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Review

Improving CRISPR Genome Editing by Engineering Guide RNAs

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CRISPR technology is a two-component gene editing system in which the effector protein induces genetic alterations with the aid of a gene targeting guide RNA. Guide RNA can be produced through chemical synthesis, *in vitro* transcription, or intracellular transcription. Guide RNAs can be engineered to have chemical modifications, alterations in the spacer length, sequence modifications, fusion of RNA or DNA components, and incorporation of deoxynucleotides. Engineered guide RNA can improve genome editing efficiency and target specificity, regulation of biological toxicity, sensitive and specific molecular imaging, multiplexing, and editing flexibility. Therefore, engineered guide RNA will enable more specific, efficient, and safe gene editing, ultimately improving the clinical benefits of gene therapy.

Highlights

Independently from engineering effector proteins, such as Cas9 and Cpf1, the engineering of guide RNAs themselves has provided useful and versatile options to improve the CRISPR technology as a genome editing toolset.

Guide RNAs can be prepared by chemical synthesis, *in vitro* transcription, or intracellular transcription systems.

Guide RNAs can be engineered in several ways, including chemical modifications, alterations in the spacer length, sequence modifications in the spacer or scaffold, fusion with additional DNA or RNA components, and partial replacement with DNA.

The engineered guide RNAs contribute to improved genome editing efficiency and target specificity, regulation of biological toxicity, sensitive and specific molecular imaging, multiplexing, and genome editing flexibility.

A Guide to Guide RNA Engineering

Genome editing took a dramatic turn with the development of the **clustered regularly interspaced short palindromic repeats** (**CRISPR**; see Glossary) system [1,2]. CRISPR is distinct from other genome editing tools, such as **zinc finger nucleases (ZFNs)**, **transcription activator-like effector nuclease (TALEN)**, and meganuclease, because it uses an RNA guide [3]. Unlike those protein-guided tools, CRISPR is a two-component system for which the **guide RNA (gRNA)** can be produced and modified independently from the effector nuclease (Box 1). This feature confers ease and flexibility to improve CRISPR as a genome editing tool. With respect to performance, CRISPR technology has also shown unsurpassed efficiency, wide applicability, and low target limitations, which are the main reasons for the fast and wide acceptance of the technology [4,5]. Furthermore, it is highly amenable to easy modifications, which has formed the basis for its remarkable technical evolution [6].

To improve CRISPR as a genetic modifier, this technology can be engineered in two ways: protein engineering of the effector nucleases, Cas9 or Cas12a (formerly Cpf1); and gRNA engineering. Engineering the catalytically inactive **dCas** [7] has made it feasible for the dead nucleases to be fused to a variety of functional enzymes for **base editing** [8–11], epigenetic regulations [12,13], **transcriptional inhibition (CRISPRi)/activation (CRISPRa)** [14,15], and library screening [16]. Moreover, Cas9 variants created by rational design or directed evolution have enabled high-fidelity gene targeting [17–20]; as such, systematic review articles covering the adopted engineering methods, applications, and prospects for Cas engineering are available [6,21,22]. Meanwhile, efforts to engineer gRNAs have been no less rigorous than efforts to engineer Cas proteins, but no comprehensive review of gRNA engineering exists yet. Thus, we review recent developments in gRNA engineering, including various types of gRNA engineering and their applications to genome editing. Current limitations and future directions are also provided to make the CRISPR system readily applicable to **gene therapy** and clinical settings.

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Multiple Approaches to Producing gRNAs

It is helpful to understand how to produce gRNAs for the purpose of gRNA engineering. The chemical identity of gRNAs is a stretch of ribonucleic acids comprising a ribose sugar and bases connected by a phosphate group. gRNAs can be produced by chemical synthesis, *in vitro* transcription, and intracellular transcription machinery. Chemical synthesis is a rapid and inexpensive platform that provides the intended gRNA at sufficient amounts and with ensured purity. It is technically unfeasible to synthesize lengthy RNAs, but gRNAs used in the CRISPR system are around 100 nucleotides (nt) or shorter. The length of SpCas9 **single-guide RNA (sgRNA)** (~102 nt) is on the borderline of ample and high-fidelity synthesis, but it may be desirable to adopt the two-guide RNA system comprising CRISPR RNA (crRNA) (~42 nt) and trans-activating crRNA (tracrRNA) (~75 nt). The use of two-guide RNA also offers efficient genome editing, nearly equivalent to sgRNA [23]. The CRISPR-Cas12a system has much lower obstacles in this regard: Cas12a requires no tracrRNA and binds only crRNA (~43 nt), which is approximately half the size of the Cas9 sgRNA [24]. In addition to productions with high purity and bulkiness, chemical synthesis allows for structural modifications with functional groups during synthesis. One additional merit is that synthetic gRNAs are complexed *in vitro* with a nuclease to form a **ribonucleoprotein (RNP)** complex in a stoichiometrically controlled manner [25,26]. Moreover, the use of RNP can provide a more specific gene editing, compared to the use of a vector system [25–27].

Other gRNA production methods rely on a biological protein expression system. One good option to produce gRNAs is an *in vitro* transcription system, which has been widely used to obtain radiolabeled and nonisotopically labeled RNA probes in blot hybridizations and nuclease protection assays. This system requires RNA polymerase, template DNA, NTPs, and Mg^{2+} in a buffer system. A linear or linearized DNA template is used to yield gRNAs with a defined terminal sequence: the phage polymerase-T7 promoter combination is a commonly used system for *in vitro* transcription, and T7 shows no defined transcriptional termination sequence, as opposed to the U6 promoter [28]. A uniform and consistent acquisition of genome editing also depends on the purification of the transcribed gRNAs with high purity and integrity. Alternatively, gRNAs can be produced intracellularly using the transcription machinery. In eukaryotic cells, a U6-gRNA cassette uses the RNA polymerase III-dependent transcription and requires 5 × or 6 × Ts in the nontemplate strand for transcriptional termination [29]. Multiple gRNA sequences can be incorporated in the 3′-untranslated region of mRNA transcripts [30], which can be trimmed by an effector protein with RNase activity, such as Cas12a [31], thereby generating multiple individual gRNAs [30].

Modality of gRNA Engineering

A limitation of protein engineering is that it depends mostly on amino acid sequence alterations, or on protein fusions. Although chemical syntheses of proteins can be performed, they pose significant hurdles in the size, homogeneity, and mass production. In contrast, gRNAs can be obtained by either chemical synthesis or biological expression *in vitro* as well as *in vivo*. This property forms the basis of the flexibility and variability of gRNA engineering and the upscaled production of engineered gRNA (Figure 1).

Covalent Chemical Modifications

The addition of functional groups, or the chemical modification of nucleotides, has been a routine practice for laboratories and commercial vendors. A variety of chemical modifications are amenable to the synthesis of gRNAs during or after a synthetic process. In particular, the phosphate backbone and the ribose sugar are useful sites for chemical modifications. Additionally, the 5′-end phosphate group can be targeted for gRNA engineering (Figure 2).

Glossary

Base editing: a genome editing technology that introduces a base change in specific sites of DNA or RNA without DNA cleavage using a base modifying enzyme fused to a catalytically inactive nuclease.
Cas13a: a Class 2 type VI-A CRISPR-Cas effector protein which can cleave target RNA and maturate crRNA.
Clustered regularly interspaced palindromic repeats (CRISPR): repetitive DNA units of a scaffold repeat and a variable sequence spacer in prokaryotic genomes that express a guide RNA from each spacer-repeat unit.
CRISPRi/a: a technique inducing target-specific inactivation or activation of gene expression by using a guide RNA and a catalytically inactive Cas nuclease fused to a transcriptional repressor or activator protein.
dCas: a catalytically inactive form of Cas9 or Cas12a that is made by mutating one or two amino acids in the endonuclease domains.
Gene therapy: medical approach to cure a disease by introducing genetic materials or gene-modified cells into a patient's body.
Guide RNA (gRNA): an RNA molecule that interacts with effector nucleases, such as Cas9 or Cas12a, and has a role in target identification by base pairing with a strand of target.
Homology-directed repair (HDR): a repair mechanism in cells for double-strand DNA breakage. Cells can precisely repair the DNA breakage in the presence of a homologue piece of template DNA.
Indel efficiency: frequency of insertion and deletion mutations triggered by double-strand DNA breakage of a target site.
Non-homologous end joining (NHEJ): a pathway to repair double-strand DNA breakage without a template DNA. This process is error-prone and thus used for gene ablation.
Off-target: a target deviation caused by the action of programmable nucleases on unintended sites.
Protospacer adjacent motif (PAM): a short nucleotide sequence of 2–6 nt flanking the 5′-upstream or 3′-downstream of the target site.

The phosphodiester groups in oligonucleotides are targets of ribonucleases in cells and blood, and replacing the phosphate backbone with nuclease-resistant groups has been attempted for RNA-based therapeutics [32]. In addition, modified phosphodiester linkages were suggested to enhance the nuclear localization of RNA molecules upon transfection using cationic lipids [33]. First, the phosphorothioate (PS) bond was extensively tested in multiple studies, which was proven to be effective *in vitro* [23,34–36] and *in vivo* [37,38]. Phosphate modifications with thiophosphonoacetate (thioPACE) or phosphonoacetate (PACE) showed a similar or better performance, compared to PS modification [34,39]. A number of sugar modifications have been made at the 2′-hydroxyl (OH) group of a ribose, and gRNAs with 2′-O-methyl (M), 2′-O-fluoro (F), S-constrained ethyl (cEt), and 2′,4′-bridged nucleic acid *N*-methylated (BNA^{NC}[N-Me]) modifications have been tested [23,34–36,38,40,41]. Modification with a bulky residue at this position including M, F, and 2′-O-methoxyethyl (ME) group stabilizes the RNA-like C3′-endo sugar pucker [42]. Additionally, M and F modifications are associated with cellular stability [43] and decreased immunostimulation [44]. Locked nucleic acid (LNA) modifications also lead to RNA stability itself [45], and RNA–DNA duplex stabilization [40]. The 5′-terminal phosphate group of a gRNA has been modified into a 5′-hydroxyl group for undesirable biological responses [46]. In theory, bases can be modified by pseudo bases, or modified ones, as with the production of mRNA [41], but this approach has not been attempted in gRNA engineering.

From the results obtained in previous studies, we can identify several general guidelines for designing gRNA: (i) the seed region of gRNAs is less tolerable to any chemical modification; (ii) engineering the phosphate and OH groups that interact with Cas9 or Cas12a for target recognition or as a scaffold may yield an unfavorable outcome. Nonparticipatory sites would be targeted for modifications, such as the tetraloop or the upper stem of the Cas9 sgRNA [47]; (iii) when appropriately combined, the phosphate and ribose modifications can have beneficial effects; (iv) each Cas9 and Cas12a ortholog may have different tolerance profiles for modification, and thus an optimal modification pattern needs to be investigated on a case-by-case basis.

Alterations in the Spacer Length

Target recognition in the CRISPR system is mediated by protospacer–**spacer** base pairing upon locating a **protospacer adjacent motif (PAM)** sequence. It is likely that prokaryotes have adopted a relatively long spacer–protospacer base pairing to precisely discriminate the invading genome from its own. The CRISPR system uses from 20 to 72 nt spacer lengths in gRNA [1,2,48]. However, the CRISPR system tolerates a mismatch of several (as many as 5 nt for SpCas9) nucleotides, which is the basis for the unwanted **off-target** activity [49]. By contrast, researchers have expanded the utility of the CRISPR system by altering the spacer length.

gRNAs have been engineered to be either a truncated or extended gRNA. The full activity of the CRISPR system tolerates truncation of a few nucleotides particularly in the PAM-distal spacer region for Cas9 [50–52], **Cas13a** [53,54], and Cas12a [55,56]. While retaining the cognate activity, the truncated gRNA creates additional effects on programmable genome engineering. However, special care should be taken when this strategy is applied to stem cells [57]. Even shorter Cas9 gRNAs result in impaired indel activity, but they can be used for orthogonal applications [51,52]. Conversely, gRNAs with an extended spacer length, by up to 4 nt, can be used to base edit a specific site with increased efficiency within a base editing window [58].

Sequence Modifications

Minor changes in the gRNA sequence can be made in either the spacer or scaffold region of gRNAs. One or two guanidines have been added 5′-upstream of the 20 nt spacer for efficient T7 *in vitro* transcription with improved specificity [59]. A predesigned mismatch can be placed in the spacer to discriminate slightly different targets, such as a single-nucleotide

This motif is recognized by effector nucleases prior to target identification.

Protospacer flanking site (PFS): a specific sequence motif which consists of a single H (not G) and is recognized by Cas13a for target identification. It acts like a PAM sequence of other CRISPR-Cas system.

Ribonucleoprotein (RNP): a gRNA-effector nuclease complex that is formed at a 1:1 stoichiometry.

Single-guide RNA (sgRNA): an artificial guide RNA that is constructed by connecting trimmed crRNA and tracrRNA with a tetraloop comprising four nucleotides.

Spacer: a DNA sequence retrieved from invasive genetic materials and incorporated into the CRISPR array in prokaryotic genomes. The newly incorporated sequence acts as a cellular memory for viral invasion.

Transcription activator-like effector nuclease (TALEN): an artificial programmable nuclease made of TAL effector DNA binding domains and a nonspecific DNA cleavage domain from FokI endonuclease.

Zinc finger nucleases (ZFNs): an artificial programmable nuclease that consists of multiple zinc finger binding domains recognizing specific DNA nucleotides and a nonspecific DNA cleavage domain from FokI endonuclease.

Box 1. How Are Guide RNAs in the Class 2 CRISPR System Constituted?

Type II, V, and VI CRISPR systems belong to the Class 2 CRISPR, which is characterized by a single monomeric endonuclease as an effector protein. Guide RNAs are constituted differently for each type (Figure I). The guide RNA for the type II CRISPR system consists of crRNA and tracrRNA, which are hybridized via a base pairing of a 3′-repeat of crRNA and a 5′-anti-repeat of tracrRNA. The hybridized gRNA binds to Cas9 forming an RNP complex, which then scans the double-stranded target DNA. The target identification is mediated by a Watson–Crick base pairing between the spacer and protospacer complementary DNA strand upon recognition of a PAM sequence by the Cas protein. sgRNA, which is an engineered form of the two RNA components connected by a tetraloop, is alternatively used for a variety of purposes [88]. The nexus or hairpins in tracrRNA constitute an additional secondary structure. Cas12a, Cas12b, and Cas12c comprise the type V CRISPR system, of which, Cas12a offers the most extensively characterized genome editing toolset [24]. In type V CRISPR, crRNA alone functions as a guide RNA without tracrRNA. The direct repeat sequence constitutes a single hairpin structure at the 5′-region of crRNA with the spacer 3′-oriented. PAM, usually a T-rich motif, is located at the 5′-end of the displaced strand of the protospacer. Type VI is an RNA-guided RNA-targeting CRISPR system with Cas13a acting as an effector nuclease [53]. The gRNA for Cas13a is similar in structure to type V crRNA. A single stem loop occupies the direct repeat, and the target identification is mediated by RNA–RNA hybridization. The **protospacer flanking site (PFS)** sequence (A, U, or C for LshCas13a) is adjacent to the 3′-end of the protospacer and affects the Cas13a-mediated RNA cleavage.

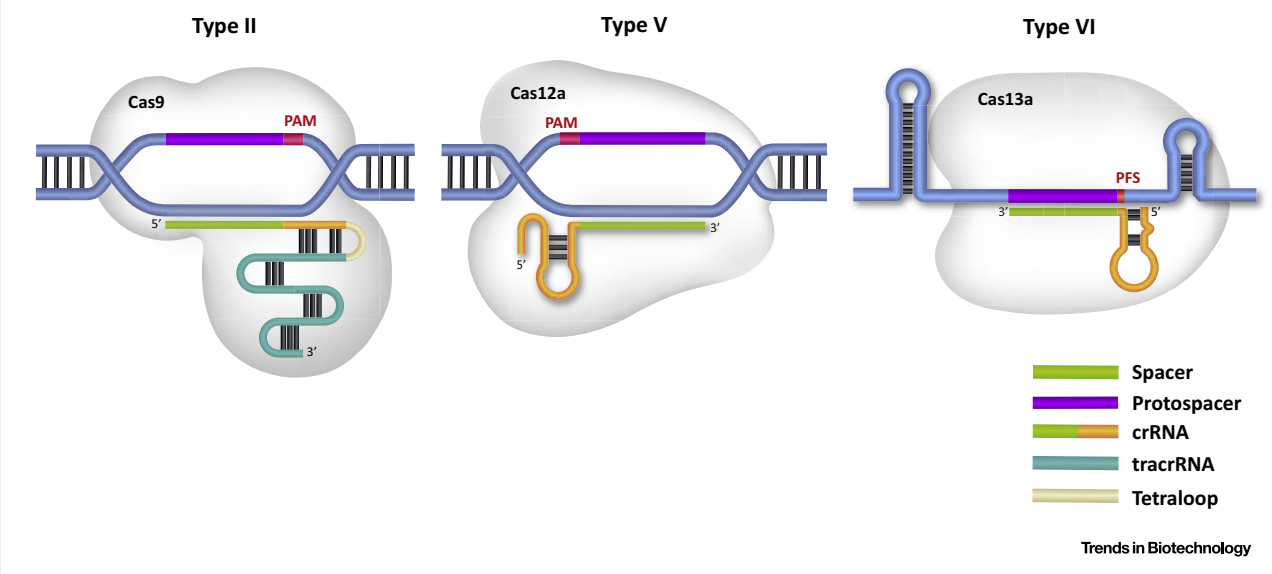

Figure I. Class 2 CRISPR Systems and the Modes of Target Modifications. Cas9 is complexed with crRNA and tracrRNA, or sgRNA in the Type II system. The RNP forms induce double-stranded breakages, rendering blunt ends. In the type V and VI systems, crRNA binds to Cas12a (or Cas12b, or Cas12c) and Cas13a, respectively. The gRNA-Cas12a complexes induce double-stranded breakages in a staggered manner, while the crRNA-Cas13a RNP complexes act as an RNA cutter.

mutant [60] or different viral lineages [61]. CRISPR-Cas12a appears to be more flexible for sequence alterations because the spacer is 3′-oriented and the essential spacer length for full activity is approximately 20 nt. The remaining three to four spacer nucleotides at the 3′-end can be further modified with an additional tail [56].

The gRNA scaffold for SpCas9 can be slightly altered in the crRNA repeat sequence with a tetra uridine by A–U flipping [62] or U-to-C (or G) conversion [63]. crRNA modifications have shown a synergistic effect when combined with an extension of the crRNA–tracrRNA duplex length by approximately 5 bp in the tetraloop region [62,63]. Alternatively, when an A–U pair in the stem loop of sgRNA was replaced with a G–C pair, this showed increased signal-to-noise values without duplex extension during cellular imaging [64]. Interestingly, even a 3′-truncated mini tracrRNA (63 nt) can retain nearly full Cas9 activity [65]. The nexus and hairpin sequences can be swapped between family members for cross CRISPR activity [66].

RNA–DNA Hybrid Guide RNA

DNA can be considered as a chemically modified form of RNA, but this natural nucleotide will be discussed separately in this section because the use of the hybrid gRNA allows for a wider

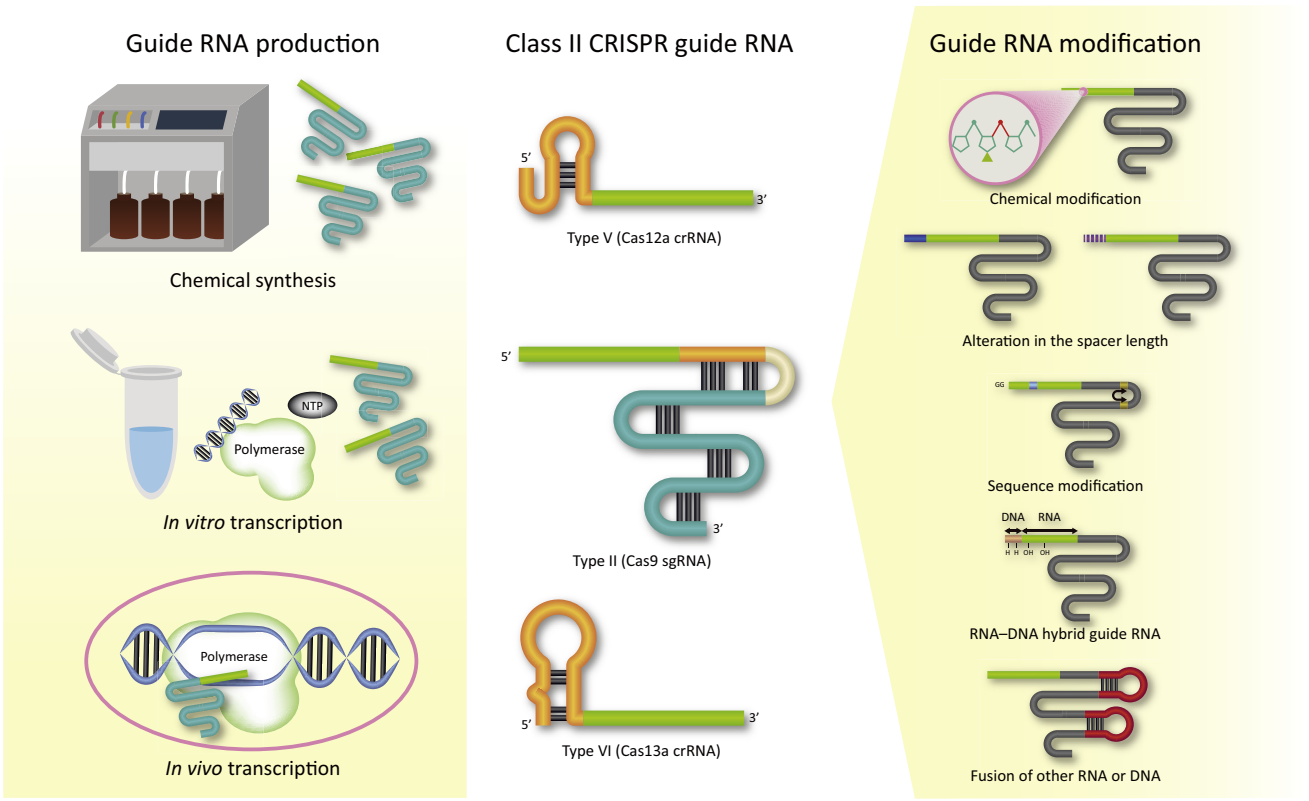

Figure 1. Guide RNAs for Class 2 CRISPR Systems and Their Production and Modification Strategies. Type II, V, and VI CRISPR systems constitute the Class 2 system, for which guide RNAs bind to monomeric endonucleases to induce modifications in nucleotides. Type II guide RNA consists of crRNA and tracrRNA, and their conjugated form, known as single-guide RNA (sgRNA), can be alternatively used. In contrast, type V and type VI systems are guided by crRNA alone. Guide RNAs can be produced through chemical synthesis, *in vitro* transcription, or intracellular *in vivo* transcription. The canonical guide RNAs can be either used directly or modified for a variety of purposes. Engineering of guide RNAs can be achieved through one or more modalities among chemical modifications, alterations in the spacer length, sequence modifications, the fusion of RNA or DNA components, and selective replacement with deoxynucleotides. The engineering modality was exemplified by Type II sgRNA for Cas9, but can be identically applicable to Type V and VI gRNAs. To visualize modification sites more clearly, the scaffold of sgRNA was shown in gray in the 'Guide RNA modification' part. Please see Figure I in Box 1 for what each color represents in guide RNAs.

range of applications than any other chemically modified gRNAs. A lower production cost and higher stability of gRNAs can be achieved by partially replacing RNA with DNA. Moreover, an RNA:DNA duplex confers a thermodynamic stability to the gRNA scaffold. The sites and lengths of a replacement can be determined after investigating the sugar moiety dependency of the Cas protein [67]. Generally, the important ribose sugar contacts for interacting with Cas9 or Cas12a should be preserved. Although it has not been investigated for a large number of targets, chimeric DNA–RNA crRNA with 8 nt DNA in the spacer plus 16 nt DNA in the scaffold, showed uncompromised genome editing efficiency in human cells [68]. The further incorporation of DNA into gRNA accordingly depends on the architecture of the gRNA–nuclease complex. Thus, a concomitant engineering of the effector protein, such as by directed protein evolution, may provide an opportunity for an advanced hybrid or even a full DNA guide.

Fusion of Independent Components

This type of modification differs from chemical modification because structural RNA or DNA sequences are incorporated within or alongside the gRNA sequence instead of chemical

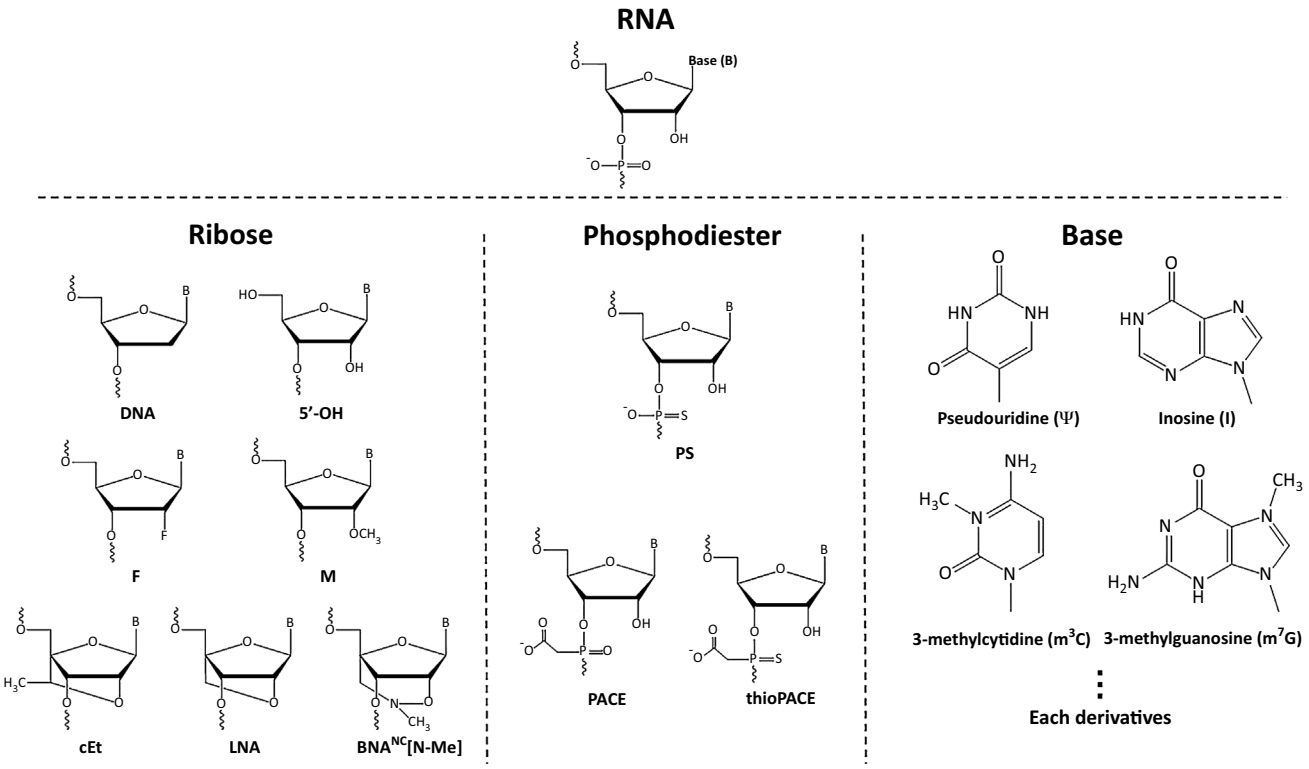

Figure 2. Chemical and Structural Modifications of Guide RNAs. Guide RNAs can be engineered using chemically and/or structurally modified nucleotides or their analogues. Basically, these modifications can be made in the ribose, phosphodiester linkage, or base. Ribose modifications are mainly made at 2′-OH. A 2′-deoxy-, 2′-fluoro- (F)-, or 2′-O-methyl- (M) ribose can substitute for an unmodified ribose. A triphosphate at the 5′-end ribose can be altered into a 5′-OH form. A locked nucleic acid (LNA) with a bridge connecting 2′-oxygen and 4′-carbon offers a modification option together with S-constrained ethyl (cEt) and next-generation bridged nucleic acids (BNA^{NC}[N-Me]). The phosphodiester can be modified into phosphorothioate (PS), phosphonoacetate (PACE), or thiophosphonoacetate (thioPACE) linkages. These modifications may affect the resistance against ribonuclease activity and thus stability, and they may affect the RNA binding properties with effector proteins. Pseudo bases or a variety of base derivatives can be used for gRNA engineering.

functional groups. MS2 and PP7 are viral RNA scaffolds that are recognized by the MCP and PCP RNA-binding proteins, respectively [69,70]. Those scaffolds can be either synthetically or transcriptionally fused to gRNA to recruit a variety of transcriptional activators, suppressors, or protein modifiers. When combined with dCas, the engineered gRNA can enable the target-specific reprogramming of gene expressions [71,72]. A structure-guided incorporation may be implemented in the sgRNA tetraloop, the hairpins, or the 3′-terminal region, through which multiplexed recruitment of specific proteins can be achieved [64,72–74]. A fused RNA sequence can act as a catalytically active enzyme [75–78] or an enzyme substrate [79]. Donor DNA can be fused to gRNA to modulate the **homology-directed repair (HDR)**-mediated gene correction efficiency [80].

Effects of gRNA Engineering

CRISPR technology has been deemed to ‘democratize’ the genome editing field due to its technically easy access to users, its high efficiency, and low cost. These merits have provided additional opportunities for users with various expertise to participate in the refinement and improvement of this technology [4,5]. The molecular engineering of gRNA, partially backed up by chemical and biological knowledge, has culminated in a significant improvement in the

genome editing field in various aspects, including editing efficiency, target specificity, avoiding undesirable biological responses, molecular imaging, multiplexing, and editing flexibility. The value created by gRNA engineering is described later and summarized in (Table 1, Key Table).

Improved Genome Editing Efficiency

Although the CRISPR system shows high genome editing efficiency for a wide variety of genomic targets, efforts to improve the efficiency are still desired, at least for certain applications. Various chemical modifications, including F, M, PS, and thioPACE, have enhanced the on-target **indel efficiency** of CRISPR-Cas9 when incorporated at tolerant positions [23,34–37]. 2′-Fluoro ribosyl repeats in up to 5 nt could also effectively improve the indel efficiency of Cas12a [41]. A polyuridinylated 3′-tail in crRNA created an improved Cas12a activity, comparable to that of SpCas9 [56]. Unless it harms the nuclease interactions, the scaffold optimization of gRNAs could also improve the **non-homologous end joining (NHEJ)**-dependent indel efficiency [62,63]. The HDR efficiency was also enhanced by a gRNA-donor DNA conjugation [80]. Fusion of multiple viral RNA hairpins has enabled the reprogramming of gene expressions [71].

Lowered Off-Target Effects and Improved Specificity

Accurate genome engineering has been achieved by the engineering of SpCas9 [17,18,81] or by the use of Cas12a [17,82]. In parallel, efforts to engineer gRNAs have contributed to 'high-fidelity' genome editing by the CRISPR system. The addition of extra nucleotides [58,59] and the truncation of nucleotides [50,55] have supported highly specific genome editing. However, the combination of engineered Cas9 and gRNA yielded unfavorable outcomes, which were also overcome by the fusion of catalytically active ribozymes to gRNAs [75]. Fused aptazymes can be used to confer tissue and cellular specificity in genome engineering [78]. RNP complex guarantees a more specific gene editing [25], and gRNA modifications can enhance specificity. Modified phosphodiester linkages, either alone or together with ribose 2′-modifications, confer significantly improved target specificity by Cas9 [35,39,40]. Improved specificity was also obtained by introducing deoxynucleotides to create an RNA–DNA hybrid guide [68].

Obviating Cellular Toxicity and the Immune Response

Toxicity and immunogenicity are among the main concerns in clinical settings, but have been relatively less explored. gRNAs that are engineered to boost the potency and specificity likely evoke undesirable cellular and immune responses because of their 'xenobiotic' nature. Currently, we can glimpse the effects through a handful of studies. Efficient genome editing was achieved by heavily modified gRNAs with the potential to reduce immunogenicity [36], and a partly modified sgRNA did not induce acute or chronic liver toxicity *in vivo* [37,38]. However, one study warned that a higher degree of phosphorothioate modification may evoke severe cellular toxicity [23]. Another study found that the 5′-triphosphate group in gRNAs can trigger innate immune responses and cellular toxicity in human and murine cells [46,83]; thus, enzymatic dephosphorylation is required to produce a 5′-hydroxyl guide for safe editing.

Specific and Sensitive Molecular/Cellular Imaging

Visualized data on genomic elements provide directly useful information about biological and pathological events inside cells. In contrast to fluorescence *in situ* hybridization, the CRISPR system using dCas supports highly specific, live cell imaging [62,84,85]. Initially, dCas9 fused with green fluorescent protein was combined with unmodified gRNA to visualize genomic loci in cells [62]. The imaging of loci was multiplexed by expanding to multiple Cas9 orthologs [86,87]. However, this approach may be hampered by the labor-intensive preparations of fusion proteins, and target limitations due to low-incidence PAM sequences. In contrast, gRNA

Table 1. Key Table

Engineering method	Guide RNA preparation method[a]			Effects created by guide RNA engineering					
	Chemical synthesis	*In vitro* transcription	*In vivo* transcription	Efficiency	Specificity	Toxicity	Imaging	Multiplexing	Other applications[e]
Chemical modification[b]	○	Δ	X	M [34–38] MS [23,34,37] MSP [34] PS [35–38] F [35–37,41] cEt [35] F, M, MS, FS, PS [37] F-cEt [35]	F, cEt [35] MP [39] BNA^{NC}[N-Me] [40]	5′-OH [46,83]			
Spacer length	○	○	○	Truncated [35]	Truncated [35,50,55]				Truncated [55] Extended [58]: Target flexibility Truncated [51,52]: Orthogonality
Sequence modification	○	○	○	Polyuridinylated [56] A–U flip [62,63] Hairpin extension [62,63] U to C/G conversion [63] A:U to G:C conversion [64]	5′-GGX20[c] [58,59] A–U flip [62] Hairpin extension [62]		A–U flip[62,63] Hairpin extension [62,63] A:U to G:C conversion [64]		Domain swap [66] :Orthogonality
Fusion	○	○	○	gRNA-donor [80] Viral hairpins [71,72] 5′-Ribozyme [75] Pairing RNA [79]	Ribozyme [75] Aptazyme [78]		Viral hairpins [64,72–74] PUF-binding site [74] MS2-binding variant [74] Spinach2 [72]	Multi-gRNAs [30,89] Viral hairpins [31,71] Ribozyme [76,77]	Tetraloop [88] :Simplicity Ribozyme [76,77] :Promoter flexibility
Hybrid guide	○	Δ	X		crHyb:tracrHyb[d] [67] crHyb (10DNA) [68]				

[a]○, compatible; X, incompatible; Δ, difficult but not impossible.
[b]M, 2′-O-methyl ribose; MS, 2′-O methyl ribose, 3′-phosphorothiate; MSP, 2′-O-methyl ribose, 3′-thiophosphonoacetate; PS, 3′-phosphorothioate; F, 2′-fluoro ribose; FS, 2′-fluoro ribose, 3′-phosphorothioate; cEt, S-constrained ethyl ribose; BNA^{NC}[N-Me], *N*-methyl substituted, 2′, 4′-bridged ribose; F-cEt, 2′-fluoro, S-constrained ethyl ribose.
[c]Addition of two guanidines 5′-upstream of the 20 nt spacer.
[d]crHyb and tracrHyb denote the partially DNA-exchanged crRNA and tracrRNA, respectively.
[e]Other applications include simplicity, flexibility, or orthogonality in genome engineering.

engineering is more manageable, and was applied to the multiplexed labeling of genomic loci and signal amplifications [64,73]. The structure-guided insertion of RNA aptamers preserved the specificity of targeting and the photostability. A biomolecular fluorescence complementation method combining dCas9 and gRNA labeling approaches was used to achieve zero-tolerance nonspecific labeling [74].

Other Applications

Additional value has been created by gRNA engineering other than that described earlier. In practice, the history of gRNA engineering began concomitantly with the development of the CRISPR technology. sgRNA is the product of trimmed tracrRNA–crRNA fusion using a tetraloop [88]. In addition to potency, the development of sgRNA has indeed significantly simplified applications of the CRISPR-Cas9 system. Multiplexing is achieved by incorporating a CRISPR array of gRNAs into a DNA expression vector [30,89], from which transcripts are produced and cleaved into individual gRNAs by the Cas12a [32], Cas13a [31,90], or fused ribozymes [76,77]. Multiple scaffolds inserted into gRNAs enable multigene regulation by the dCas9 system [71]. The flexibility in the editing window can be improved by simply altering the spacer length of the gRNA [55,58]. Ribozyme-fused gRNA can also expand the flexibility in the promoter selection [76,77]. Engineered nexus and hairpin structures in tracrRNA can direct Cas9 orthogonality [66].

Concluding Remarks and Future Perspectives

gRNAs are an equally important part of CRISPR activity along with nuclease proteins. Accordingly, gRNAs will also need to be engineered to meet the criteria for therapeutic applications. Three main elements: safety, efficacy, and integrity, are extensively investigated during drug development. Hence, gRNA has been engineered to improve genome editing efficiency [35,41,56,63], reduce off-target effects [39,50,58,75], and enhance stability in biological milieu [34,37]. However, some gRNA modifications, particularly chemical modifications, may evoke antigenicity and cytotoxicity when administered *in vivo* because the modified unnatural structures could be recognized by immune surveillance. Moreover, such modifications may induce cellular toxicity [23]. Experience in the development of siRNA therapeutics indicates that the well-established modifications in ribose (M, F, and LNA) and in the phosphate backbone (PS) do not trigger severe toxicity *in vivo* [91], but special care needs to be taken when additional or untested modifications are incorporated. Thus, gRNAs should be engineered in a multifaceted manner so that one improved attribute does not ruin the other attributes. Integrated engineering should develop a 'multipotent' gRNA that confers high specificity, efficiency, and stability, but less toxicity.

CRISPR technology has been improved by engineering effector nucleases and gRNAs. Benefits attained by engineering Class 2 Cas proteins have been similarly but independently pursued by gRNA engineering. For instance, target specificity has been improved by engineering Cas9 [17,18] and sgRNA [34,35,40,50,59,63]. However, these two improvements were made separately, and were seldomly combined. Combining these two strategies could amplify the individually obtained values in a synergistic manner or an additive manner [20]. Given the importance of specificity in genetic screening, an engineered gRNA library could facilitate genome-wide screening.

Several gRNA modifications have been structurally guided [18,80], which has informed the synthesis and testing of a limited set of gRNAs with biased or intended modifications. Very little of the proposed gRNA engineering has been backed up by a mechanistic study [36]. Furthermore, because most gRNA engineering effects have only been tested with a limited number of

Outstanding Questions

The effects of gRNA engineering have mostly been investigated for a small set of target genes. Could the effects of gRNA engineering be generalizable for every target?

What molecular, structural, and biological perturbations occur due to gRNA engineering? If such mechanistic studies are verified, will the proposed structure have room for further refinements, and to what extent?

Which gRNA engineering should be combined with protein engineering for the best synergistic effects to improve genome editing?

How can we obtain a 'multipotent guide RNA' that has a high specificity, efficiency, and stability, but less toxicity?

To what extent can guide RNA engineering contribute to the effectiveness and success rate of the CRISPR technology in genome editing-based gene therapy?

targets without large-scale validation, questions still remain that need to be addressed. Can such effects be generalizable for every target? Does the proposed structure leave no room for further refinement? What molecular, structural, and biological perturbations occur due to the gRNA engineering? Fortunately, useful techniques are available to delineate the molecular and biological events, including FRET, x-ray crystallography, kinetics tools, and others. Thus, if backed up by more rigorous mechanistic studies, engineered gRNAs should create further opportunities for improving CRISPR as a genome editing toolset.

Drugs are subjected to rigorous testing during their development with respect to pharmacokinetics and pharmacodynamics. These properties can influence the dosing, efficacy, and adverse effects of gene editing therapeutics. Surely, a gRNA, alone or in a complex with an effector nuclease, will be subjected to testing in terms of both properties. The modified gRNA will affect both the molecular behavior inside the body (pharmacokinetic properties) and the responses of the body (pharmacodynamic properties). gRNA engineering should therefore focus on both pharmacokinetic and pharmacodynamic properties. Studies on the distribution, duration, cellular uptake, or nuclear localization in target tissues or cells are particularly necessary, because they should be tightly regulated to minimize unwanted off-target effects and to foster specific editing efficiency at the intended sites.

Gene therapy has come of age, with the past several years witnessing remarkable progress. Chimeric antigen receptor-T (CAR-T) cells received FDA approval in 2017 for patients with lymphoid malignancies. In the same year, advances in gene complementation therapy culminated in their approval for the treatment of RPE65 null congenital blindness. Although we have more years (up to 10 years) to go until the first approval of gene editing-based therapeutics, several trials have been placed in clinical pipelines, including *ex vivo* [92] and *in vivo* therapies [93]. Increasingly, more clinical trials will appear, with the majority of them likely to adopt CRISPR technology. Moreover, without a doubt, the success of clinical trials using CRISPR technology will rely on how meticulously the gRNAs will be engineered (see Outstanding Questions) to edit genomes with high specificity, efficiency, and safety.

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