non-viral approaches to delivery of CRISPR/Cas9 in vivo, their lower immunogenicity than viral methods and potential to deliver larger genetic cargos make them promising candidates for CRISPR/Cas9 delivery. Non-viral methods can be broadly divided into physical and chemical delivery methods. Physical methods include electroporation and hydrodynamic injection (HDI). Briefly, electroporation involves permeabilizing the cell membrane with an electric field, allowing for the passage of exogenous material into cellular cytosol. While not suitable for systemic application, electroporation has been successfully applied as a localized delivery platform for Cas9, notably for introduction of the machinery to ocular disease models. Since electroporation is limited in its in vivo applicability however, it is considered outside the scope of this review.

HDI involves the delivery of CRISPR/Cas9 components in vivo via a rapid intravenous injection of a large volume of liquid, physically ‘pushing’ the therapeutic cargo into the cell. One of the first proof-of-concept studies by Yin et al. used HDI to deliver CRISPR components as pDNA to Fah mutant mice. The aim of the study was to correct the Fah<sup>m<sup>mut/mut</sup></sup> disease phenotype through HDR editing. The authors reported successful correction in ca. 1/250 liver cells, and expansion of the Fah-positive hepatocytes rescued the body weight loss phenotype. However, as mentioned, this study is a proof-of-concept because HDI is not a clinically-applicable strategy for delivery of therapeutics.

Chemical approaches for delivery of CRISPR/Cas9 elements in vivo have involved lipid systems, polymers, inorganic nanoparticles, or combinations of these vectors. Lipid delivery systems are primarily micellar vehicles assembled by mixing charged lipid components with nucleic acids or protein, resulting in entrapment of therapeutic material within lipid bilayers. These lipid systems fuse and incorporate within the cell membrane, allowing the cargo to be released into the cell. A recent study by Finn et al. used biodegradable lipid nanoparticles complexed with Cas9-mRNA and sgRNA to knock-out the mouse Transthyretin (TTR) gene in the liver, the murine homolog of a therapeutic target for amyloidosis in humans.
Results reported over 97% knockdown of serum TTR levels after a single systemic injection, with a corresponding 70% editing of DNA throughout the liver by NHEJ. Recently, cell-specific targeting of CRISPR/Cas9 delivery was implemented by Luo et al. with targeted delivery to macrophages in order to stop the overexpression of netrin-1 (encoded by NTN1 in macrophages), an important cause of type 2 diabetes (T2D). To accomplish this, a macrophage-specific CD68 promoter was engineered into a Cas9 expression plasmid. This plasmid was encapsulated in cationic lipid-assisted poly(lactic-co-glycolic) acid (PLGA) nanoparticles and injected intravenously to T2D mouse model. This work resulted in disruption of the NTN1 gene by NHEJ only in macrophages and their precursors, but not in other cells.

Cationic polymers are a common strategy for the in vivo delivery of CRISPR/Cas9 as they offer advantages in their chemical diversity, control of polymeric structure and ease of scale-up at relatively low cost. Cationic polymers interact and condense with negatively charged nucleic acids and proteins to form ‘polyplexes.’ A study by Li et al. used a polymeric system to deliver Cas9 plasmid to disrupt the 2-hydroxy-dATP diphosphatase gene (MTH1), (a gene commonly overexpressed in many types of cancers) by intraperitoneal injection to a peritoneal metastasis model of SKOV3 (human ovarian cancer cells) in mice. In this study, Li et al. formed a polyplex by mixing fluorinated poly(ethyleneimine) (PEI) with the plasmid, and then coated the complex with a multifunctional polymer consisting of hyaluronan grafted with PEG (poly(ethylene glycol)) side chains, and then conjugated with an RGD-R8 targeting peptide. Results reported that targeted NHEJ disruption of MTH1 in vivo significantly inhibited tumor growth. Another study by Wang et al. used PEGylated nanoparticles (P-HNPs) based on a cationic α-helical poly-peptide as illustrated in Fig. 4, for the delivery of Cas9 expression plasmid and sgRNA. After intratumoral injection into HeLa xenograft tumor-bearing mice, results of deep sequencing showed that out of 20 clones, 7 of them displayed HDR mutations near the target site. The authors reported final genome editing efficiency in polo-like kinase 1 (Plk1) tumor-associated locus as mediated by P-HNP PCas9 + sgPlk1 to be 35%, with over 71% tumor suppression and 60% improved survival rate in animal models.

Gold nanoparticles have gained significant attention in recent years as nanocarriers due to their tunable size, flexibility of surface functionalization, and modifiable rate of cellular uptake which depend on the surface functionaliza-
tion. A recent study by Lee et al. demonstrated HDR of the DMD-associated, C-X-C motif chemokine receptor 4 (CXCR4) gene using a gold nanoparticle-polymer complex. This vehicle was composed of a 15 nm gold core conjugated with thiol-modified oligonucleotides and complexed with donor DNA. Along with pre-formed Cas9 RNP, the complex was encased within the endosomal disruptive polymer PAasp (DET), poly(N-(N-(2-aminoethyl)-2-aminoethyl) aspartamide) where ‘DET’ is diethylenetriamine. The scheme for development of this complex is shown in Fig. 5. This complex, termed “CRISPR-Gold” was injected intramuscularly to mdx mice and achieved 5.4% HDR efficiency with significant improvement in the muscular agility of animals. A follow-up study by Lee et al. utilized CRISPR-Gold to edit genes in the brains of adult mice. Results of intracranial injection show CRISPR-Gold with Cas9 and CRISPR from Prevotella and Francisella 1 (CPF1) (an alternative CRISPR nuclease with structural and mechanistic differences to Cas9) RNP can achieve NHEJ editing in all major cell types in the brain, with restored neurological behavior.

5. CHALLENGES

Even though there has been huge progress in the field of delivery of CRISPR/Cas9 technologies in vitro, the field is only beginning to move to an in vivo setting. One common challenge for all CRISPR/Cas9 delivery systems is the potential for off-target mutagenesis. Although sgRNA is designed to target a specific gene of interest, Streptococcus pyogenes Cas9 (spCas9) will tolerate up to a five mismatches in its recognition, which often can interact with a significant number of off-target genes. Moreover, if the Cas9 is integrated into the host genome (such as can occur during pDNA viral delivery) the continuous expression of Cas9 will inherently increase the chance of off-target effects. Cas9 delivery as a protein or using mRNA-based delivery systems have an advantage in this regard because there is no chance of incorporation into the host genome, as a result, they offer transient exposure, for one-time editing. Common to all forms of potential delivery however is the propensity for immunogenicity. Cas9 is a bacterially-derived protein, and therefore has the potential to elicit an immunogenic response in mammalian hosts. This could be detrimental to the overall applicability of a system.

As mentioned previously a major shortcoming to mRNA is its instability, it is highly susceptible to cleavage by nucleases in the blood and in cells. To overcome this issue, some studies have utilized chemically-modified RNA, with beneficial results to stability and efficacy, but this approach inherently complicates the production process of the RNA. A recent study by Yin et al. demonstrated structure-guided chemical modification of sgRNA, followed by encapsulation within lipid nanoparticles (LNP). Results of a single intravenous injection of enhanced sgRNAs and mRNA encoding Cas9 reported over 80% NHEJ editing of Pcsk9 (a target for the treatment of familial hypercholesterolemia) in the liver, lowering cholesterol levels by approximately 35–40% in mice.

Though widely used as a delivery platform, platforms, AAV vectors suffer from their limited packaging size of ca. 4.7 kb. The most commonly used Cas9 variant spCas9 is 4.3 kb itself, and so only a single sgRNA can generally be packaged along with Cas9. There are smaller Cas9 variants from different bacterial origins (e.g. Streptococcus aureus Cas9, saCas9) that help overcome the issue of size, and which also beneficially recognize different PAM sequences. Both AV and AAV vectors are human-derived viruses, AVs cause an array of common clinical diseases, which a large proportion of people are already exposed and most adults have already developed neutralizing antibodies through pre-exposure, meanwhile, early exposure to wt AAVs result in humoral immunity starting around 2 years of age, which may limit the efficacy of AV and AAV vectors as therapeutic delivery agents. Humoral immunity is a particular concern for applications where the patient requires multiple dosages, such as in cancer therapeutics, where the patient develops an adaptive immune response to the treatment over time.

There are limitations to lipid-based systems in a therapeutic context, low stability in physiological conditions, high toxicity and inability to efficiently entrap larger molecular weight therapeutics such as larger pDNA. Kreiss et al. reported that while the physicochemical properties of liposomes are not affected by pDNA size, the transfer efficiency of the complex significantly decreases with large pDNA size. Notably, the study demonstrated that the number of pDNA molecules incorporated into each liposome was inversely proportional to the size of the plasmid. To mitigate some of these limitations researchers have tried different methods such as condensing and loading pDNA into liposomes using cationic...
Cationic polymers generally suffer from high toxicity, however factors such as molecular weight and polymer architecture also influence the final toxicity profile. While positive charge aids cellular uptake, it also promotes nonspecific interactions with nontarget cells and extracellular components such as plasma proteins and extracellular matrix. Binding with select plasma proteins is the primary mechanism for the mononuclear phagocyte system (MPS) to recognize circulating nanoparticles, which can cause most of the injected dose to be sequestered in MPS organs such as the liver and spleen with little accumulation in target tissues. Binding of plasma proteins can also mask the effect of targeting moieties for active targeting. To mitigate these issues, researchers have modified polymers with neutral species such as PEG. Reducing polymer charge using PEGylation has been reported to extend circulation times and significantly reduce protein adsorption. Moreover, including active targeting functionalities on the surface of partially PEGylated nanocarriers can allow for improved cell-specific targeting.

A recent study by Dai et al. uses a representative nanoparticle system to highlight challenges to tissue localization as a major obstacle to the field. According to this study only 0.0014% of intravenously injected doses of nanoparticles were internalized by a SKOV-3 ovarian cancer cell xenograft. The majority of nanoparticles were either trapped in the extracellular matrix of the tumor or taken up by perivascular tumor-associated macrophages. The low targeting efficiency suggests that there is room for improvement in the design of active targeting materials. Despite the relatively low uptake efficiency of ca. 2% demonstrated in this study, it is worth noting that in the field of gene therapeutics, even a low level of editing efficiency may still be sufficient to rescue disease phenotype.

6. CONCLUSION

CRISPR/Cas9-based therapeutics have advanced our understanding of the genetic influence on disease states, and thus have enabled not only the creation of genotypically-accurate animal models for disease, but the therapeutic correction of genetic disease in humans. Despite this progress, translation to in vivo studies has been hindered by various shortcomings in delivery. Though significant advances have been made to viral-based delivery of the CRISPR/Cas9 machinery, issues associated with the cost and safety profile of viral systems limit their potential for widespread use. Non-viral vehicles that can deliver the CRISPR/Cas9 machinery in its various forms are improving, while avoiding the pitfalls associated with viral strategies. Direct comparison between delivery methods is not possible, and so systematic studies must be done to compare the translational potential of different therapeutic approaches and delivery methods in relevant models.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


