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Genetic Surgery: The Revolutionary CRISPR-Cas9 Scissors

Elahe Alivaisi [1], **Ali Noori-Zadeh** [2]✉

[1] Ilam Univrsity, Department of Biology, Ilam, Iran
[2] Department of Clinical Biochemistry, Faculty of Medicine, Ilam University Medical Sciences, Ilam, Iran

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✉ ***Correspondence to:***
Ali Noori-Zadeh
Department of Clinical Biochemistry, Faculty of Medicine, Ilam University Medical Sciences, Ilam, Iran

Email:
alincbc@gmail.com

A B S T R A C T

Introduction: The CRISPR-Cas9 system, a revolutionary genome-editing tool which stems from a bacterial adaptive immune system, shows a paradigm shift in designing new therapeutics in medicine.

Aim: This review describes the CRISPR-Cas9 technology from its initial discovery in prokaryotes as a bacterial adaptive immune system to its development into a precise molecular scalpel capable of human genome editing. It explores CRISPR-Cas9 fundamental components and mechanisms, highlighting how a synergy between a guide RNA and Cas9 nuclease allows targeted DNA double-strand breaks, and harness DNA repair enzymes of the target cell for genetic code modifications.

Discussion: There is a lot of promise for using CRISPR-Cas9 technology to treat diseases. In clinical settings, it has led to breakthroughs in the treatment of monogenic disorders like sickle cell anaemia and beta-thalassemia, and it is also making good progress in oncology and other areas. The technology still has a lot of problems to solve, even with these successes. For example, it can cause mutations that aren't intended, it has technical limits, and there are moral concerns about how it could be misused, such as for heritable human enhancement.

Conclusion: CRISPR-Cas9 technology offers remarkable therapeutic potential with clinical applications already yielding breakthroughs in curing monogenic disorders such as sickle cell anemia and beta-thalassemia, and promising advances in oncology and beyond. However, it also presents significant ethical and practical risks, including the threat of heritable human enhancement (designer babies), and persistent safety concerns such as off-target mutations.

Keywords: CRISPR-Cas9, crRNA, tracrRNA, Genome Editing, Bioethics

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Introduction

The genome sequence visualisation software displayed to the doctor that the beta-globin gene locus of the patient, the HBB gene, clearly contains a single A→T mutation at codon 6 that would distort haemoglobin protein into the sickled form in erythrocytes (1). In the isolation bay, a six-year-old boy, Hasan, lay still under phototherapy lights, his slight frame connected to multiple intravenous lines delivering his regular blood transfusion. The main signs of sickle cell disease were all present: jaundiced sclera, swollen joints from vaso-occlusive crises, and the fatigue lines on his young face (2). His latest blood work showed haemoglobin levels barely above 6 g/dL (2). The doctor isolated his hematopoietic stem cells to be sent to the laboratory. The most common therapeutic strategy uses CRISPR-Cas9 to disrupt a repressor of the HbF genes (HBG1 and HBG2), specifically an enhancer region of the BCL11A gene. Knocking out this repressor allows the patient's cells to naturally produce high levels of HbF again. The CRISPR-Cas9 system was simple in design to modify Hasan's cells:

1. A 20-nt sgRNA perfectly complementary to the mutant sequence

2. The Cas9 endonuclease from Streptococcus pyogenes

3. A donor template with the corrected codon (3)

The ethics committee's concerns echoed in her mind – off-target effects, mosaicism, and germline transmission (4). But Hasan's parents had signed the consent form after reviewing all potential risks. "Let's initiate CRISPR-Cas9 delivery to hematopoietic stem cells of Hasan to boost foetal haemoglobin (HbF)," the doctor's colleague said quietly (5). The electroporator hummed as electrical pulses created transient pores in the hematopoietic stem cells' membranes, allowing the gene-editing machinery to enter (6). Somewhere in those cells, the Cas9 would make its precise double-strand break, and hopefully, homology-directed repair would incorporate the correction (7). The monitor displayed "Procedure Complete". Now came the hardest part – waiting to see if the edited cells would successfully engraft and begin producing HbF (5, 8). The real work had only just begun with the CRISPR-Cas9 system. Three months later, he underwent the transplantation of his hematopoietic stem cells modified by a new technology known as CRISPR-Cas9 to be cured of sickle cell anaemia.

Aim of the Review

The aim of this review is to provide an integrated overview of the CRISPR-Cas9 system, tracing its evolution from a bacterial adaptive immune mechanism to a programmable genome-editing tool in human cells. This review further aims to explain the core components and molecular mechanisms of CRISPR-Cas9 and to highlight its therapeutic applications, limitations, and ethical considerations.

CRISPR-Cas9 System Discovery and Development

The story of the CRISPR-Cas9 system is a remarkable example of how fundamental scientific investigation can lead to technological breakthroughs in medicine (9). As shown in Figure 1, this journey began in 1987. Yoshizumi Ishino, a Japanese researcher, and his team accidentally discovered unusual repetitive DNA sequences in Escherichia coli (10). Nonetheless, they meticulously documented nucleotide repeats interspersed with unique spacer sequences in Escherichia coli; the biological function of these elements remained unknown at that time (11). The next breakthrough was made by Francisco Mojica, who encountered similar repeating sequences in Haloferax mediterranei (12). Mojica showed these clustered repeats in diverse bacteria and proposed the term CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) for these elements. He assumed that CRISPR acts as a microbial defence system (13, 14). His pivotal discovery of viral DNA fragments within CRISPR spacers provided the first evidence that bacteria use these elements as an adaptive immune mechanism

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against bacteriophages (10). Parallel discoveries also accelerated CRISPR's molecular machinery mechanism. Bolotin and his colleagues identified an atypical CRISPR-Cas system in Streptococcus thermophilus. In these bacteria, they discovered a novel nuclease (later named Cas9) and recognised a conserved viral sequence known as protospacer adjacent motif or PAM (15, 16). The immune function was experimentally shown by Horvath and his co-workers in 2007. They demonstrated that bacteria can incorporate bacteriophage DNA into CRISPR arrays to resist future infections. In this process, the Cas9 enzyme plays a crucial role in the microbial defence against viruses (17, 18). The mechanistic details of CRISPR-Cas9 were discovered through key contributions from multiple research teams (19). In 2008, John van der Oost's group showed that CRISPR spacers are transcribed into small RNAs (crRNAs) that guide Cas proteins to target DNA and crRNAs cleave matching invaded viral DNA (20). Meanwhile, Virginijus Šikšnys and his colleagues independently characterized the RNA-guided DNA cleavage activity of Cas9. The final critical piece came in 2011 when Emmanuelle Charpentier discovered a small RNA known as tracrRNA, which is essential for Cas9 function (21). Her subsequent collaboration with Jennifer Doudna led to the engineering of a single-guide RNA system which simplifies CRISPR into a programmable gene-editing tool (22). For their groundbreaking work in adapting CRISPR-Cas9 into a precise genome-editing technology, Emmanuelle Charpentier and Jennifer Doudna were awarded the 2020 Nobel Prise in Chemistry (23). The Nobel Committee recognised their discovery of "one of gene technology's sharpest tools" that has revolutionised molecular biology and paved the way for treating genetic diseases (24). By 2012-2013, two independent teams – led by Feng Zhang and George Church – successfully adapted CRISPR-Cas9 for genome editing in human cells (21). Their work, published simultaneously in early 2013, demonstrated that CRISPR can be used to precisely modify genes in eukaryotic cells, which has countless applications in biomedicine (21).

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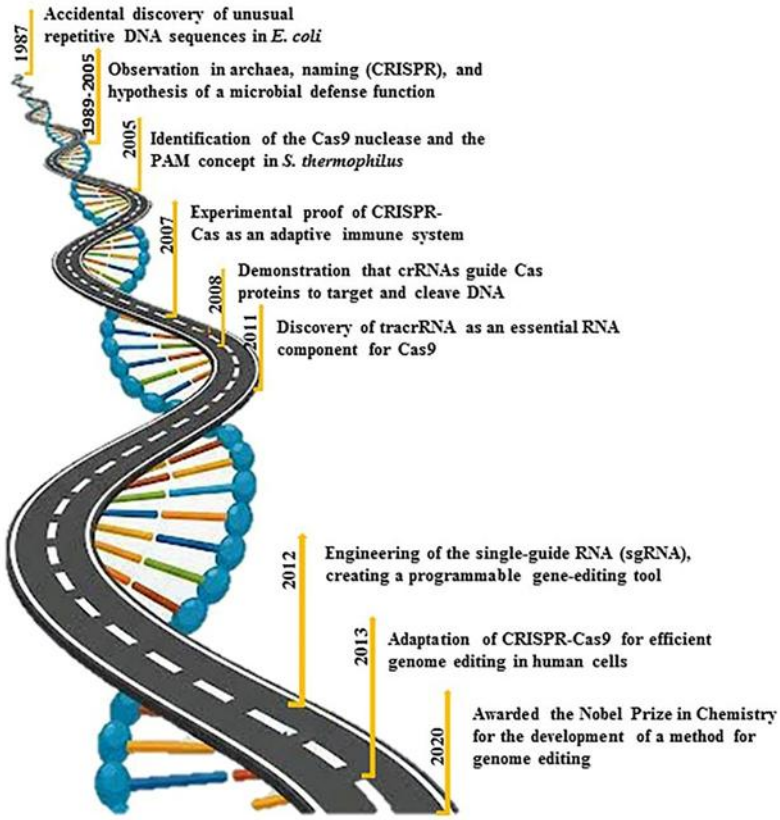

Figure 1. Time line for CRISPR-Cas9 system evolution. The CRISPR-Cas9 system evolved since the discovery of mysterious repetitive DNA sequences in bacteria (1987) into a revolutionary programmable gene-editing tool. Key milestones include the identification of adaptive immune function in prokaryotes (2005), the characterization of the Cas9 enzyme and guide RNAs, and its engineering into a two-component system (2012). Interestingly, this technology was rapidly adapted for efficient genome modifications in human cells (2013), enabling precise gene editing and earning the Nobel Prize in Chemistry in 2020.

Zhang's team established efficient genome editing in mouse and human cells, while Church's group demonstrated simultaneous multiplexed editing of several genes (25). What started as a curious observation of repetitive bacterial DNA, has become one of the most powerful tools in biology. It has far-reaching applications in medicine and biotechnology. While many researchers contributed to the CRISPR-Cas system discovery and mechanism, Charpentier and Doudna's Nobel recognition underscores the importance of transforming CRISPR from a bacterial immunity mechanism into a revolutionary tool in medicine. Moreover, Zhang and Church's contributions provided its crucial application in genome editing (26).

Immune Defense in Bacteria

Bacteria and archaea face constant threats from viral infections (27). Survival in these organisms depends on effective antiviral defenses, leading to the evolution of diverse immune strategies, which can be categorized as follows:

1. Innate immunity: Pre-existing molecular sensors detect conserved viral patterns to block infection.

2. Adaptive immunity: Specialized systems acquire memory of past infections to recognize and neutralize future threats (28).

Adaptive immunity was thought to be only present in vertebrates (29). However, the discovery of the CRISPR-Cas system revealed that microbes also

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possess a memory-based defense mechanism (10). CRISPR-Cas functions as an adaptive immune system, which can be found in 88% of archaea and a variety of other bacteria (Figure 2 and Figure 3). It stores genetic records of past infections to combat reinvasion (10). Its mechanism involves three key phases (30): 1. Spacer integration (recording viral encounters), 2. CRISPR RNA biogenesis (creating molecular guides), and 3. Targeted interference (destroying invading phages), as will be explained:

1. Spacer integration (recording viral encounters): When a phage infects a bacterium, the CRISPR-Cas system captures a short segment of viral DNA (a protospacer) and inserts it into the host genome as a spacer within a CRISPR array (31). This process, which is mediated by the Cas1-Cas2 complex, will act like a molecular archive and each spacer represents a "memory" of a past infection (31). The resulting CRISPR locus, with its alternating repeats and spacers, serves as a heritable vaccination record (31). 2. CRISPR RNA biogenesis (creating molecular guides): For activating the defense system, the stored DNA memories are transcribed into long precursor RNAs (pre-crRNAs), which are processed to produce short CRISPR RNAs (crRNAs) (18). Each crRNA contains a single spacer sequence, guiding Cas proteins to recognize matching phage DNA (32). This RNA-based system offers key advantages: a) Amplification: Because a single DNA spacer can generate many crRNA guides. b) Flexibility: Unneeded crRNAs will be degraded, conserving resources. and c) Preservation: the original DNA archive remains intact for future use (18). 3. Targeted interference (destroying invading phages): crRNA-Cas complexes scan the cell for matching viral DNA during re-infection. Upon recognition, the system cleaves the target with precision (17).

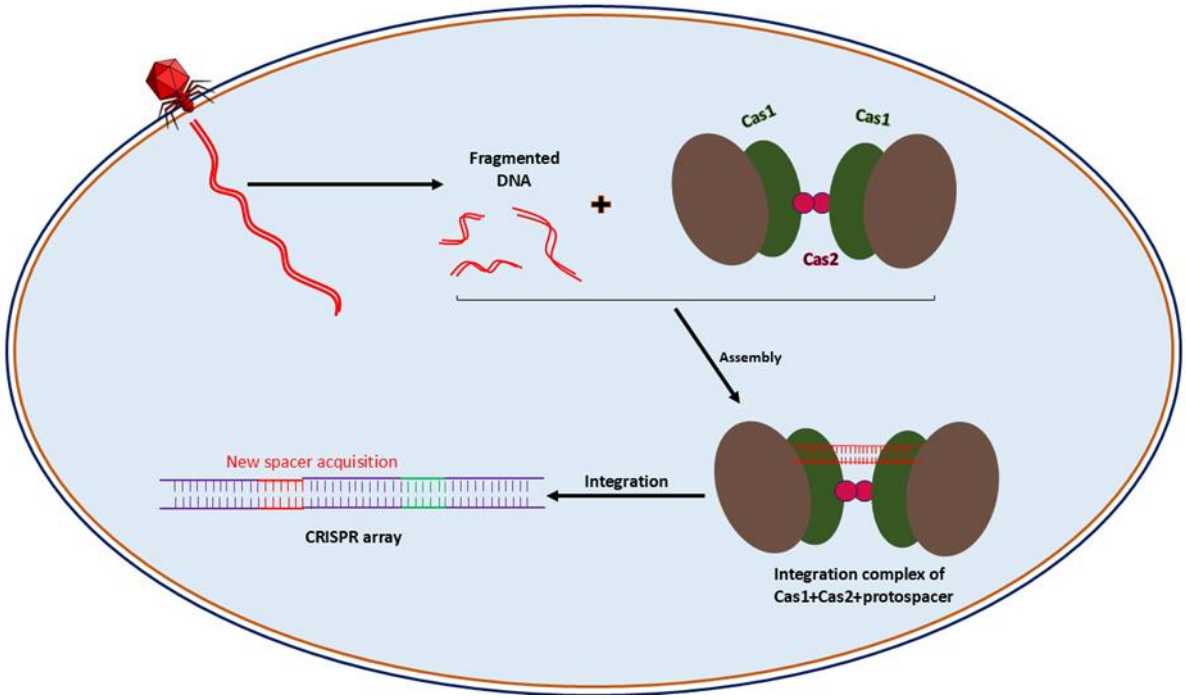

Figure 2. CRISPR-Cas system integration of new spacer from the invaded phage DNA into the genome of bacteria in adaptive immunity. The injection of phage DNA into bacterial cell (illustrated at the upper left) activates the Cas1-Cas2 proteins which excise spacer-sized fragments of phage DNA and integrated it into CRISPR array in the chromosome of bacteria.

There are three classes for cleaving: a) Class 1 systems use multi-protein complexes to degrade phage DNA (e.g., Type I). b) Class 2 systems introduce double-strand breaks (e.g., Type II, featuring Cas9), and c) Some variants also cut RNA (e.g., Type VI, with Cas13) (33). After initial

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cleavage, cellular nucleases dismantle the remaining phage DNA, thus halting the infection (33). This sequence-specific targeting ensures that only the invader is destroyed, therefore sparing the host genome (33). Thus, in CRISPR-Cas system usage by bacteria, the immunity occurs at a genetic level (34). Indeed, it equips microbes with a heritable and adaptive defense like a "genetic vaccination" system (34). Each bacterium develops lasting immunity by storing phage DNA fragments, enabling faster and stronger responses upon reinfection (34, 35). This elegant mechanism explains why CRISPR-bearing microbes often outcompete their vulnerable counterparts in the phage-rich environments (35).

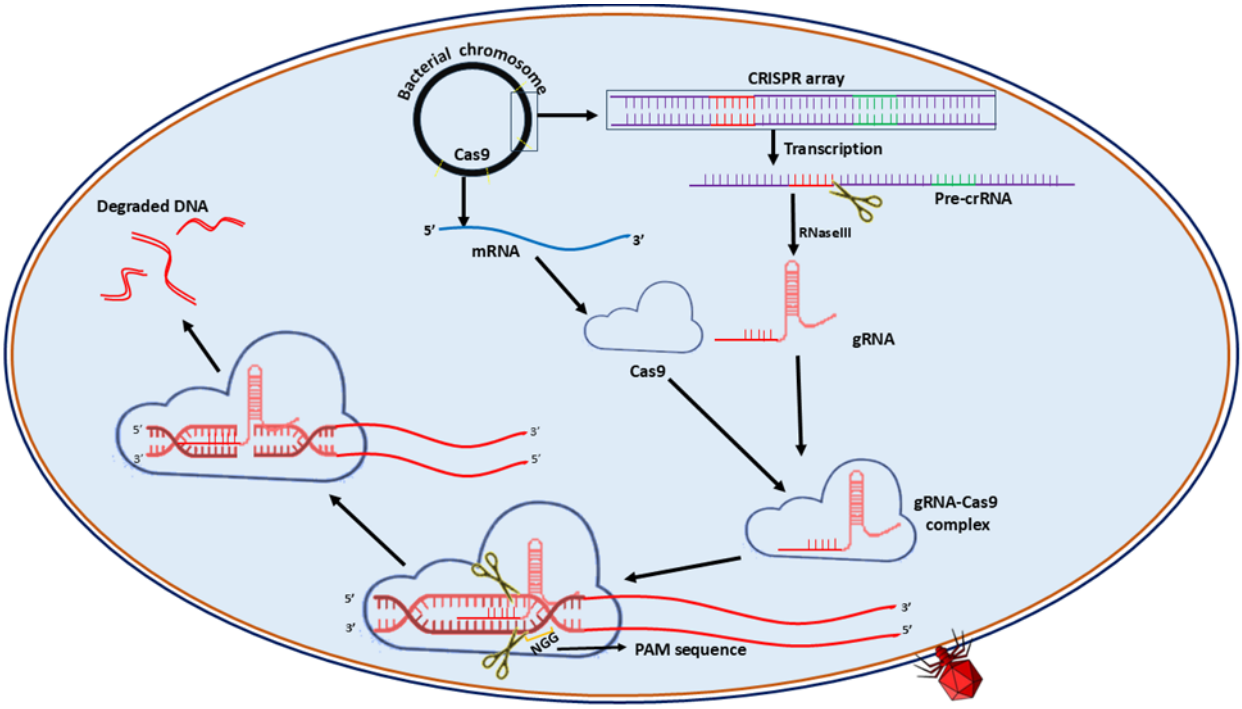

Figure 3. Activation of CRISPR-Cas system in adaptive immunity to defense against phage infections. In the bacterial genome, CRISPR array is transcribed as a pre-crRNA and then processed to generate crRNAs and tracrRNA. crRNA forms double-stranded RNA with tracrRNA through complementary base pairing. As a phage DNA with sequences matching to a CRISPR spacer enters the cell (lower right), the crRNA-tracrRNA duplex binds to Cas9, then activates and guides this enzyme to degrade sequence-matched phage DNA.

The CRISPR-Cas System Components and Mechanism

CRISPR loci contain short palindromic DNA repeats (28–37 bp), which are interspersed with spacer regions ranging from 32-38 bp (36). The latter is derived from mobile genetic elements like the genomes of bacteriophages or plasmids during prior infections and bestows the bacterium a sequence-specific immunity (36). The Cas (CRISPR-associated) genes lie adjacent to the CRISPR elements, which encode the enzymatic machinery for CRISPR-Cas function (37). Indeed, the CRISPR-Cas system operates via three core components (Figure 2 and Figure 3) (38):

1- CRISPR sequence array: A genomic archive of spacer-repeat units, often flanked by an AT-rich leader sequence.

2- gRNA (guide RNA): A hybrid of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) which directs Cas proteins to complementary DNA sequences.

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3- Cas protein: An endonuclease (e.g., Cas9) that binds CRISPR-derived gRNA to recognize the target DNA to cleave it (38).

4- Based on the enzyme mechanism, the CRISPR-Cas systems can be broadly classified into two main categories (39), including class 1 systems (types I, III, and IV), which utilize multi-protein complexes for nucleic acid modifications, and class 2 systems (types II, V, and VI), in which target recognition and cleavage mechanism depend on a single large effector protein such as Cas9 or Cas12 (39). The integration of new spacers into the CRISPR array is mediated by the conserved Cas1-Cas2 complex, which ensures the acquisition of foreign DNA fragments flanked by PAMs. Cas1 is a sequence-specific DNA integrase, and Cas2 is a nuclease that often plays a structural role, which stabilizes the complex (40). PAMs are short (2-6 bp) sequences that are critical for distinguishing self from non-self-DNA, as their absence in the host CRISPR array prevents autoimmunity for the host bacteria (39). The host's (self) DNA has the same target sequence (within the CRISPR array) but lacks the adjacent PAM. However, the invader (non-self) DNA has the correct target sequence (the protospacer) and the PAM sequence right next to it. This is because the PAM was not copied when the spacer was acquired in the host bacterium, and the Cas1-Cas2 complex only integrated the protospacer itself. Without a distinguishing feature like PAM, the Cas enzyme (like Cas9 in type II systems) would likely target and degrade the DNA in the bacteria's own CRISPR array (because it contains a perfect match to the spacer) by a returning virus, which could be fatal for the host cell. Type II enzymes, such as Cas9, only cleave DNA if a compatible PAM (e.g., 5′-NGG-3′ for Streptococcus pyogenes Cas9) is present downstream of the target sequence (39). The targeting specificity of CRISPR-Cas9 is governed by the gRNA, typically 17-24 nucleotides in length, which binds to the complementary DNA strand via hydrogen bonding of base pairing (3). The thermodynamic stability of this interaction is influenced by the GC content (40–80%) of the gRNA. Indeed, higher GC contents enhance binding affinity but potentially reduce specificity (3). Upon gRNA binding, Cas9 undergoes a protein conformational change. It transitions from an inactive state to a catalytically active form (41). These structural changes enable its RuvC and HNH nuclease domains to catalyze a double-strand break at three nucleotides upstream of the PAM sequence (41). The HNH domain cleaves the target DNA strand complementary to the gRNA. The RuvC domain hydrolyzes the non-complementary strand, typically generating blunt-ended DNA breaks (41). Although it is hypothesized that steric clashes between the RNA-DNA heteroduplex and the displaced non-target strand facilitate strand separation, the mechanism of target DNA unwinding remains an area of active investigation (42). Beyond canonical CRISPR-Cas9 systems, other Cas enzymes demonstrate distinct mechanistic properties (43). For example, the Cas12 enzyme (Type V) cleaves target DNA in a PAM-dependent manner and generates staggered ends with 5′ overhangs because of its single RuvC-like nuclease domain (43). Unlike Cas9, the Cas12 enzyme shows collateral cleavage activity, non-specifically degrading single-stranded DNA after target recognition. On the other hand, Cas13 (Type VI) enzyme targets RNA rather than DNA, induces sequence-specific RNA degradation, a feature for transcriptome engineering and viral RNA interference (44).

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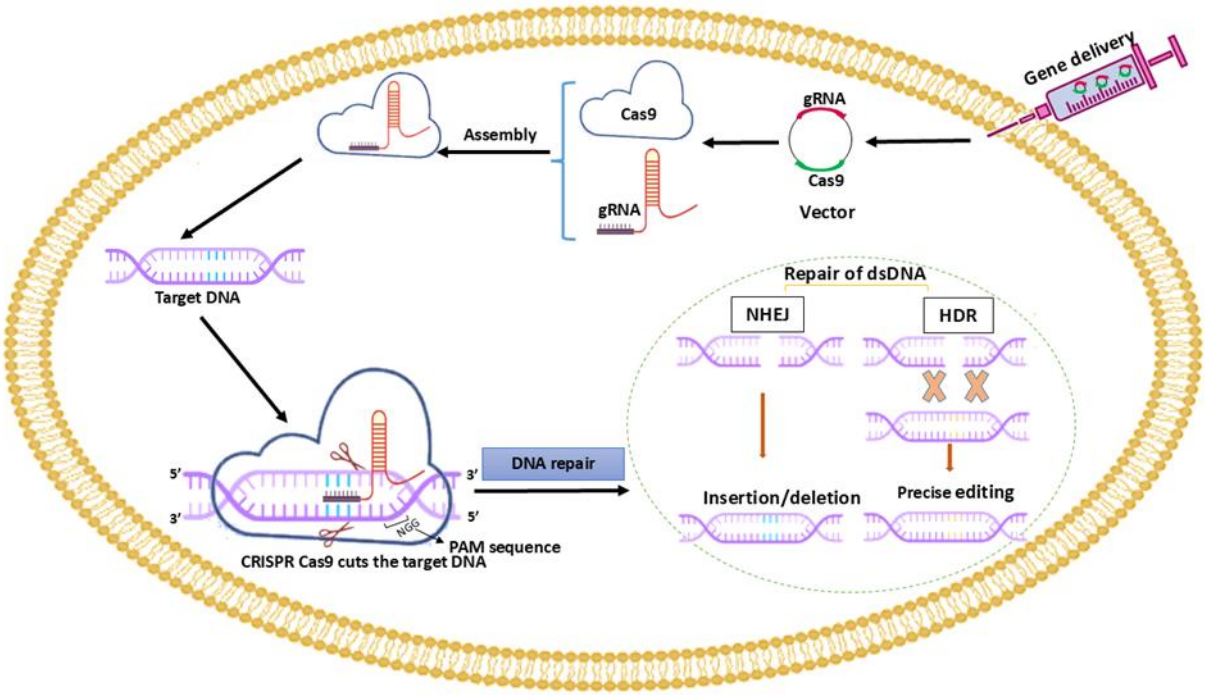

Figure 4. Composition and mechanism of CRISPR-Cas9 genome editing system. crRNA and tracrRNA form guide RNA (gRNA) through local base pairing after transcription from a vector inserted to the eukaryotic cell. The gRNA directs a Cas9 endonuclease to the target sequence of DNA in the genome. Cas9 endonuclease breaks two strands of target DNA. Then, the double-strand break generated by Cas9 endonuclease is repaired by the host-mediated DNA repair mechanisms including homology-directed repair (HDR) and non-homologous end joining (NHEJ).

Furthermore, there are other ways for modifying and editing bases, including base editing and prime editing: Base editing is used to change a single DNA "letter" (a base pair) to another without cutting the double-stranded DNA helix. A base editor is a fusion of two main parts: A CRISPR-Cas protein (such as Cas9) that is crippled or "nickase." It can still target a specific location in the genome using a guide RNA, but it cannot cut both strands of the DNA. It only "nicks" one strand. In this method, a deaminase enzyme is the actual editing machine. It performs a precise chemical reaction on a specific base of DNA (e.g., a cytosine or an adenine) to convert it directly into another base (e.g., uracil, which the cell reads as thymine). The process includes the following steps: a) The system is guided to the target DNA sequence. b) The crippled Cas protein unravels a small bubble of the DNA, exposing the target base. c) The deaminase enzyme chemically converts one base into another (e.g., C to T, or A to G). d) The cell's natural DNA repair machinery recognizes the mismatch and permanently incorporates the change into the genome. Key features of base editing include high precision and efficiency: It is excellent at making single-letter changes and is clean and safe, as it avoids double-strand breaks, which significantly reduces the risk of large, unwanted insertions or deletions ("indels") that can disrupt other genes. This method also has a limited scope. It can only perform specific transitions (C-to-T, T-to-C, A-to-G, G-to-A). It cannot repair all types of mutations and insert or delete large segments of DNA. Potential applications of it include correcting point mutations responsible for diseases such as sickle cell anemia (caused by a single A-to-T mutation), certain forms of progeria, or some types of hearing loss (45, 46). On the other hand, the prime editing method is a "search-and-replace" editor for DNA that can repair virtually any type of mutation without causing double-strand breaks. A prime editor is a fusion of two parts: a) A CRISPR-Cas9 nickase: like base editing, this Cas9 can only cut (nick) one strand of the DNA. b) A

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reverse transcriptase enzyme: an enzyme that can write RNA instructions into DNA. It uses a special guide RNA called a prime editing guide RNA (pegRNA). This pegRNA has two jobs: It guides the Cas9 nickase to the target site (the "search" function) and it contains the template for the new, desired DNA sequence that the reverse transcriptase will copy (the "replace" function). This method includes the following processes: 1- The system is guided to the target site by the pegRNA. 2- The nickase cuts one strand of the DNA. 3- The pegRNA binds to the nicked site. 4- The reverse transcriptase reads the template on the pegRNA and "writes" the new DNA sequence directly into the genome at the nicked location. The repair machinery is then tricked into incorporating and using this newly written strand as a template to permanently change the other strand. Key features of prime editing include the following: a) Extreme versatility: it can edit all base-to-base mutations, insertions and deletions. It can correct ~90% of known disease-causing genetic variants. b) High precision and fewer off-target effects: Like base editing, it avoids double-strand breaks, making it much cleaner than standard CRISPR-Cas9. c) More complex system: The pegRNA design is more complex than a standard guide RNA, which can make it trickier to be implemented. Potential applications of prime editing include correcting a vast range of genetic disorders, including those caused by point mutations, small insertions, and deletions, such as Tay-Sachs disease, cystic fibrosis, and Huntington's disease (45, 46).

Table 1. . CRISPR-Cas9 versus Base editing versus prime editing

Feature	CRISPR-Cas9 (Classic)	Base Editing	Prime Editing
Core Mechanism	Creates DSBs	Chemically converts one base to another	Uses a pegRNA and reverse transcriptase to "write" new DNA
DNA Cut	Cuts both strands	No DSB; may nick one strand	No DSB; nicks one strand
Types of Edits	Disruptions, insertions, deletions	Specific base transitions (e.g., C->T, A->G)	All 12 base changes, insertions, deletions
Precision	Lower; relies on error-prone repair	Very High	Very High
Risk of Indels	High	Very Low	Very Low
"Search & Replace"	"Cut and Hope"	"Erase and Rewrite" (limited)	True "Search and Replace"
Therapeutic Scope	Broad, but risky	Narrow (point mutations only)	Very Broad (~90% of known mutations)

DSB: Double-strand break

Discussion

Medical Hope for Gene-editing Technology

As mentioned, there is an intricate interplay among gRNA-mediated targeting, nuclease activation, and DNA repair pathways in CRISPR-Cas9-based genome editing (47). Advances in structural biology and protein engineering continue to clarify the precise mechanisms, enabling the development of high-fidelity Cas variants for genome manipulation (47). Following the introduction of genetic materials into the eukaryotic cell using genetic engineering methods such as DNA vector applications and methods like transfection and transduction, the double-strand break formed by CRISPR-Cas9 induces DNA repair (Figure 4). There are two main DNA repair pathways, including non-homologous end joining (NHEJ) and homology-directed repair (HDR) for resolving the double-stranded DNA break.

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The NHEJ is the predominant repair mechanism, which acts throughout the cell cycle and mediates direct ligation of broken DNA ends without a template (48). Due to the inherent error-proneness, the NHEJ repair system often results in small insertions or deletions (indels) that can disrupt gene function via frameshift mutations or premature stop codons (48). In contrast, HDR achieves precise genome repair as it applies homologous DNA sequences—either sister chromatids or exogenous donor templates (48). The HDR process is most active during the S/G2 phases of the cell cycle, when homologous templates are readily available (7). Thus, for CRISPR-mediated human genome editing, the HDR method requires the delivery of an exogenous donor DNA containing the desired sequence, which is incorporated via strand invasion and recombination, which occur at the double-strand break site (7). While HDR enables precise DNA editing, its efficiency is limited by the cell cycle stage, donor template availability, and competition with NHEJ (7). The advent of CRISPR-Cas9 gene editing technology has ushered in a new era of therapeutics, offering extraordinary opportunities to treat and even cure or prevent a wide range of diseases that were once considered incurable (49). The CRISPR sequence and Cas9 enzyme function as molecular scissors to make a precise induction of the genome sequence repair at a specific site (50). This revolutionary approach has rapidly transitioned from a fundamental research tool into a promising therapeutic method spanning from monogenic to polygenic genetic disorders (51). With the ability to repair mutations at the molecular level, CRISPR-Cas9 acts as a cornerstone for next-generation biomedical therapies (51).

CRISPR in Cancer Therapy: Addressing a Global Health Challenge

Recent advances in genomic investigations have elucidated that human malignancies are characterized by extensive genetic and epigenetic aberrations (such as seen in leukemias), causing uncontrolled cell proliferation, metastasis activation, and therapeutic drug resistance (52, 53). A precise genomic repairing induction with CRISPR-Cas9 application allows for the targeted correction of a tumor suppressor gene or the disabling of the oncogenic mutations at the pre-defined positions, thereby restoring normal cellular functions (54). CRISPR-Cas9-mediated gene editing represents a novel therapeutic paradigm for not just modulating tumor cells but also bestowing new abilities to immune cells (55). This technology can be used to enhance the immune system’s ability to recognize, phagocytose or induce apoptosis in malignant cells, representing an outstanding progression in immuno-oncology (56). Its excellent precision is particularly advantageous for engineering adaptive T-cell therapies, a keystone of immunotherapy (56, 57). Contemporary cancer treatment modalities encompass conventional approaches—including surgery, cytotoxic chemotherapy, and radiotherapy—as well as innovative strategies such as molecularly targeted therapy, including immune checkpoint blockade, and gene-based interventions (58). Despite these achievements, unfortunately, traditional therapies frequently impose substantial limitations, particularly due to their off-target cytotoxicity, which compromises the patients' tolerance and their long-term treatment adherence (58). Among gene-editing technologies, although zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been demonstrated to be applicable in modifying disease-associated genes for therapeutic purposes (59), their clinical translation has been hampered by some technical complexity, labor-intensive design processes, and sub-optimal efficiency (59). In contrast, the CRISPR-Cas9 system has rapidly progressed as an extraordinary genome-editing platform due to its unraveling simplicity, high precision, and broad applicability (60). Consequently, CRISPR-Cas9 has been widely adopted in both basic and clinical investigations, offering transformative advances in cancer therapy (60). The advent of this technology has facilitated unprecedented insights into the molecular

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mechanisms underlying pathophysiologic states such as tumorigenesis (55).

Beyond Cancer: CRISPR's Potential in Treating Genetic Diseases

While cancer remains a primary focus, the most profound impact of CRISPR-based therapeutics may lie in its ability to correct a variety of inherited genetic disorders (56). Monogenic diseases are particularly amenable to CRISPR-Cas9-mediated interventions (61). Promising preclinical and clinical studies have demonstrated the potential of CRISPR-Cas9 to treat a wide spectrum of genetic conditions, including hematologic, neuromuscular, metabolic, and ocular disorders (62).

1. Hematologic disorders (63):

a) Sickle cell disease (SCD) and beta-thalassemia: These diseases result from mutations in the HBB gene, leading to defective hemoglobin production (64). In milestone clinical trials, CRISPR-Cas9 has been used to reactivate fetal hemoglobin (HBG1/2) by disrupting repressor elements in the BCL11A gene. Thereby it compensates for the dysfunctional adult hemoglobin (5). The first FDA-approved CRISPR-Cas9 therapy is exagamglogene autotemcel (exa-cel). Its application has shown remarkable success in alleviating symptoms in SCD and transfusion-dependent beta-thalassemia patients (63).

b) Hemophilia: For the treatment of hemophilia A and B, CRISPR-Cas9 is being explored to restore clotting factor production (Factor VIII or IX, respectively) by correcting mutations in hepatocytes or hematopoietic stem cells, offering an incredible cure for this lifelong bleeding disorder (65).

2. Neuromuscular and Neurodegenerative Diseases:

a) Duchenne muscular dystrophy (DMD): CRISPR-Cas9 has been employed to excise the exon segments in the DMD gene for restoring dystrophin gene expression in preclinical models. Moreover, in vivo gene delivery via adeno-associated virus (AAV)-based vectors has demonstrated promising results in ameliorating muscle degeneration (66).

b) Huntington's disease: Allele-specific silencing of the mutant HTT gene using CRISPR may reduce toxic polyglutamine aggregates and delay disease progression (67).

3. Metabolic and Storage Disorders

a) Phenylketonuria (PKU): CRISPR-Cas9-mediated correction of phenylalanine hydroxylase (PAH) gene mutations in hepatocytes could restore phenylalanine metabolism to eliminate dietary restrictions (68).

b) Transthyretin amyloidosis (ATTR): Inactivation of the mutant transthyretin (TTR) gene in the hepatocytes using lipid nanoparticle-delivered CRISPR-Cas9 has entered clinical trials.

4. Ocular and Sensory Disorders

a) Leber congenital amaurosis (LCA): Subretinal delivery of CRISPR-Cas9 has been used to correct centrosomal protein 290 (CEP290) gene mutations to restore photoreceptor function in early-phase trials.

b) Hereditary hearing loss: Inner ear gene editing to rectify transmembrane channel-like 1 (TMC1) or gap junction protein beta 2 (GJB2) gene mutations may prevent progressive deafness (69).

Despite advances in CRISPR-Cas9-based therapeutics, however, this technology faces several hurdles: a) Delivery challenges: In vivo gene delivery, which would be efficient and tissue-specific, remains a bottleneck so far, particularly for non-liver tissues (69). Viral vectors (AAV, lentivirus) and non-viral systems (such as lipid nanoparticles and electroporation) are under optimization to overcome this obstacle (69). Recent studies have discussed viral and non-viral methods of gene delivery, highlighting their strengths and limitations. The reviews address practical barriers to efficient in vivo gene delivery, particularly in solid tissues such as the brain, muscle, and retina, and discuss biological barriers (endothelial barriers, extracellular matrix, and cellular uptake) and modern

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solutions such as lipid nanoparticles to enhance clinical relevance (70-72). b) Off-target effects: Unintended genomic alterations pose safety risks to the patient; high-fidelity Cas9 variants (e.g., HiFi-Cas9) and base/prime editing systems may mitigate this concern. c) Immune responses: Preexisting immunity to bacterial Cas9 may limit the efficacy as well; thus, humanized or engineered nucleases are being developed (69). d) Ethical and regulatory considerations: Germline editing remains contentious, while somatic applications are progressing cautiously under rigorous oversight (69).

Immune responses against CRISPR components

Immune responses against CRISPR components are critical to move CRISPR therapies from the lab to the clinic. Immune responses can potentially derail a treatment by eliminating the edited cells or causing severe adverse reactions, making this a cornerstone of translational relevance. One of these problems is pre-existing immunity to CRISPR components. The issue stems from the origins of CRISPR-Cas systems. The Cas9 protein commonly used (e.g., from Streptococcus pyogenes, or "SpCas9") comes from bacteria that are natural commensals or pathogens in humans. A significant proportion of the human population has been exposed to these bacteria (like the common S. pyogenes that causes strep throat). As a result, many people have pre-existing neutralizing antibodies and Cas9-specific T cells in their immune systems. If a patient's immune system recognizes the bacterial Cas9 protein, it can mount a response that destroys the therapy (if delivered via a viral vector like AAV). Also, it kills the cells that have taken up the editing machinery before the establishment of any DNA repair induced by CRISPR-Cas9. This immune clearance can drastically reduce the efficiency and durability of the editing, rendering the treatment ineffective. A potent immune response could lead to dangerous inflammatory reactions, such as cytokine release syndrome or organ-specific inflammation (e.g., hepatotoxicity if the liver is targeted). Potential strategies to mitigate immune responses can be used to overcome these barriers. Researchers are pursuing a multi-pronged approach to "de-immunize" CRISPR systems for safe clinical use, such as using novel or engineered Cas proteins, the most promising and widely pursued strategy, and "humanized" or engineered Cas variants. Cas proteins from uncommon or non-pathogenic bacteria may be one of the choices (73, 74).

Using Novel or Engineered Cas Proteins

The human immune system is unlikely to have encountered Cas proteins from bacteria that are not human pathogens or commensals. Cas proteins from Staphylococcus aureus (SaCas9), Campylobacter jejuni (CjCas9), or other exotic environmental bacteria are being explored. However, these can still have immunogenicity, and their editing efficiency and PAM requirements may be less ideal. Another option is humanized" or engineered Cas variants. In epitope mapping and its deletion method, the researchers identify the specific regions (epitopes) on the Cas9 protein that are recognized by T cells and antibodies. They can create "de-immunized" Cas9 variants that are less visible to the immune system using protein engineering to mutate or delete these immunodominant epitopes. Furthermore, the advanced computational and high-throughput screening methods are used to design Cas variants that retain their catalytic activity but have altered surface structures that are no longer recognized by the pre-existing immune factors (73).

Transient Delivery Methods

Another option for overcoming adverse immune responses is using transient delivery methods. A key factor in immune activation is the persistence of the foreign protein. Limiting its presence can reduce the chance of an adaptive immune response. Moreover, using mRNA or ribonucleoprotein (RNP) complexes can be done. Instead of using viral vectors that can lead to long-term expression of Cas9, the editing machinery can be delivered transiently (75).

One way is mRNA for Cas9 is encapsulation in lipid nanoparticles and its delivery to the targeted cells.

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The cells translate it into the protein, which performs the edit and is then naturally degraded within days. Another option is using RNP complexes. The preassembled Cas9 and its guide RNA complex are delivered directly. This is the most transient method, as the protein is active immediately and degraded quickly. The short window of expression is often sufficient for efficient editing but it is too brief to robustly prime a destructive adaptive immune response (73).

Immunosuppression

A more classical pharmacological approach for immunosuppression involves transient immunosuppressive drug usage (e.g., corticosteroids) around the time of treatment. It dampens the immune system's ability to respond to the foreign Cas proteins. While not ideal for long-term management, this can be a viable strategy for a one-time treatment, especially for ex vivo applications in which the exposure time is limited (73).

Ex Vivo versus In Vivo Editing

Ex vivo editing (cells edited outside the body): Cells like hematopoietic stem cells or T-cells are extracted, edited in the lab, and then transplanted back into the patient's body. The transient delivery of RNP is highly effective in this method. The edited cells don't express the bacterial protein when returned to the patient body, thus minimizing immune recognition. In vivo editing (editing inside the body): This is where the immune challenge is the greatest. The Cas protein is introduced directly into the patient's body, presenting a much larger antigenic challenge. The strategies above (novel Cas proteins, transient delivery via lipid nanoparticles) are essential for the success of in vivo therapies. Addressing anti-CRISPR immunity is not an optional step but a fundamental requirement for the broad clinical success of these technologies. The field has moved beyond simply proving editing is possible and is now deeply engaged in the engineering required for safe delivery (73, 74, 76).

CRISPR Babies: The He Jiankui Controversy and Ethical Aspects

The birth of the first CRISPR-Cas9-edited twins (referred to as Lulu and Nana) in 2018, engineered by Chinese biophysicist He Jiankui, ignited one of the most contentious scientific and ethical debates of the 21st century (77). By virtue of CRISPR-Cas9 gene-editing technology, He Jiankui claimed that he had modified the C-C motif chemokine receptor 5 (CCR5) gene in human embryos to provide them human immunodeficiency virus infection resistance, resulting in the live birth of genetically altered twins (77). This first-time intervention triggered a rapid and widespread condemnation from the worldwide scientific community (77). The pioneering CRISPR-Cas9 researchers, including Jennifer Doudna and Feng Zhang, criticized this experiment as ethically indefensible and scientifically premature, emphasizing well-scientifically-documented risks such as off-target genetic mutations and mosaicism (21). Critics contended that this investigation disobeyed international guidelines for human genome editing, especially the 2017 National Academies of Sciences, Engineering, and Medicine (NASEM) report, which imposes stringent restrictions on germline editing, which permits experiments for only severe and untreatable genetic diseases (78). Moreover, the informed consent approval was scrutinized, showing that the parents were imperfectly informed about the experimental risks and potential hazards of performing the procedure (78). The repercussions extended beyond academic discourse, culminating in legal sanctions against He Jiankui, who received a three-year prison sentence because of violating medical regulations (79). In response, the World Health Organization (WHO) introduced enhanced authority frameworks for human genome editing while scientific institutions reaffirmed the necessity of maintaining public trust in emerging biotechnologies worldwide (80). This powerful genome-editing tool can be fundamentally seen as a double-edged sword because on one hand, it offers transformative potential to cure

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devastating genetic diseases, saving countless human lives, and on the other hand, it simultaneously threatens to usher in ethically fraught applications such as heritable human enhancement (designer babies) and thus it poses significant risks through unforeseeable consequences (81). Furthermore, the persistent challenge of off-target mutations and unintended on-target effects (like mosaicism) introduces significant safety risks even in well-intentioned therapeutic applications (82). Ultimately, harnessing the life-saving potential of CRISPR-Cas9 while mitigating its profound risks demands not only continued scientific refinement to enhance its safety and specificity but also the establishment of comprehensive, adaptable, and globally coordinated ethical guidelines (80).

Conclusion

Based on pivotal findings from the bacterial immune mechanism, a precise molecular scissors, known as CRISPR-Cas9 technology, has been designed. It stands as a monumental breakthrough in medicine, offering unprecedented potential to cure monogenic disorders, revolutionize cancer therapy, and address a variety of human diseases by rewriting the genetic code. The coming decade will be crucial in the widespread application of this revolution, necessitating interdisciplinary collaboration among scientists, clinicians, ethicists, and regulators to ensure safe and reasonable translation.

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As this manuscript is a literature review and does not include any experiments involving humans or animals, ethical approval was not applicable. All information used in this review is derived from articles that had obtained appropriate ethical clearance by their respective authors.

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Authors' contributions

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