Journal of Molecular Biosciences and Engineering

Review Article

CRISPR Technologies in Biotechnology: Mechanisms, Applications, Advantages, Risks, and Future Prospects

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Received: 06.03.2026 Accepted: 22.05.2026 Published: 29.05.2026

https://doi.org/10.54414/GPOW1544

Abstract

CRISPR technology is an innovative technique in the field of genetic engineering. It facilitates the precise modification of DNA. The defence mechanism of bacteria against viruses served as the model for CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). Jennifer Doudna and Emmanuelle Charpentier were awarded the 2020 Nobel Prize in Chemistry for the development of the CRISPR-Cas9 system in 2012. The method cuts and modifies DNA using guide RNA and the Cas9 enzyme. The Cas9 enzyme slices the target site on DNA after the guide RNA (gRNA) finds it. Genetic engineers can introduce the desired modification (deletion, insertion, or substitution) as the cell attempts to repair this incision. This procedure is quick, inexpensive, and very accurate. CRISPR has several uses, including the treatment of genetic disorders like sickle cell anaemia, cancer immunotherapy, the prevention of viruses like HIV, the development of resilient crop types in agriculture, and the manufacture of new species in biotechnology. CRISPR is more effective and simpler than previous methods (ZFN, TALEN); however, it encounters challenges such as off-target effects (unintended incisions) and ethical issues (editing human embryos).

Keywords: CRISPR, Cas9 enzyme, gRNA

1. Introduction

Gene editing (also called genome editing) is a set of modern techniques used to modify the genetic material, i.e., DNA, of plants, animals, and microorganisms. Through these methods, it is possible to add, delete, or modify DNA sequences in certain, pre-selected parts of the genome [1]. Genome editing technologies developed in recent years have made it possible to quickly and economically make specific, predetermined changes to the genomes of various cells and organisms. These technologies are based on an approach called "targeted genome engineering" and enable highly precise modification of specific regions of the genome. The most widely used platforms in modern genome engineering include CRISPR/Cas9, TALEN (Transcription Activator-Like Effector Nucleases), ZFN (Zinc Finger Nucleases), and meganucleases [2].

This process is not carried out through natural reproduction, but artificially through human intervention. The result is genetically modified organisms (GMOs) as a form of genetic engineering. The most widely used genome editing method currently is CRISPR-Cas9 technology [1].

"CRISPR-Cas9 is a tool that has the potential to revolutionize biomedicine by permanently altering the human genome to treat genetic diseases." – Emmanuelle Charpentier [3].

The 2020 Nobel Prize in Chemistry was awarded to E. Charpentier and J. Doudna for their discovery of CRISPR technology. Genetic modifications that once seemed possible only in science fiction movies have now become a reality thanks to CRISPR. This technology offers hope for the treatment of genetic diseases caused by small changes in the genome and opens up great opportunities to improve people's quality of life [3]. Currently, more than 6,000 monogenetic diseases are known worldwide, and CRISPR/Cas-based therapies are considered a promising approach in the treatment of these diseases. Clinical studies conducted in recent

years have shown that CRISPR technology can show effective results in the treatment of sickle cell anemia, β-thalassemia, and some hereditary retinal diseases [2].

The discovery of CRISPR technology arose from an important scientific need in genetic research. Programmable nuclease systems, such as ZFN and TALEN, used before CRISPR had high technical complexity, an expensive manufacturing process, and limited flexibility. Therefore, scientists needed to develop new technologies that could modify specific parts of the genome more precisely, quickly, and effectively. This need was met with the discovery of CRISPR in the immune system of bacteria. Bacteria use this mechanism to cut and neutralize the DNA of viruses that infect them [3]. The CRISPR/Cas system functions as an adaptive immune defense mechanism in bacteria. Viral DNA fragments are stored in the bacterial genome as "spacers" and allow for recognition of those sequences during subsequent infections. CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) synthesized from the CRISPR locus direct the Cas9 enzyme to specific target DNA, ensuring the cleavage of foreign genetic material [2].

Once CRISPR's DNA-cutting mechanism was understood, this system became a powerful tool for gene editing. The CRISPR-Cas system recognizes a precise target in the genome through guide RNA (gRNA), and the Cas enzyme cuts the DNA at that point. The molecular basis of genome editing is the generation of double-stranded DNA breaks and the repair of these damages through intracellular DNA repair mechanisms. Two repair systems are mainly involved in this process: Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR). NHEJ is a rapid but error-prone mechanism, which results in the loss of gene function. On the other hand, HDR enables more precise gene correction by utilizing a donor DNA template. CRISPR is a more precise, cost-effective, and straightforward system than previous gene editing technologies due to these features [3].

CRISPR technology is significant because of its many uses. This approach creates new opportunities for researching the genetic traits of different organisms and treating genetic illnesses. Therefore, CRISPR is regarded as a significant turning point in the advancement of today's biology and medicine [2], [3].

The possibilities of genome engineering have been considerably extended in recent years by novel techniques based on CRISPR technology, such as base editing, prime editing, and epigenome editing. These technologies are regarded as one of the primary instruments for personalised medicine in the future since they enable more precise genetic alterations without causing double-strand breaks [2].

2. Historical Development of CRISPR Technologies

CRISPR clustered regularly interspaced short palindromic repeats were initially identified in the DNA sequences of the Escherichia coli bacterium and subsequently characterised by Ishino et al. from Osaka University (Japan) in 1987. The discoverers of these challenging-to-study DNA fragments were unable to comprehend their origin or significance within the bacterial cell, despite the fact that the sequencing process required several months. Despite the fact that the biological function of the CRISPR system had not yet been clarified in the early work in this field, scientists had already proposed a method to utilise the information encoded in CRISPR loci in medical research. This method involved genotyping a variety of bacterial strains, initially Mycobacterium tuberculosis and eventually Streptococcus pyogenes. As it turned out, CRISPR loci had a high degree of polymorphism in different strains of the same species of pathogenic bacteria, which enabled the identification of bacterial strains in clinical conditions [4].

In 1995, Francisco Mojica of the University of Alicante (Spain) made a breakthrough in the understanding of the biological function of CRISPR loci when he discovered analogous structures in the archaeal genome of Haloferax mediterranei. Their existence in two evolutionarily distant domains of life indicated the significant functional importance of these elements and provided a motivation for additional research. Mojica observed the similarity between the elements he described in archaea and the DNA repeats that had been previously identified in bacterial genomes. He was one of the first scientists to propose that these unusual loci contain fragments of foreign DNA and are, in fact, a component of the immune system of bacteria and archaea. In the same year as Mojica, two other laboratories simultaneously reached similar outcomes, thereby announcing the beginning of an era of active investigation into this remarkable natural phenomenon. Viral DNA fragments ("spacers"), which are 17-84 bases in length, are grouped into clusters in intergenic regions and separated by short palindromic repeats, in accordance with the theory of the prokaryotic immune system. These fragments represent a library of potentially harmful genetic information. At first, it was believed that the operation of

such a system would be facilitated by the mechanism of RNA interference. Nevertheless, Marraffini and Sontheimer's publication experimentally demonstrated for the first time that the immune system of prokaryotes actually targets foreign DNA, not mRNA. Consequently, the use of such a system in the laboratory could serve as a potent tool for genomic editing. Interestingly, later studies demonstrated that some of the described CRISPR systems do work with RNA molecules directly and, therefore, can be used to deactivate specific transcripts inside the cell in a selective way [4].

Rodolphe Barrangou and Philippe Horvath, two French food scientists, worked with yoghurt cultures of the bacterium Streptococcus thermophilus for the Danish company Danisco in 2007. Their research provided the first experimental information regarding the mechanism of action of the CRISPR system. Scientists have been able to trace the historical development of the bacterial acquisition of spacers at the CRISPR locus in response to viral attacks by bacteriophages, as a result of the company's extensive collection of bacterial strains collected since the 1980s. The addition of new spacers in this work caused acquired immunity to the corresponding new types of bacteriophages in S. thermophilus: an observation which subsequently led to the authors obtaining one of the first patents in this area and the start of bacterial cultures' "vaccination" with the use of CRISPR-based technology by Danisco in 2005 [4].

At present, CRISPR repeats have been detected in the majority of archaeal genomes and nearly half of the bacterial genomes that have been studied. However, they have not been observed in eukaryotic or viral DNA sequences. One of the earliest publications on the subject suggested the presence of CRISPR repeats in mitochondria, and the same article also, for the first time, described CRISPR in cyanobacteria. The authors used a set of previously published data on the sequencing of mitochondrial plasmids from Vicia faba L. beans, and their conclusions were further cited by Mojica et al., but these observations were not confirmed in later studies [4].

The search for early articles on the topic is currently complicated by the fact that a variety of abbreviations were used for CRISPR by individual scientific groups during early discoveries. The name CRISPR, as used today, first appeared in the work of Jansen et al. in 2002 and was proposed by Mojica in correspondence between two collaborating scientific groups. The same publication was the first to identify the existence of genes associated with CRISPR repeats (referred to by the authors as Cas1-4, CRISPR-associated genes). These genes were found to be located near CRISPR loci of various prokaryotes, and two of them contained motifs characteristic of helicase and nuclease. This supported the authors' hypothesis about the non-random association of the Cas genes with the CRISPR locus and their involvement in DNA metabolism. Also in 2002, a team of scientists led by Eugene Koonin from the NCBI Institute (Bethesda, USA) identified the same gene region, but at that time, the association of these genes with CRISPR sequences could not be determined. From the moment the genes associated with the CRISPR system were first discovered to the present day, an extraordinary abundance and diversity of genes have been found in prokaryotic cells, including helicases, nucleases, polymerases, and others. Proteins associated with this system can be assigned either to adaptive modules (primarily Cas1 and Cas2, which play a role in immunity acquisition) or effector modules (involved in the recognition and degradation of mobile genetic elements and their direct elimination); additionally, some supplementary and regulatory proteins associated with the system have also been identified. Currently, a classification method is accepted in which all known CRISPR-Cas systems are divided into 2 classes and 6 types, which are further divided into numerous subtypes. At the time of writing this review, Makarova et al. had identified more than 30 subtypes. The main difference between the classes is that the effector module of Class 1 systems is represented by a complex of several proteins, while in Class 2, it is a single multidomain protein (Cas9, Cas12, or Cas13) [4].

Of all the known Cas proteins, the most studied are those belonging to the directional cutting system of foreign DNA (and, as it was later discovered, in some cases, RNA); these are called "genetic scissors" and include the nuclease Cas9. This protein was first identified in a paper by Bolotin et al. in the context of its association with CRISPR repeats and was initially named Cas5 (other alternative names are Csn1 and Csx12). Furthermore, the authors identified the presence of the HNH motif (His–Asn–His), which is also found in other nucleases. Another significant observation made by Bolotin et al. was the discovery of a specific pattern in the nucleotide sequences on one side of the identified spacers of the CRISPR sequences, although the role of this phenomenon was only revealed in later studies. Currently, short motifs that are not present in the original spacers of the CRISPR locus but

are adjacent to the protospacers are called PAMs (protospacer-adjacent motifs). Protospacers are DNA fragments that are attacked by the immune system of prokaryotes and are identical to the corresponding spacers in the CRISPR locus except for the PAM motif. These motifs are important at the stage of recognition of potentially dangerous genetic information; their presence at the end of the sequence signals that the DNA fragment is foreign and needs to be destroyed, while the DNA sequences stored in the CRISPR locus as spacers and not containing PAM motifs are not attacked by the prokaryotic immune system [4].

It turned out that a critical element in the CRISPR-Cas9 system is a short RNA molecule, a processed product of transcription from the CRISPR locus, which directs proteins of the prokaryotic immune system to foreign molecules containing genetic information. A group of researchers led by John van der Oost from Wageningen University in the Netherlands identified the existence of these types of RNA molecules and named them crRNA (CRISPR-associated RNA). It was also noted that the initial result of transcription from the CRISPR locus is a pre-crRNA precursor molecule consisting of various interstitial segments and repeats, which are subsequently cleaved into separate fragments. In the study led by Virginijus Siksnys (University of Vilnius, Lithuania), it was shown that the length of the 20-base pair true "guide" crRNA sequence, complementary to the target DNA, is necessary and sufficient for the nuclease activity of the CRISPR-Cas complex, even if the interstitial region at the CRISPR locus is represented by a longer nucleotide sequence. This publication was one of two in vitro studies, carried out in parallel and independently in competing laboratories, that described, for the first time, how the Cas9 enzyme uses crRNA to attack foreign DNA [4].

The final and significant component of the CRISPR-Cas9 system, which makes it impossible to create a functional CRISPR-Cas9 system in a laboratory setting, is another short RNA molecule discovered by Emmanuelle Charpentier's group in 2011 in connection with its role in crRNA processing. This molecule, essential for nuclease activity, was named tracrRNA (trans-activating CRISPR RNA). Subsequent studies, ultimately confirmed by a Nobel Prize, demonstrated tracrRNA's role in the cleavage mechanism of target DNA. It was also proposed at the time that two RNA molecules, crRNA and tracrRNA, could be combined into one chimeric molecule (sgRNA-single guide RNA), which greatly facilitated the practical use of the CRISPR-Cas9 system in subsequent applications [4].

3. Molecular Basis of the CRISPR/CAS9 Mechanism

CRISPR/Cas systems are divided into two main classes based on the structure and functional properties of the Cas proteins: Class I (types I, III, and IV) and Class II (types II, V, and VI). While Class I systems are composed of complexes consisting of several Cas proteins, in Class II systems, genome editing is carried out by a single Cas protein. The CRISPR/Cas9 system, which belongs to Class II due to its simpler structure, is the most widely studied and most widely used system in genetic engineering.

The two main components of the CRISPR/Cas9 system are guide RNA (gRNA) and the Cas9 endonuclease protein. The Cas9 protein, which was first used in genome editing, was derived from the bacterium *Streptococcus pyogenes* and is called SpCas9. This protein is a 1368 amino acid, multidomain DNA endonuclease that is capable of creating double-strand breaks in target DNA. It is precisely because of this property that Cas9 is called "genetic scissors" [5].

The Cas9 protein is structurally composed of two main parts: the recognition (REC) lobe and the nuclease (NUC) lobe. The REC lobe encompasses the REC1 and REC2 domains and is primarily responsible for binding guide RNA. The NUC lobe consists of domains that interact with RuvC, HNH, and PAM. The RuvC and HNH domains create double-strand breaks by cutting each strand of DNA separately. The PAM (Protospacer Adjacent Motif) interaction domain enables Cas9 to recognize and bind to target DNA. Without the PAM sequence, Cas9 cannot bind to DNA, which ensures the high specificity of the system [5].

Guide RNA consists of two parts - CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). crRNA is 18–20 nucleotides long and recognizes and pairs with the target DNA sequence. tracrRNA consists of RNA loops that act as a structural support for the Cas9 protein. In genome editing technologies, these two RNAs are artificially combined to create a single guide RNA (sgRNA), which allows targeting of almost any gene [6].

CRISPR/Cas9 technology relies on the interaction of guide RNA (sgRNA) and the Cas9 endonuclease protein to make precisely targeted changes in the genome. Initially, an active complex is formed when the sgRNA attaches to the Cas9 protein. This complex searches for PAM (Protospacer Adjacent Motif) sequences as it travels throughout the cell's DNA. Once the PAM is found, the sgRNA pairs with the target DNA sequence, and the Cas9 protein is activated at that point [6].

The HNH and RuvC domains of the activated Cas9 protein cut both strands of DNA, creating a double-strand break. After this break, the cell activates its natural DNA repair mechanisms. As a result, the function of the gene may be disrupted, altered, or a new genetic sequence may be added. Thus, CRISPR/Cas9 technology allows for highly precise genetic modifications to be made in any area of the genome [6].

The CRISPR/Cas9 system was first discovered in bacteria as a defense mechanism against viral DNA. However, this system later became a revolutionary tool in genetic engineering. While the classic CRISPR/Cas9 technology enabled genome editing with high precision, one of its main drawbacks was the inability to regulate genes in a reversible manner and without damaging DNA [6].

To overcome this problem, MIT professor Jonathan Weissman and his colleagues created new modifications of the Cas9 protein - the CRISPRoff and CRISPRon systems. These systems are not based on classical DNA cleavage, but rather regulate gene activity through epigenetic mechanisms. The CRISPRoff system turns off the activity of genes by adding epigenetic marks to certain gene regions with the help of guide RNA, while CRISPRon removes these epigenetic changes and reactivates the genes [7].

Studies have shown that these changes remain stable during cell division and can even be inherited. Scientists have tested this system on stem cells, artificially turning off genes and transforming those cells into neurons and other differentiated cell types. The results showed that the silenced genes were not activated even after cell differentiation [7].

Thus, the molecular basis of CRISPR/Cas9 technology is not limited to DNA cutting alone, but also allows for the control of gene expression at the epigenetic level. This technology holds great promise in the study of genome functions and, especially, in the treatment of genetic and neurodegenerative diseases such as Alzheimer's disease [7].

4. Application Areas of CRISPR Technologies in Biotechnology

CRISPR/Cas9 genome editing technology has revolutionized the fields of biology and medicine in a short time since its discovery. This technology is currently widely applied in numerous fields such as gene therapy, disease treatment, medical diagnostics, gene activation, and gene silencing. In the future, CRISPR technologies are expected to be further improved and become a key tool in the treatment of many hereditary and infectious diseases.

4.1. CRISPR/Cas9 in Gene Therapy

The CRISPR/Cas9 system is considered one of the most effective and widely used technologies for genome editing in recent years. This system is based on the defense mechanism of bacteria and archaea against foreign genetic material and was later adapted for use in genetic engineering [8], [9].

The main components of CRISPR/Cas9 technology are single-guide RNA (sgRNA) and the Cas9 endonuclease enzyme. The sgRNA recognizes a specific DNA sequence and directs the Cas9 enzyme to that site, resulting in a double-strand break in the target DNA [8].

After this break, the cell's natural DNA repair mechanisms are activated. Genome editing occurs mainly through two mechanisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR). During the NHEJ mechanism, DNA is repaired imprecisely, and insertions or deletions can occur, while the HDR mechanism allows for more precise genetic changes when a suitable DNA template is available [9].

CRISPR/Cas9 technology is used in gene therapy in two main ways. In the first approach, CRISPR components are introduced directly into the body and genome editing is performed in vivo. In the other approach, cells taken from the patient are edited in the laboratory and then returned to the body, which is called ex vivo gene therapy [9].

CRISPR/Cas9 technology has shown promising results in the treatment of various genetic diseases. For example, in some experimental studies, this system has been used to correct mutations caused by diseases such as hereditary tyrosinemia, and a reduction in disease symptoms has been observed [9].

However, there are also some challenges in the clinical application of CRISPR technology. One of the main challenges is the efficient delivery of the editing system to the target cells and the occurrence of off-target effects related to the generation of off-target changes [8].

4.2. CRISPR in Cancer and Infectious Diseases

CRISPR/Cas9 technology has opened up important prospects for the research and treatment of cancer and infectious diseases. Since cancer is a disease caused mainly by genetic changes, genome editing technologies are widely used to study the molecular mechanisms of tumors [8].

The CRISPR system allows us to study the function of genes involved in the development of tumor cells and change their activity. In addition, CRISPR technology is used to create cancer models, which allows us to study the mechanisms of tumor formation and test new drugs [8].

CRISPR/Cas9 technology is also being used in the study of infectious diseases. For example, in some studies, the CRISPR system has been used to target the genome of the hepatitis B virus to reduce viral replication [10]. The cccDNA structure that causes the hepatitis B virus to remain in a stable form in cells allows the virus to persist for a long time. The CRISPR system can help reduce infection by disrupting this viral DNA [9].

However, for the clinical application of CRISPR technology, it is important to address issues such as delivering the gene editing system to target tissues and reducing off-target effects [8].

4.3. CRISPR in Medical Diagnostics

During the COVID-19 pandemic, CRISPR technology has been used not only as a potential treatment tool but also as a rapid and accurate diagnostic method. One of the most important achievements in this field has been the SHERLOCK™ CRISPR SARS-CoV-2 test kit. The test in question has received Emergency Use Authorization from US federal agencies for use in laboratory settings. The SHERLOCK system allows for the detection of the virus's genetic material with high sensitivity. In addition, the subsequently developed STOPCovid diagnostic test is also based on a CRISPR-based approach and is considered an effective method for rapid detection of the virus [5].

Another CRISPR-based COVID-19 diagnostic method is the DETECTR system developed by Mammoth Biosciences. Similar to the SHERLOCK and STOPCovid tests, the DETECTR method uses the genetic material recognition (search) feature of Cas proteins. This system mainly uses the naturally occurring Cas12 and Cas13 nucleases and identifies the viral RNA with high accuracy [10].

The diagnostic applications of CRISPR technology are not limited to COVID-19. Similar diagnostic systems based on the search function of Cas proteins have also been developed for the detection of infectious and genetic diseases. In early 2021, research led by Dr. Kiana Aran of Cardea Bio combined three Nobel Prize-winning technologies - graphene, transistors, and CRISPR to create a very small chip. This chip has the ability to detect pathogenic single-nucleotide polymorphisms (SNPs) [5].

It should be noted that approximately 50% of disease-causing mutations in humans are associated with SNPs. In this regard, the development of such CRISPR-based diagnostic technologies is considered an important scientific achievement in the field of modern medical diagnostics [5].

5. Advantages of CRISPR Technology

CRISPR-Cas9 is one of many gene editing technologies available to researchers. While it can be an extremely industrious approach for inducing changes to genetic material, it, like any other gene editing application, has several advantages and disadvantages that should be taken into consideration when designing an experiment [11].

5.1. Fast and Flexible Design

Specifically, CRISPR-Cas9 gene editing requires two components: the Cas nuclease and a guide RNA. In some cases, researchers prefer to use the two-part crRNA-tracrRNA system, while in others they use sgRNA. After the guide RNA and endonuclease bind to each other, they form a ribonucleoprotein (RNP) complex. It's within this complex that the guide RNA can bind to the target sequence, and editing can occur [11].

5.2. Multiplexed Gene Editing

CRISPR-Cas9 can edit multiple genes simultaneously when the sgRNAs used are designed to target different genetic regions. This makes CRISPR-Cas9 gene editing an attractive and efficient approach for manipulating multiple locations in the genome [11].

5.3. Cost-Effectiveness

For the reasons mentioned above, CRISPR-Cas9 can be extremely cost-effective compared to other gene editing approaches requiring protein engineering, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The simplicity of CRISPR-Cas9, relying largely on 2 molecules to induce edits, as well as the flexibility available in designing sgRNAs to bind to any/numerous target(s), means that researchers can start editing quickly and make adjustments easily [11].

6. Disadvantages of CRISPR Technology

6.1. Delivery Limitations

Successful delivery of CRISPR-Cas9 to cells is necessary to perform the desired gene editing. As with many other gene editing applications, delivering the Cas9/gRNA complex to a sufficient number of cells can be challenging because all components must be delivered to the cells at the correct concentrations and at the correct point in the cell cycle. CRISPR reagent delivery methods include electroporation, lipofection, microinjection, nanoparticles, and viral plasmids [11].

6.2. Efficiency Limitations

Another necessary limitation to consider when using the CRISPR-Cas9 gene editing method is that gene editing activity may not occur once the Cas9/gRNA complex is taken up by cells. This is especially true when the goal is to insert or insert material into a gene, a process based on homologous directed repair (HDR). However, researchers have made significant progress in improving HDR efficiency rates with CRISPR. To read more about improving CRISPR-Cas9 gene editing efficiency, check out the DECODED article, improving efficiency of homology-directed repair (HDR) [11].

6.3. Off-Target Effects

Off-target effects occur when undesirable changes occur as a result of the Cas9 nuclease editing a non-targeted part of the genome. Off-target effects are a major concern for CRISPR-Cas9 experiments and can be difficult to predict. Efforts have been made to improve in silico off-target prediction tools as well as to reduce off-target effects by improving Cas9 nucleases [11].

7. Genome Editing for Human Germline

The editing of human germline cells with CRISPR-Cas9 is prohibited for various safety reasons. However, the application of CRISPR-Cas9 to somatic cells with the aim of transferring desired characteristics to our lives is increasing. Many phenotypic traits have a genetic component independent of the environment. By exploiting this characteristic, CRISPR-Cas9 could be used to enhance athletic performance, prevent violent behavior, or reduce addiction. While gene therapy is generally used to the benefit of patients, in the future, the criminal justice system may require recidivism or dangerous criminals to correct their violence-related genes using genome editing technologies. One of the biggest dilemmas here is obtaining informed consent for a minor if the intervention is performed during zygote development. This would give parents or guardians the right to make decisions on behalf of minors for non-health-related reasons. Moreover, from a social perspective, some genetically enhanced populations or individuals may have certain advantages over others in

terms of various traits such as mental and physical capacity. Therefore, the use of CRISPR-Cas9 in genome enhancement should be seriously discussed both socially and morally [12]. The potential use of CRISPR-Cas9 for genome editing in human germ cells has raised serious ethical debates. Until 2015, all therapeutic applications in humans were performed on somatic cells using genome editing technologies. However, in 2015, the editing of the human germline performed by Chinese scientist Huang and his team with CRISPR-Cas9 raised new social, moral, and bioethical issues [12].

8. Future Perspectives

Over the past decade, significant progress has been made in the use of CRISPR, both as a tool and as a treatment method. As discussed, CRISPR-Cas has become very effective as a screening tool, but it would benefit from better genome coverage, more refined gRNA designs, and analyses that can distinguish between multigene transcripts. Clinically, as we have previously noted, CRISPR-based editing has proven effective in developing cell therapies for both ex vivo and in vivo applications. Despite the clear applications of CRISPR-based editing, several challenges remain (see Key Questions). These current challenges (e.g., immune responses, application, off-target effects, DNA damage, etc.) stem from the fact that nature optimized CRISPR nucleases to function as a bacterial defense system, not for precise genome editing; this means that much more work needs to be done to make these systems suitable for precise editing in humans. Furthermore, significant genomic heterogeneities exist within the human population, complicating treatment development. To optimize targeted activity and minimize off-target effects, gRNAs specifically designed for the patient's genetic makeup may be needed. In addition, to accelerate the development of CRISPR-based therapies, various methods for precise control of Cas9-based systems are being developed, and new editors (e.g., base and prime editors) are being created. Also, we are seeing an increase in translational dCas9-based technologies that enable dose and temporal control of a therapeutic target without double helix breaks, thus overcoming the current challenges associated with Cas9. Especially considering how far CRISPR technologies have advanced since their discovery only a decade ago, we anticipate that further engineering strategies over the next decade will better minimize and optimize the system, allowing for the development of transformational in vivo applications [13].

9. Conclusion

Considered one of the most transformative discoveries in modern molecular biology and biotechnology, the CRISPR-Cas9 methodology has created a fundamental scientific turning point in the field of genome editing. Analysis of scientific sources and conducted research clearly demonstrates that this system is based on the natural adaptive immune defense system that bacteria have developed against viral attacks during their evolution, and allows for highly precise, targeted modification of the genetic code. This innovative technology promises broad future prospects in both fundamental theoretical experiments and practical applications, as it allows for highly efficient and point-wise manipulations of the genome at specific coordinates.

The results of scientific research show that the CRISPR-Cas9 mechanism has a much simpler structure, superior accuracy rating, and a more universal application area compared to the classical genome editing methods used before it, including homologous recombination, zinc finger nucleases, and TALEN systems. In particular, the ability to perform parallel changes on several different genetic regions in the same time frame, i.e., the multiplexing function, is the most fundamental advantage that distinguishes this management system from other methodological approaches. This process, which takes place at the molecular level, is carried out by directing the nuclease enzyme called Cas9 to a specific DNA site designated as a target via a special single-stranded RNA molecule that acts as a guide. At that specific point, a double-strand break is created by the Cas9 enzyme, and immediately thereafter, the cell's own internal DNA repair mechanisms, non-homologous end joining and homology-directed repair pathways, are activated. Using these regeneration pathways, unwanted genes can be completely deactivated, existing mutations can be corrected, or new genetic sequences can be integrated into the genome, making the CRISPR-Cas9 system the most efficient genome editing tool in biology.

Simultaneously, this methodology has significantly accelerated the advancement of gene therapy in the field of health and medicine, thereby enabling the creation of innovative treatment strategies for the radical treatment of hereditary genetic diseases and the complete removal of certain viral infections from the body, particularly those that cause chronic diseases, such as Hepatitis B.

In spite of these accomplishments, the practical application of CRISPR-Cas9 technology is plagued by significant ethical dilemmas and technical challenges. The risk of off-target effects is one of the primary issues, as the system occasionally induces unknown mutations and cuts in other unanticipated, non-target DNA regions. Scientific research is being conducted in an effort to enhance the safety and specificity of the technology, as these types of random alterations have the potential to cause harm to the body.

Numerous next-generation methodologies are currently being developed to enhance and optimise CRISPR technology from a scientific perspective. Particularly, the incorporation of bioinformatics programmes and artificial intelligence tools into CRISPR design systems contributes significantly to the more precise identification of target genes and the reduction of off-target effects. At the same time, new generation genetic engineering modifications, such as the inactive Cas9 protein, base editing based on direct nucleotide replacement, and prime editing, which offers more complex editing capabilities, guarantee safer and more flawless genetic manipulations in the future.

In conclusion, CRISPR-Cas9 technology, as one of the most remarkable achievements of modern biological science, has had a strong global impact on both the development of fundamental laboratory research and the expansion of applied fields such as agriculture, medicine, and biotechnology. Further improvement of this technology in the future and the formation of completely safe application mechanisms will play a decisive role in the complete treatment of hereditary genetic pathologies, ensuring food security on a global scale, and the creation of completely different innovative directions of the biotechnology industry.

Author Contributions

The author conceived the study, conducted the literature search, analyzed and synthesized the collected information, and wrote, reviewed, and approved the final manuscript.

Conflict of Interest

The author declares no conflicts of interest.

Funding

This research received no external funding.

Acknowledgment

The author gratefully acknowledges Western Caspian University for providing an academic environment and institutional support that facilitated the completion of this review article.

Abbreviations

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), Guide RNA (gRNA), Human Immunodeficiency Virus (HIV), Transcription Activator-Like Effector Nucleases (TALEN), Zinc Finger Nucleases (ZFN), Genetically Modified Organisms (GMOs), Deoxyribonucleic Acid (DNA), CRISPR RNA (crRNA), Trans-Activating crRNA (tracrRNA), Non-Homologous End Joining (NHEJ), Homology-Directed Repair (HDR), Histidine–Asparagine–Histidine (HNH), Protospacer-Adjacent Motifs (PAMs), single guide RNA (sgRNA), Recognition (REC), Nuclease (NUC), Single-Nucleotide Polymorphisms (SNPs).

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