

SUPERSON, Maciej, SZMYT, Katarzyna, WILK-TRYTKO, Klaudia, KRASNOBORSKA, Julia, SAMOJEDNY, Sylwia, SZYMAŃSKA, Katarzyna and WALCZAK, Kamil. Applications of gene modification technologies in the treatment of inherited diseases. *Journal of Education, Health and Sport*. 2024;66:50073. eISSN 2391-8306.

<https://dx.doi.org/10.12775/JEHS.2024.66.002>

<https://apcz.umk.pl/JEHS/article/view/50073>

<https://zenodo.org/records/10992408>

The journal has had 40 points in Minister of Science and Higher Education of Poland parametric evaluation. Annex to the announcement of the Minister of Education and Science of 05.01.2024 No. 32318. Has a Journal's Unique Identifier: 201159. Scientific disciplines assigned: Physical culture sciences (Field of medical and health sciences); Health Sciences (Field of medical and health sciences).

Punkty Ministerialne 40 punktów. Załącznik do komunikatu Ministra Nauki i Szkolnictwa Wyższego z dnia 05.01.2024 Lp. 32318. Posiada Unikatowy Identyfikator Czasopisma: 201159. Przypisane dyscypliny naukowe: Nauki o kulturze fizycznej (Dziedzina nauk medycznych i nauk o zdrowiu); Nauki o zdrowiu (Dziedzina nauk medycznych i nauk o zdrowiu). © The Authors 2024;

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The authors declare that there is no conflict of interests regarding the publication of this paper.

Received: 06.03.2024. Revised: 10.04.2024. Accepted: 15.04.2024. Published: 18.04.2024.

Applications of gene modification technologies in the treatment of inherited diseases

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Abstract:

Introduction and Purpose: In last years gene modification technologies such as CRISPR/Cas9 has had a revolutionary impact on the treatment of inherited diseases. Technologies developed from bacterial defense mechanisms, has become a basic tools in scientific research and medical therapies. In our article we provided an overview of applications of gene modifications technologies, directly focusing on CRISPR/Cas9, in genetic disease treatment.

State of Knowledge: New applications of CRISPR/Cas9 are still being explored. Treating inherited diseases such as cystic fibrosis, Duchenne muscular dystrophy, thalassemia, hemophilia, Huntington's disease, Crigler-Najjar syndrome, sickle cell anemia, Marfan syndrome, and phenylketonuria, is feasible with this novel technique. A comparative analysis with other gene editing methods highlights CRISPR/Cas9's efficacy, ease of use, and multiplexing capabilities.

Summary: CRISPR/Cas9 is a groundbreaking technology with broad applications in genetic research and therapy. Its ease of use, cost-effectiveness, and ability to target multiple genes simultaneously position it as a preferred method. However, there are some challenges associated with precision issues and ethical considerations in human embryo gene editing. As CRISPR/Cas9 continues to evolve, responsible application and ethical considerations are important for maximizing its potential in treatment of genetic diseases.

Keywords: Crispr/cas9, gene modification, gene therapy, genetic diseases

1. Introduction

CRISPR/Cas9 technique is one of the most innovative technologies in genetics and biotechnology, which has been introduced in recent years. Its development is the result of years of research on bacteria and their defense mechanisms against viruses. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) was first identified in bacteria in the early 1990s [1, 2]. as part of their "immune" system, which protected bacteria from bacteriophage attacks by destroying their genome with the help of Cas9 nuclease [3].

The development of gene editing using CRISPR/Cas9 technology accelerated when we discovered how we could use this natural bacterial system to advance genetic engineering. Key science article, written by Emmanuelle Charpentier and Jennifer Doudna, published in 2012 demonstrated how the CRISPR/Cas9 system can be programmed by humans to cut DNA at specific locations, enabling precise gene editing [4]. Since then, CRISPR/Cas9 technology has rapidly evolved to become a tool widely used in scientific research, biotechnology, and medicine. Its applications include disease modeling, gene therapies, genetic modification of plants and animals, and gene function research, among others.

Gene editing technology, particularly the above-mentioned CRISPR-Cas9 system, has revolutionized the possibilities of genome modification. The possibility of precise DNA modification, make it possible to correct, among other things, genetic mutations responsible

for the occurrence of diseases such as cystic fibrosis or Duchenne muscular dystrophy, as well as many others. It gives a chance to provide a long-term or even complete cure for patients suffering from, so far, incurable genetic diseases [5, 6]. Gene editing is also enabling the development of new targeted therapies in oncology. The CRISPR-Cas9 system can be used to modify a patient's immune system cells to enhance their ability to destroy cancer cells, potentially creating an entirely new, very precise way of cancer treatment [7].

2. Fundamentals of CRISPR/Cas9 technology

The CRISPR-Cas9 system, which is currently being used for genome modification, was originally derived from the bacteria's natural defense mechanism against bacteriophage attacks, which was designed to destroy the DNA of invasive viruses by cutting. Central to this system is the CRISPR associated protein 9 (Cas9) complex, which plays the role of "molecular shears," and the guide RNA (gRNA) that directs the complex to the specific DNA sequence of the target. gRNA consists of two key parts: a CRISPR fragment that provides specificity for binding to DNA, and a trans-activating CRISPR RNA (tracrRNA) fragment that binds to Cas9, allowing DNA to be cut at the site recognized by the gRNA.[8]

The CRISPR/Cas9-mediated genome editing process begins with the design of a gRNA that is complementary to the targeted DNA sequence in the genome. Once inside the cell, the gRNA binds to the Cas9 nuclease, forming a complex capable of recognizing and binding specific DNA sequences. Once the targeted DNA sequence is located, Cas9 performs precise cutting of both DNA strands, resulting in the formation of a double stranded DNA chain break (DSB). Then, the cell uses natural DNA repair mechanisms to reconstruct the broken chain. There are two main repair mechanisms: non-homologous end joining (NHEJ), which can lead to insertions or deletions at the cut site (which can be used to "turn off" genes), and homologous repair recombination (HDR), which allows specific modifications or insertions of new DNA sequences, provided the appropriate DNA template is provided [9].

3. CRISPR/Cas9 versus other gene editing methods (ZFN, TALEN).

In addition to the CRISPR/Cas9 system, researchers are also able to use other genome modification methods, each with its own unique features, advantages and limitations that affect their application in research and therapy. The most widely used, in addition to the

CRISPR/Cas9 system described, include: ZFNs (zinc-finger nucleases) and TALEN (transcription activator-like effector nucleases) [10, 11].

3.1 Zinc-finger nucleases

ZFNs are artificially created restriction endonucleases that combine the zinc-finger proteins' ability to recognize specific DNA sequences with nuclease activity to enable accurate DNA cutting [12]. ZFNs consist of a DNA recognition module made up of several zinc-finger domains, each recognizing three nucleotides, and a FokI nuclease domain, which is responsible for cutting DNA. In order for ZFNs to cut DNA, they must operate in pairs, which ensures high specificity of sequence recognition and minimizes the risk of untargeted cuts [13].

ZFNs have been used in many biotechnology and medical applications, including generating animal models for disease research, correcting genetic mutations responsible for inherited diseases, as well as in plant engineering and the production of transgenic animals [14, 15]. One of the most notable applications of ZFNs is in gene therapy, where they have been used to correct mutations in the gene responsible for hemophilia B in animal models and in human blood precursor cells [16].

Despite their innovation, ZFNs have some limitations, including high cost and difficulty in design and the potential risk of untargeted genome modifications. Additionally, delivery of ZFNs to target cells remains a challenge. Nevertheless, due to their stability and long-term expression, ZFNs continue to be used in some therapeutic applications [17].

3.2 Transcription activator-like effector nucleases

TALENs were among the first tools enabling scientists to precisely modify genes in living organisms. Their ability to target DNA cuts at specific locations in the genome allows the deletion, insertion or modification of genes [18]. The system consists of two main components: a DNA-binding domain derived from *Xanthomonas* bacterial effector proteins, and the FokI nuclease domain, which is responsible for cutting DNA. The DNA-binding domain can be designed to recognize specific genomic sequences, which, in combination with the FokI nuclease domain, enables precise DNA cutting and initiation of genome repair by the cell [19].

TALENs have been used in a wide range of applications, from basic research on gene function, to the development of animal models of disease, to gene therapies. For example, in medicine, TALENs offer the potential to treat genetic diseases by correcting mutations in patients' genes [20, 21]. In agriculture, TALENs enable the creation of plants with desirable traits, such as pest resistance or improved nutritional value.[22]

Despite their versatility, TALENs face challenges similar to other gene editing systems, including specificity of operation and potential off-target DNA modifications. TALENs, like ZFNs, combine DNA recognition modules with FokI nuclease for DNA cutting. However, they have greater design flexibility than ZFNs, allowing them to precisely target more genetic sequences. TALENs are prized for their high specificity and efficiency, but their design is more complex and time-consuming than CRISPR/Cas9 [23].

Criterion	CRISPR/Cas9	ZFN	TALEN
Effectiveness	High	Moderate	High
Specificity	High	High	High
Ease of design	Easy	Difficult	Moderate
Cost	Low	High	High
Multiplexing capability	Yes	Not	Limited

Table 1. Comparison of genome editing methods.

CRISPR/Cas9 stands out for its ease of design, low cost and ability to target multiple genes simultaneously, making it the preferred method for many research and therapeutic applications. ZFNs and TALENs, despite their limitations, continue to be used in specific contexts where their unique features can be beneficial.

4. CRISPR/Cas9 applications in the treatment of inherited diseases.

The use of genetic modification systems, including the CRISPR/Cas9 technology, could completely transform our view of genetic diseases, previously considered incurable. Among the most promising are studies on the use of genome modification technology in diseases such as:

4.1. Cystic fibrosis

Cystic fibrosis is a genetic disease inherited in an autosomal recessive manner that affects many organs, primarily the lungs and pancreas, leading to severe complications and shortened life expectancy. The disease is caused by mutations in the CFTR (cystic fibrosis transmembrane conductance regulator) gene, which interfere with chloride channel function in cells, causing thick and sticky secretions in organs [24]. In the case of cystic fibrosis, research has focused on using CRISPR/Cas9 to correct mutations in the CFTR gene to restore its normal function [25]. The experimental use of CRISPR/Cas9 to correct the most common mutation $\Delta F508$ in the CFTR gene has shown promising results in cellular and animal models. In one study, gene editing using CRISPR/Cas9 improved chloride channel function in nasal epithelial cells from cystic fibrosis patients, indicating the potential effectiveness of this method in restoring CFTR function [26].

4.2. Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is one of the most common yet one of the most severe forms of muscular dystrophy, inherited in a recessive manner linked to the X chromosome. The disease is caused by mutations in the dystrophin gene, a protein that is crucial for maintaining the integrity and function of skeletal muscle and cardiac muscle. Patients with DMD experience progressive muscle weakness, ultimately leading to premature death [27]. In the context of DMD, research has focused on using CRISPR/Cas9 to remove mutations that cause a reading frame shift in the dystrophin gene, with the goal of restoring functional dystrophin production in patients' muscles [28]. Studies in animal models, including mice and dogs with DMD, have shown that the use of CRISPR/Cas9 can effectively restore dystrophin expression in muscle, improving muscle function and strength. In a 2016 study [29], CRISPR/Cas9 therapy led to increased dystrophin expression in the muscles of mice with DMD, resulting in significant improvements in muscle function.

4.3. Thalassemia

Thalassemia is a group of inherited blood diseases characterized by the production of abnormal hemoglobin, resulting in the appearance of severe anemia. The disease is caused by

genetic mutations affecting the globin chains that build up the hemoglobin molecule [30]. The CRISPR/Cas9 system offers a potential method to remove the disease at the DNA level by correcting the genetic mutations responsible for the disease, allowing the production of hemoglobin of normal structure to be restored [31]. Research on the application of CRISPR/Cas9 in thalassemia has focused on two main areas: alpha and beta thalassemia. For beta thalassemia, where the deficit involves the beta globin chain, the use of CRISPR/Cas9 to correct mutations in the HBB gene has shown promising results in animal models and in human cells *ex vivo* [32]. For alpha thalassemia, associated with mutations in the HBA1 and HBA2 genes, CRISPR/Cas9 has also shown potential in restoring functional expression of alpha globin [33].

4.4. Hemophilia

Hemophilia is a genetic blood disorder characterized by an increased tendency to bleed due to the absence or insufficient activity of blood clotting factor. Depending on the type of hemophilia (A or B), patients are deficient in factor VIII or IX, leading to serious and potentially life-threatening complications. Traditional treatments rely on the administration of missing clotting factors, but gene therapies, such as CRISPR/Cas9, are opening up new avenues to permanently treat the disease [34]. In the context of hemophilia, research is focusing on using CRISPR/Cas9 to correct the genetic mutations responsible for the disease or inserting copies of functional clotting factor genes directly into the patient's genome [35]. Recent studies have shown that the use of CRISPR/Cas9 to modify liver cells in mouse models of hemophilia B effectively restores factor IX production, leading to significant improvements in blood clotting and reduced bleeding tendency [36]. Similar approaches are being explored in the context of hemophilia A, with preliminary results suggesting that CRISPR/Cas9 may also be useful in restoring factor VIII activity [37].

4.5. Huntington's disease

Huntington's disease (HD) is an inherited neurodegenerative disease characterized by the progressive loss of nerve cells in the brain. It is caused by a mutation in the HTT gene, leading to an expansion of CAG repeats, resulting in the production of the toxic protein huntingtin [38]. Current treatment options for HD are limited to symptom relief. The use of CRISPR/Cas9 offers a potential treatment option by targeting deletion or correction of the genetic mutation responsible for the onset of the disease [39]. In the context of HD, CRISPR/Cas9 is used to target the expansion of CAG repeats in the HTT gene, aiming to

reduce the expression of the pathological huntingtin protein [40]. Studies in animal models have shown that the use of CRISPR/Cas9 can effectively reduce the expression of the toxic huntingtin protein, leading to reduced neurological symptoms and prolonged survival. In a study using CRISPR/Cas9 in a mouse model, HD led to a significant reduction in pathological protein accumulation and improved motor function in the mice tested [41].

4.6. Crigler-Najjar syndrome

Crigler-Najjar syndrome is a rare metabolic disorder characterized by a significant increase in serum levels of unconjugated bilirubin, leading to jaundice and risk of brain damage. The disorder results from a mutation in the UGT1A1 gene, leading to a deficiency in the enzyme responsible for bilirubin coupling [42]. Studies in animal models and cells from patients with Crigler-Najjar syndrome are focused on evaluating the efficacy and safety of this method. Preliminary studies in animal models with Crigler-Najjar syndrome have shown that CRISPR/Cas9 can effectively correct mutations in the UGT1A1 gene, leading to restoration of enzymatic function and reduced bilirubin levels [43, 44].

4.7. Sickle cell anemia

Sickle cell anemia is an inherited hematological disorder caused by a point mutation in the HBB gene, leading to the production of pathological hemoglobin S instead of normal hemoglobin A. This mutation causes erythrocytes to take on a characteristic sickle shape, leading to a number of serious complications [45]. Using CRISPR/Cas9 technology, researchers are targeting the genome of patients with sickle cell anemia to make one of two potential types of modifications: directly correcting mutations in the HBB gene, which is responsible for the production of pathological hemoglobin S, or increasing the expression of the HBG1 and HBG2 genes, which encode fetal hemoglobin. Increasing the level of HbF in patients' erythrocytes can reduce HbS polymerization and improve the clinical manifestations of the disease [46]. Clinical trials using CRISPR/Cas9 to treat sickle cell anemia have shown promising results. Dever et al showed that CRISPR/Cas9-mediated HBB gene editing in erythroid precursors from patients with sickle cell anemia effectively corrects the mutation and restores normal hemoglobin production [47]. In addition, studies of activating HbF expression as a therapeutic treatment strategy have shown that it can reduce symptoms and improve patients' quality of life [48].

4.8. Marfan syndrome

Marfan syndrome is an inherited connective tissue disease resulting from mutations in the FBN1 gene. These mutations lead to abnormal production of fibrillin-1, which affects the structure and function of connective tissue throughout the body [49]. The use of CRISPR/Cas9 to treat Marfan syndrome involves identifying and targeting specific mutations in the FBN1 gene that are responsible for the disease. This strategy can involve repairing a point mutation, deleting an abnormal DNA fragment or inserting a correct copy of the gene [50]. Preliminary studies have shown that CRISPR/Cas9 can be effectively used to correct mutations in the FBN1 gene in cells from Marfan syndrome patients and in animal models. Gene editing resulted in improvements in connective tissue structure and function, suggesting the potential efficacy of this method in treating Marfan syndrome [51, 52].

4.9. Phenylketonuria

Phenylketonuria is an inherited metabolic disorder that, if left untreated, can lead to serious health problems. The disease is caused by mutations in the gene encoding the enzyme phenylalanine hydroxylase, which leads to increased levels of phenylalanine in the blood and tissues [53]. To date, the use of CRISPR/Cas9 to treat PKU has been studied in animal models and human cells in vitro, focusing on correcting mutations and restoring normal PAH enzyme activity [54]. Preliminary studies in animal models, such as PKU mice, have also shown promising results, with improved phenylalanine metabolism following gene editing [55].

5. Challenges and limitations of CRISPR/Cas9 technology

CRISPR/Cas9 technology, despite its huge potential, poses a number of both technical and ethical challenges for researchers and the public. The following are the main technical and ethical challenges for CRISPR/Cas9 applications in the future.

5.1. Technical challenges

One of the main technical challenges is to minimize the risk of untargeted DNA modifications, known as the off-target effect, in potential future patients by ensuring that gene editing is as precise as possible. Unwanted modifications introduced during gene therapy can lead to unforeseen consequences, such as gene dysfunction or tumor induction [56]. Another challenge that is difficult to bypass is the efficient and safe delivery of components of the genome modification system to target cells and tissues in the body. Delivery methods, such as viral vectors, have their limitations, including potential risks of immunogenicity and toxicity

[57]. Moreover, CRISPR/Cas9 efficacy can vary depending on the cell type, which poses a challenge in developing universal gene editing protocols for a variety of applications [58].

5.2. Ethical challenges

The use of CRISPR/Cas9 to edit the genes of human embryos raises serious ethical concerns, especially with regard to the possibility of making heritable changes that will be passed on to future generations. These issues concern both the safety and morality of manipulating the human genome at the embryonic level [59]. Moreover, the development of CRISPR/Cas9-based gene therapies may also exacerbate existing inequalities in access to advanced medical therapies. There is a risk that such therapies will only be available to a limited number of patients, mostly in developed countries [60].

5.3. Future directions and potential of CRISPR/Cas9 technology

CRISPR/Cas9 is still under intensive research to improve its precision and efficiency. Despite its potential, CRISPR/Cas9 comes with many challenges. In response to these limitations, researchers are working to develop new strategies which improve the precision and efficiency of this technology.

5.4. Development of Cas9 variants

One line of research is to develop variants of the Cas9 protein with greater specificity to reduce the risk of off-target editing. Researchers have developed modifications of the Cas9 protein, such as eSpCas9 and SpCas9-HF1, which exhibit reduced activity at non-target sites in the genome while maintaining high cutting efficiency at target sites [61, 62].

5.5. Innovations in delivery systems

Improving CRISPR/Cas9 delivery methods to target cells and tissues is another key area of research. Strategies based on nanoparticles, viruses and virus-free delivery vectors are being developed to increase transduction efficiency while reducing immune responses. Innovative approaches, such as the use of exosomes for CRISPR/Cas9 transport, are opening up new possibilities for effective and targeted gene therapy [63].

5.6. Development of new genome editing strategies

To increase the precision of gene editing, new strategies such as prime editing are also being developed. Prime editing allows the introduction of precise mutations, insertions and deletions without creating double-stranded DNA breaks, minimizing the risk of untargeted changes and potential mutations [64].

5.7. Monitoring and control of off-target effects

Developing methods to monitor and control off-target effects is one of the most important thing for assessing the safety of CRISPR/Cas9-based therapies. Techniques such as GUIDE-seq, CIRCLE-seq, and DISCOVER-seq allow the detection of off-target cutting sites across the genome to better assess the specificity and safety of gene editing [65, 66].

6. Conclusion

CRISPR/Cas9 has an unquestionable potential in addressing many inherited diseases. It has the significant impact on genetic research and therapy, offering a new options for curing diseases once considered incurable. Despite its promising prospects, there are cautions about ethical, safety, and technical challenges that need to be addressed. As research advances, CRISPR/Cas9 stands as a hope for causal treatment of genetic diseases, emphasizing the need for continued innovation, responsible application, and ethical considerations in gene editing's future developments.

Authors contribution

Conceptualization: Maciej Superson; Methodology: Sylwia Samojedny, Validation: Katarzyna Szymańska, Kamil Walczak; Formal analysis: Katarzyna Szmyt, Maciej Superson; Investigation: Julia Krasnoborska, Klaudia Wilk-Trytko, Maciej Superson; Resources: Sylwia Samojedny; Katarzyna Szmyt; Writing - Original Draft Preparation: Kamil Walczak, Julia Krasnoborska, Katarzyna Szmyt; Writing - Review & Editing: Maciej Superson, Klaudia Wilk-Trytko, Kamil Walczak,

All authors have read and agreed with the published version of the manuscript.

Funding

This research received no external funding.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

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