

CRISPR-Cas9: A Revolutionary Tool for Genome Editing

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CRISPR-Cas9: A Revolutionary Tool for Genome Editing

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ABSTRACT

CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9) is a transformative gene-editing system that enables precise, efficient, and cost-effective changes to DNA. Developed from a natural defense mechanism found in bacteria, this method employs a guide RNA to direct the Cas9 enzyme to specific regions in the genome, where it makes targeted cuts. This allows scientists to delete, insert, or modify DNA sequences with remarkable precision. Since its emergence, CRISPR-Cas9 has revolutionized areas like biomedical science, agricultural development, and treatment of genetic disorders. It has been applied to correct mutations, improve crop varieties, and create disease-resistant animals. Advances such as base editing and prime editing have further improved its targeting accuracy. However, the technology also raises important ethical questions, especially concerning modifications to the human germline. This review discusses the CRISPR-Cas9 system's mechanism, its broad range of applications, recent developments, and the ethical challenges it presents.

Keywords: CRISPR-Cas9, gene editing, genome engineering, genetic diseases, agriculture.

INTRODUCTION

Gene editing is a rapidly evolving field within biotechnology, enabling researchers to precisely alter the DNA of an organism. This involves the insertion, deletion, or modification of specific genetic sequences and holds enormous potential for transforming medicine, agriculture, and environmental science. Earlier gene-editing technologies like zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were essential for the development of genome engineering. However, their widespread use was often restricted due to technical difficulties, high costs, and limited precision, which led to off-target genetic alterations [1].

The field experienced a major advancement with the emergence of the CRISPR-Cas9 system, which was adapted from a bacterial immune response mechanism. In 2012, Jennifer Doudna and Emmanuelle Charpentier demonstrated the system's ability to precisely edit genes in eukaryotic cells [2]. CRISPR-Cas9 utilizes a guide RNA to target the Cas9 protein to a specific location on the DNA, where it creates a double-strand break. This break activates the cell's natural repair processes, facilitating accurate genetic modifications.

Because of its simplicity, precision, and affordability, CRISPR-Cas9 has become the preferred tool for genetic engineering. It has been widely applied to correct inherited diseases, enhance

crop traits, and study gene functions [3]. Its versatility and efficiency have significantly advanced the field of genetic research across a variety of species.

This review will delve into the CRISPR-Cas9 system's mechanism, its diverse applications, recent technological advancements, and the ethical issues it raises. As the technology evolves, it fosters crucial discussions on how to use it responsibly, particularly in clinical and environmental contexts.

Mechanism of CRISPR-Cas9

CRISPR-Cas9 represents a revolutionary approach to genetic engineering, offering researchers the ability to precisely alter an organism's DNA. Originally discovered as part of a bacterial immune defense mechanism against viruses, the system consists of two main components: Cas9, an enzyme that acts like molecular scissors, and guide RNA (gRNA), which directs Cas9 to the correct DNA sequence. Below is a breakdown of the CRISPR-Cas9 mechanism:

1. Guide RNA (gRNA) Design and Targeting

The first step in the CRISPR-Cas9 process involves creating the guide RNA, a short RNA sequence complementary to the target DNA sequence. This guide RNA leads Cas9 to the exact DNA region that requires modification. The specificity of this targeting is guaranteed by the precise pairing of the gRNA with its corresponding DNA sequence.

2. Cas9 Enzyme Binding to DNA

After the guide RNA binds to the target DNA, the Cas9 enzyme is recruited to the DNA site. Cas9 is a type of nuclease that has the ability to cleave DNA. Once the target sequence is identified, Cas9 attaches to the DNA and forms a complex with the gRNA.

3. Cleaving the DNA with Cas9

At this stage, Cas9 induces a double-stranded break in the DNA at the location specified by the guide RNA. This precise cut is crucial for making targeted genetic modifications. Cas9 creates a blunt-ended break, cutting both strands of the DNA exactly at the point specified by the gRNA [4].

4. DNA Repair and Genetic Modifications

Following the DNA cleavage, the cell's natural repair processes attempt to fix the break. There are two primary DNA repair pathways:

Non-homologous end joining (NHEJ): This repair method is less accurate and can lead to insertions or deletions (indels) at the break site, which may disrupt the function of the gene, effectively "knocking out" the gene.

Homology-directed repair (HDR): This pathway is more precise and is employed when a repair template is available. The cell uses this template to repair the DNA accurately, allowing for the insertion of new genetic material or correction of the existing DNA sequence [5].

5. Types of Genetic Modifications

By exploiting these DNA repair pathways, scientists can introduce several types of genetic changes:

Gene knockout: Disrupting a gene to study its function or role.

Gene insertion: Adding new genetic material to analyze its effects or generate desired traits.

Gene correction: Fixing genetic mutations responsible for inherited disorders.

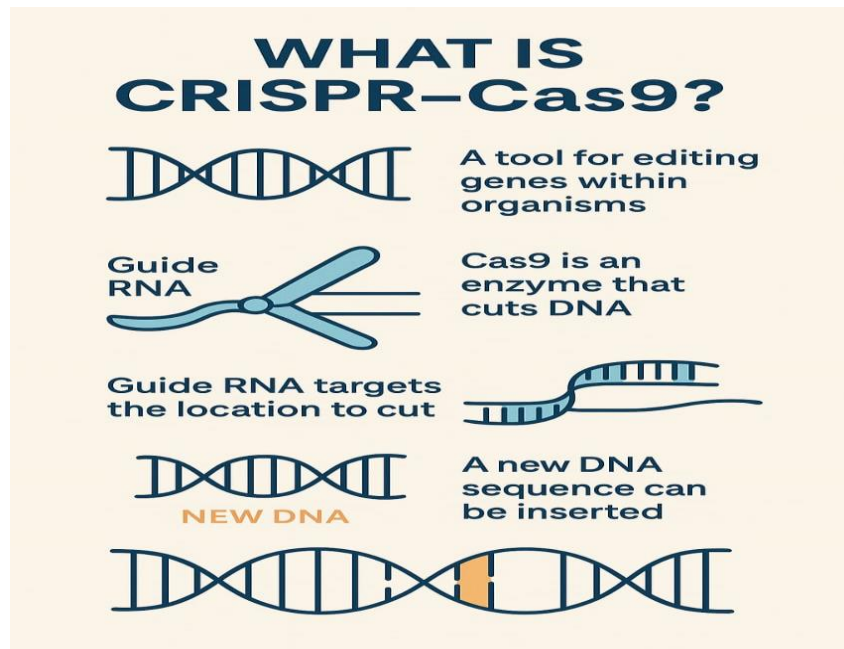


Figure 1 Mechanism of CRISPR-Cas9.

CRISPR-Cas9 Tools and Variants

1. Classic CRISPR-Cas9 (Type II)

The original CRISPR-Cas9 system was derived from *Streptococcus pyogenes*, a bacterium that employs this system as part of its defense against viral infections. The Cas9 enzyme, guided by a single-guide RNA (sgRNA), introduces a double-strand break (DSB) at a specific location in the DNA. The cell then repairs the break using its native repair mechanisms, such as non-homologous end joining (NHEJ) or homologous recombination (HDR).

Limitations

Off-target Activity: Cas9 has the potential to bind and cleave unintended genomic regions, which can result in off-target mutations. These undesired edits can reduce the precision and safety of genome modifications.

Size Constraints: The Cas9 protein (~1,400 amino acids) is relatively large, which can complicate its delivery, especially through viral vectors that have size restrictions.

Ethical Issues with Germline Editing: The use of Cas9 in modifying human germline cells (embryos or reproductive cells) raises ethical concerns. Unintended mutations could be inherited by future generations, causing long-term consequences.

2. CRISPR-Cas12 (Cpf1)

CRISPR-Cas12 (also referred to as Cpf1) is a Type V CRISPR system that functions similarly to Cas9 but with distinct mechanistic differences. Cas12 creates staggered cuts (sticky ends) in DNA, which can enhance the precision of genetic editing, especially in the case of homologous recombination. Additionally, Cas12 is smaller than Cas9, potentially improving delivery efficiency [6].

Limitations

Reduced Specificity: In certain cases, Cas12's targeting specificity may not match that of Cas9, and optimization of the guide RNA is necessary to minimize off-target cleavage.

Challenges in Delivery: Although smaller than Cas9, Cas12 still faces hurdles in efficient delivery, particularly in vivo. This often necessitates the development of advanced delivery strategies, such as nanoparticles or viral vectors.

Ethical Concerns in Germline Editing: Like Cas9, Cas12 raises significant ethical issues when it comes to modifying the human germline, especially in embryos, as these changes may be passed down to future generations.

3. CRISPR-Cas13 (C2c2)

CRISPR-Cas13 is a Type VI CRISPR system that targets RNA instead of DNA, making it a powerful tool for RNA editing. Cas13's ability to cleave RNA allows for non-permanent genetic alterations, making it ideal for studying gene function and for therapeutic RNA targeting. The system also exhibits collateral cleavage activity, where it indiscriminately cuts nearby RNA after recognizing its target [7].

Limitations

Off-target RNA Effects: While Cas13 is designed to cleave specific RNA sequences, it can also degrade unintended RNA, especially in the presence of complex transcripts, which could lead to unwanted effects.

Instability of RNA: RNA is inherently unstable and has a relatively short half-life in cells. Cas13's activity must be carefully timed to avoid excessive degradation of non-target RNA, which could induce cellular stress.

Efficiency in Mammalian Cells: Cas13's efficiency in RNA editing within mammalian cells remains a challenge. Optimization of the delivery system and protein expression levels is required for optimal performance.

4. CRISPR-Cas9 Variants for Enhanced Precision

a. High-Fidelity Cas9 (HF-Cas9)

HF-Cas9 is a modified version of the original Cas9, designed to improve targeting accuracy by minimizing off-target activity. Specific modifications to the Cas9 protein reduce its tendency to bind to unintended regions of DNA.

Limitations

Reduced Editing Efficiency: HF-Cas9, while more specific, often shows lower editing efficiency compared to the wild-type version. Achieving similar levels of editing might require higher concentrations of Cas9 or longer incubation periods.

Incompletely Eliminated Off-target Effects: Despite advancements in specificity, HF-Cas9 does not fully eradicate off-target cleavage. Off-target mutations may still occur, especially in genomic regions that share significant sequence homology with the target.

b. eSpCas9 (Enhanced Specificity Cas9)

eSpCas9 is another engineered Cas9 variant, optimized to enhance DNA binding specificity by reducing off-target interactions. This modification makes it ideal for applications requiring precise genome editing [8].

Limitations

Reduced Efficiency: Similar to HF-Cas9, eSpCas9 tends to exhibit lower editing efficiency than wild-type Cas9. Its increased specificity can sometimes hinder the enzyme's ability to cleave the intended target efficiently, especially in complex loci.

Emergence of New Off-target Sites: Even though eSpCas9 reduces off-target activity, the mutations it introduces may result in new off-target binding sites, which necessitates comprehensive validation in each application to avoid unintended edits.

5. CRISPR Base Editors

Base editors represent a class of genome-editing tools that enable the conversion of one DNA base into another without the need for double-strand breaks. These editors are composed of a catalytically dead Cas9 (dCas9) protein fused to a deaminase enzyme, which facilitates precise base substitutions, such as converting cytosine to thymine [9].

Limitations

Scope of Mutations: Base editors are limited to making specific types of mutations, particularly transitions (e.g., C to T or A to G), and cannot facilitate insertions or deletions, limiting their use for complex genetic modifications.

Off-target Mutations: Although base editors do not induce double-strand breaks, they can still cause off-target mutations, leading to unwanted genetic alterations at non-target locations.

Dependency on Repair Mechanisms: The efficiency of base editing can depend on the DNA repair processes present at the target site. Some genomic loci may not support efficient base editing, limiting the tool's effectiveness for certain applications.

6. CRISPR Prime Editing

Prime editing is an advanced CRISPR-based technology that enables precise DNA edits, such as insertions, deletions, or substitutions. This approach uses a prime editor, a fusion of a catalytically impaired Cas9 protein and reverse transcriptase, to directly edit the target DNA sequence [10].

Limitations

Challenges with Delivery: Prime editing requires the concurrent delivery of both the prime editor and a prime editing guide RNA (pegRNA), which complicates the delivery process. This remains a significant hurdle, particularly for in vivo applications where delivery technologies are still evolving.

Variable Efficiency: While prime editing provides exceptional precision, its efficiency varies significantly depending on the target site, cell type, and genomic context. In some cases, the editing process may not be as efficient as desired.

Off-target Editing Risks: Though rare, prime editing can still cause off-target effects. When the target sequence shares high homology with non-target regions, unintended edits may occur. Therefore, careful validation is required to ensure specificity.

7. CRISPR Interference (CRISPRi) and CRISPR Activation (CRISPRa)

CRISPRi and CRISPRa are systems that use dead Cas9 (dCas9) to regulate gene expression without inducing double-strand breaks. In CRISPRi, dCas9 is fused to a transcriptional repressor, while in CRISPRa, it is fused to an activator, allowing for gene silencing and activation, respectively.

Limitations

Incomplete Gene Regulation: CRISPRi may not always fully suppress gene expression, and CRISPRa might fail to achieve sufficient activation of the gene. The efficiency of these systems

can be highly context-dependent, depending on the target gene and the transcriptional regulators used.

Off-target Effects: As with other CRISPR-based tools, CRISPRi and CRISPRa are susceptible to off-target effects, particularly when guide RNAs bind to regions with sequence similarity to the intended target. This can lead to unintended changes in the expression of non-target genes.

Challenges in Delivery and Dosing: Efficiently delivering the necessary components (dCas9, guide RNA, and transcriptional regulators) remains a significant challenge. This is especially true when applying these systems in primary cells or in vivo models, where optimizing delivery strategies can be complex.

APPLICATIONS OF CRISPR-Cas9 IN GENE EDITING

1. Gene Knockout and Knock-in

Gene knockout refers to the intentional disruption or deactivation of a gene to explore its function, while gene knock-in involves inserting a specific gene or mutation into a particular site in the genome. The CRISPR-Cas9 system is commonly used for both approaches due to its ability to accurately target and modify specific genes.

Gene Knockout: This technique involves the creation of double-strand breaks (DSBs) at the site of the target gene. The cell repairs these breaks through error-prone mechanisms, which typically result in frameshift mutations that disable the gene's function. This method is often employed to study gene functions or create disease models.

Gene Knock-in: Here, CRISPR facilitates the insertion of foreign DNA or specific mutations at designated genomic locations. This is critical for creating genetically modified organisms (GMOs), which have applications in research and therapy, such as in gene therapy [11-12].

2. Gene Therapy

Gene therapy involves correcting or replacing defective genes to treat genetic disorders. CRISPR-Cas9 is a precise tool that allows for direct DNA sequence modifications, enabling the correction of genetic mutations or the insertion of missing genes.

In Vivo Gene Editing: CRISPR-Cas9 has been used in preclinical studies to correct genetic mutations that lead to inherited diseases such as sickle cell anemia and muscular dystrophy.

Ex Vivo Gene Editing: In this method, CRISPR is used to edit cells from the patient, such as T-cells or stem cells, which are then reintroduced into the patient's body to treat conditions like genetic disorders or cancer [13-14].

3. Disease Modeling

CRISPR-Cas9 plays a significant role in generating both in vitro and in vivo models of human diseases. These models are essential for studying the underlying molecular mechanisms of diseases and testing new treatments. By editing genes linked to conditions like cancer, neurodegenerative diseases, and cardiovascular disorders, researchers can create more accurate models for therapeutic research.

Cancer Models: CRISPR is used to introduce mutations that mirror the genetic changes found in human cancers, providing valuable insights into cancer progression, drug resistance, and the mechanisms of tumor formation.

Neurodegenerative Disease Models: Using CRISPR, researchers can induce mutations related to Parkinson's disease, Alzheimer's disease, and Huntington's disease, creating models that help study these conditions and develop potential therapies [15-16].

4. Agricultural Biotechnology

CRISPR-Cas9 has greatly advanced agricultural biotechnology, enabling precise genetic modifications in both plants and animals. These modifications result in organisms with enhanced traits such as improved disease resistance, better nutritional value, and increased resilience to environmental stresses.

Crop Improvement: CRISPR has been used to create genetically modified crops that can resist diseases, pests, and harsh environmental conditions, like drought. For instance, CRISPR-edited rice has been developed to improve yield and resist bacterial blight.

Livestock Engineering: In animals, CRISPR allows for the creation of livestock with beneficial traits, such as greater disease resistance, accelerated growth, and improved meat quality [17-18].

5. Epigenetic Modifications and Gene Regulation

The CRISPR-Cas9 system, particularly the dead Cas9 (dCas9) variant, is also being used to regulate gene expression without altering the DNA itself. This is achieved by linking dCas9 to transcriptional activators or repressors, providing a mechanism for precise control of gene expression in research and therapy. **CRISPR Interference (CRISPRi):** In this approach, dCas9 is fused with repressor proteins to block gene expression. **CRISPR Activation (CRISPRa):** Alternatively, dCas9 is fused with activator proteins to enhance gene expression [19-20].

6. Microbial Genome Editing

CRISPR-Cas9 is also applied in microbial genome editing, including in bacteria and yeast, to address a wide range of challenges, from basic research to industrial applications such as biofuel production, antibiotic development, and the creation of pharmaceuticals.

Bacterial Engineering: Researchers use CRISPR-Cas9 to enhance bacterial strains, enabling them to carry out processes like bioremediation, improved antibiotic production, and greater efficiency in environmental cleanup.

Yeast Engineering: In yeast, CRISPR is used to optimize fermentation processes, increase yields, and create new pathways for synthesizing chemicals and pharmaceutical compounds [21-22].

Ethical, Legal, and Social Considerations

Ethical Considerations

CRISPR-Cas9 has transformed genetic research by enabling precise and affordable gene editing. Despite its promise, it raises complex ethical issues. One key concern involves germline editing, which alters the DNA of embryos or reproductive cells, with changes passed on to future generations. Although this technology could potentially eliminate inherited disorders like Huntington's disease or cystic fibrosis, it also prompts serious ethical debates. For example, modifying traits unrelated to health, such as intelligence or physical appearance,

might lead to “designer babies” and raise concerns about a new form of eugenics, potentially increasing social inequality [23].

Informed consent poses another ethical challenge, especially since unborn embryos cannot agree to genetic modifications made on their behalf. Additionally, the possibility of unintended mutations, known as off-target effects, could result in unforeseen harm, urging caution before clinical use.

Legal Considerations

Laws regulating CRISPR-Cas9 differ widely across countries. In the United States, the use of germline editing for clinical purposes is banned federally, primarily due to restrictions on funding, although somatic cell editing continues under FDA supervision. In Europe, the Oviedo Convention explicitly bans heritable genome modifications. Meanwhile, China’s regulatory framework has been less defined, as illustrated by the case of He Jiankui, who edited the genes of twin babies and was subsequently imprisoned for breaching ethical and legal standards [24]. There is a pressing need for unified international regulations to promote ethical standards, prevent misuse, and encourage responsible scientific progress. However, differing national policies and cultural perspectives make this difficult to achieve.

Social Considerations

The social impact of CRISPR technology is significant. If gene-editing therapies remain accessible only to affluent individuals, existing health and social inequalities could worsen. Moreover, public skepticism and distrust may grow if advances outpace ethical oversight and clear communication. Experiences with genetically modified organisms (GMOs) have shown that insufficient transparency and public involvement can provoke resistance.

To address these challenges, it is essential to engage the public in open discussions and for scientists and policymakers to communicate transparently, ensuring the benefits of CRISPR are fairly distributed [25].

Challenges and Risks of CRISPR-Cas9

A key challenge associated with CRISPR-Cas9 is the occurrence of **off-target effects**, where the gene-editing tool inadvertently alters DNA sequences other than the intended ones. Such unintended modifications can lead to harmful mutations or interfere with crucial genes, potentially resulting in unforeseen health consequences [26]. Enhancing the tool’s precision and accuracy remains a vital focus in ongoing research efforts.

Another important technical issue involves the **delivery of CRISPR components**. Successfully transporting these elements into the appropriate cells without triggering immune reactions or causing tissue damage is difficult, which limits the potential for therapeutic applications [27].

Ethical and Social Risks

CRISPR-Cas9 raises significant ethical concerns, particularly regarding **germline editing**, where changes to the genome are passed on to future generations. Because these modifications are irreversible, they present unpredictable risks and complex moral questions related to altering human heredity from a social perspective, unequal access to gene-editing technology could worsen health disparities between wealthy and disadvantaged groups. Additionally, there are concerns about the potential misuse of CRISPR for enhancement purposes beyond medical necessity, which might increase social division or discrimination [28].

Regulatory and Safety Concerns

Regulatory oversight of CRISPR varies widely around the world and is often fragmented, creating challenges for the ethical and safe implementation of the technology. The absence of comprehensive international guidelines raises the possibility of unethical experimentation and premature clinical application.

Moreover, the long-term safety of CRISPR remains uncertain. There is a need for thorough monitoring and follow-up to detect any delayed adverse effects or unexpected consequences before the technology can be broadly used in humans.

Future Perspectives of CRISPR-Cas9

1. Advancements in Precision and Efficiency

Future advancements in CRISPR-Cas9 technology are focused on improving its precision and minimizing off-target effects. Innovations like base editing and prime editing are being explored to enable more accurate gene modifications without inducing double-stranded DNA breaks. These developments are expected to significantly reduce the likelihood of unwanted mutations, resulting in safer and more reliable outcomes for clinical applications [29].

2. Therapeutic Applications

CRISPR-Cas9 has the potential to transform the treatment landscape for a variety of genetic disorders. Clinical trials are already investigating its use for conditions such as sickle cell anemia, thalassemia, and certain cancers. In the future, CRISPR could be used to address complex diseases like Alzheimer's and heart disease, provided that further research helps us better understand the underlying genetic mechanisms. Additionally, personalized medicine, where CRISPR is used to customize treatments based on an individual's genetic makeup, is a highly promising area.

3. Agriculture and Environmental Use

CRISPR is also poised to make a significant impact outside of healthcare, particularly in agriculture and environmental science. It could be employed to develop crops that are resistant to diseases, improve their nutritional profile, and decrease the need for chemical pesticides. Moreover, CRISPR may play a role in environmental conservation, such as controlling invasive species or altering disease vectors like mosquitoes by editing their reproductive genes [30]. However, these uses must be carefully regulated to ensure ecological balance and ethical responsibility.

4. Global Governance and Ethical Standards

As CRISPR technologies continue to advance, establishing global regulations and ethical guidelines becomes increasingly important. A unified international framework will be essential to ensure that gene-editing tools are used in a responsible and equitable manner. For instance, initiatives like the World Health Organization's expert committee on human genome editing emphasize the need for strong governance to ensure that future research and clinical trials are conducted ethically [31].

History of CRISPR-Cas9

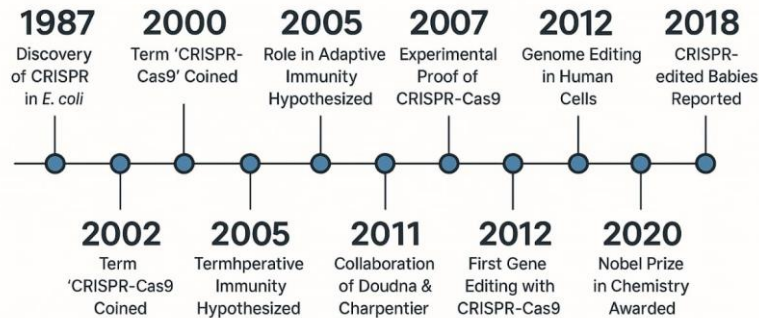


Figure 2 Advancement of CRISPR-Cas9 in Gene Editing.

Conclusion

CRISPR-Cas9 has revolutionized the field of gene editing, offering precise and efficient tools to modify genomes across various organisms. Its potential to treat genetic disorders like sickle cell anemia and thalassemia is already being tested in clinical trials (Frangoul et al., 2021), while its applications in agriculture and biotechnology continue to expand. However, the technology also raises significant concerns. Off-target effects, where unintended genetic alterations occur, remain a challenge for its precision (Kosicki et al., 2018). Ethical issues surrounding germline editing and the potential for designer babies require careful deliberation to ensure responsible use (Gyngell et al., 2017). Additionally, unequal access to CRISPR technologies could exacerbate health disparities (Jasanoff & Hurlbut, 2018). Moving forward, the future of CRISPR will depend on improving accuracy, establishing international regulatory standards, and fostering global ethical dialogues (Anzalone et al., 2019), ensuring its safe and equitable use.

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