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From Bench to Bedside: The Expanding Role of CRISPR/Cas Systems in Genome Engineering and Human Disease Research

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ABSTRACT:

upheaval in biomedical research. It makes it possible to correct errors in the genome and turn on or off genes in cells and organisms quickly, cheaply and with relative ease. It has a number of laboratory applications including rapid generation of cellular and animal models, functional genomic screens and live imaging of the cellular genome. It has already been demonstrated that it can be used to repair defective DNA in mice curing them of genetic disorders, and it has been reported that human embryos can be similarly modified. Other potential clinical applications include gene therapy, treating infectious diseases such as HIV and engineering autologous patient material to treat cancer and other diseases.[1]

At present, there are three mainstream genome editing tools in the world, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the RNA-guided CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) nucleases systems. Due to the advantages of simple design, low cost, high efficiency, good repeatability and short-cycle, CRISPR-Cas systems have become the most widely used genome editing technology in molecular biology laboratories all around the world. In this review, an overview of the CRISPR-Cas systems will be introduced, including the innovations and applications in human disease research and gene therapy, as well as the challenges and opportunities that will be faced in the practical application of CRISPR-Cas systems.[2]

History background of gene editing technologies

The unprecedented progress in the gene editing technologies in the last decade has ushered in a marvelous time for the field of genetics. The ripples created by the advent of gene editing technologies, particularly CRISPR/Cas, have been felt almost in every field of biological sciences, from model organisms, evolution, agriculture, diagnostics and therapeutics. Traditionally, genetic research has depended upon uncovering and analyzing mutations that occur spontaneously.

Currently, we have three main gene editing technologies: Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases

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(TALENs) and CRISPR-Cas. Owing to the ease of use, the CRISPR-Cas technology dominates the field of gene editing; however, the other two technologies are also used in several medical and agriculture areas. Interestingly, all three of these technologies have been derived as a result of investigations into basic biological phenomena without the intention of performing genetic editing in animal cells.[3]

Discovery

Clustered regularly interspaced short palindromic repeats, otherwise known as CRISPRs, are repeating DNA sequences in the genomes of prokaryotes, such as bacteria and archaea. CRISPRs were first identified in E. coli in 1987 by a Japanese scientist, YoshizumiIshino, and his team, who accidentally cloned an unusual series of repeated sequences interspersed with spacer sequences while analyzing a gene responsible for the conversion of alkaline phosphatase. However, due to the lack of sufficient DNA sequence data, the function of these arrays remained a mystery.

In 1993, researchers led by J.D. van Embden in the Netherlands discovered that different strains of Mycobacterium tuberculosis had different spacer sequences between the DNA repeats. They characterized M. tuberculosis strains based on their spacer sequences, a technique known as 'spacer oligonucleotide typing' or spoligotyping. Subsequently, these sequences were identified in several other bacterial and archaeal genomes. Researchers Francisco Mojica and Ruud Jansen were the first to refer to them as CRISPRs.[4]

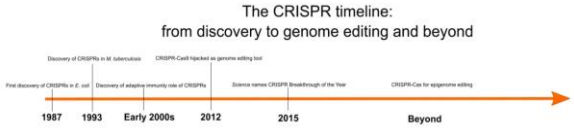

Figure 1

Components and Mechanism

Based on the structure and functions of Cas-proteins, CRISPR/Cas system can be divided into Class I (type I, III, and IV) and Class II (type II, V, and VI). The class I systems consist of multi-subunit Cas-protein complexes, while the class II systems utilize a single Cas-protein. Since the structure of type II CRISPR/Cas-9 is relatively simple, it has been well studied and extensively used in genetic engineering. Guide RNA (Grna) and CRISPR-associated (Cas-9) proteins are the two essential components in CRISPR/Cas-9 system. The Cas-9 protein, the first Cas protein used in genome editing was extracted from Streptococcus pyogenes (SpCas-9). It is a large (1368 amino acids) multi-domain DNA endonuclease responsible for cleaving the target DNA to form a double-stranded break and is called a genetic scissor. Cas-9 consists of two regions, called the recognition (REC) lobe and the nuclease (NUC) lobe.

Molecular mechanisms: adaptation, maturation and interference

The CRISPR-Cas system acts in a sequence-specific manner by recognizing and cleaving foreign DNA or RNA. The defence mechanism can be divided into three stages:

(i) adaptation or spacer acquisition
(ii) crRNA biogenesis
(iii) target interference.

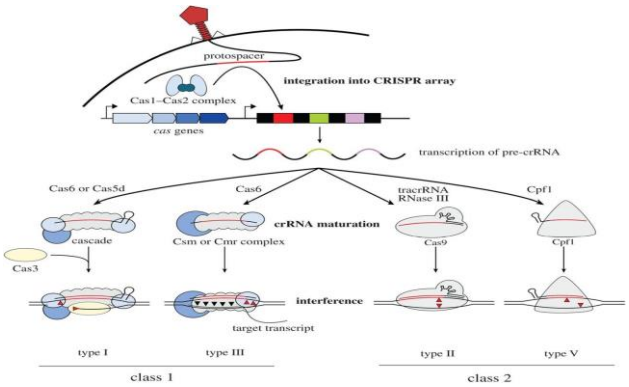

Figure 2

Adaptation

In a first phase, a distinct sequence of the invading MGE called a protospacer is incorporated into the CRISPR array yielding a new spacer. This event enables the host organism to memorize the intruder's genetic material and displays the adaptive nature of this immune system. Two proteins, Cas1 and Cas2, seem to be ubiquitously involved in the spacer acquisition process as they can be found in almost all CRISPR-Cas types. Exceptions are the type III-C, III-D and IV CRISPR-Cas systems, which harbour no homologous proteins. Moreover, type V-C shows a minimal

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composition as it comprises only a putative effector protein termed C2C3 and a Cas1 homologue. In past years, major advances have been made in revealing the biochemical and genetic principles of CRISPR-Cas immunity. However, the mechanism of spacer acquisition is still not fully understood. The selection of protospacers and their processing before integration remain widely obscure in many CRISPR-Cas types. Recent findings, however, shed light on the biochemistry of the spacer integration process. It has been demonstrated that Cas1 and Cas2 of the type I-E system of Escherichia coli form a complex that promotes the integration of new spacers in a manner that is reminiscent of viral integrases and transposases. Although both Cas1 and Cas2 are nucleases, the catalytically active site of Cas2 is dispensable for spacer acquisition. A new spacer is usually incorporated at the leader-repeat boundary of the CRISPR array while the first repeat of the array is duplicated.

The mechanisms of the different CRISPR-Cas types might be conserved only to a certain extent as several studies have shown variations regarding the requirements and targets of the adaptation machinery. While Cas1 and Cas2 are sufficient to promote spacer acquisition in most studied type I CRISPR-Cas systems, type I-B further requires Cas4 for adaptation. The type I-F CRISPR-Cas system of Pseudomonas aeruginosa additionally requires the interference machinery to promote the uptake of new spacers. Similarly, type II-A systems require Csn2, Cas9 and tracrRNA (trans activating CRISPR RNA—see further details below) for acquisition. Another, so far unique, adaptation mode was revealed for a type III-B Cas1 protein that is fused to a reverse transcriptase. Here, acquisition from both DNA and RNA was reported.

Biogenesis

To enable immunity, the CRISPR array is transcribed into a long precursor crRNA (pre-crRNA) that is further processed into mature guide crRNAs containing the memorized sequences of invaders. In type I and III systems, members of the Cas6 family perform the processing step yielding intermediate species of crRNAs that are flanked by a short 5′ tag. One exception is given by the type I-C systems, which do not code for Cas6 proteins. Here, the protein Cas5d processes pre-crRNA resulting in intermediate crRNAs with an 11 nt 5′ tag. Further trimming of the 3′ end of the intermediate crRNA by an unknown nuclease can occur and yields mature crRNA species composed of a full spacer portion (5′ end) and a repeat-portion (3′ end), which usually displays a hairpin structure in most type I systems. The maturation of crRNAs in class 2 CRISPR-Cas systems differs significantly. In type II systems, tracrRNA is required for the processing of the pre-crRNA. The anti-repeat sequence of this RNA enables the formation of an RNA duplex with each of the repeats of the pre-crRNA, which is stabilized by Cas9. The duplex is then recognized and processed by the host RNase III yielding an intermediate form of crRNA that undergoes further maturation by a still unknown mechanism to lead to the mature small guide RNA. An RNase III-independent mechanism was discovered in the type II-C CRISPR-Cas system of Neisseria meningitidis. Here, promoter sequences were identified to lie within each repeat and some were able to initiate transcription leading to intermediate crRNA species. Even though RNase III-mediated 3′ processing of the crRNA :tracrRNA duplex was observed, it was dispensable for interference. In the type V-A CRISPR-Cas system, it has been shown that Cpf1 has a dual function during CRISPR-Cas immunity. Cpf1 processes premature crRNAs and, following a further maturation event of unknown nature, uses the processed crRNAs that it has generated to cleave target DNA.

Interference

In the last stage of immunity, mature crRNAs are used as guides to specifically interfere with the invading nucleic acids. Class 1 systems employ Cascade (CRISPR-associated complex for antiviral defence)-like complexes to achieve target degradation, while in class 2 systems, a single effector protein is sufficient for target interference. To avoid self-targeting, type I, II and V systems specifically recognize the PAM sequence that is located upstream (types I and V) or downstream (type II) of the protospacer. In type III systems, the discrimination between self and non-self is achieved via the 5′ tag of the mature crRNA, which must not base pair with the target to enable degradation by the complex.

In type I systems, Cascade localizes invading DNA in a crRNA-dependent manner and further recruits the nuclease Cas3 for target degradation. Cas3 induces a

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nick on the foreign DNA and subsequently degrades the target DNA. In type II CRISPR-Cas systems, the tracrRNA:crRNA duplex guides the effector protein Cas9 to introduce a double-strand break in the target DNA. The interference machinery of type III systems comprises Cas10-Csm (types III-A and III-D) and Cas10-Cmr (types III-B and III-C) complexes, which are able to target both DNA and RNA. Intriguingly, it has been shown that interference of type III-A and type III-B systems depends on the transcription of the target DNA. More precisely, the subunit Cas10 cleaves the DNA while Csm3 and Cmr4 cleave the transcribed mRNA in type III-A and type III-B CRISPR-Cas systems, respectively. Interference in type V CRISPR-Cas systems shows similarities to interference in type II. An RNA duplex, consisting of tracrRNA and crRNA, is strictly required for target cleavage in type V-B systems. Type V-A, however, only employ crRNA for target localization and degradation.[5]

Types of Crispr system

CRISPR systems are divided into two main classes: class 1 and class 2. The main difference between the two classes is their effector molecules: Class 1 effectors contain multiple subunits while class 2 effectors are single large proteins. The specific types within each class depend on the specific Cas endonuclease responsible for cleavage and its mechanism of action.

Class 1 CRISPR systems can be further subdivided into 3 types (type I, type III, and type IV) and 12 subtypes. The class 1 system is found in 90% of the CRISPR loci in bacteria and archaea and can target both DNA and RNA.

Class 1 CRISPR systems are characterized by their multiple effector molecules. The effector molecules contain complexes that are responsible for RNA recognition and crRNA binding. Class 1 effector molecules are similar between the types, despite their distinct sequences.

Type I and type III are the more common class 1 systems. Both contain proteins responsible for pre-cRNA processing, crRNA and target binding, target cleavage, spacer insertion, and regulation. However, a close look at their detailed structure reveals differences between the two.

Type I

There are seven different subtypes of class 1, type I CRISPR systems. All class 1 type I subtypes contain cas3 loci that have the ability to unwind double-strand DNA and RNA-DNA complexes in order to facilitate target cutting. However, the different subtypes have effector molecules made up of different components. Further, type I systems can only target DNA.

Type III

Similar to type I, type III systems are all share a common cas loci, in this case, cas10. Cas10 encodes something similar to an RNA recognition motif called Palm and a cyclase domain responsible for cutting. Type III systems can recognize both DNA and RNA for cleavage. Although there are far fewer subtypes of type III than type I, each type III subtype is more diverse in terms of the operon structure.

Type IV

Type IV is a putative class 1 system, whereby relatively little is known about it compared to types I and III. In class 1, type IV systems, the effector molecular is much smaller (albeit, still multiprotein), and does not contain domains capable of target cleavage or spacer insertion.

What are Class 2 CRISPR systems?

Class 2 CRISPR systems are characterized by the presence of a single effector molecule. There are 3 types of class 2 systems, and 9 subtypes. While class 2 systems are more commonly known (Cas9 is a class 2 system), they only represent 10% of the CRISPR loci and unlike class 1, they are only found in bacteria.

Class 2 systems can target both DNA and RNA, depending on the type.

Type II

The most common class 2 system type is type II. Type II systems are characterized by the presence of Cas9, as well as ancillary proteins cas1 and cas2. Cas9, the commonly used genome engineering endonuclease is a type II system. Type II systems require a tracrRNA for function.

Types V and VI

Type V systems commonly use Cas12 as their endonuclease of choice. Like Cas9, Cas12 targets DNA

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for editing. Similar to type II systems, type V also requires tracrRNA for function. Type VI is the only class 2 system that targets RNA for editing. Cas13 is a type VI endonuclease that enables the editing of RNA.

CRISPR is being widely used and studied as a tool for addressing **disease modeling** and **drug or target screening**. However, Omar Abudayyeh and Jonathan Gootenberg, have harnessed the power of Cas13 (class 2, type VI) to make CRISPR a diagnostics tool. With the help of Cas13, **SHERLOCK (Specific High sensitivity Enzymatic Reporter unLOCKing) was created to search and detect specific sequences in the genome**. It has been successfully shown to **detect both Zika virus and certain strains of dengue fever**. Recent advancements in SHERLOCK are in progress in hopes to develop a greater breadth of disease detection.[6]

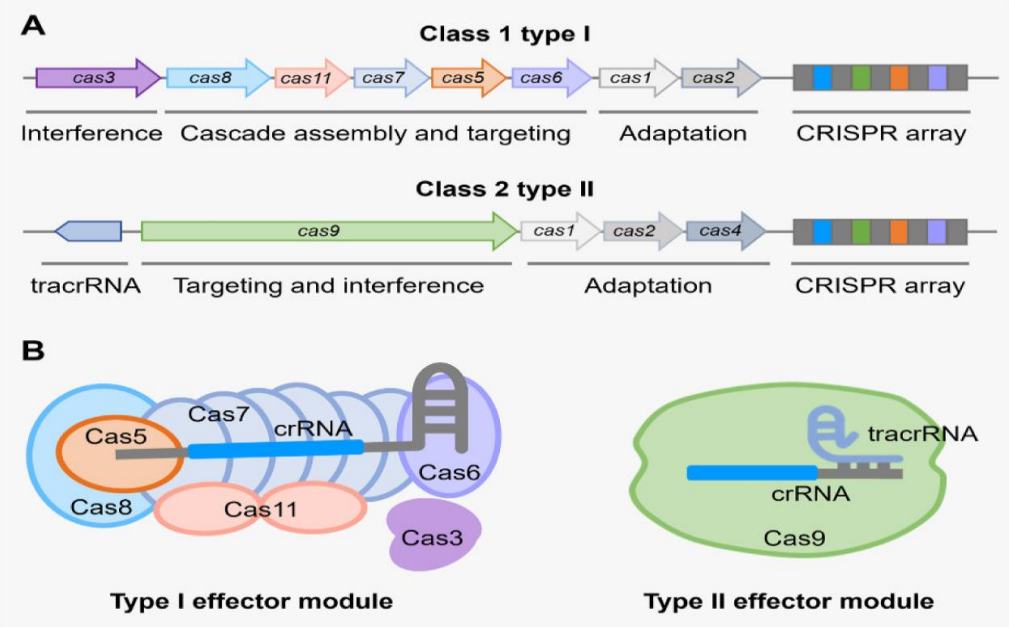

Figure 3

Variants

In the last ten years, CRISPR-Cas9 technology has gained widespread recognition as a groundbreaking influence in the domain of genome editing. It has acted as a catalyst, sparking a revolution in the field of molecular biology. This comprehensive review endeavors to furnish a thorough analysis of CRISPR-Cas9 technology, delving into its fundamental principles, encompassing its constituent components, the intricacies of the genome editing process, and its unparalleled strengths compared to other genome editing tools as shown in Table 1.[7]

Table 1

Criteria	CRISPR-Cas9	TALENs	ZFNs
Efficiency	High	Moderate to High	Moderate to High
Specificity	High	Moderate	Moderate
Ease of Use	Relatively Easy	Moderate	Complex

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Criteria	CRISPR-Cas9	TALENs	ZFNs
Design Flexibility	High	Moderate	Moderate
Off-Target Effects	Possible, but can be minimized	Possible, but can be minimized	Possible, but can be minimized
Cost Effectiveness	Cost-effective	Relatively Expensive	Expensive
Availability and Adoption	Widely Available and Adopted	Moderate Availability and Adoption	Limited Availability and Adoption

Alternatives Crispr enzymes

S. pyogenes Cas9 (SpCas9) is the most commonly used CRISPR endonuclease for genome engineering, but there are other Cas enzymes available. These enzymes have unique properties and can target different types of nucleic acids, including dsDNA (Cas9, Cas12), ssRNA (Cas13), or ssDNA (Cas14).

Efficient Editing and Multiplexing with Cas12a

One of the most popular Cas9 alternatives is Cas12a (Cpf1). There are many Cas12a orthologs, but the most common is AsCas12a (from *Acidaminococcus sp.*). The popularity can be attributed to Cas12a's many benefits.

Cas12a exhibits increased efficiency with larger edits using HDR. Cas12a-mediated DNA cleavage creates DSBs with a short 3′ overhang, allowing for directional gene transfer, which may increase the efficiency of gene editing. Cas12a also has more condensed multiplexing capabilities, as it can process multiple gRNAs under a single promoter, and can cleave ssDNA non-specifically.

Cas12a also has an expanded targeting range to AT-rich regions, as it recognizes TTTV (V = A, C, or G) PAM sites. Engineered versions of Cas12a recognize different PAM variants, with one of the most popular being EnCas12a, an engineered version of AsCas12a that recognizes TYCV and TATV PAM sites.

The DETECTR platform, developed by the Jennifer Doudna lab, can identify specific strains of pathogens or genetic mutations. In DETECTR, Cas12a is targeted to a specific genomic location, such as a viral genome, and a ssDNA-fluorescently quenched reporter is added. As this reporter is degraded by Cas12a, it will release a quantifiable signal that indicates the presence of your target DNA sequence.

Degrading DNA with Cas3

Rather than initiating DNA cleavage to form a break, Cas3 initiates single-strand DNA degradation. This degradation can continue for many kilobases in one or both directions from the target region, effectively turning Cas3 into a DNA "shredder" system that can cause large genomic deletions. The boundaries of these deletions can be defined, either with HDR repair templates or with anti-CRISPR proteins. Large deletions generated by Cas3 have fewer off-target alterations, such as small mutations or inversions, than do large deletions generated by Cas9 systems.

Cas3 must be paired with the Cascade (CRISPR-associated complex for antiviral defense) complex to initiate DNA degradation. Cascade is made of five proteins: Cas8 (Cse1), Cas11 (Cse2), Cas7, Cas5 and Cas6e. As the name implies, this system is harnessed for antiviral mechanisms in bacteria (*E. coli*). Like the other Cas enzymes, the Cascade-Cas3 system has been co-opted for use in eukaryotic cells. The Cascade complex is recruited to target DNA using a gRNA and recruits Cas3. Cas3 nicks the non-targeting strand and starts degrading the single-stranded DNA, unraveling DNA as it goes using its helicase properties, often called a **reeling** mechanism. The targeted strand is cleaved non-specifically by Cas3 along the way. Cas3 initiates a final DSB, either due to a defined boundary or running into other proteins bound to DNA, and the break can be repaired by the cell's endogenous repair mechanisms.[8]

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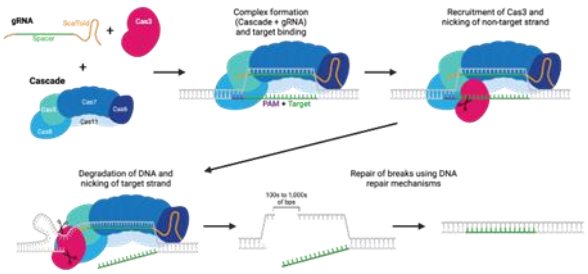


FIGURE 5

Why CRISPR-cas system used to encounter antibiotic resistance threat?

Four major classes of DNA binding proteins have been engineered to achieve effective genome editing: meganucleases originated from microbial MGEs [62], transcription activator like effectors (TALEs) derived from bacteria (*Xanthomonas*) [63], Zinc finger nucleases (ZFNs) from eukaryotic transcription factors [11] and finally the RNA guided DNA endonucleases cas9 from CRISPR-cas type II system of bacteria [48].

Genome editing by meganucleases is not widely used due to low sequence specificity for target DNA [11]. ZFNs also have limitations, as they are difficult to design for binding to a desired sequence. Furthermore, ZFNs have limited target site selection. TALENs are easy to design due to their capacity to have longer DNA binding protein domains, allowing for high specificity of targeting. However, TALENs are much larger than ZFNs, and this size poses a complication for delivery into cells [64].

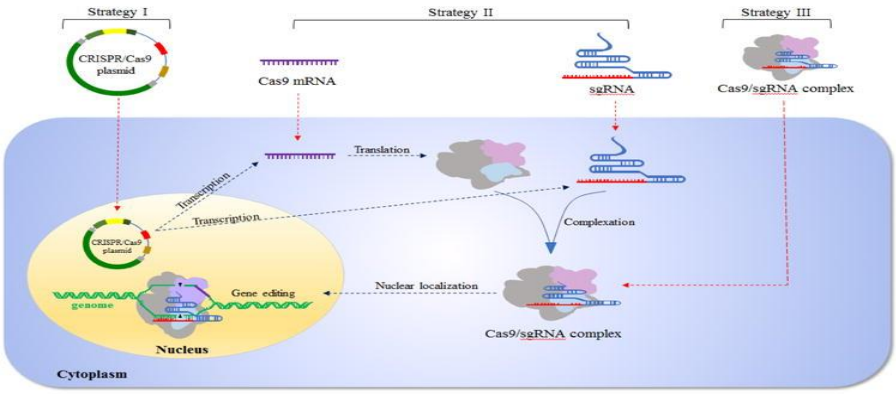
The Cas9 nuclease of the CRISPR-cas type II system uses a guide RNA to identify target DNA by Watson–Crick base pairing. Sequences present in CRISPR guide RNAs are specific to an invader sequence, meaning this sequence can be easily replaced by our desired sequence to retarget the CRISPR-cas9 nuclease [11].

Detailed study of the CRISPR-cas system has enabled researchers to insert, delete and mutate desired genes in virtually any species, and can even be used to correct genetic diseases in live animals [65]. Additionally, this system is now used in specific antibacterial preparations that can target AMR pathogens within complex populations of bacteria, allow antibacterial delivery to pathogenic bacteria, and in some cases deliver treatments to host cells infected with pathogenic bacteria. The CRISPR-cas system distinguishes between commensal and pathogenic bacterial species due to sequence specific targeting. The potential of CRISPR-cas system to counteract AMR pathogens is highlighted here.[9]

Different strategies and Delivery methods for CRISPR-Cas9

As illustrated in Figure 4, there are three strategies to edit genome using CRISPR-Cas9. The first and the most straightforward approach is to use a plasmid-based CRISPR-Cas9 system encoding the Cas9 protein and sgRNA from the same vector, thus avoiding multiple transfections of different components [26]. The second strategy is to deliver the mixture of the Cas9 mRNA and the sgRNA [27]. The third strategy is to deliver the mixture of the Cas9 protein and the sgRNA [28].

Figure 6. Different strategies to edit genes using the CRISPR-Cas9 system.



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Direct delivery of the Cas9 mRNA and sgRNA into target cells edits genome after expressing the Cas9 protein and subsequently forming the Cas9/sgRNA complex inside the cells [9,31]. The advantage of administering mRNAs is the transient expression of Cas9 protein, which limits the duration of gene-editing. In addition, delivery of mRNAs has lower off-target effects than the delivery of plasmid-based CRISPR-Cas9 system. Apart from subsiding off-target effects, mRNAs only need to enter the cytoplasm to exert their effects. Furthermore, the use of the mRNA encoding Cas9 protein shows low cytotoxicity in primary cells and cell lines [32]. However, the relatively poor stability of mRNA is an obstacle for this type of gene-editing strategy.

Direct delivery of the Cas9 protein complexed with sgRNA is the most widely studied strategy in recent years. The purified Cas9 protein is positively charged and can efficiently form a complex with sgRNA, which is called Cas9/sgRNA ribonucleoprotein complexes (RNPs). Direct delivery of RNPs has numerous advantages, including rapid action; high gene editing efficiency; no requirement of codon optimization and promoter selection; and reduced off-target effects, toxicity and immune responses.[33] Various delivery systems that have been exploited for CRISPR-Cas9 are summarized in Table 2.[10]

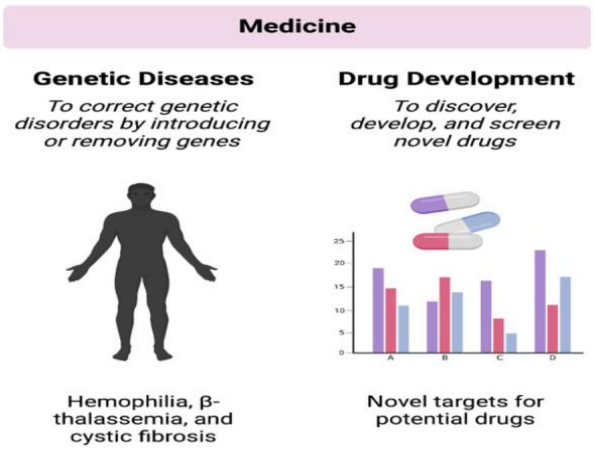
Table 2. Delivery systems for different strategies of CRISPR-Cas9.

Types of CRISPR-Case 9	Delivery strategies
Plasmid-based CRISPR-Cas9	Electroporation, Hydrodynamic injection, Microinjection, Mechanical cell deformation, Lipid Nanoparticles, AAV, Lentivirus
Cas9 mRNA and sgRNA	Electroporation, Microinjection, Lipid Nanoparticles
Cas9 protein and sgRNA	Electroporation, iTOP, Lipid Nanoparticles, Polymer nanoparticles, CPP delivery, DNA nanostructure, Gold nanoparticles

APPLICATIONS: CRISPR-Cas9 technology in medicine

CRISPR-Cas9 technology has emerged as a powerful tool with transformative implications in the field of medicine. This sub-section provides an overview of the applications and advancements of CRISPR-Cas9 in medical research and therapy. Furthermore, CRISPR-Cas9 technology has opened up new avenues for cancer research and therapy. It allows for the targeted disruption of genes involved in cancer progression or the introduction of specific modifications to sensitize cancer cells to existing treatments. In a study, CRISPR-Cas9 was used to knock out a gene involved in chemotherapy resistance, enhancing the effectiveness of the treatment in cancer cells.[12]

Figure 7



Therapeutic Role of CRISPR/Cas-9:

The first CRISPR-based therapy in the human trial was conducted to treat patients with refractory lung cancer.

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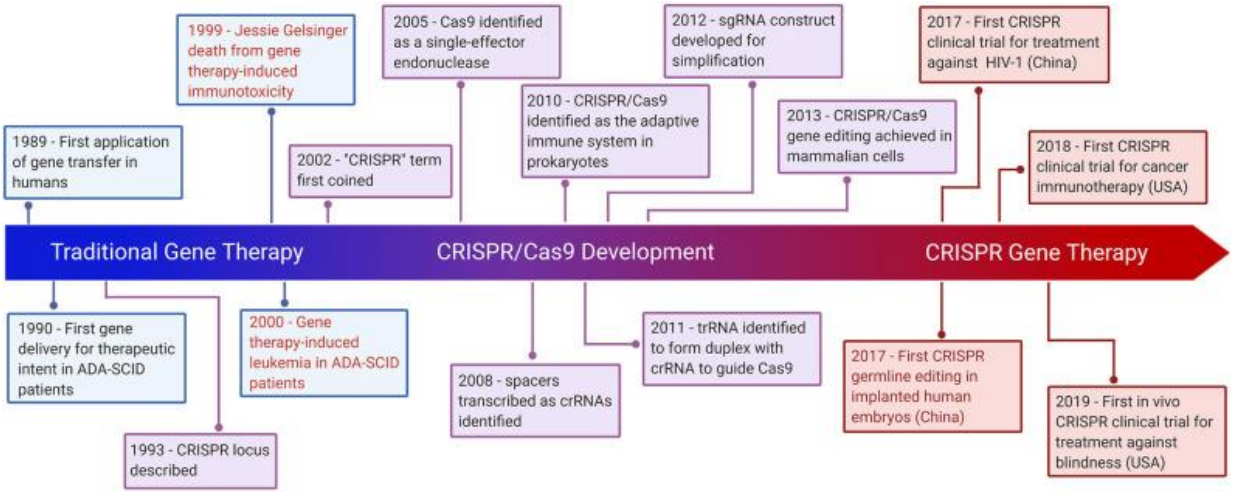
Researchers first extract T-cells from three patient's blood and they engineered them in the lab through CRISPR/Cas-9 to delete genes (TRAC, TRBC, and PD-1) that would interfere to fight cancer cells. Then, they infused the modified T-cells back into the patients. The modified T-cells can target specific antigens and kill cancer cells. Finally, no side effects were observed and engineered T-cells can be detected up to 9 months of post-infusion CRISPR/Cas-9 gene-editing technology could also be used to treat infectious diseases caused by microorganisms. One focus area for the researchers is treating HIV, the virus that leads to AIDS. In May 2017, a team of researchers from Temple University demonstrated that HIV-1 replication can be completely shut down and the virus eliminated from infected cells through excision of HIV-1 genome using CRISPR/Cas-9 in animal models. In addition to the approach of targeting the HIV-genome, CRISPR/Cas-9 technology can also be used to block HIV entry into host cells by editing chemokine co-receptor type-5 (CCR5) genes in the host cells. For instance, an in vitro trial conducted in China reported that genome editing of CCR5 by CRISPR/Cas-9 showed no evidence of toxicity (infection) on cells and they concluded that edited cells could effectively be protected from HIV infection than unmodified cells.[13]

Crispr in gene therapy for genetic disorders

Pioneering Discoveries in CRISPR/Cas9 Technology

The bacterial CRISPR locus was first described by Francisco Mojica (23) and later identified as a key element in the adaptive immune system in prokaryotes (24). The locus consists of snippets of viral or plasmid DNA that previously infected the microbe (later termed "spacers"), which were found between an array of short palindromic repeat sequences. Later, Alexander Bolotin discovered the Cas9 protein in *Streptococcus thermophilus*, which unlike other known Cas genes, Cas9 was a large gene that encoded for a single-effector protein with nuclease activity (25). They further noted a common sequence in the target DNA adjacent to the spacer, later known as the protospacer adjacent motif (PAM)—the sequence needed for Cas9 to recognize and bind its target DNA (25). Later studies reported that spacers were transcribed to CRISPR RNAs (crRNAs) that guide the Cas proteins to the target site of DNA (26). Following studies discovered the trans-activating CRISPR RNA (tracrRNA), which forms a duplex with crRNA that together guide Cas9 to its target DNA (27). The potential use of this system was simplified by introducing a synthetic combined crRNA and tracrRNA construct called a single-guide RNA (sgRNA) (28). This was followed by studies demonstrating successful genome editing by CRISPR/Cas9 in mammalian cells, thereby opening the possibility of implementing CRISPR/Cas9 in gene therapy (29) (Figure 1)[14]

Figure 8



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Crispr in cancer research and treatment

The first clinical trial (ex vivo) on non-small-cell lung cancer patients was performed in China using CRISPR/Cas9 as a tool for the editing of genes [169]. The electroporation of Cas9 and sgRNA was performed, in which the PD-1 gene present in T cells in the peripheral blood of patients was targeted and inculcated back into the patients [170]. In the peripheral blood, edited T cells were found to be present in the patients who received infusions within a very short period. As a result, they discovered that this method was efficient and secure, which improved therapeutic efficacy [15].

CRISPR in Infectious Disease Control (HIV, COVID-19, etc.)

CRISPR could help us cure HIV

The Human Immunodeficiency Virus, or HIV, is a virus that attacks the immune system, making it harder for the body to fight off diseases. According to the World Health Organization (WHO), 39 million people were living with HIV by the end of 2022, including adults and children.

Most people living with HIV have an HIV-1 infection (HIV-2 is slow to develop and does not spread as fast). The HIV virus is spread by exchanging body fluids, mainly through unprotected sexual intercourse, sharing needles or syringes, and from an infected mother to her baby during childbirth or breastfeeding.

An HIV-1 infection is dangerous because the virus attacks the immune system, the human cells that defend against infection. It can be fatal without timely medical intervention and lifelong therapy.

Unfortunately, there is still no cure for HIV. One of the main reasons for this is the ability of the virus to mutate constantly, making it challenging to develop a single treatment that can target all strains. Also, some cells infected with HIV can become dormant, only to resurface years later. This is one of the reasons why HIV-infected individuals need treatment for life.

Using CRISPR to treat HIV

Using CRISPR, scientists are editing out the HIV-infected immune cells in the body, including dormant ones. To achieve this, CRISPR is being used to cut fragments from the DNA of the HIV virus itself, rendering it unable to replicate and spread.

Early progress in this field has shown promising results, with researchers successfully using the CRISPR-Cas system to remove HIV from infected cells in laboratory experiments. However, it is still being studied in clinical trials to understand its effects on HIV-1 infections in humans.[16]

Application of Crispr Cas9 other than medicine

CRISPR has been all over the news in the past couple of years, and with reason. This gene editing tool is making gene editing easier and faster than ever, and the possibilities it has opened up go well beyond human health. You might have heard of the tremendous potential CRISPR could have in treating disease. The technology is already accelerating the research on the underlying causes of all sorts of human conditions. Furthermore, clinical trials using the gene editing tool to treat diseases ranging from cancer to blindness and AIDS are underway.

The medical applications of CRISPR have taken the spotlight, especially after the intense criticism that surged after a Chinese scientist revealed to the world the birth of CRISPR twins, the first humans to be born from a gene-edited embryo. But the technology offers endless (and less morally questionable) possibilities beyond its applications in human health.

Talking to several players in the CRISPR arena, I gathered a list of unusual applications of CRISPR that clearly show the potential that the gene editing technology has to impact many different kinds of industries

DNA ‘tape recorders’:

FIGURE 9

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Scientists at Harvard have used CRISPR to create a molecular tool called CAMERA — short for CRISPR-mediated analogue multi-event recording apparatus. The tool acts as a recorder of events in the lifetime of a cell, such as exposure to antibiotics, nutrients, viruses and light.

To achieve this, CRISPR was programmed into cells so that a specific DNA edit was made only in the presence of the signal. By counting the rate of edits, they can even determine the duration and strength of the trigger. The system works in both bacterial and human cells, and it is possible to make it record multiple kinds of signals simultaneously.

In the long term, these developments could help scientists detect environmental pollutants in the field or track the signals that determine whether stem cells grow to be neurons, muscle cells or another cell type.

Spicy tomatoes:

FIGURE 10

Scientists in Brazil and Ireland are using CRISPR to create the first tomato that is naturally spicy. It turns out that the tomato already carries many of the genes to produce capsaicin, the compound that makes chili peppers spicy. With tweaks from CRISPR, the researchers could give them the missing genes to make them spicy.

Chili peppers are hard to grow. They require very specific conditions, produce inconsistent levels of heat, and have a much lower yield than tomatoes. Thanks to CRISPR, it would be easier to obtain the spicy capsaicin by growing it in tomatoes. The researchers have already created a first hybrid of tomato and chili, and soon enough we might be using them to make spicy salsa.

Although the term sounds like it comes from a science fiction novel, it is not. Scientists are already working on bringing back animals that are extinct. The first candidate is the passenger pigeon, once a dweller of North American forests.

Using CRISPR technology, researchers plan to introduce genes from the passenger pigeons into its modern-day relative — the band tail pigeon. The hybrids will be bred for several generations until the offspring DNA matches that of the extinct species. The first generation of 'revived' pigeons is expected to hatch in 2022.

Not long after, mammoths could follow. A group at Harvard is now working on bringing back the woolly mammoth that went extinct thousands of years ago. [17]

CRISPR for Crop Improvement

CRISPR/Cas9 method of gene editing has been adopted in nearly 20 crop species so far (Ricroch et al., 2017) for various traits including yield improvement, biotic and abiotic stress management. Many of the published articles are considered as proof-of-concept studies as they describe the application of CRISPR/Cas9 system by knocking out specific reported genes playing an important role in abiotic or biotic stress tolerant mechanisms. Biotic stress imposed by pathogenic micro-organisms pose severe challenges in the development of disease-resistant crops and account for more than 42% of potential yield loss and contribute to 15% of global declines in food production (Oerke, 2005). CRISPR/Cas9-based genome editing has been utilized to increase crop disease resistance and also to improve tolerance to major abiotic stresses like drought and salinity (Figure 4). A survey of the use of CRISPR for genome editing in various crop species is presented below. [18]

Crispr in Aquaculture

Aquaculture is one of the leading industries in the global food supply, addressing world food security concerns resulting from global climate change, population growth, and resource scarcity [1]. By 2050, the world population is expected to reach 9.7 billion, necessitating an increase of 25% to 70% in food production. To overcome these obstacles, it is essential to provide rich and healthy diets, which is where aquaculture plays a role [2]. In 2018, global aquaculture

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contributed 46 percent of the overall world production of fish as well as 52 percent of human fishery product consumption [3]. The sustainable development and continued growth of the aquaculture sector rely on high-protein feeds in order to cover the requirements of an increasing world population [4,5]. Aquaculture is an important source of animal protein for human consumption [6]. The high-quality, easy-to-digest protein of fishery products containing high levels of essential amino acids (EAAs) is regarded as superior to other sources of protein such as bovine, dairy, and egg products. It is the principal source of essential fatty acids (EFAs) for a healthy human diet, including n-3 polyunsaturated fatty acids (PUFAs), namely docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3). In addition, aquacultural products also contain a wide range of vitamins, notably vitamin A, and minerals not readily available from many land-based sources [7].

Genome editing provides new opportunities, and its applications in the aquaculture industry may be a clever tool to achieve maximum efficiency and allow more cost-effective availability of nutrient-rich food worldwide [8]. Initially, zinc finger nucleases (ZFNs) and transcription activator-like endonuclease (TALEN) technologies predominated in genome engineering. Currently, the most advanced genome-engineering technique, the clustered regularly interspaced short palindromic repeats (CRISPRs) system, has become the most widely used worldwide [9,10] and is significantly more cost-effective, user-friendly, and efficient for site-specific genome editing than ZFNs and TALENs. The CRISPR/Cas approach has been used in various livestock and fish species, with no sign of a slowdown in progress [11].

The CRISPR-Cas system is an acquired immune system found in many archaea and bacteria, ensuring sequence-specific defense mechanisms to protect against detectable foreign nucleic acids. The CRISPR loci consist of the CRISPR assembly, composed of short repeats directly interspersed with short variable DNA segments (known as spacers), flanked by various Cas genes [12]. There is more than one type of CRISPR/Cas system, including CRISPR/Cas9, CRISPR/Cas12, and CRISPR/Cas13 [12]. Nevertheless, due to its notably high efficacy and accuracy, CRISPR/Cas9 is the most widely used in several scientific fields [13].

CRISPR/Cas9 enables precise genome editing to improve crucial traits, including sex determination and growth rates (including length, weight gain, and the development of muscle fibers) [14,15,16]. Furthermore, it is a powerful approach to improving fish disease resistance by addressing pathogen recognition traits or immune-associated genes, thus avoiding over-dependence on chemical therapeutics and antibiotics [17,18,19]. This technique may radically transform modern aquaculture by improving essential traits in many fish species genetically. For instance, it successfully removed the germ cells involved in sexual differentiation from reproductive cells in Atlantic salmon (*Salmo salar*) [20,21], enhanced the feed conversion ratio to boost growth rates in yellow catfish (*Pelteobagrus fulvidraco*) [22], and achieved effective genetic mutations in Nile tilapia (*Oreochromis niloticus*), reducing the occurrence of undesirable effects [23].

The protection of the environment is crucial to the sustainable development of the aquaculture sector. CRISPR/Cas9 technology is not limited to improving the desired characteristics of fish but also considers environmental sustainability and the interests of consumers. Some researchers have altered fish to minimize the amount of nitrogen-rich waste they produce, thereby reducing the environmental effects of aquaculture. Moreover, they have enhanced the fish's nutritional composition, improving n-3 PUFA levels and minimizing undesirable components. The *fads2* gene for fatty acid desaturation in rainbow trout (*Oncorhynchus mykiss*) was modified to optimize the n-3 PUFA synthesis [4]. Nevertheless, public and consumer acceptance of this technology is hampered by various factors, including negative public perception and possible ecological effects [24], which have generated ethical concerns regarding genome editing in animals, especially using gene knock-in and knockout techniques. Commercializing CRISPR/Cas9-engineered products requires risk assessment and consumer approval. Regulatory approval and consumer trust in genetically modified fish are affected by environmental considerations, safety, and health credibility [25]. To earn regulatory approval and consumer confidence, it is essential to provide comprehensive clarification on how

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fish traits are modified, meet regulatory guidelines, and provide supporting data on genetically modified fish to address these concerns [4]. The CRISPR/Cas9 system still lacks comprehensive studies on its various implementations and the limitations it presents in editing major desired traits in different fish species. Previous reviews have mainly focused on describing the principles of this technique, highlighting its benefits and applications to improve economically important traits [26]. Hence, the purpose of this review is to discuss and review research advancements aimed at enhancing various target traits in aquaculture, including growth, disease resistance, reproductive confinement, omega-3 fatty acid metabolism, and pigmentation by using CRISPR/Cas9 technology. [19]

CRISPR-Cas9 technology in environmental sciences

The application of CRISPR-Cas9 technology in environmental sciences presents exciting possibilities for addressing environmental challenges, conservation efforts, and sustainable practices [34]. This sub-section provides an overview of the use of CRISPR-Cas9 in environmental research and potential applications.

One area of interest in environmental sciences is the use of CRISPR-Cas9 for conservation purposes. Researchers are exploring the potential of CRISPR-Cas9 to mitigate threats to endangered species by editing their genomes to enhance their resilience or adaptability to changing environments. The successful use of CRISPR-Cas9 to introduce genetic resistance to white-nose syndrome in bats, a devastating fungal disease that has caused significant declines in bat populations [35,36].

CRISPR-Cas9 technology is also being investigated for controlling or eradicating invasive species. Invasive species pose significant threats to ecosystems and native biodiversity. Researchers are exploring the possibility of using CRISPR-Cas9 to genetically modify invasive species to suppress their populations or make them less harmful to native species. While still in the early stages of research, this approach has the potential to provide effective and environmentally friendly solutions for invasive species management [36].

Additionally, CRISPR-Cas9 has the potential to enhance agricultural practices and promote sustainable farming methods. The use of CRISPR-Cas9 to develop crops with improved nutrient uptake, drought resistance, and reduced pesticide reliance [34]. These genetic modifications can contribute to increased crop productivity, reduced environmental impact, and more sustainable food production [34].

Furthermore, CRISPR-Cas9 technology offers opportunities for environmental monitoring and assessment. A study stated that the detection and identification of specific organisms, pathogens, or contaminants in environmental samples [35]. This can aid in the early detection of harmful agents, helping to prevent or mitigate environmental pollution or disease outbreaks. CRISPR-based diagnostic tools have shown promise in detecting waterborne pathogens and monitoring environmental health [35].

While the application of CRISPR-Cas9 in environmental sciences is still in its early stages, it holds tremendous potential for addressing environmental challenges and promoting sustainable practices. However, it is crucial to consider potential ecological and ethical implications, and careful risk assessments and regulatory frameworks should accompany the development and deployment of CRISPR-based environmental interventions [35].

CRISPR-Cas9 technology presents exciting opportunities for environmental sciences. Its applications range from conservation efforts and invasive species management to sustainable agriculture and environmental monitoring. Continued research and responsible implementation of CRISPR-Cas9 in environmental contexts can contribute to the preservation and restoration of ecosystems and support sustainable practices [35,36].[20]

CRISPR-Cas9 technology in nanotechnology

CRISPR-Cas9 technology has the potential to synergize with nanotechnology in various ways. Nanotechnology offers precise control at the nanoscale, and when combined with the precision of CRISPR-Cas9, it opens up new possibilities for targeted delivery, imaging, and sensing applications. Here are a few potential areas where the integration of CRISPR-Cas9 and nanotechnology could be explored: (a) Targeted delivery systems. Nanoparticles can be engineered to carry CRISPR-Cas9 components, such as guide RNA and Cas9 protein, to specific cells or tissues. This

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targeted delivery approach could enhance the efficiency and specificity of genome editing while minimizing off-target effects [44]. (b) Imaging and tracking. Nanoparticles with imaging agents can be used to monitor the delivery and activity of CRISPR-Cas9 components in real-time. This allows researchers to visualize and track the editing process, providing valuable insights into its efficacy and potential optimization [45]. (c) Gene regulation and epigenetic modifications. Nanotechnology-based platforms can be employed to modulate gene expression and epigenetic modifications, either by directly targeting DNA or influencing regulatory factors. Integration of CRISPR-Cas9 with nanotechnology can enable precise control over gene expression and epigenetic regulation, offering new avenues for disease treatment and fundamental biological research [46].

While the specific references on CRISPR-Cas9 technology in nanotechnology are limited, some researchers published articles and reviews on CRISPR-based delivery systems, nanocarriers for gene editing, or nanotechnology-enabled gene regulation for further insights into the potential synergies between CRISPR-Cas9 technology and nanotechnology [21]

Future prospects

A key goal of future research will be to improve the accuracy of predictive models by incorporating additional features. Current methods for predicting target efficiency and specificity are based solely on the sequence of the target site. However, it is now accepted that chromatin environment can influence CRISPR-Cas9 activity. Early studies mapping the genome wide binding of inert Cas9 enzymes using ChIP-seq showed a preference for DNAase sensitive regions, which are typically more accessible environments. This was supported by later studies which showed that high-activity target sites were often enriched for histone modifications associated with open-chromatin environments.

A direct link between chromatin and CRISPR-Cas9 activity was shown in 2016, where a pair of studies demonstrated that the presence of nucleosomes at the target site physically blocked CRISRP-Cas9's access and reduced overall activity. The differences in chromatin environment likely explain why the same CRISPR-Cas9 target site can display different activities across cell-lines. There is also evidence that off-target activity is influenced by chromatin accessibility, with the CROP-IT pipeline including this information into their off-target model. Incorporating environmental information in future predictive models will help improve accuracy and will be critical if the technology is to be applied in the clinic. Such modeling may also allow for the selective targeting of individual tissues by leveraging the differences in chromatin environments.

Incorporation of chromatin environments would likely also improve off-target predictions, which is thought to be more susceptible to chromatin accessibility. Besides chromatin information, future off-target pipelines should also focus on including variant information. A recent study demonstrated that the variance between individuals has a dramatic effect on the off-target landscape, with point mutations creating and destroying potential off-target sites. Such information is critical for the application of CRISPR technology in almost all fields, as not taking an individual's unique genome into account could have deleterious side-effects.

Future models may also not only be able to predict the success of CRISPR-Cas9 editing, but also the outcome. By targeting sites with microhomology and exploiting the microhomology-mediated repair pathway, researchers may be able to delete specific DNA segments and thereby control the outcome of CRISPR-Cas9 editing. Additionally, a recent study found that the mutations induced by repair of CRISPR-Cas9 cleavage were non-random and determined by the target sequence. Such a finding suggests that it would be possible to predict the mutational outcome of CRISPR-Cas9 editing, allowing for researchers to make precise edits without the need of using knock-ins.

The optimal future pipeline will incorporate all of these factors into both on- and off-target activity predictions. Such a pipeline could also provide a method by which experimentally validated predictions could be reintegrated into the training data for the models, to continue to improve accuracy. Future models may also predict success of other CRISPR-Cas9 applications such as knock-ins, which involve the repair of the double strand break using a supplied template, and base-editing, where a Cas9 fusion protein converts one base into another without cleavage.[30]

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Conclusion

CRISPR/Cas9 has rapidly transformed from a naturally occurring microbial immune mechanism into one of the most powerful and versatile genome-editing technologies available today. Its simplicity, precision, cost-effectiveness, and adaptability have enabled groundbreaking advances in basic research, disease modeling, functional genomics, and therapeutic development. As innovations such as improved Cas variants, base editors, and epigenome-editing tools continue to emerge, CRISPR technologies hold immense promise for treating genetic disorders, combating infectious diseases, and engineering personalized medicine solutions. However, despite its vast potential, challenges related to delivery, off-target effects, ethical considerations, and regulatory oversight remain significant. Continued research, responsible application, and global scientific collaboration will be essential to fully realize the transformative impact of CRISPR/Cas systems in human health and biotechnology.

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