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Delivery Approaches for CRISPR/Cas9 Therapeutics *In Vivo*: Advances and Challenges

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Abstract

Therapeutic gene editing is becoming a viable biomedical tool with the emergence of the CRISPR/Cas9 system. CRISPR-based technologies have promise as a therapeutic platform for many human genetic diseases previously considered untreatable, providing a flexible approach to high-fidelity gene editing. For many diseases, such as sickle-cell disease and beta thalassemia, curative therapy may already be on the horizon, with CRISPR-based clinical trials slated for the next few years. Translation of CRISPR-based therapy to *in vivo* application however, is no small feat, and major hurdles remain for efficacious use of the CRISPR/Cas9 system in clinical contexts.

Areas Covered—In this topical review, we highlight recent advances to *in vivo* delivery of the CRISPR/Cas9 system using various packaging formats, including viral, mRNA, plasmid, and protein-based approaches. We also discuss some of the barriers which have yet to be overcome for successful translation of this technology.

Expert Opinion—This review focuses on the challenges to efficacy for various delivery formats, with specific emphasis on overcoming these challenges through the development of carrier vehicles for transient approaches to CRISPR/Cas9 delivery *in vivo*.

Keywords

CRISPR/Cas9; gene therapy; *in vivo* genome editing; therapeutic strategies; non-viral delivery; viral delivery; intracellular delivery

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1. Introduction

Despite the fact that over 7000 identified diseases have been linked to alterations in the human genome, effective treatments have only been developed for ~500.[1] In recent years, gene-based approaches to disease therapy have gained significant traction in research, as evidenced by the emerging interest in gene editing technologies including meganucleases, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs).[2] While effective, these techniques notoriously require extensive design when choosing new genetic targets and can suffer from barriers to fidelity and efficiency of editing.[3,4] Clustered regularly-interspaced short palindromic repeat (CRISPR)-related systems have become a cornerstone of current gene editing-based approaches for curing human disease. CRISPR/Cas9 systems are simple, elegant solutions for selective alteration and interrogation of the genome. These strategies benefit from flexible yet high-fidelity sequence targeting and efficacious editing, underscoring their position as perhaps the foremost technique for mammalian gene editing.[5,6]

The CRISPR/Cas9 system is composed of two main elements. The first component is the Cas9 protein, an endonuclease capable of double-stranded cleavage of DNA. The other element is single-guide RNA (sgRNA) molecules that complex with the Cas9 protein and guide it to its genomic site of action by forming complementary base pairing with the target sequence.[7] CRISPR/Cas9 systems are originally a component of the bacterial innate immune system, where they provide a ‘memory’ platform for the host’s immune response to effectively counter repeated viral infection.[8] Several Cas9 nuclease variants have been identified from different bacterial strains. These variants feature significantly different properties, including size, targeting sequence (protospacer-adjacent motif, or PAM) specificity, and the location where double stranded break (DSB) occurs. Of these systems, the most widely used Cas9 nuclease is derived from *Streptococcus pyogenes* and expressed in *E. coli* as recombinant protein. In nature, most Cas9 variants, including *sp*Cas9, require two separate RNA molecules for their targeted nuclease activity; CRISPR-RNA (crRNA) and trans-activating crRNA (tracrRNA) are both required in order to form a functional Cas9-sgRNA ribonucleoprotein complex (Cas9-RNP). The discovery that a single sgRNA molecule could replace these two molecules greatly simplified the CRISPR/Cas9 system, and today Cas9-mediated DNA cleavage can be obtained with high sequence specificity based on the facile redesign of sgRNA alone.[9,10]

In the CRISPR editing process a DSB is generated by Cas9. Endogenous cellular repair mechanisms typically repair the break, using a series of polymerases and ligase enzymes to add or remove nucleotides to the break point before re-joining the broken strands.[6] Depending on the desired alteration, different mechanisms for repair are utilized, with results that can be categorized as a) gene disruption, b) gene correction, c) gene insertion, and d) large gene deletion (Figure 1).[11] Repair after the DSB can occur through two main mechanisms, non-homologous end-joining (NHEJ) and homology-directed repair (HDR). For disruption of a gene sequence (i.e. knock-out), activity of the Cas9/sgRNA complex alone is sufficient, and as such repair typically occurs through the NHEJ pathway. The principle of NHEJ involves the incorporation of random nucleotides at the break site, until a small amount of strand overlap allows DNA polymerase enzymes to re-joint the strands.

Because this random incorporation can frequently lead to frame-shift mutations, this method can be used to knock-out gene expression.[12] Alternatively, the incorporation of a homologous 'repair template' DNA strand along with the CRISPR/Cas9 machinery provides the basis for HDR.[13] This mechanism of repair involves the incorporation of a DNA template strand as a homologous scaffold. This scaffold must contain a nucleic acid segment complementary to the desired mutation and flanked by homology arms (complementary sequences to either end of the DSB) that localize the template to the correct genomic sequence. The required length of these homology arms, as well as the structural nature of the template DNA itself is determined largely by the size of the desired insert. As an example, for correction of shorter sequences single-stranded oligonucleotide donors (ssODNs) facilitate greater editing efficiency than double-stranded donors. Following nuclease-induced DSB, the template strand either guides break repair, or is inserted into the DSB, allowing for the correction or knock-in respectively, of desired gene sequences.[14,15]

CRISPR/Cas9-based systems are quite versatile and have been extensively used for the production of knock-out/knock-in animal models, as well as *in vitro* cell line models.[16–29] Maddalo et al. have successfully established a *Eml4-Alk*-driven lung cancer mice model, which has specific chromosomal rearrangements after viral-mediated CRISPR-Cas9 delivery.[27] Per this versatility, CRISPR/Cas9 has been used as a tool for a number of applications apart from therapeutic gene editing, including functional gene interrogation, and transcriptional activation.[30–32] Of more clinical interest, the system has also been applied for the therapeutic alteration of genes in multiple somatic cell types, including immune cells such as primary T cells and hematopoietic stem cells.[33–35] Hainzl *et al.* have utilized Cas9 and Cas9-D10A nickase correcting single-cell clones from patient-derived keratinocytes.[19] In addition, Swiech *et al.* demonstrated the targeting of *Mecp2* and different other genes (*Dnmt1*, *Dnmt3a* and *Dnmt3b*) in postmitotic neurons in the adult mice *in vivo*.[21] Many methods of delivery, including electroporation or nucleofection, gene-gun delivery, lipid-based transfection, and other mechanical and non-mechanical approaches, have been applied to the delivery of Cas9 protein and sgRNA *in vitro/ex vivo*.[36–38] Effective translation of CRISPR/Cas9 technologies *in vivo* however, requires the use of biocompatible vehicles capable of both systemic circulation of the Cas9-RNP, as well as transport into cells. While mechanical methods of introduction are effective *in vitro* and *ex vivo,* their clinical application is inherently limited by accessibility of target regions.[39,40] Delivery of the CRISPR/Cas9 components into the nuclei of target cells is necessary for gene modification.[41] The components of the CRISPR/Cas9 machinery are macromolecules, and therefore are unable to spontaneously enter the cytosol, let alone the nucleus.[42] Several approaches have been taken for the cellular delivery of the CRISPR/Cas9 system (Figure 2), such as plasmid/viral vector-based approaches, Cas9-encoded mRNA, or direct Cas9 protein delivery. A number of synthetic vectors have been developed for delivering CRISPR components in vivo, including inorganic nanoparticles and polymer/lipid formulations. However, while progress has been made using each of these delivery formats, significant challenges still exist in the transition of these technologies to *in vivo* application. This review will highlight landmark publications and recent advances in the field of CRISPR/Cas9 delivery for application *in vivo,* with a focus on various approaches and vehicles used for intracellular

delivery, with a discussion of some of the hurdles still faced for translation of this technology.

2. Approaches to CRISPR/Cas9 delivery *in vivo*

Introduction of the Cas9 protein into the cytosol and subsequently the nucleus of the cell is a major hurdle to application of the CRISPR system. The CRISPR/Cas9 system can exist and be delivered in several different formats (or delivery cargos), each with their inherent promises and challenges (Table 1). Notably, plasmids for Cas9 protein and its associated gRNA can be incorporated into viral vectors, species designed by nature to carry genetic material to be expressed in the cell. Viral methods have comprised a significant portion of delivery approaches for the CRISPR/Cas9 system, so much so that delivery formats can be described as 'viral' or 'non-viral'. Viral approaches to delivery of gene editing machinery have seen success in several applications, from *ex vivo* transfection to animal model development.[43–45] Their effectiveness is largely due to the inherent ability of viral vectors to introduce exogenous genetic material into the cell, which generally results in high transfection efficiency. However, viral approaches face certain barriers to safety and practicality which will be explored more in Section 2.1. Largely for these reasons, alternative systems have been explored as carriers to bring the CRISPR/Cas9 system into the cell. These synthetic 'carrier vehicles' have been developed to encapsulate and deliver the CRISPR/Cas9 machinery in non-viral delivery formats. Non-viral formats of the CRISPR/Cas9 machinery include plasmid-based, mRNA-based, and the complexed Cas9-RNP. Of the approaches developed to introduce the CRISPR/Cas9 machinery into the cell however, few are translatable to *in vivo* application due to lack of stability or biocompatibility of the delivery vehicle and its cargo. Of the approaches which have demonstrated promise for translatability and systemic application, many have been applied for treatment of hepatic diseases.[46,47,26] This is a convenient target because of its role in the mononuclear phagocyte system (MPS) but limits the tissue specificity of these systems for non-liver-specific disease. The challenge of disease tissue specificity of systemically-delivered therapeutics is an ongoing and very significant obstacle for CRISPR/Cas9 delivery approaches but is in its infancy due to the paucity of systemic delivery strategies. In the following sections we will discuss the strengths and shortcomings of some of these potentially clinically translatable approaches, as well as their respective potential for clinical translatability.

2.1 Viral delivery methods

Viral methods are the most commonly-used approaches to CRISPR/Cas9 delivery. Genome-editing agents have been widely applied using lentiviral, adenoviral (AV) and adeno-associated viral (AAV) techniques for the integration of DNA encoding CRISPR/Cas9 machinery into the host genome as well as extrachromosomal expression of the CRISPR/Cas9 machinery.[48]

AV vehicles are effective and widely-studied transduction agents for gene delivery, with over 2,000 AV-related clinical trials approved since 1989.[49] AVs can be transduced in both dividing and nondividing cells, and generally persist in their expression extrachromosomally rather than by integrating into the host genome.[50,51] These vectors are typically known to

elicit a significant immune response in the host.[52] Despite this immunogenicity , *in vivo* proof-of-concept studies have demonstrated >22% editing efficiency of the *PTEN* gene in hepatocytes through systemic injection.[53] Mice receiving this treatment however, also showed massive hepatomegaly and significant immune response after a period of weeks as an effect of viral delivery components being expressed in the cell. A recent study by Ehrke-Shulz *et al.* demonstrated delivery of the CRISPR/Cas9 machinery using a new generation high-capacity AV vector devoid of all viral genes.[54] These CRISPR/Cas9 constructs were targeted to the human papillomavirus (HPV) oncogene *E6*, the DMD (Duchenne muscular dystrophy)-associated dystrophin gene, and the HIV co-receptor C-C chemokine receptor type 5 (CCR5) and delivered into immortalized and primary cell lines with highest indel activities up to 93%. While promising, this approach has only recently been taken, and has not yet been applied *in vivo*.

Lentiviral vectors provide a non-specific yet highly efficient integration of delivered gene into the host genome. Non-specific integration has risks associated with incorporation into random locations of the host genome, including a propensity to incorporate into vital host genes, causing insertional mutagenesis. Random integrations of genes near a protooncogene may also lead to tumorigenesis, a severe example of the dangers associated with lentiviral-based CRISPR therapies, especially *in vivo.*[55] Non-integrating lentiviral vectors have been developed to avoid these issues but have shown decreased efficiency in comparison.[56] Due to these limitations, lentiviral methods are currently suitable primarily for *in vitro* applications.[57,58] However, several reports[59–61] have demonstrated the specificity of lentiviral CRISPR editing *in vivo* however, notably for targeted editing of lung cancer tumor-suppressor genes $Kras^{LSL-G12D/+}$ and $Trp^{53fl/fl}$ in mice. [62] This study demonstrated the targeted generation of diverse adenocarcinomas, with no detectable off-target effects.

Unlike AV and lentiviral methods, AAV-based approaches to gene delivery provide controlled integration of delivered CRISPR genes into the *AAVS1* (adeno-associated virus integration site 1) locus in mammalian cells, with a wide tropism and capability to transfect both dividing and non-dividing.[63] This avoids much of the toxicity associated with random integration, making AAV-based approaches the safest of the viral delivery methods, and therefore more appropriate for *in vivo* application.[64] An interesting study by Tabebordbar *et al.* used an AAV vector to deliver CRISPR elements into mice to correct the mutated dystrophin gene in murine models of DMD by mediating NHEJ of the dystrophin gene in muscle tissue.[22] In these studies, *sp*Cas9 and sgRNA were delivered separately using two different AAV vectors into DMD model postnatal *mdx* mice via intramuscular, retro-orbital, and intraperitoneal (IP) injections. Through targeted knock-out of the defective exon 23, the authors reported a reading frame restoration with restored production of dystrophin, in a modified form which promoted skeletal muscle function more than the wild type (*wt*) protein.

AAV-based systems face inherent issues related to packaging size. AAV systems can package genes only up to ~4.7 kilobase pair (kbp). The size of the Cas9 gene itself is 4.3 kbp; therefore, the Cas9 gene and sgRNA must be delivered separately by using two individual AAV-vectors. This limitation raises further issues related to the integration of donor DNA or extra genes into the vector, significantly limiting flexibility. To overcome this

packaging size limitation, smaller Cas9 variants have been utilized (e.g. *Streptococcus aureus* Cas9, *Sa*Cas9) that allow for the packaging of genes encoding both Cas9 and sgRNA into a single vector.[65] However, *sa*Cas9 in particular has been shown to be more immunogenic than *sp*Cas9 in mammals, again hindering therapeutic application of this method.[66]

2.2 Plasmid-based CRISPR/Cas9 strategies

Delivery of DNA encoding the Cas9 protein is an attractive non-viral method for introduction of the CRISPR/Cas9 machinery into the cell. Production of DNA is well-documented and relatively straightforward, the material itself is hardy and thermostable, and persistent expression of the Cas9 protein does facilitate greater editing efficiency than other methods, despite concerns over unwanted and potentially harmful effects to the host.[67] The concept of gene delivery using synthetic vectors is conceptually similar to viral-based approaches, but avoids the issues associated with introduction of viral material into the cell.[68]

In 2014, the Anderson lab was the first to report systemic delivery of the CRISPR/Cas9 system to adult mammalian organs.[69] The researchers selected a mouse model of hereditary tyrosinemia that included a point mutation (G->A) of fumarylacetoacetate hydrolase (Fah), an essential enzyme in the tyrosine catabolic pathway, to show the potential for correction of human genetic disease. The components of the CRISPR-Cas9 system were designed and delivered in plasmid format by hydrodynamic injection. However, the correction rate was insufficient for clinical translation due to the low expression of the *wt*-Fah protein (~1/250 hepatocytes), with concurrent weight gain which was hypothesized to be a result of this correction. Additionally, the authors of this paper acknowledged the impracticality of hydrodynamic injection for clinical application and took different approaches in subsequent studies.[70,71] Regardless, this work illustrated the applicability of plasmid-based CRISPR/Cas9 in vivo.

For CRISPR/Cas9 delivery in plasmid format to be a viable option, a delivery system must exist which can compete with the high transfection efficiencies of viral vectors. To utilize the efficacious properties of viral systems without complications related to integration of viral genes, Li *et al.* synthesized a multifunctional nucleus-targeting “core-shell” artificial virus to deliver the plasmid encoding Cas9 and sgRNA.[72] This artificial viral vehicle accelerated endosomal escape and facilitated penetration of the plasmid into the nucleus without an additional nuclear-localization signal (NLS). This vehicle was targeted to ovarian cancer through dual-receptor mediated endocytosis and showed effective disruption of the *MTH1* gene (~80% decrease of gene expression *in vitro*), with concurrent decrease in tumor size.

Organ-specific targeting of CRISPR/Cas9 delivery vehicles is a challenge that has recently come to the forefront of gene editing research, but it is critically important when considering future therapeutic application. In a recent study, Liu *et al.* functionalized a cell-specific aptamer (LC09) onto a lipopolymer moiety to demonstrate selective tumor specificity in both orthotopic osterosarcoma and lung metastasis.[73] One potential advantage of plasmid-based delivery is that tissue or cell-specific targeting can also be integrated into the plasmid itself. Luo *et al.* reported tissue targeting by engineering a CD68 promoter onto the Cas9

expression plasmid for targeted delivery to macrophages. These constructs were encapsulated into cationic lipid-assisted polymeric nanoparticles and delivered by systemic injection. The netrin-1 protein was shown to have been downregulated, with subsequently improved type 2 diabetes (T2D) symptoms, concurrently with reduction of other macrophage-associated inflammatory cytokines (IL-6 and TNF-α) secretion.[74] Moreover, it is worth mentioning polyethyleneimine (PEI) as the most common cationic polymer for plasmid-based delivery. An example of PEI-mediated transfection is reported by Zuckermann et al..[75] In this work, authors have used a PEI-CRISPR/Cas9 approach for somatic gene transfer of different CRISPR plasmids deleting single (Ptch1) or multiple (Trp53, Pten, Nf1) tumor suppressor genes in the mouse brain.

2.3 mRNA-based delivery

Delivery of Cas9-encoded mRNA is another commonly used approach for introduction of the CRISPR machinery into the cell. Unlike gene-based delivery methods, mRNA-based strategies are transient in function, leading to the eventual removal of the nuclease from the cell and circumventing the risks associated with integration into the host genome.[76] mRNA-based methods also benefit from faster effect time, as mRNA is transcribed in a matter of minutes.[77] This delivery format however is limited by two major factors; inherent stability of mRNA, and the requirement for individual deliveries of each component.

A recent study conducted by the lab of D.G. Anderson reported a combinatorial delivery method, using lipid-mediated delivery of Cas9 mRNA and AAV delivery of the sgRNA/HDR template. [78] These vectors were co-administered to a murine model of hereditary tyrosinemia, aimed at correction of the *Fah* gene. Through systemic injection, this study reported correction in >6% of hepatocytes. While reasonably effective, this method did rely on viral co-delivery to supplement mRNA delivery. This is not the only barrier to efficacy faced by mRNA-based delivery approaches. RNA is more fragile than other types of genetic material and is often subject to premature degradation. This lack of stability is especially problematic for sgRNA delivered prior to complexation with Cas9. It was hypothesized that sgRNA suffered degradation during mRNA translation, significantly hindering editing efficiency. To improve efficiency by increasing sgRNA stability, further studies reported the benefits of synthetic modifications to the sgRNA, changing the RNA 2'OH group to 2'OMe and 2'F, and including phosphorothioate bonds. [79] Focusing instead on NHEJ-mediated correction of *Fah* mutant models, this study reported >80% editing in hepatocytes compared to ~40% using native RNA. This represents an effective method to overcome the stability issues associated with RNA-based CRISPR/Cas9 delivery. A similar study by Finn *et al.* packaged synthetically-modified sgRNA with *sp*Cas9-encoded mRNA into a lipid nanoparticle vehicle.[80] Following systemic administration, the researchers observed efficient knock-down of the mouse transthyretin (*Ttr*) gene in the liver, with a >97% reduction in serum protein levels, which persisted for up to 12 months.

While certain studies such as those by Finn have seen success in co-delivery, mRNA-based approaches are typically still limited by the need for multiple deliveries; generally, Cas9 mRNA is delivered independently of a second vector carrying the sgRNA, and a homologous DNA template in the case of HDR. However, a recent study by Miller *et al.*

reported the first successful non-viral co-delivery of Cas9 mRNA and sgRNA using a single zwitterionic amino lipid (ZAL) delivery vector. [81] These vehicles were engineered with a zwitterionic head group and an amine-rich linker region. Using a series of hydrophobic tails, mRNA encoding the CRISPR machinery was conjugated onto this vehicle. This study demonstrated up to 95% protein knock-down *in vitro*, with induced expression of fluorescent protein tdTomato in hepatocytes *in vivo* through intravenous injection.

2.4 Protein-based CRISPR/Cas9 strategies

Delivery of the Cas9 protein complexed with sgRNA (together, the Cas9-RNP) using synthetic delivery vehicles provides a transient, direct pathway for introduction of the CRISPR/Cas9 system.

To achieve the efficient intracellular delivery of the Cas9 protein, the lab of David Liu fused *sp*Cas9 with supercharged GFP (−30GFP) to provide effective complexation with Lipofectamine®.[82] This method achieved ~50% NHEJ editing efficiency *in vitro.* The authors further hypothesized that the anionic charge of sgRNA would be sufficient to facilitate encapsulation using cationic lipid Lipofectamine® transfection reagent (RNAiMAX). Complexes composed of Cas9 protein, sgRNA, and RNAiMax, or RNAiMax alone were injected to the inner ear of transgenic Atoh1 (Atonal BHLH Transcription Factor 1)-GFP mice, a reporter model which expresses GFP fluorescence under the control of the *Atoh1* gene. The inner ear was targeted due to its confined space and well-characterized inner-ear type. Ten days post-injection, 13% loss of fluorescence was observed in the ears of the mice. While largely proof-of-concept, this study provided insight into the concept of encapsulation and electrostatic assembly for delivery of the Cas9-RNP.

PEI polymers have also been used alone or in combination with liposomes for Cas9 protein delivery in vivo to help induce endosomal escape. Sun et al. reported a polymeric core-shell nanoparticle with a PEI coating on a DNA nanoclew loaded with Cas9-sgRNA complex.[83] In vivo delivery and 25% EGFP distruption was demonstrated using U2OS-EGFP tumor bearing mice. Interestingly, the editing efficiency was higher when the DNA nanoclew and the sgRNA guide sequences were partially complementary, suggesting that a modification of the DNA nanoclew could be used to incorporate different sgRNAs for multiplexed editing.

Recent work by the lab of Rotello demonstrated direct cytosolic delivery of Cas9 protein carrying a localized negative charge in the form of an oligo glutamic acid tag (E-tag, denoted as Cas9En) at the N-terminus.[84] In complex with its sgRNA component, this Cas9En-RNP co-assembled with positively charged, arginine-functionalized gold nanoparticles (Arg-AuNPs) to give rise to a single delivery vector. These supramolecular delivery vehicles delivered Cas9 directly to the cytosol, with nuclear accumulation *via* an attached NLS. Delivery was accomplished in ~90% of cells with efficient gene editing in the *PTEN* (30%) and *AAVS1* (29%) gene loci. While this study yielded promising *in vitro* results, systemic applicability of this delivery vector has not yet been demonstrated.

In a recent study by Lee *et al.*, CRISPR/Cas9 HDR repair vectors targeted at repair of the DMD-associated *CXCR4* gene were incorporated into a supramolecular delivery platform.[85] This platform consisted of a 15nm gold core, decorated with thiolated DNA capable of

'holding' an ssODN template through complementarity. The Cas9-RNP was electrostatically complexed within the vehicle, and the complex was incubated with the endosomal disruptive polymer PAsp(DET), where 'DET' is diethylenetriamine. This delivery vector, termed CRISPR-Gold, was administered through local injection to muscle tissue, achieving a 5.4% HDR efficiency with significant phenotypic improvement in the muscular agility of animal models in four-limb hanging tests. This success may revolutionize the treatment of some genetic diseases, for which local injection is effective, and is beneficial for patients without the use of viruses.[86] More recently, the CRISPR-Gold system was applied for intracranial injection to murine models of fragile X syndrome (FXS) which exhibited pronounced and erratic behaviors consistent with this disease.[87] Following CRISPR/Cas9-mediated knock-down of the *Grm5* gene and its related protein metabotropic glutamate receptor 5 (mGluR5, associated with FXS pathophysiology) treated animals displayed normalized behaviors and notably decreased disease symptoms after two weeks. One consideration for this method however, is the necessity for effective endosomal escape. This is a challenge to many contemporary delivery systems, and often compromises delivery efficiency.[76,88]

The transient delivery of Cas9-RNP offers reduced off-target effects, virtually no off-target mutagenesis and relatively low immune response (Table 1).[89] Because the Cas9-RNP is pre-formed, there is also no risk of loss of efficacy due to the potential degradation of free sgRNA.[90,91] Delivery of proteins into the cell is challenging however; [92] endosomal entrapment is a barrier to protein delivery systems, as most therapeutic proteins require localization in the cytosol, or (like Cas9) in the nucleus.[93,94] Further, the use of the Cas9-RNP does face challenges to translatability; expression of the Cas9 protein can be laborious, and once isolated its nuclease activity is lost in a matter of days.[95]

3. Conclusion

The delivery formats for the CRISPR/Cas9 system discussed here are potential platforms for *in vivo* application, with clinical translatability. As such, factors related to safety, efficacy, and practicality are of paramount importance in evaluating the feasibility of each approach. Significant advances have been made in recent years to mitigate shortcomings associated with viral, mRNA-based, plasmid-based, and protein-based delivery approaches for the CRISPR/Cas9 system, but each platform still faces its own barriers to translation. However, the recent advances discussed here have led to the development of delivery vehicles of unprecedented capability for delivery of the CRISPR/Cas9 technology, overcoming many obstacles that once severely hindered the translatability of this system.

4. Expert Opinion: a focus on delivery vehicle

Introduction of the CRISPR/Cas9 machinery into a higher-level organism is a challenge. Delivery of the editing machinery into the cells, the cytosol and then the nucleus is the first obstacle that must be considered when evaluating strategies. With the recent advances discussed in this review, facile delivery of the CRISPR/Cas9 editing components is closer to fruition than ever before. Nonetheless, there are certain shortcomings which severely hinder applicability, especially when considering the potential for translatability.

The first of these considerations is immunogenicity. The Cas9 protein itself has been shown to elicit a humoral immune response, which can lead to cytotoxicity in host cells. This is likely due to the presence of certain peptides in Cas9 which can act as major histocompatibility complex (MHC)-binding epitopes. It is important to note that as Cas9 is a bacterially-derived protein, it should be expected to have inherent immunogenic effects in mammals. Studies by Mali *et al.* suggested Cas9-triggered immunogenicity as a chief factor for destabilization of the host system during CRISPR/Cas9-based therapy.[66] These studies reported the intramuscular administration of AAV-CRISPR-Cas9 vectors targeted at a variety of reporter genes in muscle tissue, including *Mstn, Fst, Pd-l1,* and *Cd47*, with ~2-fold gene activation 2 weeks post-treatment. More interestingly however, this study reported edited cells enriched with a spectrum of immunological responses, which parametric examination revealed was due solely to the Cas9 protein, rather than the AAV9 vector. While obviously not severe enough alone to invalidate the significance of CRISPR/Cas9-based therapies, when combined with immunogenic effects caused by a delivery vehicle, immunogenicity could be a limiting factor for the applicability of a delivery approach. For this reason, it is important to consider the immunogenicity, off-target effects, and potential mutagenesis of various delivery vectors. These concerns are especially important for viral vectors; while AAV vectors and AV vectors avoid random integration into the host genome, production of viral material within the cell alone can be sufficient to render a delivery method simply too dangerous for clinical application.[96] The integration of multiple delivery vectors then becomes significant, implying the continued expression of viral genetic material from each delivery vector.

Another important consideration for CRISPR/Cas9 delivery formats is the persistent expression of the Cas9 protein. While a higher expression level of Cas9 in the cell will lend to a higher potential for editing, once integrated into the genome, Cas9 expression is persistent and irreversible. This continuous expression of Cas9 within the host entails questions of unintentional ‘off-target’ editing within the host genome. Further, sgRNA will tolerate up to five mismatches in recognition sequence, and while this may not seem like much in a ~100 nucleotide (nt) strand of sgRNA, this can translate to hundreds of potential off-target sites in the genome[97]. Studies by Cradick *et al.* using commercial transfection reagents for introduction of the CRISPR/Cas9 plasmid targeted at NHEJ editing of human hemoglobin beta (*HHB*) and *CCR5* genes examined off-target editing effects. 3 days post-delivery, while efficiency varied widely based on guide strand, up to >50% off-target indel was observed with some sgRNA.[98] This off-target editing represents a massive limitation to efficacious editing when the CRISPR/Cas9 system is persistently expressed and demonstrates a very real limitation to efficacy. Some studies have approached this limitation through the use of Cas9 variants such as nickases, protein variants with reduced catalytic activity that create single-stranded breaks instead of DSBs.[99] This obviously limits efficacy however and is limiting for HDR repair.

Protein-based and mRNA-based strategies for delivery of CRISPR/Cas9 have an advantage in that they are transient approaches to delivery. Because exposure is limited to a one-time ‘hit-and-run’ editing, immunogenic response is minimal compared to strategies which entail persistence of the Cas9 protein. There is also no incorporation into the host genome, which circumvents issues of off-target mutagenesis, and other potential hazards of genomic

integration. Equally important, delivery strategies for *in vivo* introduction of the Cas9 protein or mRNA are versatile- nanomaterial-based delivery vectors in particular present significant opportunity for modulation, which may facilitate tunable biodistribution, clearance, and efficacy *in vivo.* Nanomaterials-based approaches may provide suitable platforms for development of delivery vectors capable of systemic introduction *in vivo*; gold nanocarriers have been demonstrated previously as promising agents for systemic delivery of therapeutics, much due to their tunable properties, non-toxic nature, and favorable size.[100]

In our opinion, the next step forward in the field of clinical translatability for CRISPR/Cas9 machinery is the development of stable, versatile delivery vehicles for the introduction of transient editing machinery (protein or mRNA-based strategies) into the cell. The major barrier to efficacy for these methods has typically been endosomal entrapment, but recent advances in the field have overcome this obstacle with rationally-designed carrier vehicles for direct cytosolic delivery or effective endosomal escape. Translation of these methods into a practical *in vivo* approach requires not only efficacious treatment, but also a realistic method of administration (i.e. systemic introduction). Intelligently designed carrier vehicles with sufficient biocompatibility may be capable of 1) efficient delivery on a cellular level, and 2) localization to disease-relevant tissue through vehicle design. Development of vehicles capable of both of these parameters is critical to advancement in the field and should be addressed.

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Article Highlights

- CRISPR/Cas9 editing provides a powerful method for the treatment of genetic disease.
- The CRISPR/Cas9 machinery can be packaged in various formats, each with inherent pros and cons.
- Multiple biological and synthetic approaches have been used to deliver the CRISPR/Cas9 editing machinery.
- Intracellular and nuclear delivery of editing machinery is critical for efficacy.
- Delivery approaches must remain viable *in vivo* to have future clinical translatability.
- Despite advances in the field of *in vivo* CRISPR/Cas9 delivery, challenges still remain to biocompatibility, safety, and tissue specificity.

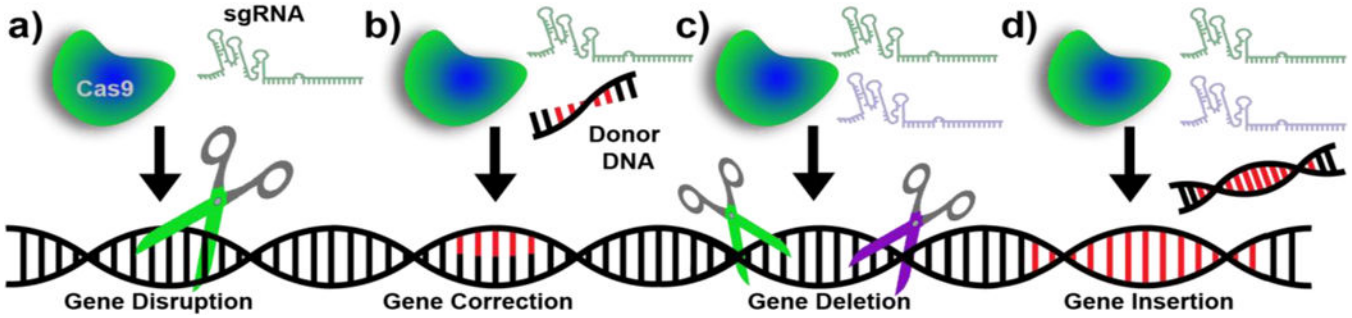

Figure 1.

Multiple components of CRISPR/Cas9 system are delivered into cells to achieve a specific function. (a) Cas9 and sgRNA for gene disruption (knock-out), (b) Cas9, sgRNA, and a template ssDNA for mutation correction, (c) Cas9, sgRNA, and a template DNA for gene insertion (knock-in), and (d) Cas9 and two sgRNAs for gene deletion.

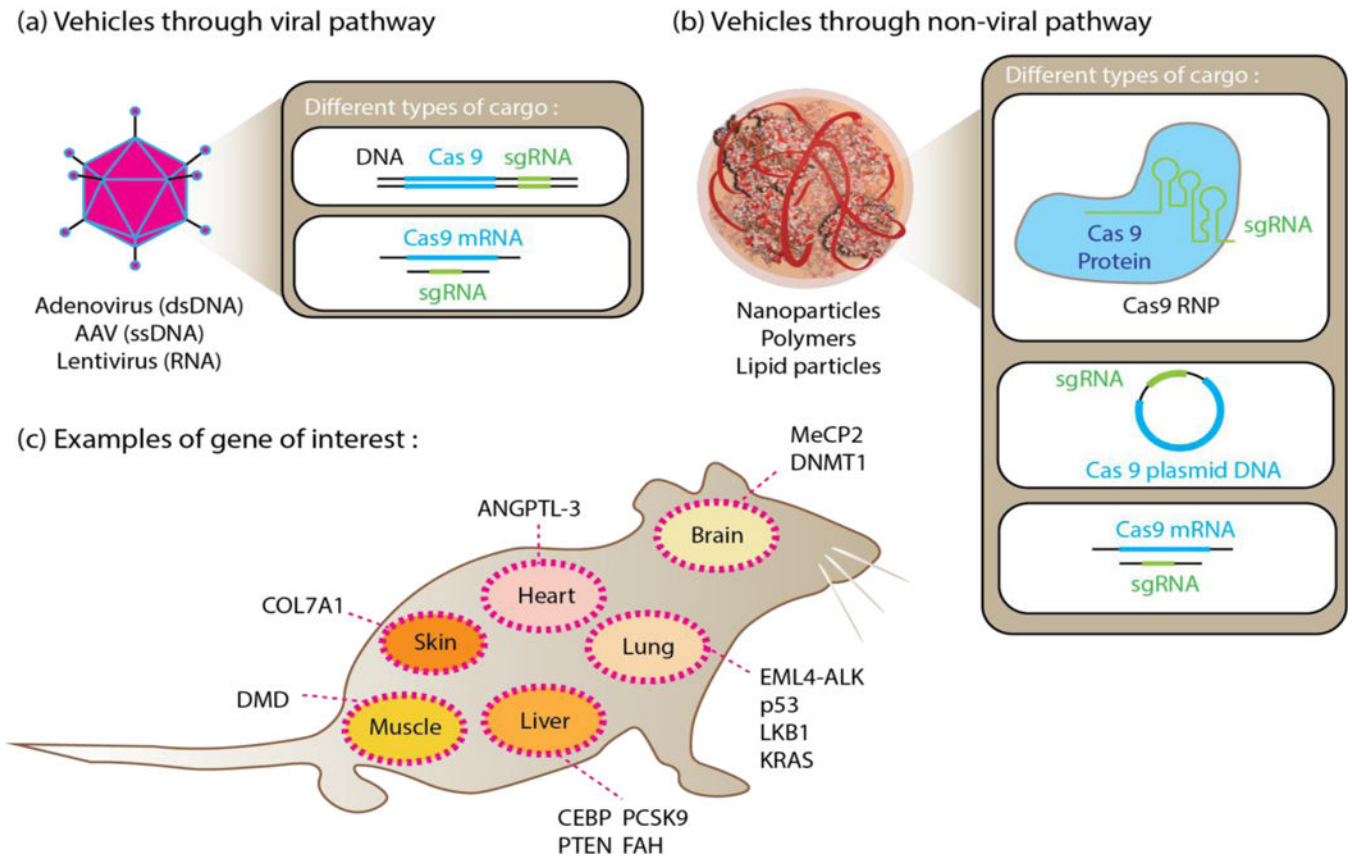

Figure 2.

Representation of different delivery formats for CRISPR/Cas9; (a) viral-based, (b) plasmid-based, mRNA-based, and direct Cas9-RNP delivery, as well as (c) some representative genes that have been used as targets for therapeutics or model development.

Table 1.

Generalized comparison of various CRISPR/Cas9 delivery formats.

	Viral	Plasmid	mRNA	Protein
Insertional Mutagenesis	High	Moderate	Low	Low
Persistence of Cas9 Protein	Long	Moderate	Moderate	Short
Off-Target Effects	High	Moderate	Moderate	Low
Immunogenicity	High	Moderate	Moderate	Low