The recent advances of CRISPR-Cas9 Technology

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Abstract

The type II CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 (CRISPR-associated nuclease 9) system is a powerful gene editing tool, and now is widely used in diverse organisms, including human beings. This sequence-specific gene editing is guided by 20-nucleotide (nt) sgRNA, creates a double-stranded break (DSB), then triggers the activation of intrinsic cellular DNA repair mechanisms, such as nonhomologous end joining (NHEJ) or homology-directed (HDR) DNA repair pathways. NHEJ is an imprecise process, it generates random insertions or deletions at the targeted sites, and HDR creates desired mutations or deletions through precise homologous recombination guided by exogenous donor DNA fragment. Besides, Cas9 can be engineered with versatile functions, such as transcription regulation and epigenetic modification. So, the CRISPR-Cas9 system provides a simple, efficient method to precisely manipulate the genome, and holds great hopes in the treatments of genetic diseases, infectious diseases and cancers. In this review, we focus on the recent development of CRISPR-Cas9 in genome editing and gene regulation.

Keywords

CRISPR-Cas9, gene editing, sgRNA, regulation, fusion protein.

1. Introduction

Before CRISPR-Cas9 system was actually understood, it took scientists decades to investigate its biological meaning and function. In 1987, Nakata and his colleagues reported a set of 29 nt repeats downstream of the iap gene during their research on the IAP enzyme, and they also found these 29 nt repeats were interspaced by five intervening 32 nt non-repetitive sequences[1]. Later, these interspaced repeat sequences were identified in many other bacterial and archaea species, and were named CRISPR (clustered regularly interspaced short palindromic repeat) by Jansen and Mojica in 2002[2]. Meanwhile, several clusters of CRISPR-associated (cas) genes were cloned and shown to be genetically conserved and adjacent to the repeat elements in the genome loci. In 2005, researchers demonstrated that the spacer sequences bear homology with bacteriophage genome, and viruses can't infect archaeal cells when the spacer sequences in archaeal were identical to their own genomes[3, 4]. At last, it was concluded that the CRISPR-Cas system may be an adaptive immune defense mechanism against bacteriophage infection[5].

Among the several CRISPR-Cas systems, the type II CRISPR-Cas9 system is the most widely studied one. Studies demonstrated that Cas9, mature crRNA and tracrRNA are three essential components for the successful reconstitution of the type II CRISPR nuclease system. Later, researchers found that a single guide RNA (sgRNA) that generated by fusing a crRNA containing the targeting guide sequence with a tracrRNA sequence, can facilitate DNA cleavage by Cas9 *in vitro[6]*.

Before the emerging and development of CRISPR-Cas9 gene editing system, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) technologies that utilizing dimerized Fok1nuclease domain to generate DSB were used to perform genome editing[7, 8], their targeted sequence specificity is decided by the zinc-finger DNA-binding motif and TALE DNA-binding motif. To the Cas9-sgRNA system, a 20 nt guide RNA sequence directs Cas9 to a specific genomic locus where Cas9 creates a DSB. So, the Cas9-sgRNA system is more simple and efficient compare to the former ones[9]. Now, the sgRNA-Cas9 system has been used for genome

editing in a variety of experimental model systems, including Arabidopsis, silkworms, mammalian cell lines, zebrafish, mouse and monkey[10]. In this review, we will summarize the advances of CRISPR-Cas9 gene editing system in recent years.

2. Controllable CRISPR-Cas9 gene editing

Improving the specificity and reducing the off-target cleavage of CRISPR-Cas9 genome editing system is of vital importance, especially to its clinical applications in the future. Much effort has been devoted to improve the specificity of the Cas9 enzyme by controlling its activity spatially and temporally.

A split Cas9 protein form has been recently reported by Zetsche and colleagues, the Cas9 protein is divided into two parts that can reassemble with the aid of rapamycin-binding dimerization domains to reconstitute the original enzymatic activity[11]. Another method that depends on the chemical-triggered excision of a function-disrupting intein has been shown to modulate the activity of Cas9[12]. However, this intein-Cas9 system is irreversible and cannot be switched off once the intein has been spliced out of the enzyme[13].

Liu and colleagues reported the development of a Cas9 variant whose activity can be switched on and off in human cells with 4-hydroxytamoxifen (4-HT) by fusing the Cas9 enzyme with the hormone-binding domain of the estrogen receptor (ERT2). This modified Cas9 nuclease showed low endonuclease activity without 4-HT but high editing efficiency at multiple loci with this chemical. Through the optimization of the duration and concentration of 4-HT treatment, the off-target cleavage also reduce to a minimal level[14].

Optically regulating Cas9 function is another way to precisely control of gene editing. James Hemphill and colleagues developed a light-activated CRISPR/Cas9 system in live mammalian cells through the site-specific incorporation of unnatural caged amino acids into a recoded amber stop codon, TAG. Through screening, the author successfully identified two lysine sites, K163 and K866, that are amenable to optically control of Cas9 function. The function of the caged Cas9 mutants in the presence and absence of UV exposure was tested using a dual reporter assay, they found Cas9 K866 mutant showed minimal background activity before irradiation and high activity after light exposure to 365nm UV[15]. So, this light-activation of CRISPR/Cas9 gene editing allow for the study of gene function with high precision and may reduce off-target cleavage by restricting the function of Cas9 to certain locations or time points.

3. Improvement on CRISPR-Cas9 gene editing accuracy

Cas9 creates DSBs at targeted genomic sites complementary to sgRNA sequences, but Cas9 can cleave at genome loci that are imperfectly complementary to the guide RNA and may induce unwanted off-target mutations, which poses a major limitation for fundamental studies and further therapeutic uses in treating human diseases.

Several methods have been reported to improve Cas9 specificity. A pioneer work was performed by Ran and colleagues, they developed an approach that combines a Cas9 nickase mutant with two paired guide RNAs to introduce two juxtaposed single-stranded DNA nicks. Because single nick in one DNA strand can be easily repaired with high accuracy, simultaneous nicking in both strands is required to form double-stranded breaks, which will greatly lower the off-target cleavage than the conventional CRISPR-Cas9 system. Results demonstrated that using this engineered nickase can reduce off-target activity by 50- to 1,500-fold in various cell lines without sacrificing on-target cleavage efficiency[16].

The crystal structure of SpCas9 in complex with guide RNA and target DNA reveals a positively charged groove, positioned between the HNH, RuvC, and PAM-interacting domains in SpCas9, which may be involved in off-target cleavage. Slaymaker and colleagues generated several SpCas9 mutants consisting of individual alanine substitutions at 31 positively charged residues within the groove and assessed changes to genome-editing specificity. They identified one variant eSpCas9(1.1)

that mutated at K848/K1003/R1060 reduces off-target effects to nearly undetectable level while maintains robust on-target cleavage with high efficiency and specificity as wild-type SpCas9 nuclease[17].

Based on the structural studies, SpCas9-HF1 (N497A/R661A/Q695A/Q926A) to reduce the direct hydrogen force to the phosphate backbone of the target DNA strand. The SpCas9-HF1 variant characterized in this report reduces all or nearly all genome-wide off-target effects to undetectable levels, while retains on-target activities comparable to wild-type SpCas9[18].

In another study, Janice S. chen and colleagues found both SpCas9-HF1 and eSpCas9(1.1) are trapped in an inactive state when bind to mismatched targets in the single-molecule Förster resonance energy transfer experiments. And they also found that the non-catalytic domain of Cas9, REC3, recognizes target complementarity and governs the HNH nuclease to regulate overall catalytic activity. Based on this structural analysis, they designed several Cas9 variants that bearing different mutations in the REC3 domain, and found a new hyper-accurate Cas9 variant (N692A/M694A/Q695A/H698A) demonstrating high level specificity without compromising on-target activity[19].

4. Reduce the PAM sequence limitation

CRISPR-Cas9 nucleases are widely used for genome editing, but the sequences that Cas9 can recognize is constrained by PAM (protospacer adjacent motif), a short nucleotide motif immediately downstream of the target DNA sequence. SpCas9, the most robust and widely used Cas9 to date, primarily recognizes NGG PAMs and is consequently restricted to sites that contain this motif, thus constrains the application of genome editing in many conditions. One potential solution to address targeting range limitations would be to engineer Cas9 variants with novel PAM specificities. Recently, Kleinstiver and colleagues adapted a bacterial-based positive and negative selection system to identify new SpCas9 variants to exhibit altered PAM specificities. Through this molecular evolution method, they demonstrated that VQR (D1135V/R1335Q/T1337R), EQR (D1135E/ R1335Q/T1337R) and VRER (D1135V/G1218R/R1335E/T1337R) variants recognize the 5'- NGA-3', 5'-NGAG-3' and 5'-NGCG-3' PAMs, respectively[20, 21]. These three SpCas9 variants showed robust genome editing activities for endogenous target sites with altered PAMs in zebrafish and human cells not currently targetable by wild-type SpCas9, thus expanded the target range in Cas9-mediated genome engineering[22]. Meanwhile, their genome-wide specificities are comparable to wild-type SpCas9.

5. Engineering Cas9 with versatile functions

The CRISPR-associated catalytically inactive dCas9 protein offers a general platform to recruit different protein effectors to DNA in a highly specific manner in human cells[23].

Gilbert reported that fusion of dCas9 to effector domains with distinct regulatory functions enables site-specific and efficient transcriptional repression or activation in human and yeast cells. They fused this modified dCas9 protein with different repressive chromatin modifier domains, including the KRAB domain of Kox1, the CS (chromo shadow) domain of HP1α, or the WRPW domain of Hes1, and transiently transfected these dCas9 fusion proteins with sgRNA into GFP+ HEK293 reporter cells. The results demonstrated that cells expressing the dCas9-KRAB fusion protein show a 5-fold decrease in GFP signal. They also observed up to 15-fold repression of GFP level in a lentivirus-based dCas9-KRAB stable reporter cells. In the next, they fused four copies of the well-characterized transcription activator VP16 or a single copy of p65 activation domain (AD) to dCas9 and transfected dCas9-VP64 or dCas9-p65AD to reporter cells, the results revealed that both dCas9-VP64 and dCas9-p65AD can effectively activate reporter gene expression[24, 25]. Based on this study, the authors further developed a robust and highly specific genome-scale CRISPR-Cas9 mediated repression and activation screening platform. Using this high-throughput screening method,

they systematically studied genes affected ricin resistance and revealed pathways and complexes that govern response to cholera and diphtheria toxin in cellular level[26].

Hilton and colleagues fused the nuclease-null dCas9 protein to the catalytic core of the human acetyltransferase p300 HAT domain. The fusion protein catalyzes acetylation of histone H3K27 at its target sites, leading to robust transcriptional activation of target genes from promoters and both proximal and distal enhancers. Gene activation by the targeted acetyltransferase was highly specific[27].

6. Conclusions and future perspectives

With the advent of CRISPR/Cas9, genome editing now becomes much easier and faster than ever before. But before we harness the full potential of CRISPR/Cas9 in research and clinical practice, many basic questions must be solved, especially the off-target cleavage, which may bring unwanted mutations into the genome, and another question is to reduce PAMs limitation. Based on the crystal structure information, researchers developed many Cas9 mutants with improved accuracy or expanded the PAMs sequence. Besides, various Cas9 fusion proteins make the CRISPR-Cas9 system spatially and temporally controllable, or turn the modified Cas9 protein as a gene regulatory tool with repression and activation activities[28].

Now, CRISPR-Cas9 gene editing tool has already revolutionized biology research in many fields, making it easier and feasible to many fundamental questions, disease studies, drugs discovery[29]. The first CRISPR-Cas9 associated clinical trials in people are underway in China and about to start in Europe and the US. In the future, CRISPR-Cas9 system may continue to surprise us with their powerful and broad applications.

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