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REVIEW ARTICLE

Engineering CRISPR-Cas9 RNA–Protein Complexes for Improved Function and Delivery

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Abstract

The use of CRISPR-derived, RNA-guided nucleases for genome editing has shown great promise for addressing genetic disease. Encouraging work in cell culture and animal models has demonstrated the capability of genome-editing enzymes to correct disease-causing loci, but clinical translation can only proceed once the corrective enzymes have been rendered safe, effective, and capable of being delivered to the appropriate cells. To address these needs, there has been rapid and extensive progress in the engineering of the RNA and protein components of Cas9, the most widely-used genome editor. Here we review advances in engineering of the enzyme Cas9 by altering its chemical or molecular composition. Such efforts have enhanced enzyme stability, improved capacity for delivery, augmented specificity, and broadened the horizons of genome manipulation.

Introduction

After more than a decade of steady development using engineered nucleases, genome editing experienced an explosion of accelerated progress due to the discovery and implementation of the endonuclease Cas9. Cas9 is a ribonucleoprotein (RNA-protein, or RNP) enzyme derived from the *Streptococcus pyogenes* (*Spy*) CRISPR* locus that is capable of cleaving a double-stranded DNA molecule bearing complementarity to the guide RNA (gRNA) bound by Cas9.[1] Cas9 proved to be a robust biochemical tool based on its effectiveness in inducing a double-stranded break (DSB) in a targeted DNA sequence, which is the initiating step of genome editing. Following a DSB, cells initiate repair pathways, resulting in either an insertion/deletion (indel) via nonhomologous end joining or incorporation of an exogenous donor DNA at the DSB via homologous recombination (HR). In contrast to the laboriously constructed proteinaceous endonucleases used by genome editing pioneers, the Cas9 protein component requires no modification for each new genetic target other than the rapid and inexpensive synthesis of a corresponding gRNA.[2]

The efficiency and convenience of the Cas9 enzyme induced a sea change in the field of genome editing, as evidenced by the modification of hundreds of cell lines or organisms for use in biomedical research, agriculture, microbial engineering, and therapeutic development. Although the inherent properties of *Spy* Cas9 have been adequate to ignite a revolution in the field, scientists have also performed creative molecular modifications to imbue the enzyme with enhanced abilities to enter cells, to resist degradation, to modify the genome in novel ways, and more. In this review, we will explore the engineering of the Cas9 RNP (protein as well as sgRNA) to enhance delivery and expand its functionality.

Although there are multiple CRISPR-derived enzymes being used for genome editing (with still more continuously being discovered), *Spy* Cas9 appears to be a serendipitously capable forerunner among CRISPR-based enzymes. None of the Cas9 orthologues (e.g. *Staphylococcus aureus*,[3] *Streptococcus thermophilus*,[4] *Neisseria meningitidis*,[5] *Geobacillus stearothermophilus*,[6] etc.) or alternative enzymes (e.g., Cas12a (Cpf1)[7]) have displaced *Spy* Cas9 as the default choice for a genome editing platform.[8] Either because of bias conferred by initial exposure and research investment or because of its

*Clustered Regularly Interspaced Short Palindromic Repeats.

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inherent qualities, *Spy* Cas9 remains the technology of choice at the heart of a rapidly evolving field. Since *Spy* Cas9 has been the subject of so many tweaks, upgrades, and uses in various contexts, it is the focus of the ensuing review. Thanks to the commonalities between *Spy* Cas9 and its CRISPR-derived ribonucleoprotein analogues, it is fortunate that many of these engineering advances will be readily transferrable between platforms. For example, the chemical modification of gRNA to render it resistant to nuclease degradation can be applied to other RNA-guided enzymes, and fusions of protein effector domains can be performed independently of the specific endonuclease in use. Therefore, the successful engineering of *Spy* Cas9 has broad implications for the improvement of all CRISPR-based biotechnology.

Cas9 Anatomy and Foundational Modifications

In its natural context of the *S. pyogenes* adaptive immune system, Cas9 consists of a 1,368 amino acid (aa) protein (158 kDa), a ~75 nucleotide (nt) tracrRNA that serves as a scaffold for RNP complex assembly, and a ~40 nt CRISPR RNA (crRNA) that contains a 20 nt spacer region that guides DNA targeting through complementary base pairing[9] (Fig. 1). The Cas9 protein bears two catalytic domains that induce a DSB in a bound DNA if it bears sufficient complementarity to the spacer sequence. The tracrRNA and crRNA bear 24 nt of complementarity to each other, forming a double-stranded RNA helix ending with a proximal pair of termini (one from each constituent RNA molecule). This property inspired the first instance of Cas9 RNP engineering: addition of a four-nucleotide loop to fuse the tracrRNA and crRNA into a single molecule known as the single-guide RNA (sgRNA)[1] (Fig. 1). This reduced Cas9 to a two-component system without impairing its function and greatly facilitating its use in biotechnology.

Another key modification was made during the earliest applications of Cas9 as a genome editing tool. To promote the enzyme's transit from the cytosol to the nucleus, the gene encoding the Cas9 protein was augmented with one or more SV40 nuclear localization signal (NLS) tags.[4,10,11] Furthermore, the Cas9 gene was codon optimized for expression in human cells. It was shown that the modified protein and RNA components of Cas9 can be encoded in a single plasmid that, following its transfection, impels the cultured cells to act as factories for the genome editing enzyme. Following translation of the engineered Cas9 protein and transcription of the sgRNA, the two components can form the RNP complex and perform efficient genome editing after Cas9 is trafficked to the nucleus of mammalian cells.

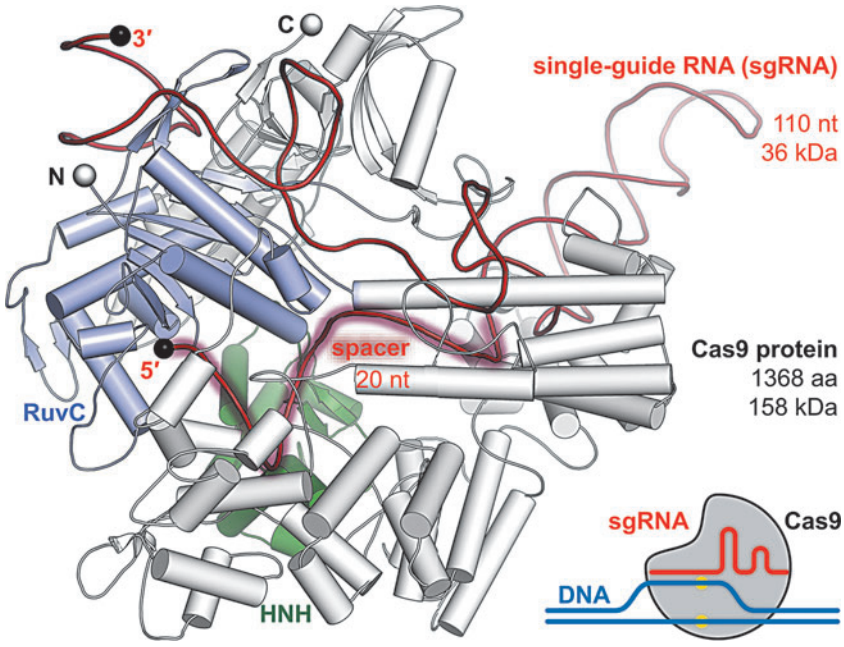


FIG. 1. CRISPR-Cas9 RNP anatomy. The *Streptococcus pyogenes* Cas9 ribonucleoprotein (RNP) enzyme consists of the Cas9 protein (white) and its guide RNA, here represented as a single-guide RNA (sgRNA, red). The termini of the respective protein and RNA compoments are labeled. The 20 nt spacer sequence responsible for DNA targeting is highlighted. The RuvC (blue) and HNH (green) nuclease domains of the Cas9 protein are shown. Inset: cartoon representation of Cas9 cleaving double-stranded DNA, with cleavage events shown in yellow.

Engineering Cas9 at the Genetic Level

Extensive engineering has been performed on the Cas9 protein at the genetic level. One key example relates to the protospacer-adjacent motif (PAM), a nucleotide cluster adjacent to the DNA sequence targeted by the spacer of the gRNA. The *Spy* Cas9 PAM is NGG (where ''N'' can be any nucleotide). The PAM is recognized by protein–DNA interactions, and its purpose in endogenous immune surveillance by CRISPR systems is to allow discrimination between the hosts *CRISPR* locus (which lacks a PAM) and the pathogenic DNA (which bears a PAM). The PAM provides a constraint when using Cas9 for genome editing, because it limits the sites that can be targeted. Mutation of the PAM-recognizing residues of Cas9 has resulted in enzyme variants with relaxed or distinct PAM specificity without sacrificing endonuclease activity, thus allowing researchers more freedom in selecting target sites in the genome.[12–15]

Cas9 cleavage at sites similar to the intended target site results in off-target cleavage, a major concern and potential source of undesired genomic changes. Accordingly, the Cas9 protein has been engineered to improve its specificity, often via mutation of positively charged residues that provide DNA affinity that is non-sequence specific.[16–19] This work has revealed that a small number of point mutations can greatly reduce the off-target activity of the enzyme, with variable impacts on on-target efficiency. The Cas9 variant that best reduces undesired editing without sacrificing efficiency embodies a single amino acid change.[19]

Internal modifications of Cas9 have also been used to reduce the overall size of the enzyme through deletions that preserve activity,[20] which can facilitate packaging of the Cas9 gene in viral delivery systems with limited capacity. Alternatively, the protein's primary sequence can be augmented to incorporate desirable functional modules at one or more sites,[21] including a chemical-sensing domain to impart allosteric control over Cas9 function.[22] More extensive protein fusions can repurpose Cas9 entirely, as discussed in the section that follows.

Cas9 as a Molecular Scaffold for Genomic Control

Repurposing the protein component of Cas9 as a hub for assembly of functional protein domains allows a vast array of new biotechnology applications (Fig. 2). Mutations to residues in the RuvC and HNH nuclease centers of Cas9 resulted in a catalytically deactivated Cas9 (dCas9) that retains its ability to bind a targeted DNA sequence without cleaving it.[23] This version of Cas9 has been utilized to control gene expression, either through inhibition (CRISPRi) or activation (CRISPRa) (recently reviewed in depth[24]) (Fig. 2a). Multiple activating[25,26] (e.g., VP64) or inhibiting[26,27] (e.g., KRAB) domain fusions have been developed, offering a gene silencing strategy that is generally more efficient and specific than RNA interference[24] as well as a totally novel approach to gene activation. Furthermore, fusion of Cas9 to a histone demethylase[28] or acetyltransferase[29] has been used to manipulate epigenetic factors. Because such modifications

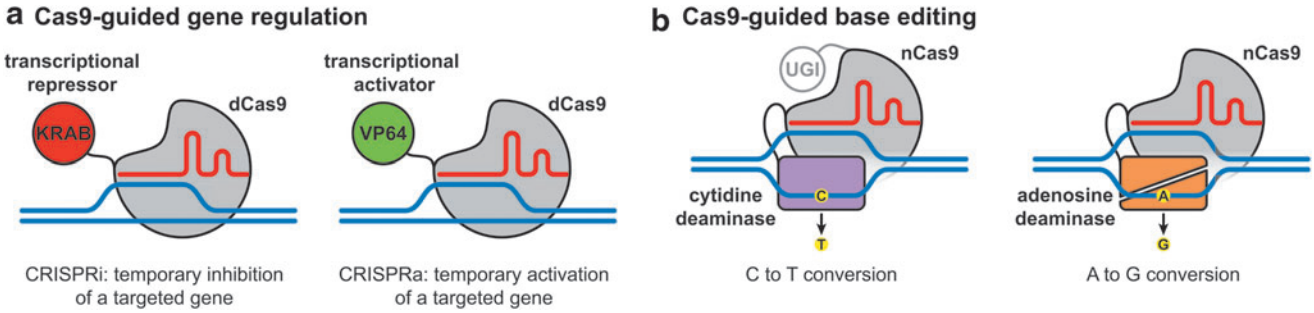


FIG. 2. Cas9 as a scaffold for transcriptional control and base editing. **(a)** Cas9 can be used for inhibition or activation of gene expression (CRISPRi or CRISPRa, respetively). For inhibition, a deactivated Cas9 (dCas9) is fused to a transcriptional repressor (e.g., a KRAB domain) and directed downstream of the transcriptional start site (TSS) of the gene of interest. An analogous strategy is used for activation, but the tethered domain is an activator (e.g., VP64), and the targeted site is optimally upstream of the TSS. Alternatively, cleavage-competent Cas9 protein can be used with a truncated sgRNA for binding without inducing a double-stranded break. **(b)** Using Cas9 for base editing, single-nucleotide changes via chemical modification followed endogenous DNA repair. Left: a cytidine can be converted to a thymine by tethering a Cas9 nickase (nCas9) to a cytidine deaminase (e.g., APOBEC). To prevent counter-productive host repair of the inosine intermediate to cytidine, the most effective platforms include one or more tethered uracil-N-glycosylase inhibitor (UGI) domains. Right: an adenosine can be converted to a guanosine by tethering nCas9 to an adenosine deaminase (e.g., the dimeric TadA).

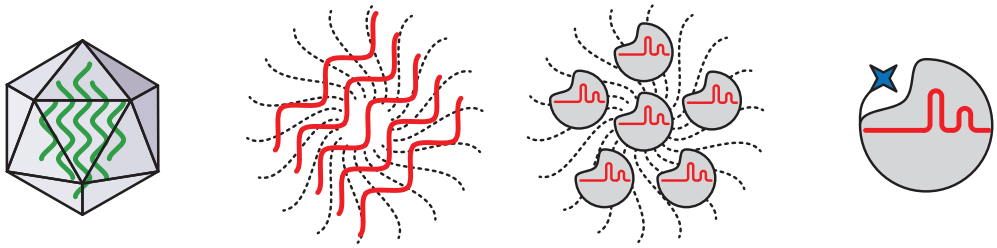
persist but do not require induction of a double-stranded break, epigenome editing may represent a safe and appealing therapeutic approach for certain diseases.

Another dCas9-based approach involves internal modification of the gRNA with sequences used to recruit RNA-binding counterparts within the cell that enable either the visualization or transcription of specific DNA sequences in the genome.[30,31] This strategy has been expanded to orchestrate elaborate transcriptional programs by using a modified gRNA as a scaffold to recruit additional effector domains.[32]

The Liu lab has pioneered the ''base editor'' approach (recently reviewed in depth[33]), which relies on deactivation of just one of the catalytic domains to generate a nickase (nCas9).[34,35] The nCas9 is tethered to enzyme domains capable of chemical modification of a proximal single nucleotide, inducing a deliberate point mutation without introducing a DSB or requiring a donor DNA template (Fig. 2b). Such a strategy can be used to introduce a stop codon or to correct a single-nucleotide polymorphism responsible for disease. One limitation of this technology is the availability of enzymes to convert one nucleotide type into another. The first reported base editor relied on a fusion to a cytidine deaminase, which promotes conversion of a cytidine to thymine.[34] To enable other interbase conversions, directed evolution was used to develop an adenine deaminase which efficiently converted adenine to guanosine when fused to nCas9.[35] By using one of these base editors, a wide array of conversions are possible: C to T, A to G, T to C, or G to A, representing 56% of all pathogenic single nucleotide polymorphisms.[35] As demonstrated by Gapinske and colleagues, base editing can also be used to program exon skipping, which has widespread therapeutic implications.[36] Importantly, it was recently shown that base editors can also be delivered in animals as an RNP,[37] an appealing format for therapeutic application as discussed later in this review. Because transversions between purines and pyrimidines are extremely difficult, it remains to be seen if additional conversions will be enabled. Analogously, recent work from Halperin and colleagues has enabled programmable directed evolution by tethering an error-prone polymerase to nCas9, producing a window of mutagenesis proximal to the targeted locus.[38]

Delivering Cas9 Encoded as DNA and/or RNA

Delivery represents a substantial hurdle for therapeutic applications of CRISPR-based genome editing, and several strategies have been pursued (Fig. 3). Initial demonstrations of genome editing in tissue culture using *Spy* Cas9 relied on a plasmid encoding protein and RNA components, with cellular uptake via chemical transfection or



Approach	DNA	RNA	RNA & protein (RNP) enzyme	Engineered RNP enzyme
Example	Adeno-associated virus (AAV) packaged with DNA encoding Cas9 protein & sgRNA	Liposomes encapsulating Cas9 mRNA & sgRNA	Cas9 ribonucleoprotein associated with carriers, e.g. cationic nanoparticles	Cas9 ribonucleoprotein modified to add desirable properties
Size	20 nm	≥50 nm	≥50 nm	12 nm
Advantages	Extremely effective; prior use with traditional gene therapy	Straightforward to prepare; low immunogenicity; effective	Short half-life limits off-target editing; can be highly efficient	Short half-life limits off-target editing; simplified manufacture
Disadvantages	Risk of increased off-target cutting & genomic integrations; can be immunogenic; capacity for DNA storage can be limiting; non-trivial manufacture	Toxicity not fully characterized; tends to accumulate in the liver, thus limiting versatility	Unknown immunogenicity profile; co-carrier molecules may be toxic	Unknown immunogenicity profile; requires additional engineering, transduction reagents, or electroporation to enter cells
Tissue Specificity	Some tropism inherent to various strains; additional tropism can be engineered	Intravenous use edits the liver preferentially; molecular targeting is being developed	Shows great promise via local injection	No inherent cell-penetrating properties: molecular targeting can be precisely engineered

FIG. 3. Delivery strategies for Cas9. The DNA- or RNA-based approaches (left two columns) rely on both or one of the RNP components to be delivered in the form of genetic instructions, while the RNP enzyme approaches (right two columns) deliver the Cas9 enzyme as a pre-assembled complex. Adapted from Wilson *et al.*[96]

electroporation.[10] This rapidly facilitated myriad reports demonstrating proof of concept for Cas9-driven correction of genetic disease, and in turn spurred the hunt for delivery methods that would allow clinical translation. This resulted in the quick adoption of previously established technologies for use with Cas9.

Traditional gene therapy research identified adeno-associated virus (AAV) as a highly effective and reasonably safe means for delivery of single-stranded DNA[39] (Fig. 3). A pair of AAV vectors has been used to package the *Spy* Cas9 gene and the sgRNA gene for tandem use,[40] and the more compact Cas9 enzyme from *S. aureus* has been encoded with their cognate sgRNA in a single AAV virion.[3] So far, AAV has been used for *in vivo* delivery of Cas9 in dozens of animal studies.[41]

Another established technology being repurposed for genome editing is the use of polymers initially developed to deliver nucleic acids such as dsDNA gene therapy vectors and small interfering RNAs.[42] Generally referred to as lipid nanoparticles (LNPs), polymers bearing cationic and hydrophobic properties can be used to encapsulate an mRNA encoding Cas9 and a sgRNA that will reconstitute the RNP enzyme *in vivo* following cellular entry by the LNP (Fig. 3). Diverse LNP-mediated approaches for delivery of Cas9 components in the form of RNA have shown great promise,[43,44] especially for liver-focused applications *in vivo*.[45,46]

Viral and nanoparticle delivery strategies are not mutually exclusive, as demonstrated by *in vivo* correction of a mouse model of human hereditary tyrosinemia that paired LNP-mediated delivery of Cas9 mRNA with AAV-mediated delivery of gRNA and a DNA donor for homologous recombination.[47] LNPs can be used for the delivery of plasmids encoding Cas9 and sgRNA[48–50] or for delivery of the Cas9 RNP, as discussed in the following section. The advantages and disadvantages of delivering Cas9 as DNA, RNA, or RNP are summarized in Fig. 3.

Delivery of Cas9 as an RNP Complex

Therapeutic delivery of Cas9 as an RNP complex is appealing because it will be degraded quickly, which decreases the incidence off-target editing that can result following prolonged Cas9 overexpression via viral vectors.[51] Importantly, the constructs producing the large fusion proteins used in base editing often exceed the packaging size limits of viral vectors. With this in mind, therapeutic delivery of base editors in RNP format becomes an enticing alternative, which may hold great promise since base editors can correct single-nucleotide polymorphisms, induce programmable exon-skipping, or knock out genes, all without the need for a double-stranded break.[33] Despite the appeal of these approaches, the Cas9 RNP has no inherent ability to enter cells, so for therapeutic use it must either be engineered to incorporate this capacity or be combined with reagents that promote cellular entry. We will review progress in developing Cas9 RNP for use *in vivo* or *in vitro* in mammalian cells.

One of the first examples of Cas9 RNP delivery employed cationic lipids, taking advantage of their known interaction with nucleic acids.[52] Although at physiological pH the Cas9 protein is positively charged, preventing interaction with cationic lipids, the RNP complex displays regions of dense negative charges due to protrusion of the sgRNA from the complex (Fig. 1). Simple co-incubation of the Cas9 RNP with a lipofection reagent and subsequent delivery to U2-OS cells achieved ~80% of disruption of an endogenous green fluorescent protein (GFP) gene. This represented a two-fold increase compared to the delivery of plasmid-encoded Cas9 RNP. Importantly, this delivery strategy succeeded *in vivo* at disrupting a GFP reporter in hair cells of the murine inner ear.

Although a systemic intravenous administration is the most straightforward approach for therapeutic genome editing, this has not yet been accomplished using Cas9 RNP. However, direct tissue injection is an appealing alternative because of the permanent nature of genome editing: it would only have to be done once. Genome editing via a local tissue injection also has the benefit of limiting exposure to the active therapeutic agents, thus mitigating potential immunogenicity. Delivery vehicles and engineered proteins have now been developed to promote Cas9 RNP-mediated editing after a direct injection into the brain, ear, or muscle tissue.[52–56] However, human organs are orders of magnitude larger than mouse organs, and it is unclear if the volume of tissue being edited after a single injection will be large enough to enable clinical translation.

Lipofectamine/Cas9 RNP complexes have been used to rescue mice from hearing disorders, via an injection in the ear.[53,55] Interestingly, these studies used 1–2 day old mice, and this suggests that genome editing at an early stage of development could be highly beneficial due to the higher cell division rate and the reduced area that needs to be edited. In addition, several new classes of cationic lipids have also recently been developed, which can also transfect the Cas9 RNP into cells and *in vivo*.[57] Collectively these studies suggest that cationic lipids have great potential for delivering the Cas9 RNP.

Nanoparticles and polymers have also been investigated for Cas9 RNP delivery. A key challenge with delivering Cas9 RNP via nanoparticles is forming a stable complex with the Cas9 RNP. Gold nanoparticle–DNA conjugates have been investigated to deliver the Cas9 RNP and take advantage of PAM-mediated binding

affinity of the Cas9 RNP to load the protein onto the DNA fragment tethered to the nanoparticle.[55] In addition, gold nanoparticle-DNA conjugates also provide a convenient strategy for performing HR *in vivo*, because they can simultaneously deliver the donor DNA and Cas9 RNP into cells. This strategy has been used to perform HR *in vivo* to correct mutations responsible for Duchenne muscular dystrophy in a mouse model. In addition, gold nanoparticles have broad tropism and also have been used for delivering Cas9 to the brain.[54] These results suggest brain diseases could eventually be treated via localized gene editing.

Engineering Cas9 RNP for Improved Cellular Delivery

Engineering the Cas9 protein to enable RNP delivery (Fig. 4) has several attractive features, such as the well-defined structure of the complex, its ease of production, and its small size, enabling delivery into a variety of otherwise inaccessible tissues. A Cas9 RNP engineered with additional NLS signals has shown great promise for therapeutic editing of the brain.[56] An engineered Cas9 RNP that bore four NLS moieties fused to its N terminus was able to edit genes *in vivo* in the striatal neurons of mice after a direct intracranial injection, with an estimated editing efficiency of 23%.

A key study envisioned the use of cell-penetrating peptides to deliver Cas9 to virtually any cell type.[58] Cell-penetrating peptides (CPP) are short protein tags that can mediate non-specific uptake in mammalian cells and/or fusion with the cell membrane. These have been successfully used to transfect cells with zinc-finger nucleases or transcription activator-like effector nucleases (TALENs).[59] In this study, the CPP "9R," comprising nine consecutive arginine and four leucine residues, was used to deliver the Cas9 protein and sgRNA in parallel (not as a preformed RNP). A 9R peptide functionalized with a thiol-active maleimide group was conjugated to a cysteine residue introduced at the C-terminus of the Cas9 protein (Fig. 4), and this was applied to cells via co-incubation with sgRNA that had been pre-mixed with an inert version of the 9R CPP. This method generated 6% editing with a fluorescent reporter and 15–20% indels at endogenous loci, suggesting assembly of the RNP inside the cell. However, editing efficiency was cell-line dependent and was considerably reduced in cancer and stem cells. No mechanistic investigation of the entry pathway was performed for this study, although it has been demonstrated that polyarginine peptides can readily cross the cell membrane[60] and that polyleucine polymers lead to pH-dependent membrane leakage in endocytic vesicles.[61]

Another study built upon the discovery of HIV TAT domain as a CPP. Here, PTD4, a synthetic transduction domain derived from TAT, was fused to CM18 (cecropin/melittin fusion peptide) and a hexa-histidine tag and simply co-incubated with Cas9 or Cas12a RNP.[62] This method generated between 12% and 27% indels at endogenous loci in a range of easy- and hard-to-transfect cells. The number of NLS at the C-terminus of the Cas protein was reduced to a single NLS, resulting in a reduced non-specific binding of the Cas RNP to the cell membrane and preventing nonspecific uptake that may occur over prolonged incubation, as observed for mouse neurons.[56] Indeed, using this specific CPP, it was demonstrated that the uptake is very fast (requiring only a 1–2 min co-incubation), suggesting interactions

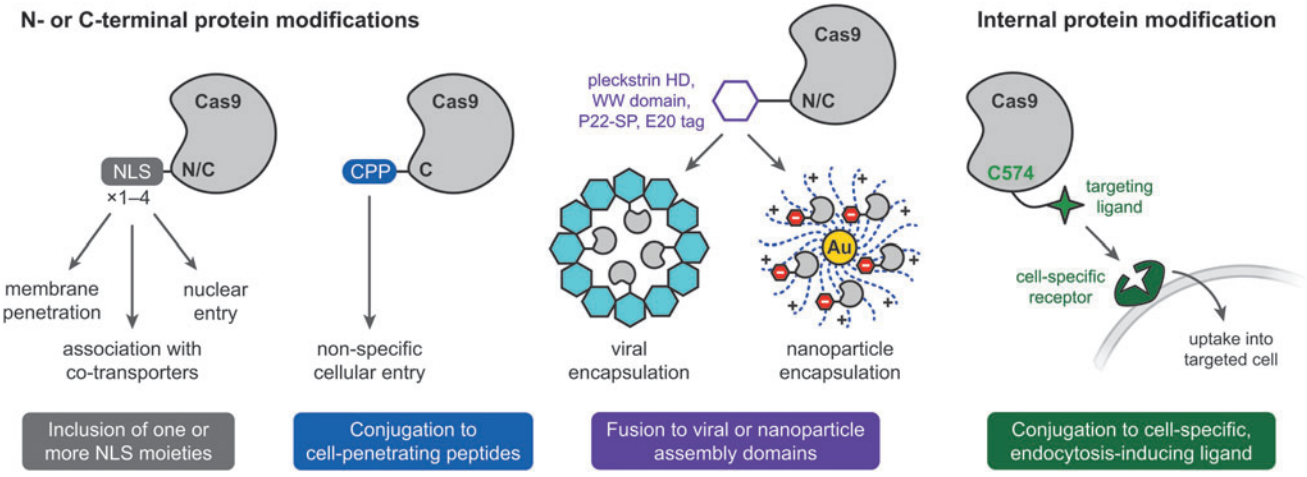


FIG. 4. Engineering of Cas9 protein. The Cas9 protein component has been modified at the genetic level (first and third panels) or post-translationally (second and fourth panels) to improve its capacity to be delivered and/or to perform more effective genome editing.

between the RNP and the CPP as well as interactions of the CPP with endocytic pathways.

Electrostatic interactions can also be harnessed to encapsulate the Cas9 RNP into nanoparticles. One study demonstrated the use of a supercharged tag to mediate nanoparticle assembly.[63] In this case, a glutamic acid tag (up to 20 consecutive residues; ''E20'') was fused to the N-terminus of Cas9 and was able to interact with cationic arginine gold nanoparticles to form nanoassemblies (Fig. 4). Upon incubation with HeLa cells, endocytic uptake of the Cas9 protein assemblies was observed within the first 30 s and reached the nucleus after just 10 min by fluorescent live cell imaging. This result translated to editing when using RNP encapsulation, with ~30% indels at two different loci in three different cell lines.

Another study utilized association mediated by complementary charge by using a trio of C-terminal NLS moieties on Cas9 to load bio-compatible black phosphorous nanosheets with RNP.[64] Following incubation with MCF-breast cancer 7 cells, endosomal localization of Cas9 RNP was observed after just 4 h and extensive (~80%) nuclear uptake occurred after 12 h. Importantly, release of the RNP was mediated by the slow degradation of the nanosheets in the acidic environment of the endosome (~25% in 12 h). Genome editing experiments by co-incubation of mammalian cells with the Cas9 RNP loaded nanosheets achieved between 17% and 32% indels across a range of cell lines.

Encapsulation of the Cas RNP in nanoassemblies is very attractive since this strategy protects the protein and gRNA from degradation before reaching mammalian cells. Choi and colleagues hijacked lentiviral biosynthesis to package the Cas9 RNP.[65] For this work, the Cas9 protein was encoded in the *gag* gene (with a protease cleavage site), upstream of the pleckstrin homology domain that recruits the Gag and Gag-Pol proteins to the membrane. Therefore, the Cas9 protein was released during the maturation of the lentiviral vesicles. To package the RNP, the sgRNA was included in a lentiviral vector under a U6 promoter. Genome editing levels were compared between encapsulated RNPs and DNA-encoded Cas9 lentiviral vectors and showed sensibly lower indels in $CD4^+$ T-cells, 15% versus 20% respectively. However, in a recapitulation of earlier findings, off-target editing was undetectable with the encapsulated RNPs. One advantage of this method is the targeting capability by varying the lentiviral pseudotype. In the aforementioned study, VSV-G envelope protein was used, which has broad infectivity across human cells; however, LCMV (lymphocytic choriomeningitis virus) glycoproteins could be employed to target pancreatic cells.[66]

Similarly, Cas9 RNP was successfully packaged in arrestin domain containing protein 1 [ARRDC1]-mediated microvesicles (ARMMs) and excreted and excreted by mammalian cells.[67] For Cas9 RNP delivery, the authors took advantage of the interaction between ARRDC1 and WW-domain containing proteins, and thus fused WW-domains at the N-terminus of Cas9. After purification of the microvesicles from HEK293T cells, Cas9 RNP loading was validated by qRT-PCR and Western blotting. Fusion of four WW-domains resulted in ~2-fold increase in RNP loading per microvesicle compared to that of two WW fusion domains. Genome editing was evaluated only by knock-out of a single copy of GFP in U2-OS cells and surprisingly resulted in ~5% and 8% editing with two and four WW fusion domains, respectively, although the amount of RNP delivered was 20-fold higher with the four WW fusion domain.

One study successfully encapsulated Cas9 RNP in enterobacteria phage P22 virus–like particles.[68] P22 phage rely on only two proteins for capsid assembly: P22 coat protein and a scaffold protein. For RNP packaging, Cas9 was genetically fused to the scaffold protein in an expression plasmid and expressed in *E. coli* together with a plasmid encoding the P22 coat protein and sgRNA. Presence of the Cas9 protein and sgRNA in the virus-like particles was validated by polyacrylamide gel electrophoresis, and endonuclease activity by *in vitro* cleavage of double-stranded DNA and linearized plasmids. To date, these RNP virus-like particles have not been evaluated for delivery in mammalian cells. In addition, P22 particles encapsulate additional nucleic acids that may trigger innate immune reactions.

Another innovative study employed ultrasound-propelled nanomotors to deliver Cas9 RNPs inside mammalian cells.[69] The nanomotors used here are gold nanowires that are capable of mammalian cell penetration under an ultrasound field. In this study, the motors were functionalized with thiol groups and allowed to react with naturally occurring cysteine residues on the surface of the Cas9 RNP to create disulfide bonds. Surprisingly, upon ultrasound-mediated delivery in GFP-expressing melanoma cells, a 60% decrease in GFP fluorescence was observed already after 30 min and 80% after 2 h. In addition, this knockout was concentration-independent with a mere 0.6 nM concentration being sufficient for 60% decrease in fluorescence after 2 h, significantly outperforming lipofectamine-mediated delivery of Cas9 RNP (by almost 100%).

Engineering Cas9 RNP for Cell-Specific Targeting

A key drawback of most delivery methods is the poor specificity/selectivity for desired tissues or cell types. To address this, one recent study investigated whether

Cas9 RNP could be shuttled into cells by harnessing specific transport receptors.[70] To this end, a ligand that is specifically recognized with high affinity by the asialoglycoprotein receptor (present mainly on hepatocytes), was conjugated onto cysteine residues on the surface of Cas9 (Fig. 4). To increase avidity, an additional cysteine was introduced at the N-terminus of Cas9, allowing for two ligands to be conjugated (including the natural cysteine at position 574), and validated by mass spectrometry. Live cellular uptake imaging showed very rapid uptake only in a hepatocarcinoma cell line, although a high number of nuclear localization signals and/or mCherry fluorescent protein were found to promote slow non-specific uptake. Removal of two NLS and mCherry (leaving only one NLS moiety fused to Cas9) significantly increased the hepato-selective uptake, with a 9-fold relative increase in uptake after 4 h between the conjugated and unconjugated RNP. In addition, the authors identified a small endosomolytic peptide that allows escape of the internalized RNP from the endosome to the cytosol, enabling cellular entry without sacrificing receptor-mediated specificity. Simple co-incubation of the conjugated Cas9 RNP and the endosomolytic peptide with hepatocarcinoma cell line HepG2 generated about 5% editing at an endogenous locus, 10-fold more than what was observed for the unconjugated Cas9 RNP. Thus far, efficiency of this delivery method remains to be seen *in vivo*.

Engineering Cas9 RNP for Tethering a Donor DNA

Homologous recombination (HR) is the preferred mechanism of DNA repair following a DSB when the intention is to insert a novel or corrective DNA sequence (contained in a ''donor DNA'' reagent), as in the generation of reporter lines or when correcting the mutation underlying a monogenic disease. Because HR is typically a rare outcome following a DSB, the development of new biochemical strategies promoting HR is of major importance in the field of genome editing.

Tethering the DNA donor template to the Cas9 RNP is a promising approach: increasing the local concentration of the donor template at the break site would be expected to promote the HR pathway. A variety of methods have been deployed to conjugate the donor DNA to the Cas9 RNP. For example, several Cas9 fusion proteins have been developed, which can recognize tags on the donor DNA. In this vein, Savic and colleagues used SNAP-tag technology to conjugate O^6-benzylguanine–labeled donor DNA to Cas9 protein with a SNAP tag fused at its C terminus.[71] The protein–oligo conjugate was mixed with *in vitro* transcribed sgRNAs to generate the Cas9 RNP-DNA complex. A 33.1% mean correction efficiency of the RNP-DNA system was observed at the *CXCR4* locus in human embryonic kidney (HEK293T) cells, which is a 24-fold increase compared to the classical CRISPR-Cas9 system delivered with an unconjugated single stranded DNA repair template.

Lovendahl and colleagues developed another approach to covalently link proteins and DNA using HUH-endonuclease domains as the fusion tag (HUH-tags), and has the benefit of being able to react with DNA devoid of any chemical modifications.[72] They applied this system to conjugate a single stranded donor DNA to Cas9 protein fused with a HUH-tag.[73] The PCV (porcine circovirus 2 rep protein) HUH-tag can be fused at either termini of Cas9 and covalently conjugated to a donor DNA extended at its 5′ terminus with a 13 nt recognition sequence. Up to a 30-fold enhancement of HR to indel ratio was demonstrated in both HEK293T and U2-OS cell lines at multiple target loci and was more pronounced at low concentrations of RNP. Interestingly, the N-terminus fused Cas9 (PCV-Cas9) shows higher homology-directed repair efficiency than the C-terminus fusion (Cas9-PCV); although the reason remains unclear.

Lee and colleagues have described the direct conjugation of the donor DNA to the gRNA, forming a single molecule termed the ''gDonor''.[74] Following LNP-mediated delivery of a Cas9 RNP incorporating the gDonor, they observed a 3-fold increase in HR compared to RNP delivery with donor DNA provided *in trans*. A similar strategy was used to tether the gRNA to a donor DNA: Carlson-Stevermer and colleagues modified an internal loop of the sgRNA to incorporate the S1m aptamer, which binds to streptavidin with high affinity.[75] Using streptavidin as an adaptor, this modified gRNA could tether a biotinylated donor DNA, proving improvements in HR to indel ratios from 2.7-fold to 18-fold depending on locus and donor identity. While these relative improvements seem promising, it is important to note that the absolute rates of HR seldom surpass in 10% in these contexts, so there is still much room for improvement.

Engineering gRNA for Stability and Fidelity

Since it takes two to tango, it is important to highlight the utility in modifying the gRNA to promote efficient genome editing. The roadmap for modification was established in part by a study examining the roles and impacts of various modules constituting the gRNA.[76] Several engineering advances have since been deployed to enhance gRNA quality, stability, and versatility (Fig. 5).

A typical method of generating gRNA is via *in vitro* transcription (IVT) from a DNA template bearing an appropriate promoter for the enzyme of choice, typically T7 RNA polymerase. Although this is an affordable and convenient way to prepare gRNAs in the laboratory, it has

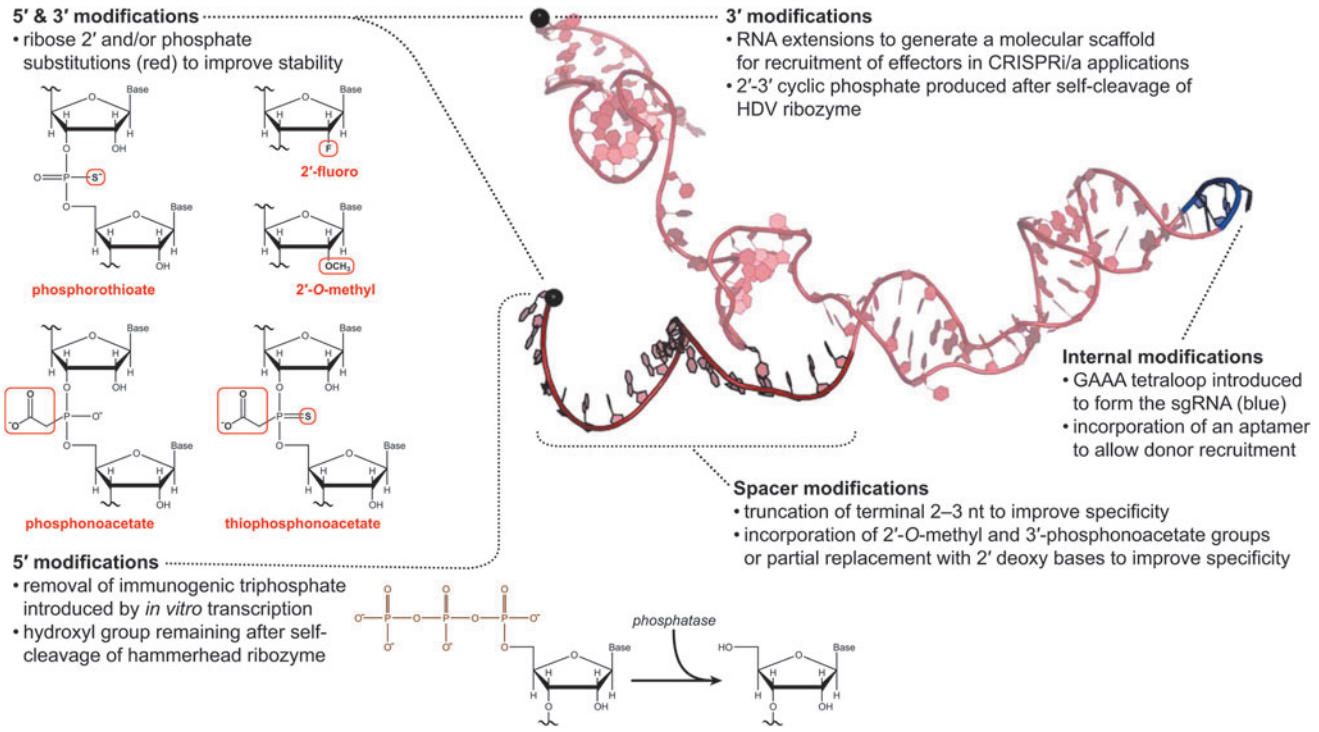


FIG. 5. Engineering of the Cas9 guide RNA. The RNA that guides targeting of Cas9 has been modified chemically, enzymatically, or genetically to promote stability, efficacy, and to prevent immune responses from the cells being editing.

some disadvantages. First, gRNAs produced by T7 RNA polymerase will bear a guanosine triphosphate at the N-terminus. This ''naked'' triphosphate is distinct from the capped terminus of mammalian transcripts and thus triggers a potent innate immune response via recognition by RIG-I.[77,78] Removal of the 5′-triphosphate by treating sgRNAs with a phosphatase ameliorates inflammatory signaling and the toxicity associated with genome editing in T cells[77] and hematopoietic stem cells.[78]

An optimized *in vitro* transcription that sidesteps the immunogenic triphosphate addition involves an IVT template with hammerhead ribozyme[79] and hepatitis delta virus ribozyme[80,81] sequences placed at the 5′ and 3′ termini of the sgRNA, respectively. This is known as an RGR (ribozyme-gRNA-ribozyme) strategy.[82] Following transcription of the RNA, the ribozymes perform self-catalysed cleavage that releases an sgRNA bearing a hydroxyl group at the 5′ terminus and a 2′-3′ cyclic phosphate at the 3′ terminus. This strategy eliminates the requirement for a 5′ G nucleotide associated with T7 RNA polymerase (which constrains selection of genetic targets) and can be used to avoid the 3′-terminal heterogeneity associated with run-off transcription, resulting in exceptionally pure and homogeneous gRNAs for RNP assembly.[70]

One elegant method of preventing degradation by cellular RNases relies on chemical alterations to synthesized sgRNAs. Hendel and colleagues discovered that including 2′-*O*-methyl, 3′-phosphorothioate, or combined 2′-*O*-methyl 3′-thiophosphonoacetate modifications at three terminal nucleotides of the 5′ and 3′ termini enhanced the genome editing efficiency in different cell types when co-delivered with Cas9 mRNA or protein.[83] These modified gRNAs demonstrated a longer lifespan in cells and promoted improved editing when Cas9 was co-delivered as an mRNA or in the context of a pre-formed RNP. Some vendors have incorporated this approach into their manufacturing options, resulting in robust and reliable gRNAs with enhanced stability and editing efficiency *in vitro* and *in vivo*.[44,46] Work by Basila and colleagues used modified nucleotides in a dual-guide system to improve stability and efficacy,[84] and a recent study by Mir and colleagues incorporated 2′-fluoro nucleotides and performed extensive modification scanning to identify gRNAs with increased stability and potency.[85]

The length of the gRNA spacer is a mediator of specificity that can be manipulated to alter the activity of Cas9. One of the first attempts to improve specificity for the target by Cas9 was to elongate the spacer sequence from 20 to 30 nt. Interestingly, this appeared to

result in a subsequent processing back to the natural 20 nt length.[86] In contrast, shortening the guide sequence proved to be a successful strategy for promoting stringent editing. Spacer sequences of 17 or 18 nt have been reported to mediate more precise genome editing, potentially by increasing the sensitivity of Cas9 binding to mismatches within the shorter complementary sequence,[87] while spacer sequences shorter than 16 nt still mediate DNA binding but prevent DSB.[88] Another simple modification adds two additional mismatched guanine nucleotides to the 5′ terminus of the guide RNA.[89] Several studies using such strategies have demonstrated specificity improvements of 10-fold to over 100-fold, although the on-target efficiency varies from comparable to severely reduced.[89–91] Furthermore, recent work from Yin and colleagues demonstrated that the partial replacement of nucleotides in the spacer portion of the gRNA with DNA reduced off-target activity in human cell lines,[92] while Ryan and colleagues observed improved specificity following incorporation of 2′-*O*-methyl and 3′-phosphonoacetate groups into the spacer.[93]

Engineering gRNA for *In Vitro* Protein Evolution

Analogous to the work from Halperin on repurposing dCas9 for random mutagenesis, a prior study named CRISPR-X modified the sgRNA to incorporate two MS2 hairpin binding sites.[94] Following electroporation of sgRNA and MS2-AID fusion proteins in K562 cells expressing dCas9, the authors successfully evolved GFP into EGFP and identified mutations in the 20S proteasome core subunit PSMB5 that rendered K562 cells resistant to the therapeutic cancer drug bortezomib. One could easily imagine electroporating Cas9 RNP directly with the MS2-AID in mammalian cells or deliver it to specific tissues.

Summary and Outlook

The field of nonviral genome editing has made tremendous advances in the last 5 years. However, several remaining challenges must be adequately addressed before more rapid clinical progress can be made. For example, some of the most compelling applications of genome editing involve correcting gene mutations via HR, which requires a variety of cellular factors to be present within cells and it is unclear if adult human tissue will be amenable for HR. In addition, the toxicity of double stranded DNA breaks in cells will also need to be carefully studied, and may pose additional problems in clinical trials.[95] Finally, the Cas9 protein is a foreign protein and may cause an adaptive immune response after multiple injections.[77] However, despite these limitations, genome editing therapeutics have several unique aspects that make it likely that they will ultimately generate significant clinical benefits. For example, the majority of diseases being considered for genome editing based therapies are devastating genetic diseases with no cure. In addition, genome editing therapies only need to be administered once because editing is permanent and can potentially be administered via a local tissue injection or with targeting to the select cell population requiring correction. Under such circumstances, genome editing therapeutics will likely provide significant clinical benefits.

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Author Disclosure Statement

N.M. is a co-founder of GenEdit Inc. The remaining authors declare no competing interests.

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