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CRISPR-Cas9: A Precise Approach to Genome Engineering

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Abstract

In the last few decades, genomic manipulation has made significant progress as a result of the development of recombinant DNA technologies; however, more often than not, these techniques have been costly and labor intensive. In contrast, recently developed next-generation sequencing (NGS) technologies have provided a cheaper, faster, and easier process to study genomics. In particular, an NGS technique emerged from bacterial CRISPR-associated protein-9 nuclease (Cas9) as a revolutionary method to modify, regulate, or mark specific genomic sequences on virtually any organism. A later adaptation of this bacterial defense mechanism that successfully and permanently edits dysfunctional genes and corrects missing proteins has resulted in a new era for disease genetic engineering. Clinical trials using this technique are already being performed, and the applicability of CRISPR-Cas9 techniques is actively being investigated using in vivo studies. However, the concept of genome correction poses great concerns from a regulatory perspective, especially in terms of security, so principles for the regulation of these methodologies are being established. We delved into CRISPR-Cas9 from its natural and ortholog origins to its engineered variants and behaviors to present its notable and diverse applications in the fields of biotechnology and human therapeutics.

Keywords

CRISPR-Cas9, NGS, bacterial adaptive defense system, genetic engineering, clinical trials

Background

Since the 1970s, the development of recombinant DNA technology has been revolutionary in biology by increasing knowledge on how specific genes and regulatory elements work.[1] Genomic manipulation of model organisms, such as yeast, flies, zebrafish, and rodents, relied on the use of chemical DNA mutagens to isolate aberrant phenotypes for study. However, these early methods have shortcomings related to causal mutations in selected clones that are initially unknown and are high cost and labor intensive.[2]

CRISPR (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats) sequences have been detected in diverse bacteria and archaea[3] by predicting their probable roles in gene regulation or DNA repair.[4,5] In 2005, a crucial point was the discovery that many spacer sequences in CRISPRs derive from viruses or plasmids.[6,7] Later, thanks to Ruud Jansen from Utrecht University, it was observed that prokaryote repeat clusters include a group of homologous genes that encode CRISPR-associated systems or cas genes, and 4 types have been discovered so far.[8] A spacer component was also discovered in CRISPR loci, leading to a theory that the CRISPR-Cas system could serve as an adaptive defense system in bacteria[9] by using antisense RNA strands as memory sequences of past invasions.[9]

Additionally, RNA interference (RNAi) techniques were used to understand how Cas9 works. Methods using this type of RNA tool have provided information on gene function. Nevertheless, incomplete gene knockdown and a considerable amount of off-target activity have generated complicated interpretations of phenotypic alterations. Now, sequencing-specific programmable nucleases have been developed to enable targeted modification of the DNA itself[2] and are included in next-generation sequencing (NGS) technologies. Notably, the RNA-guided endonuclease Cas9 from CRISPR has provided a useful system to produce targeted mutagenesis in eukaryotic cells.[10] For this reason, Cas9 associates the stable mutagenic nature of habitual mutagens with the programmability of RNAi.[2]

CRISPR-Cas9 Structure and Functionality

After CRISPR was proposed as part of the adaptive bacterial defense system,[11] adaptive immunity was classified into 3 stages: (1) adaptation: insertion of a short sequence of the invading DNA occurs in the CRISPR array as a spacer sequence; (2) expression: transcription of precursor crRNA

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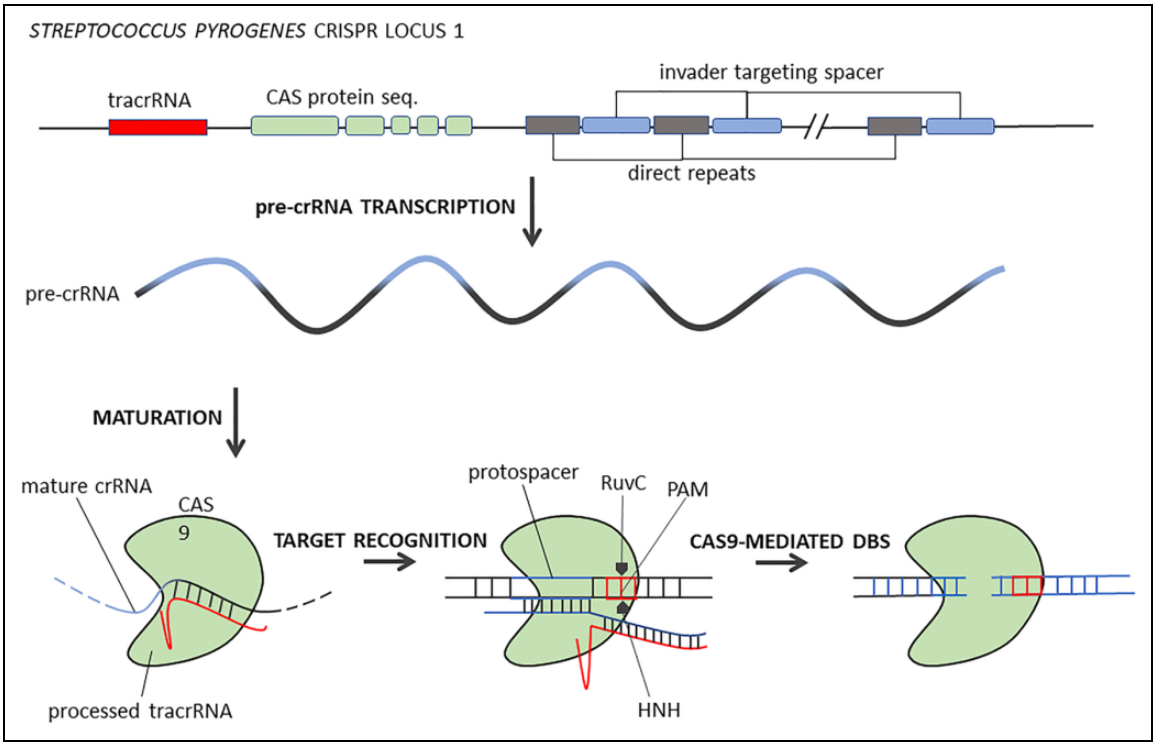

Figure 1. Maturation of the type II CRISPR-Cas system from *Streptococcus pyogenes*.

(pre-crRNA) ensues to generate individual crRNAs composed of an invader targeting spacer portion and a repeat portion; and (3) interference: crRNA-directed cleavage of invasive nucleic acids occurs by the Cas proteins at sites complementary to the crRNA spacer sequence. During this process, three CRISPR-Cas systems (type I, type II, and type III) could be differentiated based on their distinct use of molecular mechanisms to achieve the recognition of nucleic acids and their cleavage.[10,12] The type II system was proven to be especially useful for genomic engineering applications because it requires only a single protein for RNA-guided DNA recognition and cleavage.[13,14]

A short-sequence motif adjacent to the crRNA-targeted sequence on the invading DNA called protospacer adjacent motif or PAM plays a fundamental role in the adaptation and interference stages in type I and type II CRISPR-Cas systems and in the binding stage of Cas to foreign DNA.[15-18] Canonically, PAM comprises a 5′-NGG-3′ or a 2- to 6-base-pair DNA sequence.[19]

A functional CRISPR-Cas locus is composed of an operon of cas genes that encodes for Cas protein constituents and a CRISPR array of DNA-targeting spacers encoding for crRNA components intercalated with identical repeats.[20,21] In type II CRISPR-Cas variants of *Streptococcus pyogenes* strains, a trans-activating crRNA (tracrRNA) was reported as an upstream small trans-encoded RNA that plays a pivotal role in crRNA maturation by Cas9 and ribonuclease III and in specific immunity against certain parasites.[19] In 2012, this protein was exposed as part of the tracrRNA: crRNA duplex[19] that directs the DNA cleavage[13] (Figure 1).

On the other hand, Cas9 was primarily identified through bioinformatic analyses as a large multifunctional protein[4] with 2 nuclease domains named RuvC-like[11] and HNH[6,11,22] domains. The Cas9 protein HNH domain cleaves the DNA strand complementary to the crRNA 20-nucleotide sequence, whereas the RuvC-like domain cleaves the DNA strand opposite to the complementary strand[13,14] (Figure 1). A variant protein with single-stranded DNA cleavage activity can be generated by mutating either the HNH or RuvC-like domain, whereas mutating both domains produces a catalytically inactive DNA-binding protein with RNA guiding.[13,14]

In 2007 and 2011, genetic studies showed that Cas9 from *Streptococcus thermophilus* strains is essential in defense against viral invasion[19,23] and might be responsible for introducing double-strand breaks (DSBs) into plasmids and phages invading the cell[24] through the active HNH and RuvC domains.[25]

The ability of the catalytically deactivated CRISPR-Cas protein to specifically bind an RNA-guiding sequence and PAM without permanently modifying DNA is a key property that enabled the application of CRISPR-Cas as a target gene regulator at the genomewide scale by blocking transcriptional elongation via transcription factor or RNA polymerase binding, depending on the binding site. This protein variant was referred to as CRISPR interference (CRISPRi). *Escherichia coli* experiments with CRISPRi demonstrated that the sequencing of

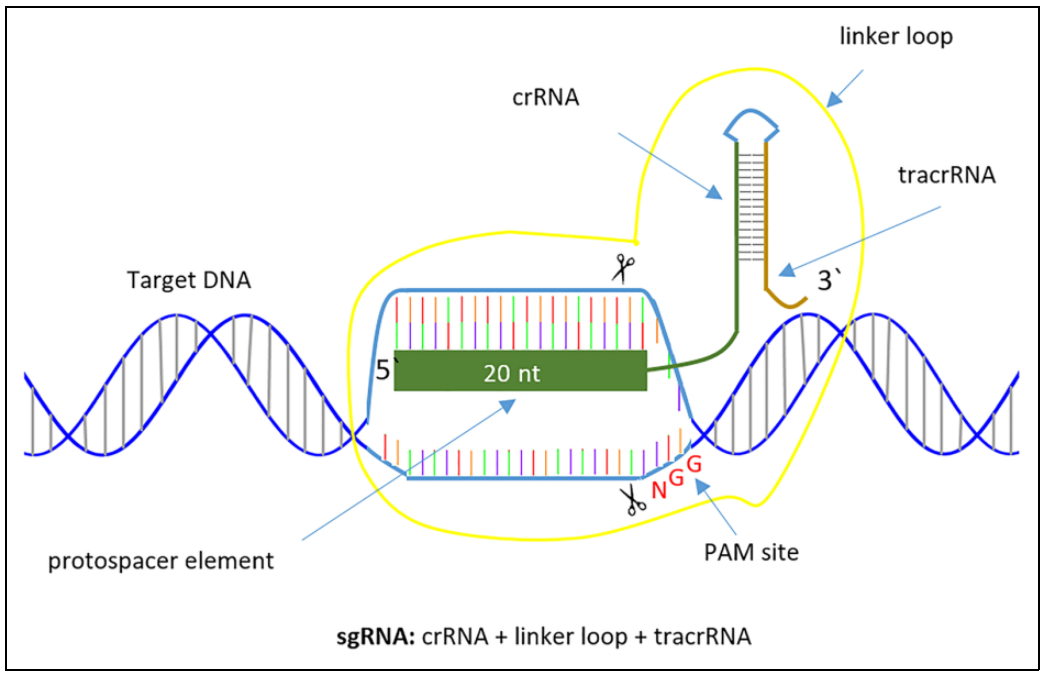

Figure 2. The Cas9 enzyme breaks the double-stranded DNA through catalytic centers that cleave each strand at a target site next to the PAM site and the single guide RNA (sgRNA) matching its 20-nucleotide sequence.

whole genomes had no detectable off-target associations,[26] and further tests with RNA-seq analysis showed that CRISPRi-directed transcriptional repression is highly specific, thus opening the door for CRISPRi to be used to simultaneously repress multiple genes with reversible effects.[26-29]

Meanwhile, DNA target recognition by CRISPR-Cas requires pairing of the crRNA guiding sequence to foreign DNA and the presence of a PAM sequence adjacent to the targeted sequence in the DNA[13,14] (Figure 2). The tracrRNA: crRNA duplex involved in the base pairing was then engineered as a single-guide RNA (sgRNA) while retaining 2 main features: the double-stranded structure now linked by a loop at the 3′ side of the guide sequence that binds to Cas9 and the 20-nucleotide RNA guide sequence at the 5′ end of the sgRNA that defines the DNA target site. This simple system can thus be used to target any DNA sequence of interest adjacent to a PAM by changing the guide sequence of the sgRNA.[13]

CRISPR-Cas9 technology has been widely adopted to target, edit, and modify a myriad of genomes using the *S pyogenes* variant or type II (Cpf1). In vivo and in vitro experiments[13,30,31] have shown that wild-type Cas9, even when closely related to the target sequence, can use distinct tracrRNA : crRNA transcripts as a guide; thus, a large collection of CRISPR-Cas9 systems for multiple gene targeting exist and have been used successfully for genome editing in human cells.[32,33]

Genome editing technology using Cas9 produces a DSB at a target locus in the DNA.[34,35] After this cleavage, two repair pathways are possible to obtain the desired editing sequence: nonhomologous end joining (NHEJ) or homologous directed repair (HDR). In cases without a repair template, breaks in dsDNA are ligated through NHEJ. The repaired strands are not restored to the original sequence because a determined sequence was removed by nuclease digestion. This process is useful for producing gene knockouts,[36] and the stimulation of multiple DSBs can also mediate many deletions in the genome.[33,37] On the other hand, although HDR occurs at lower frequency than NHEJ, its results are more useful for generating precise modifications. HDR is part of the repair mechanism that uses the homologous DNA template of the damaged sequence to recover missing or corrupted information, and this mechanism can be triggered to create precise and planned modifications at a target locus by externally supplying an engineered repair template functioning as a conventional double-stranded DNA sequence with homology arms that flank the insertion sequence.[37] Permanent modifications can be added to the target genome through the innate cell repair mechanism using this method. Fortunately, it is possible to stimulate higher levels of HDR using an introduced repair template in the form of dsDNA.[37]

Genome Targeting

For Cas9 to achieve nuclease activity or block transcriptional elongation, it must initially recognize and bind to a PAM sequence in the target DNA. Once bound, Cas9 undergoes a conformational change that leads to unwinding of the target dsDNA and guides the complementary RNA strand to bind with the unwound DNA. In the absence of PAM, target sequences, even when they are fully complementary to the

guide RNA sequence, are not recognized by Cas9, proving that PAM is essential for initial DNA binding.[38]

Additionally, Cas9 target binding behavior was estimated by the number and type of binding sites to target DNA by high-throughput sequencing and chromatin immunoprecipitation. Results in human embryonic kidney cells and mouse embryonic stem cells showed that an active Cas9 seldom cleaves DNA at off-target binding sites. This result implies that in addition to the presence of PAM, almost perfect guide RNA/target DNA complementarity is necessary for Cas9 to cleave DNA.[38] Cas9 binding was also more likely to occur in open chromatin areas than in compact chromatin areas.

Uses in Engineered Cells—Examples of Applied Uses

The efficiency with which genetic modification can be performed and the high degree of success in producing phenotypic expressions promise numerous applications that have not been seen with other genetic manipulation techniques. Some of the most relevant applications of the CRISPR-Cas system have been proved in the analysis of mammalian gene functions.[1] In January 2013, some studies demonstrated that CRISPR-Cas9 can edit human germline cells.[32] Its capacity to introduce DBSs at specific positions facilitates the production of human cell lines and primary cells with chromosomal translocations that can be found in cancers, such as lung cancer[39] and acute myeloid leukemia.[40] Other important examples include the ability to correct genetic mutations that may cause inherited disorders.[1] The gene responsible for cataracts in mice (Crygc) was successfully corrected in mice using CRISPR-Cas9.[41]

Other uses of CRISPR-Cas9 are based on the gene drive principle in which the inheritance of a particular gene tends to rapidly increase its prevalence in a population.[42] Austin Burt, an evolutionary biologist at Imperial College London, along with his research team, has rendered infertile females of a malaria-carrying mosquito that could quickly wipe out a population.[43] Furthermore, the multifunctionality of Cas9 enables the study of various human diseases, such as diabetes, schizophrenia, or heart disease.[1] Modification of one to several alleles in rodents and monkeys has also been possible by injecting the corresponding sgRNA with Cas9 into fertilized zygotes.[44]

Clinical Trials of Gene Editing With CRISPR-Cas9–Based Therapeutics

An increasing number of clinical studies are starting to use gene therapy with CRISPR-Cas9 genes, which creates the need to address regulatory concerns that can affect clinical applications.[45] However, it is not possible to clearly delineate all potential normative concerns relevant to genetic editing. The main problems regarding these therapies include safety, efficacy, and quality control. In fact, genetic materials for the delivery of gene editing nucleases are not markedly different from those of conventional ex vivo or in vivo gene therapies.[46]

The Committee for Advanced Therapies of the European Medicines Agency has discussed the prospects of CRISPR-Cas9 products. In addition, the FDA's Office of Pharmaceutical Quality has an Emerging Technology Team that helps companies develop clinical trials. These international organizations review the most relevant existing guidelines for gene editing therapeutics.[47]

The CRISPR / Cas9 system has recently been used in clinical trials in China that were designed to knock out PDCD1 (programmed cell death protein 1). Currently, eight therapeutic products based on the editing of CRISPR-Cas9 genes are undergoing clinical trials worldwide (http://www.clinicaltrials.gov), as shown in Table 1.

Considering CRISPR-Cas9 promise for correcting disease-causing mutations; however, concerns persist regarding secondary mutations in regions not targeted by the sgRNA. As a recent study in 11 blind mice treated with CRISPR-Cas9 and CRISPR-Cpf1 for sight restoration has proven unexpected mutations in certain cases after CRISPR-Cas9 editing in vivo.[48] In this study, whole-genome sequencing (WGS) was used to assess the presence of insertions and deletions (indels), while choosing the highest-activity sgRNA for the CRISPR. It reported 117 indels and 1397 SNVs that were detected in two CRISPR-treated mice, which indicated nonrandom off-targeting of the sgRNA. The reported mutation rate in the CRISPR-treated mice was substantially higher than that generated by spontaneous germline mutations and as additional control, each of the variants was compared to the mouse dbSNP database and the Mouse Genome Project. None of the CRISPR-generated off-target mutations were found in any of these strains, which further confirmed that these WGS-identified single-nucleotide variants (SNVs) were the result of CRISPR-Cas9 off targeting.

Thus, the study brings attention on the importance of carefully assaying specific gRNA and Cas9 through the use of WGS to determine the presence of off-target mutations in vivo, and also on the imperative necessity to increase the fidelity of CRISPR-Cas9 techniques regarding off-target mutations before its use without risk in clinical settings.[48]

In 2015 the US Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and the US Department of Agriculture (USDA) in an effort to update the Coordinated Framework for Regulation of Biotechnology regulation policies about CRISPR-Cas9 technology and other gene-editing systems raised questions about how they should be regulated and under what authorities, considering these technologies are treated as genetic engineering as well as a form of mutagenesis, with the former being regulated while the latest is not. Because products developed using CRISPR-Cas9 usually do not fit wholly into the established categories that have been regulated in the past 30 years, the details on its regulation remain uncertain.[49]

Table 1. Therapeutic Gene Editing With CRISPR/Cas9 in Clinical Trials.

NCT Number	Title	Conditions	Interventions	Study Types	Enrollment	Dates	Locations
NCT03166878	A Study Evaluating UCART019 in Patients With Relapsed or Refractory CD19+ Leukemia and Lymphoma	B cell leukemia B cell lymphoma	Biological: UCART019	Phase 1 Phase 2	80	May 25, 2017	Biotherapeutic Department and Hematology Department of Chinese PLA General Hospital, Beijing, Beijing, China
NCT03167450	Examining the Knowledge, Attitudes, and Beliefs of Sickle Cell Disease Patients, Parents of Patients With Sickle Cell Disease, and Providers Towards the Integration of CRISPR in Clinical Care	Sickle cell disease		Observational	90	May 30, 2017	National Human Genome Research Institute (NHGRI), Bethesda, Maryland, United States
NCT03081715	PD-1 Knockout Engineered T Cells for Advanced Esophageal Cancer	Esophageal cancer	Drug: Cyclophosphamide Drug: Interleukin-2 Other: PD-1 knockout T cells	Phase 2	21	March 16,=2017	Hangzhou Cancer Hospital, Hangzhou, Zhejiang, China
NCT02863913	PD-1 Knockout Engineered T Cells for Muscle-invasive Bladder Cancer	Invasive bladder cancer stage IV	Biological: PD-1 Knockout T cells Drug: Cyclophosphamide Drug: IL-2	Phase 1	20	August 11, 2016	Department of Urology, Peking University First Hospital, Beijing, Beijing, China
NCT02867345	PD-1 Knockout Engineered T Cells for Castration Resistant Prostate Cancer	Hormone-refractory prostate cancer	Biological: PD-1 knockout T cells Drug: Cyclophosphamide Drug: IL-2	Phase 1	20	August 15, 2016	Department of Urology, Peking University First Hospital, Beijing, Beijing, China
NCT02867332	PD-1 Knockout Engineered T Cells for Metastatic Renal Cell Carcinoma	Metastatic renal cell carcinoma	Biological: PD-1 knockout T cells Drug: Cyclophosphamide Drug: IL-2	Phase 1	20	August 15, 2016	. Peking University, Cell Biotech Co, Ltd, Beijing, China
NCT02793856	PD-1 Knockout Engineered T Cells for Metastatic Non-small Cell Lung Cancer	Metastatic non-small cell lung cancer	Drug: Cyclophosphamide Other: PD-1 knockout T cells Drug: Interleukin-2	Phase 1	15	June 8, 2016	West China Hospital, Sichuan University, Chengdu, Sichuan, China
NCT03044743	PD-1 Knockout EBV-CTLs for Advanced Stage Epstein-Barr Virus (EBV) Associated Malignancies	Stage IV gastric carcinoma Stage IV nasopharyngeal carcinoma T-cell lymphoma stage IV Stage IV adult Hodgkin lymphoma Stage IV diffuse large B cell lymphoma	Drug: Fludarabine Drug: Cyclophosphamide Drug: Interleukin-2	Phase 1 Phase 2	20	February 7, 2017	The Comprehensive Cancer Center of Nanjing Drum Tower Hospital, Nanjing, Jiangsu, China The Comprehensive Cancer Center of Nanjing Drum Tower Hospital, Nanjing, Jiangsu, China

ClinicalTrials.gov [Internet]; US National Library of Medicine, Bethesda, MD; US National Institutes of Health, US Department of Health & Human Service.

By its side, the European Commission has established a dedicated Advanced therapy medicinal products (ATMP) Regulation containing updated definitions of gene therapy medicinal products and cell therapy medicinal products.[50] To consider the nature of these medicinal products, a new Committee for Advanced Therapies (CAT) was established that is responsible for all regulatory procedures concerning ATMP in the EU. The classification, certification, and scientific evaluation in centralized marketing authorizations is evaluated for each product and therapy independently.[51]

It is probably not an exaggeration to say that with CRISPR-Cas9 and the developments that are under way to improve genetic editing, we are entering a new stage in the history of the human dominion over life. The applications of this domain reach all forms of life. No one doubts that genetic editing can contribute to substantially improving the health and living conditions of people and the environment.[52]

Conclusions

The simple structure and mechanism of the CRISPR-Cas9 system along with its complementary base-pairing guiding system is a flexible, user-friendly designer nuclease technology that has already stimulated innovative applications in biology. The power of this technology, which was adapted from a prokaryotic viral defense system, can already be seen in studies that have reproduced chromosomal translocations associated with tumors, developed sgRNA libraries to systematically analyze gene functions in mammalian cells, and corrected inherited genetic disorders. The CRISPR-Cas9 system enables the engineering of refined Cas9 orthologs with smaller sizes, increased specificity, faster editing, and alternative ways of cell insertion by continuing to explore the natural evolution of Cas9 in bacteria and by delving into the complete mechanism of its variants and is thus creating solutions that have not yet been imagined for precise gene modification in unexplored corners of nature.

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Supplemental Material

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