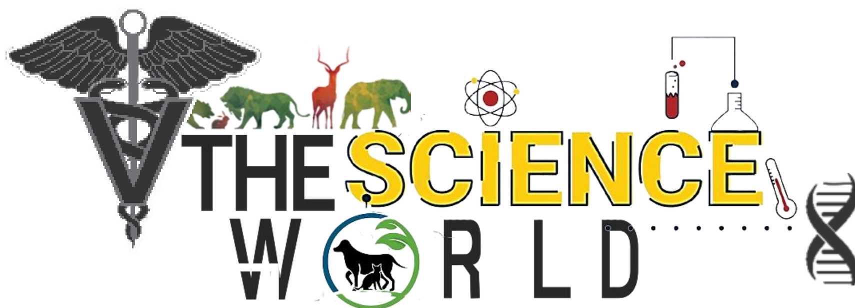

Popular Article

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Crispr Cas9: The Era of Search and Replace

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Abstract

Because of its straightforward design, low cost, high efficiency, ease of use, and ability to edit numerous loci at once, CRISPR/Cas9 technology has emerged as the most studied gene editing technique in recent years. Additionally, it can be completed without the use of plasmids, avoiding many of the problems that plasmids pose. The study of genes or genomic functions in bacteria, plants, animals, and humans has demonstrated the immense potential of CRISPR/Cas9.

Introduction

The process of altering a specific DNA target sequence in the genome to accomplish DNA fragment knockout, insertion, or other sequence alterations is known as "gene editing technology." Homologous recombination was utilized in the early stages of gene editing to exchange sequences between two homologous DNA strands. However, this technique is ineffective and prone to mistakes. Later, nuclease was artificially modified to create Meganuclease, ZFNs, and TALEN, which were utilized for gene editing. The modified nuclease is a chimera made up of a non-specific DNA cleavage domain and a specific DNA binding domain. The primary issue with gene editing was resolved by these technologies, which produced double-strand breaks (DSBs) at particular genomic locations. However, each time a new site is identified, the binding domain sequences must be modified. Furthermore, the experimental procedure is time-consuming and the structures' design is intricate (Shah et al. 2019). Since CRISPR/Cas9 technology has been shown to be more effective in construction than earlier gene editing methods, it was swiftly put into production. Research in the life sciences has reached new heights thanks to the sustainable development of the CRISPR/Cas9 system, which has demonstrated considerable application value in gene function research, gene therapy, and genetic enhancement.

The Discovery of the CRISPR/Cas9 System

In 1987, the repeated tandem arrays were first identified in Escherichia coli (E. Coli). The genomes of bacteria and archaea contain a sequence of highly conserved DNA repeats that are spaced apart. These distinct sequences were eventually discovered to be present in roughly 40% of bacterial genomes and 90% of archaeal genomes. Clustered regularly interspaced short palindromic repeat (CRISPR) is the formal name given to this distinct family of repeating tandem arrays in 2002. The CRISPR-associated protein (Cas), a helicase or nuclease that functions similarly to CRISPR, was subsequently identified. There is up to 100% homology between the CRISPR and phage sequences. This suggests that phages may be the source of the CRISPR sequences. Scientists began paying more attention to CRISPR in 2005 when three study groups discovered that it might be connected to bacteria' immunity. The scientists hypothesized that CRISPR might play a role in bacterial defense mechanisms. They postulated that the CRISPR may identify and remember the exogenous nucleic acids that are invading cells by using anti-sense RNA. The RNAi mechanism of eukaryotic self-immune function is comparable to this defense system. An experiment employing Lysozyme to infect Streptococcus thermophiles quickly demonstrated the self-immune function mediated by the CRISPR/Cas system. It is thought that CRISPR/Cas is the "acquired immune system" those bacteria or archaea developed to fend off invading DNA from plasmids or phages.

The Basic Structure of the CRISPR/Cas9 System

There are three forms of CRISPR/Cas systems: type I, type II, and type III. The complexity of types I and III is comparatively high. However, Streptococcus pyogenes SF370 has a very basic type II CRISPR/Cas system that simply uses Cas proteins. Specifically, Cas9 is a ~160KD protein that can autonomously target and cleave DNA. Its six domains are Rec I, Rec II, Bridge Helix, RuvC, HNH, and Protospace Adjacent Motif Interaction (PI). After being refined, the type II CRISPR/Cas9 system has grown to be a potent tool for gene editing because of its simplicity. A trans-activating CRISPR RNA (tracrRNA) region at the 5′ end, a number of Cas genes (Cas9, Cas1, Cas2, and Csn2) that encode proteins necessary for immune response, and a CRISPR region at the 3′ end that is made up of numerous spacers and direct repeats make up the type II CRISPR/Cas locus (Shah et al. 2019). To recognize a particular DNA sequence, tracrRNA (encoded by the tracrRNA area) and crRNA (CRISPR RNA, encoded by the CRISPR region) combine to create the crRNA: tracrRNA complex (Figure 1). To accomplish site-specific DNA cleavage, the Cas gene's nuclease is directed by crRNA. The recognition (REC) lobe and nuclease (NUC) lobe are two crucial components of the Streptococcus pyogenes Cas9 (spCas9) protein. The two REC domains, REC1 and REC2,

which are the functional domains of spCas9 when interacting with repeat: anti-repeat duplex, make up the REC lobe, which is composed of a long alpha helix. PI, RuvC nuclease, and HNH nuclease domains make up the NUC lobe. The spCas9 protein's amino terminus contains the HNH domain, which is in charge of cleaving the complementary DNA sequence to crRNA. The third nucleotide upstream of the Protospace Adjacent Motif (PAM) is where the cleavage site is found. Through a surface with positive charges created by the interaction of the RuvC domain and PI domain, the RuvC domain interacts with sgRNA. Short artificial RNA, or sgRNA, can take the place of crRNA to direct the spCas9 protein to cleave target DNAs at particular locations. To find the PAM sequence on the target DNA, the REC lobe first joins forces with sgRNA. The target DNA can then be coupled with sgRNA to direct the HNH and RuvC domains in the NUC lobe to cleave the target DNA's two strands. While the RuvC domain cleaves the complimentary strand, the HNH domain cleaves the DNA strand identified by sgRNA. The area where sgRNA directs spCas9 to cleave the target DNA is a positively charged groove that forms between the REC and NUC lobes. The primary purpose of the PAM, a crucial targeting component, is to aid Cas9 in precisely differentiating between its own DNA and foreign DNA with the same sequence. In order to achieve targeted cleavage of foreign DNA, this indirectly shields its own DNA from nuclease attack (Shah et al. 2019). The Cas9 protein is inactive on its own, but when it comes into contact with sgRNA, its structure drastically changes, allowing it to become active and break the target DNA (Figure 2).

The Mechanism of the CRISPR/Cas9 System

Three primary steps make up the process by which bacteria fend off the invasion of foreign nucleic acids: expression, interference, and adaptability. When a virus initially infects a bacterium, the bacteria will recognize its unique PAM sequences, digest the virus DNA into spacer sequences of the right size, and incorporate these sequences into its CRISPR spacer region, allowing the bacteria to memorize the invading virus. The bacteria can identify the same type of virus and convert its spacer sequences into pre-crRNA when it invades them again. With the aid of CnsI and RNaseIII, the pre-crRNA will be coupled with tracrRNA and transformed into mature crRNA. Guide RNA (gRNA) is another name for crRNA, which uses complementary sequences to identify and bind foreign DNA. Cas nuclease is unable to cleave foreign DNA on its own, but when it forms a ribonucleo-protein complex with mature tracrRNA and crRNA, it can be guided by crRNA to cleave the invading DNA by identifying its PAM site, destroying the foreign DNA and achieving self-defense. The CRISPR/Cas system is capable of gene editing due to the precise target sequence recognition capability of

crRNA, the DNA cleavage activity of Cas nuclease, and the DNA repair processes of cells. In order to repair DNA damage and prevent cell death, cells will initiate their own repair pathways when DSBs occur. Non-homologous end joining (NHEJ) and homology-directed repair (HDR) are the two repair processes. In order to repair double-strand breaks (DSBs) through homologous recombination—the integration of homologous segments into DNA—the cells typically engage the HDR mechanism if homologous sequences are available. By creating a repair template (donor) DNA fragment with homologous arms and co-transforming it with a gene editing vector, we can use this repair process to integrate donor DNA into cell genomic DNA and accomplish gene knock-in. The cells typically start the NHEJ pathway, which directly connects the damaged DNA, when homologous DNA is unavailable. Base pair insertions or deletions (indels), which can result in gene frameshift mutations and gene knockout, are common in this repair method (Figure 3), (Mladenov et al. 2011 and Wang et al. 2013). Cas9 and gRNA can be assembled into a single vector or two distinct vectors with matching promoters, terminators, replicons, and selection markers for gene editing. The synthetic gRNA is a chimera RNA that combines all of the necessary tracrRNA and crRNA components. The gRNA's front section, known as sgRNA, is in charge of identifying the target site. The subsequent section serves as a scaffold to attach to the Cas9 protein. Every time a

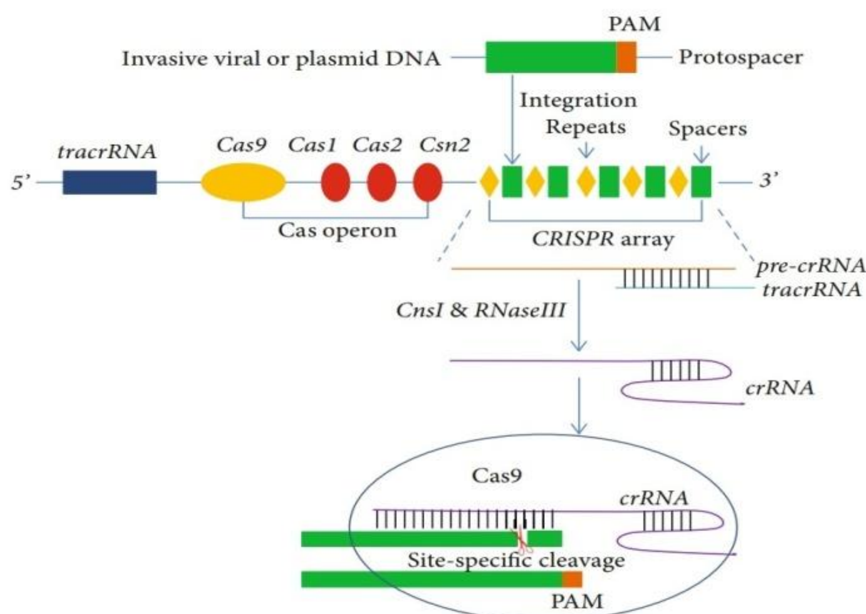

Figure 1: Bacterial adaptive immunity mediated by CRISPR/Cas9. Cas genes (colored ellipses), repetitions (yellow diamonds), and spacers (green rectangles) make up a typical type II CRISPR/Cas locus. A cell that has been infected by phages will absorb and incorporate the DNA fragments from the phages into the CRISPR array. After being translated into crRNA precursor (pre-crRNA) (brown line), spacers are linked with tracrRNA (blue line) so that RNase III may cleave them into mature crRNA. Through complementary pairing, crRNA identifies foreign DNA and directs Cas9 to break foreign DNA at specified sites (Jiang et al.2017).

new target is identified, sgRNA must be altered. The ideal sgRNA length is 20 nt, which facilitates the creation of the CRISPR/Cas9 vector. There are numerous potential targets that are appropriate for CRISPR/Cas9 because a potential target can be located in an average of every 8 bp DNA sequence. These elements have made the CRISPR/Cas9 system the most widely used gene editing tool. Target cells that have been infected with CRISPR/Cas9 vectors will express Cas9 and gRNA, which can modify genes (Shah et al. 2019). The first instance of CRISPR/Cas9 gene editing in eukaryotic cells occurred in 2013. Using CRISPR/Cas9, researchers were able to modify the Th gene of mice as well as the human genes EMX1, PVALB, PPP1R12C, CLTA, and CCR5. Thus, CRISPR/Cas9's ability to modify genes was verified. The CRISPR/Cas9 system is one of the most significant scientific discoveries of the past ten years because of its ease of use, which has drawn a lot of researchers to fully utilize it.

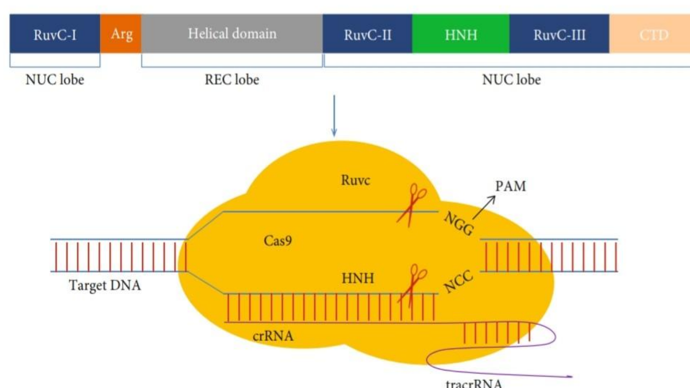

Figure 2: The Cas9 protein's general structure and the Cas9–sgRNA–target DNA complex schematic diagram in a type II CRISPR/Cas system. The NUC and REC lobes make up the Cas9 protein. The REC lobe has two REC domains and a lengthy alpha helix. The PI, RuvC, and HNH domains make up the NUC lobe. With the help of the crRNA: tracrRNA complex, the cleavage functions of the RuvC and HNH domains may identify the PAM site and cleave the target DNA (Nishimasu et al. 2014 and Jiang at al. 2017).

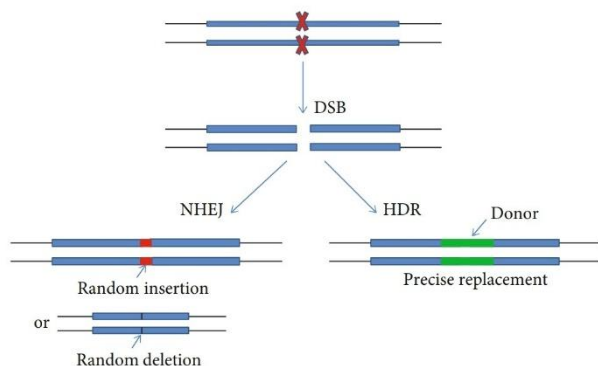

Figure 3: NHEJ and HDR mediated by CRISPR/Cas9. Cells will use either the NHEJ or HDR pathway to repair damaged DNA. The NHEJ method can be used to knock off genes by directly connecting the fragmented DNA. The HDR method can be used for gene knock-in and repairs damaged DNA by homologous recombination (Menchaca et al. 2020).

The Application of the CRISPR/Cas9 System

CRISPR/Cas9 Gene Editing in Animals: CRISPR/Cas9 technology is essential for character modification, gene function research, and the creation of new life substances

because gene knockout, gene insertion, and other DNA sequence modifications mediated by this technology may result in changes in gene expressions or phenotypes of living organisms. CRISPR/Cas9 gene modification has been used in animals and is important for cattle breeding and strain enhancement. Animals ranging from the lower Caenorhabditis elegans (Friedland et al. 2013) to the higher primate Cynomolgus monkeys (Niu et al. 2014) have all undergone successful gene editing. Using CRISPR/Cas9 technology, three labs were able to successfully remove a single gene, double genes, and multigenes of mouse cells in 2013. Additionally, researchers have employed D10A-Cas9- Using nickase technology, chicken primordial germ cells were altered to create a myostatin–knockout (MSTN–KO) chicken, which greatly expanded the skeletal muscle in its legs and chest. For KFC and everyone who enjoys eating chicken, this is truly fantastic news. In order to meet the growing need for food from humans and lessen the negative effects on the environment, researchers are also attempting to use CRISPR technology to modify and create animals (Menchaca et al. 2020).

sgRNA Library: A potent instrument for methodical genomic study is sgRNA library screening technique. It is a crucial research technique for studying the roles of genes, RNA, and proteins as well as for rapidly screening for potential therapeutic targets and breeding new species of microbes, plants, and animals. CRISPR knock-out, knock-in, activation, and inhibition libraries are the primary sgRNA libraries now in use that enable high-throughput screening of the entire genome. On a chip, tens of thousands of sgRNA covering all or a portion of the genome are created. After that, the sgRNAs are reassembled into the proper vectors to create plasmids that are tracked using high-throughput sequencing. Following lentivirus packaging, plasmids are converted into cells in the host to introduce different mutations in genes. Lastly, phenotypic changes are used to identify candidate genes of the host cells. Currently, numerous human and mouse genomic libraries have been successfully created by scientists, and they are continually being improved. In order to screen and analyze every gene in the human genome, Yilmaz et al. created 180,000 distinct mutations using a sgRNA library. This resulted in a blueprint of the human genome and revealed the roles of the genes in the occurrence of disease, which is crucial for the treatment of many human diseases. Cell transplantation or in vitro culture models were used to screen the majority of the sgRNA libraries. However, in vivo screening of cells and targets has recently been accomplished by researchers, allowing for the quick creation of patient-specific avatars to direct precision medicine. The creation of sgRNA libraries in multiple species generates significant economic value and advances biological research (Li et al. 2019, Yilmaz et al. 2018 and Greenberg et al. 2022).

Gene Editing without Plasmids Remained: For CRISPR/Cas9 gene editing, plasmids are typically inserted into target cells. However, the plasmids that are left in the cells after transformation will have a negative impact on further research. Cas9 expression that is persistent will raise the possibility of off-target. Additionally, the plasmids' selected marker precludes researchers from using the same marker in subsequent plasmids. The aforementioned issues will be resolved if the plasmids are removed either during or after gene editing. Transforming the gRNA and the Cas9 protein—both of which are expressed in vitro—directly into the target cells provides a definitive solution. Transforming the plasmids into target cells to accomplish gene editing and then transforming a produced gRNA that targets the plasmids into the cells to remove the plasmids is another method. Because gRNA transcription in vitro is expensive, scientists have created alternative techniques, like include incorporating a temperature-sensitive replicator into the plasmids so that, upon gene editing, the plasmids are eliminated by altering the temperature. In addition, while gene editing is taking place, a target DNA fragment can be inserted into the plasmids so that the sgRNA can direct the Cas9 protein to target and eliminate the plasmids (Liu et al. 2018).

In addition to the above, CRISPR/Cas9 technology plays vital roles in SNP detection and multiplexed CRISPR editing.

Conclusion

Because of its excellent effectiveness and ease of use, CRISPR/Cas9 technology has significantly aided the development of numerous biological fields. Because the position effect of conventional transgenic technology is eliminated, a high level of biological safety is attained. Instead of adding an entire gene to the genome, researchers can alter a few base pairs, which will allay public fear. However, because we still don't fully understand the CRISPR/Cas9 system, its potential hasn't been fully realized. More unexpected discoveries and useful applications could result from further CRISPR/Cas9 system development.

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