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EMERGING APPLICATIONS OF CRISPR–CAS9 BEYOND GENE EDITING

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ABSTRACT

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR CAS9) system is a powerful tool in genome editing and has evolved as a core part of molecular biology, biotechnology, biomedical research, diagnostics and therapeutics and spreading its vastness in other fields like agriculture, life sciences, industrial biotechnology and synthetic biology. CRISPR CAS9 does not only repair the cellular DNA but also works as the internal defense mechanism by adapting, expressing, recognizing the target and performing actions. This review discusses about the mechanism followed by CRISPR CAS9 in gene editing and various other possible applications of CRISPR CAS9.

Keywords: CRISPR-Cas9, DNA repair, Drug discovery, Gene Regulation, sgRNA.

1 INTRODUCTION

Researchers discovered clustered regularly interspaced short palindromic repeats within prokaryotic life first. CRISPR sequences plus Cas proteins function as internal defense mechanisms for bacteria plus Achaea. This natural immunity stops harmful DNA coming from phages or plasmids. This system represents a modern breakthrough in genetic science because researchers find usage simple and effective. Success with these experiments provides paths for clinical application. Scientists aim to fix human genomes by targeting specific sequences soon [1].

CRISPR Cas9 editing shows great worth. You want higher physical accuracy inside living biological units. Research demands improved timing during specific gene shifts. Stopping genomic mutations while cells divide stops structural faults. Refined gene tools help guarantee success as cells mature into grown forms correctly [2].

Genomes in CRISPR loci contain spacers taken from virus DNA plus other foreign parts between repetitive units producing tiny RNAs. These specific segments block infections by previous attackers since microbial cells actively learn through storing genomic snippets following every viral interaction. Bacterial immunity fluctuates through adding or deleting segments [3].

2 MECHANISM OF CRISPR CAS9

CRISPR-Cas9acts as biological defense found inside bacteria and archaea by neutralizing harmful genetic invaders like bacteriophages or plasmids.

The functional steps in the molecular action of CRISPR cas9 include initial viral adaptation period, expression stage, and the enzymatic target DNA interference event [4].

2.1 Adaptation (Spacer Acquisition)

During this phase, proteins chop up foreign DNA from invading pathogens. Enzymes insert segments called protospacers into the host genome. These pieces integrate within the CRISPR locus between repeat regions. This helps the organism identify and respond when the same pathogen returns to cause infections.

2.2 Expression and RNA Biogenesis

The CRISPR locus transcribes a long precursor RNA strand. This transcript holds spacer sequences along with repeat regions. Inside the Type II system, a Trans activating RNA links with repeat segments on the precursor transcript. RNase III proteins process this duplex into mature guide RNA sequences. These strands are fused into single guide RNA molecules, which improve performance while keeping target specificity intact.

2.3 Target Recognition and Interference

The guide RNA forms a complex with Cas9 enzymes. This assembly finds complementary DNA targets using standard pairing rules. Its recognition requires a specific sequence the target area, often referred to as the PAM sequence in the standard Streptococcus system.

When proteins bind the PAM site, Cas9 unwinds the local DNA to test complementarity. After matching, Cas9 undergoes a shift. Two specific enzyme domains break the strands. One HNH section cuts the matched DNA, and a RuvC domain cleaves the partner strand. This coordination results in a precise double strand break near the PAM site.

2.4 Cellular DNA Repair Mechanisms

Once a break occurs, natural cell repair pathways initiate responses. Two dominant repair paths address these severed ends.Non homologous end joining directly connects broken DNA strands. This repair process often introduces errors, creating insertions or deletions at the site. These mutations may disrupt gene function which helps to knock out genes for experimental study. Homology repair sequences insert donor strands for specific modifications during break repair. Specific insertion sites can be selected for gene changes or mutation corrections [5].

3 CRISPR CAS9 IN GENE EDITING

CRISPR- Cas streamlined genetic alteration by employing a solitary guide RNA molecule to locate DNA sequences. Researchers consider editing cycles smoother, swifter, and improved versus traditional molecular techniques. Adding this RNA component broadens tool reach while helping scientific teams formulate lab strategies effortlessly with superior clarity regarding the complex target identification selection procedure.

One critical role involving CRISPR- Cas gene editing is adaptation in selecting genomic sequences. Designing compact RNA lets scientists command editing instruments toward almost every requested chromosomal site. Extreme targeting precision allows exact molecular swaps while diminishing accidental shifts away from markers intended for modification by our current standard lab practices. The field has embraced CRISPR-Cas as its dominant tool due to its simplified efficiency and design. Scientific departments adopted specific tools because reliability, pace, and lower expenses outweigh old-fashioned methods. In comparison with ancient tools, CRISPR-Cas technology asks for minor protein modifications and yields results rapidly with improved data outputs inside specific clinical biological laboratory settings [6].

Different variations across class two styles display changing specificity, sequence recognition habits, plus cut signatures. Such traits allow researchers flexibility for genome work through choosing correct CRISPR sets dedicated toward individual research targets. Scientific adaptation substantially enhanced accuracy along with utility throughout the sphere regarding our latest contemporary gene-modifying diagnostic tool improvements. Widespread reliance on CRISPR instruments during genetic work arrived following the discovery of the type IIA protein from Streptococcus progenies bacteria cultures. SpCas9 cemented CRISPR as strong, reliable genomic platforms inside eukaryotic organisms. Since simplicity paired with strong target identification works, SpCas9 emerged as primary equipment within current genetic work circles across laboratory teams. Cas9 facilitates precise genome surgery through accurate

location selection plus binding DNA markers. Higher specificity raised exact standards while perfecting molecular modification speed, making CRISPR- Cas essential equipment surrounding modern research environments. Success hinges upon targeted molecular accuracy during experimental phases because outcomes provide clear signals concerning genetic research progression inside biology. Furthermore, it is possible to highlight another benefit of CRISPR-Cas technology—compatibility with genetic screening and functional genomics investigations. It provided scientists with opportunities to study gene function on a broad scale; therefore, it contributed to further development in the field of molecular biology and genomics [7].

The problem of off-target editing, in spite of all advantages of CRISPR-Cas technology, becomes one of the most significant obstacles in genome editing. When the CRISPR system edits unintended nucleotide sequences, undesired mutations take place. In order to avoid such negative outcomes and increase the efficiency and specificity of CRISPR-Cas systems, some modifications were made to them. Specifically, there are a number of strategies that enhance target recognition by modified guide RNAs and lower the rate of off-target effects through modification of the Cas9 protein, which becomes highly efficient in recognizing targets. Delivery approaches were also improved since they make genome editing much more specific and accurate due to control over the activity of the Cas9 protein [8].

4 APPLICATIONS OF CRISPR CAS9 BEYOND GENE EDITING

4.1 Gene regulation

Better knowledge about gene regulation will greatly help us to know and control the identities and functions of cells. In order for stem cells to differentiate, they need to go through the gene regulation process just like in development. Improved understanding of gene regulation will enable better manipulation of stem cell differentiation and reprogramming. Aside from cellular engineering, most of the genetic predisposition to disease is due to changes in non-coding parts of the genome sequence, which are believed to work by gene regulation. Once again, principles of gene regulation will assist us to predict and possibly correct non-coding sequence changes [9].

Apart from the nuclease function, Cas9 is able to act as an excellent platform through which one can recruit proteins and RNAs to specific sites on the DNA sequence. In addition, Cas9 has been converted into potent gene regulators which are sequence specific. This is done by fusing transcription activators and repressors to dCas9. The dCas9 retains its DNA binding specificity through binding the sgRNA and target sequence, though without the nuclease activity.

While dCas9 alone is sufficient for the effective inhibition of target genes' transcription via sterically hindering the transcriptional machinery, this newly described approach has been named as CRISPR interference (CRISPRi), meaning that the approach interferes with transcription of RNA. While CRISPRi is highly efficient in prokaryotic cells, dCas9-sgRNA fusion alone might be not very efficient at silencing mammalian genes. However, CRISPRi in mammalian cells might be improved through the fusion of dCas9 to the transcriptional repressor domain (e.g., Kox1 KRAB domain), leading to successful inhibition of reporter and endogenous genes [10].

4.2 Epigenetic modifications

Epigenetic is a field that focuses on the inheritable modifications of gene expressions without changes in DNA sequence1–3. Epigenetic changes are brought about through age, health conditions and environmental factors. DNA methylation, histone modification and non-coding RNA related gene silencing have been established as key epigenetic mechanisms. DNA methylation takes place through addition of a methyl (CH3) group to the 5-carbon of the cytosine ring of the DNA, hence controlling gene expression8–10. DNA methylation plays a critical role in many biological functions such as genomic imprinting, X-chromosome inactivation, chromosome stabilization, and suppression of transposable elements among others. Epigenetic researches have provided crucial information concerning DNA methylation, but much more studies have to be carried out in this area.

Two strategies were designed to use the CRISPR/Cas9 technique in epigenetic in order to regulate the de-methylation of Oct4 promoter and to induce Oct4 expression in differentiated cells. The role of Oct4, the transcription factor which is found only in stem cells and is responsible for self-renewal and pluripotency in stem cells has been proved. As the hypo-methylation of promoter region of Oct4 in stem cells is responsible for its expression, the hyper-methylation in differentiated cells results in a complete shutdown of Oct4 27,28. First, we modified genetically the cells so that the CpG dinucleotides in the promoter region become non-methylated dinucleotides through knock-in strategy using CRISPR/Cas9 technique. In addition, to avoid the side effects that may arise due to mutation in the sequence of promoter, we used CRISPR/dCas9 based system of demethylation 29 and tried to improve the system by performing chromatin remodeling. In doing so, we have used catalytically inactive dCas9 fused with the catalytic domain of Ten-Eleven Translocation dioxygenase 1 (Tet1), which converts 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC) .We assessed the consequences of the selective demethylation of this dCas9-Tet1 complex targeting the methylated CpG dinucleotides within the Oct4 promoter locus together with the synergetic effects of histone

modifications and DNA methylation. In this research, we tried to assess the usefulness of the selective demethylation tool and its possible applications in the treatment and editing of hypermethylated genes.

The significance of the epigenetics study lies in providing information on how genes are regulated. Particularly, much effort has been made in the regulation of gene expression through DNA methylation. Nonetheless, it has not been easy to manipulate the methylation status of DNA selectively. Conventional demethylation has been based on the use of various inhibitors of DNMTs, but it is not applicable in the manipulation of a particular gene due to the absence of specificity of the inhibitors. Thus, finding ways of selectively modifying DNA methylation is significant in gene regulation and further application in therapy [11].

4.3 Imaging

Another possible method to realize the living cell imaging was the fusion of Cas9 with the fluorescent protein (GFP, eGFP or mCherry) to further label and characterize the targeting DNA. Targeted DNA labeled by sgRNA could be recognized by the Cas9-GFP to conduct imaging of living cells. The CASFISH system that included dCas9 with Halo tag and Halo tag ligands conjugated with fluorescent dyes, were a fast and efficient imaging method and was applied in the detection of primary tissue section. Similar to CASFISH method, (Po)STAC system relied on scFv-dCas9 fusion protein and GFP-scFv antibody to generate the fluorescence signal. To provide the PAM as part of the oligonucleotide (PAMmer), constructed the CRISPR-Sunspot system that is suitable for imaging low-abundant mRNAs efficiently. Based on the SunTag system, the dCas9 could be associated with 24× GCNs (polypeptides that enable recruitment of particular proteins to facilitate signal amplification) which can be recognized by scFv-GFP proteins and result in signal amplification. CRISPR-Sunspot was used to identify the co-localization of Camk2a mRNA and its regulatory protein Xlr3b in neurons, thus providing a novel strategy for uncovering molecular mechanism of diseases induced by aberrant mRNA molecules. The dCas13b-eGFP fused with sgRNA can also be used for tracing and investigating the dynamics of nuclear domain-related lncRNAs.

Modifications of CRISPR-Cas9-based imaging systeModification on Cas9-based imaging systems, including Cas9 fused with fluorescent protein (FP), Halo Tag or Sunspot system. In the first line, the FP-labeled Cas9 was employed to recognize and label the specific targeting DNA with the assistance of sgRNA. In the second line, Cas9-Halo Tag system was illustrated. Cas9 was labeled by Halo tag, and with the guidance of sgRNA, the FP fused Halo tag ligand recognized the complex of Cas9-sgRNA-DNA, and marked the location of DNA with fluorescence. The third line shows the modified principle of CRISPR-Sunspot system, which was consistent with that of Cas9-Halo Tag system. (B) Modification of sgRNA-based imaging system, including sgRNA directly fused with fluorescent dye or RNA aptamer. In the first line, sgRNA with fluorescent dye attached was used to label target DNA and direct Cas9 to edit genome. In the second line, RCAS-FISH method was introduced. MS2-labeled sgRNA recognized the complex of MCP-fused fluorophore and characterized the target DNA with the aid of Cas9. (C) Modification on Cas9/sgRNA-based imaging system. The FP labeled dCas9 and fluorescent dye-labeled sgRNA complexes bound to the specific targeting DNA to enable multiple color imaging. (D) Reporter gene imaging system by CRISPR-Cas9 modification. The reporter gene was inserted into CRISPR-Cas9 and bound to the fluorescence probe or radionuclide probe for imaging [12].

5 CRISPR- CAS TECHNOLOGY IN SYNTHETIC BIOLOGY

5.1 CRISPR-Cas in Designing Genetic Circuits

The creation of genetic circuitry is one of the main achievements brought by CRISPR-Cas technologies to synthetic biology. The conventional methods of gene regulation often use transcription factors, but these methods might lack certain flexibility and programmability. CRISPR approaches eliminate this problem by providing a means of precise regulation of the targeted genes with the help of the guide RNA. With the help of CRISPR-based gene circuits, complex regulatory networks can be created that will be able to carry out logic operations and regulate cell function.

5.2 CRISPR-Cas in Synthetic Biology – Future Prospects

The future of synthetic biology is closely associated with further progress in the field of CRISPR-Cas technologies. In particular, the integration of precise genome editing, programmable regulation of gene expression, and high-throughput genetic screening will contribute to acceleration of the design-build-test-learn cycle in synthetic biology. The use of CRISPR-Cas will probably become more widespread in the creation of genetically engineered microorganisms, bioproduction processes, healthcare services, and applications in the environment. With the continuous improvement of the technology, there will be new chances for solving global problems in the fields of medicine, agriculture, energy, and biotechnology [13].

6 ROLE OF CRISPR-CAS IN DRUG DISCOVERY

6.1 Identification of Novel Drug Targets through CRISPR-Cas

One of the vital roles of CRISPR-Cas technology in the drug discovery process is the identification of novel drug targets. It is crucial to have knowledge about genes and molecular mechanisms related to disease development in order to develop an efficient treatment. With the help of genome editing and screening techniques using CRISPR technology, scientists are able to conduct the analysis of gene functions and discover genetic factors responsible for the development of different diseases. Thus, with the help of CRISPR, it is possible to accelerate the process of drug target discovery.

6.2 CRISPR-Cas in Drug Resistance Studies

Resistance to drugs remains one of the main issues in treatment of many diseases including cancer and infections. Using CRISPR-Cas technology, researchers are able to discover genes and mechanisms responsible for the drug resistance. Through the genome-wide screening approaches, it is possible to understand how genetic differences affect the response to treatment.

6.3 CRISPR-Cas in Drug Screening and Validation

The CRISPR-Cas technology has helped improve the efficiency of drug screening and validation procedures. Once a possible target for a drug is found, CRISPR methods can be employed to validate the importance of the target biologically and assess its appropriateness for use in drug development. The use of CRISPR enables researchers to accurately assess the function of the target and reduces the possibility of failing in the latter phases of drug development. Therefore, the CRISPR-Cas technology helps create reliable and cost-efficient drug discovery programs.

6.4 Future Prospects of CRISPR-Cas in Drug Discovery

In the future, the CRISPR-Cas technology is likely to play an even more significant role in drug discovery. Owing to ongoing advances in genome editing precision, screening techniques, and target validation approaches, the potential of the technology is likely to expand in the field of biomedical research. It has immense potential in identifying new targets, studying diseases, and creating new therapies. As CRISPR technologies develop further, their contribution in discovering new drugs is likely to increase [14].

7 CONCLUSION

Conversion of the CRISPR system into a genetic engineering technology has made a number of revolutionary breakthroughs in the field of life sciences. Genetic editing techniques have added more versatility to the CRISPR system, giving scientists more efficient methods to study living organisms and human diseases. The CRISPR based techniques might be the means of treating humans from genetic diseases in the near future. Undoubtedly, the CRISPR-based technologies will continue making changes to both basic and applied research in science. So, when the CRISPR-Cas9 system is going to be tested in clinical trials, this aspect should be considered. Investigating and understanding such challenges will help us identify ways of overcoming their limitations.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of conflict of interest

The authors declare no conflicts of interest regarding this manuscript.

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