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DEVELOPMENT OF CRISPR/CAS9 GENE EDITING TECHNOLOGY

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ABSTRACT:

CRISPR-Cas systems for genome editing have revolutionized life science research and hold significant potential for disease treatment and biotechnology advancements, transforming the way life science research is conducted. This ancient anti-viral immune system, found in bacteria and archaea, allows for precise targeted mutations, corrections, or gene modulation, enabling researchers to create targeted treatments. CRISPR/Cas9 may target genomic loci and be employed in cancer research to investigate tumourigenesis and development pathways. Prokaryotes have evolved defense mechanisms against viral predators, such as clustered regularly interspaced short palindromic repeats (CRISPR) and associated proteins (Cas), which have an adaptive immune system that remembers previous infections by incorporating invading genome sequences into the CRISPR locus.

This article goes into the introduction, history, discovery & development, mechanism, applications, challenges, and essential components of CRISPR-Cas9, such as the Cas9 protein and guide RNA. It explores its applications in genetic manipulation, precision genome editing, personalised medicine, gene therapy, and agriculture. The purpose of this review is to enlighten readers about this novel prokaryotic adaptive immune system, and we expect that in the near future, this technology will be widely employed in clinics and research.

Keywords: CRISPR/Cas 9,Genome editing, Gene therapy, Off target effects, RNA therapy, DNA, Cancer research, Animal model

INTRODUCTION:

Genome editing has risen as a revolutionary field in molecular biology, with the CRISPR-Cas9 system standing out as a leading-edge technology, garnering significant interest. This innovative approach has captured the spotlight for its potential to precisely modify genetic sequences with unprecedented accuracy [1]. The advent of CRISPR-Cas9 has heralded a new era in genome editing, characterized by unparalleled precision and versatility. This groundbreaking technology has surpassed previous methodologies such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) in terms of cost-effectiveness and user-friendliness, representing a significant leap forward in the realm of genetic manipulation. Its potential applications are diverse, spanning across medical research, human gene therapy, plant science, and beyond [2].

The CRISPR-Cas9 system consists of two essential elements: the Cas9 protein and the guide RNA (gRNA). Acting as a molecular cutter, Cas9 precisely cleaves the DNA at specified locations, guided by the gRNA, which matches with the target DNA sequence. Originally sourced from bacterial adaptive immune systems, this molecular apparatus has been repurposed for genome editing in a wide array of organisms, including humans, due to its simplicity and versatility [3]. The process of genome editing with CRISPR-Cas9 involves several crucial stages. The Cas9-gRNA complex is guided to the target DNA sequence, where Cas9 creates a double-stranded DNA break. Following this, cellular repair mechanisms introduce alterations at the target site through either non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways. The precise and customizable nature of CRISPR-Cas9 allows for accurate genome modifications, making it indispensable for fundamental research, therapeutic applications, and improving crop traits [4].

CRISPR-Cas9 offers a plethora of benefits over alternative genome editing tools, fueling its broad adoption and influence. It demonstrates superior efficiency and precision, reducing off-target effects and improving editing results in comparison to older methods such as zinc finger nucleases (ZFNs) and TALENs. The straightforwardness and affordability of CRISPR-Cas9 democratize access for

the scientific community, enabling gene function investigations, disease modeling, and potential therapeutic applications [5]. Nonetheless, CRISPR-Cas9 encounters challenges that demand careful attention. Off-target effects, where Cas9 may inadvertently cleave unintended DNA sites, can result in undesired genetic alterations. Research efforts are focused on enhancing target specificity through modified Cas9 variants and improved gRNA design utilizing bioinformatic tools. Moreover, the efficient delivery of CRISPR-Cas9 components into target cells and tissues remains a hurdle that researchers are addressing by exploring various delivery systems, including viral vectors and nanoparticles. Ethical considerations are paramount, particularly concerning the editing of the human germline, necessitating responsible use and robust regulatory frameworks [6]. CRISPR-Cas9 emerges as a potent instrument for genome editing, where the Cas9 protein and gRNA collaborate seamlessly to intricately alter target DNA sequences. Its capacity to uncover genetic intricacies and craft therapeutics is immense. However, obstacles such as off-target effects, delivery efficacy, and ethical concerns demand vigilant resolution through sustained investigation and conscientious application. By doing so, the scientific community can unlock the full capabilities of CRISPR-Cas9, driving progress across varied domains and enriching our comprehension of biology [7].

CRISPR-Cas systems serve as a critical genome editing tool widely employed across a range of organisms, including bacteria, yeast, and tobacco. These systems are divided into two classes: class 1, characterized by multiple effector nucleases, and class 2, distinguished by a single effector nuclease. Notably, class 2 includes type II Cas9 and type V Cas12a, which are notable for their effectiveness in genome editing within eukaryotic cells. Apart from DNA modification, CRISPR-Cas systems demonstrate proficiency in RNA editing. Within class 2, Type VI CRISPR-Cas13 systems employ a single RNA-guided Cas13 protein with ribonuclease activity to precisely cleave target single-stranded RNA (ssRNA). Various editing methodologies, such as base editing and prime editing, have been developed. Base editing systems, which integrate dCas9 coupled with cytosine deaminase (CBE) or adenosine deaminase (ABE), enhance the efficiency of site-directed mutagenesis. Prime editing, a recent advancement, utilizes a fusion protein comprising Cas9 nickase and reverse transcriptase to directly encode new genetic information at a specific DNA site [8]. The historical roots of CRISPR-Cas9 research can be traced back to its discovery as an adaptive immune defense mechanism in bacterial cells, where it serves as a formidable defense against foreign DNA intrusion.

Scientists have skillfully harnessed CRISPR-Cas9 for precise genome editing across a wide spectrum of organisms, leading to significant breakthroughs and a deeper understanding of its mechanisms. Its applications span various fields, notably contributing to enhancements in agronomic traits [9].

Essential components of CRISPR/Cas9 system (Cas9 protein, guide RNA):

The CRISPR-Cas9 system represents a revolutionary tool for genome editing, hinging on two pivotal constituents: the Cas9 protein and the guide RNA (gRNA). A more profound comprehension of this system can be achieved by delving into the structure and function of these components [3]. The Cas9 protein, derived from bacterial immune systems, serves as a molecular tool for precise DNA cleavage at predetermined target sites.

It consists of two nuclease domains responsible for cutting DNA strands and a recognition domain that interacts with the guide RNA (gRNA). When bound to both the gRNA and the target DNA sequence, Cas9 undergoes a structural change, resulting in the formation of a double-strand break (DSB) at the specified location [10, 11]. The guide RNA (gRNA), acting as a guiding beacon, directs the Cas9 protein to the intended location within the genome for editing. This artificial RNA molecule consists of a scaffold region and a customizable guide sequence. The guide sequence is meticulously crafted to match the target DNA sequence, enabling Cas9's binding and DNA cleavage. Practical guidance on gRNA selection and design, considering factors such as target specificity, the protospacer adjacent motif (PAM) sequence, and effective gRNA design criteria, will greatly assist researchers in understanding and utilizing this essential component [12]. Utilizing the cutting precision of the Cas9 protein and the specificity of the guide RNA (gRNA), the CRISPR-Cas9 system enables accurate genome alterations. The process of genome editing with CRISPR-Cas9 unfolds through several stages. Initially, the CRISPR-Cas9 components are introduced into the target cells, typically through viral vectors or direct injection. Once inside the cells, Cas9 and the gRNA combine to form a complex that traverses the genome, searching for the target DNA sequence guided by the complementarity of the gRNA [13, 14].

After identifying the target site, the Cas9 protein initiates a precise double-strand break (DSB) at that position. DSB repair involves two main pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ, known for its error-prone nature, often results in minor insertions or deletions (indels) at the DSB site. Conversely, HDR depends on a template DNA molecule to facilitate precise modifications [15]. Discussing off-target effects and proposing strategies to mitigate them via gRNA design, specificity enhancement, and experimental optimization offers a nuanced view on the constraints of CRISPR-Cas9 technology [16].

By integrating the proposed enhancements, this review will emerge as a definitive source, providing a more elucidated insight into the CRISPR-Cas9 system, its benefits, and potential drawbacks. Consequently, researchers can utilize this potent genome editing tool with greater efficacy and accountability, thereby advancing the boundaries of molecular biology. The comprehensive comprehension of the CRISPR-Cas9 system and its constituents, as explored in Section A, establishes the basis for a comprehensive SWOT analysis (Strengths, Weaknesses, Opportunities, and Threats) of CRISPR-Cas9 technology [17].

HISTORY OF GENETIC ENGINEERING:

The CRISPR-Cas9 system, which consists of short DNA repeats, was discovered in 1987 in bacterial genomes and 1995 in archaea. In 2005, CRISPR loci were proposed to protect prokaryotes from foreign genetic information, and the Cas9 protein was first described.

The complex contains two RNA molecules, crRNA and tracrRNA, discovered in 2007 and 2011, respectively. The Nobel Prize-winning work in 2012 combined the two RNA molecules into one strand to make the system more usable.

CRISPR/Cas9 technology, first introduced in 2012, has become a dominant tool in biology for gene editing. Although not the first technology, it has evolved over time, with the first gene-targeting system being zinc finger nucleases (ZFN) in 2005. Transcription activator-like effector nucleases (TALENs) were introduced in 2010 [18], and Streptococcus pyogenes was genetically engineered in 2012. CRISPR-mediated technologies can be used to personalize gene therapy, target pathogens, and treat infectious diseases [19].

However, challenges include delivery strategies, control over repair pathways, and ethical considerations [20]. The technology has the potential to personalize gene therapy, target pathogens, and treat infectious diseases.

Table 1: Comparison of genome engineering tools.

Properties	ZNFs	TALENs	CRISPR
DNA-binding moiety	Protein	Protein	Protein
Nuclease	FokI	FokI	FokI
Target recognition size	18–36 nucleotides	30–40nucleotides	22nucleotides
Toxicity	Variable to high	Low	Low
Ease of targeting multiple targets	Low	Low	High
Complexity of design	Very complex	complex	Simple
Off-target effect	Moderate	Low	Variable

History of TALENs & ZFNs:

The transcription activator-like effectors (TALEN) programmable nucleases were the first to successfully adapt CRISPR/Cas9 for genome editing in eukaryotic cells. They were used to edit different genome loci in human and murine cells [21]. Feng Zhang obtained a patent in his own name before Doudna [22]. In 2010, another class of sequence-specific nucleases was created by fusion of TALEs and FoxI endonuclease [23]. TALEs were first identified as the product of plant pathogen bacteria in the genus Xanthomonas. TALENs recognize specific DNA sequences through tandem repeats of DNA-binding domains containing 33-35 amino acids [24]. They are easier to use, have lower cytotoxicity, and target every human gene [25].

1.Transcription activator-like effector nucleases (TALENs):

ALEs are type III effector proteins found in Xanthomonas spp., a group of Gram-negative plant pathogens [26]. They have an N-terminal bacterial secretion and translocation signal, a distinct DNA binding domain called repeat 0, a central repeating modular DNA-binding domain, two or more C-terminal nuclear localization signals, and a highly conserved acidic AD. TALEs have been used as artificial transcription factors and nucleases due to their simple DNA-binding code and ease of engineering. TALENs, like ZFNs, are formed by combining the FokI nuclease element and a TALE DNA-binding domain. TALENs have less nuclease-associated cytotoxicity, possibly due to longer DNA target sites and thymine requirements [27].

Advantages include quick assembly, powerful resources, cross-species flexibility, and high success rates. Make TALEN technology an easy choice for genome editing applications. Recent years have seen a surge in TALEN usage reports, surpassing previous decades of artificial nuclease-based technology applications. TALEN technology has been used in various organisms, including yeast, plants, algae, protozoans, nematodes, fish, insects, mammals, and even human cells [28]. TALEN technology has proven to be simple and widely applicable, as evidenced by its rapid adoption across various species.

2. Zinc finger nucleases (ZFNs):

Zinc finger nucleases (ZFNs) are restriction enzymes that edit the genome at specific sites. They are hybrid proteins with multiple zinc finger domains and a Fok1 [29]. endonuclease domain that promotes DNA double-strand breaks. The fusion of these domains creates highly specific "Genomic scissors" that can target any gene in any organism. ZFNs with more zinc fingers have increased specificity, efficiency, and targeting. However, they have drawbacks such as context-dependent specificity and the ability to target only one site at a time, which limits knockout editing [30].

DISCOVERY & DEVELOPMENT OF CRISPR/CAS9 TECHNOLOGY:

The CRISPR/Cas system is an essential component of bacteria and archaea's adaptive defense processes, helping to resist foreign genetic plasmids and phage invasion. It was first found in the genome of Escherichia coli in 1987 [31], then in additional bacteria and archaea in 2000.

In 2002, these repetitions were dubbed CRISPR. In 2005, researchers revealed that CRISPR may be linked to microorganism immunity [32], prompting a focus on its involvement in bacteria's defensive mechanism.The CRISPR system, which employs antisense RNA to identify invading nucleic acids, was thought to work similarly to eukaryotic self-immune function [33, 34]. In 2007, Barrangou et al.

revealed the CRISPR system as an adaptive immune system, indicating that phage gene sequences may modify bacteria's resistance to phage infections. In 2008, Brouns et al. showed that non-coding RNA from the CRISPR proto-interregional sequence can direct Cas protein to DNA targets for defensive purposes. Deltcheva et al. (2011) discovered that trans-coding crRNA (tracrRNA) plays an important role in pre-crRNA processing and maturation, revealing novel crRNA maturation mechanisms [35].

In 2012, the Cas9-crRNA combination was shown to cut target DNA in vitro, making it the first gene-editing tool. Charpentier and Doudna's groups then integrated crRNA and tracrRNA into a single sgRNA, which improved Cas9 editing efficiency. In 2013, Zhang's group created the CRISPR/Cas9 system to edit genomes in eukaryotic cells, a method extensively employed in life sciences such as gene therapy, animal and plant genetic trait improvement, and biological breeding [36,37,38]. Emmanuelle Charpentier, a French microbiologist, and Jennifer Doudna, an American biologist, received the Nobel Prize in Chemistry in 2020 for their innovative work.

Contributions of Charpentier and Doudna:

Natural CRISPR/Cas9 has been used to convert bacteria, and its functioning mechanism has been identified. The first CRISPR gene sequence was introduced to E. coli in 2011 by Siksnys et al., proving that CRISPR/Cas systems may be employed as a host less defensive mechanism against foreign infection [39, 40].

In 2012, Charpentier and Doudna isolated Cas9 from S. thermophilus and Streptococcus pyogenes, allowing for in vitro DNA cleavage [41]. They clarified the CRISPR/Cas9 process, finding that the cleavage location is determined by a seed sequence in the crRNA and needs PAM participation. The mechanism can act as a gene silencer, allowing for gene targeting and editing by changing the nucleotide seed sequence.

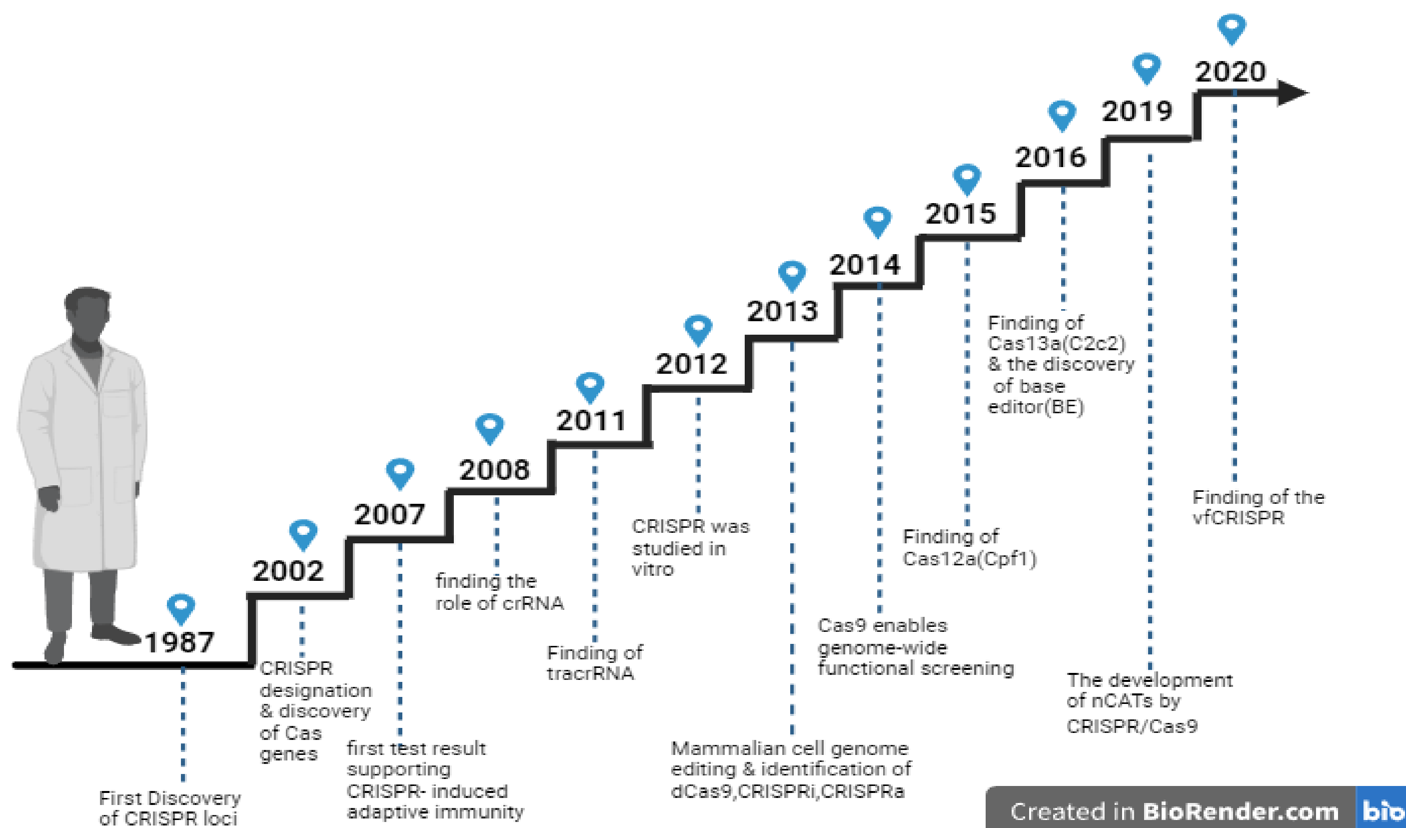

Fig.1- CRISPR/Cas9-based gene editing tools were created in 1987 and dubbed in 2002. In vitro investigations in 2012 demonstrated that mature crRNA and tracrRNA produced a double-stranded RNA structure, guiding Cas9 protein to create a double-stranded fracture on target DNA. In 2013, the type II Cas system was used to break DNA in mammalian cells, opening the path for CRISPR/Cas9 gene editing technology. By 2020, many CRISPR/Cas9-based technologies have been developed for DNA and RNA levels.

First study employing CRISPR technology for illness treatment:

CRISPR/Cas9 technology has been used to edit genes in a variety of animals and plants, including mice, fruit flies, rats, rice, and wheat [42, 43, 44]. Wu et al. (2013) presented a work that employed CRISPR/Cas9 to repair cataracts in a mouse model induced by base deletions [45]. They co-injected mRNA expressing Cas9 with sgRNA into fertilized mouse eggs, and ten animals had the mutant allele. Two NHEJ-induced animals as well as four mice with corrected cataracts via HDR induction were cured [46]. This demonstrates how CRISPR/Cas9 may edit the DNA to address genetic disorders. In another work, Schwank et al. obtained intestinal stem cells from two patients with cystic fibrosis transmembrane conductor receptor (CFTR) mutations and used CRISPR/Cas9 to fix the disease-causing

mutation. They presented a procedure for altering genetically altered stem cells in vitro and then introducing them into the body to cure illness [47, 48].

Adaptations:

Recent advances in understanding CRISPR-Cas systems, particularly the interference phase, have resulted in a better knowledge of the molecular intricacies involved in spacer acquisition. Cas proteins grab DNA prespacers [49], which are then guided to the CRISPR locus, where a repeat sequence is replicated and a new spacer is incorporated. Cas1 and Cas2 genes are crucial for adaptability and are found in most CRISPR-Cas systems [50]. Cas1 binds strongly to the CRISPR locus and integrates additional spacers, whereas Cas2 acts as an adapter protein, bridging Cas1 proteins and binding and stabilizing prespacer DNA [51].

A CRISPR repeat and a leader sequence are required for the incorporation of a new spacer. The AT-rich leader sequence is located upstream of the CRISPR locus, which includes the transcription promoter. Repressor proteins may mute transcription in some species, and the CRISPR-Cas system may also influence Cas2 activity [52]. The integration of sequences into the CRISPR locus suggests the acquisition of spacers targeting the bacterial chromosome (self-DNA) instead of foreign DNA. However, there is a bias towards acquiring new spacers from foreign DNA compared to self-DNA. Degradation products of the bacterial RecBCD complex, which processes double-stranded DNA breaks (DSBs), are one source of protospacers for the Cas1–Cas2 complex. Bacterial genomic DNA is rich in Chi sites, indirectly protecting it from spacer acquisition [53].

The CRISPR-Cas system needs particular components to handle prespacers for integration. Cas2 is fused to a DnaQ domain in type I-E systems, where it degrades prespacers at the 3′ end. Cas4 chops 3′ overhangs until it encounters a bound Cas14-Cas22 complex, which generates spacers with a particular length and the right PAM [54, 55].

PRINCIPLE OF CRISPR/CAS9 SYSTEM:

Any gene or genomic area may be edited using the CRISPR/Cas9 system, which enables site-directed editing techniques like mutation, knockout, insertion, and deletion (indel). It is made up of a sgRNA and a Cas9 nuclease that attaches to the target DNA fragment and initiates double-stranded DNA cleavage to create a DSB. The homology-directed repair (HDR) route or non-homologous end joining (NHEJ) DNA replication are the two methods used to repair the DSB. Protein function loss may arise from a frameshift mutation affecting the transcription and translation of the target gene in the protein-coding region. When a foreign sequence enters a cell, the HDR pathway inserts the foreign gene into certain cell genome locations, correcting the target gene's indel [56]. The genome editing of mammalian cells, animals, plants, and even viruses has made extensive use of the CRISPR/Cas9 system possible due to its versatile and controlled selection of guide RNA target locations.

CRISPR SYSTEM CLASSIFICATION:

CRISPR systems are categorized into six types based on the sequence and structure of Cas proteins. Type II, which includes the Cas3 protein, is commonly used in gene therapy due to its simplicity and multifunctionality [57]. Type III, which includes the Cas10 protein gene, destroys both RNA and DNA. Researchers are exploring new RNA-guided Cas proteins to develop CRISPR-based technologies, aiming to shrink nuclease size for easier gene delivery [58]. Cas14, a miniature CRISPR system, targets single-stranded DNA without PAM and can operate on larger RNA scaffolds. The CRISPR/Cas13a system can recognize and cleave single-stranded RNA molecules [59].

Table 2: Classification of CRISPR endonucleases

Class	Type	Protein
1	I	Cas3
	III	Cas10
	IV	Scf1
2	V	Cas9, Cas1, Cas2, Cas4
	VI	Cas13

MECHANISM OF CRISPR/CAS9:

Genome editing is a genetic engineering approach that involves inserting, deleting, or altering DNA within live cells. Prokaryotes employ CRISPR, a short, repeating DNA sequence, to defend themselves against viruses and bacteriophages [60]. The CRISPR-Cas9 system comprises of a single-guide RNA crRNA array, a trans-activating crRNA, and a Cas9 nuclease known as PAM. The sgRNA array binds to target DNA, whereas the tracrRNA base pairs with the gRNA to produce a functional guide RNA, and the conserved sequence motif, PAM, is critical in recognizing and cleaving the target DNA close to the crRNA-targeted region [61].

The foreign gene is introduced into the host and converted into protospacers, which are DNA fragments. Cas1, Cas2, and Csn2 proteins are all involved in this process. These protospacers are chosen and combined to create a CRISPR array, with selection based on protospacer adjacent motifs (PAMs), which are short sequences next to target regions in the exogenous genome [62]. This integration makes it easier to discover related gene invasions. The Cas9 nuclease has two critical domains, RuvC-like and HNH, which are responsible for cleaving one DNA strand. Its tracrRNA region controls Cas9 and directs it to certain infectious sequences [63].

The CRISPR/Cas complex involves four steps: 1) Inserting spacers from phage or foreign DNA into the CRISPR sequence using both Cas1 and Cas2 proteins. 2) Adding additional spacers at the beginning of the CRISPR sequence. 3) CRISPR loci are transcribed into pre-crRNA via a noncoding region rich in AT (adenine and thymine). 4) Converting pre-crRNA to crRNA, each having a single spacer between two half-repeats. Finally, crRNA is utilized to eliminate foreign genetic material [64].

The CRISPR system is a genetic engineering technique that includes tracrRNA, Cas genes, a leader sequence, and a CRISPR array. It transcribes tracrRNA, which is complementary to the CRISPR array's repeat sequence. The CRISPR array transcribes repeat and spacer sequences to generate crRNA precursor (pre-crRNA), which eventually forms a mature tracrRNA-crRNA complex. This complex is broken down by RNase III and other nucleases [65]. The CRISPR/Cas9 system activates nucleases via tracrRNA and crRNA, preventing the foreign genome from invading. The 20-nt crRNA identifies the target sequence, and double-stranded DNA is cleaved 3 nt upstream of the PAMs [66]. This interference shields the organism from invasion and enables gene editing.

The CRISPR/Cas system is responsible for gene editing due to its capacity to recognize target sequences, Cas nuclease's DNA cleavage activity, and cell DNA repair processes. When DSBs develop, cells use their own repair mechanisms to fix DNA damage and prevent cell death, such as non-homologous end joining (NHEJ) and homology-directed repair (HDR) [67]. If homologous sequences are present, cells can repair DSBs using HDR, allowing for gene knock-in. If not, cells employ NHEJ, which directly connects damaged DNA but is vulnerable to base pair insertions or deletions, potentially resulting in gene knockout [68]. CRISPR/Cas9 vectors, when converted into target cells, express Cas9 and gRNA, allowing for gene editing [69], drawing several scientists for further research and notable scientific achievements during the past decade.

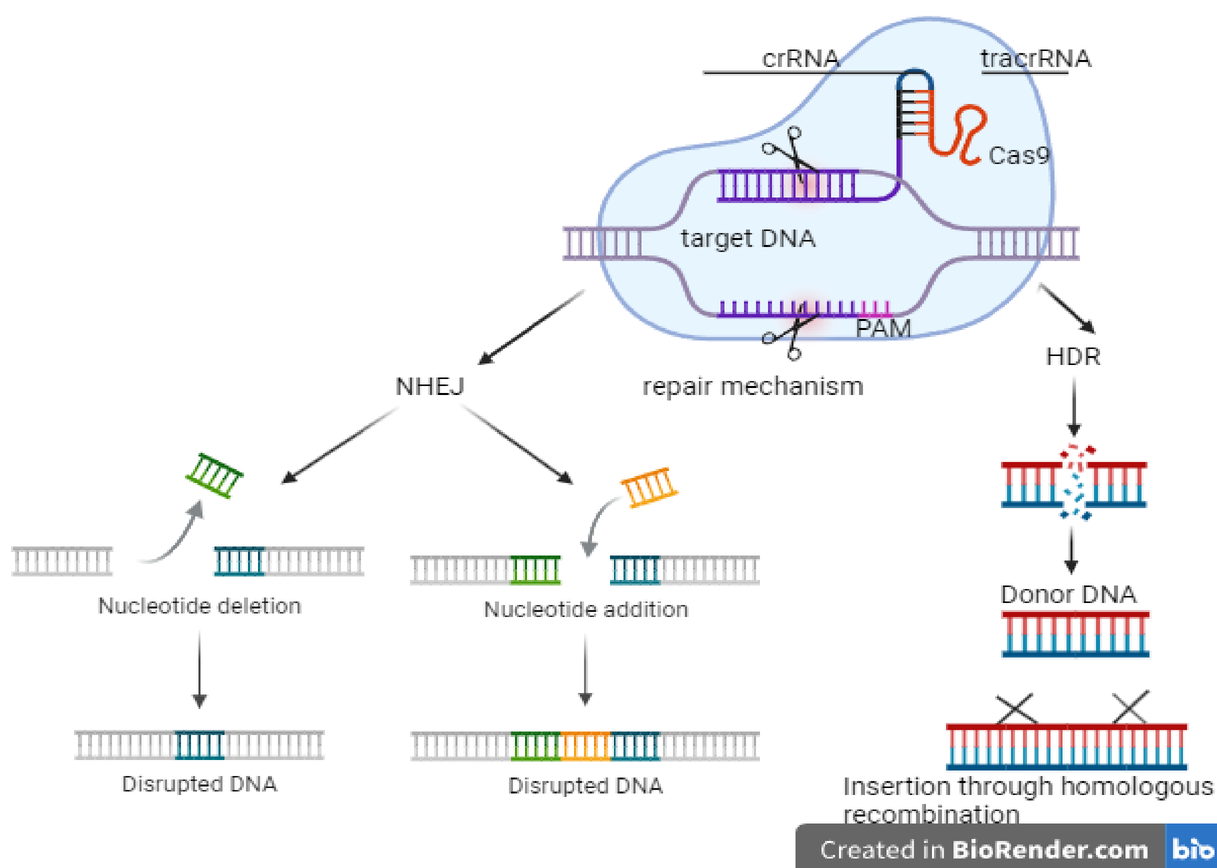

Fig.2- Mechanism of CRISPR/Cas9 gene editing technology. The Cas9 complex identifies the protospacer when paired with sgRNA, which can only be changed by hybridizing with the crRNA and binding to the Cas9 protein. This results in the CRISPR-Cas9/sgRNA complex, which unwinds dsDNA and anneals sgRNA's corresponding sequence to one of the DNA strands. Endonuclease domains break both DNA strands three bases upstream of the PAM sequence, resulting in a double-strand break (DSB) that can be repaired via homology directed repair (HDR) or non-homologous end joining (NHEJ).

The role of cas9 protein & guide RNA in CRISPR technology:

1. **Cas9 protein -** The Cas9 protein is a type of molecular scissors that are sourced from the immune systems of bacteria. Its function is to precisely cut DNA at specified target places. It is made up of two nuclease domains and one recognition domain that interacts with gRNA. When Cas9 binds to both gRNA and target DNA, it causes a double-strand break, resulting in SpCas9, which cuts the target DNA 3-5 bp upstream of the PAM region. Its bilobed structure consists of a recognition lobe and a nuclease lobe linked by linker sequences [70].

2. gRNA - The gRNA directs the Cas9 protein to the appropriate genomic region for editing. It has a scaffold area and a configurable guide sequence. Understanding gRNA selection and design, including target specificity and PAM sequence, will assist researchers in using this critical component [71]. The CRISPR-Cas9 system enables precise genome changes by using the Cas9 protein's cutting prowess and gRNA selectivity.

THE APPLICATIONS OF THE CRISPR/CAS9 SYSTEM:

1.CRISPR/Cas9 genes:

CRISPR/Cas9 technology is a tool used to edit genes in microorganisms, plants, and animals, resulting in changes in gene expression and phenotype. It is crucial for gene function research, character modification, strain improvement, and crop and livestock breeding. It was first used in bacteria in 2013, allowing them to repair DSBs using HDR [72]. To increase efficiency, host cells should use the λ-Red recombination system or Recent [73]. Cas9 and gRNA can be expressed using one, two, or multiple plasmid systems. However, CEP should be used cautiously due to potential toxicity [74].

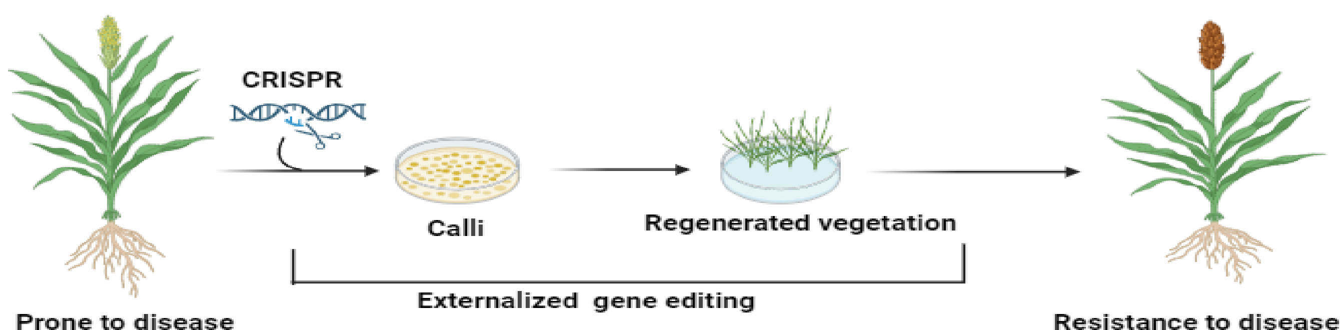

Fig.4- An elite variety that is susceptible to illness is the starting point for plant genome editing. Targeted changes are then performed to this elite variety using CRISPR technology, making it resistant to disease.

2. Precision medicine:

Scientists are interested in gene editing because of its potential use in precision medicine, which seeks to cure genetic defects and severe illnesses that conventional therapy cannot treat. CRISPR/Cas gene editing has been successfully used in mammalian models to precisely observe phenotypes and pathogenesis, gain a better understanding of pathophysiology, promote gene therapy, and create new treatment methods. CRISPR/Cas technology has been shown in studies to treat cataract mice and genetic tyrosinemia caused by Fah gene mutations. In 2014, the CRISPR system was injected into the liver of adult mice via the tail vein to knock out two primary tumor suppressor genes, resulting in a mouse liver tumor model. In 2016, Kang et al. utilized CRISPR/Cas9 technology to develop a human gastric A cancer model was created by knocking out the RUNX3 and IL2RG genes in pigs. The technology has been successfully applied to brain, lung, and liver disease models [75]. Chang et al. discovered a safe knockout site in monkey embryonic kidney epithelial cells, potentially increasing vaccine production.

3. SgRNA Library:

SgRNA library screening technology is crucial for systematic genomic analysis, enabling rapid investigation of gene, RNA, and protein functions, drug targets, and species breeding. CRISPR knock-out, knockin, activation, and inhibition sgRNA libraries are already available, enabling high-throughput screening of the whole genome.Tens of thousands of sgRNAs are synthesized on a chip, recombined to form vectors and plasmids, and high-throughput sequencing is used to track plasmids. Scientists have created numerous human and mouse genomic libraries, with ongoing improvements [76]. Yilmaz et al. used a sgRNA library to analyze 180,000 mutations in the human genome, providing a blueprint for disease development. Recent advances in in vivo screening have allowed for the creation of patient-specific avatars for precision medicine. This study promotes biological research and economic value [77].

4. Gene Editing without Plasmids Remains:

Plasmids are often used in CRISPR/Cas9 gene editing, but this can lead to off-target effects and hinder future experiments [78]. Researchers can remove plasmids after or during gene editing, either by directly transforming the Cas9 protein and gRNA into target cells or by transforming plasmids before transforming a transcribed gRNA that targets the plasmids. Methods to eliminate plasmids include including a temperature-sensitive replicator in plasmids or inserting a target DNA fragment into plasmids for the sgRNA to guide the Cas9 protein [79].

5. Cas9 Modifications:

The Cas9 protein, when fully functional, can cause DSBs in the genome. Inactive domains convert Cas9 into Cas9 nickases (Cas9n), which cause single-strand breaks. Cas9n can be used for heterozygous and base editing when combined with deaminase and DNA polymerase [80]. When both HNH and RuvC domains are inactive, Cas9 transforms into dead Cas9 (dCas9) [81], which can activate or inhibit gene transcription using CRISPRa or CRISPRi techniques. It can also be used for fluorescence imaging when combined with fluorescent proteins to observe DNA changes and identify interesting sites [82].

6. CRISPR/Cas9 system in cancer research:

Cancer starts and progresses due to mutations and dysregulated gene expression, including oncogenes, tumor suppressor genes, chemoresistant genes, metabolism-related genes, and cancer stem cell-related genes [83]. Treatment aims to slow tumor growth and progression by correcting mutations and restoring gene expression, with the CRISPR/Cas9 gene editing system showing promising results.

7. DNA-based knockout/in Oncogenes:

Oncogenes, regulated differently than normal genes, can cause malignant transformation of cells. The CRISPR/Cas9 system can be used to delete, interfere with, and modify oncogenes, inhibiting tumor growth [84]. Knocking out CD133, miR-3064, and EGFR alleles reduced vimentin expression in colon cancer cells, slowed cell proliferation, and inhibited cell migration and invasion. The system has been effective in identifying oncogenes and assessing their therapeutic potential in various cancers, such as NSCLC cells carrying KRAS mutations [85].

8. Tumor suppressor & Chemotherapy resistance genes:

Tumor suppressor genes are crucial for cancer initiation and progression [86]. Mutations in these genes can activate oncogenes, leading to tumor formation. The CRISPR/Cas9 system has revolutionized cancer research by enabling rapid gene validation in vitro and in vivo. Mutations in the NF2 gene have been found to inhibit tumorigenesis in malignant pleural mesothelioma (MPM), and a human mesothelial cell line lacking NF2 has shown increased migration and invasion ability [87]. The deletion of multiple tumor suppressor genes in the mouse brain has led to glioblastoma development. CRISPR/Cas9 technology can identify tumor suppressor genes [88].

Chemotherapy resistance is a significant challenge in cancer treatment due to dysregulation of chemoresistance-related genes [89]. CRISPR/Cas9 technology has been used to identify and validate these genes, which are crucial for cancer treatment. Knockdowns of NRF2, SKA3, RSF1, ERCC1, and aurora B have increased sensitivity to cisplatin and carboplatin in lung cancer cell models [90], while knockdowns of ERCC1 and aurora B reduce sensitivity to cisplatin and paclitaxel [91].

9. Metabolism-related genes:

Tumor cells need energy to grow, migrate, and invade, and metabolic reprogramming, which regulates energy metabolism, is the new hallmark of cancer. Cancer cells frequently favor the "Warburg effect," which promotes aerobic glycolysis even in the presence of oxygen. Other bioenergy metabolism pathways found in cancer cells include lipid metabolism, amino acid metabolism, and mitochondrial biogenesis [92]. Understanding these energy metabolism mechanisms may aid in cancer treatment by targeting energy production pathways. Glucose transporter-1 (GLUT-1) and hypoxiainducible factor-1α (HIF-1α) are important markers of cellular hypoxia, influencing glucose uptake and glycolysis in laryngeal cancer cells. Lu et al. discovered that removing the HIF-1α and GLUT-1 genes from HEp-2 cells significantly reduced proliferation, migration, and invasion. Reduced glucose uptake and lactic acid levels [93].

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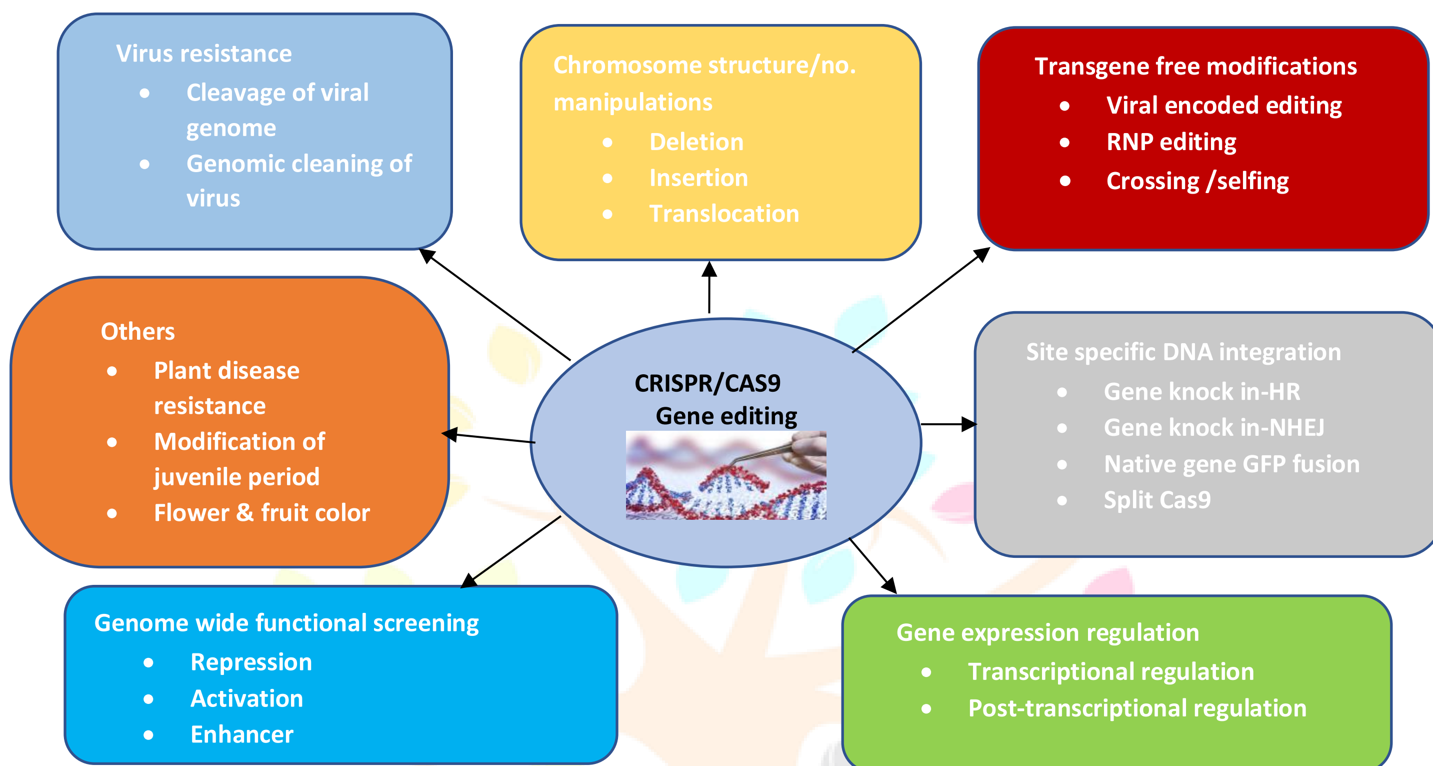

Fig.4 – Applications of CRISPR/Cas9 System

LIMITATIONS AND CHALLENGES:

The precise delivery of CRISPR/Cas gene therapies to the body holds promise for treating diseases both in research settings and clinical practice. Its advantages, including high specificity, efficacy, and ease of manipulation, position it as a highly coveted technology for the future. Nonetheless, scientists have encountered unforeseen circumstances when employing CRISPR technology for gene editing. Off-target effects arise from base mismatches between sgRNA and non-target sequences, potentially resulting in unintended mutations. The occurrence of even a single or multiple unknown mutations while rectifying one error is evidently unacceptable [94]. When sgRNA binds to the DNA strand, the seed sequence at the proximal end of the PAM adheres to the target strand strictly through base complementary pairing. In instances of mismatching, the distal three to five bases sometimes do not detach as anticipated. Instead, they form an unconventional duplex conformation under significant forces [95].

The mechanism permitting mismatches likely evolved in bacteria as a defense against mutations in invading phages. Techniques such as whole-genome sequencing and Guide Seq have been devised to identify instances of off-target target effects. Enhancing the specificity of sgRNAs and dissociating them from the DNA strand upon encountering mismatches is crucial for addressing this issue. The REC3 domain of Cas9 plays a pivotal role in detecting mismatches occurring at the distal end of the PAM. Researchers have introduced mutations in the REC3 domain to identify variants that could enhance the deliberately crafted to enhance the precision of Cas9, representing significant advancements in genome editing technology [96].

However, these mutants inadvertently compromised the interaction between mutated REC3 and the PAM-distal duplex, thereby diminishing Cas9's efficiency. By targeting the residues responsible for stabilizing this structure, they engineered a Cas9 variant, SuperFi-Cas9, which exhibited a remarkable 500-fold decrease in DNA duplex cleavage efficiency specifically at the 18th–20th base mismatch of the sgRNA. Importantly, this modification did not impair sgRNA-mediated double-strand cleavage when fully complementary bases were present. Moreover, enhancing sgRNA specificity by incorporating specialized structures is a viable strategy. Kocak et al. devised a hairpin structure at the 5′ end of sgRNA, effectively reducing energy during mismatches and preventing R-loop formation upon mismatch occurrence. This innovation holds promise for further improving the precision and fidelity of Cas9-mediated genome editing. The R-loop plays a vital role in activating Cas9, making it indispensable for DNA duplex cleavage. Consequently, the introduced structure not only impedes mismatch-induced R-loop formation but also hinders DNA duplex cleavage when mismatches are present. Off-target effects stand as the primary hurdle to the broad adoption of CRISPR-based gene-editing technology [97]. Enhancing sgRNA specificity through modifications in conjunction with Cas9 offers a promising avenue to mitigate these concerns and potentially avert the emergence of unforeseen mutations.

Table 3: Limitations & Challenges of CRISPR/Cas9 system

Limitations	Challenges and Solution
1. Off-target effects and specificity issues:	- Challenge: CRISPR may mistakenly modify genes that are related to the target, resulting in unwanted modifications. - Solution: To boost specificity, scientists employ upgraded guide RNA design, bioinformatics tools, and high-fidelity Cas9 enzymes [98].
2. Delivery efficiency and cell/tissue specificity:	- Challenge: Efficiently delivering CRISPR components to target cells is essential. - Solution: Researchers investigate viral vectors, nanoparticles, and various strategies to improve delivery precision [99].
3. Immune response and safety concerns associated with viral vectors:	- Challenge: Viral vectors used in CRISPR applications may provoke immune responses and safety concerns. - Solution: Strategies include utilizing gutted vectors, non-integrating vectors, and rigorous preclinical safety testing [100].
4. Delivery to specific tissues/organs:	- Challenge: Precisely delivering CRISPR to specific tissues within an organism is challenging. - Solution: Researchers look at targeting ligands, tissue-specific promoters, and other delivery strategies [101].
5. Ethical considerations and regulatory challenges:	- Challenge: CRISPR raises ethical questions and necessitates robust regulatory frameworks. - Solution: Addressing ethical challenges involves ongoing public engagement, strong guidelines, and risk assessment [102].
6. Off-target detection methods:	- Challenge: Accurately detecting off-target effects is crucial for safety. - Solution: Ongoing efforts focus on enhancing detection methods such as high-throughput sequencing and in silico tools [103].
7. Control of gene expression and spatiotemporal regulation:	- Challenge: Achieving precise control over gene expression is vital. - Solution: Optogenetic instruments, synthetic biology techniques, and inducible and reversible CRISPR systems are some of the strategies [104].
8. Delivery to non-dividing cells:	- Challenge: There are particular difficulties in delivering CRISPR to non-dividing cells. - Solution: Research focuses on alternative repair mechanisms and cell-cycle-dependent promoters for non-dividing cells [105].

Milestones in CRISPR/Cas9 research:

CRISPRs, initially identified in 1987 as odd repeated sequences [106], were popular in the 1990s and early 2000s. They were first thought to be involved in cellular DNA repair and replicon partitioning mechanisms. In 2005, researchers discovered that most

interspersed sequences between identical repetitions were obtained from extrachromosomal DNA, notably phage genomes and conjugative plasmids. This theory was later verified when scientists demonstrated the integration of additional spacers into a CRISPR-Cas locus of Streptococcus thermophilus, which exhibited perfect complementarity to sequences on the phage genome and conferred resistance against that specific phage upon future infection [107].
Research interest in the CRISPR area grew, resulting in new findings that helped us grasp the fundamental mechanics of the immune system. It was experimentally confirmed in 2008 that the CRISPR transcript is transformed into mature crRNAs, which direct the Cascade complex of the E. coli type I-E system and target DNA instead of RNA [108]. This prompted scientists to consider the prokaryotic immune system's possible usefulness as a DNA modification tool.

CRISPR-Cas9 is currently widely used for genome editing, with significant advances in understanding the underlying biochemical mechanisms in RNA-guided Cas9. In 2010, researchers discovered that Cas9 causes a single double-stranded break at a specific location on the target DNA [109]. TracrRNA, which was previously known to be involved in crRNA maturation, was discovered in 2012 to be a crucial component of the DNA cleavage complex. A single-guide RNA fusion of tracrRNA and crRNA led Cas9 to cleave DNA based on sequence [110].

BIOMEDICAL APPLICATIONS OF CRISPR/CAS9 SYSTEM:

Researchers have created engineered Cas9 variants to reduce off-target effects (OTEs) in CRISPR/Cas9 gene therapy. These variants, such as Cas9 nickase, SpCas9-HF1, evoCas9, HiFiCas9, and Cas9_R63A/Q768A, use the "excess-energy" model to maintain editing efficacy. They aim to reduce Cas9 and DNA interactions and strengthen the DNA: RNA hetero duplex in facilitating edits. Optimizing guide designs can also reduce OTEs in sgRNA by adjusting seed sequence, GC content, and modifications. A new platform, sg Designer, has outperformed other tools in six independent datasets.

1. DNA damage toxicity:

CRISPR-induced DSBs can trigger apoptosis, posing potential safety concerns in clinical applications. Using CRISPR in human pluripotent stem cells (hPSCs) has shown that p53 activation often triggers apoptosis, leading to oncogenic cell suppression [111]. Variations of Cas9, such as catalytically inactive endonuclease dead Cas9 (dCas9), can provide therapeutic utility while mitigating DSB risks. Cas9n induces SSBs instead of DSBs, and further alterations to Cas9 have led to the invention of base and prime editors [112].

2. Immunotoxicity:

CRISPR/Cas9 gene therapy faces technical limitations and immunogenic toxicity concerns. Over half of human subjects have preexisting anti-Cas9 antibodies against commonly used bacterial orthologs, SaCas9 and SpCas9. AAV vectors are widely used for CRISPR gene therapy. Three Cas9 orthologs (SpCas9, SaCas9, and CjCas9) showed robust editing efficiency and tolerated repeated administration due to reduced immunogenic toxicity. However, CjCas9 is the only option for this cohort due to pre-existing immunity in humans against SpCas9 and SaCas9. Further investigation is needed for clinical use [113].

3. Delivery of CRISPR Gene Therapy:

CRISPR gene therapy is primarily delivered using AAV vectors due to their efficiency and safety. The toolkit can be packaged as plasmid DNA or mRNA of Cas9 and gRNA and introduced to target cells via electroporation/nucleofection or microinjection. However, microinjection is technically challenging and only suitable for ex vivo delivery [114]. Electroporation is mainly used for ex vivo but can be used in vivo for certain target tissues. AAV delivery of CRISPR components can increase Onset Transient Errors (OTEs), but delivering Cas9 protein and gRNA as RNP complexes reduces OTEs while maintaining editing efficacy. CRISPR/Cas9 edits can be facilitated ex vivo or in vivo [115].

4. Potential for CRISPR Therapeutics during the COVID-19 Pandemic:

CRISPR technology has the potential to significantly aid in the COVID-19 pandemic by providing a faster and more accurate method for testing respiratory infections. Current testing methods, such as real-time RT-PCR and serological testing, have a high false negative rate and turnaround time. A CRISPR Cas12-based assay called SARS-CoV-2 DETECTR has been developed for COVID-19 detection with a 95% accuracy rate. CRISPR also offers therapeutic options for COVID-19 patients, with techniques like PAC-MAN targeting multiple regions for RNA degradation. This could help combat high mutation and re-combination rates of SARS-CoV-2, allowing CRISPR/Cas machinery to be used as a virus-battling system during the pandemic [116].

Therapeutic approaches for genetic & infectious diseases:

Genetic diseases, caused by inherited mutations, can cause symptoms. The CRISPR-Cas9 system is being used to treat defective genes by correcting genes in blood and somatic cells taken from the patient's body. Repeated treatment can increase the percentage of normal cells in the body, potentially leading to a complete cure or alleviating symptoms [117]. This method is widely used to treat various genetic diseases, including cystic fibrosis, Duchenne muscular dystrophy, Huntington's disease, hemophilia, and hematopoietic diseases.

Traditional virus defense methods include vaccine prevention and antiviral treatment, but some viruses, like HIV, herpes simplex, Epstein-Barr, human cytomegalovirus, and Kaposi's sarcoma-associated herpesvirus, cause latent and chronic infections. Targeting the viral genome in these cases is challenging due to its retention inside the host cell. The CRISPR-Cas9 system offers a new defense technique, allowing for a more detailed study of virus-host interaction and developing faster, more accurate diagnostic techniques .This technology aids gene editing for prevention and therapy, targeting both host and viral genomes [118].

The CRISPR-Cas9 system has four major strategies to avoid viral infection-

1) Modifying receptors for viral entry

2) Segmenting host viral factors

3) Inducing host transcriptional restriction factors

4) The integrated viral genome is removed and deleted. These mechanisms aim to prevent viral replication, silence essential genes, and reduce viral RNA.

1. Cystic fibrosis:

Cystic fibrosis (CF) is a disease caused by a defect in CTFR proteins, affecting multiple organs and causing loss of control over electrolytes and osmole. Genetic engineering-based treatment is being explored, with CRISPR-cas9 found to fix the F508 deletion in the CFTR gene in iPSCs. Methods for accurately and safely differentiating ex vivo edited cells are being studied, and new approaches like the double nickase approach and repair template homology arms are continuously being developed [119].

2. Huntington's disease:

Researchers have used the CRISPR-Cas9 system to disrupt the mutant HTT gene expression in a mouse model of Huntington's disease, resulting in a 50% reduction in neuronal inclusions, improved life span, and some motor impairments [120].

3. Hemophilia:

Hemophilia, a congenital hemorrhagic disease caused by mutations in blood coagulation genes, is a promising target for gene therapy. Researchers are using the CRISPR-Cas9 system to correct the defective coagulation factor gene [34], and clinical trials are underway for hemophilia A and B. However, limitations exist, and various methods are being proposed to overcome these issues [121].

4. Hemoglobinopathies:

Researchers have developed a CRISPR-Cas9 genome-editing strategy to study hemoglobinopathies, such as β-thalassemia and sickle cell disease. They deleted a 200 bp genomic region within the human erythroid-specific BCL11A enhancer, leading to a significant increase in γ-hemoglobin expression in K562 cells. This suggests reactivating fetalhemoglobin could alleviate mild clinical symptoms in patients with reduced oxygen transport capacity [122].

5. Cancer Therapeutics:

Cancer, a genetic disease caused by genetic/epigenetic errors, could be treated using the CRISPR-Cas9 system, which could repair oncogenic genome/epigenome aberrations, improving precision and safety of existing anticancer therapies and broadening applications, including drug development research, as cancer requires a series of genetic alterations [123].

6. Genome Editing of Cancer Cells:

CRISPR-Cas9 is a widely used genome editing tool due to its specificity, efficiency, and accuracy in identifying oncogene roles in cancer cells. It has been demonstrated for cancer gene edition and subsequent cancer therapeutics [124].

7. Gene therapy:

CRISPR/Cas systems have shown great potential in treating inherited diseases like Duchenne muscular dystrophy, sickle cell disease, β-thalassemia, hemophilia A and B, and recessive dystrophic epidermolysis bullosa (RDEB). The CRISPR/Cas9-based technique can fix exon 44 deletion mutations of the dystrophin gene, treat SCD by targeting the enhancer region of B-cell lymphoma/leukemia 11 A gene in hematopoietic stem and progenitor cells, correct human hemoglobin beta gene mutations, ameliorate symptoms in mice with Hemophilia A mutations, and repair an in-frame deletion in exon 2 of the factor IX gene linked to hemophilia B [125]. CRISPR/Cas systems also play a central role in curing RDEB, a severe inherited skin disorder [126].

8. Cancer modeling:

CRISPR/Cas systems are utilized in cancer modeling for various types of cancer, including breast, colorectal, pancreatic, lung, and liver. A Cre-dependent Cas9 knock-in mouse is used to produce mutations in tumor suppressor genes and proto-oncogenes, allowing for multiple mutation modeling in carcinogenesis. The technology is also used to target tumor suppressor genes for liver cancer models, create breast cancer organoids, generate colorectal cancer models, and generate pancreatic and Wilms' tumor models [127].

9. Cancer therapy:

The CRISPR/Cas9 gene-editing technology has revolutionized cancer treatment by killing cancer cells without affecting normal cells. Dai et al. developed a telomerase-activating gene expression system that kills cancer cells while sparing normal ones. The system

improves T-cell immunotherapy by knocking out the programmed death-1 gene, suppresses lncRNA UCA1 expression, and enhances macrophage phagocytosis. Clinical trials have shown promising results [128].

10. Viral and bacterial pathogen detection:

The CRISPR/Cas13a system has proven effective in detecting various viruses and bacteria. A molecular detection system called Specific High-Sensitivity Enzymatic Reporter Unlocking (SHERLOCK) has been developed for Zika and Dengue viruses with low detection limits and costs [129]. Fozouni et al. designed an amplification-free CRISPR/Cas13a test for the direct detection of the SARS-CoV-2 outbreak in 2019. Liu et al. developed a room temperature influenza A H7N9 detection system using the CRISPR/Cas13a system. A unique CRISPR/Cas13a system was designed to identify the DNA of the hepatitis B virus with high sensitivity and specificity. A CRISPR/Cas13a-based isothermal diagnostic method enables rapid detection of Ebola virus [130]. The system also plays a significant role in bacteria detection, such as Salmonella Enteritidis detection and mix-and-read detection of viable bacteria [131]

11. Therapeutic Role of CRISPR/Cas-9:

CRISPR/Cas-9 gene-editing technology has been used to treat refractory lung cancer and other infectious diseases caused by microorganisms. Researchers extracted T-cells from three patients' blood and engineered them to delete genes that interfere with cancer cell fight. The modified T-cells can target specific antigens and kill cancer cells without side effects. The technology could also block HIV entry into host cells by editing chemokine co-receptor type-5 (CCR5) genes. In May 2017, Temple University researchers demonstrated that HIV-1 replication can be completely shut down and eliminated from infected cells.

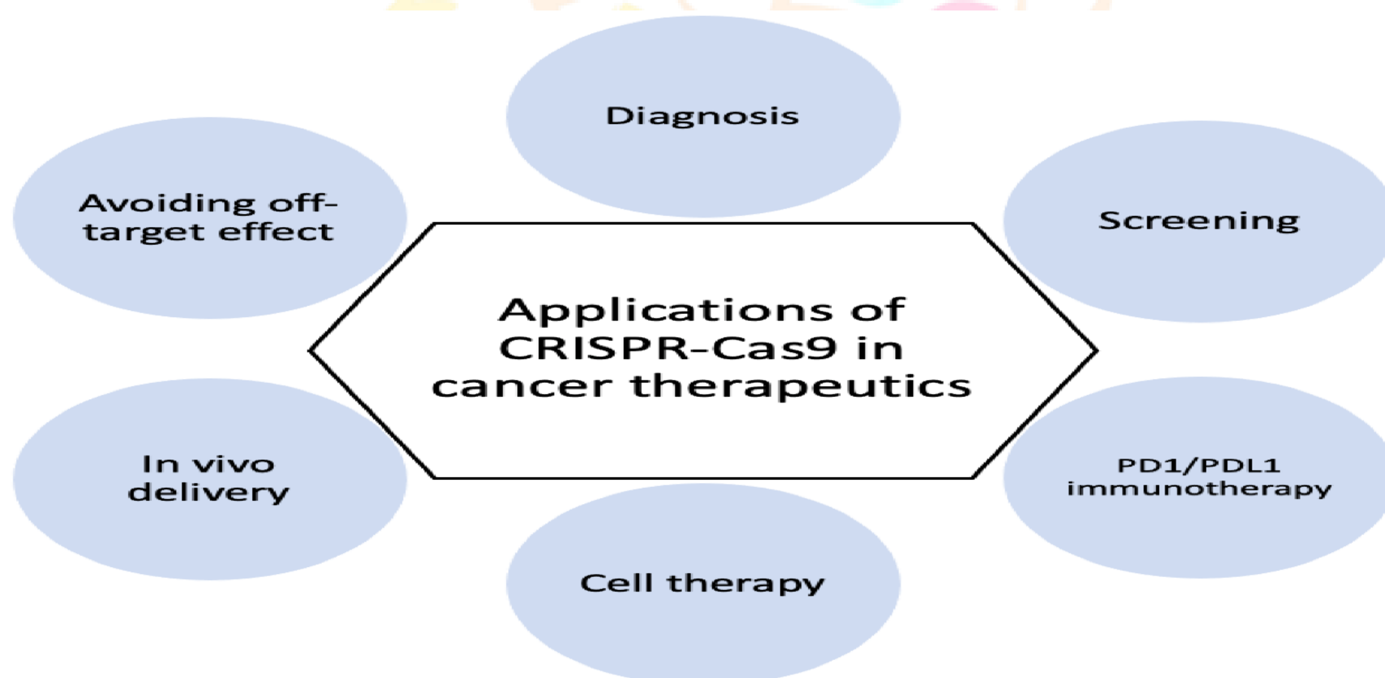

Fig.5- Applications of CRISPR/Cas9 in Cancer Therapy

ETHICAL AND REGULATORY CONSIDERATION:

MRT, a potential treatment for multiple sex pregnancies, faces ethical challenges in both theory and practice. These issues hinder further research and clinical studies to assess its efficacy in humans. Risks to donors and offspring, as well as concerns about "designer" kids, are key issues to address before implementing MRT. The term "tri-parental offspring" was previously used to describe a form of bacterial conjugation [132].

Donor eggs are often used with assisted reproductive technologies to help women with insufficient oocytes conceive. Babies may inherit genetic material from both the donor and father, but also be influenced by epigenetic factors from the carrier mother. This method is not considered "three-parent IVF" due to the small number of genes in mtDNA, which makes up only 0.1% of the total DNA.Organ donors and cytoplasm donors have no claim to donated organs or infants. Oocyte donors must sign release and consent documents waiving parental rights to offspring. Despite a 50% contribution to the offspring's genetic makeup, there is no case in the U.S. where an egg donor is granted parental status after giving up rights to eggs and ensuing offspring [133].

Concerns about germ line editing:

1. Unintended germ line editing:

Ethical concerns arise from inadvertent changes in germ cells during in vivo gene-editing therapy, similar to gene addition techniques. Human gene therapy aims to prevent genetic alteration of the human germ line. Preclinical investigations in rodents and animal models showed no persistent gene transfer to egg or sperm during early clinical trials using viral-mediated gene delivery. The preclinical safety assessment should demonstrate that a specific in vivo gene-editing approach won't detectably modify germ cells in preclinical models, reducing the risk of inadvertent germ line modification in patient germ cells. However, there's also the possibility of spontaneous germ line abnormalities in embryos due to ex vivo interventions and genome-editing reagents [134].

2. Intentional germ line editing:

Hereditary illnesses are severe and require research to cure or prevent them. One theoretical solution is genome editing of germ cells or zygotes to fix disease-causing mutations in all cells, preventing the formation of these diseases. Modern medicine's goal is to cure genetic abnormalities in somatic cells, but germ cell-forming tissues like testes or ovaries may also be gene-modified, potentially passing it down to future generations. This unintended consequence of modern medicine is that it indirectly changes the allele frequency of some disease-causing mutations in the human gene pool. Ethical concerns arise with the intentional manipulation of germ lines to eliminate harmful mutations, potentially transferring modified genomes into the human gene pool through childbearing. Other ethical considerations include the intervention's goal of disease cure or genetic enhancement, and whether the change will re-create naturally found human genetics or create a non-normal human genome, such as adding a gene to a specific genomic location. The current understanding of the genome presents risks, including the uncertainty of describing an improved genome. Preimplantation genetic diagnosis cannot fully replace germ line genome editing in some cases. However, in some cases, preimplantation genetic diagnosis may be ineffective, such as when couples with a homozygous recessive condition produce children or one parent is homozygous for an autosomal-dominant disease. Overall, understanding and addressing these risks is crucial for effective genome editing.Existing zygote editing technologies in rodents are wasteful, requiring numerous zygotes to be modified, and implanted to produce a mosaic genetically edited child. Additionally, it is impossible to ensure that unintentional heritable alterations do not occur, potentially leading to long-term consequences like cancer or negative developmental impacts. To address these safety issues, germ line stem cells should be modified, and sequencing should be conducted to prevent unintentional mutations. This approach could potentially alleviate these seemingly insurmountable safety concerns. No human germ cell transplantation or production from germ line stem cells has been performed without alteration, despite potential safety and ethical issues associated with altering human zygotes and implanting them into individuals [135].

The ethical issue with using editing in human zygotes for research is that it would require the generation of specific 1-cell zygotes, as these are already at the multicellular blastocyst stage, which is beyond the earliest stages of zygote development. Editing strategies during this stage could result in a mosaic of modified cells with varying genetic alterations, confusing experimental results. The Human Fertilization and Embryology Authority in the UK has granted permission for in vitro studies using CRISPR/CRISPR-associated protein 9 [136].

Address the ethical implication of CRISPR/Cas9 technology:

CRISPR, or clustered regularly interspaced short palindromic repeats, was discovered in Escherichia coli bacterium DNA sequences and published by Ishino et al. in 1987[137].

In 1987, the CRISPR/Cas system was discovered in prokaryotes as an adaptive immune system against bacteriophages or plasmids. Researchers discovered 32 nucleotide non-repeat sequences and tandem repeats downstream of the IAP gene, which became known as CRISPR in 2000 [138]. The bacterium's CRISPR/Cas system neutralizes viral genomes during phage attacks, acting as a defense mechanism. Its Cas library monitors invading viral sequences and destroys them when attacked again. Traditional crop improvement methods use mutation breeding with chemicals like EMS or gamma radiation [139].

Recent research has shown that the use of endogenous RNA Pol III promoters, specifically U3 and U6, in the CRISPR/Cas9 system can enhance genome editing efficiency in various plant species. These promoters transcribe guide RNAs that direct Cas9 nuclease to specific genomic areas, improving editing efficiency and precision. The use of species-specific U3/U6 promoters holds promise for further genome editing advancements [140].

CONCLUSION:

CRISPR-Cas9 technology has had a substantial influence on several sectors, including genome editing, animal research, medical, human gene therapy, plant biology, and medicine. It has transformed cancer research, agriculture, biotechnology, and biomedicine by improving drug delivery and therapy using precise gene editing. However, issues persist in assuring safe and effective clinical delivery. The discovery of biological ideas can lead to the generation of new molecular tools that improve life comprehension and manipulation.

CRISPR/Cas9 is a potent gene editing technique that takes advantage of bacteria and archaea's adaptive immunological defenses against DNA invasion. It is simpler, more efficient, less expensive, and has lower cytotoxicity than older methods like as ZFNs and TALENs. Its successful application to precise genome alteration in microbes, plants, and mammals opens up new avenues for biological science study. Researchers are working to create a more efficient and accurate CRISPR/Cas9 method to assess editing efficiency with reducing off-target risk. CRISPR/Cas9 is utilized in biology to help create novel vaccines and treatments, study gene functions, customize disease therapy, and accelerate animal and crop breeding [141]. It is preferred by researchers for its efficiency, simplicity, and cheap experimental cost, especially in cancer research because to its reduced off-target effects. It introduces a novel genetic therapy for hepatocellular carcinoma, a highly aggressive condition with few therapeutic alternatives. Although most CRISPR/Cas9 gene treatments are still in the experimental stage, they require considerable investigation and scientific explanations to fully realize their promise or potential.

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