Gene Editing

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Modified RNAs in CRISPR/Cas9: An Old Trick Works Again

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In the last few years, a new targeted genome-editing technology based on the adaptive immune system of prokaryotes, known as clustered regularly interspaced short palindromic repeats (CRISPR),[1] has become a favoured technique for genome engineering. Its ease of use and flexibility compared to previous methods [e.g., zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs)],[2] enables the selective manipulation of almost any gene, even in mammals. This system involves the formation of a complex between the Cas9 endonuclease and an RNA strand that activates and directs the complex to the target DNA region, where double-strand breaks are induced (Figure 1 A).[3]

The CRISPR/Cas9 system bears some similarity to one of the defence mechanisms present in eukaryotic cells, namely RNA interference (RNAi).[4] In RNAi, a protein complex known as RISC cleaves mRNA sequences guided by a single RNA strand to inhibit the expression of the targeted genes (Figure 1 B). Nevertheless, the CRISPR/Cas9 system is more versatile and can be used to reduce[5] or increase[6] the expression of genes, and more importantly, to edit genes, which can occur through two mechanisms. The most common is non-homologous end joining (NHEJ), which leads to insertions or deletions (indels) at the break site. In contrast, when homologous recombination (HR) takes place in the presence of an exogenous DNA template, new sequences (e.g., functional genes) can be introduced at the target site.

Another similarity between RNAi and CRISPR/Cas9 is that they both require RNAs to activate the enzymatic complex and localize their target region. However, in the case of RNAi, short RNA duplexes of 19–21 nucleotides (nt), known as small interfering RNAs (siRNA), are required to initiate the process. On the other hand, the guide RNAs (gRNAs) that activate and direct the Cas9 complex to the target DNA region are much longer, single-stranded, and composed of two RNAs, termed CRISPR RNA (crRNA, 42 nt) and trans-activating RNA (tracrRNA, 80 nt). The two

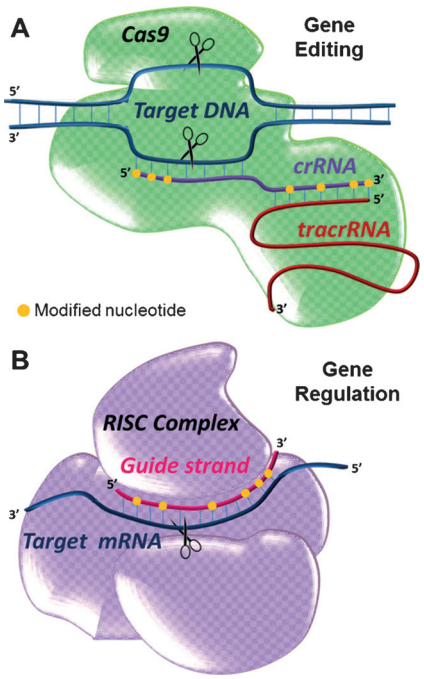

Figure 1. Schematic representation of the ribonucleoprotein complexes involved in CRISPR/Cas9 (A) and RNAi (B). In both systems, modified nucleotides can improve the activity and selectivity of the complex. In CRISPR/Cas9, the target DNA duplex is cut and edited, whereas in RNAi, the messenger RNA is cut and destroyed.

sequences partially hybridize to form the gRNA that binds to Cas9 through the tracrRNA region (Figure 1 A). The crRNA domain then hybridizes to the complementary target DNA, which should be placed next to a sequence known as a protospacer adjacent motif (PAM). Interestingly, the efficiency of the process can be improved by using a chimeric structure composed of crRNA and tracrRNA, termed single guide RNAs (sgRNAs), which are usually 100 nt long.

An essential difference between CRISPR/Cas9 and RNAi is that the gene editing achieved by the first system is permanent and inheritable. On the other hand, the regulation mediated by RNAi is transient and depends on the presence of siRNAs. Moreover, Cas9 species define the tracrRNA and PAM sequences, whereas in RNAi, siRNA sequences are independent of the RISC complex. The most widely used Cas9 nuclease is obtained from *Streptococcus pyogenes* and

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can be employed in the modification of almost any gene owing to the simplicity of its PAM sequence (NGG).

In addition, implementing this system in eukaryotic cells is not as easy as in the case of RNAi, since the Cas9 endonuclease is not expressed in these types of cells. For this reason, the whole protein or nucleic acids encoding it (e.g., plasmids or mRNA) need to be internalized into the cell, which can be accomplished by using different strategies.[7]

Owing to the inherent similarities between CRISPR/Cas9 and RNAi, they share some drawbacks and limitations that affect their efficiency. These include the low stability of RNA in serum and some activity at non-targeted regions (off-target effects). Recently, inspired by previous works on RNAi,[8] two groups have evaluated the impact of modification of the RNAs required in CRISPR/Cas9 to overcome these limitations.

Porteus and co-workers[9] synthetized three sgRNAs bearing in the last three nucleotides of both ends one of the following chemical modifications: 2′-*O*-methyl (M), 2′-*O*-methyl 3′ phosphorothioate (MS), or 2′-*O*-methyl 3′ thioPACE (MSP) (Figure 2). These modifications are commonly employed in antisense and RNAi technologies to improve stability against RNAses and to increase binding to the complementary target sequences.

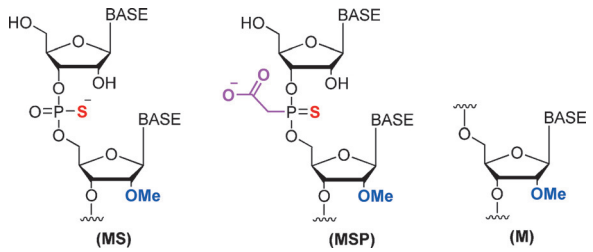

Figure 2. Structures of the modified nucleotides incorporated into sgRNA, with 2′-*O*Me (M) modification of the sugar ribose, and phosphorothioate (MS) and 2′-*O*Me 3′ thioPACE (MSP) modifications of the phosphate bond.[9] BASE = nucleobase.

Initially, they evaluated the activity of the Cas9–sgRNA complex by using an in vitro DNA cleavage assay, which confirmed that the modifications did not reduce the endonuclease activity. The activity and selectivity were then assessed in different established human cell lines and human primary T cells. In the latter case, the results are worth noting because the editing of human primary T cells, particularly when non-stimulated, can be problematic.

The activity of the CRISPR/cas9 system when using unmodified sgRNA was measured in terms of indels, and showed 2.4% of these in the target DNA. However, when the RNA strand was modified with M, MS, or MSP the frequency of gene disruption was raised to 13.5%, 68.0%, and 75.7%, respectively. Interestingly, when the quantity of sgRNA was augmented from 1 to 20 μg (per million cells), the values for the last two samples increased to 75.3% and 83.3%, which is comparable to the indel frequencies obtained when the Cas9 and sgRNA were encoded in a plasmid.

They also evaluated the efficiency regarding the insertion of an exogenous fluorescent gene through HR, and again the modified sgRNAs were better than unmodified sgRNA. The MSP-modified RNA gave rise to the most active system, and when a higher amount (20 μg) was used, the activity was similar or even superior to that obtained through expression of the CRISPR/Cas9 system from a plasmid.

Subsequently, the specificity of these chemically modified sgRNAs was assessed by measuring off-target mutations at different sites. However, the results showed variable off-target activity depending on the site, gene, and sgRNA evaluated. Further studies with other modifications must be carried out to ensure that chemically modified sgRNAs can reduce the above-mentioned off-target effects.

In another report by Cleveland and co-workers,[10] crRNA and tracrRNA structures were used instead of the long chimeric sgRNA. Modifications were introduced into the crRNA, which was more convenient owing to its shorter length. These modifications included phosphorothioate (PS) groups in the RNA backbone, and 2′-*O*-methyl (M), 2′-fluoro (F), and *S*-constrained ethyl (cEt) groups in the ribose sugar (Figure 3). As before, these modifications had previously been employed in the modification of siRNAs to increase their stability and binding to the target mRNA.

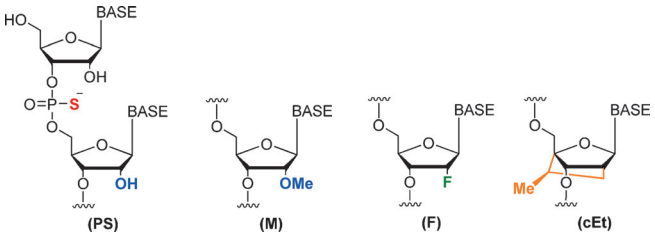

Figure 3. Structures of the modified nucleotides incorporated into crRNA, with 2′-*O*Me (M), 2′-fluoro (F), and *S*-constrained (cEt) modifications of the sugar ribose, and phosphorothioate (PS) modification of the phosphate bond.[10] BASE = nucleobase.

First, they assessed the activity of the system when phosphorothioates were placed along the whole crRNA. Remarkably, it turned out to be four times higher than with unmodified crRNA.

The introduction of additional modifications to the ribose sugar was then systematically evaluated. In this case, the presence of M or F groups in the last five nucleotides of both ends provided activity seven times higher than that obtained with the unmodified crRNA. Nonetheless, this was still half the activity achieved when the chimeric sgRNA was employed.

After testing different combinations, Cleveland and co-workers found that replacement of the ten nucleotides at 5′-end with F-modified ones and introducing five cET modifications along the last fifteen nucleotides of the 3′-end in the crRNA yielded the most active CRISPR/Cas9 system. In this system, they achieved 75% of the activity obtained with the long sgRNA. Finally, they truncated modified crRNAs to find out the minimal length required to achieve efficient editing. They discovered that the gene-editing activity when a 29 nt derivative was employed was slightly better than that obtained when unmodified sgRNAs were involved. Remark-

ably, when modified crRNAs were employed in CRISPR/Cas9, the editing was four times more selective than that obtained when using sgRNA, thus considerably reducing off-target disruptions.

In summary, these two recent reports highlight the potential use of chemically modified RNAs in CRISPR/Cas9. It is worth mentioning that introducing chemical modifications into siRNAs has afforded outstanding results in the past. Particularly, the activity was maintained in vivo and the off-target effects reduced. Now, these two reports confirm that chemical modifications can indeed be employed in the CRISPR/Cas9 system and might expand the use of CRISPR/Cas9 to more cell lines and facilitate its clinical translation. Porteus and co-workers have demonstrated that modified sgRNA can provide similar results to thoseobtained when the RNAs are expressed from plasmids. However, the preparation of long RNAs (100 nt) in high quantities (20 μg for cell cultures experiments) can be costly and in some cases, technically challenging, particularly when non-natural modifications are introduced. To address this, Cleveland and co-workers reduced the crRNA from 42 to 29 nucleotides while both retaining the activity of an unmodified sgRNA and achieving four-times higher selectivity.

These are just the first examples, but more research needs to be done involving other modifications. In this respect, the lessons learned using antisense, siRNAs or microRNAs will be invaluable, where systematic studies with different modifications have provided remarkable, and in some cases, unexpected results.[11]

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