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Review of CRISPR Cas9 in the treatment of diseases by analytical method

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Abstract

CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) systems serve as an adaptive immune defense in bacteria, protecting against invading nucleic acids from bacteriophages or plasmids. This prokaryotic mechanism has been adapted for use in molecular biology and has become one of the most powerful and versatile tools for genome editing. CRISPR/Cas9, in particular, offers a straightforward and rapid method for efficiently modifying endogenous genes across various species and cell types. Additionally, a modified version of the CRISPR/Cas9 system, equipped with transcriptional repressors or activators, enables precise regulation of gene expression by repressing or activating target genes. The simplicity and efficiency of CRISPR/Cas9 have made it widely popular across diverse fields, including basic research, biotechnology, and biomedicine. Owing to its high specificity and efficiency, CRISPR/Cas9 is extensity used for human diseases treatment, especially for cancer, which involves multiple genetic alterations. However, significant challenges remain for its clinical applications. The greatest challenge for CRISPR/Cas9 therapy is how to safely and efficiently deliver it to target sites in vivo. Here, we have examined CRISPR-Cas9 and its mechanism of action, and discussed its applications in the treatment of several diseases that are based on gene disorders.

Keywords: CRISPR/Cas9, Genome editing, Off-target effect, Guide RNA, disease treatment

INTRODUCTION

Bacteria and archaea have developed a wide array of defense mechanisms to ensure survival when exposed to foreign genetic materials such as viral genomes and plasmids. These strategies include preventing phage adsorption, hindering phage DNA injection, employing abortive infection systems, and utilizing restriction-modification systems. These methods have been documented in various studies (Chibani-Chennoufi et al. 2004; Chopin et al. 2005; Forde and Fitzgerald 1999). The discovery of the adaptive microbial immune system has further enhanced this arsenal. This system is based on clustered regularly interspaced short palindromic repeats (CRISPR) along with CRISPR-associated (Cas) genes. CRISPR sequences were first identified in 1987 within the genome of *Escherichia coli*. Ishino et al. discovered repeat sequences with an unknown function located downstream of the iap gene. Since then, CRISPR loci have been observed in approximately 40% of sequenced bacterial genomes and nearly 90% of sequenced archaeal genomes (Sorek et al. 2008). Barrangou et al. (2007) also contributed to advancing knowledge in this field. CRISPR, along with its associated Cas genes, has been shown to constitute an adaptive immune system that protects against bacteriophage infections. This CRISPR/Cas mechanism offers a highly versatile and inheritable form of resistance by integrating short sequences from viruses or other mobile genetic elements into the host's CRISPR locus. These sequences are then transcribed and processed into small RNAs, which guide the degradation of invading nucleic acids (Charpentier and Marraffini 2014).

CRISPR/Cas9 is a powerful gene-editing tool extensively utilized within the scientific community. It originated naturally in bacteria and archaea as a defense mechanism against phage infections and plasmid transfers. When these microorganisms first encounter a foreign phage or plasmid, they capture a segment of the invader's DNA and insert it into the CRISPR spacer region. Should the organism be reinfected with the same or similar DNA, it will begin transcribing the CRISPR region to mount a defense. Through a series of processing and maturation stages, a single guide RNA (sgRNA) is generated to direct Cas9 toward cleaving the DNA strand at the homologous spacer region, thereby disrupting it. This guiding process relies on the recognition of protospacer-adjacent motifs (PAMs), which are short, guanine-rich sequences. Streptococcus pyogenes Cas9 (SpCas9) specifically favors NGG as its PAM sequence, a motif commonly found in the genomes of most organisms. This abundance facilitates the application of CRISPR technology across diverse fields, including plant and animal science as well as biomedicine. By modifying the nucleotide sequence within a small section of the guide RNA, CRISPR/Cas9 enables precise targeting of nearly any genomic locus, making it a powerful tool for correcting disease-causing mutations or silencing genes linked to disease development. Nonetheless, certain highly chromatinized regions of the

genome can be challenging to access with this technology. Potential applications include treating cancers, cardiovascular diseases, sickle cell anemia, and neurodegenerative disorders.

This review explores the advancements in CRISPR technology, providing an overview of the various gene-editing tools introduced in recent years. It also examines delivery systems used to administer CRISPR-based solutions within the body, with particular attention to developing more tailored systems for specific diseases. Additionally, the review highlights potential challenges encountered when applying CRISPR technology for disease treatment and presents corresponding strategies to address these issues. Ultimately, this approach holds promising potential for delivering highly effective gene therapy options across a range of diseases.

DISCOVERY AND DEVELOPMENT OF CRISPR TECHNOLOGY

CRISPR-based gene-editing technology is currently among the most prominent tools in the field of biology. Since 2013, research on CRISPR has experienced remarkable growth, with tens of thousands of related studies published. In October 2020, the Nobel Prize in Chemistry was awarded to French microbiologist Emmanuelle Charpentier and American biologist Jennifer Doudna for their pioneering work in developing a revolutionary approach to genome editing. This technique had been under scientific investigation for nearly three decades before it gained widespread recognition. (Fig.1). Early detection through CRISPR technology involves a distinctive sequence characterized by repeated intervals. Similar to many groundbreaking advancements, the discovery of CRISPR technology stemmed from an unforeseen observation. In 1987, Nakata and colleagues, while examining the iap gene of E. coli, identified an unusual sequence located in the 3′ end structural domain of the gene. This sequence featured five highly similar sections, each comprising 29 nucleotides, separated by segments of 32 nucleotides.[13]

Over the next ten years, this specific repeating sequence was identified across various bacteria and archaea..[14-18] In 2002, Janson and colleagues presented a comprehensive overview of the specific repeats that had been discovered. They categorized these repeats as a family and introduced the acronym CRISPR, which stands for clustered regularly interspaced short palindromic repeats.[19]

Furthermore, previous studies have identified several CRISPR-associated proteins (Cas), including Cas1 through Cas4, as critical components of bacterial and archaeal defense mechanisms. In 2005, researchers found that the spacer sequences within CRISPR are not exclusive to individual organisms..[31] Mojica et al. discovered that the majority of spacer sequences originated from external DNA sources, while only a small portion appeared unrelated to external elements. They also observed that viruses were more likely to infect cells lacking matching spacer sequences..[31] They hypothesized that CRISPR plays a role in bacterial defense against infections caused by external phages and in the transfer of plasmids..[20,32] The conjecture was confirmed 2 years later.[21-23]

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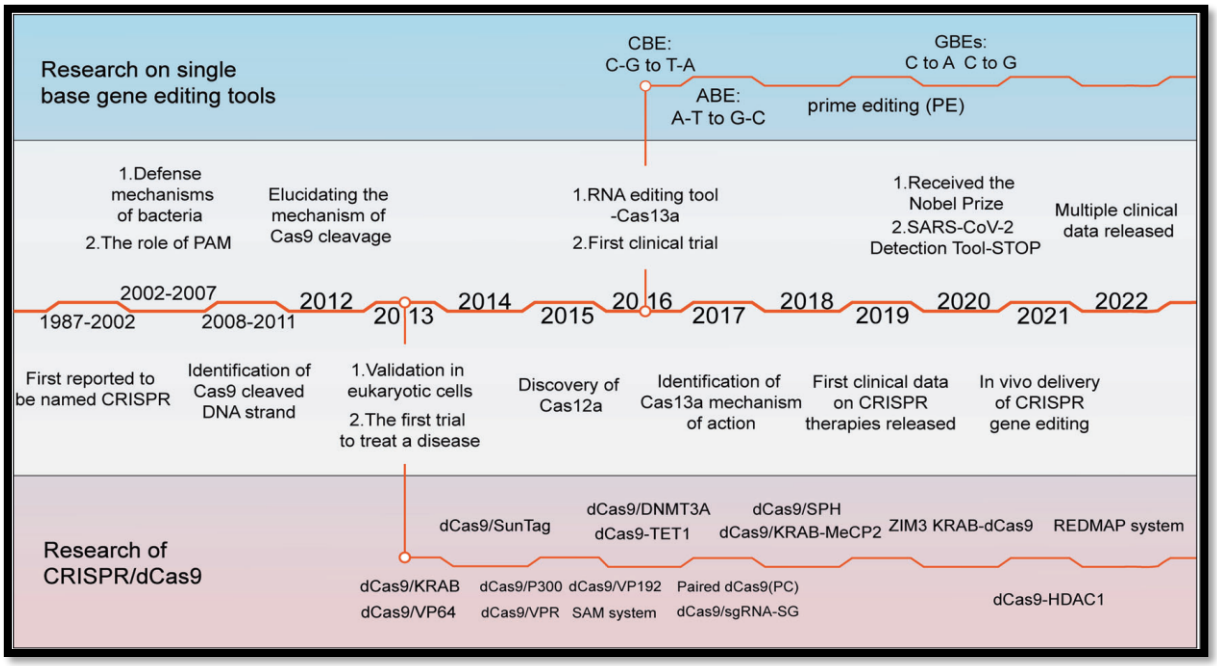


Fig. 1 Timeline of major events in the development of CRISPR/Cas technology and representative Cas9 variants. In 1987, the CRISPR sequence was first reported. The mechanism by which Cas9 cuts DNA double strands was reported in 2012, and Cas9 was subsequently used for gene editing in mammalian cells. Since then, CRISPR technology has developed rapidly, and multiple Cas9 variants with

specific functions have been identified. The representative variants are single-base substitution tools (e.g., CBE and PE) and transcriptional regulatory tools (e.g., dCas9-effector). Since 2016, CRISPR-based gene-editing technologies have been successively used in clinical treatment with great success. CRISPR clustered regularly interspaced short palindromic repeats, Cas CRISPR-associated, dCas9 dead Cas9, PAM protospacer-adjacent motifs, CBE cytosine base editors, ABE adenine base editors, GBE glycosylase base editors. (Figure wascreated with Adobe Illustrator).(yang et.al. *CRISPR/Cas9 therapeutics: progress and prospects* , 2023).

When first confronted with phage or plasmid infestation, bacteria containing CRISPR sequences acquire a segment of their DNA sequence, which serves as a spacer region between special repeat sequences. CRISPR RNA (crRNA) then undergoes a series of transcription and maturation processes to produce a single crRNA containing a protospacer sequence of 20 bases that binds to the invading DNA via complementary base pairing.[24,25] Recognition of the exogenous sequence by crRNA alone does not protect it from the phage; it also must be inactivated by disrupting the exogenous sequence through the cleavage activity of the Cas protein.[26,27] The CRISPR/Cas family of proteins is divided into two categories based on genomic and protein structure information, and the bestknown protein Cas9 is among the Class II CRISPR/Cas systems.[28,29] Class I features a sizable Cas9 protein complex responsible for DNA strand cleavage, whereas Class II relies on just one protein to perform the shearing. The Cas9 protein itself is defined by the presence of two distinct ribonuclease structural domains: a RuvC-like nuclease domain near its amino terminus and an HNH nuclease domain located in the central region of the protein. Both domains play a crucial role in cleaving the DNA strand..[30] Notably, protospacer sequences are not randomly acquired from exogenous sequences but are always accompanied by a guanine-enriched sequence called protospacer-adjacent motifs (PAMs).[22] Subsequent studies have shown that PAM sequences play an important role in the acquisition of the spacer region, where Cas proteins perform cleavage

CRISPR-BASED GENE-EDITING TOOLS

CRISPR gene-editing technology enables precise modifications of genes in eukaryotic cells. Scientists have extensively studied the mechanism of action of the Cas9 protein, leading to the development of Cas9 variants with diverse functions, as well as additional derivative gene-editing tools through specific modifications. Furthermore, the discovery of other Cas proteins within the Cas9 family has expanded the range of genes that can be targeted using CRISPR technology. To enhance its application, researchers have also designed vectors to facilitate the safe and efficient delivery of the CRISPR system into the body.

a.Composition of CRISPR/Cas9. sgRNA. When invaded by exogenous phages or plasmids, bacteria and archaea containing CRISPR obtain a foreign DNA

fragment inserted into the spacer region.[32] Re-entry of the foreign nucleic acid homologous to the spacer region into the bacteria activates transcription of the CRISPR array to produce pre-crRNA. Pre-crRNA contains sequences with complementary base pairing to tracrRNA, the repeat region of the CRISPR array.[26,34] TracrRNA initially attaches to the Cas9 protein following transcription. Subsequently, complementary base pairing between pre-crRNA and tracrRNA results in the formation of a double-stranded RNA, allowing the pre-crRNA to bind with Cas9. Once this binding is established, RNase III processes the pre-crRNA during the primary stage, while Cas9 trims any surplus repetitive and spacer sequences in the secondary stage.[25,35] After the two processes, the crRNA matures and gains the ability to target the DNA strand. The backbone RNA (tracrRNA) and crRNAs that target specific sequences together comprise the sgRNA.[26,33,67]

Researchers constructed a crRNA-tracrRNA fusion transcript to simplify the aforementioned process and facilitate the application of the CRISPR/Cas9 system in eukaryotes, which greatly simplified the process of crRNA processing and maturation.[33,36] By designing a crRNA targeting sequence of only 20 bp of bases next to the PAM site, almost any position containing the PAM site can theoretically be targeted. The major difference between CRISPRbased gene-editing technology and ZFNs and TALENs is that CRISPR-based gene-editing technology relies on the RNAmediated recognition of the target DNA.[27,68]

The design of the sgRNA plays a crucial role in determining the success of CRISPR gene editing at the target site. Serving as a guide, the sgRNA directs the gene-editing system to the specific DNA sequence, while the Cas9 protein carries out the modification of the target strand. With SpCas9 as a reference, its ability to bind to the target DNA relies on recognizing the PAM sequence located downstream of the target site. This recognition initiates the separation of the double-stranded DNA, enabling the editing process to proceed..[38,39] The 10 bases proximal to the PAM on crRNA are called the seed sequence, and the seed sequence first binds to the DNA strand through complementary base pairing to begin forming its R-loop structure.[40] The distal DNA of PAM interacts with the structural domains of REC2 and REC3 of Cas9 to accelerate the formation of the R-loop, and the formation of the intact R-loop promotes the activation of the structural domains of the HNH and RuvC nucleases that catalyze the cleavage of the double-stranded DNA.[39,41-46] When using wild-type Cas9 for gene editing, such as SpCas9 (S. pyogenes Cas9) and SaCas9 (Staphylococcus aureus Cas9), off-target effects, chromosomal translocations, large segment deletions, and other abnormalities often occur.[47-49] Due to the limitations of the PAM, the CRISPR/Cas9 gene-editing system often fails to target the proper sites. Therefore, the modification of Cas9 focuses on two goals: enhancing the security of Cas9[50-59]and freeing it from the limitations of PAM[60-66] (Tables 1, 2).

b.Method for CRISPR delivery. Plasmid DNA (pDNA) serves as an excellent vector for delivering the CRISPR system due to its resistance to degradation, its

ability to be amplified in significant amounts, and its ease of modification. Upon entering the cell, the plasmid containing CRISPR/Cas9 is guided into the nucleus with the help of NLS, where it facilitates the transcription of mRNA encoding both Cas9 and sgRNA.[73,74]

This process is very tedious, and loading CRISPR/ Cas9 tools on mRNA may greatly simplify this process. However, mRNA is easily degraded and has low stability. In particular, geneediting tools that deliver Cas9 to function in concert with effector proteins are difficult to apply because the number of bases in the mRNA encoding Cas9 and effector proteins is too large.[75,76] Cas9 RNPs, known as ribonucleoproteins (RNPs), are complexes formed by fusing purified Cas9 with sgRNA in vitro, and RNPs function immediately after entering cells.[77,78]

Table 1. Cas9 variants that have been modified to broaden the scope of application

Variant name	Resources (PAM)	Selection strategy	PAM	Ref.
spCas9-NG	SpCas9 (NGG)	Elimination of the interaction between Cas9 and the third position of PAM	NG	115
xCas9	SpCas9	PACE	NG, GAA, GAT	116
Cas9-NRNH	SpCas9	PANCE and PACE that evolved by enabling SpCas9 to bind to specific sequences with non-G PAMs	NRNH	114
SpG	SpCas9	Structure-guided engineering	NGN	117
SpRY	SpCas9	Structure-guided engineering	Almost unlimited	117
Cas9-VQR	SpCas9	Bacterial selection system	NGAN NGCG	341
Cas9-EQR	SpCas9	Bacterial selection system	NGAG	341
SaCas9-KKH	SaCas9 (NNGRRT)	Molecular evolution and bacterial selection system	NNNRRT	112
eNme2-C	NmCas9 (NNNNGATT)	PANCE, ePACE, and BE-PPT	Almost unlimited	113
eNme2-C.NR	NmCas9	PANCE, ePACE, and BE-PPT	Almost unlimited	113
eNme2-T.1	NmCas9	PANCE, ePACE, and BE-PPT	NTN	113
eNme2-T.2	NmCas9	PANCE, ePACE, and BE-PPT	NTN	113

Table 2. Cas9 variants that have been modified for increased security

Variant name	Resources	Selection strategy	Mutation domain	Ref.
SpCas9-HF1	SpCas9	Reduce the interaction between Cas9 and nontarget DNA sites	HNH and REC3 domains	342
eSpCas9	SpCas9	Neutralize the positive charges of Cas9 and DNA links and sites.	HNH and PAM-interacting domains	343
Sniper-Cas9	SpCas9	Sniper screen, an *E. coli*-based selection method		101
HypaCas9	SpCas9	REC3 and DNA complementation control HNH domain activation	REC3 domain	103
evoCas9	SpCas9	Screening method using a yeast reporter strain	REC3 domain	102
Cas9TX	SpCas9	Prevent the perfect repair of DNA	Carry optimized TREX2	110
HscCas9-v1.2	SpCas9	Substitution of amino acid residues	Multiple domains	105
superFi-Cas9	SpCas9	When mismatched, sgRNA, and DNA chains form RuvC loop	RuvC loop	104
efSaCas9	SaCas9	Construction of an SaCas9 variant library and directional screening system	REC3 domain	106
SaCas9-HF	SaCas9	Modify that residues where the distal region of PAM is linked to the target DNA	Recognition lobe domain and RuvC domain	108

However, RNPs are relatively difficult to deliver into cells due to their complex composition and charge properties, whereas proteins and nucleic acids are usually delivered using electroporation with the assistance of cell-penetrating peptides.[79,80] With continuous innovations in delivery vectors, scientists have identified exosomes as a promising approach to deliver Cas9 RNPs.[81,82]

c.Functional categories of CRISPR tools. The CRISPR/Cas9 system was originally studied for its robust ability to cleave double-stranded DNA. The single-guide RNA (sgRNA) directs the Cas9 enzyme to a specific target site, where double-strand breaks (DSBs) are generated, producing blunt ends through the action of the HNH and RuvC nuclease domains. These DSBs then activate DNA repair pathways, primarily non-homologous end joining (NHEJ) and homologous directed repair (HDR)..[70,83,84] The repair of double-strand breaks (DSBs) through non-homologous end joining (NHEJ) is often imprecise, frequently causing base mutations that lead to targeted genetic alterations. In contrast, homologous-directed repair (HDR) is a more complex yet precise mechanism, capable of accurately restoring broken DNA strands. This results in a repaired DNA strand that is identical to the target sequence and, as a result, will be cleaved again by Cas9 until the single-guide RNA (sgRNA) no longer matches. However, the likelihood of HDR occurring in mature cells is significantly lower compared to NHEJ..[71] Cas9 efficiently cleaves double-stranded DNA, but in practice, the sgRNA often mismatches with double-stranded DNA, leading to offtarget effects.[69] In addition, a more efficient method to mediate mutational inactivation of genes is needed to enhance the efficiency of gene knockdown and reduce unnecessary cleavage. Cas9 nickase (Cas9n), a Cas9 variant with mutations in the nuclease structural domain RuvC (D10A) of Cas9, only creates breaks in DNA strands complementary to the crRNA.[85] DNA single-strand breaks are repaired through the high-accuracy base excision repair (BER) pathway. To leverage this, two adjacent sgRNA/Cas9n

complexes are engineered to target and cleave a single site. This approach effectively minimizes Cas9-induced damage to off-target DNA while significantly improving the precision of Cas9 activity..[86] An offset of an appropriate distance between two Cas9ns facilitates the efficiency of gene editing. Zhang Feng and colleagues designed an online tool (http://www.genome-engineering.org/) for the design of two Cas9n sgRNAs to facilitate follow-up research.[85]

d.Carriers for delivering CRISPR technology. Plasmids or mRNAs carrying CRISPR/Cas gene-editing systems can be introduced into cells in vitro using standard methods for nucleic acid delivery, such as transfection reagents, virus-based transfection, and other related techniques. Ribonucleoproteins (RNPs) can also be delivered into cells via electroporation. However, these methods are generally less effective for use in animals or humans. To achieve efficient in vivo delivery, CRISPR tools must navigate a multi-step process comprising three main phases: (1) the delivery carrier must remain stable in the bloodstream, avoiding degradation or immune clearance; (2) the carrier must accumulate in the target tissue and initiate cellular uptake through endocytosis; and (3) the CRISPR system must escape the lysosome and reach the cytoplasm to perform genome editing or regulate gene expression. The second phase, involving targeted tissue accumulation, is particularly crucial for successful delivery. This intricate process relies heavily on specialized delivery vehicles to ensure its success. (Fig.2).

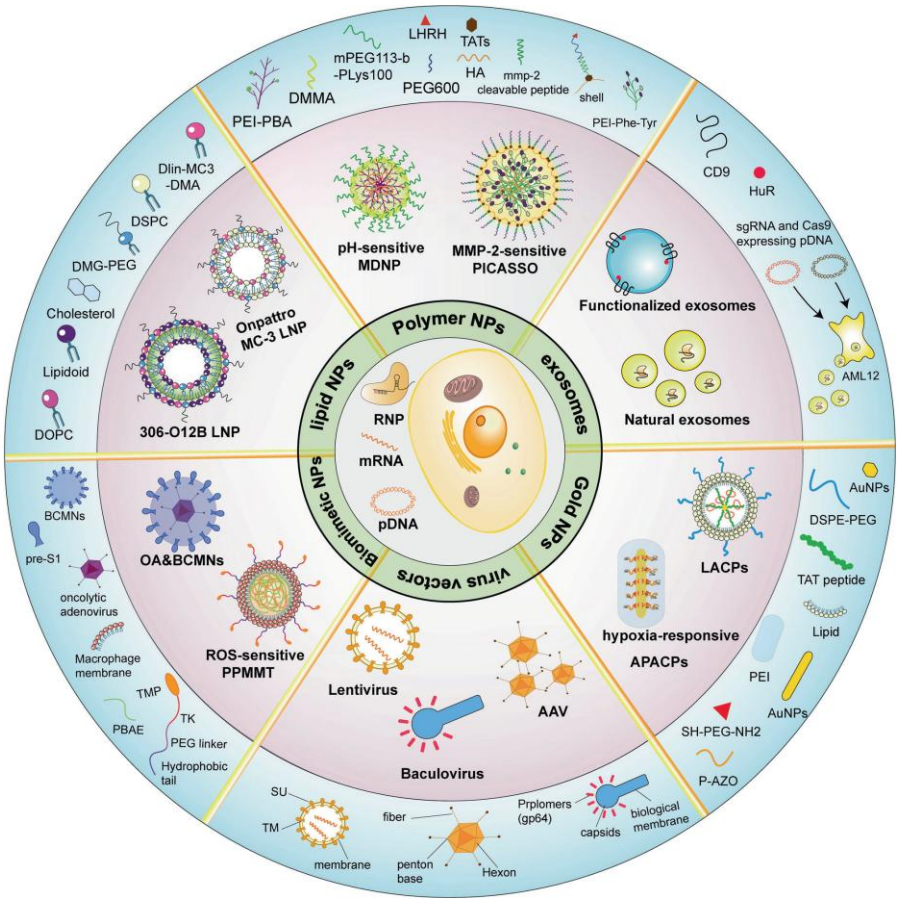


Fig. 2 Schematic diagram showing multiple types of vectors for the in vivo delivery of CRISPR systems. The central region shows three forms of CRISPR action: pDNA, mRNA, and RNP. The middle circle section shows examples of delivery carriers, and the outermost area shows how the carriers are produced or the components. SU surface envelope protein, TM transmembrane envelope protein. (Figure was created with Adobe Illustrator and Biorender.com) .(yang et.al. *CRISPR/Cas9 therapeutics: progress and prospects* , 2023).

CRISPR/CAS9 MECHANISM

CRISPR/Cas9 is a system that evolved naturally in bacteria and archaea as a defense mechanism against viral infections. These microorganisms integrate short segments of viral DNA into their genome, which are later transcribed into RNA known as CRISPR RNA (crRNA). The crRNA interacts with a second RNA molecule, the trans-activating crRNA (tracrRNA), and the CRISPR-associated protein 9 (Cas9)—all of which are encoded within the bacterial genome.. If this complex encounters viral (bacteriophage) DNA which is complementary to the crRNA sequence it will bind to it. Cas9 is an endonuclease which cleaves double-stranded DNA, slicing through the viral DNA and preventing transcription [87,89].

Researchers quickly recognized that this system could facilitate precise gene editing within the genome of a selected cell. By providing a cell with Cas9, tracrRNA, and a crRNA tailored to the desired target, Cas9 can cut the specific DNA region. Following this breakthrough, the crRNA and tracrRNA were combined into an artificially engineered single guide RNA (sgRNA), which can be customized to target specific sequences. [89]. The final component required for CRISPR/Cas9- mediated gene editing is the presence of a protospacer adjacent motif (PAM) site adjacent to the target region[90]. A PAM site is a short DNA sequence (usually between 3 to 8 bp in length) that Cas9 binds to, inducing the double-stranded break approximately 3 bp upstream of the PAM. These sites are naturally present on viral DNA and the exact sequence of the PAM is dependent on the species the Cas9 is isolated from. The most commonlyused Cas9 to date has been SpCas9 from *Streptococcus pyogenes*, which requires a PAM of 5'-NGG-3', where N is any nucleotide [91].

Several bioinformatics tools are available for screening DNA sequences to identify PAM sites. After selecting an appropriate PAM, the sgRNA is designed to include a region complementary to the sequence just upstream of the PAM, usually ranging from 18 to 25 base pairs in length. Introducing a specific sgRNA along with an active Cas9 protein into a cell results in a double-stranded break at the targeted site. When this occurs within the host genome, the cell attempts to repair the damage using one of two main pathways: Non-Homologous End Joining (NHEJ) or Homology-Directed Repair (HDR). (Fig.1).

When no homologous (identical or complementary) DNA sequence is available, the cell resorts to the non-homologous end joining (NHEJ) pathway. Since this repair process lacks a DNA template, it is relatively random and highly prone to errors, often causing small insertions or deletions (indels) at the cleavage site. Should these indels lead to a frame-shift mutation in an exonic region, they can potentially disrupt the gene, resulting in the production of a nonfunctional protein. In contrast, the homology-directed repair (HDR) pathway enables precise editing of host DNA. After a double-stranded break occurs, any DNA molecule with significant homology to the target site may be incorporated into the genome. By providing a donor DNA template that carries the desired mutation flanked by homologous arms matching the target region, the likelihood of the HDR pathway being activated and successfully introducing the mutation into the cell increases. Another application of the CRISPR/Cas9 system involves using an artificially-engineered inactive version of Cas9 (dCas9) to modulate gene expression at the transcriptional level through a technique known as CRISPR interference (CRISPRi). Although dCas9 retains its ability to bind DNA through the single-guide RNA (sgRNA) and PAM site, it lacks endonuclease activity. When directed to key regulatory elements of a target gene, dCas9 can physically block transcriptional machinery, such as transcription factors or RNA polymerase, ultimately reducing the gene's transcription level.[92]. This effect can be enhanced by fusing dCas9 with a transcriptional inhibitor, such as Krüppel associated box [93].

Alternatively, fusing dCas9 with a transcriptional activator can upregulate expression via CRISPR activation (CRISPRa)[94](Fig.3).

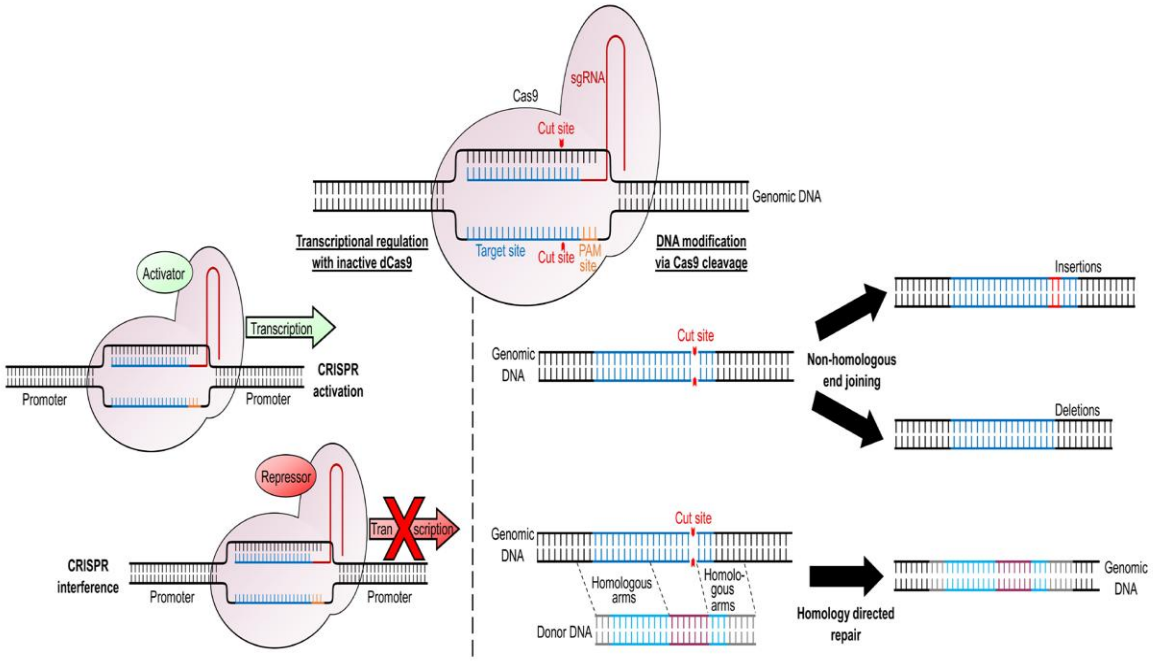


Fig 3. Applications of CRISPR/Cas9 for transcriptional regulation and genomic modification**.** (Caroline F. Peddle.et.al *The Application of CRISPR/Cas9 for the Treatment of Retinal Diseases*, 2017)

Following Cas9 binding, cleavage of both DNA strands allows DNA modification. In the absence of any homologous sequences, the cell will undergo non-homologous end joining, resulting in small insertions or deletions around the cut site. If donor DNA is supplied which has homologous arms matching the genomic DNA it will be incorporated into the genome via homology directed repair. Catalytically inactive dCas9 can be targeted to a promoter to alter transcriptional regulation. Fusing a transcriptional activator to dCas9 will upregulate gene expression (termed CRISPR activation) while fusing a transcriptional repressor to dCas9 will downregulate gene expression (termed CRISPR interference).

Application of CRISPR cas9 in treatment of diseases

1.cancer's disease. The main cause of tumors is the dysregulation of cell growth, including the activating of proto-oncogenes and the inactivating of tumor-suppressive genes [95,96]. Therefore, genome engineering technique offers a new hope for cancer treatment and the ability to edit multiple genes endows the CRISPR/Cas9 with great potential in cancer treatment. According, the knock-out of oncogenes by CRISPR/Cas9 technology is a very helpful method to inhibit the tumor growth. On another hand, repairing tumor suppressor genes and restore their function can also

inhibit the development of tumors[97]. Currently, CRISPR/Cas9 based gene therapy have been actively researched in lung cancer [98], breast cancer [99], head cancer [100], colorectal cancer [101], hepatocellular carcinoma [102], etc. Various target genes are implicated, including EGFR (epidermal growth factor receptor), p53 (tumor protein p53), FAK (focal adhesion kinase), Nestin, BRCA (breast cancer gene), HER2/Neu (human epidermal growth factor receptor 2), TERT (telomerase reverse transcriptase), ALK (anaplastic lymphoma kinase), KRAS (Kirsten rat sarcoma viral oncogene homolog), BRAF (v-raf murine sarcoma viral oncogene homolog B), NOTCH1 (notch homolog 1), PTEN (phosphatase and tensin homolog), among others.

CRISPR/Cas9 technology is capable of targeting specific disease-causing genes to create cell or animal models reflecting corresponding gene mutations. This approach facilitates a deeper understanding of the mechanisms behind disease development and helps refine treatment strategies. For instance, Cheng-Chi Chao and colleagues utilized CRISPR/Cas9 to establish two KRAS-driven cancer systems: one involved genome editing through CRISPR/Cas9 to eliminate KRAS G12S mutant alleles, while the other employed dCas9-KRAB for transcriptional regulation to inhibit KRAS expression.[103]. Experimental results shown that using CRISPR/Cas9 technology to specifically target oncogenic KRAS mutant alleles could significantly inhibit tumor growth [104]. Furthermore, CRISPR/Cas9 technology is also a good tool to study the pathogenesis of the diseases and identify the role of new oncogenes or tumor suppressor genes in the disease process. For instance, small cell lung cancer (SCLC) which is prone to recurrence is a high-grade neuroendocrine tumor, accounting for about 15% to 20% of lung cancer [105]. Studies have shown that p107 and p130 (members of the retinoblastoma protein family) have repeated mutations in approximately 6% of human SCLC tumors[106]. The results were verified by Tyler Jacks and coworkers who simulated the deletion of p107 and p130 in Trp53/Rb1 via an SCLC mouse model constructed using CRISPR/Cas9 system[107] The research above proves the potential of the functions of other candidate genes frequently mutated in SCLC and will also help to verify future SCLC therapeutic targets. To date, the main research findings of cancer-related mutation by CRISPR/Cas9 in cancer treatment are shown in Table4.

The CRISPR/Cas9 systems not only directly target oncogenes to suppress tumor growth but also play a crucial role in large-scale cancer gene screening, thereby enhancing the efficiency of anti-cancer drug development. Recently, researchers from the Wellcome Sanger Institute, GlaxoSmithKline (GSK), EMBL-EBI, and OpenTargets utilized genome-wide CRISPR screening technology to construct a novel resource library for cancer-dependent genes. They also developed a gene scoring framework aimed at identifying potential new targets. The research team performed CRISPR/Cas9 screenings on over 300 human cancer cell lines across 30 distinct cancer types. By integrating adaptive cellular responses, genomic biomarkers, and drug development targets, they systematically prioritized promising new targets tailored to specific tissues and genotypes. Through this

approach, the team successfully demonstrated that Werner syndrome ATP-dependent helicase (WRN) could serve as a synthetic lethal target for multiple cancer types exhibiting microsatellite instability. [108].

2.Cardiovascular diseases. Cardiovascular disease is one of the major causes of death in humans.[120,121] Common cardiovascular diseases include atherosclerosis, myocardial hypertrophy, heart attack, and aortic dissection.[122-126] However, unlike tumors and liver diseases, blood flow in the heart and blood vessels is faster and blood pressure is greater, posing a challenge for nanoparticle enrichment at the lesion site[127] (Fig.4). During fetal development, the number of cardiomyocytes expands rapidly, whereas cardiomyocytes gradually lose their ability to proliferate with aging.[128] Evidence of cardiomyocyte renewal has been observed in many mammals; however, it remains insufficient to compensate for the loss of cardiomyocytes caused by cardiomyopathy. Promoting the expression of genes linked to cardiomyocyte proliferation, such as myocyte enhancer factor 2D (Mef2d) and Krüppel-like factor 15 (Klf15), in the cardiomyocytes of adult animals could potentially have a beneficial impact on treating diseases related to cardiomyocytes..[129,130] Direct delivery of CRISPRa systems into cardiomyocytes is relatively difficult and may also lead to widespread off-target effects. Schoger et al. first constructed a dCas9/VPR transgenic mouse, and this sequence was inserted after the myosin heavy chain (Myh) 6 promotor and transcribed in concert with Myh6.[131] Since Myh6 is a cardiomyocyte-specific gene, expression of the dCas9/VPR system occurs only in cardiomyocytes.[132] The subsequent administration of AAV9 containing sgRNA induces the transcription of genes associated with cardiomyocyte proliferation within the targeted cells. Through this methodology, a cardiomyocyte-specific gene expression activation system was successfully established, allowing precise temporal control over transcriptional activation. This advancement offers a valuable framework for cardiovascular research, as well as investigations involving other organs or tissues that are inherently challenging to access directly.

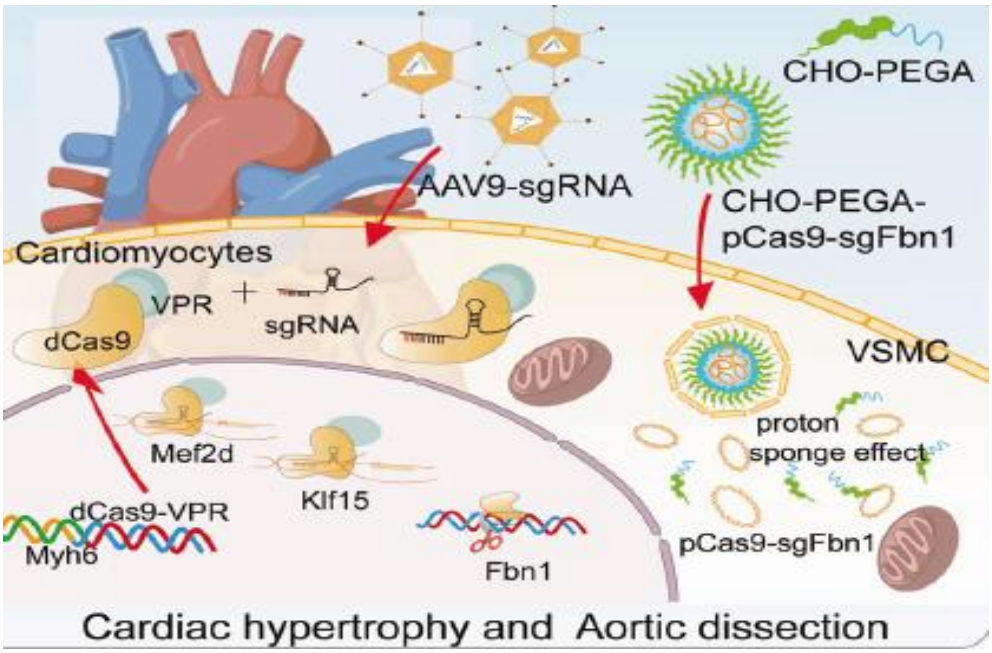


Fig.4 AAV9 delivered sgRNAs targeting Mef2d and Klf15 into dCas9-VPR transgenic mice. dCas9-VPR was synergistically transcribed with Myh6 and therefore specifically activated the expression of Mef2d and Klf15 in cardiomyocytes. Lipid nanoparticles CHO-PEGA deliver CRISPR/Cas9 to vascular smooth muscle cellsin aortic coarctation to knockdown Fbn1. .(Li et.al. *CRISPR/Cas9 therapeutics* , 2023).

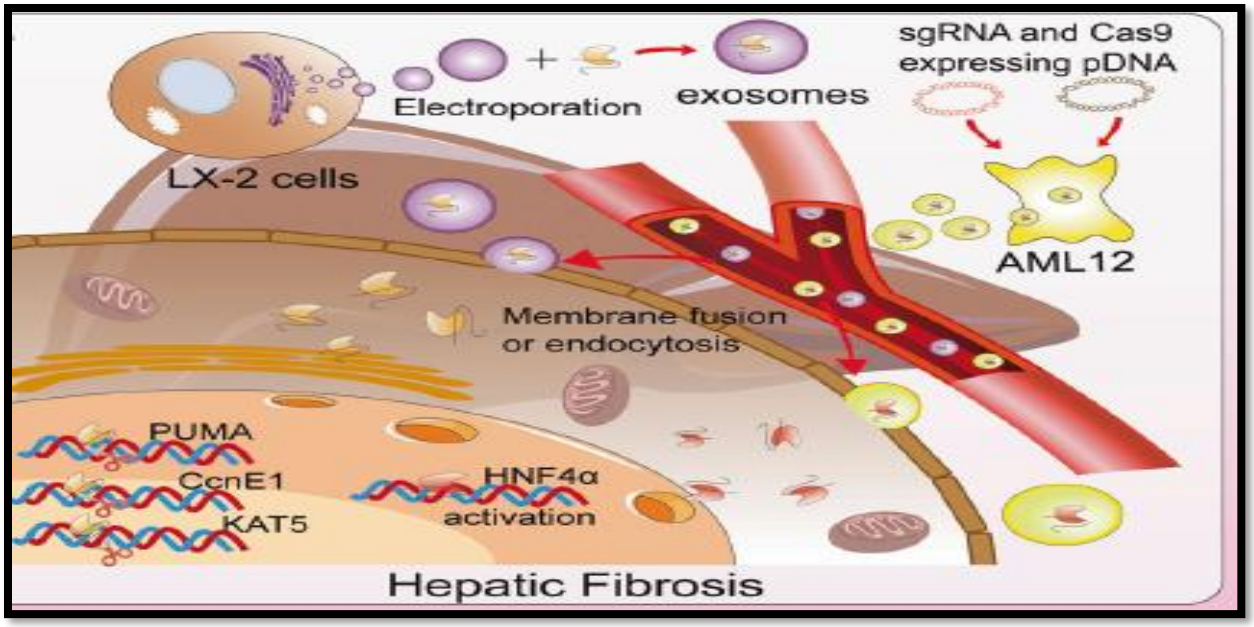
The development of aortic disease is usually accompanied by the inflammation of vascular endothelial cells and the phenotypic transformation of smooth muscle cells in the vascular mesoderm. [133,134] Zhang et al. constructed a hydroxyl-rich lipid nanoparticle capable of delivering CRISPR/Cas9 to vascular smooth muscle cells.[135] In another study, Zhao et al. combined lipid nanoparticles and polymer nanoparticles to construct a delivery vehicle for endothelial cells.[136] The CRISPR-based gene therapy was effectively administered to vascular smooth muscle cells (VSMCs) and endothelial cells. However, a significant proportion of the nanoparticles was still sequestered in the liver. Furthermore, CRISPR/Cas9 technology has been extensively employed in various medical applications, including bone regeneration and the treatment of conditions such as cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction, Alzheimer's disease, obesity, and other disorders. [109,110,112,137,138].

3.Liver diseases. The liver is the main organ in the body that metabolizes lipids, and liposomes and lipid nanoparticles without special modifications are more likely to be enriched in the liver.[113,114] In addition, many cells in the liver secrete large amounts of exosomes, and these hepatocyte-derived exosomes with a homologous

tissue targeting ability will be more easily enriched in the liver.[115] Unlike synthetic lipid nanoparticles, naturally occurring exosomes serve as carriers for the intercellular transport of proteins, nucleic acids, and various other molecules. As a result, natural exosomes are exceptionally safe and are seldom targeted for clearance by the immune system. (Fig.5)

Various cell types in the liver produce or take up exosomes.[116] Exosomes derived from hepatic stellate cells (HSCs) have a well-documented role in promoting liver fibrosis. However, hepatocyte-derived exosomes exhibit potential as therapeutic carriers for targeted delivery to HSCs. Endogenous liver exosomes obtained from the AML12 mouse hepatocyte cell line demonstrate enhanced safety and efficacy as delivery vectors. Specifically, encapsulation of the CRISPR-dCas9-VP64 system within AML12-derived exosomes resulted in the successful activation of hepatocyte nuclear factor 4α (HNF4α), a critical transcriptional regulator of hepatocyte differentiation, within HSCs and a mouse model of liver fibrosis. This activation notably mitigated fibrosis severity. In another study aimed at treating liver fibrosis, Luo and colleagues transfected plasmids encoding dCas9/VP64 and single guide RNA (sgRNA) into the AML12 mouse hepatocyte cell line..[116] The presence of dCas9 was detected in exosomes, suggesting that RNPs with transcriptional activity can be loaded in exosomes.

Phenylketonuria (PKU) is an autosomal recessive liver disease in which phenylalanine hydroxylase (PAH) enzyme deficiency results in decreased phenylalanine metabolism, causing hyperphenylalaninemia.[117] The repair of mutated bases through a single-base editor, which converts CG base pairs into T-A base pairs, successfully restores PAH expression and lowers elevated phenylalanine levels in the blood. Villiger and colleagues employed an AAV vector to deliver a single-base gene editor to the Pahenu2 mouse model. This approach led to 63% of the mRNA displaying the corrected base sequence, demonstrating the efficacy of this gene-editing system for treating PKU.Hepatitis B virus (HBV) is a serious threat to people's health, and long-term treatment with drugs such as interferon may lead to a significant increase in viral resistance.[118] Moreover, these treatments do not eliminate HBV covalently closed circular DNA (cccDNA), and targeted destruction of cccDNA using Cas9 is an effective method for treating HBV. Wang et al. designed an infrared light-responsive bionanoparticle for delivery of the CRISPR/Cas9 system to HBV-infected cells.[119]



.Fig.5 The exosomes secreted by LX-2 cells were extracted, and RNPs were loaded into the exosomes by electroporation. In studies targeting the knockdown of PUMA, CcnE1, and KAT5, exosomes were effective at alleviating liver diseases such as liver fibrosis. In vitro transfection of plasmids encoding sgRNA and dCas9/VP64 into mouse liver AML12 cells resulted in the secretion of AML12 exosomes carrying the CRISPR/ dCas9 system. Delivery of these exosomes to HSCs elevated HNF4α expression and prompted cell differentiation into hepatocytes. (Figure was created with Adobe Illustrator and Biorender.com) .(yang et.al. *CRISPR/Cas9 therapeutics: progress and prospects* , 2023).

This device has demonstrated efficacy in the inactivation of HBV cccDNA. The application of CRISPR-based genome editing technology has proven highly

effective in addressing numerous liver diseases, representing a promising and innovative approach for their future treatment.

4.Sickle cell disease and β-thalassemia. Sickle cell disease (SCD) and transfusion-dependent β-thalassemia (TDT) are both caused by mutations in the hemoglobin β subunit gene and are among the most common single-gene genetic disorders worldwide.[139,140] Sickle cell disease is marked by an imbalance in the hemoglobin chain and hemolytic anemia, commonly managed through blood transfusions and iron-chelation therapy. Individuals with β-thalassemia have sickle-shaped red blood cells with reduced oxygen-carrying capacity and often endure pain. As a result, their treatment typically includes hydroxyurea, pain management medications, and blood transfusions. Bone marrow transplantation is another treatment option for both conditions; however, finding suitable matches remains challenging..[111] The investigation into the pathogenesis of two hematological disorders at the genetic level revealed the transcription factor BCL11A as a crucial suppressor of fetal hemoglobin and γ-globin protein expression. Enhancing the expression levels of these proteins has been shown to significantly reduce the symptoms associated with sickle cell disease and β-thalassemia. This indicates that targeting BCL11A could provide a therapeutic avenue for improving patient outcomes in these conditions by promoting the production of fetal hemoglobin, which has beneficial effects on red blood cell function and overall health. Such findings underscore the importance of genetic factors in the management of hematological diseases and highlight the potential for innovative therapies that leverage our understanding of gene regulation..[141]

In 2019, Wu et al. used CRISPR/Cas9 to cleave the BCL11A enhancer sequence in HSCs and successfully downregulated its expression without inducing significant side effects.[142] In December 2020, clinical data were released for a gene therapy called CTX001, a one-time therapy for SCD and TDT developed by CRISPR Therapeutics in association with Vertex Pharmaceuticals.[111]

This study explored the application of Cas9 technology to specifically edit the BCL11A enhancer region in hematopoietic stem and progenitor cells (HSPCs). Th e goal of this genetic editing was to reduce the expression of BCL11A, a protein t hat suppresses the production of hemoglobin and fetal hemoglobin. By targeting t his enhancer, scientists aimed to promote the production of these types of hemogl obin, which are beneficial for patients with blood disorders. After the genetic mod ification, the altered HSPCs were transplanted into two patients suffering from sic kle cell disease (SCD) and betathalassemia (TDT). This approach seeks to provide a novel treatment strategy by enhancing the body's own ability to produce healthi er hemoglobin.

Twelve months posttransplantation, both patients showed a notable rise in fetal hemoglobin levels. Further examinations at 18 and 15 months confirmed that thes e levels reached normal in both cases. Encouragingly, similar treatments conducte d on eight more patients resulted in analogous successes, underscoring the widesp

read potential and efficacy of this treatment method. Nonetheless, it is crucial to a cknowledge that the procedure is not entirely free of risks. The initial two patients experienced some mild side effects, which, although not severe, were effectively managed and resolved with proper medical care. In a separate clinical study target ing sickle cell disease (SCD), a technique involving RNA interference to reduce B CL11A expression was explored.[143] They constructed a lentiviral vector carrying short hairpin RNA (shRNA) and used this lentivirus to transduce CD34 + cells from SCD patients, and clinical success was also achieved.

5.Huntington'sDisease. The use of the CRISPR/Cas9 technique is pivotal in ad dressing genetic disorders by enabling precise gene editing.

This approach involves generating and analyzing patientspecific induced plurip otent stem cells (iPSCs) from individuals with particular genetic conditions. Thro ugh cuttingedge translational research, scientists can produce human iPSCs reflect ing the genetic makeup of these diseases. iPSCs play a crucial role in in vitro studi es of neurodegenerative diseases like Alzheimer's, Parkinson's, and Huntington's diseases. Additionally, the CRISPR/Cas9 system is instrumental in creating anima l models that mimic human genetic disorders, which are essential for understandin g disease pathology and developing new treatments. The goal of CRISPR/Cas9 th erapy is to directly correct the genetic mutations responsible for diseases within a living organism, offering a promising avenue for therapeutic intervention. [144].

6.**Alzayamar'sDisease.**Genetic mutations are responsible for about 1% of fami lial Alzheimer's disease (FAD) cases. This suggests that genome editing using CR ISPR/Cas9 could be beneficial for FAD, although its impact on sporadic Alzheim er's disease (SAD) might be limited or negligible. Both FAD and SAD involve dis rupted amyloidbeta (Ab) metabolism, so reducing Ab production could provide a t reatment strategy applicable to both types of Alzheimer's, regardless of whether th e onset is familial or sporadic. A summary of studies illustrating the potential of C RISPR/Cas9 as an experimental treatment for both FAD and SAD is provided in Table 2. In models of earlyonset Alzheimer's, using CRISPR/Cas9 might not be s uitable since most cases of Alzheimer's are sporadic and triggered by unknown fa ctors. Only a small number of Alzheimer's cases occur before the age of 60, classi fying them as early-onset Alzheimer's. The CRISPR/Cas9 technique can significantly correct these autosomal dominant mutations. Recent studies also support the potential of this gene editing system,

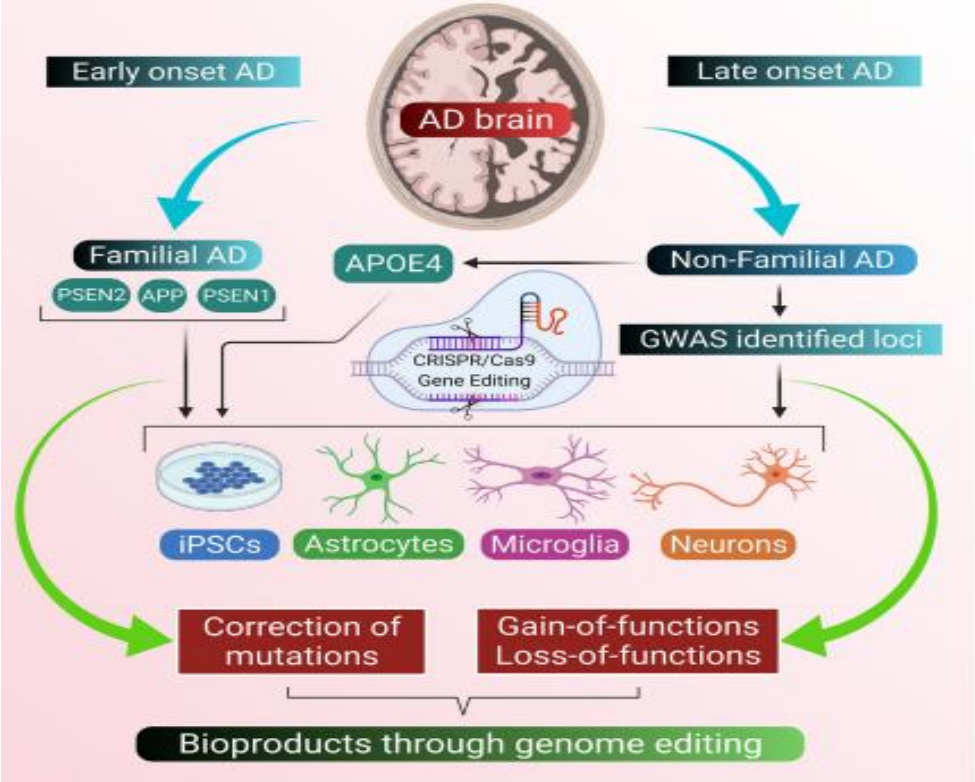


Fig.6. Illustration showing the possible CRISPR/Cas9 mediated gene editing approach in AD. GWAS, genome-wide association studies. (Shanu Bhardwaj et.al, *CRISPR/Cas9 gene editing: New hope for Alzheimer's disease Therapeutics*,2022)

which has been reported to correct similar types of mutations. For instance, the CRISPR/Cas9 system was utilized in basal forebrain cholinergic induced pluripotent stem cells (iPSC)-derived neurons from a PSEN2N141I mutated individual for the correction autosomal dominant mutations[145], resulting in correction and stabilization of the Ab42/40 ratio. Furthermore, the PSEN2 mutation corrected by this editing system also reversed electrophysiological deficits. Previous studies wherein CRISPR/Cas9 was utilized to fix PSEN gene mutations in FAD using iPSCs derived from the patient further supported these results[146,147].

Conclusions

The CRISPR Cas9 technology has emerged as a groundbreaking tool in the fiel d of genetic engineering, offering promising avenues for the treatment of various diseases. This innovative approach allows scientists to precisely edit genes, openi ng up possibilities for correcting genetic mutations responsible for hereditary con ditions.

By utilizing this method, researchers can target specific DNA sequences withi n the genome, enabling them to disrupt or modify genes linked to diseases such as

cystic fibrosis, muscular dystrophy, and certain types of cancer.The versatility of CRISPRCas9 lies in its ability to be tailored for different therapeutic applications For instance, by digning specific guide RNAs,scientists can direct the Cas9 enzym e to cut at preciselocations within the DNA, facilitatg the repair or replacement of faulty genes. This precision reduces the risk of off.target effects, which has been a significant challenge in earlier geneediting technologies.Moreover, the potential o f CRISPR Cas9 extends beyond treating singlegene disorders. It holds promise for complex diseases where multiple genes are involved, providing opportunities for developing multifaceted treatment
strategies. The ongoing research and clinical trials are continually expanding our understanding of how CRISPR can be harnessed to address a broad spectrum of h ealth issues, potentially transformig personalized medicine.

Despite its incredible potential, challenges such as ethical considerations, deliv ery mechanisms, and longterm effects need to be addressed to ensure the safe and effective use of CRISPR Cas9 in clinical settings. As the scientific community co ntinues to explore these dimensions, CRISPR Cas9 stands at the forefront of a new era in medical science, poised to reshape the landscape of disease treatment.

Discussion

The CRISPR-Cas9 system is considered a tool with high potential for treating genetic diseases, as it allows for precise modification of gene machinery and provides definitive treatments. However, its clinical application faces serious challenges;These include the high risk of off-target effects, challenges in safely delivering the system to target tissues, as well as complex ethical considerations, particularly in germline genome editing.However, newer generations of editors (such as Base/Prime Editors) and early successes in clinical trials have made the future outlook much brighter, and future research should focus on increasing safety, efficiency of delivery, and developing ethical frameworks to prepare this technology for widespread use.

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