

CRISPR/Cas Systems for Therapeutic Purposes: Applications in Disease Prevention, Mechanism Study, and Treatment

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Abstract. CRISPR/Cas systems were novel multifunctional genome editing techniques, and the modification of Cas proteins endowed them with more efficient and precise properties. Herein, we systematically outlined the principles and applications of CRISPR/Cas systems in disease prevention, mechanism research, and gene treatment of diseases. Specifically, we detailed the systems widely utilized, containing the Cas9, Cas12a, and Cas13a systems, and summarized the current status of the medical applications of these systems in terms of vaccine design, trait improvement, animal models of human diseases, organ transplantation, gene therapy, and gene detection. We concluded that CRISPR/Cas systems had great potential to advance medical research and practice but also should be carefully regulated and supervised to ensure their safety and efficacy.

Keywords: CRISPR/Cas systems, gene modification, therapeutic applications, gene testing.

1. Introduction

Gene editing is a revolutionary discovery that has opened up new possibilities and challenges for the manipulation of life at the molecular level. Feng Zhang applied the CRISPR/Cas9 system for the first time in 2013, and Emmanuelle Charpentier and Jennifer Doudna received the Nobel Prize in Chemistry in 2020 for their work on developing the sharpest tools in gene technology [1]. This new genetic engineering technique allows for the relatively precise modification of a target gene's location within the genome of an organism. An increasing number of Cas proteins were found and exploited to complement the technology category due to their widespread existence in bacteria and archaea. They are critical components of immune defense mechanisms, respectively, which are adaptive defense systems against foreign mobile genetic elements (MGEs) [2], as shown in Figure 1. Great advantages, overwhelming other gene editing techniques, were endowed, including high efficiency and simplicity. It has versatile applications in the life sciences. Hitherto, they had been applied in advanced disease prevention and facilitated research on revealing deep-seated mechanistic studies. And they were promising in terms of application in gene treatment.

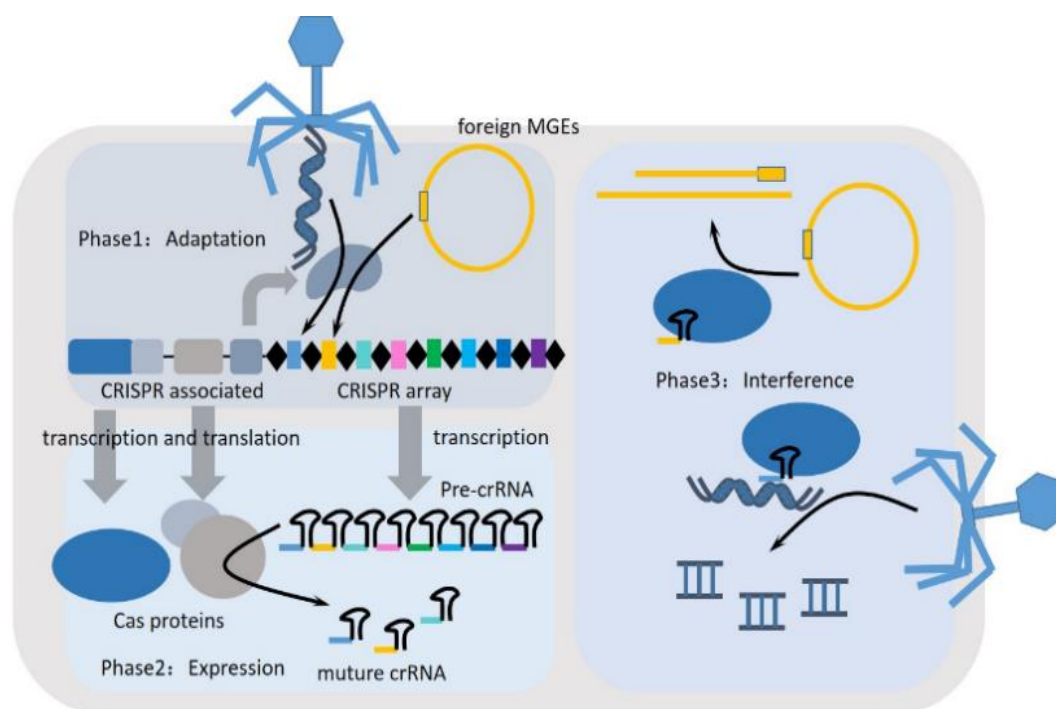


Figure 1. Immune defense mechanism of CRISPR/Cas systems

2. The overview of medical applications

2.1. Disease prevention

2.1.1. Genetic testing

CRISPR/Cas systems were widely used in disease detection. CRISPR genetic testing technology had provided simple, accurate, and fast ways to detect diseases in breeding, medical treatment, and other fields. The use of this technology had made grassroots testing and even on-site testing possible. The testing process was simplified; professionals and expensive instruments would gradually no longer be necessary.

Cas13a is an RNA enzyme belonging to type VI in the second class of CRISPR/Cas systems [3]. Cas13a has "incidental cleavage" capabilities. For example, Zhao et al. [4] designed a special RPA primer and CRISPR RNA (crRNA) for reacting with the LwCas13a system. They established a CRISPR/Cas13a detection method for the novel coronavirus (SARS-CoV-2). This quarantine method was unique, accurate, and fast. The detection could be conducted by test strips within two hours. This conduction method further reduced the dependence on real-time PCR instruments.

Since the amplification effect and principle were similar to those of RPA, the recombinase-mediated isothermal amplification technology RAA could also be combined with CRISPR-Cas13a to detect pathogenic bacteria. Su et al. [5] achieved rapid detection of *Staphylococcus aureus* by designing RAA-specific primers and crRNA, amplifying sample nucleic acids using RAA, and performing CRISPR-Cas13a molecular detection. The RAA-Cas13a method could complete the test within 30 minutes. This method is suitable for primary laboratories and easy to operate.

Cas12a was also a second class of Cas proteins with incidental cleavage activity. The CRISPR-Cas12a system could also be used for virus detection. Zhang et al. [6] used RPA combined with CRISPR-Cas12a to detect decapod iridescent virus I in shrimp samples within 40 minutes. Cas13a-PRA and Cas12a-PRA detection procedures were shown in Figure 3. A comparison of different CRISPR detection methods was shown in Table 1.

The application of CRISPR-Cas in genetic testing was not yet mature. Many bottlenecks needed to be broken. In the future, if the CRISPR quarantine technology could be combined with nucleic acid-free extraction technology, rapid detection methods suitable for site use would be obtained.

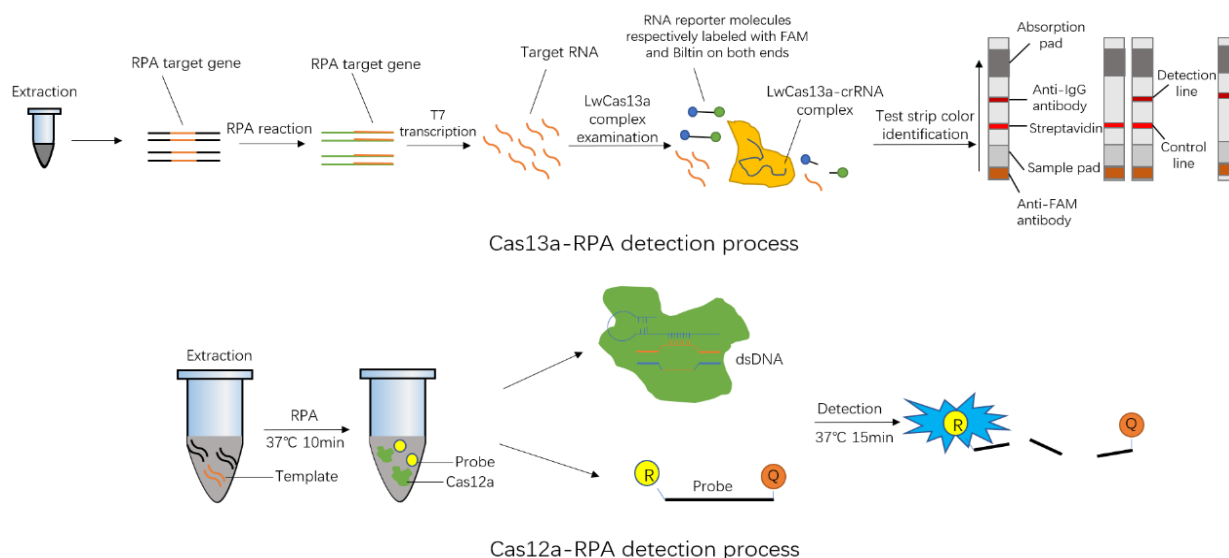


Figure 2. Cas13a-RPA and Cas12a-RPA detection process

Table 1. different CRISPR disease examination method comparison table

| Method | Target | Detection Method | Time | Sensitivity |
|----------------------|----------------------------------|------------------------|--------|---------------------------|
| RPA-Cas13a | SARS-CoV-2 | Test paper | 80min | 10 ⁴ copies/ml |
| RAA-Cas13a | Staphylococcus aureus | fluorescence detection | 30min | 1copy/μL |
| RPA-Cas12a | DIV1 | fluorescence detection | 40min | 10CFU/ml |
| Not amplified Cas13a | H7 subtype avian influenza virus | fluorescence detection | <60min | 100pmol/L |

2.1.2. Disease resistance

Economic animals were more susceptible to pathogen infection due to large-scale breeding. Therefore, targeted screening of disease-resistant animals could effectively identify the disease-resistance animals. The CRISPR/Cas9 system's two traditional routes for gene editing are non-homologous end joining (NHEJ) and homology-directed repair (HDR). Despite the high fidelity of HDR, mammalian cells prefer to use NHEJ over HDR through a number of ways [7]. First off, HDR is only active during the S/G2 phases of the cell cycle while NHEJ is active all throughout. Furthermore, NHEJ is quicker than HDR. Third, NHEJ suppresses the HDR process. However, NHEJ is error-prone and unpredictable. Knocking out virus target genes, genes for receptor proteins, and proteins required for virus proliferation and knocking in anti-infection genes via the CRISPR/Cas9 system were feasible ways to foster animal disease resistance via related methods. For instance, knocking out the CD163 domain conferred resistance to one specific virus, which is called PRRSV, in pigs [8]. Xu et al. [9] knocked out the genes of receptor proteins CD163 and the pAPN for the species to acquire complete resistance to PRRSV and transmissible gastroenteritis virus (TGEV).

2.1.3. Vaccine preparation

Live attenuated vaccines modified by the Cas9 protein were of good medical value in preventive medicine. Live attenuated vaccines, which reduced the virulence of pathogenic microorganisms by artificially directed mutations, could induce the desired immune response. Previously, the preparation process for live attenuated vaccines required a lot of time and labor. Generating random mutations in the viral genome via the CRISPR toolkit and selecting live attenuated viruses adapted to non-permissive cells could effectively increase the efficiency and reduce the cost of vaccine production.

NHEJ and HDR pathways were applied to virus attenuation, e.g., Fan et al. [10] prepared the M3 strain using Cas9 protein triple knockout HSV-1. FAdV4-egfp-rF2 and FAdV4-RFP_F1 attenuated vaccines were respectively produced by HDR recombination at different genetic loci of Fowl adenovirus 4 [11-12].

Recombinant vaccine was a vaccine made by using DNA recombinant biotechnology to directionally insert natural or artificially synthesized genetic materials from the coat protein of

pathogens that can induce immune responses in the body into target cells. The vaccine was made after the cells expressed and purified the genetic material. They had the advantages of being safe, effective, eliciting a long-lasting immune response, co-immunizable, and easy to implement. The recombination method could be divided into two types: insertion into the genome of the pathogen and free-state recombination. The former had stability, while the latter was more flexible. For example, Tang et al. [13] inserted ILTV, gDgI, and H9N2 AIV hemagglutinin genes into different positions of the assemblage HVT-IBDV VP2 virus genome, preparing a triple-insertion HVT-VP2-gDgI-HA recombinant vaccine. Moraes et al. [14] used Cas9 technology to generate markerless mutations in the *lysA* gene to obtain a lysine-nutrient-deficient recombinant BCG vaccine and co-express the LTAK63 antigen with a *Mycobacterium* vector.

2.2. Disease mechanical research

2.2.1. Disease models

This technology was increasingly used to create genetically modified animal models for research purposes. The establishment of animal models of human diseases could reduce the uncertain damage of drug treatment to the human body. The complexity of modeling was significantly simplified by the use of the CRISPR/Cas9 system, so it was widely used. An ideal disease model should accurately summarize the relevant disease phenotypes and mechanisms, providing a reliable platform for drug development and gene screening research. Zebrafish were suitable for constructing models of cardiovascular disease (CVD) and developmental diseases, for example, the *thpo* loss-of-function model, the YARS2(KO) model, the HEG1 mutant, etc. However, mice had high homology with humans and were suitable for constructing models for genetic and metabolic diseases, for instance, the 252T>C human ARSB mutation knock-in model, the *Ces2* (KO) model, and the *Slco1b2* knockout (KO) rat model. For animals with distant kinship, the animal gene needs to be humanized before constructing the model; otherwise, simulation distortion might be caused. CRISPR/Cas systems could also be used to target specific tissues or organs to induce pathogenesis based on delivery systems to study localized diseases or to culture human organs in animal organisms for modeling. For example, a non-human primate (NHP) model of retinitis pigmentosa (RP) was successfully established by knocking out the *RHO* gene in the macaque retina using AAV-SaCas9 technology [15]. Wu et al. [16] used the same delivery system to inject into the brains of macaques to study the association between aberrant behavior in non-human primates and acute manipulation of pathogenic genes in the brain. The SCNT system and the CRISPR/Cas9 system together generated FG double knockout pigs with severe immunodeficiency and later colonized hepatocytes to obtain an animal model with human hepatocytes [17].

2.3. Therapy

2.3.1. Organ transplantation

The insufficient supply of donor organs hindered the wide application of allotransplantation. On January 7, 2022, the world's first gene-edited pig heart transplant was urgently approved by the US FDA and completed, and the patient did well after surgery [18], marking the gene-edited animal organ as a promising therapeutic tool. Besides, it's promising to provide an efficient and precise means of xenotransplantation as an adjunct to the former. This technology was mainly applied to knock out cellular antigenic protein genes, endogenous viral genes in swine, and knock in anti-immune protein genes.

Utilizing the CRISPR/Cas9 system, combined with immunosuppressive therapies, allowed for long-term biological function of porcine heart, kidney, and islet grafts in NHPs. However, the liver is maintained in NHPs for only 25 days *in vivo*. Therefore, the use of CRISPR/Cas systems to develop porcine organ donors that are more compatible with humans could improve transplantation success rates and reduce late maintenance costs. However, most studies had only found that transferring human cell surface protein genes to donor animals reduced the immune response. But these proteins could only relieve it rather than eliminate it, so further research on the surface proteins of cancer cells

in vivo or the identity proteins of normal cells recognized by immune cells was needed. Additionally, the requirement for cross-match testing prior to transplantation would undoubtedly narrow the audience. Therefore, it was necessary to expand the pool of eligible recipients by establishing organ reserves and producing multiple matches, using the flexibility and efficiency of the CRISPR/Cas9 system.

2.3.2. Gene therapy

Despite the continuous improvement of cancer treatment strategies, the cure rate for cancer patients was still not satisfactory. In recent years, gene editing using the efficient and stable CRISPR/Cas9 tool had been widely considered for cancer treatment research.

The use of genetic diagnosis to identify sensitive genes was the main means of preventing cancer. Gootenberg [19] et al. set up a diagnostic system according to CRISPR called SHERLOCK. The system consists of RNA-guided RNase Cas13a (inducing strong nonspecific single-stranded DNA (ssDNA) reverse cleavage as a side effect) and reporting signals (RNA cleavage and release), which were highly sensitive to the detection of mutations in BRAFV600E and EGFR L858R in mammalian cells.

At the same time, large-scale genomic screening could detect mutated genes in various cancers. The CRISPR/Cas9 system exhibited better gene editing ability, fewer miss-target effects, and stronger adaptability. Compared to conventional methods using RNAi or cDNA libraries, it could influence either coding or non-coding parts of the genome and take many different designs and implementations [20]. The complete gene CRISPR/Cas9 knockout library was created by Shalem et al. [21] and targets 18080 genes in the human genome (GeCKO).

One of the pathogenic sources of cancer is oncogenic virus infection, usually including hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), and so on. Using Cas9 sgRNA could specifically identify the viral genome and directly target oncogenes and genes needed to maintain viral replication, causing mutations in the viral genome and inhibiting oncogene expression.

In recent years, the CRISPR/Cas9 system had been developed as an effective gene editing technology for the treatment of HIV-1 infection. It could be targeted for use in the HIV-1 genome or cell cofactors to reduce HIV-1 infection and eliminate the provirus, and to eradicate the virus by inducing the activation of transcription in the latent virus gene pool. Meanwhile, CRISPR-Cas9 may be able to target HIV-1's life cycle, shown in Figure 3.

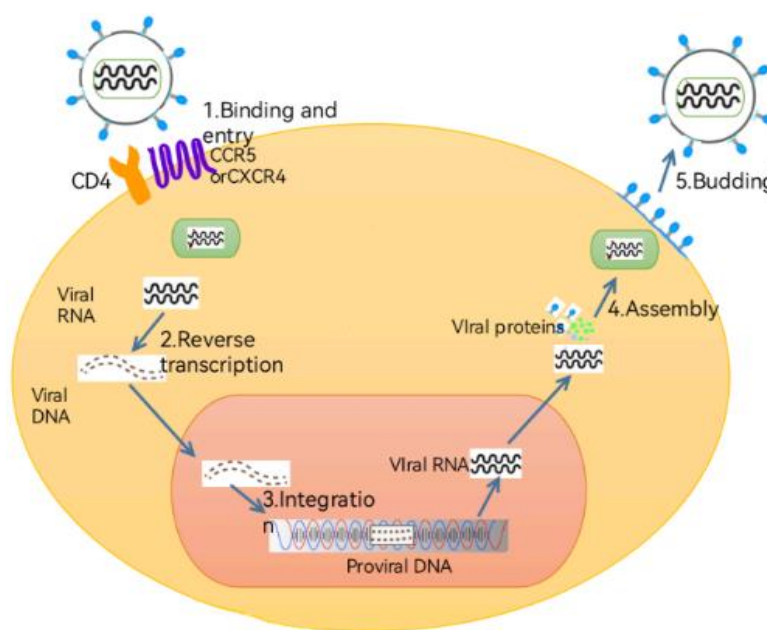


Figure 3. The Life Cycle of HIV-1

By reactivating dormant viruses in host cells through HAART, inducing cell killing and activating antiviral immune responses, the goal of eradicating latent HIV-1 reservoirs was achieved. Zhang et

al. [22] created 20 sgRNAs in 2015 that specifically target the LTR-U3 regions of the HIV-1 promoter and efficiently screen them for localization in NF- κ B targeting two targets near or near the binding site. Specific target sgRNA could induce reactivation of HIV-1 provirus in HIV-1 latent cell lines. Seven sgRNAs were created by Limsirichai et al. [23] to target important functioning components of the HIV-1 LTR. These sgRNA types, including two different sgRNA types and NF- κ B overlapping binding sites, have been shown to activate the HIV-1 LTR promoter and cause the production of putative HIV-1 genes.

2.3.3. Potential therapeutic application

In primary human B cells, CRISPR-Cas9 has been utilized to substitute endogenously encoded antibodies with antibodies that target certain pathogens such as RSV, HIV, influenza virus, or EBV [24]. The endogenous regulatory components that support typical antibody development and secretion effectively controlled the expression of the modified antibodies in primary B cells. It's a less harmful and more precise method of treatment. It's a less harmful and more precise method of treatment. Nonetheless, the option suffered from longer study times and the need for gene editing for each individual, entailing a deeper understanding of the antibody structure.

3. Conclusion

The CRISPR/Cas9 system allowed accurate editing of target sequences in the fields described above. However, it was used in a limited way, mainly for recombining exogenous genes and creating gene knockouts. Nevertheless, many genes in animal cells are not simply knocked in and will be expressed automatically, but are subject to very strict and precise regulation. Moreover, knocking out genes might cause irreversible modifications to the gene pool of the cells, which was not conducive to further modification and research. Additionally, the off-target effect could not be completely circumvented. Based on the above points, the application of base editing (BE), prime editing (PE), epigenetic editing, and other new expansion technologies on Cas proteins without generating double strand breaks (DSBs) was increasingly likely to result in a momentous revolution. The Cas12a- and Cas13a-based genetic diagnostic systems had been widely used in the detection of human and animal diseases. However, the former had the limitation of PAM sequence identification and the high off-target property of both of them, so there was still room for exploration and development.

Nevertheless, since CRISPR/Cas systems lowered the scientific threshold for genetic engineering, this technique also caused a series of ethical issues, such as uncertainty in the outcome of the modification, disruption of the well-being of animals, and irreversible contamination of the gene pool of the research species. Additionally, a quantity of technical barriers needed to be overcome, including optimization of delivery methods, ensuring precise knockout or integration of genes, and extending application targets. On the one hand, it's time to reinforce the censorship and management of this double-edged technique and to standardize the scientific implementation criteria. On the other hand, it's essential to exploit novel, harmless, reversible Cas proteins and their derivative operating systems endowed with a lower probability of off-target effects.

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