CRISPR-Cas9 Genome Editing Technology: Development and Applications

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Abstract: *The discovery of an ancient adaptive immune system in bacteria, called the CRISPR/Cas system, has revolutionalized the modern science. The bacterial proteins that identify and target viral DNA for degradation have been characterized and manipulated for a variety of uses in agriculture, molecular biology and medicine. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system provides a robust and multiplex genome editing tool, enabling researchers to precisely manipulate specific genomic elements and facilitating the elucidation of target gene function in biology and diseases. CRISPR-Cas9 gene editing technique has set the stage for remarkable developments. This technology is used to modify, regulate, or mark genomic loci in a wide variety of cells and organisms from all three domains of life. These results highlight a new era in which genomic manipulation is no longer a bottleneck to experiments, paving the way toward fundamental discoveries in biology, with applications in all branches of biotechnology, as well as strategies for human therapeutics.*

Keywords: *CRISPR-Cas9, DSB, Genetic Engineering, crRNA-tracrRNA complex*

I. INTRODUCTION

The development of recombinant DNA technology in the 1970s marked the beginning of a new era for biology. The study of natural DNA repair pathways in bacteria and yeast, as well as the mechanisms of DNA recombination, revealed that cells have endogenous machinery to repair double-strand DNA breaks (DSBs) [1]. Thus methods for introducing precise breaks in the DNA at sites where changes are to be introduced were recognized as a valuable strategy for targeted genomic engineering. Early approaches of genome engineering were oligonucleotide coupled chemical cleavage and nucleic acid base pairing by self splicing introns [2]. Although these approaches did not lead to robust methods, they demonstrated the utility of base pairing for site-specific genome modification. At around the same time, the initial reports of zinc finger–mediated DNA binding [3] led to the creation of modular DNA, recognition proteins that, when coupled to the sequence-independent nuclease domain of the restriction enzyme *FokI*, could function as site specific nucleases[4]. Although ZFNs are effective genome editing reagents for some experiments, they were not widely adopted because of the difficulty inherent in designing and validating such proteins for a specific DNA locus of interest. Thus the field was primed for the first reports of transcription activator–like (TAL) effectors, which occur naturally in bacteria that infect plants, enabling rapid creation of *FokI* coupled versions that could be used similarly to ZFNs for site-directed genome editing [5,6]. Such TAL effector nucleases (TALENs) were easier than ZFNs to produce and validate, generating widespread excitement about the possibility of facile genome editing that would be fast and inexpensive. But difficulties of protein design, synthesis, and validation remained a barrier to widespread adoption of these engineered nucleases for routine use. The field of biology is now experiencing a transformative phase with the advent of facile genome engineering in animals and plants using RNA-programmable CRISPR-Cas9. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system provides a potential platform for targeted gene regulation [7]. About 40% of bacteria and 90% of archaea possess CRISPR/CRISPR-associated (Cas) systems to confer resistance to foreign DNA elements [8]. The CRISPR associated protein Cas9 is an endonuclease that uses a guide sequence within an RNA duplex, tracrRNA:crRNA, to form base pairs with DNA target sequences, enabling Cas9 to introduce a site-specific double-strand break in the DNA. The simplicity of CRISPR-Cas9 programming, together with a unique DNA cleaving mechanism, the capacity for multiplexed target recognition, and the existence of many natural type II CRISPR-Cas system variants, has enabled remarkable developments using this cost-effective and easy-to-use technology to precisely and efficiently target, edit, modify, regulate, and mark genomic loci of a wide array of cells and organisms[9].

II. APPLICATIONS OF CRISPR/Cas TECHNOLOGY

Genetic and epigenetic control of cells with genome engineering technologies is enabling a broad range of applications from basic biology to biotechnology and medicine. Causal genetic mutations or epigenetic variants associated with altered biological function or disease phenotypes can now be rapidly and efficiently recapitulated

in animal or cellular models. Additionally, precise genetic engineering of important agricultural crops could confer resistance to environmental deprivation or pathogenic infection, improving food security while avoiding the introduction of foreign DNA. Sustainable and cost-effective biofuels are attractive sources for renewable energy, which could be achieved by creating efficient metabolic pathways for ethanol production in algae or corn. Direct *in vivo* correction of genetic or epigenetic defects in somatic tissue would be permanent genetic solutions that address the root cause of genetically encoded disorders and engineering cells to optimize high yield generation of drug precursors in bacterial factories could significantly reduce the cost and accessibility of useful therapeutics. Few applications of this technology are described below.

Application of CRISPR in Eukaryotic Organisms

The CRISPR/Cas9 system has been found to work at a high efficiency for genome editing in most eukaryotic model systems [10]. Moreover, modified versions of this system can be used to knockout genes, insert new exogenous DNA into the host genome, and to block RNA transcription for a variety of applications. CRISPR could be used to delete harmful disease causing mutations in the human genome, especially in IVF embryos. Alternatively, the system could be used to knockout specific genes in model organisms in order to study diseases. The applications of CRISPRCas9 *gene* knockout system as therapy of HIV [11] and for creation of multiple gene knockout model organisms [12] are studied. CRISPR/Cas Nickase gene knocking system is used in autologous stem cell therapies. Schwank *et al.* (2013) studied the use of CRISPR gene knock-in as a therapy for Cystic Fibrosis. They succeeded in correcting mutant CFTR Delta-F508 alleles (alleles with a mutation that causes cystic fibrosis) using the CRISPR/Cas9 mediated homologous recombination in intestinal stem cells. They showed that the corrected genes could function normally in an organoid system. Other studies have revealed that mouse organoids grown *in vitro* can be successfully transplanted into living mice [13].

CRISPR interference (CRISPRi)

Qi *et al*. (2013) created a catalytically dead *Cas9* (d*Cas9*), lacking endonuclease activity, in order to function in gene silencing as opposed to gene editing through DSBs. This method, called CRISPR interference (CRISPRi) halts mRNA synthesis by blocking RNA polymerase at the promoter region of the DNA. CRISPRi has the ability to silence multiple genes at the same time. This method can be compared to RNAi. However, CRISPRi has the possible advantage of working earlier than RNAi by silencing the gene before mRNA is created rather than simply degrading the mRNA. CRISPRi Gene Knockdown is inducible and reversible. CRISPRi has many possible applications in medical research. For example, Kearns *et al*. (2014) researched the ability of dCas9 to influence the differentiation state of human pluripotent stem cells. It was determined that dCas9 could be used to positively or negatively regulate the expression of particular target genes that influence cell differentiation. CRISPRi could therefore be useful to investigate stem cell differentiation pathways [14].

CRISPR/Cas9 in the generation of animal models

Many recent studies have shown that CRISPR-Cas9 technology could be used for rapidly generating targeted genome modifications in the germ lines of various model organisms [15] which will significantly advance the functional genomics. Microinjection of Cas9-encoding mRNA and customizable sgRNA into one-cell stage embryos of zebrafish is able to efficiently modify the target genes *in vivo* in a simple, rapid and scalable manner [16]. Co-injection of Cas9 mRNA and sgRNAs targeting different genes into mouse zygotes generates mutant mice with bi-allelic mutations, confirming that CRISPR/Cas-mediated gene editing could be used for the simultaneous disruption of multiple genes with high efficiency [17]. CRISPR-Cas9 technology has been used for efficient genome engineering in many other model organisms, including *Drosophila* [18] *Caenorhabditis elegans* [19] *Xenopus tropicalis* [20] *Rattus rattus* [21] and *Sus domesticus* [22]. The application of CRISPR-Cas9 technology for genome editing in a wide range of organisms will promote our understanding of development and disease and help develop animal models and therapeutic strategies for human diseases.

CRISPR/Cas9 in Functional Genomic Screening

A CRISPR-Cas-based knockout library has been applied to identify the host genes mediating the cellular responses to anthrax and diphtheria toxins. A series of studies has demonstrated that CRISPR mediated repression (CRISPRi) and CRISPR-mediated activation (CRISPRa) are powerful tools for functional genomics screening. A CRISPRi system consisting of a catalytically inactive Cas9 and a guide RNA has been shown to specifically and efficiently repress the transcription of target genes in *Escherichia coli* and mammalian cells[23, 24] whereas a catalytically inactive Cas9 fused with a transcriptional activation domain has been used to activate the expression of specific endogenous genes [25, 26]. Genome-scale CRISPRi and CRISPRa libraries that specifically target transcriptional repressors or activators to endogenous genes have been successfully used for screening essential genes for growth, tumor suppression, differentiation regulation, and cellular sensitivity to a cholera diphtheria toxin, suggesting that CRISPRi and CRISPRa are valuable tools for mapping complex pathways [24]. Therefore, it demonstrates that CRISPR-Cas9 technology can be a promising functional genomic screening tool for discovering essential genes in various biological processes.

CRISPR-Cas9 in Correction of Genetic Disorders

One of the most exciting applications of the CRISPR-Cas9 is the possibility of curing genetic diseases. A study has shown that the CRISPR-Cas9 system can be used to modify an EGFP transgene or the endogenous *Crygc* gene in spermatogonial stem cells (SSCs). The modified SSCs carrying a corrected *Crygc* mutation can undergo spermatogenesis and produce offspring with the corrected phenotype at an efficiency of 100% [27]. The injection of Cas9, sgRNA, and homology-directed repair template into mouse zygotes has been shown to correct the dystrophin gene mutation responsible for muscular dystrophy in the germ line and prevent the development of muscular dystrophy in mutant mice [28]. Interestingly, a similar strategy using the CRISPR-Cas9 technology has successfully corrected the cystic fibrosis transmembrane conductor receptor (CFTR) locus by homologous recombination in cultured intestinal stem cells of cystic fibrosis human patients [29] demonstrating that primary adult stem cells derived from patients with a single-gene hereditary defect could be corrected by CRISPR/Cas9 mediated homologous recombination, suggesting a promising strategy for gene therapy in human patients.

CRISPR-Cas9 in the Treatment of Infectious Diseases

Considering that the CRISPR-Cas system originally functions as an antiviral adaptive immune system in bacteria, this system could be used for treating infectious diseases by eradicating pathogen genomes from infected individuals. Recently, studies have shown that the CRISPRCas9 system can eliminate the HIV-1 genome and prevent new HIV infection when transfected into HIV-1 provirus-integrated human cells, a sgRNA expression vector targeting the long terminal repeats (LTR) of HIV- 1 efficiently cleaves and mutates LTR target sites and suppresses LTR-driven viral gene expression. In addition, this system has been shown to delete viral genes from the host cell chromosome[11]. The high specificity of Cas9/sgRNAs in editing the HIV-1 target genome has also been recently demonstrated. Cas9/sgRNAs efficiently inactivate HIV gene expression and replication in latently infected cells, including microglial, promonocytic and T cells. Significantly, Cas9/sgRNA mediated genome editing has been shown to immunize cells to prevent HIV-1 infection [30]. These results indicate that the CRISPR-Cas9 technology can serve as a potential tool for clinical applications to cure infectious diseases.

RNA-Guided Genome Editing in Plants Using CRISPR–Cas System

It is important to test the functionality and utility of the CRISPR–Cas system for genome editing and gene targeting in plants. As a proof of concept, targeted gene mutation was successfully achieved in three specific sites of a mitogen-activated protein kinase gene in rice genome. Furthermore, the mutation efficiency and off-target effect have been assessed for the RNA guided genome editing in plants. To adapt the CRISPR–Cas9 system for plant genome editing, two *R*NA-guided *G*enome *E*diting vectors (pRGE3 and pRGE6) were created for expressing engineered gRNA and Cas9 in plant cells. In both vectors, CaMV35S promoter was used to control the expression of Cas9 which was fused with a nuclear localization signal and a

FLAG tag. Rice protoplast transient expression system was used to test the engineered gRNA–Cas9 constructs. The efficient transformation of rice protoplasts was demonstrated with a plasmid construct carrying the Green Fluorescence Protein (*GFP*) marker gene. Following the transformation of empty pRGE3 vector and the pRGE3–PS1/2/3 gRNA constructs into rice protoplasts, the Cas9 nuclease was successfully expressed as revealed by the immunoblot analysis [31]. Mutagenesis continues to play essential role for understanding plant gene function and, in some instances, provides an opportunity for plant improvement. CRISPR/Cas9 technology could be used for targeted mutagenesis, gene replacement and stacking of genes in higher plants. Its applications are not restricted to mutagenesis and target site cleavage can be exploited to promote sequence insertion or replacement by recombination [32].

III. APPLICATION IN GENE THERAPY

Precisely genome editing has the potential to permanently cure diseases through disrupting endogenous disease-causing genes, correcting disease-causing mutations or inserting new protective genes [33, 34]. CRISPRCas9 provides a novel highly efficient genome editing tool for gene therapy studies. For instance, Ebina *et al*. (2013) disrupted the long-terminal repeat promoter of HIV-1 genome using CRISPR/Cas*9*, which significantly decreased HIV-1 expression in infected human cells. The integrated pro-viral viral genes in host cell genomes can also be removed by CRISPR/Cas9 [11]. With the rapid development of induced pluripotent stem (iPS) cells technology, engineered nucleases are applied to genome manipulation of iPS cells [35, 36]. The unlimited self renewing and multipotential differentiation capacity of iPS cells make them very useful in disease modeling and gene therapy. Using CRISPRCas9, Horii *et al.* (2013) created an iPS cell model for immunodeficiency, centromeric region instability, facial anomalies syndrome (ICF) causing by DNMT3B gene mutation [37]. The iPS cells were transfected with plasmids expressing Cas9 and gRNA, which disrupted the function of DNMT3B in transfected iPS cells. Using the same hPSC lines and delivery method, Ding *et al*. (2013) compared the efficiencies of CRISPR/ Cas9 and TALENs for genome editing of iPS cells. They observed that CRISPR/Cas9 was more efficient than TALENs [36]. Sarcoma is believed to develop as a result of genetic alterations in mesenchymal/ progenitor stem cells, CRISPR/Cas9 systems can mediate genome editing, epigenetic regulations and trancriptome modulations. It holds extensive application potential in sarcoma models and gene therapies and could be used to explore drug treatment and resistance of sarcoma [38]. However, it is still a long road to clinically applying CRISPR/Cas9 for gene therapy. The high specificity of CRISPR/Cas9 for target sites and eliminate possible off target mutations with negative effects must be ensured.

IV. LIVE IMMAGING OF CELLULAR GENOME

Studying the interactions of specific genes given changing chromatin states would require a robust method to visualize DNA in living cells. Traditional techniques for labeling DNA, such as fluorescence in situ hybridization (FISH), require sample fixation and are thus unable to capture live processes. Fluorescently tagged *Cas9* labeling of specific DNA loci was recently developed as a powerful live-cell-imaging alternative to DNAFISH [39]. Advances in orthogonal *Cas9* proteins or modified sgRNAs will build out multi-color and multi-locus capabilities to enhance the utility of CRISPR based imaging for studying complex chromosomal architecture and nuclear organization.

V. APPLICATION IN BIOMEDICINE AND BIOTECHNOLOGY

The CRISPR/Cas*9* system of genome editing and manipulation has proved to be an exciting new development for the fields of molecular biology and translational medicine. This system is used to study various aspects of genome, including importance and functions of genes and regulatory compounds that control them using the ability of RNA guided endonucleases to target virtually any area of organism's genome. The ease of design and testing of Cas9 may also facilitate the treatment of highly rare genetic variants through personalized medicine. Supporting these tremendous possibilities are a number of animal

model studies as well as clinical trials using programmable nucleases that already provide important insights into the future development of Cas9-based therapeutics [9].

VI. FUTURE PROSPECTS

Though CRISPR technology has come a long way in a very short amount of time, there are still many challenges that must be overcome in order for its value in applications to be fully realized. The foremost problems that must be overcome are addressing CRISPR specificity and developing effective and safe delivery systems. The era of straightforward genome editing raises ethical questions that will need to be addressed by scientists and society at large. How can we use this powerful tool in such a way as to ensure maximum benefit while minimizing risks? It will be imperative that nonscientists understand the basics of this technology sufficiently well to facilitate rational public discourse. Regulatory agencies will also need to consider how best to foster responsible use of CRISPRCas9 technology without inhibiting appropriate research and development [9]. The direct and precise genome editing raises ethical concerns, such as gene modification of human germline cells using the CRISPR/Cas9 system to create "engineered babies" [40] which creates arguments and queries among scientists and public. Nonetheless, the discovery of CRISPR is an immediate step change improvement for researchers, with long-term implications that are promising, potentially risky, but currently undetermined.

VII. CONCLUSION

CRISPR/Cas9 is a novel technique with a bright future in genomic editing. It has the potential to be useful in a broad range of applications from simplifying research to acting as a new form of gene therapy for patients with HIV and genetic diseases and genome editing in plants. CRISPR is still a young system and more research must be completed in order to overcome its constraints. While there are many challenges ahead before CRISPR/Cas9 can be utilized as a safe and reliable gene therapy, these challenges do not seem insurmountable. Research in the area of CRISPR/Cas9 is gaining speed and this system could very well be the solution to many genetic issues faced today.

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