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Review Article

CRISPR gene therapy: a review

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ABSTRACT

The adaptive immune system of prokaryotes is the source of CRISPR-Cas9, a ground-breaking genome editing technique that uses RNA-guided nucleases to precisely alter DNA sequences. An summary of CRISPR/Cas9's discovery, structural elements, mode of action, therapeutic uses, and present limitations is given in this article. The Cas9 nuclease and guide RNA work together to identify particular DNA targets and cause double-strand breaks in the system. Cellular processes like homology-directed repair or non-homologous end joining fix these defects, allowing for gene disruption or correction. Numerous genetic abnormalities, such as hemoglobinopathies including sickle cell disease and β-thalassemia, hereditary retinal diseases, muscular dystrophies, liver metabolic disorders, congenital lung diseases, and genetic deafness, have showed great potential for treatment with CRISPR/Cas9. Clinical applicability is limited by issues such off-target effects, PAM sequence restrictions, DNA damage-induced toxicity, and immunological responses to Cas proteins, despite its wide therapeutic potential. These obstacles are being addressed by improvements in delivery methods and high-fidelity Cas9 variations. All things considered, CRISPR/Cas9 is a revolutionary development in molecular medicine and gene therapy, providing strong prospects for accurate genome engineering and upcoming clinical uses in personalized medicine.

Keywords: CRISPR-Cas9, Gene editing, Gene therapy, Genome engineering, Genetic diseases, Off-target effects

INTRODUCTION

In a wide range of cell types and creatures, genome editing refers to the alteration of genomic DNA at a particular target site by insertion, deletion, and replacement of DNA, which can lead to the inactivation of target genes, the acquisition of new genetic features, and the correction of dangerous gene mutations.

Genome editing technology has emerged as the most effective way to study gene function, investigate the pathophysiology of genetic disorders, create new targets for gene therapy, breed crop varieties, and more in recent years due to the quick development of life sciences.[1]

Technologies that may add, remove, and alter an organism's or cell's DNA sequence make it possible to analyze the roles of particular genes and regulatory components in life sciences research. A larger-scale examination of gene or protein networks may be made possible by multiplexed editing. Similar to this, altering chromatin states or transcriptional regulation at specific loci can show how genetic material is arranged and used inside a cell, shedding light on connections between the structure of the genome and its activities.[2]

Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the RNA-guided CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) nuclease

systems are now the three most popular genome editing methods available. The most popular genome editing method in molecular biology labs worldwide is CRISPR-Cas systems because of their straightforward design, low cost, high efficiency, strong repeatability, and quick cycle.[3,4] An overview of CRISPR-Cas systems will be presented in this study, including with its advancements and uses in gene therapy and human illness research, as well as the challenges that will arise in their practical use.

DISCOVERY AND DEVELOPMENT OF CRISPR TECHNOLOGY

One of the most popular biological tools at the moment is CRISPR-related gene editing technology. Tens of thousands of articles pertaining to CRISPR technology have been published since 2013, indicating an extraordinary expansion in the field. In October 2020, American biologist Jennifer Doudna and French microbiologist Emmanuelle Carpenter received the Nobel Prize in Chemistry for "developing a new approach to genome editing." Before the technique gained global attention, scientists had been researching it for about thirty years.

The development of CRISPR technology was the result of an unforeseen circumstance, as is the case with many significant discoveries. When researching the IAP gene of *E. coli* in 1987, Nakata et al discovered an odd sequence in the 3′ end structural domain of the gene. Five extremely homologous segments with 29 nucleotides spaced 32 nucleotides apart made up the sequence. This specific repeat sequence was found in a number of bacteria and archaea over the course of the following ten years.[1]

Researchers found in 2005 that not every organism uses the same spacer sequences in CRISPR. Mojica et al discovered that viruses were more likely to infect cells lacking homologous spacer sequences and that the majority of spacer sequences were obtained from foreign DNA, with only a small fraction unrelated to the outside world. They hypothesized that CRISPR plays a role in both plasmid transfer and bacterial resistance to exogenous phage infection. Two years later, the theory was verified.[2]

Natural CRISPR/Cas9 has been quickly used to change bacteria, and the functioning mechanism of CRISPR/Cas9 has been steadily discovered. The first report of CRISPR/Cas9 functioning in a non-host bacteria was made in 2011 when Siksnys et al transferred the first CRISPR gene sequence from *Streptococcus thermophiles* to *E. coli*. The recipient *E. coli* effectively resisted plasmid transformation.[3] According to this discovery, CRISPR/Cas systems can be employed as a defense mechanism against external infection and their hosts are not required for the CRISPR system to operate.[4,5]

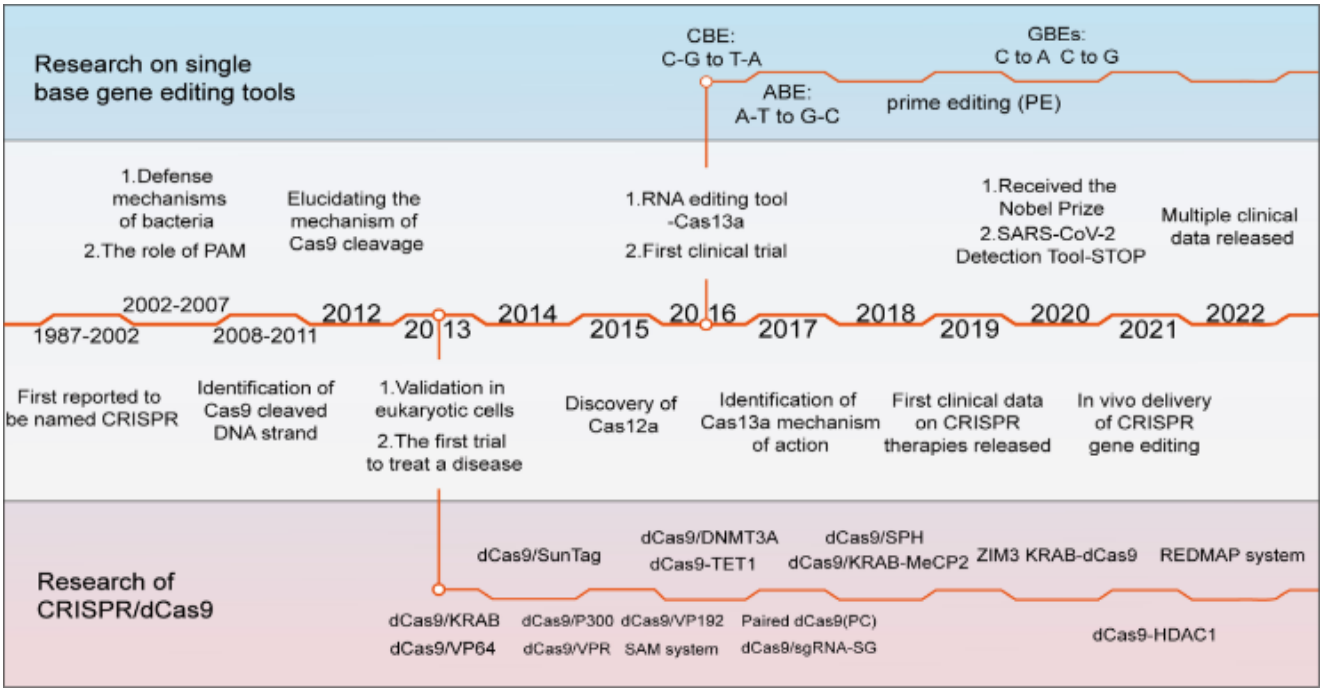

Figure 1: Timeline of major events in the development of CRISPR/Cas technology and representative Cas9 variants.

Components of CRISPR/Cas-9

Prokaryotes use CRISPR and its related protein (Cas-9) as an adaptive immunity mechanism to protect themselves from viruses or bacteriophages. The CRISPR/Cas system can be classified into Class I (type I, III, and IV) and Class II (type II, V, and VI) based on the structure and functions of Cas-proteins. Class II systems use a single Cas-protein, whereas class I systems use multi-subunit Cas-protein complexes. Type II CRISPR/Cas-9 has been thoroughly

researched and utilized in genetic engineering due to its comparatively simple structure.CRISPR-associated (Cas-9) proteins and guide RNA (gRNA) are the two key components of the CRISPR/Cas-9 system (10). The first Cas protein utilized in genome editing, Cas-9, was isolated from Streptococcus pyogenes (SpCas-9). The recognition (REC) lobe and the nuclease (NUC) lobe are the two sections that make up Cas-9. The RuvC, HNH, and Protospacer Adjacent Motif (PAM) interaction domains make up the NUC lobe, while the REC lobe's REC1 and REC2 domains bind guide RNA. CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) are the two components of guide RNA. Guide RNA is used to target viral DNA in prokaryotes, but it may be synthetically created in a gene-editing tool by combining tracrRNA and crRNA to create single guide RNA (sgRNA) that can target nearly any gene sequence that needs to be altered.[1]

MECHANISMS OF CRISPR/CAS-9 GENOME EDITING

In nature, the CRISPR/Cas9 system recognizes and breaks down invasive genetic sequences to protect bacteria or archaea. Three general phases comprise the mechanism of CRISPR/Cas-9 genome editing: recognition, cleavage, and repair.

Recognition

For precise cleavage, the target sequence must be accurately recognized. In the interactions between sgRNA and Cas9, the REC domain of Cas9 was crucial. Following the creation of the sgRNA-Cas9 complex, the target sequence is recognized and bound by the Cas9 nuclease. Through RNA-DNA base pairing, spCas9 might be directed to any target of interest upstream of a necessary 5'-NGG PAM. For sgRNA-Cas9 to bind to the correct region of the target gene, PAM is crucial. Complementary base pairing reactions are used by sgRNA-Cas9 to read out and capture the target DNA and achieve site-specific binding when Cas9 detects PAM.

In order to create a sgRNA-target DNA heterodupex and initiate R-loop formation, the CRISPR-Cas9 nuclease selectively binds a target DNA bearing a conventional 5'-NGG-3' PAM and unzips DNA corresponding to the sgRNA seed sequence.[2]

Cleavage

The creation of double strand breaks (DSBs) and the ensuing cellular DNA repair process are essential for CRISPR/Cas9-mediated genome editing. The target dsDNA became unstable at the PAM motif when the target sequence was identified and an RNA-DNA heteroduplex was formed. The two functional nickase domains (HNH and RuvC) of Cas9 are catalytically activated by these events. PAM motif is also necessary for Cas9's double-stranded endonuclease activity. Cas9 acts as a scissor during target DNA cleavage, with the RuvC domain cutting the displaced strand and the HNH nuclease domain nicking the DNA strand corresponding to the guide RNA to produce a site-specific DSB.[3]

Repair

Nuclease-induced double-strand breaks (DSBs) in DNA trigger two primary repair mechanisms: the homology-directed repair route and the non-homologous end joining (NHEJ) pathway. Both ends of a double-strand break (DSB) are processed by endogenous DNA repair machinery and reconnected in the NHEJ-mediated error-prone DNA repair process, which can result in random indel (insertion and deletion) alterations at target sites. Furthermore, DSB can start HDR-mediated DNA repair, which needs a donor dsDNA sequence or ssDNA that contains homology as a repair template. High fidelity and accurate editing are made possible via the HDR approach.[4]

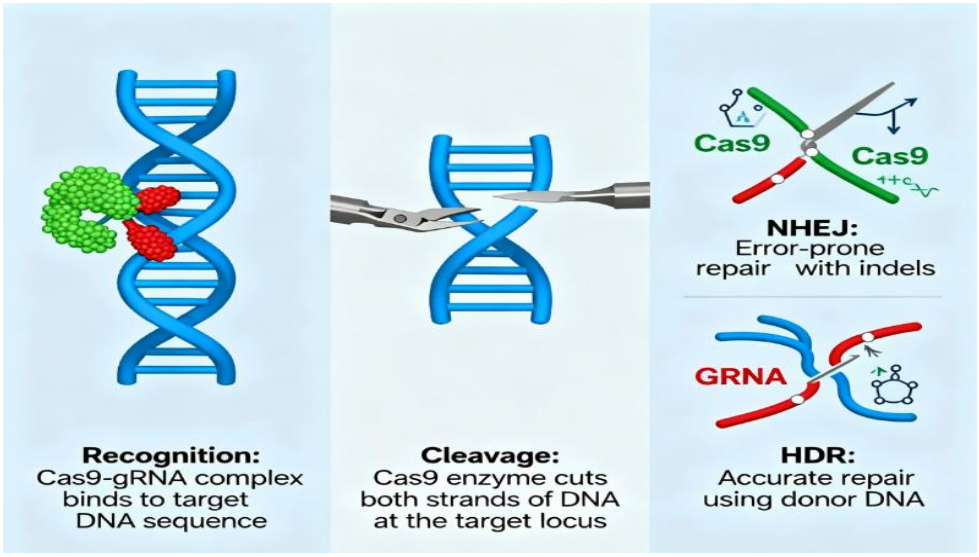

Figure 2: Mechanism of action of CRISPR/Cas9 genome editing.

APPLICATIONS OF CRISPR IN GENETIC DISEASES

Hemoglobinopathies

gene therapy can alter the causal gene in autologous hematopoietic stem cells (HSCs) and fix the hematopoietic system; inherited blood diseases are ideal candidates for gene therapies. Two inherited blood disorders are sickle cell disease and β-thalassemia. β-thalassemia is caused by a variety of mutations in the β-globin gene, such as minor insertions, single point mutations, or deletions, which lead to decreased or absent β-globin synthesis. A Glu->Val mutation in the hemoglobin β-globin subunit results in aberrant hemoglobin S, which is the etiology of sickle cell disease.[1]

Inherited eye disease

A uncommon genetic eye condition called Leber congenital amaurosis (LCA) causes profound vision loss at birth or in infancy. The severe retinal degeneration known as LCA10 is brought on by mutations in the CEP290 gene. The CEP290 gene (~7.5 kb) is too big to fit into one AAV. In order to get over this restriction, Editas Medicine created EDIT-101, a potential genome editing treatment that uses subretinal administration to fix the CEP290 splicing deficiency in human cells and humanized CEP290 mice. This method eliminates the aberrant splice donor produced by the IVS26 mutation using the SaCas9.[2]

Muscular genetic disease

The most prevalent type of progressive muscular dystrophy, DMD is brought on by mutations in the dystrophin gene and is marked by muscle weakness, loss of ambulation, and early mortality. Following local or systemic delivery of CRISPR/Cas components via AAV, several labs have employed NHEJ to circumvent an early stop codon in exon 23 and restore dystrophin production in neonatal and adult mice.[3] One of the neuromuscular illnesses, congenital muscular dystrophy type 1A (MDC1A), typically manifests at birth or early infancy. Amyotrophy, myasthenia, and hypotonia are its primary symptoms. Loss-of-function mutations in LAMA2, which codes for laminin-α2, cause MDC1A.

Ronald D. Cohn and his associates employed CRISPRa to upregulate LAMA1, which encodes laminin-α1 and is structurally similar to laminin-α2, in order to make up for the loss of laminin-α2. In the MDC1A mouse model, upregulation of LAMA1 reduces paralysis and muscle atrophy and offers a unique mutation-independent method of disease repair.[4]

Genetic liver disease

Patients with loss of function FAH mutations who have hereditary tyrosinemia type I (HTI) accumulate harmful compounds that harm their livers. In the HTI mouse model, FAHmut/mut has been corrected using CRISPR/Cas-mediated HDR by hydrodynamic injection of plasmids encoding CRISPR/Cas components or by combination delivery of AAV carrying HDR template and sgRNA and of nanoparticles with Cas9 Mrna.[5] A hazardous gain-of-function mutant allele causes liver illness in patients with alpha-1 antitrypsin deficiency (AATD), and the loss of AAT antiprotease action causes progressive lung disease. AAT point mutations have been corrected using HDR, while mutant AAT has been disrupted using the CRISPR/Cas-mediated NHEJ to lessen the pathologic liver phenotype.[6]

Congenital genetic lung disease

Cystic fibrosis and hereditary surfactant protein (SP) disorders are examples of congenital genetic lung illnesses. Mutations in the SP genes of the pulmonary epithelium generate monogenic lung disorders, which manifest as either chronic widespread lung disease with limited treatment options or prenatal catastrophic respiratory failure death. In order to inactivate the mutant SFTPCI73T gene through NHEJ, researchers carefully timed intra-amniotic introduction of CRISPR/Cas9 components into a prenatal mouse model with the human SP gene SFTPCI73T mutation using a CRISPR fluorescent reporter system. In SFTPCI73T mutant mice, prenatal gene editing restored lung development, saved lung pathophysiology, and raised the survival rate to 22.8%.[7]

Genetic deafness

Genetic mutations and genetic inheritance account for at least half of all occurrences of significant congenital deafness. There are just a few treatments available to reduce or reverse hereditary deafness, despite the identification of about 120 genes linked to deafness. In the humanized transmembrane channel-like 1 (Tmc1) Beethoven (Bth) mouse model, David R. Liu's team recently used cationic lipid-mediated in vivo delivery of Cas9-guide RNA complexes to disrupt the dominant deafness-associated allele and improve the animals' hearing loss.After screening 14 Cas9/sgRNA combinations, David P. Corey's team discovered that SaCas9-KKH/gRNA could specifically and safely identify mutant Tmc1 but not wildtype allele in vitro and in vivo. This finding offers a method to effectively and selectively disrupt the dominant single nucleotide mutation rather than the wild-type alleles.[8]

LIMITATIONS OF CRISPR/Cas9

Off-target effects

Off-target effects refer to the possibility that genome editing systems will cleave DNA not only at the target location but also in other areas. This could restrict the use of Cas proteins or have negative consequences.Off-target effects could lead to chromosomal rearrangements, which

could unintentionally affect some genomic regions that are not well matched and restrict the use of CRISPR-Cas editing systems for therapeutic purposes.[1] Off-target effects can also cause crucial function loss and interfere with the operation of essential human genes, leading to a variety of physiological or signaling problems.

Additionally, Cas9 variations that are especially designed to lower OTEs while preserving editing efficacy have been created by researchers. One of these high-fidelity variations, SpCas9-HF1, takes advantage of the "excess-energy" theory, which postulates that an excess affinity between Cas9 and target DNA may be facilitating OTEs.[2] SpCas9-HF1 has been demonstrated to have no discernible off-target activity when compared to wildtype SpCas9 by introducing mutations to four residues implicated in direct hydrogen bonding between Cas9 and the phosphate backbone of the target DNA. evoCas9 and HiFiCas9 are two more Cas9 variations that have been created; they both include modified amino acid residues in the Rec3 domain, which is crucial in nucleotide recognition. Desensitizing the Rec3 domain reduces OTEs while preserving editing efficacy because it increases the reliance on specificity for the DNA:RNA heteroduplex to cause DSBs.[3]

Protospacer adjacent motif requirement

The bacteria's Cas9 One of the most often utilized Cas9s is Streptococcus pyogenes (SpCas9), which has a relatively short canonical PAM recognition site: 5′NGG3′, where N can be any nucleotide. Nevertheless, SpCas9 is rather large and challenging to package into AAV vectors, which are the most widely used gene therapy delivery vehicle. As an alternative, RNA-targeting Cas9 variants have been created, which also increase the range of genes that can be targeted by reducing the limitations imposed by PAM requirements. By supplying a short oligonucleotide with a PAM sequence, referred to as a PAMmer, S. pyogenese Cas9 (SpyCas9) can be engineered to target RNA, hence removing the requirement for a PAM site inside the target area.[1]

DNA-damage toxicity

Instead of the desired gene change, CRISPR-induced DSBs frequently cause apoptosis. Using this method in human pluripotent stem cells (hPSCs) raised additional safety concerns because it showed that p53 activation in response to the harmful DSBs induced by CRISPR frequently results in subsequent apoptosis.[2]

A significant safety concern for clinical uses of DSB-inducing CRISPR therapy is further highlighted by the numerous reports of massive deletions spanning kilobases and intricate rearrangements as unintended outcomes of on-target activity. Other Cas9 variants, like catalytically inactive endonuclease dead Cas9 (dCas9), which has deactivated nuclease domains, may be therapeutically useful while lowering the danger of double-strand breaks.

Immunotoxicity

Like conventional gene therapy, CRISPR/Cas9 still raises concerns about immunogenic toxicity in addition to technological restrictions. More than half of the human participants in Charlesworth et al study had preexisting anti-Cas9 antibodies against the most widely employed bacterial orthologs, SaCas9 and SpCas9. Pre-existing human immunity to two of these orthologs-SpCas9 and SaCas9-is a significant drawback, making CjCas9 the only available treatment for this patient group. Nevertheless, compared to the other two orthologs, this one has not received as much research and will require more study to demonstrate its safety and effectiveness for clinical application.[4]

CONCLUSION

In nature, the CRISPR/Cas-9 system identifies and breaks down exogenous genetic elements to shield prokaryotes from invasive viruses. Adopted from acquired immunity in prokaryotes, CRISPR/Cas-9 gene editing consists of two components: Cas-9, a protein that basically cuts the DNA at the position indicated by guide RNA, and guide RNA, which locates (binds) the target DNA to be edited. Finding the target gene that determines the desired phenotype and creating the guide RNA are essential steps in the CRISPR/Cas-9 gene-editing process. These days, molecular biology is entering a new era with a wide range of uses, from clinical applications to fundamental molecular research. Even though a lot of work has been done, there are still some difficulties with practical applications, and different advancements are required to go past these difficulties in order to guarantee the greatest benefit while lowering the risk.

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