Off-Target Effects Of CRISPR/Cas9 and Their Solutions

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Abstract. In the continuous exploration, the superiority of CRISPR/Cas9 system has been discovered. Its ability to edit target genes with high specific and low cost has greatly increased the convenience of constructing biological models, accelerated the screening of targets and effectively improve the gene editing technology. However, when CRISPR/Cas9 system is used in the studies, these studies are frequently constrained by the off-target effects, which may result in mutations in the experimental products and erroneous experimental data. Therefore, this paper mainly introduces CRISPR/Cas9 and the principle of its off-target, and discusses the effects of off-target from PAM sequence, sgRNA and other aspects. To more thoroughly study the off-target effects' mechanism, this paper also lists three kinds of detection technologies of off-target effects. Finally, three solutions including designing sgRNA, changing the abundance of Cas9/sgRNA and improving Cas9 protein were analyzed and discussed from the effects of off-target, which provided ideas for reducing the off-target effects and improving genome editing technology.

Keywords: Genome Editing, CRISPR/Cas9, Off-Target, sgRNA.

1. Introduction

CRISPR/Cas9 is a bacterial immune system which is used to fend off the invasion of exogenous genetic material. Current research found that CRISPR/Cas system provides acquired immunity for bacteria. For example, when viruses or foreign plasmids infect bacteria, a small segment of foreign DNA will be intercepted as a "spacer" through Cas1-Cas2 protein, and integrate it into CRISPR locus thus generating relevant "memory". The next time virus or foreign plasmids invades, CRISPR-Cas9 system will identify the invader with the relevant "memory", and use Cas9 to cut the specific position of foreign DNA to protect itself. The mechanism of CRISPR/Cas9 system is shown in Figure 1.

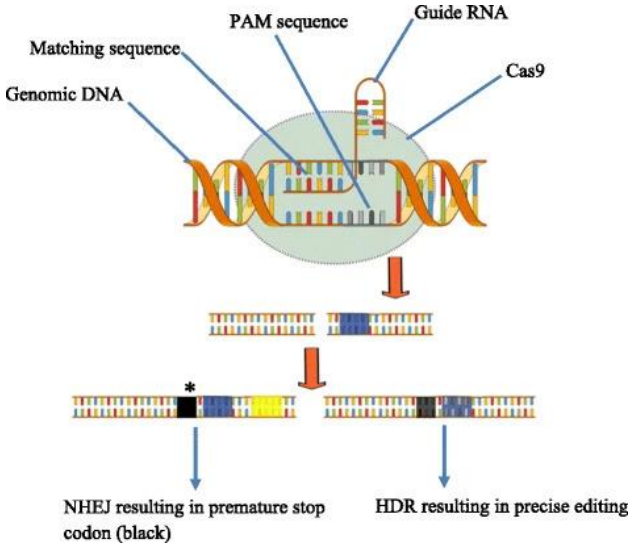

Figure 1. CRISPR/Cas9 facility. The essential parts in the system include Cas9 and gRNA. A molecular cutter, which is performed by the nuclease Cas9, could cut the DNA strands. The gRNA acts as a guide in order to inform the Cas9 a specific position in sequence to cleave. After the joining of DNA, NHEJ or HDR are 2 possible situations [1].

The benefits of the CRISPR/Cas9 system have steadily come to light via ongoing study and discovery. The convenience of developing biological models, the speed at which targets are being screened, and the efficiency with which gene editing technology is being developed have all been significantly improved by its highly precise fixed-point editing capability for target genes and cheap cost. Although the CRISPR/Cas9 system can carry out gene editing swiftly and effectively, further in-depth study has revealed that the method may sometimes have off-target effects. Cas9 may cut unintended locations, leading to unstable gene sequence and the impaired function of other normal genes [2,3]. The widespread use of CRISPR/Cas9 technology is significantly constrained by off-target. As a result, the focus of this technology study has shifted to the influencing elements, detection techniques, and remedies of the CRISPR/Cas9 system off-target effects. It is possible for sgRNA to pair with non-target gene sequences to form base mismatches and bulges of DNA or RNA when CRISPR/Cas9 technology is used to identify sgRNA and target genes. This phenomenon, known as the off-target effect, occurs when gene mutation occurs outside the scope of the experimental design. The settings that result in the off-target and the outcomes following the off-target are both influenced by a variety of factors [4,5]. The structure and length of sgRNA, the concentration of Cas9/SgRNA throughout the reaction process, and the PAM sequence specifically identified by Cas9 can all affect the likelihood and outcome of the off-target. As a result, this research explores the occurrence principle, influencing variables, and detecting technology of the miss effect and suggests a number of remedies.

2. Off-target effects

Three main reactors in CRISPR/Cas9 system, which is shown in Figure 2, are found the main influence factor of off-target effect.

2.1. Protospacer adjacent motif (PAM)

Cas9 will recognize the PAM sequence before cutting. PAM sequence mainly affects the cutting efficiency of CRISPR/Cas9 on DNA. NGG (canonical PAMs) has the best cutting efficiency, but in the other 15 non-NGG sequences, NAG has a higher cutting efficiency than NGA (non-canonical PAMs) [6]. NGA has a higher cutting efficiency than NAG at specific places in HEK-293 cells, according to various studies that thoroughly examined the impact of PAM sequence on the cutting efficiency utilizing the green fluorescence reporting technique.

2.2. Single guide RNA (sgRNA)

The target specificity of the CRISPR/Cas system is determined by the 20nt sequence region of the sgRNA and target genome determines. Many studies have suggested that the sequence of sgRNA may led to off-target effects. Kuscu et al. used ChiIP-seq technology to combine 12 different sgRNAs in HEK293T cells with dCas9 to draw a genome-wide map of binding sites [4]. Target sites bound by dCas9 range in number from 10 to over 1000, which depends on the sequence of sgRNA. In addition, the length of sgRNA will have certain impacts on the miss effect or cutting efficiency.

2.3. Other effects

There are other aspects that can easily lead to mismatch, according to some literature. More than 700 sgRNAs were created in the study by Hsu et al. in the HEK293T and HEK293FT cell lines in accordance with various target locations to assess their off-target effect [7]. The findings demonstrated a relationship between the quantity and spatial distribution of mismatches and the Cas9 protein's tolerance to mismatched bases [7]. SgRNA can be separated into proximal and distal non-seed regions based on the distance between the base and PAM sequence. Higher tolerance for mismatch in the distal non-seed area is demonstrated by the CRISPR/Cas9 system. Furthermore, mismatch will also have an impact on the nucleic acid cleavage activity of the Cas9 protein.

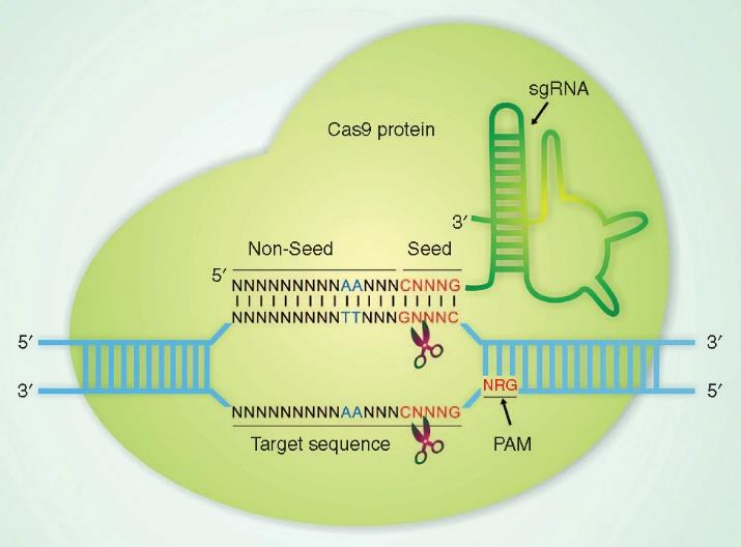

Figure 2. Three main reactors in CRISPR/Cas9 mechanism. Cas9 protein is performed in green-yellow. Red sequences represent PAM and seed sequences. Guide RNA is performed in dark green. Unrelated base sequences are performed in blue. Scissors indicate cleavage sites.One single letter represents a specific deoxynucleotide.(N=T, A, C, G; R=A or G) [8].

3. Off-Target detection technology

Numerous studies have shown that the incidence of off-target effects in the application of Crispr/Cas9 technology is generally high, so the detection of off-target Effects is important to confirm the editing accuracy and to investigate the mechanism of off-targeting in various systems.

3.1. Traditional off-target detection

Traditional detection consists of software prediction and sequencing. This technique is an initial targeted detection of off-target effects, which first predicts the possible off-target sites in the reaction by some off-target prediction software such as Cas-OFFINDER and CHOPCHOP, and then amplifies and sequences the predicted sites to determine whether off-target effects are generated [9]. Xie et al. used different detection software such as CRISPR Design, Cas-OFFINDER, Cas9 Design, etc. to evaluate off-target effects [10]. For example, Cas-OFFINDER is only able to detect off-target effect, while CRISPR Design and Cas9 Design could help in design progress. In Figure 3 the working principle of CRISPR-OFFinder is specifically introduced. However, program prediction is often limited by its logic itself and insufficient information. Therefore, missing off-target sites or misclassifying is unavoidable.

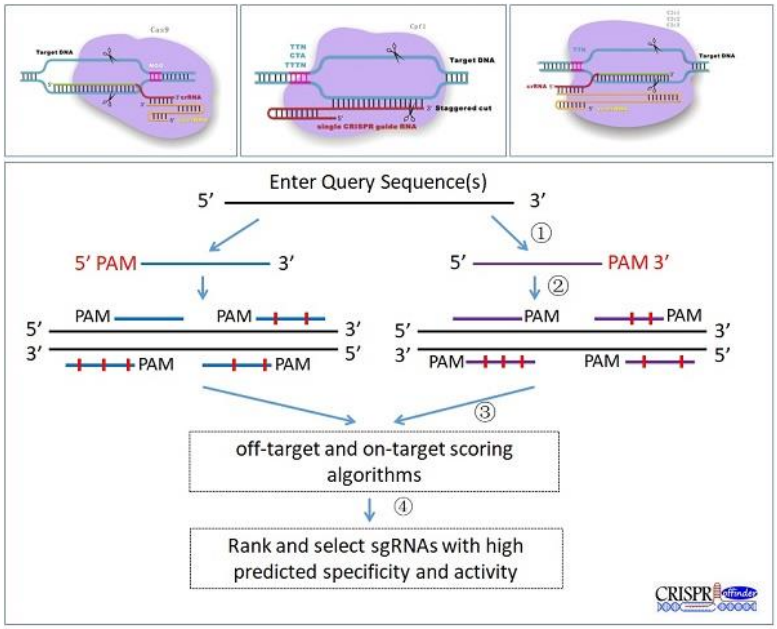

Figure 3. Working form of CRISPR-offinder: (1) Designing a specific sgRNA, while user-defined PAMs are put in use ; (2) Predicting possible off-target locations; (3) Confirming the predictions and evaluating the activity according to the support vector machine model, which is led by sgRNA Scorer 2.0. Moreover, the specific sgRNAs, which shows that low off-target activity will be marked by Cas-Offinder and CFD (Off-Target Cutting Frequency Determination); (4)Selecting the results with minimal off-target locations among specific sgRNAs [11].

3.2. Marking method detection

Gene-wide unbiased detection is achieved by tracking the reactions or reactants that occur during the application of CRISPR/cas9. For example, by integrating specific double-stranded nucleotide sequences into the DNA break sites, extracting genomic DNA and then amplifying and sequencing the specific sequences could be possible to analyze the off-target effects [12]. However, the exemplified method could only detect DSBs only at the time of the break [12].

3.3. The rest of the detection methods

There are still many studies that have explored the remaining types of detection methods, predicting and evaluating the location of the off-target effect by using deep learning to build models with software programs only, to off-target detection by blue-white screening-- and so on [13,14]. Blue-white screening: To investigate the effect of PAM site on CRISPR/Cas9 efficiency, the target sites with mutated bases at PAM site were cloned into LacZ region of PMD19-T vector respectively, keeping the original reading frame coding status, and under the induction of X-Gal and IPTG, the above vector could produce blue colonies when transferred into *E.coli* [14]. The color change of the colonies was observed by cotransforming the target and CRISPR vector containing the corresponding gRNA, and the CRISPR/Cas9 efficiency was quantified by combining with lysis assay. Most of methods are similar to the two types of methods in 3.1 and 3.2 and could be only at the experiment stages without being put into practical use.

4. Solutions and discussion of off-target effect

The off-target effect undoubtedly causes experimental errors and excess workload. After detecting the off-target loci, the decrease of the off-target incidence is an important measure to improve the efficiency of CRISPR/Cas9-related experiments. At the present stage of research, modification of the participating reactants is a relatively mainstream method.

4.1. Design of sgRNA

Modifying sgRNAs that bind directly to target genes can lead to increased response specificity. Minimizing the similarity between sgRNA and non-target genes, increasing the mismatch between them, and modifying the mismatch spacing and the ratio of G and C sequences can effectively reduce the off-target efficiency [4]. Futhermore, in Fu et al.'s study, reducing sgRNA length is also a feasible means [15]. However, what needs to be mentioned is that the best length of SgRNA for the reaction is under 20 base pairs. There is not much space for reduction.

4.2. Changing the abundance of Cas9/sgRNA

A high abundance of protein complexes increases the possibility of their binding to the correct target, but also increases the probability of off-target effect [5]. Therefore, according to different reaction systems, experiments are needed to determine a suitable Cas9/sgRNA concentration. This could reduce the occurrence of off-target effects by keeping the concentrations of abundance as low as possible while not affecting the cleavage efficiency. In addition, the use of different promoters during transfection to influence the transcripts can also produce similar effects [16].

4.3. Improvements of Cas9

Researchers have made important improvements to Cas9 protein, and Cas9 protein has significantly improved its specific binding with target after improvement. The improvement methods include developing mutants of Cas9 protein and searching for orthologous protein of Cas9. For example, eSpCas9 and HF modified Cas9 can significantly reduce the off-target effects and improve the targeting specificity by modifying the structure of Cas9 In addition, after the Cas9 protein is mutated into the incision enzyme nCas9 protein, the use of paired nCas9 can also induce the editing of target sites and effectively reduce the occurrence of off-target [17]. Therefore, further research and modification of Cas9 protein is essential to make it better play its flexible and precise advantages.

5. Conclusion

CRISPR/Cas9 has become a mainstream gene editing tool in further research and exploration, and has demonstrated its convenience of modification and potential for continued development in medical, agricultural, and other biological research such as screening for disease-causing genes, improving the species of plants and building plant and animal cell models. It is consequently important to study and reduce the off-target rate of this system in order to improve the experimental efficiency. Collectively, most of the mainstream off-target countermeasures nowadays are based on the study of the influencing factors of off-target efficiency. After the detection of off-target locus, the enhancement of recognition between sgRNA and target genes or the Cas9 protein and PAM sequences has achieved significant reduction of Off-Target rate, but some specific modifications will reduce the original cutting efficiency. Moreover, although the measures against main impression factors are efficient, the other side factors such as the reacting environment and the enzymatic activity are also worth-exploring. As a result, in the process of off-target detection and specific modification, how to detect off-target sites more efficiently and accurately, and how to achieve a lower off-target rate while maintaining the original cleavage efficiency will be the main direction of future research. If the accurate detection without experiments in advance and the comprehensive off-target countermeasures are available, it will take CRISPR/Cas9 system to greater heights.

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