The 2nd International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/4/20220645

Development and Prospect of Gene Editing Technology

Kai Gao[1,†], Haomin Hu[2,4,†], Siyu Li[3,†]

[1]Faculty of Life Sciences, Nanjing Agricultural College, Nanjing, 210000, China
[2]Bioscience, Tianjin Normal University, Tianjin，300387，China
[3]Ealing international school, Shenyang, Liaoning, 110000, China

[4]1930170177@stu.tjnu.edu.cn
[†]These authors contribute equally.

Abstract. Gene editing technology, as an important technology in the field of science, can accurately and stably modify the genome sequence, bringing revolutionary changes to various fields of life sciences, so it has attracted wide attention from researchers in various countries. This article makes a brief introduction to ZFN and TALEN technology, and at the same time makes a detailed introduction to CRISPR/Cas9 technology, focusing on the limitations, application, and development of CRISPR/Cas9 system. This research found that the CRISPR/Cas9 system and its related technologies are going deep into various fields of life sciences and have achieved remarkable results, providing more ideas and possibilities for human disease treatment, crop gene control and other fields.

Keywords: gene editing, technology, CRISPR/Cas9.

1. Introduction

Gene editing technology is a technique for precise modification and directional editing of genomes by artificially causing insertion, deletion or replacement of specific genes. Gene editing technology has developed rapidly in the past two years. With the emergence of new transposon editing tools, genome exact editing, double base editing and single editing have made great progress.

The earliest gene editing technology is Homologous recombination (HR), which achieves gene editing by introducing foreign genes and exchanging between homologous sequences, but the efficiency is very low. Later, the gene editing technology of nuclease appeared, which can modify specific genes without introducing exogenous genes. However, traditional nucleases such as restriction endonucleases can only cut short and simple gene sequences, and the efficiency of complex organisms is low. Therefore, a more accurate and simple gene editing technology based on artificial nucleases has emerged [1-2].

With the continuous advancement of technology, scientists are increasingly aware of the great ability of gene editing technology, which can not only improve the insect resistance and high yield of crops, but also cure difficult diseases. However, gene-editing technology poses challenges. Genetics is a lot of uncharted territory, and there's still a lot of uncertainty about whether or not it's possible to do some gene-editing under ethical issues, and whether it can accurately solve the problem without causing potential risks. Therefore, this paper will briefly discuss the development, application and current challenges of gene editing technology, trying to arouse readers' thinking.

DOI: 10.54254/2753-8818/4/20220645

This report focused on recent developments in gene editing and envisages challenges and opportunities.

2. Principles Of Gene Editing

As a common molecular biological event, in mitotically active mammalian cells， DNA double-strand breaks (DSBs) happen every day. After DSBs occur, cells can be repaired in a variety of ways, including classical NHEJ [3]. a-EJ, SSA and HR [4]. HR can be accurately repaired, but it requires the existence of homologous templates; NHEJ is a direct ligation of two DNA ends that are largely non-homologous to achieve repair. In this process, the two ends will undergo several nucleotide deletions in most cases, which is an inaccurate repair mechanism. As an auxiliary repair mechanism, both a-EJ and SSA require more extensive end single-strand excision, which also leads to the loss of genetic information [5]. According to DNA cleavage repair, if you provide modified homologous recombination templates in cells, and the target DNA is naturally generated or artificially induced to produce DSBs, triggering homologous recombination repair, it is possible to change specific DNA sequences or insert foreign genes. Gene mutation and knockout can be achieved by using the inaccurate repair mechanism of NHEJ, a-EJ and SSA without providing homologous templates. Traditional gene editing utilizes occurring DSBs to achieve replacement, gene knockout, integration [6-7].

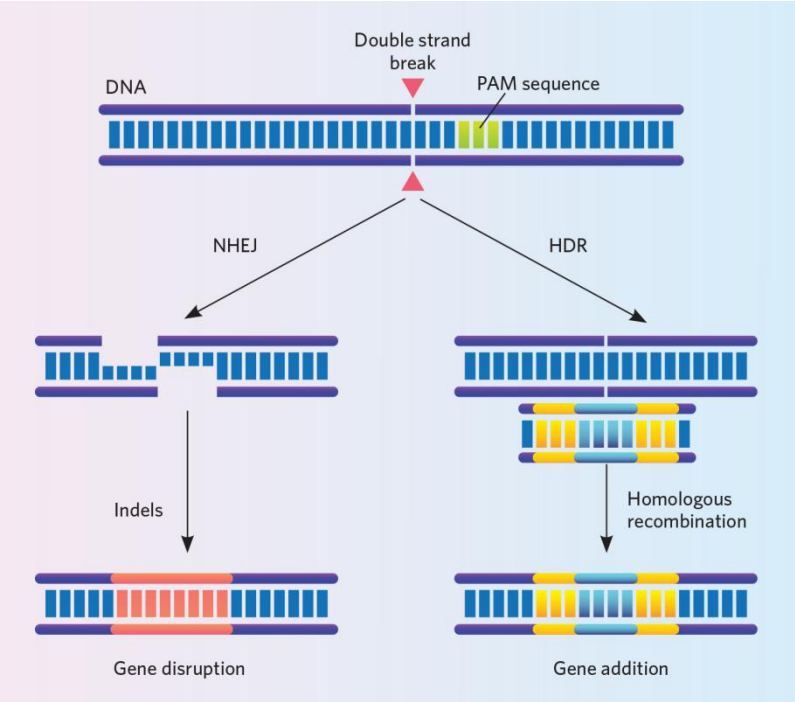

Figure 1. The mechanism of gene disruption and addition.

3. Recombinant Nuclease-Mediated Gene Editing Technology

3.1. Zinc-finger nucleases

ZFNs is the original gene editing, a landmark breakthrough. ZFN is composed of ZFP and FokI nuclease structures that recognize and cleft DNA, respectively. ZF can recognize three consecutive base pairs (Figure 2a), and the number of ZFS in series affects the recognition power of ZFN. The FokI is connected to the ZFP through the N-terminus. FokI constitutes a segment of the dimer connected to the ZFP, which requires a pairwise design when used.

DOI: 10.54254/2753-8818/4/20220645

3.2. TALENs Technology

ZFN technology is expensive and difficult to edit multiple targets. The discovery of the TALE motif gave birth to Talens. TALE motifs are concatenated to form DNA recognition target module base pairs, which are linked to the FokI domain. (FIG. 2b), there is a one-to-one correspondence between the tandem TALE motif and the recognized base pairs. It was found that TALENs had similar cleavage efficiency as ZFNs, but its toxicity was usually lower than ZFNs and it was easier to construct. However, TALENs is much larger than ZFNs, has more repeats and is difficult to edit in E.coli [9].

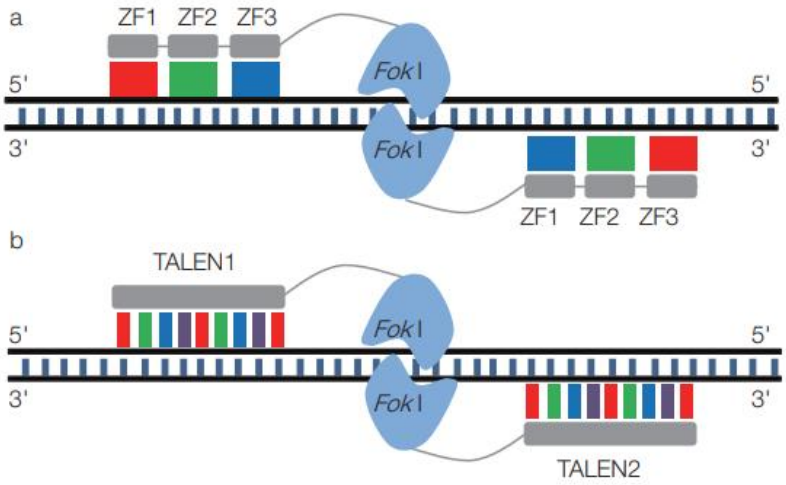

Figure 2. (a) Zinc finger nuclease gene editing technology(ZFN); (b) TALEN gene editing technology [10].

4. CRISPR/Cas9 Technology

4.1. CRISPR mechanism in gene editing

Protein Cas9 protein not only has helicase activity, which can unwind DNA chains, but also has nuclease activity, which can cut DNA chains.

crRNA and tracrRNA form a single guide RNA together, and then Cas9 protein combines with sgRNA to form a complex, thus recognizing and cutting specific regions of DNA. First, it recognizes and binds to PAMs, and after successful binding, the sgRNA partially melt the double-stranded DNA, and because the sgRNA strand is designed, its sequence can bind to the genomic target sequence. Because it contains two nuclease domains, it can cleave two single strands of DNA separately. This double-stranded DNA breaks, and then the cell changes its DNA sequence as it repairs itself.

There are four steps for SpCas9 to find the target site. First, apo-Cas9 will interact with DNA by random collision and then separates quickly after binding. The formation of the Cas9 RNP upon binding of apo-Cas9 to sgRNA alters the morphology and function of SpCas9, allowing the target to be rapidly separated from the DNA by a unidirectional search of about 27 BP (non-target strand DNA-3 'to 5') along the double-stranded DNA, unless PAM is encountered. A stabilizing interaction with PAM then prompts the formation of a stabilizing R loop on the complementary substrate, thereby activating Cas9 for DNA cleavage [11].

DOI: 10.54254/2753-8818/4/20220645

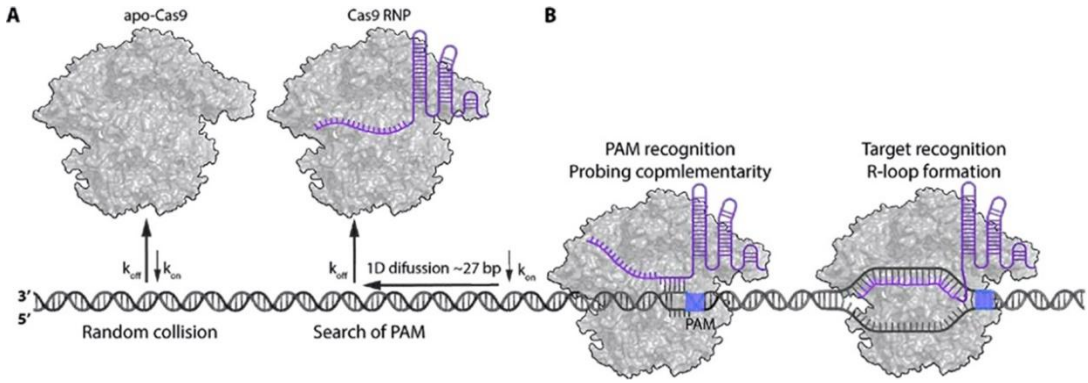

Figure 3. SpCas9 target search pathway.

4.2. Nuclease Cas9 cleavage mechanism

Cas9 protein relies on two domains with cleavage activity: the HNH domain, which cleaves the DNA strand complementary to the crRNA, and the RuvC domain, which cleaves the non-complementary strand. The RuvC domain can be divided into three subdomains. RuvC I is close to the amino terminus of Cas9, and RuvC II and RuvC III flank the HNH domain When Cas9 binds to the target gene locus, a conformational change occurs, and the nuclease functional region performs positional cleavage on the reverse strand of the target DNA [12].

5. Applications Of CRISPR

5.1. Applications in crop genome engineering

In the crop genome editing work based on CRISPR/cas9 technology, the application of CRISPR/cas9 technology in the targeted editing of different plant genomes such as tobacco, sorghum, rice, corn and so on, can obtain genome editing plants with high mutation rate and stable inheritance, and cause double-stranded breaks of DNA sequences at target sites through their own endonuclease activity. The mutation is then caused by NHEJ or homologous recombination-mediated repair. Cas9 is suitable for expression in rice by codon optimization techniques and uses a strong promoter to drive its expression. The researchers selected two genes for CRISPR-Cas site-specific mutations, namely CAO1 of rice chlorophyll synthesis gene and LAVE of tillering angle control gene, and the results showed that 83.3% of the CAO1 gene loci in the first generation of transgenic plants were mutated, while up to 91.6% of transgenic plants had corresponding loci mutations in the LAZY gene, of which the proportion of LAZY gene mutant homozygotes reached 50%, showing the phenotype of tiller angle enlargement. This suggests that the CRISPR-Cas system can be used to achieve effective site-directed mutations in rice-specific genes. CRISPR-Cas site-directed gene mutation technology can greatly accelerate the study of rice functional genome, thereby accelerating the cultivation of high-yield, high-resistance and high-quality rice varieties and improving the yield and quality of rice.

Not only that, but CRISPR-Cas9, its derived base editor, and CRISPR activation system greatly assist in genomic engineering in plants. A multifunctional CRISPR-Combo platform based on a single Cas9 protein has been developed for simultaneous genome editing and gene activation in plants. Achieving rice regeneration without exogenous plant hormones through the application of CRISPR-Combo is a new method of primarily enriching heritable targeted mutations. It also shows that CRISPR-related technologies have broad application prospects in crop breeding [13].

5.2. Application on gene delivery vectors

In the field of gene delivery vector research and development, by applying CRISPR/cas9, scientists have developed a new gene delivery vector----MyoAAV.

DOI: 10.54254/2753-8818/4/20220645

Hereditary muscle diseases cause progressive muscle wasting and there are few treatments. Some use harmless viral vectors to deliver functional copies of disease-causing genes into cells, which have shown promise in targeted clinical trials, but also face challenges.

Viral vectors carrying functional copies of genes need high doses to reach muscles throughout the body, and those used in clinical trials tend to go more to the liver. Resulting in high levels of viral vectors in the liver and even death in the participants. In a new study, Pardis C. Sabeti, a professor and member of the Institute from research institutions such as the Broad Institute and Harvard University, Mohammadsharif Tabebordbar of Sabeti's lab, and Amy Wagers, A new family of adeno-associated viruses (AAV) was jointly developed to improve targeting to muscle tissue. It is a safe and effective method. This group of viral vectors was more than 10 times more efficient at reaching the muscle. Because of this increase in efficiency, MyoAAV can deliver therapeutic genes at about 100 to 250 times lower doses, potentially reducing the risk of liver injury side effects. The research team constructed MyoAAV by modifying the outer protein shell of AAV.

With MyoAAV, therapeutic genes or CRISPR-Cas9 gene-editing systems are delivered specifically to muscle cells. Improved muscle function in mouse models of Duchenne muscular dystrophy and X-linked myotube myopathy. Tubular myopathy is a relatively rare hereditary muscle disease. MyoAAV can efficiently deliver gene therapy to nonhuman primate muscles and human muscle cells.

5.3. Application in cancer treatment

The traditional method of making mouse models is to introduce mutations into mouse embryonic stem cells, and then inject the stem cells into blastocyst to form chimeric mice, and then another generation to obtain homozygous mutated mice. CRISPR/Cas9 technology effectively improves the efficiency of making mouse models. Jaenisch's research group used CRISPR/Cas9 to knock out 5 genes simultaneously in mouse embryonic stem cells, and then they injected Cas9 mRNA and sgRNA targeting Tet1 and Tet2 into the fertilized eggs of mice, and established mice that knocked out two genes at the same time.

In addition to enabling simultaneous editing of multiple genes in mouse embryonic stem cells and germ cells, the CRISPR/Cas9 technology is no less capable of editing in mouse somatic cells. The researchers used the virus-mediated CRISPR/Cas9 system to edit multiple genes of primary hematopoietic stem cells in mice and successfully established an acute myeloid leukemia (AML) model. In a mouse model of lung cancer expressing the KrasG12D gene, researchers used CRISPR/Cas9 to conduct functional screening of a series of possible tumor suppressor genes in human lung cancer patients, and studied the synergistic effect of these genes and the proto-oncogene KrasG12D in the occurrence and development of lung cancer. In 2014, Jacks' team destroyed the tumor suppressor genes Pten and p53 in the liver of mice by injecting the CRISPR/Cas9 plasmid into the tail vein to produce a mouse model of liver tumors, and the cancer mice produced by this technology have similar cancer phenotypes to mice constructed by traditional Cre-loxp technology.

When doing in vivo editing, the DNA encoding Cas9 and sgRNA is too large and inefficient to pack with viruses. In order to solve this problem, Zhang Feng's research group integrated Cas9 into the Rosa26 site to construct an inducible expression of Cas9 mice, Cas9 and CAG promoter between a loxP-stop (33 polyA signal)-loxP sequence to block the expression of Cas9, and then use the tissue-specific promoter to express Cre recombinase, you can remove the interference sequence starting Cas9 expression. Some researchers used AAV virus to transduce sgRNA targeting KRAS, p53 and LKB1 genes into the lungs of Cas9 mice, and successfully established a mouse lung cancer model. At this point, researchers can use induced Cas9-expressing mice for genetic manipulation to quickly establish cancer models in vivo.

DOI: 10.54254/2753-8818/4/20220645

6. Limitation And Future Development

6.1. The limitation of CRISPR

The first problem with Crisper is the off-target effect. [14] This is because genomes are extremely complex, and gRNAs may be locally matched to non-targeted sequences. This local match also activates Cas 9 endonuclease activity, resulting in an off-target effect. Cas9 can not only identify standard or non-standard PAMs, but also cause off-target effects to a certain extent. More than three mismatches between the target sequence and the 20-nucleotide sgRNA result in off-target effects [15] The off-target effect may affect the functional expression of normal genes, even activate carcinogens and inhibit tumor suppressor genes, causing potential safety hazards, which greatly hinders the further clinical application of this technology. At present, we mainly improve the specificity of the CRISPR/Cas9 system by optimizing gRNA design, modifying Cas9 protein, using RNP delivery methods, and other strategies to reduce the off-target effect. The second problem with Crispr is that the Cas9 protein is too bulky. Many applications currently require gene editing machines to fuse with other enzymes and functions, but most Cas9 proteins are too large to be delivered by viral vectors.

6.2. CRISPR double nickase

Zhang's team introduced targeted double-strand breaks by combining Cas9 Nickase mutants with pairwise guide Rnas. Because individual cleavages can be repaired with high fidelity, proper offset to guide RNA to synchronous cleavages is required for double-strand breaks. [16]

Well, as shown in figure 4, WTCas9 can cut double and double strands, while Cas9nickase on the right can only cut one strand, and Zhang Feng's team took advantage of this feature, and he came up with the idea of using two CAS nine Nick to work together on a gene locus to form a double row break. On the other hand, Nonspecific binding does not cause double-strand breaks, which reduces nonspecific mutations.

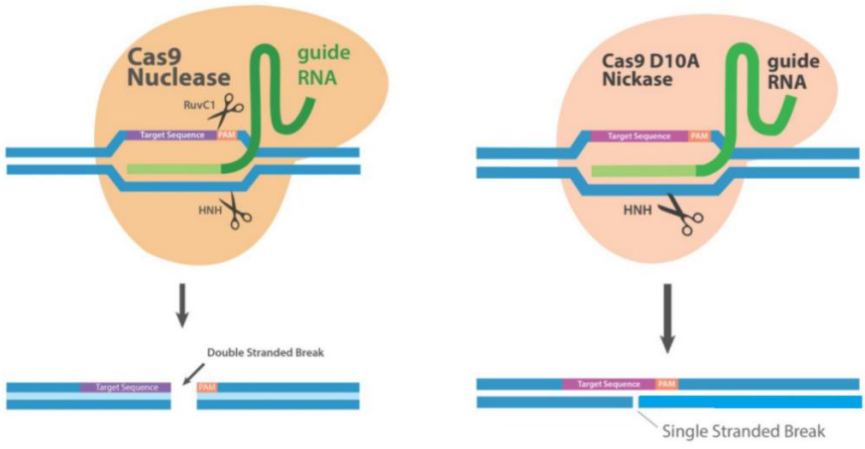

Figure 4. The mechanism of Cas9.

Taking advantage of the fact that Cas9 nickase can only perform single strand splicing, Zhang Feng's team thought of combining two Cas9 nickases on a gene site to form a double strand break (DSB), while non-specific binding will not cause DSB, thus reducing non-specific mutations.

After testing, the team found that the offset in figure 5 below was between - 8 and 8 bp, which caused the best NHEJ. The effect of more than 8bp to 100bp is also acceptable.

DOI: 10.54254/2753-8818/4/20220645

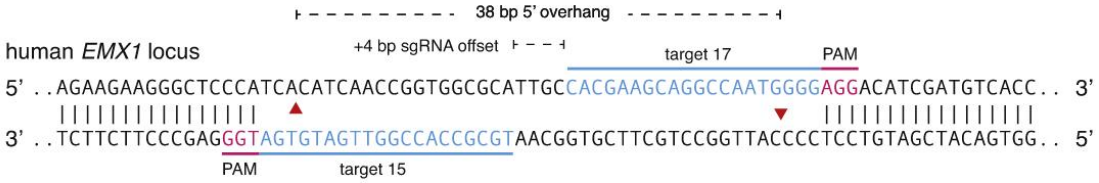

Figure 5. Representative sequences of the human EMX1 locus targeted by Cas9n [14]

6.3. Iscb-ωrna system

On May 24, 2022, a team of researchers from Cornell University proposed a solution. In this new study, the researchers found that the IscB-ωRNA system works in a similar way. By replacing the protein component of the larger Cas9 with RNA, the IscB protein is compressed into the core chemical reaction center to cleaves the target DNA. Achieve smaller sizes [17].

6.4. future development

In the direction of drug development and gene diagnosis technology for human diseases, AGN-151587 (EDIT-101), developed by Editas Medicine in collaboration with Allergan, The CAS9-encoding gene and two guide Rnas (grnas) were loaded into AAV5 viral vectors and are currently in phase 1/2 clinical trials in LCA10 patients. [18]

SHERLOCK is a CRISPR-based diagnostic tool developed by Dr. Zhang's team that can detect genetic "fingerprints" of multiple organisms or sample types up to the single-digit Amor level, which may indicate the presence of a single DNA or RNA molecule in the sample. Recently, Professor Zhang Feng's team has developed a method to detect the RNA of the new coronavirus using CRISPR-based technology, which only requires a purified nucleic acid molecular sample, and can be completed in 1 hour in a simple three-step process. However, this technology is still relatively preliminary, has not yet been tested in a real patient sample, and cannot be used clinically to diagnose the new crown virus infection. In the future, more new drugs can be developed based on CRISPR/cas9 technology, and the speed and effect of genetic diagnosis technology also have great potential for improvement.

The off-target effects of Cas nucleases are particularly dangerous in the specificity and delivery of CRISPR-Cas systems compared to off-target effects of small-molecule drugs or antibody therapies, as they cause permanent changes in the genome. Therefore, improving the specificity and targeted delivery of Cas is particularly critical. Researchers have made great strides in improving Cas enzyme and sgRNA design, greatly increasing the specificity of nucleases. Furthermore, tools to predict on-target outcomes and spatiotemporal control of gene expression provide comprehensive coping strategies to reduce off-target effects.

Optimizing the vector for delivering cas systems remains a major challenge, especially when the human body can generate an immune response to sgRNA and Cas. In the lab, researchers can use electroporation, transfection, direct injection, and more to introduce DNA, mRNA, or RNA protein complexes encoding the Cas system into cells. However, many of these methods are not applicable in clinical settings. Moreover, loading individually larger Cas nucleases into viral vectors formed by binding to guiding RNA remains a challenge. One strategy for solving this problem is to utilize individually smaller Cas homologues, or to minimize the system in order to load them into viral vectors. Another approach is to use nanomaterials to accomplish directional transport of specific cell species. Recent studies have shown that direct injection of nanoparticles carrying Cas9-sgRNA in mouse models can correct DMD mutations, leading to clinical symptom relief. The success of future CRISPR-based therapies will depend heavily on further aspects of delivering Cas system carriers

Cystic fibrosis is an incurable human genetic disease, and scientists have been fighting against it, especially for some mutations that cause cystic fibrosis; Recently, scientists from the University of Trento and other institutions said through research that they might be able to use gene editing technology to promote the heritability of CRISPR Cas treatment for cystic fibrosis.

The related research results are expected to open a new perspective for the development of new treatment for cystic fibrosis; Cystic fibrosis is caused by a special gene mutation, which can produce cystic fibrosis transmembrane conduction regulator (Cftr). Its abnormal function will affect the function of multiple organs, especially the lung. The technology is called SpliceFix. Because it can restore the mechanism of protein production while repairing genes. This technology also named as AsCas12a genome editing as they use a single crRNA and the Acidaminococcus sp. BV3L6, AsCas12a.

7. Conclusion

Through research, we found that the rapid development of crispr/cas9 editing technology in recent years is different from the generation of zinc finger nuclease technology and the second generation of transcriptional activator-like effector nuclease technology, with relatively small cost, relatively small size, more accurate and reliable principles and mechanisms, Cas9 protein not only has helicase activity, can unlock DNA strands, but also has nuclease activity, can cut DNA strands. Excellent results have been achieved in the fields of human genetic disease treatment, cancer disease radical treatment, crop gene control, etc., but at the same time, the crispr/cas9 system also faces problems such as the inaccurate matching of sgRNA with target DNA and the inability to select a suitable delivery vector due to the excessive volume of the cas9 protein. Corresponding solutions are constantly being invented, such as the IscB- ω RNA system and the CRISPR double-incision enzyme protocol of Zhang Feng's team. In the future, gene editing technologies such as CRISPR/Cas9 systems will inevitably be applied to more and more fields, and there will be broader development and achievements.

References

[1] H.I. Ahmad, et al. A review of CRISPR-based genome editing: survival, evolution and challenges. Curr Issues Mol Biol, 2018, 28: 47–68.
[2] K. Musunuru. The hope and hype of CRISPR-Cas9 genome editing: a review. JAMA Cardiol, 2017, 2(8): 914–919.
[3] G. M. Martin, et al. Increased chromosomal aberrations in first metaphases of cells isolated from the kidneys of aged mice. Isr J Med Sci, 1985, 21(3): 296-301.
[4] M. R. Lieber, et al. Ageing, repetitive genomes and DNA damage. Nat Rev Mol Cell Biol, 2004, 5(1): 69-75.
[5] M R. Lieber. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem, 2010, 79: 181-211.
[6] H. H. Y. Chang, et al. Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nat Rev Mol Cell Biol, 2017, 18(8): 495-506.
[7] F. Paques, J. E. Haber. Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. Microbiol Mol Biol Rev, 1999, 63(2): 349-404
[8] S. Chandrasegaran, D. Carroll. Origins of programmable nucleases for genome engineering. J Mol Biol, 2016, 428(5): 963-989
[9] Y. Liu, et al. Development and challenges of gene editing technology. Chinese Journal of Bioengineering,2019,35(08):1401-1410.
[10] A. Lapinaite, et al. DNA capture by a CRISPR-Cas9–guided adenine base editor. ence, 2020, 369(6503):566-571.
[11] C. Huai, et al. Structural insights into DNA cleavage activation of CRISPR-Cas9 system. Nature Communications, 2017, 8(1):1375.
[12] C. Pan, et al. Boosting plant genome editing with a versatile CRISPR-Combo system. Nat. Plants 8, 513–525 (2022).
[13] C.A. Lino, et al. Delivering CRISPR: a review of the challenges and approaches. Drug Deliv. 2018;25 (1):1234–1257.
[14] X.H Zhang, et al. Off-target effects in CRISPR/Cas9-mediated genome engineering. Mol TherNucleic Acids. 2015;4(11):2162–2531.

[15] F. A. Ran, et al. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell, 154(6), 1380–1389.
[16] G. Schuler, et al. Structural basis for RNA-guided DNA cleavage by IscB-ωRNA and mechanistic comparison with Cas9. Science. 2022 Jun 24;376(6600):1476-1481.
[17] Z.G. Liu. Research progress of genome editing mediated by CRISPR/Cas9 system. Journal of Animal Husbandry and Veterinary Medicine,2014,45(10):1567-1583
[18] G. Maule, et al. Allele specific repair of splicing mutations in cystic fibrosis through AsCas12a genome editing. Nat Commun. 2019 Aug 7;10(1):3556.