EXPERT REVIEW

CRISPR therapy: A revolutionary breakthrough in genetic medicine

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Keywords: CRISPR/Cas9 therapy; gene; DNA; RNA; healthcare management; healthcare technologies;

ABSTRACT

Gene editing with CRISPR-Cas9 technology revolutionizes modern medicine by enabling precise DNA modifications. Initially discovered as a bacterial defense mechanism, the Cas9 enzyme, guided by RNA, can target and cut specific DNA sites, allowing for gene editing. Applications include genetic engineering, functional studies, and potential treatments for genetic diseases like cancer. Notably, in 2023, CRISPR-Cas9 was approved for treating sickle cell anaemia with significant results, despite challenges like long-term security and high costs. CRISPR-Cas9 technology allows for correcting genetic defects, treating diseases, and improving agricultural crops. It can regulate gene transcription through the CRISPRi system, using an inactive Cas9 to interfere with gene expression without permanently altering DNA. This gene-editing tool shows promise in gene therapy, potentially curing diseases like HIV-1, sickle cell disease, and haemophilia B. However, challenges include off-target mutations and efficient delivery of CRISPR/Cas9. Precise target site selection and dosage control are crucial, with tools like CasOT helping identify and prevent unwanted mutations. CRISPR-Cas9 requires a PAM sequence to function, narrowing its targets in the genome but increasing specificity. Production of gRNA faces challenges due to mRNA processing, with alternatives like the artificial gene RGR showing promise. Efficient delivery methods are still needed, with current techniques involving DNA and RNA injection. Future applications include treating genetic diseases and agricultural improvements, with ongoing research essential for overcoming challenges and ensuring safety and accuracy.
Global collaboration is vital for the ethical use of this technology.

Contribution to evidence-based healthcare: CRISPR therapy represents an exciting frontier in genetic medicine, allowing for precise gene editing and opening up new possibilities for treating incurable diseases.

International Healthcare Review (online)
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eISSN: 2795-5567
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How to Cite
da Silva Barreto, G., Brito do Nascimento, J., Cristina Santos de Almeida, K., Carlos Marcolino Neto, J., & Lucas Ferreira Luz da Silva, S. (2022). The CRISPR therapy: A revolutionary breakthrough in genetic medicine. International Healthcare Review (online). https://doi.org/10.56226/88
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Published online: 16/December2024
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What do we already know about this topic?
In 2023, it was approved to treat sickle cell anemia, showing significant results. However, it faces challenges such as long-term safety and high costs. CRISPR-Cas9 technology is a revolutionary tool for genome editing, allowing easy modification of DNA sequences and gene functions. It uses guide RNA (gRNA) to direct the Cas9 enzyme, which cuts DNA, and cellular repair mechanisms to introduce precise modifications.

What is the main contribution to Evidence-Based Practice from this article?
CRISPR/Cas9 gene editing holds promise for gene therapy, allowing diseases to be cured by correcting mutations or inserting protective genes. Examples include HIV-1, sickle cell disease, and hemophilia B.

What are this research's implications towards health policy?
CRISPR-Cas9 technology requires a PAM sequence to function, which restricts its targets in the genome but increases specificity. The production of gRNA faces challenges due to mRNA processing, with alternatives such as the artificial gene RGR showing promise. CRISPR/Cas9 delivery still needs to be developed more efficiently, with DNA and RNA injection being the current techniques. Future applications include treating genetic diseases and improving agriculture, and continued research is essential to overcome challenges and ensure safety and accuracy. Global collaborations are essential to the ethical use of the technology.

Authors' Contributions Statement:
GSB: Formal analysis, Software, Writing– original draft. JBN: Data curation, Writing– original draft. KCSA: Data curation, Writing– original draft. JCM: Writing– original draft. Software, Conceptualization., SLFS: Methodology, Writing– review & editing. validation,

Introduction

Gene editing has long been a field of great interest in modern medicine, and the technology of cluster-interspaced regularly interspaced short palindromic repeats (CRISPR)-Cas9 has emerged as a transformative tool that allows for precise modifications to DNA, enabling significant advances in the treatment of genetic diseases (Li et al., 2023).

This system is described as an RNA-mediated adaptive immune system defence that is detected in bacteria and archaea. This system prevents the invasion of viruses and plasmids into these organisms (Paul & Montoya, 2020, Jinek et al., 2012). Cas9, belonging to the CRISPR Type II system, has attracted the interest of many scientists. Cas9 encodes a guide RNA (gRNA), forms a direct bond to the target DNA with the Watson–Crick base pairing, and promotes its cleavage. The host cell responds to this double-stranded break with two different mechanisms: (a) junction of nonhomologous ends (NHEJ) and (b) homology-directed repair (HDR) that lead, respectively, to insertion/deletion and frame-shift mutation in the target DNA and HDR that offers a donor DNA as a template for homologous recombination (Gasiunas, Barrangou, Horvath and Siksnys, 2012; Guernet and Grumolato, 2017; Zhan, Rindtorff, Betge, Ebert and Boutros, 2018).

Cas9 has many applications in genetic engineering, such as gene editing, gene expression, and functional gene studies. Based on these characteristics, Cas9 has attracted a lot of attention in the treatment of many diseases caused by mutations. Thus, it seems that Cas9 has made a revolution in the treatment of diseases such as cancer (Hsu, Lander, and Zhang, 2014; Jia et al., 2018).

Initially discovered in bacteria as a defence mechanism against viruses, CRISPR-Cas9 technology uses RNA-guided Cas9 proteins to locate and cut specific DNA sequences. In 2007, researchers discovered that these

sequences were part of a bacterial immune system. The bacteria used CRISPR to defend against viruses by embedding segments of viral DNA into their sequences to recognize and destroy invading viruses in future infections. This process allows for the removal, insertion, or modification of target genes, providing a highly accurate approach to gene editing (Paul & Montoya, 2020, Barrangou & Horvath, 2007). One of the most promising applications of CRISPR is in the treatment of genetic diseases. In 2023, the approval of CRISPR-based treatments such as Casgevy for sickle cell anaemia and beta-thalassemia marked a significant breakthrough. These therapies correct genetic mutations in bone marrow stem cells, allowing for the production of functional haemoglobin, which is crucial for patients suffering from these conditions (FDA, 2023).

Despite its revolutionary potential, CRISPR therapy faces considerable challenges. Long-term safety is a primary concern, given the possibility of off-target effects that can result in unwanted mutations. In addition, the high cost of therapies limits access, raising questions about health equity (Gene, 2017).

Working Principle

Generally, the CRISPR-Cas system is composed of a clustered set of CRISPR-associated genes (Cas) and a CRISPR matrix (repeated sequences and single spacer sequences; Hsu et al., 2014). Diversity in Cas genes and their positioning is the basis of the CRISPR-Cas classification (Figure 1; Makarova, Wolf et al., 2015). Cas genes are responsible for encoding functional proteins known as effector complexes. CRISPR-Cas systems are divided into two classes and each class has several types and subtypes (Makarova, Wolf et al., 2015). Class 1 is found in bacteria and archaea (hyperthermophiles), while Class 2 is detected only in bacteria (non-hyperthermophiles; Chylinski, Makarova, Charpentier and Koonin, 2014).

Figure 1 demonstrates the schematic structure of different classes and types of the CRISPR-Cas system. Generally, the structure of functional proteins is simpler in Class 2 compared to Class 1. Thus, the act of functional proteins (Cas proteins) in Type II and Type V is performed by Cas9 and Cpf1, respectively. Cas9 and Cpf1 are single, large proteins. However, functional proteins in Class 1 are multi-subunits and consist of multiple proteins (CASCADE complex for Type I; Cmr or Csm RAMP complexes for Type III; Makarova, Wolf et al., 2015). The Cas1 and Cas2 genes are observed in all types except Type IV (Makarova, Wolf et al., 2015). Different Cas proteins have various roles in the CRISPR-Cas system. The Cas1 protein is a well-known integrase enzyme that is required for the specific breakdown of a CRISPR matrix to insert a newly identified spacer (Paul & Montoya, 2020, Nuñez, Lee Engelman, & Doudna, 2015; Wiedenheft et al., 2009). The role of the Cas2 protein is unclear; however, this protein has RNase and DNase activities and is required for the adaptation phase in Escherichia coli (see below; Makarova, Wolf et al., 2015; Nam et al., 2012).

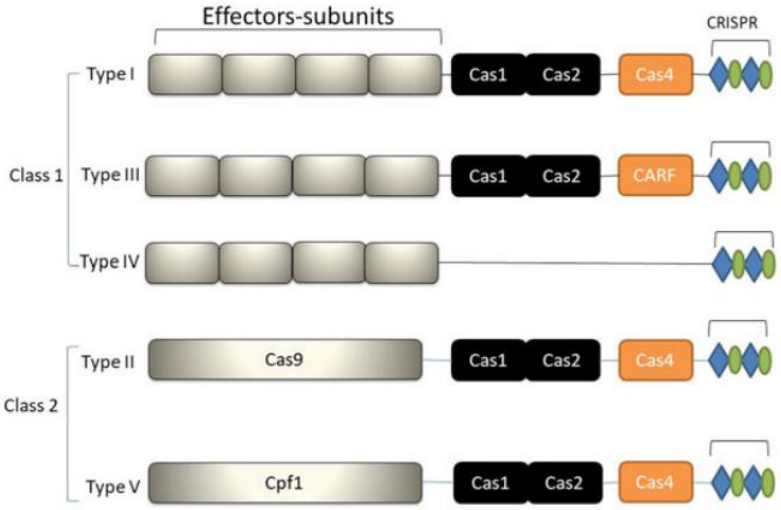

Figure 1 The schematic illustration of the classification of the CRISPR-Cas system. Blue rhombus: repeated units; green circle: spacer sequences; grey rectangle: effector modulus complex. CRISPR-Cas: clustered regularly intercalated short palindromic repeats/CRISPR-associated nuclease.

The CRISPR tool is powerful for editing genomes. It allows researchers to easily alter DNA sequences and modify the function of genes. Its potential applications include correcting genetic defects, treating and preventing the spread of diseases, and improving crops.

- Guide RNA (gRNA): This is a specially designed RNA that guides the Cas9 enzyme to the specific DNA sequence that one wishes to edit. Guide RNA is complementary to the target sequence in DNA, allowing it to couple precisely.
- Cas9: An enzyme that cuts DNA. When guided by the guide RNA, Cas9 makes a double-strand cut in the target DNA sequence. After cutting, the cell's DNA repair mechanisms kick in, making it possible to insert, delete, or modify DNA sequences.

Editing Process:

- Recognition and binding: gRNA binds to the target DNA sequence, thanks to the complementarity between RNA and DNA bases.
- DNA cutting: Cas9, guided by gRNA, cuts the two strands of the DNA molecule at a specific position.
- DNA Repair: Cells have natural DNA repair mechanisms that are activated after cutting. This repair can be used to introduce precise changes to the genome, such as inserting new DNA sequences or correcting mutations.

Applications of CRISPR

Genome editing

CRISPR/Cas9 provides a robust, multiplexable genome editing tool, allowing researchers to precisely manipulate specific genomic elements and facilitating the elucidation of the function of target genes in biology and disease. Through the co-delivery of plasmids that express Cas9 and crRNA, CRISPR/Cas9 has been used to induce specific genomic modifications in human cells (Manghwar, Lindsey, Zhang & Jin, 2019, Cong et al., 2013, Jinek et al., 2013, Cho et al., 2013, Mali et al., 2013, Hou et al., 2013). By integrating several distinct gRNAs with Cas9 into a CRISPR matrix, CRISPR/Cas9 can simultaneously induce multiple mutations in mammalian genomes (Cong et al., 2013). In addition to mammalian

genomes, CRISPR/Cas9 also demonstrates its potentiality in editing the genome of zebrafish (Hruscha et al., 2013, Jao et al., 2013, Chang et al., 2013, Hwang et al., 2013), mice (Wang et al., 2013, Shen et al., 2013), drosophila (Manghwar, Lindsey, Zhang & Jin, 2019, Bassett et al., 2013, Yu et al., 2013), caenorhabditis elegans (Friedland et al., 2013), Bombyx mori (Wang et al., 2013), and bacteria. For example, Bassett et al. provided an enhanced RNA injection-based CRISPR/Cas9 system, which was highly efficient in creating the desired mutagenesis in the Drosophila genome (Manghwar, Lindsey, Zhang, & Jin, 2019, Bassett et al., 2013).
Through direct injection of mRNA and gRNA Cas9 into the embryo, they successfully induced mutagenesis at target sites in up to 88% of the injected flies. The generated mutations were transmitted stably to 33% of the total offspring through the germline (Cheng, Fan, Wen Du, 2020, Bassett et al., 2013). CRISPR/Cas9 is also used to induce desired genomic changes in plants to generate specific traits, such as valuable phenotypes or disease resistance (Cheng, Fan, Wen Du, 2020, Xie et al., 2013). To validate the application of CRISPR/Cas9 in plants, Jiang et al. transferred the green fluorescence protein gene to the genomes of Arabidopsis and tobacco, and bacterial rust susceptibility genes to the rice genome. Miao et al. illustrated the robustness and efficiency of CRISPR/Cas9 in rice genome editing. By modifying crop genomes, CRISPR/Cas9 can be used to improve crop quality as a new breeding technique in the future (Miao et al., 2013).

Transcription regulation

The regulation of gene transcription in living organisms is very useful for studies of gene function and transcriptional networks. By disrupting functional sites related to transcription, CRISPR/Cas9 can regulate the transcription of specific genes. However, this process is irreversible due to permanent modifications to DNA. Recently, a modified CRISPR/Cas9 system called CRISPR inference (CRISPRi), was developed for regulation of RNA-guided transcription. Qi et al. generated a catalytically defective Cas9 (dCas9) mutant with no nuclease activity. dCas9 was co-expressed with gRNA to form a recognition complex, which could interfere with transcriptional elongation, RNA polymerase, and transcription factor binding (Qi et al., 2013). With two gRNAs targeted, respectively, a red fluorescent protein (RFP) gene and a green fluorescent protein (GFP) gene, Qi et al. observed that CRISPRi could simultaneously repress RFP and GFP expression without crosstalk in Escherichia coli (Qi et al., 2013). However, the degree of repression of gene expression achieved by CRISPRi was modest in mammalian cells. Qi et al. fused repressive effector or activating domains to dCas9, which together with gRNA could implement precise and stable transcriptional control of target genes, including transcription repression and activation (Qi et al., 2013). Chen et al. illustrated the performance of CRISPRi to individually or simultaneously regulate the transcription of multiple genes. CRISPRi provides a new, highly specific tool to switch gene expression without genetically altering the target DNA sequence (Cheng, Fan, Wen Du, 2020).

Gene Therapy

Precisely, genome editing has the potential to cure diseases permanently through the disruption of endogenous disease-causing genes, correcting disease-causing mutations, or inserting new protective genes (Ebina et al., 2013). Using ZFNs-induced HDR, Urnov et al. corrected the disease-causing genetic

mutation in human cells for the first time. Subsequently, ZFNs were used to correct the genetic mutations that cause sickle cell disease and haemophilia B. Through disabling virulence genes or the insertion of protective genes, ZFNs have been used to induce resistance to virus infection in human cells and increase the efficiency of immunotherapies (Torikai et al., 2012). Like the newest engineered nucleases, CRISPR/Cas9 provides a new, highly efficient genome-editing tool for gene therapy studies. For example, Ebina et al. disrupted the HIV-1 genome long terminal repeat promoter using CRISPR/Cas9, which significantly decreased HIV-1 expression in infected human cells. Proviral viral genes integrated into host cell genomes can also be removed by CRISPR/Cas9 (Cheng, Fan, Wen Du, 2020, Torikai et al., 2012).

With the rapid development of induced pluripotent stem cell (iPS) technology, modified nucleases are applied to the manipulation of the genome of iPS cells (Hockemeyer et al., 2009). The unlimited capacity for self-renewal and multipotential differentiation of iPS cells makes them very useful in disease modelling and gene therapy. Using CRISPR/Cas9, Horri et al. created an iPS cell model for immunodeficiency, centromeric region instability, and facial anomalies syndrome (CIF) syndrome caused by mutation of the DNMT3B gene. In this study, iPS cells were transfected with plasmids expressing Cas9 and gRNA, which disrupted DNMT3B function in transfected iPS cells. Using the same hPSC lines and delivery method, Ding et al. compared the efficiencies of CRISPR/Cas9 and TALENs for genome editing of iPS cells. They observed that CRISPR/Cas9 was more efficient than TALENs (Ding et al., 2013). However, it is still a long way to apply CRISPR/Cas9 clinically for gene therapy. We must ensure the high specificity of CRISPR/Cas9 for the target sites and eliminate possible off-target mutations with negative effects. Careful selection of target sites, delicate gRNA design, and genome-wide potential research. Off-target sites are mostly needed (Cheng, Fan, Wen Du, 2020, Ding et al., 2013).

Challenges

Despite the great potential of CRISPR/Cas9 in genome editing, there are some important issues that need to be addressed, such as off-target mutations, PAM ependency, gRNA production, and CRISPR/Cas9 delivery methods.

Off-target mutations

Off-target mutations are a major concern about CRISPR/Cas9-mediated genome editing. Compared with ZFNs and TALENs, CRISPR/Cas9 has a relatively high risk of off-target mutations in human cells (Conant et al., 2022, Fu et al., 2013). Large genomes often contain multiple DNA sequences that are identical or highly homologous to the target DNA sequences. In addition to the target DNA sequences, CRISPR/Cas9 also cleaves these identical or highly homologous DNA sequences, which leads to mutations at unwanted sites, called off-target mutations. Off-target mutations can result in cell death or transformation. To reduce the cellular toxicity of CRISPR/Cas9, more and more efforts are being made to eliminate off-target mutations of CRISPR/Cas9 (Conant et al., 2022, Fu et al., 2013). To ensure the specificity of CRISPR/Cas9, it is best to select the target sites with the fewest off-target sites and incompatibilities between the gRNA and its complementary sequence. Xiao et al. recently developed a flexible CasOT search tool, which could identify potential off-target sites in whole genomes (Hendriks, Clevers & Artegiani, 2020, Xiao et al., 2014). The dosage of CRISPR/Cas9 is another factor that affects off-target mutations and

must be carefully controlled. Methylation of the targeted DNA sequences did not appear to affect the specificity of CRISPR/Cas9. In addition, converting Cas9 to nickase can help reduce off-target mutations while maintaining the on-target cleavage efficiency implemented by CRISPR/Cas9 (Conant et al., 2022, Xiao et al., 2014).

Dependence on PAM

Theoretically, CRISPR/Cas9 can be applied to any DNA sequence via engineered programmable gRNA. However, the specificity of CRISPR/Cas9 requires a 2~5 nt PAM sequence located immediately downstream of the target sequence, in addition to gRNA/target sequence complementarity (Laurent, Geoffroy, Pavani & Guiraud, 2024, Jinek et al., 2012). The PAM sequences identified vary between different Cas9 orthologs, such as NGG PAM from Streptococcus pyogenes, NGGNG and NNAGAAW PAM from thermophilic Streptococcus, and NNNNGATT PAM from Neisseria meningitidis (Hendriks, Clevers & Artegiani, 2020, Jinek et al., 2012). Recently, Hsu et al. reported a NAG PAM, which had only approximately 20% efficiency of NGG PAM to guide DNA cleavage. For one, the PAM-dependent manner of CRISPR/Cas9-mediated DNA cleavage constrains the frequencies of targetable sites in genomes. For example, it is possible to find a target site for 8 nucleotides for NGG PAM and NAG PAM, while for 32 and 256 nucleotides for NGGNG PAM and NNAGAAW PAM. On the other hand, dependence on PAM also increases the specificity of CRISPR/Cas9. CRISPR /Cas9 off-target mutations requiring long PAM should be smaller than CRISPR/Cas9 mutations requiring short MAP.

gRNA production

gRNA production is another important issue for CRISPR/Cas9-mediated genome editing. Due to the extensive post-transcriptional processing and modification of the mRNA transcribed by RNA polymerase II, it is currently difficult to apply RNA polymerase II for gRNA production. RNA polymerase III, U3, and U6 snRNA promoters are currently used to produce gRNA in vivo. However, the snRNA U3 and U6 genes are ubiquitously expressed maintenance genes, which cannot be used to generate specific tissues and gRNA cells. The lack of commercially available RNA polymerase III also limits the application of U3- and U6-based gRNA production. Gao et al. designed an artificial RGR gene, whose transcribed mRNA contained desired sequences of gRNA and ribozyme at both ends of the gRNA. After self-catalyzed cleavage, mature gRNA was successfully produced and induced sequence-specific cleavage in vitro and yeast (Laurent, Geoffroy, Pavani & Guiraud, 2024, Jinek et al., 2012).

Delivery methods

Questions also remain about the methods of delivery of CRISPR/Cas9 into organisms. DNA and RNA-based techniques are used for CRISPR/Cas9 delivery, such as injection of plasmids expressing Cas9 and gRNA and injection of CRISPR components as RNA. The efficiency of delivery methods depends on the types of target cells and tissues. More attention should be paid to the development of new robust delivery methods for CRISPR/Cas9 (Laurent, Geoffroy, Pavani & Guiraud, 2024, Jinek et al., 2012).

Future Applications

The possibilities for CRISPR's application are vast, including the treatment of genetic diseases, improvements in crops, and advances in biotechnology. However, ongoing research

is crucial to overcoming current challenges, and ensuring that gene editing is safe, accurate, and ethical.
With continued research, the accuracy and safety of CRISPR edits are expected to improve, allowing for broader and safer applications in medicine and biotechnology. Global collaborations will be essential to regulate and guide the ethical use of technology (Laurent, Geoffroy, Pavani & Guiraud, 2024).

Conclusion

CRISPR therapy represents one of the most exciting frontiers of genetic medicine. While significant challenges remain, the ability to precisely edit genes opens up new possibilities for treating hitherto incurable diseases. As technology advances, CRISPR is expected to transform the global health landscape, offering hope and new therapeutic options to millions of people around the world.

RECEIVED: 28/November/2024 ● ACCEPTED: 3/December/2024 ● TYPE: Expert Reviewe ●FUNDING: The authors received no financial support for the research, authorship, and/or publication of this article ● DECLARATION OF CONFLICTING INTERESTS: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. ● Availability of data and materials data is available from the corresponding author on reasonable request ● Ethics approval and consent to participate: Not required for the methodology applied

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