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CRISPR–Cas9 and In Vivo Genome Editing: Transforming the Future of Therapeutics

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ABSTRACT

One of the finest ways to change genomes is via CRISPR-Cas9. This approach lets us modify DNA exactly and without making any mistakes. We want to emphasize how crucial CRISPR-Cas9 is to human health in this review, especially when it comes to applying it in vivo for diseases like cancer and degenerative ones. Some recent developments include cancer genome editing using patient-derived xenografts (PDXs), treating atherosclerosis with adeno-associated virus (AAV) CRISPR, and repairing photoreceptor degeneration with Cas9-RecA. The study discusses base editing, prime editing, and CRISPR-integrated synthetic biology as potential future directions, along with other Cas systems, the regulatory and ethical landscape, and more subjects. We have added tables and figures to help you compare gene-editing methods with the drug development processes that are already in use. CRISPR is set to alter current medicine by making medicines that weren't possible before. But they are still working out how to deal with off-target consequences, delivery modalities, and germline ethics.

KEYWORDS: *CRISPR, Cas9, Diagnosis, Genome editing, Gene therapy, Strain engineering*

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INTRODUCTION

For a long time, the unspoken code of DNA has determined how healthy people are. Our genes usually determine how likely we are to get a wide range of diseases and conditions, from common ones like cancer, diabetes, heart disease, and neurodegenerative disorders to rare ones like hemophilia, sickle cell anemia, and cystic fibrosis.[1,2] For decades, medicine has excelled at treating symptoms, slowing disease progression, or providing temporary relief. Yet the root causes of the faulty instructions written within the genome remain largely untouched. The dream of modern biomedicine has been to correct errors at their source, offering not just management but genuine cures.

The dream of modern biomedicine has been revising the previous mistakes at their sources offering not just management but genuine care. These dreams began to take tangible form the first genome editing tools. If we talk about the difference between all the genome editing tools like ZFNs and TALENs describe that DNA could be cut and rewritten in a targeted way, and CRISPR were complete system required some scalability and efficacy, and they proved that fact that genome editing was possible.

In 2012, Jinek and colleagues gave a new insight into the defense mechanism of bacterial genomes and said that CRISPR based techniques required a short guide RNA to direct the Cas proteins to target any DNA sequences. Came across editing in the genome not only efficient but also more accessible.[3] Laboratories around the world adopt the technology without years of specialized training, sparking an explosion of new discoveries. The impact of CRISPR has been compared to the invention of the polymerase chain reaction (PCR) in the 1980s, a universal tool that fundamentally reshaped molecular biology.

From the long period of time since its discovery, CRISPR has been used as a prominent tool for various fields of life science. In the decade since its discovery, CRISPR Cas9 has transformed research and entered real-world applications. In agriculture, it has been used to engineer crops with improved resilience and nutritional profiles. In biotechnology, it has streamlined synthetic biology and microbial engineering. But it is in medicine that CRISPR's potential feels most revolutionary. For the first time in history, clinicians are not limited to treating the downstream effects of mutations; they can contemplate correcting or silencing the mutations themselves. There are two primary approaches used ex-vivo genome editing involves the removal of desired cells from the patient's body and editing them outside the body, and reinfusing them. This approach very helpful to

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treat sickle cell Anemia and β-thalassemia. However, ex vivo strategies are best suited for blood and immune cells, which can be extracted and manipulated relatively easily.

On the other hand, in vivo genome editing also has great affinity to cure different genetic diseases, in which the machinery used in genome editing is directly delivered inside the patient's body to rectify the mutations at the source. This approach is also helpful to expand the editing at the tissue level, like the liver, kidney, muscles, and retina. As well as it works for the central nervous system. So could you imagine this fact that one time injection that silences any gene driving heart disease, repairs a faulty enzyme causing metabolic dysfunction, or restores vision in retinal disorders. The potential is staggering.

Gillmore and their colleagues gave the first evidence after the delivery of CRISPR Cas9 into the patients with transthyretin amyloidosis, and this alleviates to disease-causing protein levels.[4] This statement proved that CRISPR was not bound at a laboratory level; however, it jumped into the clinical level. Since then, research has accelerated across multiple fronts, from cardiovascular medicine to neurology and oncology.

However, there are many problems associated with the gene delivery system, but reaching the gene of interest at the correct site is the crucial challenge in front of scientists. While viral vector-associated delivery systems like Adeno-associated virus (AAV) have been studied and applied but they face size-related issues, preexisting immunity, and concerns about the long-term safety.[5] There are some non-viral mediated transfer systems also available in which lipid Nanoparticles (LNPs) and polymer-based carriers give better alternative options. Beyond delivery, scientists must confront risks of off-target mutations, unintended immune reactions, and questions about durability and reversibility.

If we talk about the human DNA, it is crucial to think about the ethical and social considerations because it raises profound questions; where we make the limit across the treatment, therapies, and enhancements. It also raises concerns about ensuring fair and equal access to genome editing technology, so that these treatments do not worsen existing health disparities. Moreover, strong safeguards are necessary to prevent potential misuse, especially in germline editing, where genetic changes could be inherited by future generations.[6] These debates are not abstract; they shape regulatory frameworks, guide public trust, and determine how quickly new therapies can reach patients. CRISPR-based therapies are not only for rare genetic diseases but also for common conditions like cholesterol, muscular dystrophy, and certain cancers. This technology does not stop here, with evolving new iterations like base editing, prime editing, etc., giving unintended effects.[7]

This review focuses on the most exciting and challenging frontier: in vivo CRISPR–Cas9 genome editing in human health. We aim to collect recent scientific progress, highlight the biological and technical obstacles that remain, and explore the future directions of this field. Ultimately, the question is not whether CRISPR will change medicine; it already has, but how responsibly, safely, and equitably this transformation will unfold in the years ahead.

ORIGINS OF CRISPR

The origin of the CRISPR system can be traced back to 1987, when Ishino and colleagues first reported unusual DNA repeats in Escherichia coli, although their biological role remained unclear at the time.[8]

These sequences were later termed Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) by Jansen and co-workers in 2002, who also identified the presence of associated Cas (CRISPR-associated) genes.[9] Subsequent comparative genomic studies demonstrated that the unique "spacer" sequences within CRISPR loci were derived from bacteriophage and plasmid DNA, suggesting a role in microbial defense.[10] This hypothesis was experimentally confirmed by Barrangou and colleagues in 2007, who showed that CRISPR provides adaptive immunity against phages in Streptococcus thermophilus.[11] The transformative breakthrough came in 2012, when Doudna and Charpentier demonstrated that the Cas9 protein could be programmed with a guide RNA to cleave DNA at specific sites, thereby repurposing the bacterial immune system into a versatile genome-editing tool.[3]

Components and Mechanisms of the CRISPR-Cas System

CRISPR-Cas has a diverse array and consists of different Cas proteins. Bacteria and archaea use CRISPR technology as their immune system against phages and foreign DNA. CRISPR arrays are made up of three main parts: A leader section (next to the first repeat and works like a promoter to start the CRISPR system). Short repeated sequences (that fold into hairpin shapes to keep the RNA stable), and spacers (unique pieces of foreign DNA that were taken from viruses or other sources). Mainly, CRISPR can be divided into two classes, class 1 and class 2, class 1 consisting of multiple Cas effector proteins, and these proteins bind to crRNA and produce target interference. Class 2 requires only types 2, 5, and 6, and it encompasses only a single multidomain large Cas effector protein to form a complex with crRNA in the interference process.[12]

Commonly used Cas proteins

Cas 9 is most widely used; alternative Cas proteins offer expanded functionality:

Cas12a (Cpf1): CRISPR-Cas12 is a part of the class 2 system. It contains Cas 12a protein along with CRISPR RNA. It does not require any RNA or other proteins for the pre-crRNA processing. It has the ability to cleave the target without tracer RNA, Cas12a identifies double-stranded DNA via a single crRNA. Cas12a identifies double-stranded DNA regions with a T-rich PAM sequence using the guidance of its crRNA. Once bound, it unwinds the DNA strands. The target strand pairs with the crRNA, triggering a conformational change that exposes the RuvC domain. This domain first cleaves the non-target strand (NTS) and then cuts the target strand (TS). This sequence of action is also known as the cis-cleavage activity of Cas12a.[13]

Cas 13: Cas 13 does not need any Protospacer Adjacent Motif (PAM) to recognize its targets. However, they depend on a protospacer flanking site (PFS). The nucleotides that are upstream of the spacer sequences must contain A, C, or U. It is used for editing and antiviral applications. Actually, it is an enzyme that targets and cleaves single-stranded RNA sequences instead of DNA, allowing applications like RNA knockdown, gene editing, and diagnostics. Here, it's RNA sequence works as a guide to find a specific RNA sequence, and CAS13 cleaves it. Cas 13 also has a unique feature to collateral and trans cleavage activity, in which they cleave many RNA in addition to their targets, and this works in SHERLOCKS diagnostic tests.[8]

Cas-14: Cas-14 is also referred to as Cas12f. It was recently discovered and identified from uncultivated archaea in 2018. Like Cas 9, which largest double-stranded DNA needs PAM sequences but Cas 14 cleaves single-stranded DNA without PAM sequence requirements, and it contains RUVC nuclease domain that is required for its activity. It is approximately a 400-700 amino acid sequence, making it feasible to deliver into cells. Due to its high specificity, Cas14 has been applicable in diagnosis, including the development of fluorometric sensors and double aptamer biosensors for the determination of bacteria.[14,15] Mainly, it explores the genome editing tool by expanding the target of (ssDNA) region that may be inaccessible to CRISPR system. Cas-14 is a versatile tool for both genome and molecular diagnosis.

CRISPR Cas9 Function

Cas-9, guided by a synthetic single-guide RNA (sgRNA), introduces double-stranded breaks (DSBs) at target DNA sites. These breaks are repaired mainly by three methods, figure 1 & 2 represent stepwise mechanism and their role respectively.

Non-homologous end joining (NHEJ) repair is prone to errors and results in insertion or deletion (indels). It is a fast but perhaps error-prone method that binds broken DNA double-strand ends directly without the need for a homologous template. It is a key repair pathway in vertebrates. Ku 70/80, DNA Ligase IV, nuclease, and polymerases are required for the processing and ligation. This process gives results in little insertion and deletion at the repair site, and it causes mutation and instability to genomic those who necessary for the survival of the cell.[16]

Homologous direct repair (HDR) uses a template for the correct insertion and an accurate DNA repair mechanism that uses a guide template to rectify a double-stranded DNA break. Scientists utilize HDR, causing changes in DNA by providing a template such as CRISPR Cas gene editing. Unlike non-homologous end joining (NHEJ), which can introduce errors, HDR is an error-free pathway that is more active during the S and G2 phases of the cell cycle when a homologous template is available. Researchers utilize HDR to make specific changes in DNA by providing a custom template, such as with CRISPR Cas9 gene editing.[13]

Microhomology-mediated end joining (MMEJ) is an alternative repair with short homology arms. It is an error-prone DNA repair pathway that fixes double-strand breaks by aligning short, similar DNA sequences (microhomologies) at the broken ends to seal them. DNA end resection, gap filling, annealing of micro homologous sections, excision of DNA "flaps," and ligation are all steps in this process. MMEJ is crucial in situations where other repair routes are impaired, yet it is mutagenic, causing deletions and insertions, even if it is effective at restoring DNA integrity.[10] It is a different type of repair mechanism that requires short homology arms by comparable DNA at the broken ends near them, this process repairs double-strand breaks.

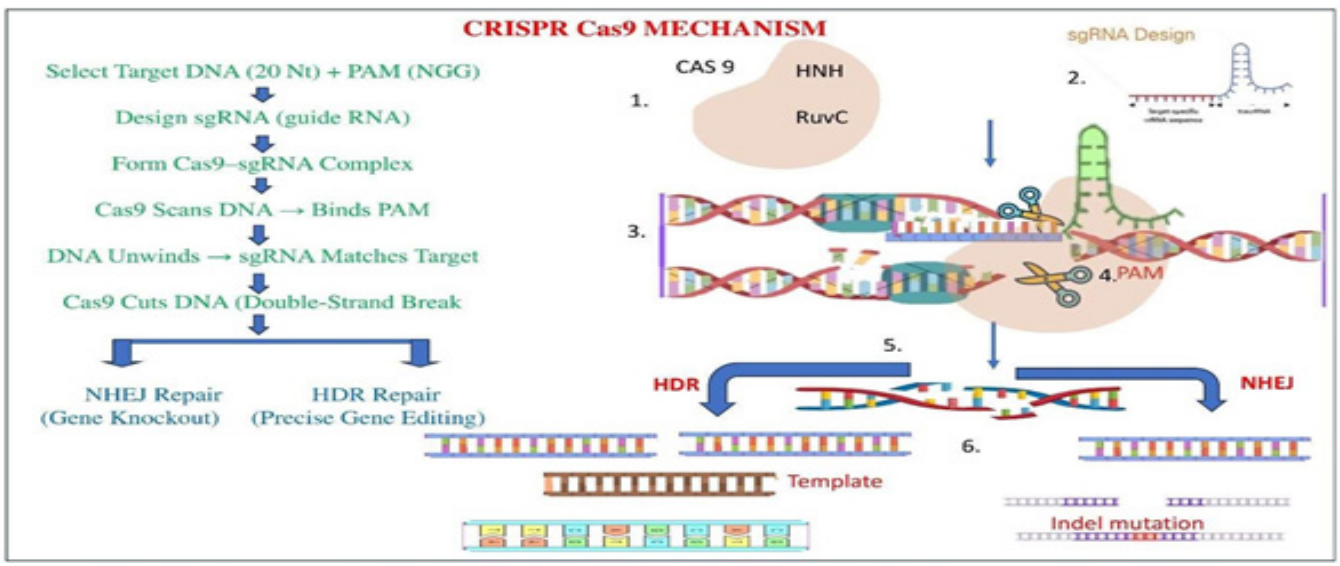

Figure 1: Stepwise mechanism of CRISPR Cas9 shows spacer acquisition from phage DNA, processing of CRISPR RNA, and Cas9 guided targeting that cleaves invading nucleic acids.

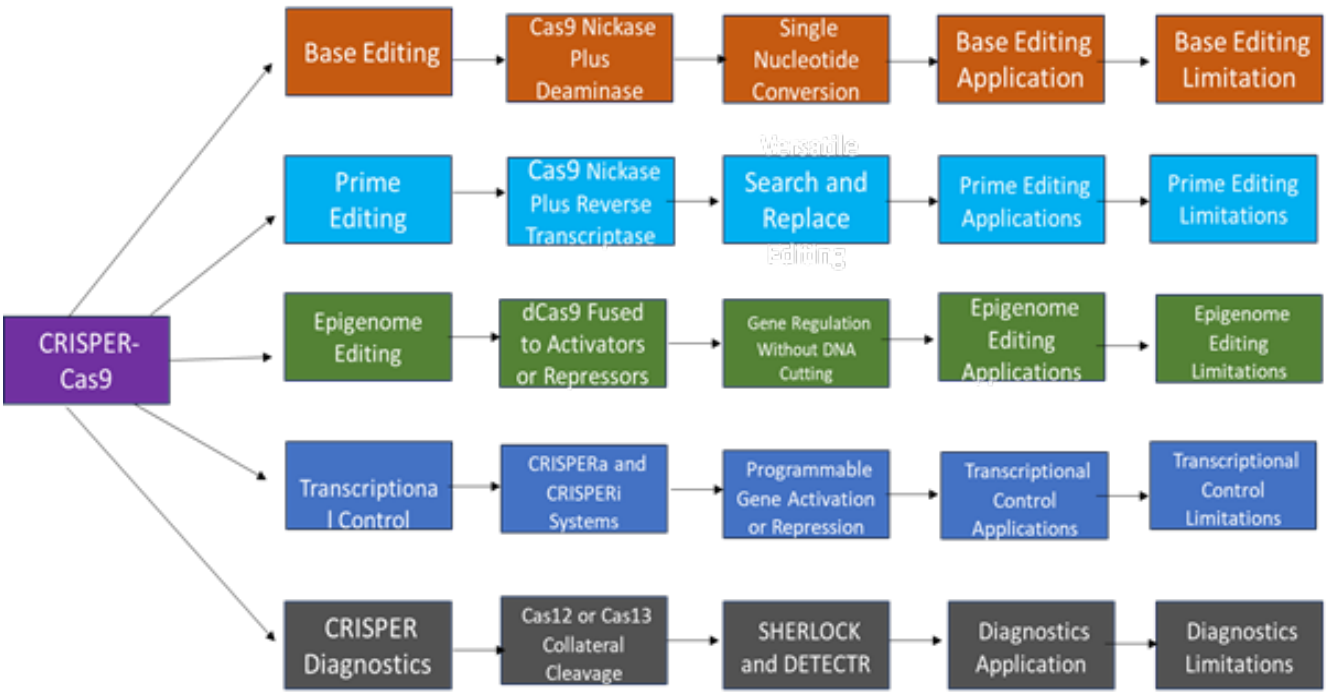

Figure 2: Expanding horizons of CRISPR Cas9: Diverse strategies, applications, and limitations. This flow chart illustrates the determining functions of CRISPR-Cas 9 technology. Primary branches consist of base editing, prime editing, epigenome editing, transcriptional control, and CRISPR-based diagnostics. Every pathway describes the molecular mechanism, and its applications, along with limitations, give an integrated overview of CRISPR Cas 9 as a versatile genomic tool in the field of biotechnology.

Therapeutic Applications of CRISPR Cas in different fields of life Science

Clinical trials aimed at modifying T-cells and other hematopoietic cells are currently in progress or have been announced. These approaches use ex-vivo genome editing, where a patient's cells are altered outside the body and then reintroduced. Compared with in-vivo strategies, ex-vivo therapies provide distinct advantages, like genome-editing tools that can be delivered into cells more easily, the likelihood of unintended editing in non-target cells is reduced, and properly edited cells can be isolated or enriched before transplantation, thereby enhancing both the safety and overall effectiveness of the treatment.

Development of Organoid Culture

Organoids are aggregates of cells, and they are a self-organized structure that grows from stem cells. CRISPR could be applied to a mouse model and a human intestine organoid through gene knockout or to rectify the disease-causing mutation. Adult epithelial stem cells allow researchers to study the development of organoid culture. Scientists found that the epithelial lining of the human intestine expanded indefinitely in-vitro when it found a basic matrix and essential growth factor.[17]

Coronary Heart Diseases

A promising way to prevent coronary heart disease (CHD) is to block certain genes that cause harmful cholesterol problems. These genes include PCSK9, ANGPTL3, and APOC3. People who naturally have mutations that turn off these genes usually have lower blood fat (lipid) levels and a much lower risk of CHD, for example, in the case of PCSK9, the risk can be reduced by up to 88%. Even people who inherit two such mutations and completely lose the gene's function remain healthy without negative effects. Because of this, these genes are now considered important targets for therapy, and some treatments based on them are already available or being tested in clinical trials.1 CRISPR technology can play an important role in this type of gene therapy.

Advancement in Plant Biology

With the help of CRISPR-Cas technology, researchers have found a new way to improve crop quality and productivity through successful application and alteration in the metabolic pathway of the plant. Type 2 CRISPR has a terrifying impact on the field of bioengineering and molecular biology. Jiang et al. (2024) created a variety of binary vectors with distinct Cas9 and sgRNA combinations and used Agrobacterium or PEG-mediated transfection to study their transitory expression in Arabidopsis, tobacco, rice, and sorghum.[18] Their research verified that target genes in these plants can be edited by the CRISPR Cas9 system. Similar to this, Jia and Wang used Xanthomonas citri subsp. citri (Xcc)-mediated agroinfiltration to target the CsPDS (phytoene desaturase) gene in sweet oranges.[4,22]

(Yip, 2020) This method successfully induced mutations in the target gene without any discernible off-target effects. By showcasing the effectiveness and specificity of CRISPR Cas9 in plant gene editing, Yin et al. provided additional evidence for these conclusions.[19]

Applications of Cas9 in Research, Medicine, and Biotechnology

Cas9 can be used for many different types of targeted genome editing. The normal (wild-type) Cas9 enzyme has made it possible to efficiently change the DNA of many species that were difficult to modify with older genetic methods. One big advantage is that Cas9 can be easily reprogrammed just by designing a short guide RNA. which makes it useful for large-scale experiments to study gene functions or discover which genetic changes cause certain traits. If the cutting ability of Cas9 is turned off, it becomes a "dead" Cas9 (dCas9), which still finds DNA sequences but does not cut them. Scientists can then attach other proteins or RNAs to dCas9, allowing it to perform many new tasks.

Epigenetic Control

Epigenetic modifications such as DNA methylation and acetylation regulate transcription and other biological functions by changing chromatin states. Zinc Fingered Nuclease and TALEN are used in limited studies to target epigenetic enzymes for a specific DNA site. Now, epigenetic CRISPR offers a more sustainable approach that permits precise addition and deletion of the marks at the interested loci to examine their impact on the genetic regulations. The only way to tackle this problem is to use prokaryotic epigenetic enzymes to reduce such crosstalk.

Cancer therapy

CRISPR Cas technology is a rapid and most powerful tool in cancer research and therapy due to its precision editing and modulate molecular pathways. The best applications of this technology are targeted delivery, where CRISPR knockout and activation screen has been applied for the determination of oncogenes and tumor suppressor genes. It has great advancement in cancer immunotherapy, for example, T-cells can be engineered to alleviate the anti-tumor potential through the interruption in different checkpoints of the cell cycle, such as PD-1, by enhancing the efficacy of CAR-T cells and TCR-T cell therapeutics. The next area is drug resistance mechanism, where research easily determined the pinpoint genes that are involved in chemotherapy resistance.[20] Although direct in-vivo therapeutic editing of oncogenes or tumor suppressor genes is still in experimental stages, early findings highlight the transformative potential of CRISPR Cas systems in developing personalized and more effective cancer treatments.[21] Tables 1 and 2 highlight some of the prominent CRISPR-based therapies currently being evaluated in clinical trials. It includes information on the target disease, therapeutic approach, CRISPR system used, delivery method, and clinical trial phase. These therapies primarily focus on correcting or silencing disease-causing genes in disorders such as hematological diseases, genetic blindness, sickle cell disease, β-thalassemia, and certain cancers. Tables 1 and 2 emphasize the rapid translation of CRISPR genome-editing technologies from the laboratory to human applications, demonstrating the potential of CRISPR to provide curative solutions where conventional treatments are limited.

Table 1: Selected CRISPR-Based Therapies in Clinical Trials

Disease/Condition	Target Gene/Mechanism		Delivery Method		Status/Stage (2024)
Sickle Cell Anaemia	BCL11A enhancer disruption		Ex-vivo edited HSCs		Ongoing Phase I/II clinical trials
β-Thalassaemia	BCL11A enhancer disruption		Ex-vivo edited HSCs		Ongoing Phase I/II clinical trials
Leber Congenital Amaurosis	CEP290 mutation correction		AAV–CRISPR direct delivery		Early-stage clinical trials
Familial Hypercholesterolemia	PCSK9 gene disruption		In-vivo AAV–CRISPR		Pre-clinical (animal models)
Cancer (multiple types)	TCR/CAR-T CRISPR enhancement		Ex-vivo T-cell modification		Multiple early-stage trials
HIV Infection	Excision of proviral DNA		Ex-vivo immune cell editing		Pre-clinical research

Limitations

CRISPR Cas9 is very powerful at changing DNA, but this strength also brings some problems. The biggest concern is that CRISPR Cas9, like other genome-editing tools, can cut DNA in the wrong places, not just the target gene. These unwanted cuts, called off-target effects, can cause harmful changes in the genome. Research studies on how serious this problem is have shown mixed results; some show it is a big issue, while others find it less concerning.

CRISPR Cas9 requires a specific short DNA motif (the PAM, eg. "NGG" for SpCas9) adjacent to the target site. If a suitable PAM is not available near the desired edit location, it may be difficult or impossible to target that region. Cas9 occasionally makes cuts in locations that are comparable to the target but not exactly the same. Unwanted DNA alterations, deletions, or rearrangements may result from these off-target double-strand breaks (DSBs). The guide RNA's sequence, length, and mismatches have a significant impact on the risk of off-target activity as well as on-target efficiency. Some positions in the gRNA are more critical than others (mismatch near the PAM "seed" region is especially problematic).[1]

Table 2: Emerging CRISPR Cas Innovations, Therapeutic Applications, and Current Status

CRISPR Innovations	Mechanism	Therapeutic Example	Delivery Strategy	Current Status	Limitations	Reference's
Base Editing	Cas9 Nickeas fused to cytidine/adenine deaminase makes precise point mutations without DSBs	PCSK9 editing to lower cholesterol in primates	LNP delivery of base editor mRNA +sgRNA	Preclinical (animal models)	Limited to C, T, and A, G transition by stander edits is possible.	[22]
Prime Editing	Cas9 Nickeas fused to reverse transcriptase guided by peg RNA for search and replace edits	Correction of sickle cell mutation in human stem cells.	Lentiviral/peg RNA systems.	Proof of concept, human cells.	Large pegRNA design, efficiency still low *in- vivo*	[7]
Epigenome Editing	dCas9 fused to KRAB/VP64 to silence or activate genes	Silencing the MYC oncogene in cancer	AAV delivery of dCas9 fusions	Experiment al (lab models)	An effect often temporary requires sustained expression.	[6]
Transcription Control (Crispr)	CRISPR interference or activation with dCas9	Regulating metabolic genes in diabetes models.	dCas9- sgRNA constructs.	Preclinical, functional genomics screens	Limited therapeutic use so far.	[23]
Diagnostic s (Cas12, Cas13, Cas14)	Collateral cleavage of reporters after RNA/DNA target binding	Sherlock for COVID-19, Detector for HPV/HBV	Lateral flow Cas enzymes	Deployed in pandemic settings, validation ongoing	Sensitivity/ specificity vary in mainstream clinical yet	[23]
HDR-Enhanced CRISPER	Alternative nucleases with unique cutting patterns (Cas12: Cas13: RNA targeting: Cas14: ultra small)	RNA editing for viral infection (Cas13)	Viral and non-viral delivery	Preclinical models	Delivery and specificity challenges	[5]
In-vivo AAV-CRISPR	AAV-based systemic delivery of Cas9-sgRNA	Sickle cell anemia, β thalassemia (BCL11A enhancer disrution)	*Ex-vivo* editing autologous transplant	Ongoing Phase ½ clinical trials	Limited to accessible blood cells.	[24]

Cont. Table 2: Emerging CRISPR Cas Innovations, Therapeutic Applications, and Current Status

Nanoparticle le--Based CRISPR Delivery	LNP encapsulation of CRISPR components	PCSK9 disruption in the liver	LNP delivery	Preclinical primates	Tissue targeting, immune response	**[22]**
Synthetic Biology with CRISPR	Cas is integrated into gene circuits and biosensors	Biosensor detection of toxins/pathogens	Engineered microbes with CRISPR circuits	Research lab	Safety and regulation concerns	**[6]**

Future Prospective

The next phase of CRISPR Cas systems will involve not just improvement but transformation, pushing the limits of precision, safety, delivery, and accessibility. As research continues, several transformative directions are expected to change the role of CRISPR in modern treatments. Genome editing accuracy is improved with the help of a broad CRISPR toolbox, and the next generation editing Cas 9 variants, such as Cas 9, Cas12, Cas 13, and Cas14 are also giving opportunities to editing in complex genomics regions.[25] Ongoing improvements supporting double-stranded break repair, lowering genotoxic risk with high fidelity, efficient, and targeted delivery at the specific site, are the key challenges of the CRISPR technology, and it resolved by the tissue-targeted delivery platforms.[19,25] While Adeno-Associated Virus (AAV) and lentivirus-mediated deliveries were also widely used to trigger an immune response, and limited packaging capacity. Now scientists are focusing on the non-viral mediated delivery system, such as polymer designing for the specific organ.[19]

Most especially promising innovation involves a self-regulating and feedback-controlled CRISPR System. These highly specific and smart therapeutic approaches would automatically recognize the harmful molecular signal and genome editing. Along with these rapid advances, there are many challenges in front of scientists.26 Next-generation system will encourage the guide RNA design algorithms, integrated, catalytical inactive Cas 9 proteins (dCas) infused with regulatory domains, use anti-CRISPR proteins, and optimize delivery timing.[26]

Reversible epigenetic editing changes the gene expression without changing in DNA sequence. In the upcoming generation, CRISPR has highly ethical concerns that require oversight and fair distribution. In the developing countries, the infrastructure and funding systems are limited, so the clinical progress is slow due to this reason, global coordination and sharing knowledge are crucial. In summary, CRISPR has evolved from a natural "cut and fix" into a precise, programmable, and highly stable therapeutic platform.[19]

CONCLUSIONS

CRISPR Cas system always gives a new insight in the field of molecular biology, which offers a versatile, stable, and effective tool for accurate genome editing. CRISPR, which was naturally found in bacterial genomes with the help of nuclease and guide RNAs has now been modified to certain DNA and RNA sequences. different Cas proteins like Cas9, Cas 12, Cas13, Cas14, provides RNA targets and diagnosis. CRISPR is used in diverse areas like personalized medicine to rectify the mutation, develop new therapies for inherited diseases, cancer, and infectious diseases.

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