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Advances in CRISPR/Cas9 Gene Editing Technology, its applications and Immunotherapy in India: A Review

Dr. Nidhi Vadhavekar[1], Dr. Manu Pandya[2], Yuvraj Sawant[3], Saloni Chatwani[4], Dr. Ameesha Gupta[5], Dr. Aanya yalamanchi[6], Jasmit Kaur Puri[7], Dr. Anant Patil[8]

[1,2,3,5,6,7]MBBS, Padmashree Dr. D.Y.Patil School of Medicine
[4]MBBS intern, Dr. Vasantrao pawar Medical College
[8]MBBS, MD pharmacology, Padmashree Dr. D.Y.Patil School of Medicine

ABSTRACT

BACKGROUND: This review explores CRISPR/Cas9 gene editing, detailing its mechanism and broad applications. It covers bacterial genome modifications and significant clinical advancements, including its role in cancer immunotherapy (e.g., CAR-T cells, viral targeting) and treating genetic blood disorders. Recent innovations like Prime Editing and advanced delivery methods are highlighted. The review also examines CRISPR's status and challenges in India, noting regulatory gaps despite progress in diagnostic tool development. It concludes by emphasizing the need for continued innovation, ethical guidelines, and robust regulation to fully realize CRISPR's potential amidst ongoing technical hurdles.

Methodology: This comprehensive narrative review synthesized information from a targeted search of scientific literature across databases like PubMed, Scopus, and Google Scholar, using specific keywords to identify advancements, applications, and the Indian context of CRISPR/Cas9 gene editing. Information was then critically analyzed and organized thematically.

Results: The review extensively explores the significant clinical advancements achieved through CRISPR/Cas9, including its pivotal role in cancer immunotherapy—specifically in developing chimeric antigen receptor T (CAR-T) cells and targeting oncogenic viral infections (HBV, EBV, HPV, JCV)—and its noted potential in treating hereditary blood disorders like sickle cell anemia and \beta-thalassemia. Recent technological innovations, such as Prime Editing (PE) and Prime Editing and Template Integration (PASTE) for precise insertions, the application of catalytically inactive dCas9 (CRISPRi/a) for gene regulation, and various advanced delivery systems, are also discussed as key outcomes of ongoing research and development. Furthermore, the analysis of CRISPR's status in India highlights progress in developing diagnostic tools for infectious diseases (e.g., Tata MD CHECK SARS-CoV-2 Kit 1.0, FELUDA) and improvements in Cas9 enzyme engineering.

Conclusion: CRISPR/Cas9 is a revolutionary gene-editing tool with transformative potential across bacterial engineering and diverse clinical therapies, including cancer immunotherapy and genetic disorder treatment. Despite remarkable advances, persistent challenges like off-target effects, delivery hurdles, and, specifically in India, the urgent need for clear ethical and regulatory frameworks, must be addressed.

Realizing CRISPR's full benefits demands continued scientific innovation alongside responsible ethical guidance and robust policy development.

Keywords: CRISPR, CRISPR/Cas9, IMMUNOTHERAPY, GENE EDITING, CANCER

1. Introduction

Recent discoveries related to the Clustered regularly interspaced short palindromic repeats (CRISPR), CRISPR-associated (Cas) proteins have redefined and largely extended the applications as well as the approaches of gene therapy[1]. Gene therapy is defined as the ability of causing genetic enhancement by rectification of mutated or altered genes and site specific amendment which target a therapeutic treatment[2]. The main aspect in genome editing is the generation of double stranded break(DSB) in the DNA, at a particular locus within the genome; which is achieved using “molecular scissors”[3]. CRISPR is defined as the naturally existing RNA mediated adaptive immune system defense in the bacteria and archaea that protects them against phages[4,5]. This system renders immunity against both the bacteria and the viruses, killing them by eradicating the genetic material[4]. CRISPR/Cas system domain was classified into two classes. CRISPR/Cas Class I system is multimeric and has effector complexes which consist of numerous Cas proteins, whereas CRISPR/Cas Class II system is monomeric, effector complexes have a single, multi-domain Cas protein[5]. The CRISPR/Cas9 gene editing system which has been a source of tremendous amounts of discoveries in the last decade is a type II CRISPR system and two component domain[1,4]. The two components of this gene editing system are the single guide RNA(sgRNA) and Cas9 nuclease. This two component domain has consistently shown to have high specificity, sensitivity and efficacy which allowed its application into the research for treatment of tumors and other disorders, infectious diseases[4]. Few advantages of CRISPR/Cas9 domain that prove its superiority over other forms of CRISPR/Cas9 domains and other forms of immunotherapy are-its ability to be expanded into various therapeutic measures, its flexibility, its cost effectiveness and the wide range of applications that it can be easily incorporated into[5,6]. It was employed to identify genes that affect cancer cell- survival, multiplication, drug resistance and migration[7]. Immunotherapy has come out as a promising approach in treating various diseases, including cancer and carcinogenic viral infections; in order to overcome drug resistance[4].

This review article aims at providing a comprehensive overview of the variants of the CRISPR gene editing system, with a specific focus on the CRISPR-Cas9 domain, mechanism, and its applications. Additionally, this article highlights the recent advances in immunotherapy in India, discussing the challenges and potential solutions in its application.

2. Methodology

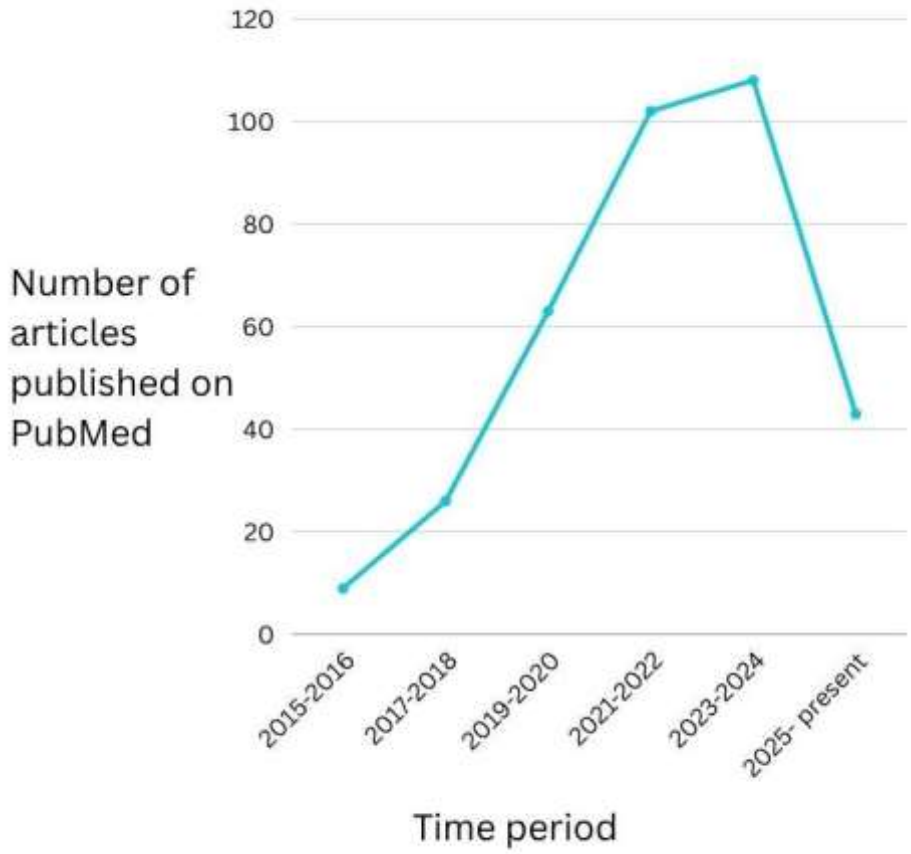
This review synthesizes current knowledge and recent advancements pertaining to CRISPR/Cas9 gene editing technology, its diverse applications, and its emerging role in immunotherapy, with a particular focus on the landscape within India. The methodology employed for this comprehensive narrative review involved a targeted and systematic search of peer-reviewed scientific literature and relevant policy documents.

2.1Search Strategy:

A thorough search was conducted across prominent electronic databases, including PubMed, Scopus, and Google Scholar. The search queries were formulated using combinations of keywords such as "CRISPR-

Cas9," "gene editing," "genome engineering," "clinical applications," "immunotherapy," "CAR T-cells," "viral infections," "genetic disorders," "Prime Editing," "PASTE," "CRISPRi/a," "delivery systems," and "India." No specific date restrictions were applied to capture the breadth of advancements since the inception of CRISPR technology, up to the present. Reference lists of highly relevant articles and key reviews were also scrutinized for additional pertinent sources.

Figure No.. 1: Trends of the Number of articles published on PubMed about CRISPR over the last 10 years



2.2Inclusion Criteria:

Articles, reviews, and reports were included if they provided significant insights into:

The fundamental mechanisms and recent technological enhancements of CRISPR/Cas9.

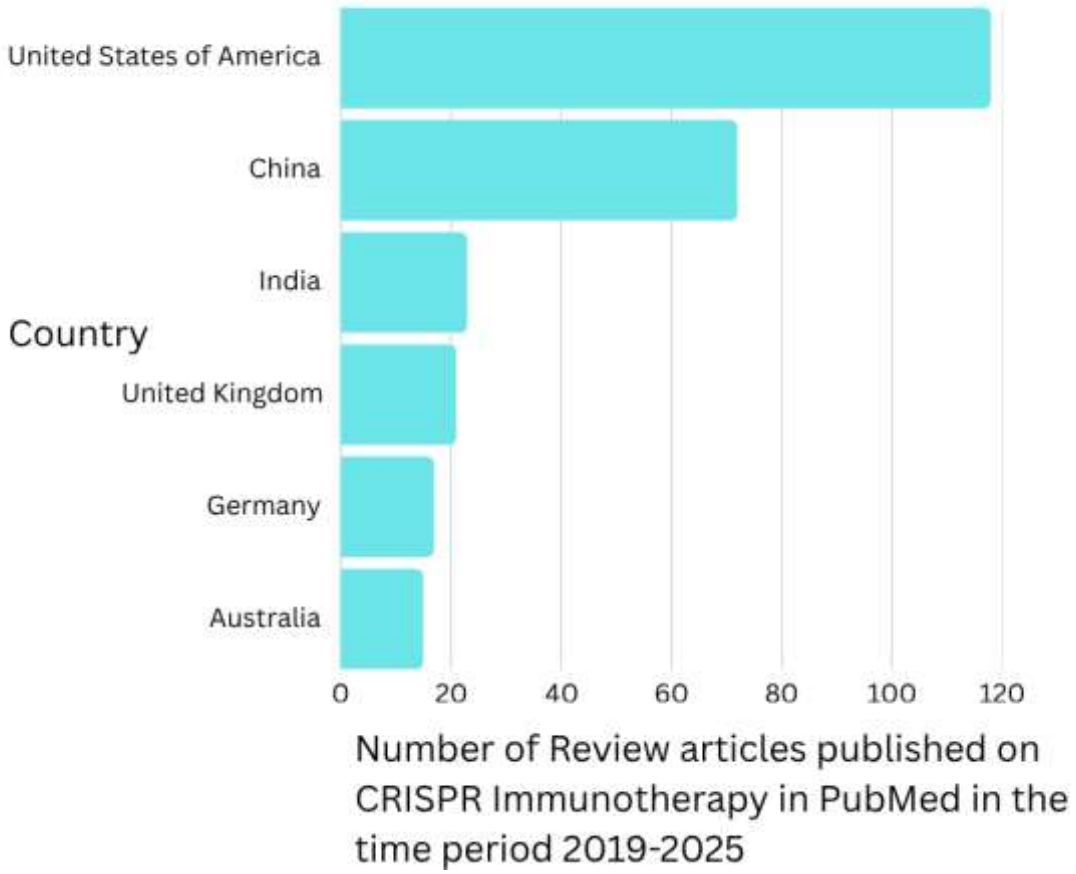
Diverse applications of CRISPR/Cas9 in bacterial and eukaryotic systems.

Specific clinical applications, especially in oncology and hematology.

Novel delivery methods for CRISPR components.

The status, challenges, and ethical considerations of CRISPR technology in India.

Figure no. 2. Trends of the number of review articles published over last 6 years in various countries on PubMed



2.3Data Extraction and Synthesis:

Information from the identified literature was systematically extracted, summarized, and critically analyzed. The extracted data included details on CRISPR mechanism, technological innovations (e.g., Prime Editing, PASTE), various application areas (e.g., bacterial genome editing, CAR-T cell development, targeting viral infections, treatment of blood disorders), and the regulatory and developmental landscape specific to India. The synthesized information was then organized thematically to provide a coherent and comprehensive overview of the current situation of CRISPR/Cas9 technology, its applications, and its implications, highlighting both progress and persistent challenges.

3. Discussion

3. 1. Mechanism of CRISPR

The CRISPR/Cas system consists of- sgRNA, a combination of crRNA (which binds to the target DNA) and tracrRNA (which activates Cas), along with the Cas endonuclease that forms double-strand DNA breaks (DSBs). The sgRNA guides Cas9 to a particular site near a PAM chain, with the break occurring about three base pairs upstream of this pattern. The prevalent PAM chain for Cas9 is 5'-NGG-3', with "N" representing any nucleotide. After binding, Cas9 separates the DNA strands and forms a RNA-DNA hybrid.It later activates its nuclease domains: the HNH domain cleaves the DNA strand complementary to the sgRNA, and the RuvC domain cuts the non-complementary strand, producing blunt ends. When a

homologous DNA template is available, the cell can utilize homology-directed repair (HDR) to fix the break, therefore allowing accurate genetic alterations.The genome editing process involves recognition, cleavage, and repair stages and varies based on the target nucleic acid and Cas variant used[9].

3.1.1 CRISPR/Cas9 based Genome editing

3.1.1.1 In E.coli

E. coli is a widely studied bacterium in biology due to its simple genome, rapid growth, and low cost, making it an ideal model for CRISPR-Cas9 applications. Sapranauskas et al. showed that a foreign CRISPR-Cas9 system could be effectively introduced into E. coli, therefore conferring immunity against plasmids and phages. Jiang et al. were the first to achieve precise site-specific mutations in E. coli using a dual RNA-guided Cas9 system. Researchers further utilized CRISPR-Cas9 to delete or insert entire genes. The Cas9 nickase (nCas9), produced via D10A mutation, which cuts only one DNA strand, enabled the development of base and prime editing technologies for introducing point mutations without double-strand breaks (DSBs). Despite these advances, challenges like low efficiency for editing long sequences, difficulties in multiplex editing, and limited applications in some strains persist [10].

3.1.1.2The editing of longer DNA sequences

Researchers have advanced the engineering of large DNA fragments in E. coli. Li et al. had achieved over 90% efficiency in deleting approximately 12 kb of genomic DNA using double-stranded donor DNA, compared to less than 20% with single-stranded DNA. This improvement is due to decreased mismatch repair correction during homologous recombination, as single-stranded DNA edits near the replication forks often trigger mismatch repair, thereby decreasing efficiency. Using double-stranded DNA minimizes these errors and enhances chances of success. Bassalo et al. noted an increased editing efficiency by mutating the upstream homology arm to disable the PAM sequence, preventing Cas9 from repeatedly cutting the same site, enabling the insertion of a 10 kb pathway in a single step. Wang et al. engineered the REXER system, combining CRISPR-Cas9 with λ-red recombination in order to facilitate the insertion of more than 100 kb of synthetic DNA into the E. coli genome [10].

3.1.1.3 Multiplex genome editing

CRISPR-Cas9 systems have been modified to enable simultaneous editing of multiple genomic sites in E. coli, reducing effort and time. Liu et al. combined CRISPR-Cas9 with λ-red recombination to enable the modification of two genes at once and achieving 88.0% efficiency for double-point mutations, but only 38.7% for deletions or insertions at two loci. Li et al. developed a single-plasmid CRISPR-Cas9 system expressing multiple gRNAs to target different sites, allowing the concurrent codon replacements in three genes with an efficiency of only 23.0% [10].

3.1.1.4 Expanding scope of strains

Efforts have been taken in order to expand the range of E. coli strains compliant to CRISPR-Cas9 editing. E. coli BL21, is a frequently used natural product for synthesis. It has been facing challenges due to leaky gRNA expression, which can cause plasmid loss and reduce editing efficiency. Researchers addressed this by replacing or removing the promoter responsible for gRNA expression, resulting in improved genome editing in BL21. Additionally, Rubin et al. engineered the DNA-editing all-in-one RNA-guided CRISPR-Cas transposase (DART) system, which allows precise editing of particular E. coli subspecies within complex microbial communities without a need for isolating individual species. Hence, ultimately overcoming traditional limitations of genome editing [10].

3.1.1.5 In other bacteria

CRISPR-Cas9 systems have been used to genetically engineer diverse bacterial species, like- Streptococcus pneumoniae, Tatumella citrea, Bacillus subtilis, Streptomyces coelicolor, Saccharopolyspora erythraea, and Corynebacterium glutamicum, for the production of natural compounds. The CRISPR interference (CRISPRi) system has been utilized in Methylorubrum extorquens and C. glutamicum, while the CRISPR activation (CRISPRa) system has also been employed in B. subtilis and Pseudomonas putida. In C. glutamicum, Yao et al. has constructed a single-plasmid CRISPR-Cas9 system that includes the cas9 gene, gRNA, and homologous arms on a temperature-sensitive plasmid, achieving a high genome editing efficiency, of over 95.7% [10].

3.1.1.6 Manual CRISPR/Cas9 genome editing

Despite the availability of high-frequency and automated CRISPR-Cas9 systems, manual protocols are still essential for gene function studies. This is mainly seen in suspension cell lines such as Jurkat, K562, and U937, which are extensively used in immunology and cancer research [10]. These manual methods allow precise control as well as customization of editing conditions for various gene targets[11].

The process begins with designing sgRNAs using tools like CRISPOR or Benchling to predict on-target efficiency and off-target risks. The sgRNAs are synthesized or transcribed in vitro and then combined with recombinant Streptococcus pyogenes Cas9 protein to form RNP complexes[11].

RNP delivery is preferred over plasmid or viral methods due to its transient activity, reducing off-target effects and preventing foreign DNA integration [11]. Electroporation is more commonly employed to introduce RNPs into human suspension cells, as it temporarily permeabilizes the cell membrane without the use of vectors [10].

Screening typically involves PCR amplification of the target site which is followed by Sanger sequencing, along with analytical tools like TIDE or ICE, which used to quantify indels and assess editing efficiency [12]. In order to enhance precision, inducible Cas9 systems regulated by doxycycline, tamoxifen, or small molecules are being increasingly used for temporal control of gene editing [11]. High-affinity Cas9 variants like- eSpCas9 and SpCas9-HF1, have also been developed to decrease off-target effects without compromising efficiency.

Overall, manual CRISPR-Cas9 remains a fundamental technique in human gene editing, particularly for research requiring high customization. It underpins models of blood cancers, immune deficiencies, and complex diseases, and contributes to the development of gene therapies and cell-based treatments [13].

3.1.1.7 Automated CRISPR/Cas9 genome editing

Automated CRISPR-Cas9 genome editing marks a major advancement in functional genomics, allowing high-frequency, reproducible gene modifications across various human cell types which is more efficient. Unlike manual approaches which mainly require individual optimization, automation which involves integration of robotics, software-guided design, and liquid handling to streamline workflows [11]. Guides are synthesized and assembled with Cas9 into ribonucleoprotein complexes via robotic systems, ensuring consistent reagent handling and reducing human error [14]. Delivery methods include automated electroporation or microfluidic platforms that standardize parameters such as voltage, pulse duration, and sample volume [15]. Automated cell handling systems facilitate uniform plating and incubation, while robotic FACS or clone-picking systems enable rapid isolation of edited clones [13]. Post-expansion, automated genotyping processes perform PCR, thermal cycling, and sequencing without manual input, with data analysis tools like CRISPResso or ICE integrated into laboratory information management systems (LIMS) to evaluate editing efficiency across large sample sets [14]. These advancements have

driven large-scale CRISPR screens and accelerate the development of cell-based models for drug discovery, immunotherapy, and personalized medicine [15].

3.2. Classification of CRISPR-Cas Systems

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) systems are adaptive antivirus immunity mechanisms found in most archaea and many bacteria, functioning on the principle of self-nonself discrimination [16]. A diverse array of Cas (CRISPR-associated) proteins are integral to various stages, including the processing of CRISPR loci transcripts, the cleavage of target DNA or RNA, and the integration of new spacers [17-19]. The current classification of CRISPR-Cas systems broadly includes three common types (I, II, and III) and a less common type IV [17, 20, 21]. This classification primarily relies on their respective signature genes and the typical organization of their loci [22].

3.2.1 Type I CRISPR-Cas Systems

All Type I loci are characterized by the presence of the signature gene cas3. This gene encodes a large protein that possesses a helicase domain with single-stranded DNA (ssDNA)-stimulated ATPase activity, facilitating the unwinding of DNA-DNA and RNA-DNA duplexes [22]. Frequently, this helicase domain is coupled with an HD family domain, which exhibits endonuclease activity and is involved in the cleavage of targeted DNA [22, 23].

Type I systems are typically encoded by a single operon containing the cas1 and cas2 genes, along with genes for the subunits of the Cascade or effector complex, which include a large subunit and a small subunit (often fused to the large subunit). Type I systems are further subdivided into six subtypes: I-A to I-F, each distinguished by its own signature gene and unique operon organization. Subtypes I-E and I-F are notable for lacking the cas4 gene, unlike other subtypes. The relationships between these subtypes are based on Cas1 phylogeny (Subtypes I-A, I-B, I-C, I-E, and I-F), largely corresponding to previously proposed classifications [24], with the exception of the Hmari and Tneap subtypes, which have been combined into subtype I-B [17].

3.2.2 Type II CRISPR-Cas Systems

The defining characteristic of Type II CRISPR-Cas systems is the signature gene cas9. This gene encodes a multidomain protein that consolidates all the functions of the effector complexes and is crucial for target DNA cleavage and the maturation of the crRNA [25]. Type II systems are also commonly referred to as "HNH" systems. They are currently categorized into three subtypes: II-A, II-B, and II-C. The recently proposed Type II-C CRISPR-Cas systems are relatively simple, possessing only three protein-coding genes (cas1, cas2, and cas9), and are frequently observed in sequenced bacterial genomes [20, 26, 27].

3.2.3 Type III CRISPR-Cas Systems

All Type III systems contain cas10 as their signature gene. This gene encodes a multidomain protein with a palm domain structurally similar to those found in cyclases and polymerases of the PolB family [28, 29]. Cas10 functions as the large subunit of the effector complexes in Type III systems. Each Type III locus also encodes other subunits of these effector complexes, typically including one gene for a small subunit, one gene for a Cas5 group RAMP protein, and usually several genes for RAMP proteins of the Cas7 group. Interestingly, Type III CRISPR-Cas systems often do not encode their own cas1 and cas2 genes, instead utilizing crRNAs generated from CRISPR arrays associated with Type I or Type II systems [30, 31]. However, in numerous genomes that lack Type I and Type II systems, cas1, cas2, and cas6 genes are directly linked to a Type III system, suggesting it is fully functional independently [7]. Currently, there are two recognized subtypes within Type III: III-A (formerly the Mtube subtype or Csm module) and III-

B. While clearly related, these subtypes can be differentiated by the presence of distinct genes for the small subunits of effector complexes, namely csm2 for III-A and cmr5 for III-B. Type III-A loci frequently possess cas1, cas2, and cas6 genes and have been demonstrated to target DNA, whereas most Type III-B systems lack these genes and are consequently dependent on other CRISPR-Cas systems within the same genome. Type III-B CRISPR-Cas systems have been shown to target RNA [21, 30, 32-34].

3.2.4 Type IV CRISPR-Cas Systems

Type IV CRISPR-Cas systems are found in several bacterial genomes, often located on plasmids. Similar to subtype III-A, this system typically lacks cas1 and cas2 genes and is often not associated with CRISPR arrays. The Type IV systems possess an effector complex composed of a highly reduced large subunit (csf1), two genes for RAMP proteins of the Cas5 (csf3) and Cas7 (csf2) groups, and, in some cases, a gene for a predicted small subunit [35]. The csf1 gene can be considered a signature gene for this system. There are two distinct subtypes of Type IV systems: one contains a DinG family helicase csf4 [36], while the second subtype lacks DinG but typically includes a gene for a small alpha helical protein, presumed to be a small subunit [35].

3.3. Clinical Applications of CRISPR/Cas9

CRISPR-Cas9 mediated genome editing presents a revolutionary approach to gene therapy, with immense potential for treating a wide range of diseases. The provided text highlights several promising applications and advantages of this technology:

3.3.1 Advantages and Applications of CRISPR-Cas9 in Gene Therapy

- Broad applicability of Programmable Nucleases: Zinc-finger nucleases, TALEs, and CRISPR-Cas9 systems are powerful tools for genome editing and engineering [4].
- Cancer Immunotherapy:
  - CAR-T cell therapy enhancement: CRISPR-Cas9 can be used to engineer autologous T cells ex vivo to express cancer-specific antigens, making CAR-T cell therapy more effective and safer [40]. It can expand the patient population eligible for this therapy.
  - Overcoming MHC mismatches: CRISPR-Cas9 can knock out genes like B2M in human primary CD4+ T cells, abrogating surface MHC-I expression. This allows for the creation of "universal" T cells transferable to many cancer patients regardless of HLA genotypes, as demonstrated by CAR19 T cells showing robust anti-leukemic responses in B-cell malignancy patients [4].
  - Targeting Cancer Genome Sequences: It can directly alter cancer-causing mutations [4].
  - Epigenome Mapping: Useful for understanding and potentially modulating gene expression in cancer [4].
  - Identification of Immune-Regulatory Genes: CRISPR-Cas9 screening can precisely identify genome-wide immune-regulatory genes that modulate the tumor microenvironment or enhance anti-tumor immune cell activity. This is crucial for developing next-generation cancer immunotherapies [37]. Examples include validating PD-L1 and CD47 as known immune-escape molecules, and discovering that interruption of IFN-γ signaling and knockdown of DEAH-Box Helicase 37 (DHX37) can enhance T cell responses [42]. It also identified ZNF143 and NUDT21 as CD19 regulators [42].
- Targeting/Inactivating Cancer-Causing Viral Infections:
  - HBV suppression: CRISPR/Cas9 can directly target HBV covalently closed circular DNA (cccDNA) and integrated HBV DNA, or indirectly degrade HBV RNAs or target host proteins, offering a promising therapeutic approach for HCC [38].

- HPV16 and HPV18: The system can selectively target cell lines transformed by HPV, which induce cervical carcinoma due to viral proteins E6 and E7 [4].
- EBV ablation: CRISPR/Cas9 can target and ablate EBV gene expression, providing a method to reduce EBV infection during its latent phase and offering therapeutic potential for EBV-related cancers (Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, gastric cancer) [4].
- JCV cleavage: CRISPR/Cas9 has the potential to cleave JCV genomes, offering a promising therapeutic approach to defeat progressive multifocal leukoencephalopathy (PML) [4].
- HSV-1 treatment: The CRISPR-Cas9 genome editing approach can target both actively replicating viruses and latent viral reservoirs of HSV-1, addressing a significant public health challenge by targeting immediate early genes like ICP0 and ICP27 [4]. Utilizing multiplex gRNA, researchers created successful deletions of large viral genome fragments, blocking the possible creation of escape mutant viruses [4].

- Treatment of Blood Disorders: CRISPR-Cas9-based gene therapy has shown promising results for severe sickle cell anemia and transfusion-dependent β-thalassemia, offering hope for other blood disorders with a genetic predisposition to hematologic cancers [41].
- Disease Modeling and Diagnostics: Beyond therapy, CRISPR-Cas9 is also valuable for creating disease models and developing diagnostic tools [39].
- Confirmation of Gene Encoding: Genome-wide CRISPR/Cas9 screening confirmed that the MS4A1 gene encodes CD20, the target of rituximab, and found that interferon regulatory factor 8 (IRF8) affects anti-CD20 antibody-induced cytotoxicity and phagocytosis [42].

3.3.2 Challenges to Full Realization in India

While the prospects are exciting, several significant challenges need to be recognized and addressed for CRISPR-Cas9 gene therapy to be fully realized in India:

1. Safety Concerns:
   - Off-target effects: CRISPR-Cas9 can cause unintended genetic alterations at sites other than the intended target. While precision has improved, unintended edits can lead to cell death, large deletions, chromosomal disorganization, or even malignant tumors.
   - Host Immunogenic Responses: The delivery of CRISPR components (Cas9 protein, viral vectors) can trigger an immune response in the patient, potentially reducing efficacy or causing adverse reactions.
2. Delivery Methods: Current delivery methods for CRISPR/Cas9 can be inefficient and inconsistent, especially for in planta transformation or targeting specific cell types in vivo.
3. Cost and Accessibility: Gene therapies are often very expensive. Ensuring affordability and scalability of these therapies is crucial for them to benefit a larger population in India.
4. Regulatory Framework and Governance: Gene therapy products are complex and require rigorous scientific evaluation. While India has made progress with guidelines and rules (like the "National Guidelines for Gene Therapy Product Development and Clinical Trials 2019" and the New Drugs and Clinical Trials Rules 2019), the regulatory process can still be ambiguous. There's a need for a strong and evolving framework that keeps pace with rapid scientific advancements and ensures ethical, scientific, and safe implementation.
5. Ethical Considerations:
   - Germline vs. Somatic Cell Editing: India, like many countries, currently prohibits germline gene therapy (heritable changes) due to significant ethical and social reasons, focusing on somatic cell gene therapy.

- Genetic Discrimination and Eugenics: Concerns exist that gene editing could lead to genetic hierarchies and potentially a preference for certain traits.
- Public Perception and Trust: Transparent communication and public engagement are vital to build trust and ensure societal acceptance of gene editing technologies, especially given varying health literacy levels.

6. Infrastructure and Expertise: Developing and delivering advanced gene therapies requires specialized infrastructure, highly skilled personnel, and robust manufacturing capabilities, which might be limited in various parts of India.
7. Long-term Effects: The long-term effects of CRISPR-Cas9 mediated gene editing are still being studied, requiring extensive long-term follow-up in clinical trials.

Addressing these challenges will require a multi-faceted approach involving scientific advancements, ethical discussions, robust regulatory frameworks, public education, and strategic investments.

3.3.3 CRISPR In Cancer

Immunotherapy stands as a promising therapeutic strategy, demonstrating impressive clinical responses in various tumors. Among the most compelling applications of CRISPR-Cas9-mediated genome editing in gene therapy is its role in developing chimeric antigen receptor-T (CAR-T) cells. This process involves harvesting a patient's own (autologous) T cells, genetically modifying them ex vivo to enable them to target specific cancer antigens, and then reintroducing these modified cells back into the patient – a fundamental component of CAR-T cell therapy [39]. The integration of genome editing via the CRISPR-Cas9 system in creating CAR-T cells is poised to significantly broaden the pool of cancer patients who can benefit from this therapy, while simultaneously enhancing the effectiveness and safety of traditional anticancer treatments [39].

The CRISPR-Cas9 system has also been applied to replace large major histocompatibility complex (MHC) alleles at their native loci, presenting a novel approach to correct MHC mismatches encountered in cellular transplantation [39]. This strategy, capable of generating tens of millions of transferable T cells, has been utilized in patient cohorts with diverse cancer types, irrespective of their HLA genotype [39]. CAR19 T cells generated using this method have exhibited potent anti-leukemic effects in patients diagnosed with B-cell malignancies [39]. Notably, the application of TALENs-engineered CAR19 T cells recently achieved successful clinical outcomes in treating an infant suffering from refractory relapsed acute B-lymphoblastic leukemia (B-ALL) [4]. The clinical success of TALENs-engineered CAR-T cells further paves the way for the broader application of the CRISPR-Cas9 system in clinical settings [39].

Beyond its direct role in CAR-T cell generation, the CRISPR-Cas9 system has been employed in studies to correct major MHC mismatches in cellular transplantation by replacing large MHC complexes [4]. One referred study utilized the CRISPR-Cas9 system to knockout the B2M gene by targeting a specific region in primary human CD4+ T cells. This intervention resulted in a complete loss of MHC-I surface expression, leading to the production of a substantial number of transferable T cells [4]. These T cells were subsequently used in patients with various types of cancer, independent of their human leukocyte antigen genotype, thereby facilitating the creation of "universal" CAR-T cells [4]. As noted, CAR19 T cells derived from this approach demonstrated significant antileukemic activity in patients with B-cell malignancies [4].

While adoptive cell therapy and immune checkpoint blockade (ICB) have proven effective in cancer treatment, their efficacy is often limited by the restricted persistence and proliferative capacity of T cells within the tumor microenvironment (TME) [44]. Exhausted T cells (Tex), though the most abundant

cytotoxic T lymphocyte subset in tumors and primarily responsible for tumor cell killing, eventually lose their proliferative ability over time and become less responsive to current immunotherapies compared to progenitor exhausted T cells (Tpex) [44]. Crucial advancements in immunotherapy necessitate the regulation of mechanisms governing the differentiation from Tpex into Tex cells and the development of strategies to restore Tex cell functionality, thereby aiding in the identification of critical immuno-oncology targets by characterizing various cell types [44]. CRISPR screens are instrumental in identifying CAR-T cells with enhanced characteristics, including high proliferation and division rates, increased anti-tumor activity, reduced exhaustion levels, and superior capacities for tumor infiltration [45].

CRISPR-Cas9 has been extensively utilized to investigate the natural course of Colorectal Carcinoma (CRC) and to unravel the sequence of mutations involved in tumorigenesis [46]. Genomic CRISPR–Cas9 knockout screens have pinpointed genetic alterations that influence CRC progression, particularly in the context of KRAS activation, thereby highlighting critical metabolic vulnerabilities that are amenable to therapeutic targeting [46]. Furthermore, the correction of β-catenin pathway mutations using CRISPR–Cas9 in HCT-116 colon carcinoma cells successfully restored Wnt phosphorylation, diminished the nuclear translocation of β-catenin, and hindered tumor growth in mouse models [46]. CRC-associated genes, including APC, P53/TP53, KRAS, and SMAD4, have also been manipulated using CRISPR–Cas9 technology in human intestinal stem cells; transplantation of these modified cells induced adenocarcinoma-like tumors in mice. These advancements validate its application in in vivo genome editing and organoid-based cancer research [46]. Additionally, CRISPR–Cas9 has facilitated the discovery of other crucial driver mutations in genes such as Acvr1b, Acvr2a, Arid2, and the MUC5AC-CD44 axis [46].

Given the critical role of the androgen receptor in prostate cancer, therapeutic strategies targeting this pathway have been developed, encompassing RNA therapies (e.g., small interfering RNA), AR signaling inhibitors, bone-targeting agents, and PARP inhibitors [47]. A referred study showcased the application of the CRISPR/dCas9-KRAB system, wherein scientists successfully suppressed PSA transcription and subsequently reduced tumor growth, migration, and induced apoptosis [47]. This demonstrates the therapeutic potential of targeting PSA expression through CRISPR-based strategies in prostate cancer treatment [47].

Despite the progress in treating CRC, current therapies often fall short of achieving optimal outcomes, and the adverse effects of immunotherapy limit its widespread application [46]. Nevertheless, CRISPR-Cas9 appears highly promising in refining immunotherapeutic strategies such as CAR-T, CAR-NK, and TCR-T cell therapies to devise more effective cancer treatments [46]. As immunotherapy continues to emerge as a primary treatment for various diseases, CRISPR-Cas9 technology holds the potential to significantly enhance its effectiveness, particularly in advancing cancer therapies.

3.4 Newer advances of CRISPR technology

Newer advances of CRISPR technology : CRISPR-Cas9 has revolutionized cancer cellular therapy by enhancing the precision and effectiveness of treatments like CAR-T cell therapy. It enables strategic gene editing to “arm” immune cells with improved tumor-targeting features, such as inserting genes for cytokines (IL-12, IL-15), chemokine receptors (CXCR2), or chimeric switch receptors (PD1/CD28), while knocking out immune checkpoints (PD-1, CTLA-4, LAG-3, Tim-3) and suppressors like TGF-β signaling components to counteract immune exhaustion and suppression within the tumor microenvironment. CRISPR also improves the safety and durability of cell therapies by preventing fratricide (e.g., CD7

knockout), reducing cytokine release syndrome risk (e.g., GM-CSF knockout), and allowing targeted CAR insertion (e.g., into the TRAC locus) to avoid random viral integration. It facilitates the development of off-the-shelf allogeneic CAR-T cells by eliminating alloreactivity through multiplex knockouts (e.g., TRAC, B2M, PD-1), and extends to TCR-T cells and tumor-infiltrating lymphocytes, where targets like GATA3 and Regnase-1 are edited to enhance function [48]. Beyond immunotherapy, CRISPR/Cas9 has driven key advances across in vivo, germline, and ex vivo genome editing. In vivo, therapeutic correction has been achieved for the Fah gene in tyrosinemia, PCSK9 in cholesterol regulation, and hepatitis B virus via targeted cleavage of viral DNA, supported by AAV vectors and paired nickases for enhanced delivery and specificity. Germline editing via zygote injection has enabled heritable corrections such as dominant Crygc-related cataracts and dystrophin restoration in Duchenne muscular dystrophy models, with attempts to correct HBB mutations in human embryos illustrating feasibility despite ethical concerns. Ex vivo, CRISPR has corrected β-thalassemia in iPSCs, CFTR mutations in intestinal organoids, and DMD defects in myoblasts using exon-skipping and HDR strategies. HIV resistance has also been induced through CCR5 disruption or viral excision in T cells and HSPCs, with the advantage of clonal selection and functional validation prior to transplantation [49]. These advances in CRISPR/Cas9 applications have been greatly facilitated by the evolution of targeted genome insertion technologies. Classical methods, such as Homology-Directed Repair (HDR)-mediated knock-ins, utilize programmable nucleases like ZFNs, TALENs, and CRISPR-Cas to insert sequences ranging from 1 to 10 kb, but they require homology arms and are predominantly effective during the S/G2 phase of the cell cycle. More recent technologies, such as Prime Editing (PE) and Prime Editing and Template Integration (PASTE), represent major advances by enabling precise insertions of up to 50 bp or more without the need for double-strand breaks (DSBs), and with minimal genotoxicity and reduced off-target effects. Additional systems like Transposon-based methods and CRISPR-associated Transposase (CAST) provide alternatives for genome insertion, enabling larger knock-ins (1 kb to over 100 kb) without homology arms or DSB generation. These advances offer a broader range of potential applications for CRISPR/Cas9, extending beyond cancer immunotherapy to gene therapy and other clinical settings, marking a new era of efficient and precise genetic engineering [50]. In parallel, the catalytically inactive dCas9 variant has expanded CRISPR's utility by enabling reversible gene regulation rather than permanent editing. When fused with transcriptional repressors like KRAB or activators, dCas9 can target specific promoters to modulate gene expression (CRISPRi/a), offering a tunable strategy for reprogramming immune or tumor cells. These systems can silence suppressive pathways or activate beneficial ones, contributing to more refined control in immunotherapy. Moreover, the high cargo capacity of baculoviral vectors has resolved delivery challenges associated with multiplex CRISPR constructs, enabling efficient, large-scale modulation of gene networks critical to cancer and immune function. [51] Carrier-independent CRISPR/Cas9 delivery methods have advanced by utilizing physical and mechanical strategies to permeabilize cell membranes for direct cargo uptake. Techniques like electroporation, nucleofection, and iTOP create temporary pores or induce macropinocytosis for effective CRISPR delivery. Mechanical methods, including microfluidic platforms and filtration systems like TRIAMF, apply shear forces or membrane disruption to facilitate intracellular delivery. These non-viral approaches are valuable for hard-to-transfect cells and reduce insertional mutagenesis, making them suitable for clinical applications. Additionally, carrier-dependent systems such as nanoparticles (LNPs, PLGA, AuNPs), extracellular vesicles (EVs), virus-like particles (VLPs), and viral vectors (AdVs, AAVs, LVs) enhance CRISPR delivery. LNPs offer efficient and low-immunogenic delivery, while PLGA and AuNPs provide controlled release and improved in vivo editing. EVs, including

exosomes and microvesicles, are biocompatible and can be engineered for targeted delivery. VLPs and viral vectors, such as AAVs and LVs, offer high efficiency and long-term expression, with the latter integrating stably into host genomes for durable CRISPR expression. These delivery systems expand the range of CRISPR applications, particularly in gene therapy and clinical settings. [52]

3.4.1 Current status of CRISPR Technology, the advancements in its research in India

India currently lacks specific legal regulations for the widely recognized CRISPR gene editing technology, a matter of global ethical debate. This absence necessitates the urgent development of India-specific ethical standards, considering its unique sociocultural norms, including preferences for traits like fair skin and a demand for sex selection, which could unfortunately drive misuse. While CRISPR research is implicitly underway in India, there's a critical need to align its priorities with the nation's significant public health burdens, such as tuberculosis, malaria, and diarrheal diseases. Ultimately, India must responsibly advance its gene-editing research by establishing its own ethical and legal frameworks, ensuring that its applications benefit the population equitably and address its most pressing health challenges[53]. This legal void places CRISPR-related inventions under general clauses of the Indian Patents Act (1970), particularly Section 3(b), which excludes inventions deemed contrary to public morality or harmful to human, animal, or environmental health. The Indian Patent Office further clarifies this through guidelines that restrict patents on processes such as human cloning, germline modification, and genetically altering animals in ways likely to cause suffering without therapeutic benefit. Additionally, applications involving human embryos or genetic modifications with potential ecological harm are similarly restricted. These legal constraints, though not CRISPR-specific, indirectly shape the ethical landscape for gene editing in India[54]. The Indian Council of Medical Research (ICMR), through its Regional Medical Research Centre (RMRC) in Dibrugarh, Assam, has initiated the development of CRISPR-based point-of-care diagnostic tools aimed at low-resource settings. These tools are designed for rapid and specific detection of infectious diseases such as drug-resistant tuberculosis and malaria. By adapting CRISPR technology for scalable diagnostic use, these efforts align with national priorities in infectious disease control and offer a potential model for integrating gene-editing platforms into primary healthcare in India[55]. A CRISPR-based diagnostic assay, the Tata MD CHECK SARS-CoV-2 Kit 1.0, was evaluated in a tertiary care setting in India to assess its performance and feasibility as an alternative to conventional RT-PCR testing. Conducted at KEM Hospital, Mumbai, this study compared the TMC-CRISPR assay with RT-PCR using identical RNA samples from over 2,300 clinical specimens. The results demonstrated a significantly lower sensitivity of 44% for the CRISPR test compared to RT-PCR, although specificity remained high at 99%. Notably, the discordance between the two methods increased with higher Ct values, indicating reduced performance at lower viral loads. Operational feasibility assessment revealed that while the assay did not require major infrastructure changes, post-PCR steps such as CRISPR reaction setup and lateral flow strip handling demanded additional training. Furthermore, although manual and app-based result interpretations were largely comparable, issues like faint band detection, app reliability, and data security emerged as concerns. Overall, the current iteration of the TMC-CRISPR test was found to be suboptimal for clinical diagnostics, underscoring the need for significant refinements before widespread adoption[56]. The FNCas9 Editor-Linked Uniform Detection Assay (FELUDA), developed by CSIR-IGIB, is a CRISPR-Cas9-based diagnostic tool designed for rapid and affordable detection of SARS-CoV-2. Unlike Cas12 or Cas13 systems that rely on trans-cleavage, FELUDA uses a high-fidelity Cas9 enzyme for sequence-specific binding, enabling detection via a lateral flow paper strip, similar to a pregnancy test. Clinical studies report 96% sensitivity and 98% specificity across various viral loads. With minimal

infrastructure requirements and low cost, FELUDA represents a major advancement in scalable point-of-care diagnostics during pandemics, especially in resource-limited settings [57]. Scientists at CSIR-IGIB have developed an enhanced version of the FnCas9 enzyme (enFnCas9) with improved DNA-binding affinity and reduced off-target effects, offering greater precision and efficiency in genome editing compared to conventional CRISPR systems. By modifying amino acids involved in PAM recognition, the engineered enzyme can access previously difficult-to-target genomic regions. Functional validation showed superior DNA cleavage activity and diagnostic range. In therapeutic studies, enFnCas9 was used to correct a mutation in the RPE65 gene responsible for Leber Congenital Amaurosis Type 2 (LCA2), a genetic form of blindness. Induced pluripotent stem cells derived from a patient were successfully edited, and the corrected cells, upon differentiation into retinal tissue, restored normal protein expression. These results mark a significant step toward the development of high-fidelity CRISPR tools for clinical use in India. [58] Taken together, the current landscape of CRISPR research in India reflects a promising but nascent trajectory, with major strides in diagnostic development, enzyme engineering, and early therapeutic explorations. While efforts like FELUDA, TMC-CRISPR, and enFnCas9 highlight India's scientific potential and adaptability, significant challenges remain in terms of regulatory clarity, clinical translation, and equitable access. For CRISPR to become a transformative tool in Indian healthcare, sustained investment, robust policy frameworks, and interdisciplinary collaboration will be essential. These foundational advancements now pave the way for exploring CRISPR's broader clinical and biomedical applications in the Indian context.

3.5. Limitations and challenges

Despite significant progress, several limitations remain. In vivo, low HDR efficiency restricts CRISPR/Cas9 to gene disruption or cases with a survival advantage. Off-target mutations, even with improved specificity, pose safety risks. Germline editing introduces ethical concerns, especially with high off-target rates and mosaicism in human embryos. Ex vivo, extended cell culture can lead to genomic instability, and challenges in long-term expansion and tissue-specific transplantation persist [49]. Additionally, CRISPR/Cas9 faces delivery issues due to its large size, and SpCas9's limited PAM recognition reduces targeting flexibility. Off-target effects remain a safety concern, and regulatory and economic barriers hinder the broader clinical application of CRISPR technologies [59]. While significant advances have been made, these genome insertion technologies still face notable limitations. HDR-mediated knock-in requires precise homology arms and primarily functions during the S/G2 phase of the cell cycle, which can limit its efficiency and is associated with genotoxicity, indels, and off-target editing. SSA-mediated knock-in is similarly constrained by the requirement for homology arms and is limited to smaller insertions, leading to lower efficiency and potential toxicity. NHEJ-mediated knock-in operates independently of the cell cycle but can result in unwanted effects like indels, inversion of donor DNA, and duplication. MMEJ-mediated knock-in, although capable of handling short homology arms and smaller sequences, still faces challenges related to DSB generation and high indel rates, with limited off-target editing. Integrase-coupled programmable nucleases, despite avoiding DSBs, suffer from low efficiency and potential off-target effects due to pseudo-landing pads. Prime Editing and PASTE, while minimizing genotoxicity and offering high precision, are limited in terms of insertion size, with PASTE showing minimal efficiency for large insertions. Finally, transposon-based methods and CAST systems offer promising alternatives but typically exhibit low efficiency and semi-random integration patterns, with some systems facing challenges like non-specific DNA integration or associated genotoxicity[52].

4. Conclusion:

The research paper underscores CRISPR/Cas systems' role as a powerful tool for gene therapy, enabling precise genetic modifications with high specificity and efficacy. It meticulously details the mechanism of CRISPR/Cas9, its various classifications (Type I, II, III, and IV), and its diverse clinical applications, particularly in enhancing CAR-T cell therapy, correcting MHC mismatches, and combating viral infections. It also highlights the system's broad applicability in disease modeling, diagnostics, and targeted gene insertion technologies like Prime Editing (PE) and Prime Editing and Template Integration (PASTE). CRISPR/Cas9 technology stands as a profoundly transformative innovation, revolutionizing genome editing with its remarkable precision and versatility. As detailed in this review, its utility spans from sophisticated bacterial genome modifications—enabling complex edits and expanding strain manipulability—to groundbreaking advancements in clinical therapies, particularly in cancer immunotherapy and the treatment of genetic blood disorders. The continuous evolution of this technology, exemplified by novel insertion methods like Prime Editing and advanced delivery systems, further underscores its immense potential to reshape biological research and medical practice.

Despite its vast promise, the widespread application of CRISPR/Cas9 is accompanied by considerable challenges. Issues such as potential off-target mutations, the complexities of efficient delivery, and the necessity for improved efficiency in certain contexts remain areas of active research. Critically, the ethical implications of germline editing and the absence of clear, specific legal frameworks—especially highlighted within the Indian context—underscore the urgent need for robust regulatory oversight and thoughtful ethical discourse. Moving forward, continued scientific innovation, coupled with a commitment to addressing these technical and ethical hurdles, will be essential for realizing the full, safe, and equitable benefits of CRISPR/Cas9 technology.

Crucially, the review also addresses the significant challenges hindering the full realization of CRISPR-Cas9 gene therapy in India. These include safety concerns (off-target effects, immunogenic responses), delivery methods, cost, accessibility, regulatory hurdles, ethical considerations (germline vs. somatic cell editing), and the need for robust infrastructure and expertise. Despite these challenges, the paper emphasizes the immense potential of CRISPR in revolutionizing cancer immunotherapy and other therapeutic areas.

In conclusion, while CRISPR/Cas9 technology offers unprecedented opportunities for treating various diseases and advancing immunotherapy, particularly in the Indian healthcare landscape, addressing the identified challenges—ranging from scientific and technical to ethical and regulatory—will be paramount to harness its full therapeutic potential and ensure its equitable and safe application. Continued research, robust regulatory frameworks, and increased investment in infrastructure and training will be essential for CRISPR/Cas9 to transform healthcare in India and globally.

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