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Progress in human diseases treatment CRISPR system

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Abstract. Clinical medicine and biological sciences make extensive use of the gene editing method symbolized by CRISPR-Cas9. With the rapid development of molecular biology technology in recent years, the CRISPR-Cas system—which stands for clustered regularly interspaced short palindromic repeats—has become a potent tool for gene editing. Due to its high efficiency, precision, and flexibility, it has surpassed last two generations in its application within the field of molecular biology. This article provides a detailed overview of the development history, working mechanism, and applications of this technology. The CRISPR-Cas system achieves specific site modifications by introducing insertions, deletions, or single base substitutions at particular genomic sites. It has made significant contributions to research and treatment in areas such as the therapies of tumors and genetic diseases. However, challenges such as off-target effects need to be addressed before its practical clinical utility can be rigorously validated through further research and clinical trials.

Keywords: CRISPR/Cas System, gene editing technology, off-target effect, cancer, genetic disease.

1. Introduction

In 1987, upon identifying the alkaline phosphatase isozyme gene within the bacteria, the researchers of Osaka University occasionally discovered that certain identical sequences were flanked by an apparently disorganized sequence, which consisted of five homologous repeats, each comprising 24 nucleotides.[1] In 1993 Francisco Monica found this repeating structure in over 40% of bacteria and 90% of archaea, and initially named it Short Regularly Spaced Repeats (SRSRs) [2]. Later, they renamed it CRISPR. In 2002, Ruud's team discovered homologous genes located near the CRISPR sequences, which they named the CRISPR-associated genes [3]. The proteins encoded by these genes were referred to as Cas9 proteins. From that point onward, CRISPR and Cas became closely linked.

Then in 2005, researchers discovered that these spacer sequences matched the DNA of viruses that had infected the bacteria, which revealing the relationship between CRSIPR sequences and the bacterial defense against viral infection [4]. In 2007, Horvath's research group proposed that CRISPR and cas genes together provided Streptococcus thermophilus with resistance against bacteriophages. Meanwhile, this specificity depended on the spacer sequences within the CRISPR array, marking the first experiment demonstration of CRISPR's immunological function in bacteria [5]. Subsequent research confirmed that the Protospacer Adjacent Motif (PAM) plays a crucial rule to recognize and function properly [6]. To further study the immune system, Charpentierm, during her research on Streprococcus pyogenes,

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discovered the molecule tracrRNA [7]. This system enables bacteria to cut viral DNA and resist viral invasion.

In 2011, Siksnys and his colleagues were the first to demonstrate that the Type II CRISPR system could be transferred from Streptococcus thermophilus to Escherichia coli [8]. Following this, in 2012 Charpentier/Doudna and Siksnys confirmed that CRISPR-Cas9 system could perform as an in vitro genome editing technique [9] [10]. Cas9 was effectively exploited by Feng Zhang and George Church in 2013[11] to modify the genomes of mammalian cells. Numerous more Cas proteins, such as Cas12, Cas13, and Cas14, have been identified as CRISPR technology develops. For their work on CRISPR-Cas9 genome editing, Jennifer Doudna and Emmanuelle Charpentier were awarded the 2020 Nobel Prize in Chemistry.

2. The Development of Gene Editing

There were two approaches to cleave site-specific double-strand DNA before CRISPR-Cas9 was developed: ZFNs [12] and TALENs [13].

2.1. ZFNs

The ZFNs technology was the first generation of gene editing. These are modular enzymes made up of the DNA-cleavage domain and the zinc-finger protein (ZFP) domain [14]. ZFP is the most prevalent class of DNA-recognition proteins in eukaryotic species, and creating new DNA sequence-specific binding proteins requires first elucidating ZFP's DNA-binding domain. Zinc ions fix around thirty amino acids in a conserved ββα conformation within each ZFP. With differing degrees of selectivity, several amino acids on the surface of an a-helix. The DNA binding domain in ZFP generally contains three independent Zinc finger (ZF) repeats, each of which can recognize three consecutive bases, so a zinc finger DNA binding domain can identify a specific 9bp sequence (ZFN dimer, which contains six zinc fingers can recognize specific sequences of 18bp length). At the carboxyl terminal of nonspecific endonucrenase FokI the ZFN cleavage domain, which consists of 96 amino acid residues, is linked to the binding domain's C-terminal [15]. Each FokImonomer can be linked to a ZFP, thus forming a specific site for ZFN recognition; when the two recognition sites are separated by 6 ~ 8 bp, two Fok I can be polymerized into dimer, which can perform enzyme digestion and lead to double strand break, and then gene editing is performed.[16][17]

However, the Capability to target all potential genomic loci is limited by the accessibility of ZFPs. Since each ZFP recognize and bind to a specific 3-base pair(bp) sequence, 64 DNA triplet permutations exist. Presently, not all of the triplets' permutations have corresponding ZFPs. Therefore, ZFNs have relatively high off-target effects and may eventually lead to DNA mismatch and sequence change, resulting in strong cytotoxicity. When these adverse effects accumulate too much and exceed the range of cell repair mechanisms, cell apoptosis will be caused. In addition, due to the complexity of protein synthesis techniques, the generation of new ZFPs is relatively slow and expensive and it is necessary to ensure the proper sequence and folding of each ZFP.[14]

2.2. TALENs

After ZFNs, TALENs is another focused editing method that is more effective and has less off-target effect [18]. Plant pathogenic Xanthomonas bacteria produce TALEs protein. They attach to the desired promoters in the plant cell, and produce genes. Each TALEs has 34 amino acids that each recognizes a single bp and two Repeat variable diresidue (RVD) at position 12 and 13. TALE is able to identify specific targets through RVD due to its function of recognition specificity [19]. FokInuclease catalytic domains combine with TALE repeat sequences to form the TALEN protein, which can recognize and cleave specific DNA sites. When a pair of TALEN proteins recognize the target sequences on opposite strands, the catalytic domains of the FokI nuclease form a dimer, enabling the cleavage of the DNA sequence between the recognition sites (typically 12 to 20 bp apart) and breaking double-strand at specific locations [20], which can then be repaired through homology-directed repair (HDR) or non-homologous end joining (NHEJ), leading to gene editing [17].

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However, TALENs have some downsides. One such drawback is their larger size, as one TALE repeat is corresponding to a single nucleotide, and may result in less specificity [21]. The typical TALEN is encoded by around 3 kbp of cDNA. This may complicate the delivery and in cellular expression of a pair of TALENs. Moreover, therapeutic applications that necessitate distribution via viral vectors with constrained cargo size may find their increased size to be a constraint [22].

3. CRISPR/Cas System

The CRISPR/Cas system, which was first identified in bacteria and archaea, is a widely used defense mechanism against foreign genetic material [2]. To protect target DNA from foreign DNA invasions, it introduces site-specific double-strand breaks using RNA-guided DNA.

3.1. Components of CRISPR/Cas System

This system consists of CRISPR Array, an upstream leader and CAS Protein. DNA fragments in the CRISPR array are composed of Repeats, which are joined by Spacers [1]; repeat lengths within arrays are usually the same as sequence lengths, however there are occasionally minor variations [23]. Repeats typically range in length from 23 to 47 bp, and subtypes I-C, I-E, I-F, and II have a consensus sequence [24]. On the other hand, the majority of the spacer sequences in a genome are distinct. These spacers, which provide sequence-specific protection against those extra-chromosomal agents, are DNA fragments from a foreign virus or phage [4][5]. The majority of spacers most likely originate from plasmids or phages. In this system, the spacer sequence is essential. A crRNA (CRISPR-derivedRNA) is produced upon transcription, and once mature, it identifies the foreign target sequence and directs the cleavage-active Cas9 protein to attach to it in order to carry out the cleavage function. Additionally, the leader sequence can be found upstream of the initial repetition [3]. This 200–500 bp long AT-rich segment contains the promoter that starts the array's transcription.[25]

Genes encoding the Cas proteins are next to the CRISPR array. Cas proteins target and obtain new spacers from invading elements [25]. Currently, CRISPR/Cas systems are categorized into three types [24]: Type I and Type III systems require the involvement of multiple proteins to function effectively, whereas Type II system relies on Cas9. It facilitates the incorporation of spacer sequences into the CRISPR array to accurately identifies and cleaves foreign DNA or RNA [26].

3.2. Mechanism of CRISPR/Cas System

When foreign nucleic acids first invade, the bacteria recognize a specific sequence within it and integrate it as a spacer sequence into the CRISPR locus. If the same plasmid or virus invades again, this spacer sequence is transcribed into CRISPR-derived RNA (crRNA), and Transactivating crRNA (tracrRNA) is also transcribed.

In eukaryotic cells, the CRISPR/Cas9 system comprises a single guide RNA (sgRNA) and the Cas9 protein. The sgRNA is formed by combining tracrRNA with guide RNA (gRNA). This Cas9 protein acts as a nuclease, enabling precise DNA editing at designated locations as directed by the gRNA. To target a specific gene, the sgRNA first binds to its corresponding sequence in the gene, thereby directing the Cas9 protein to its intended site. Subsequently, the Cas9 recognizes a Protospacer Adjacent Motif (PAM), which consists of NGG nucleotides located next to the target region. Once this recognition occurs, the Cas9 protein becomes activated and cleaves the target gene. Once the gene is disrupted by the Cas9 protein, two repair pathways are initiated: Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR). The NHEJ repair pathway directly joins the broken DNA ends, often leading to nucleotide deletions that disrupt the gene's open reading frame, causing frameshift or nonsense mutations, which result in gene inactivation and achieve gene knockout. In contrast, the HDR repair pathway requires the introduction of a repair template that is complementary to one strand of the DNA. This template can be integrated into the target sequence through base pairing, enabling gene knock-in [27][28].

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4. Application of CRISPR

4.1. Using CRSIPR in Tumour Therapy

CRISPR/Cas9 is an effective gene-editing technique that has opened up a lot of possibilities for cancer treatment due to its ease of use and efficiency. As of right now, the CRISPR/Case gene editing method has demonstrated encouraging curative benefits in cases of bladder cancer, colorectal cancer, lung cancer and other cancers [29, 30, 31].

In recent years, utilizing the CRISPR/Cas9 technology to optimize CAR-T (chimeric antigen receptor T-cell) therapy in immunotherapy has achieved substantial progress, making it one of the important directions for future research development. CAR-T cell therapy, as a novel form of immunotherapy, works by modifying T cells into CART-T cells that carry chimeric antigen receptors. Then, they can specifically target and kill tumor cells through the immune activation effects of T cells.

T cells are frequently isolated and subsequently altered from tumor patients' peripheral blood in clinical trials including CAR-T treatment. CAR-T therapy's use is limited, nevertheless, as many patients are unable to obtain an adequate quantity of high-quality T cells. Allogeneic T cells can compensate for the deficiencies in both quantity and quality of autologous T cells; however, disparities in major histocompatibility complex (MHC) and T cell receptors (TCR) between donors and recipients may lead to significant allogeneic reactions and graft-versus-host disease (GVHD) when donor-derived T cells are administered to recipients. CRISPR/Cas9, with its ability for multiplex genome editing, can simultaneously target multiple sites to create universal CAR-T cells that lack endogenous MHC and TCR expression [32].

Programmed death-1 (PD-1) serves as a critical immunological checkpoint that inhibits T cell activation through its interaction with the ligand PD-L1. This mechanism is vital for managing normal immune responses and allowing tumors to evade immune detection. By obstructing the PD-1/PD-L1 signaling pathway, tumor immunotherapy can reactivate T cells, thereby boosting their ability to target cancer cells [33]. Recent advancements have shown that CAR-T cells lacking PD-1 not only enhance cell survival—remaining viable in vivo for almost nine months—but also minimize clinical toxicity.

Rupp et al. conducted a study wherein T cells were electroporated to introduce Cas and sgRNA targeting exon 1 of the PD-1 gene. Subsequently, these cells were transduced with lentiviruses to express the CD19 CAR gene. The results indicated that CD19 CAR-T cells with deleted PD-1 genes effectively eradicated tumor cells in a mouse model containing PD-L1+CD19+ tumors. This finding underscores the significant role of the PD-1/PD-L1 signaling pathway in regulating T cell functionality [34].

4.2. Using CRSIPR in Therapy of Genetic Diseases

The pathogenic gene for Duchenne muscular dystrophy (DMD), an X-linked recessive neuromuscular illness, is the dystrophin gene with 79 exons. It is known as the largest human gene as it is about 2.2Mb long. Mutations in the DMD gene primarily manifest as exon deletions or duplications. Some researchers have used CRISPR/Cas9-based targeted gene activation (gain-of-function) to supplement dystrophy for the treatment of DMD [35]. Considering the capacity of AAV vectors for gene therapy, the researchers optimized the SAM system by targeting sgRNAs that are 14 nt or 15 nt to guide Cas9 to the target gene site. The study showed that reducing the number of nucleotides in the sgRNA that match the target sequence from 20 to 14 or 15 could mediate effective activation, while also inhibiting Cas9's endonuclease activity and preventing the formation of indels, thus being called lethal sgRNAs (dead sgRNAs, dgRNAs). Meanwhile, the researchers modified the framework structure of dgRNAs by changing the G and C content in the MS2 stalk-loop structure adapter region to determine the optimized dgRNA framework structure. By packaging dgRNA and MPH (MS2-p65-HSF1) separately with AAV and co-transfecting them into DMD mice. Therefore, the muscle function of mice is significantly improved and is effectively alleviated the symptoms of DMD.[36]

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5. Limitation of CRISPR/Cas System

Despite the broad utility of CRISPR in cancer biology, the biggest drawback of this technology is off-target effects, which vary across different species. It is essential to identify the causes of off-target effects and find ways to ensure that Cas9 proteins accurately cut the target sequence. Reducing off-target effects remains a major challenge in the application of this technology. Off-target effects refer to the phenomenon where Cas9 proteins, in addition to cutting the target sequence, also act on other sequences similar to the target sequence. These effects occur because the sgRNA can partly mismatches when binding with DNA sequences, leading the CRISPR/Cas9 system to cut non-target DNA sequences, resulting in unwanted mutations. There are two situations in which sgRNA may cause mismatches at non-target sites: (1) Only base pair mismatches exist between the non-target DNA sequence and the sgRNA, both of which are the same length. (2) Base pair mismatches and the development of DNA or RNA bulges are caused by the uneven length of the non-target DNA sequence and the sgRNA [37][38].

In order to reduce the off-target effect, several optimization strategies have been developed, such as Double-nickase measures and dCas9 protein binding to FokInuclease [28]. Off-target effects can be reduced by controlling the concentration of the Cas9-sgRNA complex. By regulating the expression levels of either Cas9 protein or sgRNA, off-target effects can be minimized. Pattanayak and colleagues found that shorter, less active sgRNAs were more specific than longer, more active ones [39]. Moreover, high concentrations of sgRNA-Cas9 complexes could cut sites near or within the PAM sequence, leading to off-target effects. However, reducing these concentrations may also decrease the overall genome editing capability. As such, achieving a balance between the effectiveness of gene editing and off-target effects is imperative. According to recent studies, a gRNA to Cas9 complex ratio of 2:1 or 3:1 efficiently minimizes off-target effects while producing high target gene knockdown efficiency. [38]

6. Conclusion

As a third-generation of gene-editing tool, the CRISPR system surpasses ZFNs and TALENs in various aspects. It is expected to become the primary gene-editing tool in the future. As the developers of this technology have stated, "The application of CRISPR technology is limited only by our imagination." In summary, the CRISPR system has already made a significant impact molecular biology. Many of the fundamental and perplexing concerns have already started to be addressed by the swift development and advancements in CRISPR technologies. However, its application requires continued exploration, and its potential still entails further development.

References

[1] Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., & Nakata, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. Journal of bacteriology, 169(12), 5429–5433.

[2] Mojica, F. J., Juez, G., & Rodríguez-Valera, F. (1993). Transcription at different salinities of Haloferax mediterranei sequences adjacent to partially modified PstI sites. Molecular microbiology, 9(3), 613–621. https://doi.org/10.1111/j.1365-2958.1993.tb01721.x

[3] Jansen, R., Embden, J. D., Gaastra, W., & Schouls, L. M. (2002). Identification of genes that are associated with DNA repeats in prokaryotes. Molecular microbiology, 43(6), 1565–1575. https://doi.org/10.1046/j.1365-2958.2002.02839.x

[4] Mojica, F. J., Díez-Villaseñor, C., García-Martínez, J., & Soria, E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. Journal of molecular evolution, 60(2), 174–182. https://doi.org/10.1007/s00239-004-0046-3

[5] Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A. , & Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science (New York, N.Y.), 315(5819), 1709–1712. https://doi.org/10.1126/science.1138140

[6] Mojica, F. J. M., Díez-Villaseñor, C., García-Martínez, J., & Almendros, C. (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology (Reading, England), 155(Pt 3), 733–740. https://doi.org/10.1099/mic.0.023960-0

DOI: 10.54254/2753-8818/74/2024.LA18768

[7] Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J., & Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature, 471(7340), 602–607. https://doi.org/10.1038/nature09886

[8] Sapranauskas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P., & Siksnys, V. (2011). The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. Nucleic acids research, 39(21), 9275–9282. https://doi.org/10.1093/nar/gkr606

[9] Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science (New York, N.Y.), 337(6096), 816–821. https://doi.org/10.1126/science.1225829

[10] Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proceedings of the National Academy of Sciences of the United States of America, 109(39), E2579–E2586. https://doi.org/10.1073/pnas.1208507109

[11] Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science (New York, N.Y.), 339(6121), 819–823. https://doi.org/10.1126/science.1231143

[12] Hansen, K., Coussens, M. J., Sago, J., Subramanian, S., Gjoka, M., & Briner, D. (2012). Genome editing with CompoZr custom zinc finger nucleases (ZFNs). Journal of visualized experiments: JoVE, (64), e3304. https://doi.org/10.3791/3304

[13] Hockemeyer, D., Wang, H., Kiani, S., Lai, C. S., Gao, Q., Cassady, J. P., Cost, G. J., Zhang, L., Santiago, Y., Miller, J. C., Zeitler, B., Cherone, J. M., Meng, X., Hinkley, S. J., Rebar, E. J., Gregory, P. D., Urnov, F. D., & Jaenisch, R. (2011). Genetic engineering of human pluripotent cells using TALE nucleases. Nature biotechnology, 29(8), 731–734. https://doi.org/10.1038/nbt.1927

[14] Carroll D. (2011). Genome engineering with zinc-finger nucleases. Genetics, 188(4), 773–782. https://doi.org/10.1534/genetics.111.131433

[15] Wah, D. A., Bitinaite, J., Schildkraut, I., & Aggarwal, A. K. (1998). Structure of FokI has implications for DNA cleavage. Proceedings of the National Academy of Sciences of the United States of America, 95(18), 10564–10569. https://doi.org/10.1073/pnas.95.18.10564

[16] Gaj, T., Gersbach, C. A., & Barbas, C. F., 3rd (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in biotechnology, 31(7), 397–405. https://doi.org/10.1016/j.tibtech.2013.04.004

[17] Chen L Q, & Li Q. (2018). Application of Zfns, talens and crispr-cas gene editing technology in tumor therapy. Pharmaceutical Biotechnology, 25(6), 5.

[18] Beumer, K. J., Trautman, J. K., Christian, M., Dahlem, T. J., Lake, C. M., Hawley, R. S., Grunwald, D. J., Voytas, D. F., & Carroll, D. (2013). Comparing Zinc Finger Nucleases and Transcription Activator-Like Effector Nucleases for Gene Targeting in Drosophila. G3: Genes, Genomes, Genetics, 3(9), 1717-1725. https://doi.org/10.1534/g3.113.007260

[19] Boch, J., & Bonas, U. (2010). Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annual review of phytopathology, 48, 419–436. https://doi.org/10.1146/annurev-phyto-080508-081936

[20] Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J. A., Somia, N. V., Bogdanove, A. J., & Voytas, D. F. (2011). Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic acids research, 39(12), e82. https://doi.org/10.1093/nar/gkr218

[21] Guilinger, J. P., Pattanayak, V., Reyon, D., Tsai, S. Q., Sander, J. D., Joung, J. K., & Liu, D. R. (2014). Broad specificity profiling of TALENs results in engineered nucleases with improved DNA-cleavage specificity. Nature methods, 11(4), 429–435. https://doi.org/10.1038/nmeth.2845

DOI: 10.54254/2753-8818/74/2024.LA18768

[22] Gupta, R. M., & Musunuru, K. (2014). Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. The Journal of clinical investigation, 124(10), 4154–4161. https://doi.org/10.1172/JCI72992

[23] Mojica, F. J., Díez-Villaseñor, C., Soria, E., & Juez, G. (2000). Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Molecular microbiology, 36(1), 244–246. https://doi.org/10.1046/j.1365-2958.2000.01838.x

[24] Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F. J., Wolf, Y. I., Yakunin, A. F., van der Oost, J., & Koonin, E. V. (2011). Evolution and classification of the CRISPR-Cas systems. Nature reviews. Microbiology, 9(6), 467–477. https://doi.org/10.1038/nrmicro2577

[25] Richter, C., Chang, J. T., & Fineran, P. C. (2012). Function and regulation of clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR associated (Cas) systems. Viruses, 4(10), 2291–2311. https://doi.org/10.3390/v4102291

[26] Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., Barrangou, R., Brouns, S. J., Charpentier, E., Haft, D. H., Horvath, P., Moineau, S., Mojica, F. J., Terns, R. M., Terns, M. P., White, M. F., Yakunin, A. F., Garrett, R. A., van der Oost, J., Backofen, R., … Koonin, E. V. (2015). An updated evolutionary classification of CRISPR-Cas systems. Nature reviews. Microbiology, 13(11), 722–736. https://doi.org/10.1038/nrmicro3569

[27] Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. Nature biotechnology, 32(4), 347–355. https://doi.org/10.1038/nbt.2842

[28] Dong Weipeng, Wang Junshi, Zhang Shaohua, et al. CRISPR system and its application in the research progress of mice [J]. Journal of biotechnology bulletin, 2018, (5) : 57-63. DOI: 10. 13560 / j. carol carroll nki biotech. Bull., 1985.2017 0530.

[29] Cao Y, Qian R L, Yang C, Wang C & Qin W X. (2022). Application of CRISPR-Cas9 functional gene screening technology in the study of liver cancer. Chinese Journal of Cell Biology (04), 663-671.

[30] JIANG Weihua, GAO Yongshan, Liu Na, Zhang Yuan, Yang Yanjun, WANG Guigang... & Zhang Z M. (2023). Knockout of PD-1 by CRISPR/Cas9 effectively enhances the antitumor activity of EGFR-CAR T cells against lung cancer. Chinese Journal of Gerontology (14), 3483-3487.

[31] Tang D D. (2023). Transmucosal CRISPR-Cas9 delivery of fluorpolysine targeting DAD1 gene for bladder cancer perfusion therapy (Ph. D. Dissertation, Lanzhou University). Dr. Https:// link.cnki.net/doi/10.27204/d.cnki.glzhu.2023.000268doi:10.27204/d.cnki.glzhu.2023.000268.

[32] Ren, J., Liu, X., Fang, C., Jiang, S., June, C. H., & Zhao, Y. (2017). Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition. Clinical cancer research : an official journal of the American Association for Cancer Research, 23(9), 2255–2266. https:// doi.org/10.1158/1078-0432.CCR-16-1300

[33] Hu, W., Zi, Z., Jin, Y., Li, G., Shao, K., Cai, Q., Ma, X., & Wei, F. (2019). CRISPR/Cas9-mediated PD-1 disruption enhances human mesothelin-targeted CAR T cell effector functions. Cancer immunology, immunotherapy: CII, 68(3), 365–377. https://doi.org/10.1007/s00262-018-2281-2

[34] Rupp, L. J., Schumann, K., Roybal, K. T., Gate, R. E., Ye, C. J., Lim, W. A., & Marson, A. (2017). CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. Scientific reports, 7(1), 737. https://doi.org/10.1038/s41598-017-00462-8

[35] Liao, H. K., Hatanaka, F., Araoka, T., Reddy, P., Wu, M. Z., Sui, Y., Yamauchi, T., Sakurai, M., O'Keefe, D. D., Núñez-Delicado, E., Guillen, P., Campistol, J. M., Wu, C. J., Lu, L. F., Esteban, C. R., & Izpisua Belmonte, J. C. (2017). In Vivo Target Gene Activation via CRISPR/Cas9-Mediated Trans-epigenetic Modulation. Cell, 171(7), 1495–1507.e15. https://doi.org/10.1016/ j.cell.2017.10.025

DOI: 10.54254/2753-8818/74/2024.LA18768

[36] Li W, Liang S Q, Bai J & Qin H Y. (2019). CRISPR-dCas9 transcriptional regulatory system and its application in the treatment of genetic diseases. Life science (6), 628-636. The doi: 10. 13376 / j. carol carroll BLS / 2019075.

[37] Lin, Y., Cradick, T. J., Brown, M. T., Deshmukh, H., Ranjan, P., Sarode, N., Wile, B. M., Vertino, P. M., Stewart, F. J., & Bao, G. (2014). CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. Nucleic acids research, 42(11), 7473–7485. https://doi.org/10.1093/nar/gku402

[38] Guo Q J, Han Q J & Zhang J. (2018). Off-target effects and optimization strategies of CRISPR/ Cas9 technology. Advances in biochemistry and biophysics (08), 798-807. The doi: 10.16476 / j. ibb. 2018.0013.

[39] Pattanayak, V., Lin, S., Guilinger, J. P., Ma, E., Doudna, J. A., & Liu, D. R. (2013). High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nature biotechnology, 31(9), 839–843. https://doi.org/10.1038/nbt.2673