Review Article

An Overview of the CRISPR-Based Genomic- and Epigenome-Editing System: Function, Applications, and Challenges

Abstract

Developing a new strategy for an efficient targeted genome editing has always been a great perspective in biology. Although different approaches have been suggested in the last three decades, each one is confronting with limitations. CRISPR-Cas complex is a bacterial-derived system which made a breakthrough in the area of genome editing. This paper presents a brief history of CRISPR genome editing and discusses thoroughly how it works in bacteria and mammalians. At the end, some applications and challenges of this growing research area are also reviewed. In addition to moving the boundaries of genetics, CRISPR-Cas can also provide the ground for fundamental advances in other fields of biological sciences.

Keywords: *Bacterial immune system, CRISPR-Cas systems, DNA breaks, double-stranded, targeted gene repair*

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History of Genome-Editing Systems

Around three decades ago, when investigating on the *iap* gene, which is involved in biosynthesis of alkaline phosphatase in *Escherichia coli*, a Japanese scientist named Nakata along with his colleagues figured out that there were repeated sequences of DNA downstream of the gene.[1] After a while, further, a Spanish scientist named Mojica accidentally came across these sequences and began to study their function. In 1995, scientists identified that other prokaryotes also contained these sequences. They first named them as short regularly spaced repeats, but it was then turned into CRISPR.[2-4] In 2005, it was eventually discovered that CRISPR sequences exerted a defensive role in bacteria, protecting these microorganisms against phages and other external pathogens.[5]

On the other hand, after the discovery of the homologous recombination (HR) mechanism in 1989, the first step in gene editing was taken. Investigators figured out that if a fragment of DNA containing homologous arms at both sides gets into the cell, it can be inserted to the host genome through the mechanism of HR and can dictate desired changes to the cell.[6] Despite the promising results obtained at the beginning, the number of cells with inserted fragments in their genome was low, making it difficult to detect a modified cell among millions of cells. It was a tangible need to develop a procedure by which scientists can promote output.[7]

Scientists have found that if they enter DNA restriction enzymes into the cell with external oligonucleotide fragments, both the HR and nonhomologous end joining (NHEJ) can be promoted.[7,8] Meganucleases were the first enzymes used for this purpose.[8,9] However, regarding their inability to cut the host gene at a specific location, they could not be utilized in genome editing.

A suitable enzyme for genome editing must contain two important characteristics: first is to recognize a specific sequence in the genome, and second is to act as a restriction nuclease. Considering factors involved in gene transcription, scientists in 2001 designed a chimeric nuclease called zinc finger nuclease (ZFN), in which zinc finger domains were responsible for DNA identification and the restrictor enzymatic part was derived from fok1 nucleases.[10]

Although this approach was appealing, it suffered from disadvantages for common

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applications. The first disadvantage is a time-consuming design which may take months for amateurs to design a pair of ZFN against a specific region.[11] The second disadvantage is the low number of potential target regions in the genome. Actually, for every 50 nucleotides, there is only one locus that can be targeted through this mechanism.[12] Although the low number of loci does not usually make a problem for knocking-out editing, it faces limitation for knocking-in manipulation.[12]

This limitation provided the ground for the appearance of a new generation of nucleases which were able to target a specific region in the genome. In 2009, a number of scientists figured out that gall-forming pathogens containing transcription activator-like effectors (TALEs) were able to bind specifically to their targets in plant genomes and cause diseases.[13] In the next steps, other investigators combined TALEs and nuclease fok1 and created a new gene-editing system named transcription activator-like effector nucleases (TALENs) for better gene targeting.[14]

It is used for DNA double-strand breaking (similar to ZFN) as well as for knocking out/knocking in. Compared to the ZFN, two important advantages for this editing tool have been mentioned: (1) a simple design and (2) a low number of off-target breaks.[15] However, it is confronting with some limitations, such as longer cDNA in comparison to ZFN (1 kb) and difficult vector cloning.[16] Furthermore, similar to ZFN, it causes off-target breaks leading to unwanted changes and toxicity in the genome.[17] However, the number of off-targets is low in TALENs compared to ZFN.[15]

After the discovery of CRISPR as the bacterial required immunity system, a few groups of scientists simultaneously came to this conclusion that CRISPR could be utilized for targeted double-stranded DNA breaking and thereafter for gene editing in mammalians.[18] A benefit of this editing tool is to reduce the length of the site-specific sequence (20 nucleotides) compared to that of other three ones described earlier (500–1000 nucleotides), resulting in a simple gRNA design against a specific point in the genome.

CRISPR Is an Adaptive Immune System

There are different types of innate immunity in bacteria, including abortive infection, receptor mutation, and restriction-modification.[19] As mentioned above, an acquired immune system of CRISPR discovered in bacteria protects the microorganism against viruses, plasmids, and other external pathogens.[20] At first, investigators demonstrated that the bacterial genome is identical at some genomic regions with viruses and invasive plasmids. Thereafter, a hypothesis appeared indicating that these identical regions were thought to be for immunity against viruses and external agents.[20,21]

CRISPR protects the bacterium in two phases: immunization and immunity [Figure 1].[22] In the immunization phase,

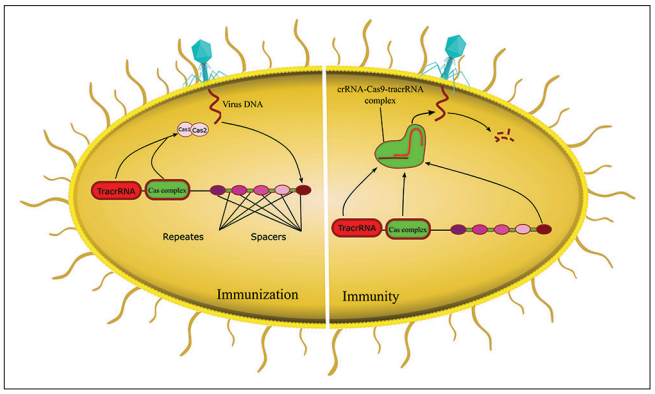

Figure 1: The bacterial-acquired immune system through the CRISPR mechanism

Cas proteins (Cas1 and Cas2) create a complex which is capable of breaking the viral genome. Then, this external nucleic content inserts the bacterial genome as repeat-spacer units.[22] In the immunity phase, following the secondary viral contamination, repeat-spacer units are transcribed into the pre-CRISPR RNA (pre-crRNA). Afterward, the Cas9 endonuclease and transactivating crRNA are bound to the pre-crRNA. Transactivating crRNA directs Cas9 toward the crRNA.[22] Eventually, a complex of crRNA-Cas9-tracrRNA is generated, which is then broken by RNA polymerase III and utilized to target the external DNA.[22]

Mechanism of CRISPR in Genome Editing

CRISPR system is composed of two components: (1) a nuclease such as Cas and Cpf1 which are responsible for DNA double-strand breaking and (2) a ribonucleotide called gRNA which is responsible for directing the nuclease to the target site in the genome [Figure 2].[18,23] Regarding the complexity of the nuclease and its function, two classes of CRISPR system have been identified: Class 1 and Class 2. Each of these two classes is divided into three subtypes, respectively. Class 1 systems consist of types 1, 3, and 4 in which the effector complex is generated of several Cas protein subunits; while class 2 systems consist of types 2, 5, and 6 in which the effector complex is made of only one multidomain protein such as Cas9 protein. In gene manipulation studies, CRISPR systems of Class 2 are more applicable than Class 1 because the enzymes of Class 1 need to make a complex for better activity, while the enzymes of Class 2 do not.[24]

After binding of the ribonucleotide/endonuclease complex to its target in the genome, the nuclease enzyme begins to make a double- or single-stranded breaking of DNA.[18] Downstream of the target sequence, there is a short motif with three nucleotides called protospacer adjacent motif (PAM) which plays a critical role in identification and restriction of the target sequence by nuclease.[23] The sequence depending on the type of nuclease is different, and each enzyme can identify a specific PAM. For instance,

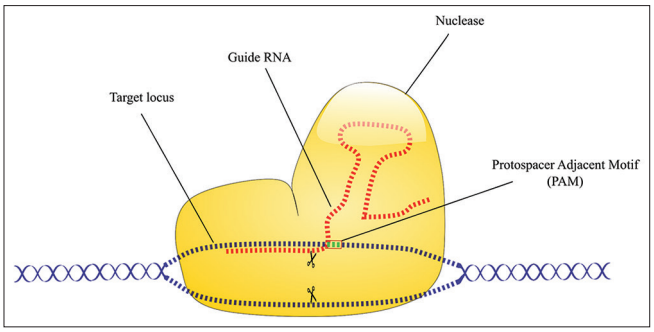

Figure 2: Structure and function of CRISPR. After binding of the ribonucleotide/nuclease complex to the target region in the genome, the nuclease cleavages the DNA at a certain distance from protospacer adjacent motif sequence through its HNH and RuvC domains. Then, the broken strands are repaired through the homologous recombination or nonhomologous end joining mechanisms

spCas9 makes a double-stranded break (DSB) at three base pairs upstream the NGG sequences.[17]

After restriction, the DNA can be repaired through two ways: HR repair and NHEJ.[25] NHEJ is an error-prone mechanism in that the chance of randomly addition and deletion of the bases is high and causes framework mutations.[26] This mechanism can be used for knocking-out studies. On the other hand, the HR way exerts more accuracy in comparison to NHEJ and is commonly used for knocking-in studies.[27,28] This mechanism needs a similar pattern, which is the homologous chromosome, while in gene correction studies, this pattern is manipulatingly inserted into the cell to dictate desired changes. One of the limitations in this procedure is to be activated only in proliferating cells, adding that the level of activity depends on cell type and target gene locus.[29]

Application

The ability of mentioned system to target and manipulate the genome of living organism has been appealing to many scientists worldwide. Their interest in this technology has improved its capabilities. Some of the most important applications are as follows:

Gene editing

Base editing can be considered as one of the most important advances in medicine, which provides the ground to treat many types of diseases. In primary experiments, the knocking-in procedure was used for this purpose. Due to low output of HDR (homology directed repair) compared to NHEJ, the knocking-in mechanism cannot be freely applied. Therefore, researchers began to investigate on another generation of base-editing tools, which were a combination of CRISPR and cytidine deaminase. In this approach, there was not any need to break DNA and produce oligonucleotide. In addition, in comparison to knocking-in system, it exerted a higher output with lower off-targets.[30,31] initiately, these editing tools were able to do nucleotide transitions only from C:G to A:T. The next generation of this base-editing tools were able to do nucleotide transitions from A:Tto C:G.[32]

Gene expression regulation

CRISPR system can also be utilized to alter the expression of one or several genes.[33] To postulate this, investigators first make mutations in the Cas nuclease in its two effector domains, HNH and RuvC. Although the resulting nuclease is not able to break the DNA (dead Cas or dCas), it can be directed to the specific DNA site by an sgRNA. They merge some gene expression activators such as VP64 and p65 with the dCas and design new types of CRISPR-based gene activators called CRISPRa, through which gene expression can easily be upregulated.[34] On the other hand, the combination of dCas9 with a site-specific sgRNA can result in blocking the transcription elongation. Furthermore, combining gene expression inhibitors such as Krüppel-associated box with the inactivated Cas9 has led to create a specific type of gene inhibitors, which are called CRISPR interference (CRISPRi) and downregulate gene expression.[35]

Epigenome editing

Epigenomic mechanisms occur in different cellular levels.[36] For instance, DNA methylation and histone deacetylation and methylation are two of the most common epigenomic changes inside the cell.[36] Investigators have been able to alter methylation and acetylation in target points through the CRISPR system.[37,38] One way to achieve this goal is to combine histone demethylase lys-specific histone demethylase 1 (LSD1) or the catalytic core of the human acetyltransferase p300 with dCas9 (dCas9–LSD1 and dCas9–p300Core). This purpose can also be achieved through targeting the regulatory regions (for example, enhancer region), instead of the target gene itself.

Screening on genome level

The ability of CRISPR system to modify any regions in the genome has led to the use of this system in functional studies on a wide scale. Before the CRISPR system was introduced, scientists performed the RNA interference (RNAi) procedure to alter gene expression levels in functional studies.[39] An important limitation for the RNAi system was to cause off-targets and also incapable in complete inactivation of target genes.[40,41] On the one hand, the simple design and cloning of CRISPR system have made it possible to knock out the genes of interest for genome-scale loss-of-function screens. On the other hand, as mentioned above, the dead Cas9 systems can also be used for both loss-of-function (through CRISPRi) and gain-of-function (through CRISPRa) screening studies. For these purposes, a set of different types of gRNAs are packed into viral vectors and transduced into the cells. Then, using the deep sequencing, we can determine which genes to be targeted in the cell (each gRNA barcode is different from the other

one), and consequently, its effects (knockouted, activated, or repressed) can be studied. According to new studies, scientists have been able to do screening successfully and conduct functional study on genome levels with the help of CRISPR system and next-generation sequencing (NGS) technology.[42]

Gene drive technology

Gene drive technology is a genetic engineering method in which, unlike Mandel's laws, genetic traits are inherited from parents to offspring in a high proportion.[43,44] In engineered gene drives, the target organism's germ line cells are transformed with a cosset consisting of the encoding gene for the Cas9 nucleases and sgRNA and desired donor sequence. The sgRNA directs Cas9 to produce a DSB in another homologous chromosome target site. Then, by activating the HDR cell repair mechanism, the entire construct is copied in the DSB site and the newly edited chromosome can be inherited to offspring. Through this way, the editing changes will happen in the next generation. This newfound technology can be utilized to eradication of diseases such as malaria (through entering malaria-resistance gene or females' sterility genes as a desired donor sequence) and Zika as well as undesired animal or plant species. However, due to the possible abuse of this technology in terrorist attacks against plants, animals, and even human beings, it has faced huge obstacles.[45]

Visualization of genomic loci

In recent investigations, it has been found possible to track and identify gene loci by merging dead nucleases with fluorescent proteins. This technique can also be employed to track the copy numbers of one specific locus in various diseases, disorders, and cancers as well as to track the activated and inactivated expression regions of the chromatin in the three-dimensional space of the nucleus.[46,47]

Detection

CRISPR system can also be used to detect viruses and bacteria inside the cell. A group of scientists from Harvard University has recently succeeded to merge CRISPR system and fluorescent reporter RNAs and create a system called specific high-sensitivity enzymatic reporter unlocking (CHERLOCK) by which a lowest number of pathogens (attomolar levels) are detected.[48] This system benefits from a type of nuclease enzymes which can target RNA instead of DNA (usually Cas13a). After binding of gRNA to the viral RNA, the nuclease is activated, and then it binds to the fluorescent reporter RNA and breaks it. The fluorescent component is then released from the complex and the pathogen is detected.[48] These techniques have been promising to diagnose different types of cancers in primary stages as well as to determine genetic disease-related polymorphisms.

Challenges

Despite the potential application for the treatment of many diseases, these systems still confront with some limitations. The first of them is to cause off-target breaks in the host genome.[49] For a gRNA, many similar sequences depending on the genome size of the species can be existed.[50] Regarding that where, in the genome, these sequences are located, their breaks could lead to malignancies and even death.[50] Various mechanisms have been developed to reduce off-target breaks; among of which truncated sgRNA,[51] the use of nickase enzymes instead of nucleases,[52] direct delivery of CRISPR constructs, molecule-triggered nucleases,[53] dimer nucleases,[54] and binding of ubiquitination signals to Cas9 can be mentioned.[55]

The second challenge in the area of gene therapy is the low output of HDR in comparison to NHEJ after target cleavage. As mentioned before, NHEJ causes unwanted mutations in the cleavage site, and it can be problematic when purposed to do precise editing of a locus in the genome. Therefore, we need mechanisms to increase the output of HDR. This purpose can be achieved through manipulating the components of cellular repair machinery and optimizing the procedures and delivery times of CRISPR constructs.[27] Scientists can also promote the output of HDR by manipulating cell cycle proteins.[27,56,57] In addition, a new nuclease called Cpf1 has recently been discovered which exerts higher HDR output compared to the previously-introduced nucleases.[54]

Another challenge for this gene-editing tool is to require to PAM.[58] Dependence of this approach on PAM limits the number of target loci, and on the other hand, it can reduce off-target breaks.[50] This technique requires specific PAM sequences to act functionally. For this purpose, another type of specific PAM-containing nucleases has been developed which compensate this limitation. Genetic engineering and enzyme changing have also been able to resolve the limitation.[59]

The host immune response to Cas protein is regarded as one of the most important challenges in the clinical trials of CRISPR. Since almost half of the human population has been immunologically resistant to nuclease-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus pyrogenes* and are not sensitive to nuclease, it is difficult to push this technique from *in vitro* to *in vivo*.[60] To handle this problem, scientists have been seeking for new types of nucleases.[61]

The last challenge of the mentioned system is to choose the right method to transfer the constructs of gRNA and nuclease into the cell. Although the direct transfer of construct plasmids sounds easy, it is not considered as an appropriate procedure. To be more elaborated, the probability of random plasmid insertion to the host

genome increases, and consequently, the transfection output decreases.[62] The next choice is to use viruses. For this purpose, the AAVs (adeno associated viruses) are more suitable because their effect on the immune system is less than other viruses, making them able to target nonproliferating cells.[63] However, with regard to the problem in carrying DNA fragments, these vectors also sound inappropriate. The large size of spCas9 (around 4.2 kb) has been found to be a problem for cloning in viral vectors.[64]

The discussing problem was solved after the discovery of nmCas9 and saCas9 (3.2 kb) (the Cas9 enzymes derived from *Neisseria meningitides* and *Staphylococcus aureus* bacteria, respectively).[65] In addition to plasmid and viral transfer, the direct delivery of gRNA and already-expressed nucleases to cell targets are other strategies proposed for this purpose.[66] To deliver these fragments to target cells, nonviral techniques such as lipofection, microinjection, and electroporation can also be utilized.[67] In addition, these techniques can reduce off-target cleavages. Despite all the limitations, this newfound technology is still on its way to progress.

Future Perspectives

As discussed above, CRISPR gene-editing system is still buckling up with problems. Finding a suitable way to transfer the system into the body in addition to those mentioned in the text is among the problems. Along with the growing advances of this technology and related sciences, ethical concerns about it are still increasing. Despite all these limitations, CRISPR is relatively applicable and has evidently proved to correct the mutations which are associated with different diseases such as thalassemia, cystic fibrosis, and Duchenne muscular dystrophy.[68] CRISPR has also demonstrated promising results in treating lethal diseases such as AIDS and cancer.[69,70] Resurrecting extinct creatures, manufacturing engineered products, and eradicating human diseases are not too far to happen through the CRISPR system.

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Conflicts of interest

There are no conflicts of interest.

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