INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES
[ISSN: 0975-4725; CODEN(USA): IJPS00]
Journal Homepage: https://www.ijpsjournal.com

OPEN ACCESS

Mini Review Article

CRISPR-Cas 9-Breakthrough in Gene Editing-A Review

Chakraborty Manas*

Flemming College of Pharmacy

ARTICLE INFO

Published: 02 Dec. 2025
Keywords:
CRISPR-Cas 9, Gene Editing
DOI:
10.5281/zenodo.17786988

ABSTRACT

The advancement of CRISPR-Cas9 technology has marked a profound transformation in gene editing with significant implications for fields like health, agriculture, and biotechnology. This review provides an overview of the historical significance and fundamental components of CRISPR-Cas9, notably the Cas9 protein and guide RNA, underscoring its major role in genetic manipulation. It emphasizes CRISPR-Cas9's involvement in precise genome editing, driving breakthroughs in personalized medicine, gene therapy, and agriculture. Of paramount importance is the integration of nanomaterials, encompassing lipid-based and polymeric nanoparticles, alongside viral vectors, serving as potent vehicles for CRISPR-Cas9, augmenting delivery efficiency and precision. Strategies are aimed at enhancing CRISPR-Cas9 through nanomaterials, also addressing ethical and regulatory considerations. Offered also a nuanced perspective on the present state of the field, highlighting the potential for transformative progress in research and current therapy. CRISPR-Cas9 stands on the brink of unlocking new possibilities in genome editing, providing innovative solutions to address current global challenges in different critical disease field.

INTRODUCTION

Gene editing is a technology that precisely modifies the genome sequence to induce insertions, deletions, or base substitutions in the field of gene modifications. Many diseases are accompanied by changes in gene expression in vivo, particularly some genetic diseases caused by mutations in a single gene, and gene-editing technology is expected to control the occurrence of diseases at the genetic level. Till date, gene-editing technology has undergone three main generations of development: the first generation of gene-editing technology was zinc-finger nucleases (ZFNs); the second generation was transcription activator-like effector nucleases (TALENs); and the most widely used third generation gene-editing technology is clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR

***Corresponding Author:** Chakraborty Manas
Address: *Flemming College of Pharmacy*
Email ✉: mchakraborty115@gmail.com
Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

associated protein 9 (Cas). , CRISPR technology directs Cas proteins to a specified location in the genome by changing the base sequence of a small segment of guide RNA, which improves the gene sequence considerably forming new gene segments replacing defective genes to a great extent. Bacteria and Archaea form a defence mechanism against phage infection and plasmid transfer. They acquire a segment of their DNA sequence to insert into the CRISPR spacer region when first infiltrated by an exogenous phage or plasmid. If reinfected with homologous DNA, the bacterium will initiate transcription of the CRISPR region. After a series of processing and maturation processes to generate a single guide RNA (sgRNA), the sgRNA guides Cas9 to shear the DNA strand that disrupts the homologous spacer region. The recognition process of the sgRNA requires the involvement of protospacer-adjacent motifs (PAMs), a short guanine-enriched sequence. The preferred PAM by *Streptococcus pyogenes* Cas9 (SpCas9) is NGG, which is common in the genomes of most organisms, thereby facilitating the use of CRISPR technology across the fields of plant and animal science, together with biomedicine.[10,11,12,13,14] By changing the nucleotide sequence of a small segment of guide RNA, CRISPR/Cas9 allows the accurate targeting of almost any desired genomic locus for the purpose of correcting disease-causing mutations or silencing genes associated with disease onset.[5,15] However, some highly chromatinized regions in the genome may not be accessible to CRISPR/Cas9. Promising applications for this technology include the treatment of cancers, cardiovascular diseases, sickle cell anemia, and neurodegenerative disease Wild-type Cas9 only cuts double-stranded DNA to form double-strand breaks (DSBs), which are repaired through DNA repair mechanisms, namely, homology-directed repair (HDR) and nonhomologous end joining (NHEJ).[20,21,22] The base sequence of the original gene is damaged, resulting in inactivation, but the inactivation of a single deleterious gene cannot address the complex processes of all disease events.[23] Therefore, researchers searched for possible ways to modify Cas9 by elucidating the physicochemical structure of Cas9, the mechanism of action by which Cas9 cleaves double chains, and other properties. They endowed Cas9 with new functions by mutating the structural domain of Cas9 and introducing effectors, including transcriptional regulatory tools such as dead Cas9 (dCas9) effectors and single-base substitution tools such as cytosine base editors (CBEs), adenine base editors (ABEs), and prime editors (PEs). Moreover, RNA recognition and cleavage functions can be performed by Cas13a isolated from *Leptotrichia shahii*.[24,25,26,27,28] These Cas9 variants and derivatives enrich the gene-editing paradigm and can be adapted to additional types of diseases. Each disease has different characteristics, and our aim is not to develop a universal delivery vehicle but to develop multiple vehicles applicable to different types of diseases. Therefore, studying the pathogenesis of diseases and the pathological characteristics of disease cells and tissues and constructing environment-responsive and ligand-recognizing nanoparticles based on these characteristics will further enrich gene-targeting drugs in diseased tissues.[33] In addition, exosomes and cell membranes from immune cells or diseased organs can effectively avoid immune clearance, and the abundant membrane proteins on the surface enable gene-targeting drugs to be delivered to diseased cells.

Discovery and development of CRISPR technology

CRISPR-related gene-editing technology is currently one of the hottest biological tools. Since 2013, explosive growth has been recorded in the

study of CRISPR technology, with tens of thousands of CRISPR-related articles published. In October 2020, the Nobel Prize in Chemistry was awarded to French microbiologist Emmanuelle Charpentier and American biologist Jennifer Doudna for "developing a new approach to genome editing". The method had been studied by scientists for nearly three decades before it received widespread attention (Fig. 1).

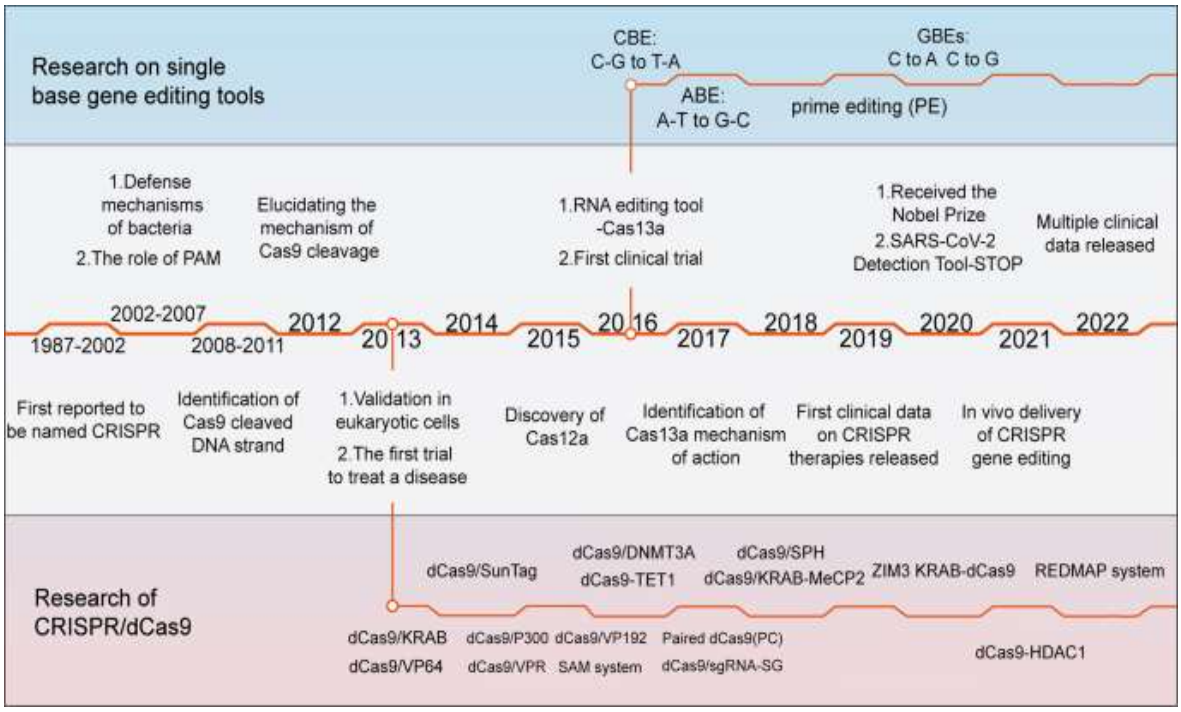

Fig. 1

Timeline of major events in the development of CRISPR/Cas technology and representative Cas9 variants. In 1987, the CRISPR sequence was first reported. The mechanism by which Cas9 cuts DNA double strands was reported in 2012, and Cas9 was subsequently used for gene editing in mammalian cells. Since then, CRISPR technology has developed rapidly, and multiple Cas9 variants with specific functions have been identified. The representative variants are single-base substitution tools (e.g., CBE and PE) and transcriptional regulatory tools (e.g., dCas9-effector). Since 2016, CRISPR-based gene-editing technologies have been successively used in clinical treatment with great success. CRISPR clustered regularly interspaced short palindromic repeats, Cas CRISPR-associated, dCas9 dead Cas9, PAM protospacer-adjacent motifs, CBE cytosine base editors, ABE adenine base editors, GBE glycosylase base editors. (Figure was created with Adobe Illustrator)

Early detection using CRISPR technology

A special sequence of repeated intervals

Like many great discoveries, the discovery of CRISPR technology was born out of an unexpected event. An unusual sequence identified in the 3′ end structural domain of the *iap* gene was first reported by Nakata et al. in 1987 while studying the *iap* gene of *E. coli*. The sequence consisted of five highly homologous sequences containing 29 nucleotides separated by 32 nucleotides. Over the next decade, this particular repeat sequence was detected in a variety of bacteria and archaea. In 2002, Janson et al.

provided a generalized summary of the specific repeats that have been identified, naming these repeats as a family and using the acronym CRISPR for clustered regularly interspaced short palindromic repeats. In addition, multiple CRISPR-associated proteins (Cas)-Cas1 to Cas4-have been revealed in previous studies.

Bacterial and archaeal defence weapons

In 2005, researchers discovered that the spacer sequences in CRISPR are not unique to each organism. Mojica et al. found that most of the spacer sequences were derived from exogenous DNA, with only a small fraction unrelated to the outside world, and they found that viruses were more likely to infect cells without homologous spacer sequences. They conjectured that CRISPR is involved in bacterial resistance to infection by external phages and in plasmid transfer. The conjecture was confirmed 2 years later. When first confronted with phage or plasmid infestation, bacteria containing CRISPR sequences acquire a segment of their DNA sequence, which serves as a spacer region between special repeat sequences. CRISPR RNA (crRNA) then undergoes a series of transcription and maturation processes to produce a single crRNA containing a protospacer sequence of 20 bases that binds to the invading DNA via complementary base pairing. Recognition of the exogenous sequence by crRNA alone does not protect it from the phage; it also must be inactivated by disrupting the exogenous sequence through the cleavage activity of the Cas protein. The CRISPR/Cas family of proteins is divided into two categories based on genomic and protein structure information, and the best-known protein Cas9 is among the Class II CRISPR/Cas systems. Class I is characterized by a large Cas9 protein complex that shears the DNA strand, while Class II requires only a single shearing protein. Cas9 is characterized by the presence of two ribonuclease structural domains, a RuvC-like nuclease domain near the amino terminus and the HNH nuclease domain in the middle of the protein, both of which have the function of cleaving the DNA strand. Notably, protospacer sequences are not randomly acquired from exogenous sequences but are always accompanied by a guanine-enriched sequence called protospacer-adjacent motifs (PAMs). Subsequent studies have shown that PAM sequences play an important role in the acquisition of the spacer region, where Cas proteins perform cleavage. In 2005, researchers discovered that the spacer sequences in CRISPR are not unique to each organism. Mojica et al. found that most of the spacer sequences were derived from exogenous DNA, with only a small fraction unrelated to the outside world, and they found that viruses were more likely to infect cells without homologous spacer sequences.8 They conjectured that CRISPR is involved in bacterial resistance to infection by external phages and in plasmid transfer. The conjecture was confirmed 2 years later. When first confronted with phage or plasmid infestation, bacteria containing CRISPR sequences acquire a segment of their DNA sequence, which serves as a spacer region between special repeat sequences. CRISPR RNA (crRNA) then undergoes a series of transcription and maturation processes to produce a single crRNA containing a protospacer sequence of 20 bases that binds to the invading DNA via complementary base pairing. Recognition of the exogenous sequence by crRNA alone does not protect it from the phage; it also must be inactivated by disrupting the exogenous sequence through the cleavage activity of the Cas protein. The CRISPR/Cas family of proteins is divided into two categories based on genomic and protein structure information, and the best-known protein Cas9 is among the Class II CRISPR/Cas systems. Class I is characterized by a large Cas9 protein complex that shears the DNA strand, while Class II requires

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Contributions of Charpentier and Doudna

The functional mechanism of CRISPR/Cas9 has been gradually revealed, and natural CRISPR/Cas9 has been rapidly applied to bacterial transformation. In 2011, Siksnys et al. transferred the first CRISPR gene sequence from *Streptococcus thermophilus* to *E. coli*, and the *E. coli* that received the CRISPR gene sequence successfully resisted plasmid transformation, which was the first report that CRISPR/Cas9 functioned in a nonhost bacterium. This finding suggested that CRISPR/Cas systems can be used as a defence mechanism against external infection and that their hosts are not necessary for the CRISPR system to function. In 2012, Charpentier and Doudna purified Cas9 from *S. thermophilus* and *Streptococcus pyogenes*, enabling the cleavage of prokaryotic DNA in vitro. They also elucidated the mechanism by which CRISPR/Cas9 works, noting that the cleavage site of Cas9 is controlled by a seed sequence in the crRNA and requires the involvement of PAM. Additionally, by altering the nucleotide sequence of a seed sequence, the system can function as a gene silencer in a variety of situations, providing gene targeting and gene editing by changing a nucleotide seed sequence.

The boom in CRISPR technology

Gene editing in mammalian cells

Previous research on CRISPR/Cas9 has focused on prokaryotic cells, and CRISPR technology started to be used in medicine, agriculture, and other fields in a paper published by Zhang Feng et al. in 2013. They used human-derived 293 T cells, into which they integrated trans-activating crRNA (tracrRNA), pre-crRNA, host factor ribonuclease (RNase) III, and Cas9 from *S. pyogenes* and added the respective promoters and two nuclear localization signals (NLSs) to ensure the entry of the structure into the nucleus. This experiment targeted 30 base pairs located before the PAM at the human empty spiracle homeobox 1 (EMX1) locus, and the results showed that cleavage of EMX1 was achieved with the inclusion of at least spCas9, tracrRNA and pre-crRNA. Additionally, the function of Cas9 from *S. thermophilus* was verified by Zhang Feng et al. and produced consistent results. In another paper published the same year, Church et al. constructed crRNA-tracrRNA fusion transcripts that became single guide RNAs (sgRNAs) and shrank crRNAs to 20 bp.58 These studies had significant implications, both confirming that CRISPR motifs function in mammalian cells and simplifying the CRISPR gene-editing system, thereby providing more possibilities for the use of CRISPR.

First clinical trial of CRISPR/Cas9 technology

The first clinical trial of CRISPR/Cas9 technology was conducted by Lu and colleagues at West China Hospital in Sichuan, China. In October 2016, Lu et al. injected CRISPR/Cas9 gene-edited T cells back into patients, the world's first human injection of gene-edited cells. The T cells used for

gene editing were derived from patients, and plasmids encoding Cas9 and sgRNA targeting the PD-1 gene were transfected into the cells by electroporation. The data showed a significant reduction in PD-1 expression in the gene-edited T cells. Follow-up studies of patients who received T-cell injections showed that the patients did not experience significant adverse effects due to receiving gene-edited T cells, and two of them were in a stable condition. This study indicated the feasibility and safety of the clinical application of gene-editing technology, which is very important to promote the clinical application of gene-editing technology.

CRISPR-based gene-editing tools

CRISPR gene-editing technology facilitates gene editing in eukaryotic cells. Researchers have studied the mechanism of action of Cas9 and have obtained Cas9 variants with different functions and some other derivative gene-editing tools through special modifications and have discovered other Cas proteins in the Cas9 family, enriching the types of genes that can be edited using CRISPR technology. Researchers have developed some vectors to assist in transport and safely deliver the CRISPR system to the body.

Method for CRISPR delivery

Plasmid DNA (pDNA) is an ideal vector for loading the CRISPR system because it is not easily degradable, can be amplified in large quantities, and can be easily modified. After entering the cell, the plasmid carrying CRISPR/Cas9 enters the nucleus with the assistance of NLS and transcribes the mRNA encoding Cas9 and sgRNA. This process is very tedious, and loading CRISPR/Cas9 tools on mRNA may greatly simplify this process. However, mRNA is easily degraded and has low stability. In particular, gene-editing tools that deliver Cas9 to function in concert with effector proteins are difficult to apply because the number of bases in the mRNA encoding Cas9 and effector proteins is too large. Cas9 RNPs, known as ribonucleoproteins (RNPs), are complexes formed by fusing purified Cas9 with sgRNA in vitro, and RNPs function immediately after entering cells. However, RNPs are relatively difficult to deliver into cells due to their complex composition and charge properties, whereas proteins and nucleic acids are usually delivered using electroporation with the assistance of cell-penetrating peptides. With continuous innovations in delivery vectors, scientists have identified exosomes as a promising approach to deliver Cas9 RNPs.

Application of gene-editing tools

Cancer:

Nano particulate Drug Delivery system is used to target cancer cell. After reaching the target they can silence cancer expressed gene to stop the progress of abnormal cell growth.

Liver Disease:

Crisper derived exosome can protect liver, an important metabolic organ, from different hepatocellular disorders like liver fibrosis etc.

Cardiovascular Disease:

Crispr technology can be successfully used for detection & treatment of cardiac problems based on gene editing methods. In addition, CRISPR/Cas9 technology is also widely used for bone regeneration and the treatment of CFTR, Alzheimer's disease, obesity, and other disease.

Limitations:

Delivery limitations:

Target site should be chosen with extreme specificity to deliver nano delivery to specific site.

Efficiency limitations:

Sometimes gene editing does not occur because of low efficiency of Crispr gene, so selection of proper Cas9 protein is important.

Off-target effects:

Off-target effects occur when the Cas9 nuclease edits an untargeted section of the genome, resulting in unwanted alterations. Off-target effects are a major concern for CRISPR-Cas9 experiments, and they can be challenging to predict.

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HOW TO CITE: Chakraborty Manas*, CRISPR-Cas 9-Breakthrough in Gene Editing-A Review, Int. J. of Pharm. Sci., 2025, Vol 3, Issue 12, 271-277. https://doi.org/10.5281/zenodo.17786988