Modern Genome Editing With Crispr-Cas9 In Medicine, Agriculture, And Biotechnology: A Revolutionary Tool For Precise Gene Editing

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1. ABSTRACT

CRISPR-Cas9 is a powerful genome-editing tool that enables precise modification of DNA sequences. Originating as a bacterial defense system, it comprises the Cas9 protein for cutting DNA and a guide RNA that directs it to specific targets. Its applications span medicine, agriculture, and environmental sciences—offering prospects for treating genetic disorders, developing resilient crops, and advancing sustainable practices. Despite its promise, concerns about off-target effects and ethical issues, especially in germline editing, demand strict regulation and transparent debate. Overall, CRISPR-Cas9 is a transformative technology with vast potential, requiring responsible oversight for safe global use. In conclusion, CRISPR-Cas9 represents a revolutionary tool in genome editing, combining precision with broad applicability. While it holds immense promise for advancing health care and agricultural innovation, careful oversight and ethical governance remain central to its continued development and global adoption.

KEYWORDS-CRISPR–Cas9; gene editing; prime editing; molecular biology; molecular genetics.

2. INTRODUCTION

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) were first observed in *Escherichia coli* in 1987 and later identified in various other bacterial species. Initially, the function of these repeating sequences was unknown. It was not until 2005 that researchers discovered their similarity to viral DNA fragments, leading to the proposal that CRISPR functions as part of an adaptive immune defense system in bacteria. In this system, bacteria and archaea use CRISPR-associated nucleases to recognize and cleave viral genetic material with remarkable specificity. By reprogramming this natural defense mechanism to target desired genetic sequences, scientists developed a powerful platform for highly precise genome modification, paving the way for therapeutic and diagnostic applications.

Efficient and accurate genome editing is a cornerstone of modern genetic engineering. Before CRISPR, methods such as Transcription Activator-Like Effector Nucleases (TALENs) and Zinc Finger Nucleases (ZFNs) were among the primary tools for inducing targeted genetic changes. CRISPR, however, offers a more streamlined, flexible, and widely applicable system. The unique organization of short, partially repetitive DNA units in prokaryotic genomes, first described by Yoshizumi Ishino and colleagues in 1987 during their study of *E. coli* genes, was a key discovery. Although its biological role was not identified at that time, follow-up studies by Francisco Mojica in the 1990s linked the sequences to microbial adaptive immunity and ultimately led him to coin the term CRISPR.[1]

The advancement of this knowledge culminated in the development of the CRISPR/Cas9 gene- editing system, a breakthrough that was recognized with the Nobel Prize in Chemistry in 2020. Today, CRISPR/Cas9 represents one of the most effective, versatile, and accessible genetic tools in biomedical research and clinical innovation. Its applications span from studying gene function and disease mechanisms to exploring new therapeutic strategies.[2]

However, the practical use of CRISPR/Cas9 faces critical challenges, particularly the effective and safe delivery of its components into the target cell nucleus. Since the system must access nuclear DNA, barriers such as cell membranes and tissues must first be overcome. Current delivery strategies include non-viral vectors, viral vectors, and physical methods. Among these, viral vectors remain the most commonly utilized, as they can efficiently transfer the CRISPR/Cas9 complex into host cells. Yet, virus-based approaches carry risks such as insertional mutations, immune responses, and the potential induction of carcinogenesis.

Therefore, ongoing research is directed toward developing safer and more efficient delivery techniques to fully harness the potential of CRISPR-based therapies

2.1 CRISPR ETHICS -

Moral decision-making in biomedicine often involves a careful profit–loss assessment, requiring consideration of potential risks, probabilities of outcomes, and the reasoning behind different ethical stances. In 2017, the U.S. National Academies of Sciences released a review on human gene editing, emphasizing that germline modifications intended to create new humans should not be permitted. However, they acknowledged that such interventions might be negotiable under specific medical circumstances.[3]

The prospect of human germline modification using CRISPR-based gene editing has generated intense bioethical debate. A key concern lies in the incomplete understanding of human germline mutagenesis and its possible consequences. While some scientists, such as Sharma and Scot, argued for its "appropriate and justified use" under strictly regulated conditions, they suggested its allowance only in embryos cultured for fewer than 14 days, in line with established ethical guidelines. In contrast, others, including Lander, firmly opposed germline editing, stating that it should only be considered when addressing otherwise untreatable genetic diseases. He further argued that given the uncertainty surrounding unintended genetic changes and long-term implications, the application of CRISPR to the human germline should remain prohibited.[2] Beyond ethics, the CRISPR-Cas9 system's rise has been marked by intellectual property disputes reflecting the technology's immense financial promise. CRISPR-Cas9 is recognized as not only a cost-effective and precise genome-editing tool, but also a means to advance numerous scientific and medical investigations. The first major patent dispute began in 2012, when the University of California, Berkeley (Doudna), the University of Vienna, and Emmanuelle Charpentier filed a joint application. Almost simultaneously, Zhang and the Broad Institute submitted, sparking years of legal battles, appeals, and disputes over licensing rights.[7]

By 2019, both parties had secured patents, resulting in a divided licensing landscape. Companies utilizing CRISPR-Cas9 for gene editing in human cells had to obtain licenses either from the Doudna team or from Zhang. The conflict intensified in 2022 when the U.S. Patent and Trademark Office Appeal Board reaffirmed Zhang and the Broad Institute's priority claims, leading to disappointment among stakeholders aligned with Doudna and financial challenges for companies licensed under her team. However, Doudna and Charpentier secured victories in parallel disputes across Europe and other global regions, including the U.K., China, Japan, Australia, New Zealand, and Mexico, consolidating their influence outside the United States.[2,7]

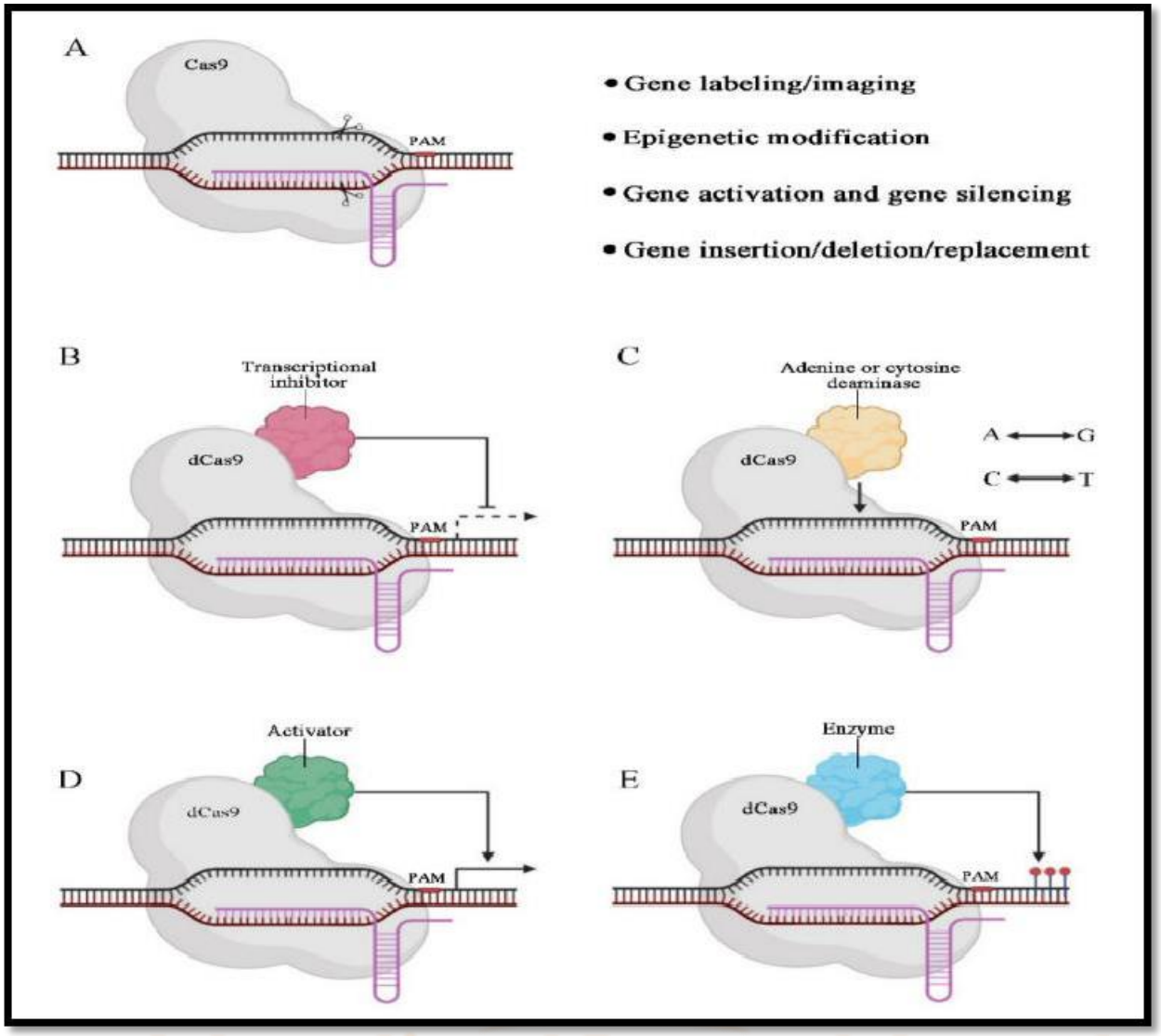

Fig. 1. Applications of CRISPR/Cas9 system.

(A) Schematic illustration of the molecular mechanism of CRISPR/Cas9 system.

(B) The dCas9 fused to a transcriptional inhibitor (red shape) can repress transcription.

(C) A DNA base editor consists of a dCas9 and an adenine or cytosine deaminase (yellow) that converts A to G or T to C, respectively.

(D) The dCas9 fused to transcriptional activator (green) can boost transcription.

(E) The dCas9 fused with enzymes (blue) that can modify epigenetic marks of DNA can be used to change gene expression status.[1,2]

3. LITERATURE SURVEY

1. **Edyta Janik, Marcin Niemcewicz, etal :**

   CRISPR–Cas9, a breakthrough in genome engineering discovered as a bacterial immune system, enables precise gene editing and has wide applications. However, it raises ethical and biosafety concerns.

2. **Saeid CHEKANI-AZAR, Ehsan GHARIB MOMBENI, etal:**

   CRISPR/Cas, first identified in E. coli in 1987, revolutionizes genome editing by enabling precise and cost-effective modifications, enhancing disease diagnosis and treatment. This review explores its history, mechanisms, and potential in studying mutations and manipulating the genome.

3. **Chenya Zhuo, Jiabin Zhang etal -**

   CRISPR/Cas9 gene editing technology offers a powerful platform for treating genetic and infectious diseases but faces challenges with off-target effects and precise tissue targeting. This review discusses emerging strategies for enhancing spatiotemporal control of CRISPR/Cas9, their pros and cons, and future directions for clinical application.

4. **Zhuan Qin, Zhiyi Xu, etal :**

   The CRISPR/Cas9 gene-editing system holds promise for genome manipulation, but its clinical application is hindered by low delivery efficiency. This review highlights advancements in nanoparticle-based delivery systems, particularly exosome-based approaches, and discusses their advantages, challenges, and potential for therapeutic use.

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5. **Arif NM. Ansori, Yulanda Antonius, etal-**

   CRISPR-Cas9 is a groundbreaking tool for precise genetic modifications, utilizing a single guide RNA to direct the Cas9 nuclease for targeted DNA edits. This review explores its fundamental principles, applications across diverse fields, ongoing research, and the ethical considerations surrounding its use.

6. Misganaw Asmamaw Mengstie Belay Zawdie Wondimu etal :

CRISPR/Cas-9 is a highly effective and precise genome editing tool utilized across fields like medicine and agriculture, relying on guide RNA and Cas-9 proteins for targeted DNA modification. Despite its potential for treating diseases and improving crops, challenges such as off-target effects, delivery issues, and ethical concerns must be addressed for broader clinical application.

7. Congting Guo, Xiaoteng Ma,etal :

Gene editing, particularly with the CRISPR/Cas9 system, has revolutionized precision modification of DNA, enabling promising clinical applications.

4. APPLICATIONS OF CRISPR/CAS9 -

4.1 Stem Cell Research

CRISPR/Cas9 technology has become an essential tool in biological and biomedical research by enabling precise gene editing techniques such as gene knockout, gene knock-in, and gene activation. This advancement has notably propelled stem cell research, which plays a critical role in tissue repair and regeneration. Stem cell-based therapies have already gained clinical approval for several conditions, with ongoing trials demonstrating encouraging therapeutic outcomes. The combination of CRISPR/Cas9 with stem cell studies is enhancing both fundamental and translational research, thereby accelerating progress in regenerative medicine and novel treatments for various diseases.[4]

4.2 Genetic Diseases

Although significant progress has been made in identifying genes responsible for monogenic human diseases, numerous challenges remain in correcting these conditions. Monogenic disorders, caused by mutations in a single gene, represent a large portion of rare diseases, with estimates of 5,000 to 8,000 such conditions. Over 75,000 pathogenic genetic variants have been catalogued in databases like ClinVar. For example, Leber congenital amaurosis (LCA), a severe inherited retinal disease, is often linked to mutations in the CEP290 gene. The gene-editing therapy EDIT-101, developed by Maeder et al., has shown success in restoring CEP290 function in mouse models. Similarly, Duchenne muscular dystrophy (DMD), caused by mutations in the dystrophin gene, was treated in mice by Nelson et al. through excision of exon 23 using an adeno-associated virus (AAV)-based CRISPR strategy, resulting in significant protein recovery. In sickle cell disease (SCD), caused by a β-globin mutation, Wu's team targeted the BCL11A

gene with CRISPR/Cas9 to elevate fetal γ-globin expression, offering a promising therapeutic approach. Additionally, CRISPR has been used to selectively disrupt a mutant Tmc1 allele responsible for hearing loss without affecting the wild-type version, demonstrating potential for treating genetic hearing impairments[5]

4.3 Cancer Therapy

Despite progress in anti-cancer treatments, cancer remains a leading cause of death worldwide, responsible for over nine million fatalities each year, highlighting the critical need to advance understanding of cancer biology. CRISPR/Cas9 technology presents promising avenues for developing highly targeted cancer therapies. Current clinical trials are exploring engineered T- cells, such as CAR T-cells, for immunotherapy, though challenges like safe gene delivery and tumor heterogeneity continue to limit effectiveness. For example, Koo et al. successfully used CRISPR/Cas9 to target mutations in the epidermal growth factor receptor (EGFR), resulting in significant tumor size reduction. Moreover, CRISPR-engineered CAR T-cells that recognize specific cancer antigens have achieved high remission rates in patients with relapsed acute lymphoblastic leukemia (ALL), demonstrating the technology's potential in precision oncology.[8]

4.4 Agriculture

CRISPR-Cas9 technology is revolutionizing agriculture by enabling precise genetic modifications to improve crops and livestock. Its applications include enhancing traits such as disease resistance, yield, nutritional quality, and stress tolerance. For instance, researchers have utilized CRISPR-Cas9 to boost rice resistance to bacterial blight, as well as to increase grain yield by editing genes associated with grain size. Beyond crops, CRISPR is becoming an increasingly valuable tool in livestock breeding, targeting improvements in meat quality, disease resistance, and animal welfare. Although still in early stages, these advances have the potential to transform agricultural productivity. However, regulatory challenges and societal concerns about genetically modified organisms (GMOs) necessitate clear ethical guidelines and public engagement to ensure responsible adoption. Overall, CRISPR-Cas9 offers precise and efficient avenues to enhance agricultural sustainability and contribute to global food security.[6]

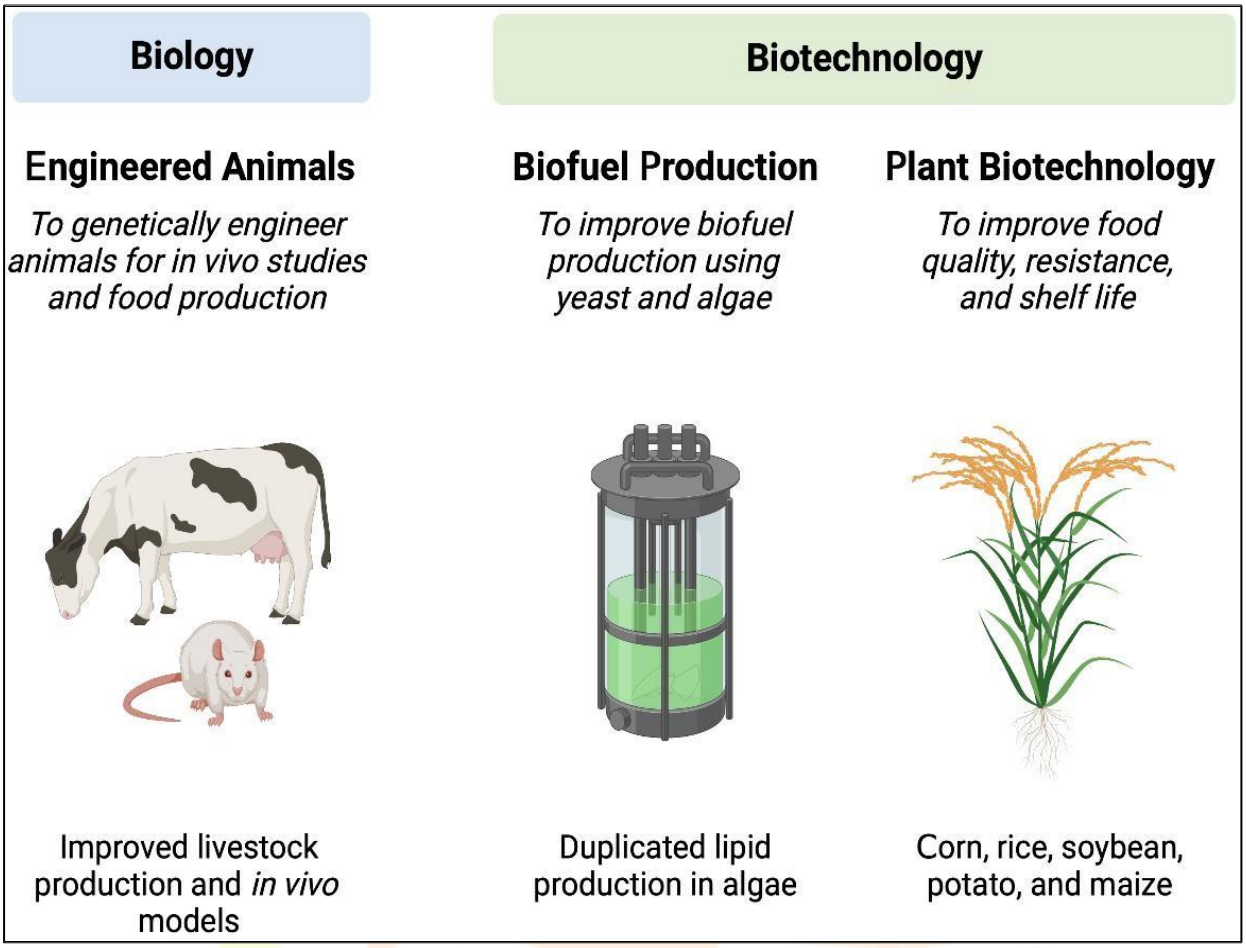

Figure 2: Applications of CRISPR-Cas9 technology for genome editing in the field of agriculture.

4.5 Medicines

CRISPR-Cas9 technology is transforming medicine by enabling precise manipulation of genes to study their functions, unravel disease mechanisms, and develop gene therapies. Researchers use CRISPR to modify genes in both human cells and model organisms, which assists in identifying key genes involved in diseases such as cancer. For example, CRISPR-Cas9 has been employed to knockout genes linked to cancer cell survival and resistance to chemotherapy, improving treatment outcomes. The technology also facilitates gene correction in patient-derived cells, including those affected by genetic disorders like β-thalassemia. Besides therapeutic applications, CRISPR-based platforms such as SHERLOCK and DETECTR offer powerful diagnostic tools for rapid and sensitive disease detection. The integration of CRISPR with stem cell research allows for the creation of accurate disease models, advancing efforts in therapeutic development. Despite its tremendous potential, challenges related to off-target effects, efficient delivery systems, and ethical considerations—particularly concerning germline editing—

remain to be fully addressed. Nevertheless, CRISPR-Cas9 holds great promise for deepening understanding of human diseases, enabling targeted therapeutic strategies, and advancing personalized medicine approaches**.** [8]

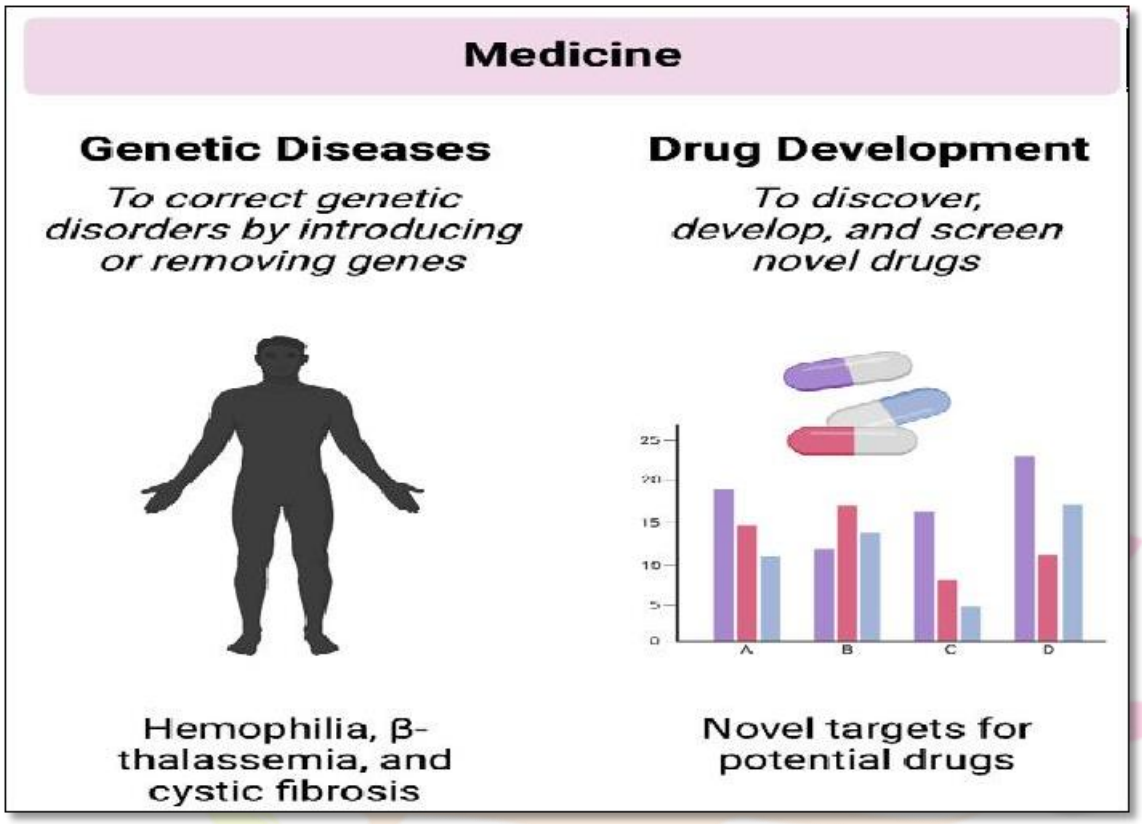

Figure 3 : Applications of CRISPR-Cas9 technology for genome editing in the field of medicine

4.6 Cell Lines –

Cell-line experiments have traditionally relied on ectopic gene expression and RNA-based repression techniques, but these methods can yield non-physiological results and lack specificity. While ZFN and TALEN technologies have been used to model gene mutations, they are labor- intensive and not commonly applied in cell lines. CRISPR-Cas9 technology simplifies gene manipulation, leading to extensive publications. It enables targeted gene editing, with variants like NmCas9 and SaCas9 offering improved specificity and efficiency. CRISPR also facilitates precise insertions via homology-dependent recombination (HDR), showing promise for gene therapy, such as correcting mutations in cystic fibrosis. Modified versions of Cas9 (like dCas9) can activate or repress genes effectively. [5]

5. CLASSIFICATION -

CRISPR Cas systems are divided into two major classes, six types and 33 sub-types. Class 2 is defined by a single, multi-domain, multifunctional effector Protein.Those classes are divided into **three types** each. In class 1, there are I, III and IV types, and class 2 includes II, V and VI types. Class 1 and 2 both use protein effector complexes, the only difference is that while Class1 uses a multi-protein complex, Class 2 uses a single protein complex. Class 1 is additionally separated into various kinds I, III, and IV, while class 2 incorporates types II, V, and VI. It can likewise be separated into 19 diverse sub-types, and it is probably going to keep on extending as new CRISPR/Cas frameworks are continuously being discovered. Numerous Cas-proteins included in Type I and III CRISPR loci form a complex with crRNA. These complexes are essential for target nucleic acid recognition and destruction. There are very few Cas proteins in the type II systems, amid these kinds, the class 2 type II CRISPR/Cas 9 framework is the most well-developed and well-studied gene-editing tool

All types are distinguished by different architectures of the effector modules, which contain unique signature proteins. Each type is also classified into numerous sub-types that are characterized by subtle differences in locus organization and encode sub type-specific Cas proteins. The primary features that define the type and sub-type of CRISPR–Cas systems are *cas* genes and the proteins they encode, which are genetically and functionally diverse. That illustrates the number of biochemical functions that they perform at various steps of CRISPR- mediated immunity. The RNA recognition motif (RRM) is prevalent in numerous Cas proteins and many of the Cas proteins' families contain functional domains which interact with nucleic acids, helicase and nuclease motifs. Genetically, *cas1* and *cas2* are commonly present in different types and sub-types, while signature genes such as *cas3*, *cas9* and *cas10* have been determined for types I, II and III, respectively.[9,10]

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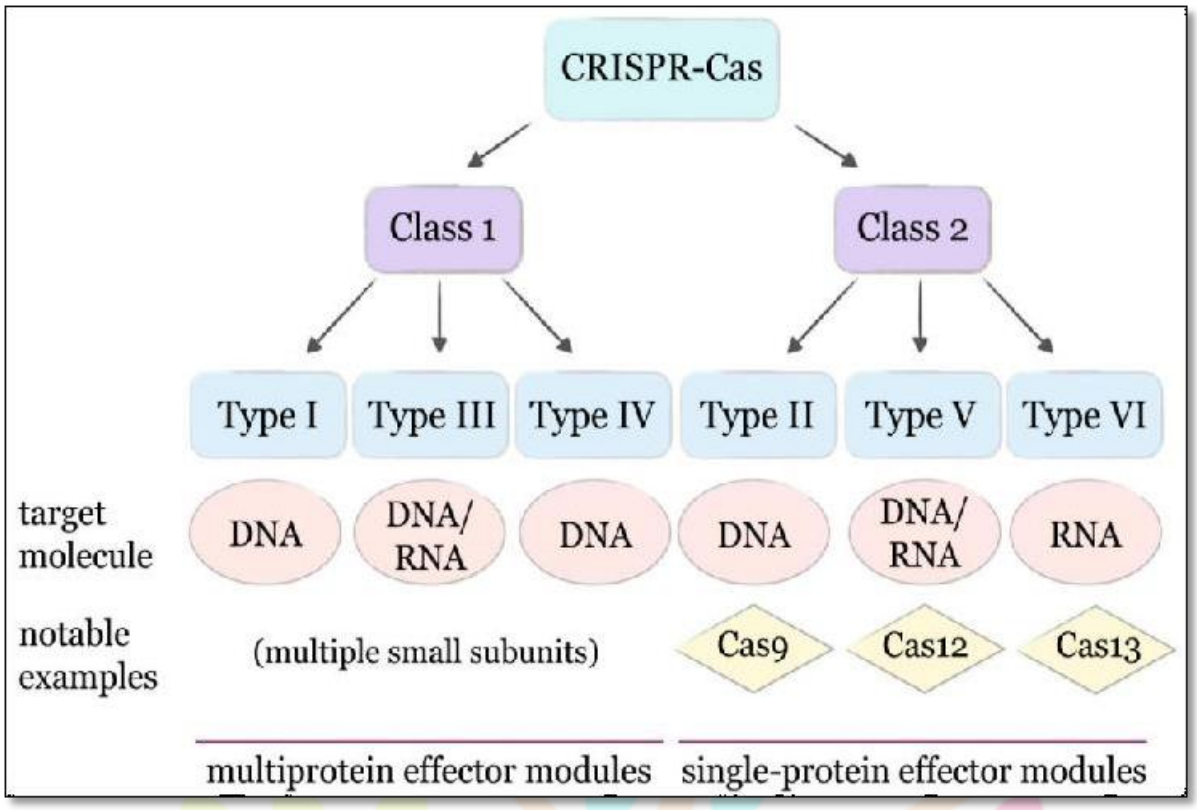

Figure 4 : Conventional classification of known CRISPR–Cas systems

6. MECHANISM OF ACTION -

The CRISPR–Cas immune response includes three steps: adaptation, expression and interference. In the adaptation step, a complex of Cas proteins encounters a short protospacer adjacent motif (PAM), binds to an invading DNA molecule and causes two double-strand breaks in it. The released short DNA fragment of invading phages or plasmids (termed protospacer) is integrated between two repeats of CRISPR array and becomes a spacer.

In the expression stage, *cas* genes' expression and transcription of the CRISPR into a long precursor CRISPR RNA (pre-crRNA) occurs. Cas proteins and accessory factors process pre- crRNA into short mature crRNA. In the interference step, the combined action of crRNA and Cas proteins recognizes and mediates the cleavage of the foreign nucleic acid, consequently protecting the host cells from the infection.[11]

The consequence of pairing the protospacer with the 50 -end 20 nt sequence and the binding of Cas9 to PAM is the formation of a DSB which triggers DNA repair. There are two major endogenous repair mechanisms in eukaryotes: non-homologous end joining (NHEJ) and

homology directed repair (HDR). In the NHEJ mechanism, protein factors rejoin DNA strands directly or do so by including nucleotide deletions or insertions. Nevertheless, this repair mechanism is an error-

prone process which can result in the semi random deletion or addition of DNA base pairs. [13]

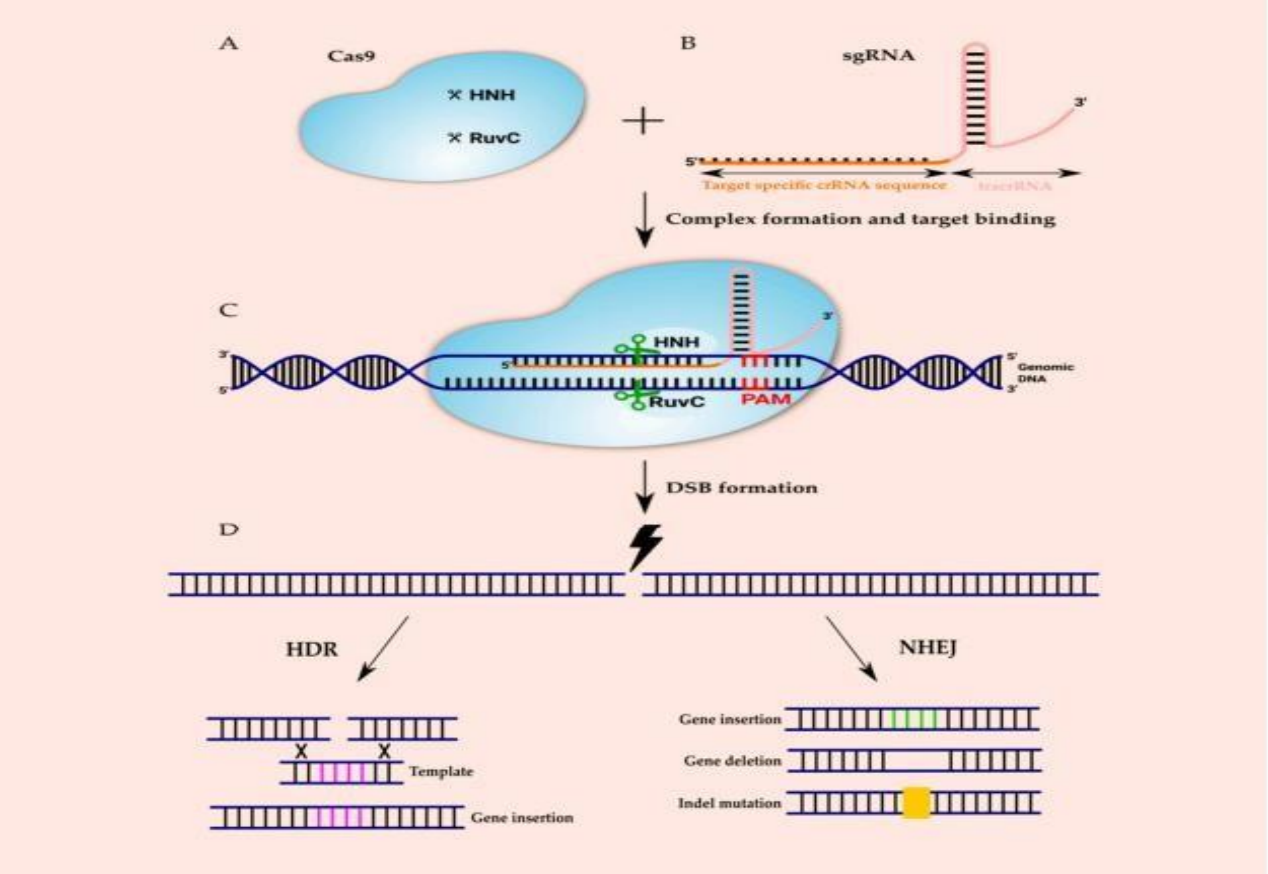

Figure 5 : Schematic diagram of the CRISPR–Cas 9 system molecular mechanism

7. MODERN TARGETED GENOME-EDITING TECHNOLOGIES

Genome-editing-tools has enabled scientists to make changes in their choice of organisms at the genomic level. Most commonly employed technologies for this purpose are transcription activator like effector nucleases (TALENs), homing-endonucleases or meganucleases, zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR) CRISPR associated protein 9 (Cas9)[24]. Engineered nucleases, such as ZFN, TALEN, and Cas9, can induce DNA double-strand breaks (DSBs) at specific genomic loci, which are subsequently repaired by one of at least two endogenous cellular DNA repair pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR).[12]

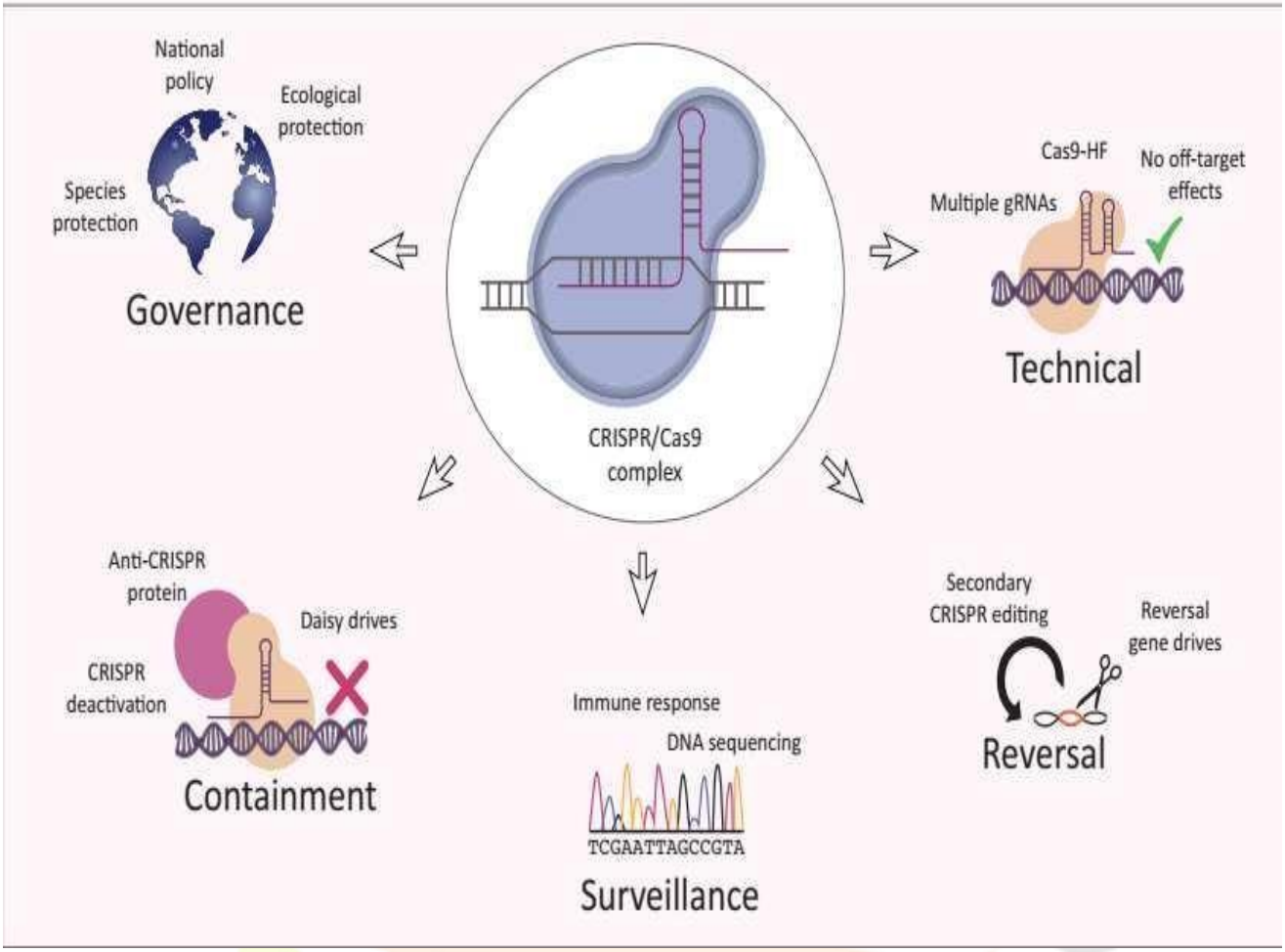

Figure 6 : Promoting safety in genome editing.

7.1 ZFN'S

These artificially made restriction enzymes are the most generally utilized endonucleases. They have zinc finger proteins known as ZFPs, which also act as transcription factors in eukaryotes, and work as DNA binding domains. ZFNs additionally have nucleotide cleavage area (Folk1) derived from *F. okeanokoites*. Based on the target side, the cleavage domain is usually surrounded by 4–6 zinc finger proteins. For efficient gene editing at the target site, ZFPs have a target specificity of 18 base pair (bp). A typical, ZFP is 30 amino acids in length with an alpha- helix structure that is opposite to two anti-parallel β-sheets. This technology uses Homology- directed-repair or Homologous-recombination and non-homologous end joining repair mechanism, for successful gene editing in prokaryotes and eukaryotes.[8]

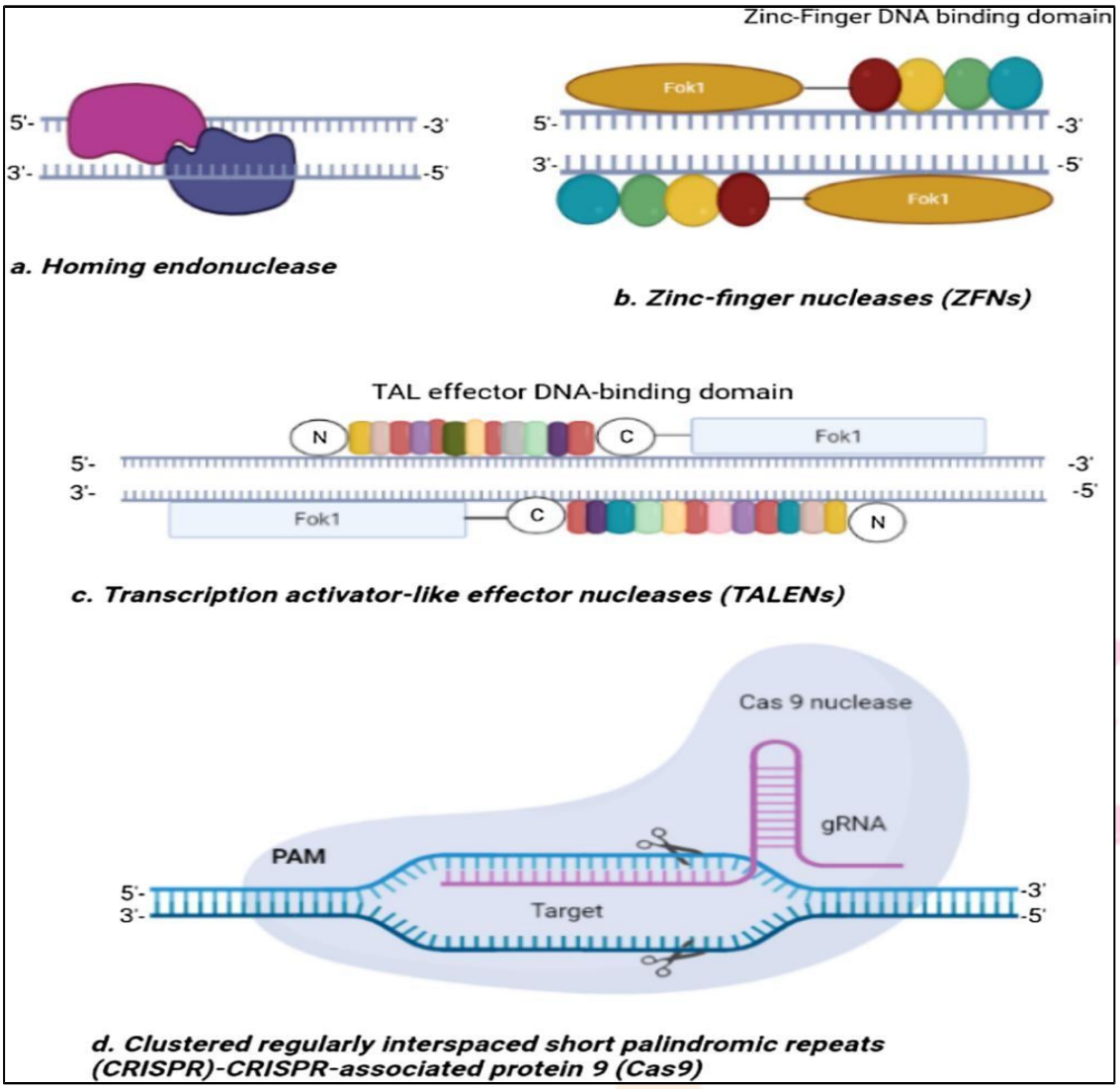

Figure 7 : General assembly of major genome-editing technologies

7.2 PRE-CLINICAL STUDIES OF CRISPR-CAS9 FOR DIFFERENT DISEASES -

CRISPR-Cas9 research has been applied for the treatment of different human diseases, which are discussed below:

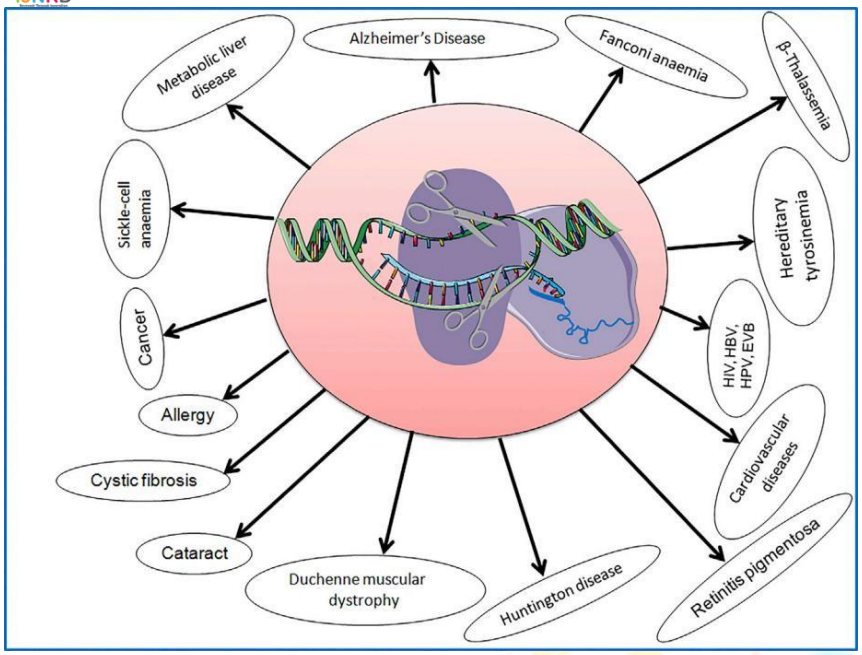

Figure 8 : CRISPR-Cas9 System Dealing for Treatment of Multiple Human Diseases.

A. Cancer -

Cancer is a major health issue often linked to changes in gene activity (epigenetics). Traditional chemotherapy struggles with targeting specific cells and patients can develop resistance to drugs. Researchers are exploring new targets for cancer treatment, especially mutated genes that could be manipulated to promote cancer cell death.

Recent studies have shown promising results using CRISPR-Cas9 technology to modify or correct genes involved in cancer. For instance:[14]

A.1) Tumor Suppressor Genes: Altering genes like hBax, E-cadherin, and p21 has led to reduced bladder cancer cell growth and increased apoptosis (cell death).

A.2) Myeloid Malignancies: Correcting the ASXL1 gene using CRISPR significantly reduced leukemia growth in mouse models.[3]

A.3) B Cell Lymphoma: Targeting the MCL-1 gene induced apoptosis in Burkitt lymphoma cells, suggesting it could be a new therapeutic target.

A.4) Cell Cycle Regulators: Inhibiting cyclin-dependent kinases (CDKs) such as CDK11 and CDK7 has shown potential in treating osteosarcoma and triple-negative breast cancer.[7]

B. Allergy and Immunological Disorders -

CRISPR-Cas9 technology is being explored for treating allergic and immune conditions. Here are some key findings:

B.1) Chronic Obstructive Pulmonary Disease (COPD) and Asthma: The MUC18 gene, which increases during infections, was targeted using CRISPR. Knocking out this gene lowered levels of interleukin (IL-8), a pro-inflammatory molecule, in airway cells, suggesting a way to reduce inflammation.

B.2) Immune Response Enhancement: The PD-1 gene, which helps regulate T cell activation, was disrupted in T cells from cancer patients. This led to increased production of interferon-gamma (IFN-γ) and stronger immune responses, indicating potential for new cancer treatments using checkpoint inhibitors.[15,16]

C. Cardiovascular Disorders (CVDs) :

The PCSK9 gene is crucial for controlling cholesterol levels in the body. Mutations in this gene can lead to high cholesterol (hypercholesterolemia) and increase the risk of heart disease (atherosclerosis). Researchers are using CRISPR-Cas9 technology to target the PCSK9 gene for treatment.

C.1) Mouse Studies: In experiments with mice, scientists have used CRISPR-Cas9 to disrupt the PCSK9 gene, which could help lower cholesterol levels. They also studied the impact of altering the low-density lipoprotein receptor (Ldlr) gene alongside PCSK9.

C.3) Zebrafish Research: Other studies have used CRISPR-Cas9 in zebrafish to correct genetic issues linked to cardiovascular disorders.

These findings suggest that CRISPR-Cas9 could be a promising tool for treating heart-related conditions, particularly those caused by genetic lipid disorders.[17,21]

D. Neurological Disorders -

Huntington's disease (HD) and Alzheimer's disease (AD) are genetic neurological disorders that can be targeted using CRISPR-Cas9 technology.

D.1) Huntington's Disease (HD): Caused by the expansion of CAG repeats in the huntingtin (HTT) gene, HD can be selectively suppressed using CRISPR-Cas9 in mouse models, showing promise for potential treatments.

D.2) Alzheimer's Disease (AD): This neurodegenerative condition is linked to mutations in the

presenilin 1 (PSEN1) and PSEN2 genes, which affect the production of amyloid-beta (Aβ), a protein associated with AD. For example, the A79V mutation in PSEN1 increases harmful Aβ levels. Researchers have used CRISPR-Cas9 to correct these mutations in induced pluripotent stem cells (iPSCs) from AD patients, replacing the faulty nucleotides with healthy ones. [18]

D.3) APP Gene Mutations: Mutations in the amyloid precursor protein (APP) gene can lead to increased Aβ production. CRISPR-Cas9 has been employed to correct these mutations, thereby reducing the risk of AD.

E. Metabolic Disorders -

Metabolic liver diseases (MLD) arise from defects in transporter proteins, affecting how the body metabolizes carbohydrates, proteins, and fats. Recent advancements in CRISPR-Cas9 technology show promise for treating various genetic liver disorders:

E.1) Phenylketonuria (PKU): Researchers corrected mutations in the phenylalanine hydroxylase (Pah)enu2 gene in mice using CRISPR-Cas9.[4,8,17]

8. CHALLENGES AND FUTURE PERSPECTIVES -

In the future, the use of base editors and core editors in human experiments will have a significant impact on health issues, because there are many inherited diseases caused by point mutations that can be cured by modern types of genome editors with extraordinary levels of minimal undesired off-targets. Some researchers are trying to expand the potential of base and core editors. A significant increase in the number of clinical trials using the CRISPR technique for genome editing has been seen in recent years. In this part, some challenges related to the CRISPR technique will be discussed. Due to the speed of research, a solution to these problems and complications will be also provided.

A) Challenges of CRISPR/Cas9 -

1. **Off-Target** - A significant challenge in gene editing with CRISPR/Cas9 is off-target effects due to mismatches between guide RNA (gRNA) and genomic DNA. The likelihood of off-targets varies by gene and cell type, but recent studies show low off-target incidence in human stem cells. Strategies to reduce off-targeting include optimizing gRNA design, using Cas9 nickase for more precise cuts, and exploring alternatives like the CRISPR/Cpf1 system, which shows better specificity without detected off-targets.[19]

2. **Editing Efficiency** - CRISPR/Cas9 is a powerful gene manipulation tool, but its efficiency in human stem cells needs improvement for clinical applications. Strategies to enhance this include optimizing gRNA and Cas9 delivery, using modified gRNAs for stability, and preprocessing host cells with compounds that improve HDR efficiency. Additionally, utilizing alternative repair pathways like microhomology-mediated end joining (MMEJ) and systems like PiggyBac transposons can significantly boost editing efficiency. However, further experimental validation is necessary to confirm these methods' effectiveness in stem cell research.[3]

3. **Delivery Routes -** Effective delivery methods are crucial for CRISPR/Cas9 gene manipulation in human stem cells, which are sensitive and often resistant to common techniques. Transient expression methods like micro-injection, electrotransfection, and nucleofection can achieve high efficiencies but are limited by fragility and scalability. Lentivirus offers stable, efficient delivery but poses risks of random genome integration.

   AAV provides high infection efficiency without genome integration, though its smaller insert capacity and potential immunogenicity are concerns. Emerging strategies, such as micro-constriction chips and cell-penetrating peptides, aim to improve delivery while minimizing toxicity, though some still face efficiency challenges.[3,8]

4. **PAM Limitation -** As it was reviewed, the PAM sequence is fundamental for CRISPR/Cas9 and is largely dependent on Cas9 specificity. PAM sequence limits the design of sgRNA and reduces the adaptability of CRISPR/Cas9 with other systems. Even though an expanding number of CRISPR sorts are found, more PAM sequences have been discovered. The obligatory PAM addition still influences the design of sgRNA in a few circumstances. Subsequently, how to create a designable PAM is critical to broadening the application of CRISPR/ Cas9.

5. **Immune Response -** Even though there are not numerous reports about the extreme immune responses caused by Cas9, the antibodies against Cas9 have been broadly recognized in human bodies, which recommends the potential hazard of aggravation against CRISPR/Cas9-based gene therapies. Scientists pay more attention to the immunogenicity caused by vectors, particularly viral vectors, since the human body may have been exposed to them before. Collectively, the immune responses caused by CRISPR/Cas9 gene-editing framework are one of the major hazard components within the improvement of CRISPR-based gene therapy in vivo.[20]

B) Future Perspective -

CRISPR/Cas9, initially a microbial immunity system, has evolved into a powerful gene-editing tool capable of treating genetic diseases through precise gene modification. Its applications include targeting

monogenic and X-linked disorders, with some therapies reaching clinical trial stages. While CRISPR/Cas9 has shown promise in treating cardiovascular and neurological diseases, challenges remain in cancer therapies due to the complexity of gene mutations and off- target effects.[9]

Delivery methods play a crucial role in the success of CRISPR/Cas9 therapies. Strategies such as transient expression, lentivirus, and AAV-based systems have different advantages and limitations, especially regarding stability and potential integration issues. Innovative methods like micro-constriction chips and cell-penetrating peptides aim to enhance delivery efficiency while minimizing toxicity.

Despite its potential, CRISPR/Cas9's clinical application faces hurdles, particularly off-target effects and the intricacies of multiple gene editing in cancers. Alternative CRISPR systems, including Cas12a and Cas3, are being explored for their unique benefits, such as increased adaptability and efficiency in targeting specific DNA [14]

sequences. Overall, while CRISPR/Cas9 offers significant therapeutic possibilities, further improvements in safety and delivery mechanisms are needed for effective in vivo gene therapy.

Since its introduction in 2013, the CRISPR/Cas9 genome-editing system has been rapidly developed and widely used in all human stem cell studies. All gene-manipulating capacities (e.g., knockout, knockin, knockdown, and expression activating) are incredibly integrated in one technique. It is user-friendly, efficacious, and economical such that genome manipulation ceases to be a challenge for new researchers. Undoubtedly, the CRISPR/Cas9 genome-editing system has revolutionarily changed the fundamental and translational stem cell research. Although solutions are still required to reduce the off-target effect, improve editing efficiency, and exploit novel delivery strategies at a low cost and with high safety for clinical stem cell studies, CRISPR/Cas9-mediated preclinical studies have made remarkable progress and offer huge potential in human stem cell and regenerative research.[2,8,21]

9. CONCLUSION –

CRISPR-Cas9 technology represents a significant leap forward in our ability to edit genomes with precision and efficiency. Its potential to revolutionize medicine, agriculture, and various scientific fields is immense, offering solutions to some of the world's most pressing challenges, such as genetic disorders, food security, and sustainable energy production. The capacity to modify genes quickly and accurately could lead to groundbreaking therapies for conditions that were once deemed untreatable, enhance crop resilience in the face of climate change, and drive innovations in biotechnology. However, the rapid advancement of this technology necessitates a careful consideration of ethical, regulatory, and safety aspects. The potential for unintended consequences, such as off-target effects or ecological disruptions,

underscores the importance of robust research protocols and comprehensive risk assessments. As we continue to explore the possibilities of CRISPR-Cas9, fostering an inclusive and informed discourse will be essential to ensure responsible use and governance of this powerful tool. A collaborative approach can help navigate the complexities of genetic editing, balancing innovation with caution. The future of genetic editing is bright, and with it comes the responsibility to harness its power wisely, ensuring that its benefits are shared equitably across society while minimizing potential risks. By doing so, we can unlock the full potential of CRISPR-Cas9 technology for the greater good, paving the way for a healthier, more sustainable future.

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