

REVIEW

Gene Editing Technology in the Treatment of Cancers and other Genetic Disorders

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SUMMARY

The field of molecular biology has been transformed by the development of CRISPR/Cas9 gene editing technology, which has opened up previously unimaginable possibilities for the treatment of a variety of genetic diseases, including cancer. The development and potential uses of CRISPR/Cas9 gene editing technology to treat cancer and other genetic disorders are summarized in this abstract.

The CRISPR/Cas9 system, which includes the Cas9 enzyme and guide RNA, precisely targets and modifies specific DNA sequences by directing Cas9 to a particular DNA sequence with the aid of a guide RNA molecule. This gene-editing tool is based on a natural bacterial defense mechanism and can perform modifications like gene knockout, gene insertion, or gene correction. The system allows for the selection of a specific DNA sequence based on location, function, or association with a particular gene or genetic trait. In particular, CRISPR/Cas9 technology holds great promise for understanding the underlying genetic mechanisms of tumorigenesis and devising innovative therapeutic strategies for cancer treatment. The detection and confirmation of oncogenes and tumor suppressor genes is one use of CRISPR/Cas9 in cancer research. Researchers can clarify the functional roles of particular genes in the initiation and growth of tumors by methodically focusing on these genes in cancer cells. Our understanding of cancer biology is improved by this information, which also offers potential targets for therapeutic intervention. Additionally,

CRISPR/Cas9 gene editing has demonstrated significant promise for the creation of individualized cancer treatments. This technology can disrupt or correct cancer-related mutations through targeted gene knockout or correction, regular cellular functions. In the realm of genetic diseases, CRISPR/Cas9 gene editing offers a revolutionary approach to correct disease-causing mutations. Researchers hope to create effective treatments by precisely identifying and altering the genetic flaws that cause various inherited disorders. However, before CRISPR/Cas9 gene editing is widely used in medicine, there are still a number of obstacles to overcome. The main areas that need more research and improvement include off-target effects, delivery methods, and ethical considerations. However, the rapid development and ongoing improvements in CRISPR/Cas9 technology hold great promise for the creation of precise and targeted treatments for cancer and genetic disorders.

Introduction

The ability to precisely target particular nucleotide sequences has long been a goal in both academic and industrial circles, with potential applications in gene functional study, gene therapy, and precision breeding of domesticated plants and animals. This ideal became a reality as a new age of targeted genome editing began with the discovery of genome-editing meganucleases in the 1990s. Endodeoxyribonucleases, which include meganucleases, are a class that naturally occurs in a wide range of diverse organisms (Silva et al., 2011). They work by identifying and cleaving particular double stranded DNA (dsDNA) sequences, which are typically >14 bp in length and whose sequence differs between different meganucleases. Meganucleases were the first class of molecular DNA "scissors" that were successfully utilized to precisely edit genetic sequences, making it possible to replace, remove, and modify DNA in ways that were previously unreachable. Meganucleases were also able to exhibit great target specificity and minimal off-target effects due to the extended length of the recognition site. However, the limited availability of pre-defined targets has

presented challenges in locating naturally occurring meganucleases that can effectively target specific DNA sequences. Although scientists have tried to alter meganucleases to increase the potential of the editing site, the construction is difficult, and the success has been modest (Opiyo and Sinha, 2021).

Since then, additional nucleases have been discovered and altered for use in genome editing. These include transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs). ZFNs and TALENs have both been successfully utilized to target and edit particular genes, with some applications in therapeutic treatments.

(Ellis et al., 2013; Xu et al., 2014; Aravalli and Steer, 2016; Bauls et al., 2020). Despite this, both have drawbacks for genome editing. Similar to meganucleases, engineering ZFNs and TALENs to target specific sequences can take some time and frequently calls for specialized knowledge (RAZA et al., 2022).

The next breakthrough in genome engineering was quick to appear. In 2012, a natural bacterial immune system was harnessed to modify DNA in a programmable manner,

leading to the development of the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) system as a genome engineering tool. Bacteria were first used in this field 20 years ago as part of basic research, with the first description of repeat sequences present in bacteria that were later shown to constitute an adaptive immune system against viral infections and invading DNA (Torres-Ruiz, et al 2017).

The study of molecular biology and medicine has undergone a revolution since CRISPR-Cas system was discovered recently. Cas9 causes a double-strand break that is then repaired via either homology-directed repair (HDR) or non-homologous end joining (NHEJ) methods in CRISPR-mediated genome editing. Although HDR can be used to insert a specific DNA template for accurate DNA sequence restoration, this pathway is characterized by limited efficiency and high rates of undesired insertion or deletion (indel) mutations that nullify the potential benefit from repairing the mutation (Kantor, McClements and MacLaren, 2020). How does CRISPR/cas9 function? How can genome editing be achieved using this method? How can it be a treatment for cancer and genetic diseases? And what are the limits and challenges of this technology?

CRISPR/Cas9 mechanism

The CRISPR/Cas system was first discovered in bacteria and archaea, where it functions as a form of adaptive immunity against viruses (Nakata et al., 1989; Mojica et al., 2005). More than 13 distinct CRISPR-Cas systems have been identified, which are divided into three primary groups (I, II, and III) and at least 12 subtypes (A-F) based on the phylogeny of the Cas gene, crRNA (CRISPR Ribonucleic Acid) biogenesis, and mechanism of nucleic acid cleavage (DNA and RNA) (Makarova et al., 2011).

Recent studies on Cas proteins demonstrate their utility in the initial identification and excision of attacking viral DNA genomes. The RNA-guided DNA breaks are elucidated by understanding the crystal structure of SpCas9 (*Streptococcus pyogenes* Cas9) and constructing the truncated Cas9 mutant that facilitates its *in vivo* therapeutic application by providing a mechanism of its packaging in size-restraint viral vectors (Nishimasu et al., 2014). The way this works for bacteria is as follows: The unique sequences that are nestled in between the palindromic repeats, which are called spacers, are bits of DNA that are foreign and do not belong to the bacterium but instead originate from mobile genetic elements (MGEs), such as bacteriophages transposons or plasmids that have previously infected the prokaryotes. This was revealed by sequencing the spacers found in the CRISPR system, which led to the hypothesis that this could be a defense mechanism employed by bacteria to recognize foreign DNA elements during a viral infection. Bacteria acquire a small piece of foreign viral DNA and integrate it into the CRISPR locus to generate CRISPR arrays. These consist of duplicate sequences, which are palindromic repeats belonging to the bacterial genome flanked by variable sequences or spacers, which again are from foreign genetic elements. In this way, bacteria retain a memory, so to speak, of a past infection (Khan et al., 2016). As we can see in **Figure 1**, once the viral DNA is injected into the cell, a section of it can be incorporated into the bacterial genome. As we mentioned, it will be inserted between the repeated palindromic sequences. This will now be called a spacer. Therefore, here, we can see three different spacers potentially from three different viruses sandwiched between the repeated palindromic sequences. Now, we have what is called a CRISPR array. This CRISPR array can undergo transcription to form crRNA, although this

longer strand is called pre-crRNA, then the protein cas9 becomes involved. Cas refers to CRISPR-associated nuclease proteins, and nucleases are enzymes that are capable of cleaving DNA at specific nucleotide linkages, similar to a pair of scissors. In particular, Cas9 is one of the nucleases found in *Streptococcus pyogenes* and is one of the most extensively researched and characterized

CRISPR-associated nuclease proteins. Therefore, this is the one we will be looking at here inside this bacterium. Now, along with cas9, there are also molecules of tracrRNA (trans-activating CRISPR RNA), which have sections that are complementary to and can anneal to the palindromic repeats (Wang et al., 2022).

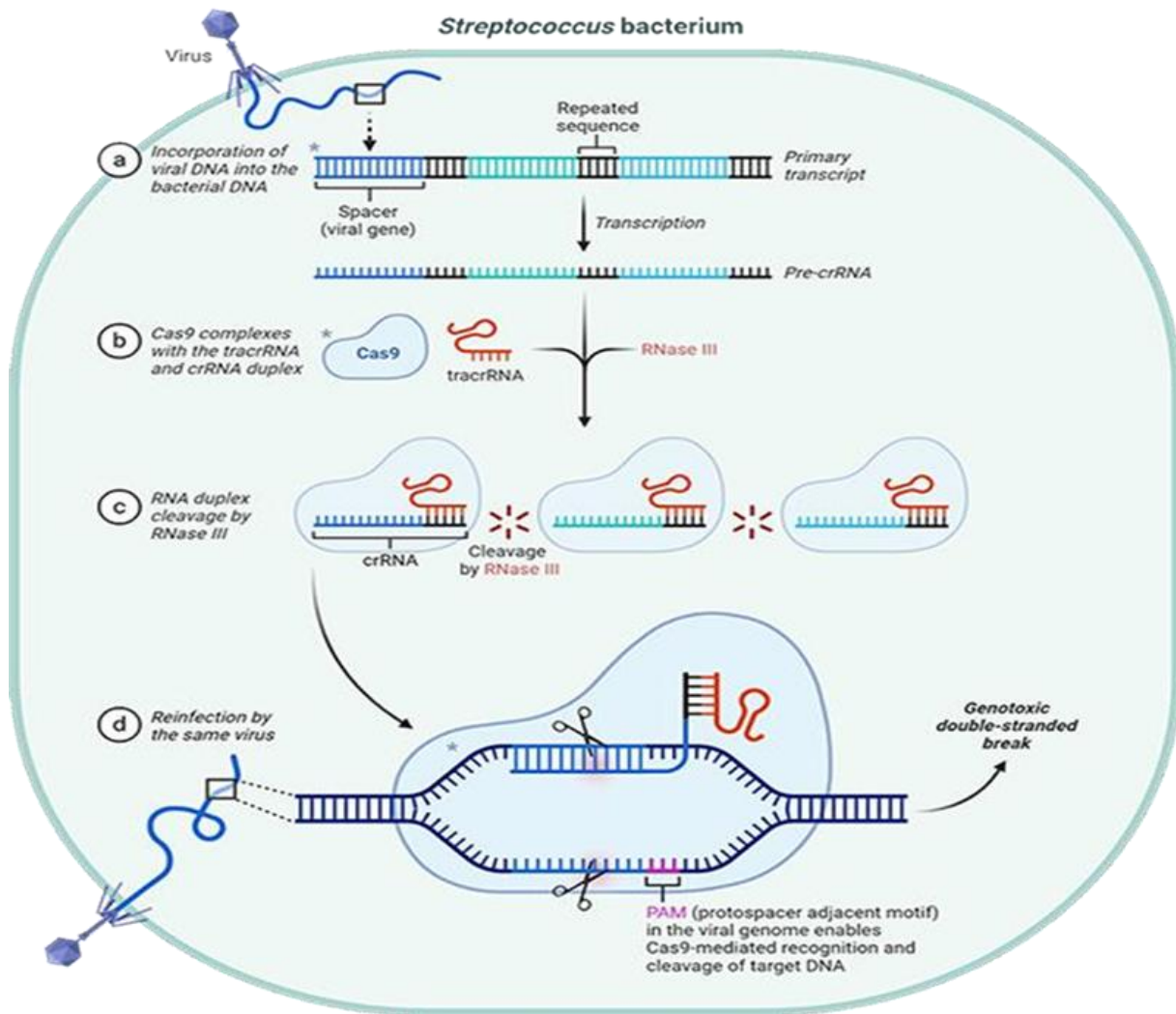


Figure 1 : Mechanism of type II CRISPR/Cas9 system in *Streptococcus bacterium*.

Therefore, for each spacer and palindromic repeat, we end up with a complex consisting of that segment of pre-crRNA, a tracrRNA and a cas9 protein. Then, another enzyme called ribonuclease three or RNase3 cleaves the strand between these complexes, leaving us with individual crRNA complexes, which we can call effector complexes. With these effector complexes formed, the cell is now ready to defend against the invader whose genome produced that crRNA. If this complex encounters a section of viral DNA that has a sequence that is complementary to this crRNA, the nuclear enzyme will coordinate, and if it recognizes a short sequence unique to the viral genome called a protospacer adjacent motif (PAM), then it will snip both strands of the DNA, just a few base pairs upstream from the PAM. In doing so, it will neutralize the virus because its genome can no longer be transcribed properly to create more viral particles.

This gives us a reasonable understanding of how CRISPR is employed by prokaryotic organisms as a natural defense. Now, it is time to understand how this phenomenon can serve as the basis for a bioethical application. This began in 2012 when Jennifer Doudna, a molecular biologist from the University of California, Berkeley, along with French microbiologist Emmanuelle Charpentier, were the first to propose that the bacterial CRISPR-cas9 system could be used as a programmable toolkit for genome editing in humans and other animal species. They eventually received the Nobel Prize in Chemistry for their work in 2020.

The first thing we need to understand is that in bacteria, crRNA and TracrRNA are separate molecular entities. The first major breakthrough arrived when it was realized that the roles of these molecules can be combined into a single

molecule by fusing them together with a linker to generate something called single guide RNA (sgRNA), which can be synthesized in the lab (Jiang and Doudna, 2017).

If the sgRNA complexes with a cas9 protein, this two-components system will be able to cleave DNA just as the three-components system does in bacteria. What this means was that it was then possible to determine any sequence of approximately 20 bp as a target for editing, and all that has to be done is to synthesize the appropriate sgRNA with the complementary sequence and insert that into a cell along with the cas9 protein, which has been sourced from *Streptococcus pyogenes* (Jiang and Doudna, 2017).

The complex reads the DNA until it finds the appropriate sequence along with a PAM sequence. Binding will occur, and DNA cleaved at precisely the desired location.

The Cas9 Enzyme

S. pyogenes Cas9 (hereafter referred to as SpyCas9) is a large (1,368-amino-acids) multidomain and multifunctional DNA endonuclease. It snips dsDNA 3 bp upstream of the PAM through its two distinct nuclease domains: an HNH-like nuclease domain that cleaves the DNA strand complementary to the guide RNA sequence (target strand) and a RuvC-like nuclease domain responsible for cleaving the DNA strand opposite the complementary strand (nontarget strand) (**Figure 2**) (Jiang and Doudna, 2017). In addition to playing a crucial role in CRISPR interference, Cas9 also takes part in crRNA maturation and spacer acquisition.

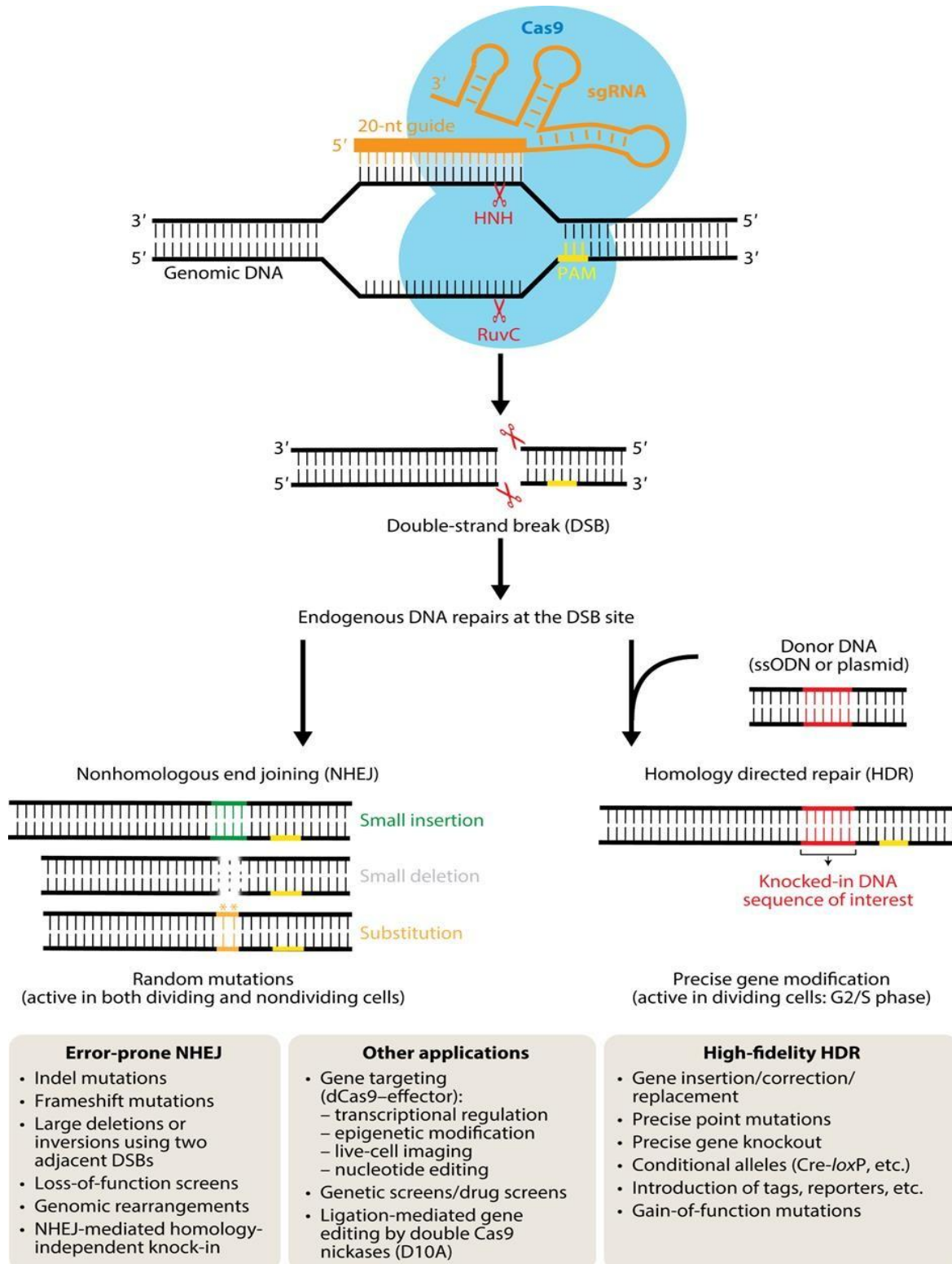


Figure 2 : Cas9 double stranded DNA breaks are subsequently repaired by cellular DNA repair machinery via the NHEJ or HDR pathway. (Wang et al., 2022).

1. Bilobe architecture of the apoenzyme

Structures of Cas9 in the apo state have two distinct lobes, the alpha-helical recognition (REC) lobe and the nuclease (NUC) lobe containing the conserved HNH and the split RuvC nuclease domains as well as the more variable C-terminal domain (CTD) (Figure 3a). The two lobes are further connected through two linking segments, one formed by the arginine-rich bridge helix and the other by a disordered linker. The REC lobe has no structural

resemblance to any other known proteins and is made up of three alpha-helical domains (Hel-I, Hel-II, and Hel-III). Additionally, the extended CTD exhibits a Cas9-specific fold and comprises sites that interact with PAM and are necessary for PAM interrogation. However, the apo-Cas9 structure exhibits a significant amount of disorder in this PAM-recognition region, indicating that the enzyme is held in an inactive state and unable to recognize target DNA before attaching to a guide RNA (**Figure 3**).

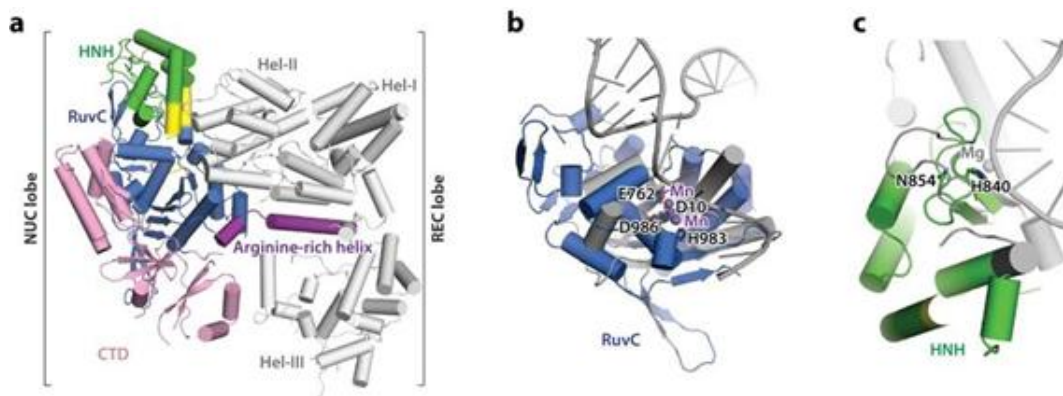


Figure 3 : structure of *Streptococcus pyogenes* Cas9 (SpyCas9) in the apo state. (a) Ribbon representation of the crystal structure of SpyCas9. (b) Close-up view of the SpyCas9 RuvC domain. (c) Close-up view of the active site of the SpyCas9 HNH domain (Wang et al., 2022).

2. HNH and RuvC Nuclease Domains

The Cas9 RuvC nuclease domain shares structural similarities with retroviral integrase superfamily members, which are distinguished by an RNase H fold, according to structural comparison of Cas9 nuclease domains to homologous structures of DNA-bound nucleases (**Figure 3b**). This suggests that RuvC is likely to use a two-metal-ion catalytic mechanism for cleavage of the nontarget DNA strand. For target-strand DNA cleavage, the HNH nuclease domain is most likely to use a one-metal-ion mechanism because it adopts the distinctive -metal fold used by other

HNH endonucleases (**Figure 3c**). The hallmarks of one-metal-ion-dependent and two metal-ion-dependent nucleic acid cleaving enzymes are a conserved general base histidine and an absolutely conserved aspartate residue, respectively (Jiang and Doudna, 2017). This is in accordance with Cas9 mutagenesis studies, which demonstrate that altering either the HNH (H840A) or the RuvC domain (D10A) turns Cas9 into a nickase (modified form of the Cas9 enzyme capable of creating a single-strand break (nick) in the DNA instead of a double-strand break), whereas altering both nuclease domains of Cas9

(also known as "dead Cas9" or dCas9) preserves its RNA-guided DNA binding ability intact while eliminating endonuclease activity. However, these postulated catalytic pathways still need to be supported by experimental evidence.

Target search and recognition

The CRISPR/Cas system consists of a number of parts with vastly varying modes of action that have the potential to be therapeutic through direct genome contact and/or editing. Despite the intricacy of the Cas family, all systems require CRISPR RNA (crRNA) for defined target specificity, while type II variants have an additional requirement for trans-activating RNA (tracrRNA), which forms a scaffold structure. (Hidalgo-Cantabrana and Barrangou, 2020) The two CRISPR RNAs mentioned above are combined into a single small guide RNA (sgRNA) for gene editing applications, considerably simplifying delivery. The Cas9: sgRNA complex randomly interrogates DNA in the cell, searching first for the appropriate PAM.

One constraint of Cas9 is its dependency on the aforementioned PAM sequence to bind DNA. The native PAM sequence for the commonly used SpyCas9 is 5'-NGG-3', where N can be any of the four DNA bases. When a suitable PAM sequence is present, target identification occurs through three-dimensional collisions, in which Cas9 quickly separates from DNA that does not contain it. The length of the dwell period depends on the complementarity between the guide RNA and nearby DNA when this occurs (Palermo et al., 2016). When Cas9 finds a target site with the right PAM, it causes local DNA melting at the PAM-adjacent nucleation site. This is followed by RNA strand invasion to create an RNA-DNA hybrid and a displaced DNA strand (referred to as an R-loop) from PAM-proximal to PAM-distal ends (Palermo et al., 2016). For Cas9 to

effectively target and cleave DNA, the seed region of the target DNA must match perfectly, whereas poor base pairing in the non-seed region is far more tolerable for target binding specificity.

Researchers are actively working on new approaches to improve the targeting precision and reduce the off-target effects of the CRISPR-Cas9 system along with broadening the targeting breadth of CRISPR tools (Vakulskas et al., 2018). Cas9 variants that have evolved to boost targeting specificity have been documented in several investigations.

Model of CRISPR–Cas9-mediated DNA targeting and cleavage

We can create a thorough model of Cas9 activation following guide RNA binding and target DNA recognition based on recent structural and mechanistic research (**Figure 4**). According to this concept, the Cas9 enzyme undergoes a significant conformational rearrangement upon attachment to the guide RNA, which changes the enzyme's state from inactive to DNA recognition-competent. For target binding and strand invasion, the RNA seed sequence is preordered in an A-form conformation, and the PAM-recognition sites are prepositioned for PAM interrogation (Sternberg et al., 2014). Cas9's first attachment to PAM sequences enables it to quickly search nearby DNA for potential target sequences (Makarova et al., 2011). Cas9 will start duplex unwinding and continue sampling the remaining target sequence once it discovers a viable target with the right PAM. To allow the first base of the target DNA sequence to flip and rotate up toward the guide RNA for base pairing, the phosphate lock loop stabilizes the unwound target DNA strand (Jiang et al., 2016). Cas9 also interacts with flipped bases on the nontarget strand to assist duplex unwinding (Jiang et al., 2016). The guide–target base

pairing and accompanying conformational changes of Cas9 facilitate guide RNA strand invasion beyond the seed region. Base pairing spreads to the 5' end of the guide sequence as the nonseed region gradually emerges from the constraint (Jiang et al., 2016). Until Cas9 is in an active state, this increasing base pairing causes additional coordinated conformational changes. HNH eventually achieves a stable, active conformation for cutting the target

strand following full annealing of the guide RNA and target DNA. The loop linkers undergo a significant conformational change in response to this conformational change in HNH, which in turn directs the nontarget strand to the RuvC catalytic core for coordinated cleavage (Jiang et al., 2016). After cleavage, Cas9 stays firmly attached to the target DNA until other cellular factors displace the enzyme for recycling.

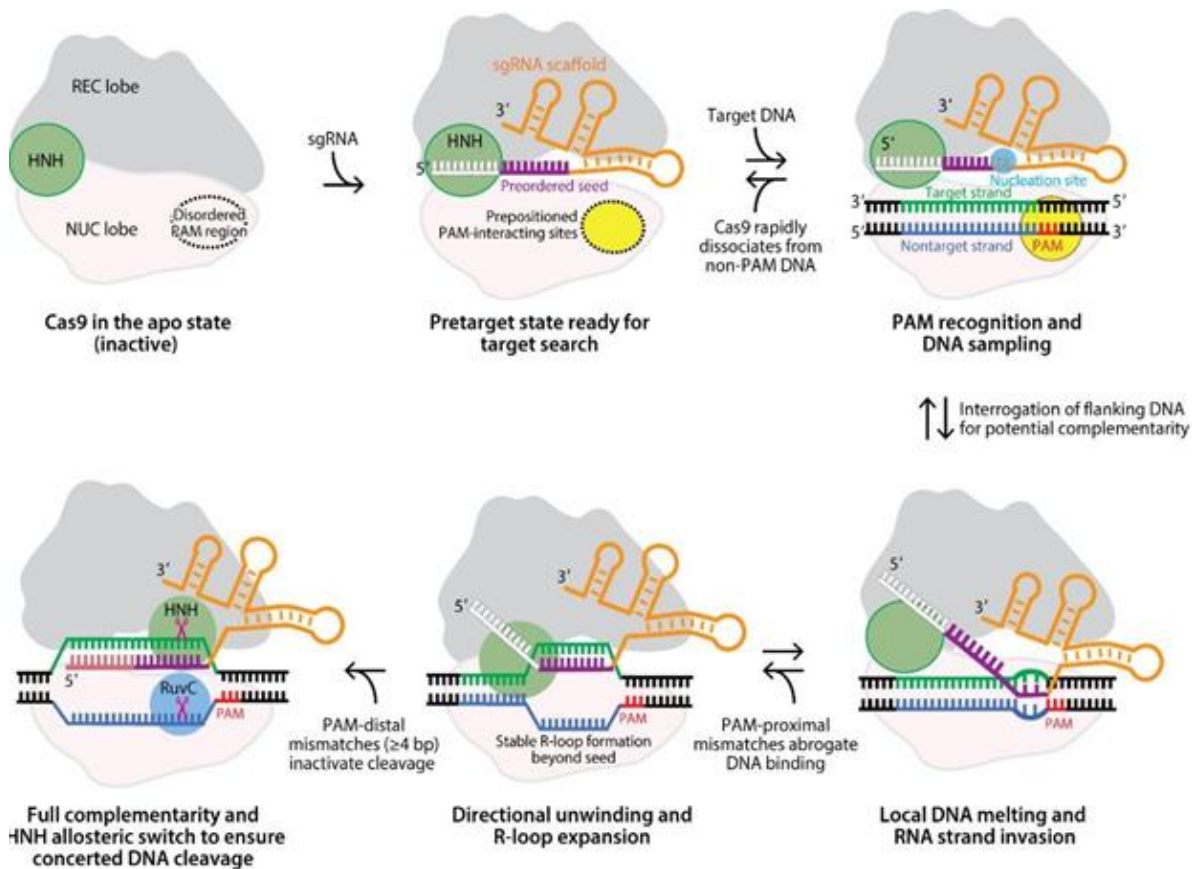


Figure 4 : Schematic representations of the proposed mechanisms of CRISPR-Cas9-mediated target DNA recognition and cleavage (Wang et al., 2022)

Delivery strategies of the CRISPR/CAS9 complex within cancer cells

The most common cause of death in the world, cancer, is caused by several genetic and epigenetic abnormalities. For this complex disease, the present treatment plans have

some drawbacks, which highlights the need for the deployment of very effective alternative methods. The Cancer Genome Atlas, which provides information on more than 15,000 tumors, documents the complexity of cancer. Most cancer patients have various genetic mutations or aberrations, which can dramatically influence tumor's development and sensitivity to therapeutic interventions (Allemailem et al., 2022).

The right delivery technique will enable CRISPR/Cas9 to affect target cells for therapeutic efficacy with little biodegradation. The three basic types of delivery methods are physical, viral, and non-viral (Allemailem et al., 2022). Each method of delivery has limitations, advantages, and difficulties. sgRNA and Cas9 can be delivered within target cells as plasmids, RNPs or a combination of sgRNA and Cas9 mRNA. Plasmids (4.2 kbp SpCas9 gene) with a significant negative charge are generally difficult to deliver (Ran et al., 2015). For traditional viral and non-viral delivery systems, the large size of sgRNA (31 kDa, 130 bases) and Cas9 (160 kDa, 4300 bases) presents a challenge (Kim et al., 2014). Additionally, after transfection, plasmids need to undergo transcription and translation processes, which typically delay editing.

1. CRISPR/Cas9 delivery by physical methods

The physical strategies include hydrodynamic injection, microinjection, electroporation, and laser irradiation for delivering and concentrating a Cas9/sgRNA complex inside a specific cell. These tactics typically require much experience and mostly harm target cells (Glass et al., 2018).

The physical methods of targeting the CRISPR/Cas9 system are schematically illustrated in **Figure 5**, and some characteristics, advantages and limitations of physical and

virus- mediated methods (Chen et al., 2016; Guan et al., 2016; Long et al., 2016; Chuang et al., 2017; Voets et al., 2017) are described in **Table 1**.

2. CRISPR/Cas9 delivery by viral particles

Viruses are naturally occurring transducers that can transmit their own genes into host cells. Some viruses can also be used to transfer other genes of therapeutic interest (Chew et al., 2016). The extrachromosomal episomes of viral vectors, including adenovirus, adeno-associated virus (AAV), and herpes virus, can remain in the nucleus or integrate into the host genome, such as lentiviruses or oncoretroviruses (Ortinski et al., 2017). Lentiviral vectors have grown in popularity in therapeutic applications due to their capacity to handle large DNA payloads to sustain high expression in both non-dividing and dividing cells. (Allemailem et al., 2022). Lentiviral vectors can pass through an undamaged nuclear membrane's nuclear pore. However, the constitutive production of Cas9 and sgRNA by lentivirus vectors can result in unfavorable off-target effects and non-specific RNA-DNA interactions (Zhang et al., 2021). Additionally, CRISPR/Cas9 off-target insertional mutagenesis can result from the high integration capacity of retroviral vectors. Additionally, the use of retroviral vectors during mass vector production can result in recombination events that produce replication-competent vectors (Allemailem et al., 2022).

Although they possess a high transfection efficiency, viral vectors suffer from some limitations, such as large- scale processing, complexity of synthesis, limited packaging size, and carcinogenic and immunogenic possibilities. These limitations shifted the targeting approach of genetic elements of interest to non- viral vectors (Zhang et al., 2021; Allemailem et al., 2022).

Table1 : Different approaches of CRISPR/Cas9 delivery by physical and viral methods elucidating their advantages and limitations. Abbreviations : Cas9, CRISPR associated protein 9 ; sgRNA, single guide RNA ; RNP, ribonucleoprotein ; AAV, adeno- associated virus ; kb, kilobase

Delivery vehicle and method	Most common cargo	Capacity	Advantages	Limitations
Microinjection by needle	DNA plasmid; mRNA(Cas9 + sgRNA); protein (RNP)	nmol/L levels of Cas9 and sgRNA	Guaranteed delivery into cells of interest	Time-consuming, difficult and generally in vitro only
Electroporation and nucleofection by electric current	DNA plasmid; mRNA(Cas9 + sgRNA)	nmol/L levels of Cas9 and sgRNA	Delivery to cell population; well-known technique	Generally in vitro only and some cells are not amenable
Hydrodynamic delivery by high- pressure injection	DNA plasmid; protein(RNP)	nmol/L levels of Cas9 and sgRNA	Virus-free; low cost; easy	Non-specific and traumatic to tissues
AAV by non-enveloped ssDNA	DNA plasmid	<5 kb nucleic acid	Minimal immunogenicity	Low capacity
Adenovirus by non-enveloped dsDNA	DNA plasmid	8 kb nucleic acid	High-efficiency delivery	Inflammatory response and difficult scaled production
Lentivirus by enveloped RNA	DNA plasmid	Almost 10 kb, up to 18 kb nucleic acid	Persistent gene transfer	Prone to gene rearrangement and transgene silencing

3. CRISPR/Cas9 delivery by non-viral vectors

Widespread clinical use of viral gene transfer is still hindered by safety concerns, as mentioned above. Non-viral vectors have been investigated as an alternative for the treatment of cancer because of their low

immunogenicity, high biocompatibility, outstanding deliverability, and affordable cost for mass production (Pack et al., 2005). Nanotechnology-based drug delivery systems will increase safety and widen the uses of CRISPR/Cas9 therapy, offering a workable solution to the problems posed by viral vectors (**Table 2**).

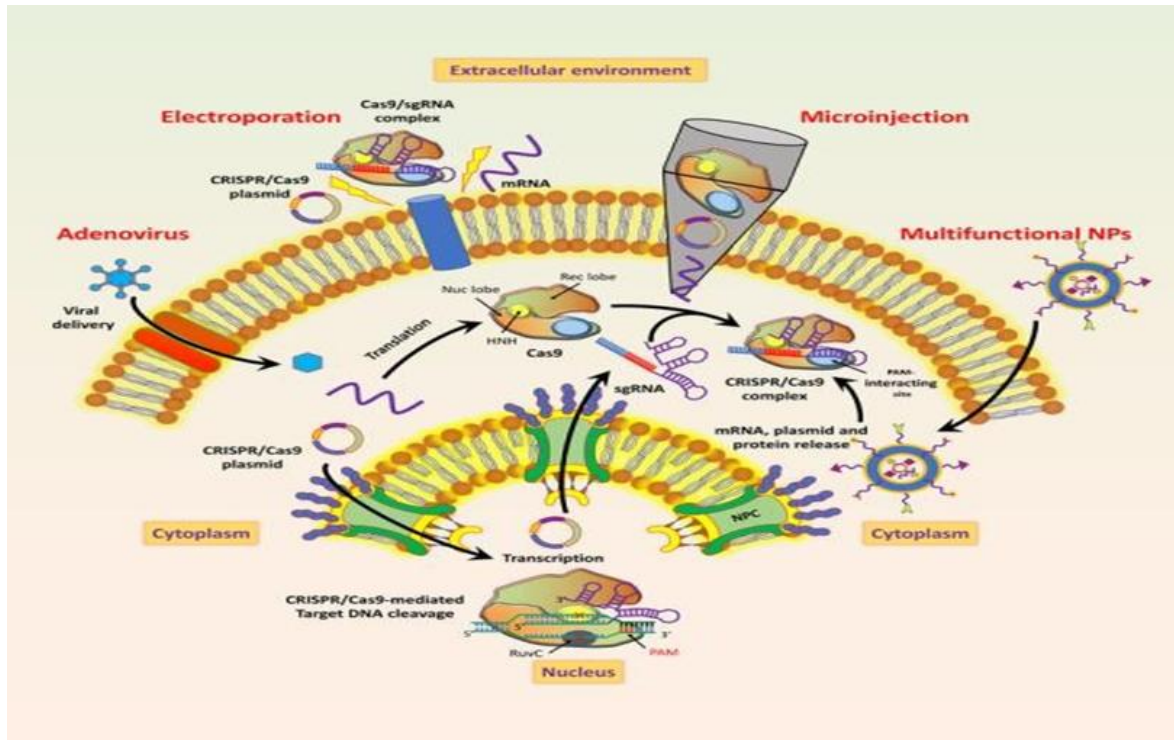


Figure 5 : The physical methodes in delivering Cas9/sgRNA inside a specific cell.

Table 2 : Nanotechnology- based delivery system for CRISPR/Cas9 (Wang et al., 2022). Polymer nanoparticles

Delivery system	Cargo options	Advantages	Disadvantages
Lipid nanoparticles	RNP plasmid DNA RNP Complex Cas9 mRNA sgRNA Donor DNA	High biocompatibility Low immunogenicity Reduce off-target effects Can be mass produced Low cost	Degradation in vivo
Polymer nanoparticles	RNP plasmid DNA RNP Complex Cas9 mRNA sgRNA Donor DNA	High biocompatibility Low Immunogenicity Reduce off-target effects Can be mass produced Low cost	Toxicity Limited delivery efficiency
Golden nanoparticles	RNP plasmid DNA RNP Complex Cas9 mRNA gRNA Donor DNA	High biocompatibility Low immunogenicity Reduce off-target effects Can be mass produced Low cost	Limited efficiency delivery

Lipid nanoparticles (LNPs)

LNPs are hydrophobic and hydrophilic amphiphilic systems made up of a variety of cationic or ionized lipids, neutral lipids such as phospholipids or cholesterol, and

polyethylene glycol-lipids. Due to the absence of a continuous lipid bilayer and a sizable internal pool, LNPs vary structurally from liposomes (Witzigmann et al., 2020).

LNPs were developed as carriers to transport a range of chemicals to cells, with significant advantages in the delivery of nucleic acids. Nucleic acids cannot easily move through the cell membrane because they are very unstable outside of the cell and carry many anions (Wang et al., 2022) (**Table 3**). Even so, nucleic acids can be delivered to cells with ease when they are enclosed in cationic liposomes. There is much interest in LNPs for the administration of anticancer medications because they have distinct advantages over standard drug therapy, such as avoiding drug degradation, permitting targeted drug delivery, and lowering drug toxicity. Pre-clinical studies have demonstrated that LNPs may effectively deliver Polymer materials are regarded as effective delivery tools because of their lengthy blood circulation, high drug bioavailability, outstanding biocompatibility, and degradability (Song et al., 2018; Wang et al., 2022). Traditional techniques of sgRNA delivery, such as Cas9 RNPs, are ineffective and poorly stable against cell proteases. A new type of nanocapsule made up of a protein core and a thin, permeable polymeric shell can be artificially engineered for stability or breakdown at various Another method of distributing CRISPR/Cas9 is via GNPs. GNPs are very biocompatible, can penetrate a wide range of cell types, and can be coupled with other components, including polymers, lipids, or nucleic acids (Wang et al., 2022). Functional diversity can be easily attained by including diverse functional components on the surface of the particle, such as glycoproteins and nucleic acids. Additionally, GNP size, shape, charge, and surface modification can all be changed to alter their pharmacokinetics (Ahmad et al., 2017). A new approach for genomics research uses GNPs with modified Cas9 protein and sgRNA, which can achieve 90% intracellular

siRNA or mRNA (Mehnert and Mäder, 2001; Pardi et al., 2017), indicating that they are a secure and reliable delivery method.

LNPs have been used extensively in preclinical studies of CRISPR/Cas9 delivery throughout the last few years. For LNP delivery of CRISPR/Cas9 components, there are two basic approaches: either Cas9 and sgRNA plasmid DNA or mRNA delivery or Cas9:sgRNA RNP complex delivery (Wang et al., 2022). Cas9 mRNA and sgRNA may effectively mediate mouse transthyretin (Ttr) gene editing by being loaded on LNPs and properly delivered to the liver of mice (Finn et al., 2018).

pH levels. The outer shell of the capsule is destroyed during capsule disintegration, allowing the core protein to enter the cell and carry out biological functions. This approach opens up a new avenue for the administration of sgRNA:Cas9 RNP for the treatment of cancer because it has low toxicity and can effectively transport a range of proteins to cells (Yan et al., 2010). Gold nanoparticles (GNPs)

delivery and 30% gene editing efficiency (Lino et al., 2018).

Role of CRISPR/CAS9 in cancer research and therapy

Thanks to CRISPR/Cas9 technology, it is now possible to diagnose and cure cancer by different tools (**Figure 6**) (Shojaei et al., 2022), including (a) the CRISPR/Cas9-based diagnostic systems SHERLOCK and DETECTR for cancer diagnostics, (b) TCR knockout (KO) CAR-T cells (universal CAR-T cells), (c) KO of inhibitory receptors such as PD-1 and LAG-3 to promote the capability of cancer immunotherapy, (d) elimination of oncogenic virus-like HPV, and (f) establishment of an *in vivo* tumor model

with multiple gene mutations with CRISPR/Cas9 gene editing tools (**Table 3**).

Table 3 : CRISPR/Cas9 applications in cancer treatment

Cancer	Target gene	Cell line	Result	REFERENCE
Liver cancer	NSD1	Huh7, Hep3B, SMMC-7721, HepG2, and SK-Hep1	Downregulation of the NSD1/H3/Wnt10b signaling pathway prevents tumor growth	(Zhang <i>et al.</i> , 2019)
Lung cancer	PD-1	T cells	Targeting exon 2 of the PD1 gene and reducing lung cancer size can both be accomplished via PD1 gene knockout.	(Lu <i>et al.</i> , 2020)
Breast cancer	miR-23b,miR27-b	MCF-7	knockout (KO) of miR- 23b and miR-27b alleviate tumor growth in breast cancer cells	(Hannaf on <i>et al.</i> ,2019)
Prostatecancer	GPRC6A	PC-3, LNCap and DU145	significant reduction in cell growth and aggressiveness	(R <i>et al.</i> ,2017)

1 Application of the CRISPR/Cas9 System in the Treatment of Liver Cancer

By employing the CRISPR/Cas9 system to target specific genes in liver cancer cells, it has recently been shown that it may be possible to reduce both cell proliferation and metastasis. A unique sgRNA was created by Zhang and colleagues in 2019 to specifically target nuclear receptor-binding SET domain-containing protein 1 (NSD1) in HCC cell lines. The HCC tumorigenic Wnt/-catenin signaling

pathway is the target of the NSD1 histone lysine methyltransferase. They discovered that in vitro and in vivo, NSD1 KO HCC cells showed less proliferation, migration, and invasion (Zhang et al., 2019). Wnt10b expression was reduced as a result of better H3K27me3 methylation and decreased H3K36me2 methylation after NSD1 ablation. Therefore, the Wnt/catenin signaling axis in nude mice and in vitro may be negatively regulated by the CRISPR/Cas9 tool to prevent HCC oncological events.

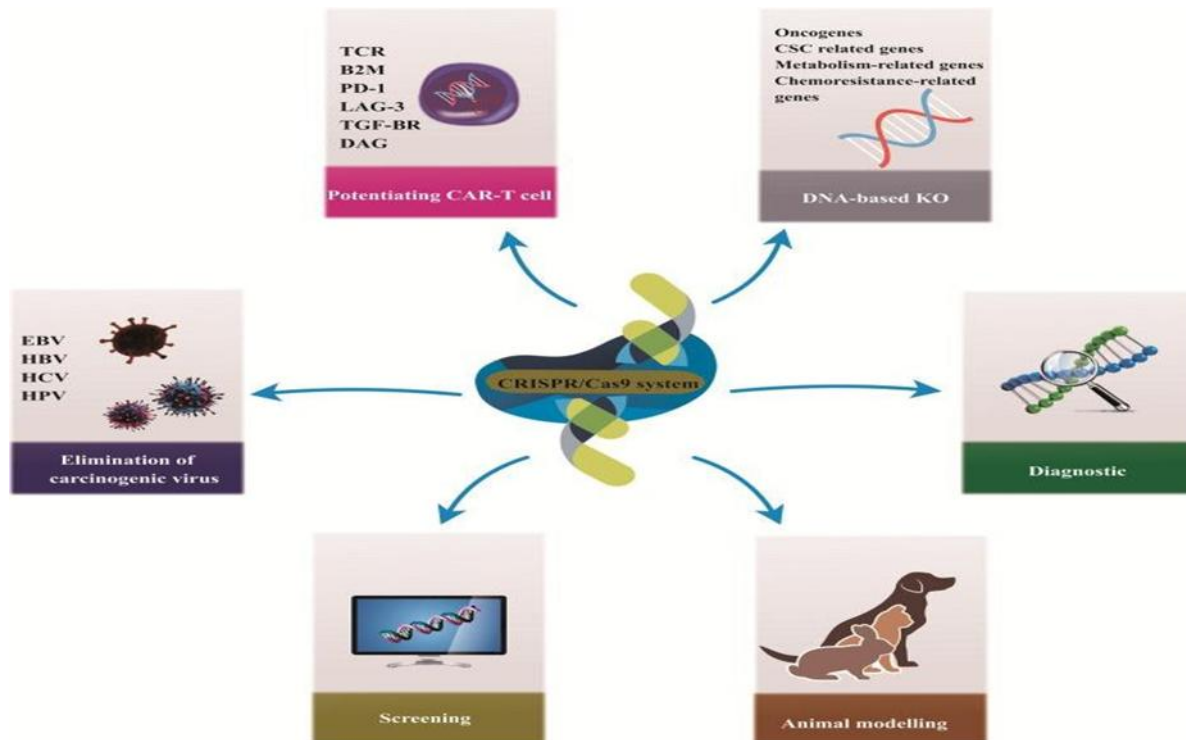


Figure 6. CRISPR/Cas9 applications in cancer research and therapy. Knockout (KO), T-cell receptor (TCR), chimeric antigen receptor (CAR)-T cell, $\beta 2$ -microglobulin (B2M), programmed cell death protein 1 (PD1 or PDCD1), lymphocyte activating gene 3 (LAG-3), transforming growth factor-beta receptor (TGF- β R), diacylglycerol (DAG), Epstein–Barr virus (EBV), human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), cancer stem cell (CSC)

2. The Application of the CRISPR/Cas9 System in the Treatment of Lung Cancer

A novel approach to treating lung cancer involves looking for immune cells such as lymphocytes. T cells were taken from the blood of lung cancer patients who were undergoing treatment trials, and the CRISPR/Cas9 system was used to knock down the gene encoding the PD1 protein (Doudna, 2020). The same patients' bloodstreams would be injected with these cells carrying altered genes again (Doudna, 2020). Because there will be less contact between the tumor ligand and the receptor on lymphocytes that do not express PD-1, the T-cell receptor will be better

equipped to recognize the troublesome cells and carry out its function (Castillo, no date). The knockdown of PD- 1 in immune cells is necessary for caspase activation, which is needed to induce programmed death in cancer cells (Zhao et al., 2018).

3. Application of the CRISPR/Cas9 System in the Treatment of Breast Cancer

Strong evidence supports the idea that altered miRNA expression contributes to the development of breast cancer (Kayani et al., 2011). Accordingly, miR-23b and miR-27b encourage the growth of certain human malignancies and may even accelerate angiogenesis in these conditions.

Through the overexpression of ST14 (suppression of tumorigenicity 14), recent studies in MCF7 breast cancer cells showed that miR-23b and miR-27b gene expression knockout utilizing CRISPR systems reduced tumor growth in xenograft nude mice (Hannafon et al., 2019). Since ST14 normally reduces breast cancer cell proliferation and invasion (Dai et al., 2021), enhancing ST14 activity may be necessary to achieve antitumor effects following miR-23b and miR-27b inactivation. The development and progression of breast cancer may also be influenced by dysregulated fatty acid synthase (FASN) expression, which complicates the endogenous synthesis of fatty acids and the modification of estrogen receptor signaling (J. Zhang et al., 2021). It has been proven that genetic FASN knockdown via CRISPR/Cas9 reduces the aggressive characteristics of breast cancer MCF-7 cells, as seen by reduced cell viability, proliferation, and migration (Gonzalez-Salinas et al., 2020). Importantly, transcriptome research has shown that FASN loss has a more pronounced deleterious impact on genes related to proliferation than lipid metabolism (Gonzalez-Salinas et al., 2020).

4. Application of the CRISPR/Cas9 System in the Treatment of Prostate Cancer

Recent studies have provided new evidence suggesting that the development of prostate cancer is associated with increased expression of G protein- coupled receptor family C group 6 member A (GPRC6A) (Pi and Quarles, 2012). The upregulation of GPRC6A facilitates the growth of prostate cancer cells in response to dietary and bone-derived ligands (Pi and Quarles, 2012). However, when the expression of GPRC6A is knocked down (KD), it diminishes the invasive properties of prostate cancer cells by attenuating the epithelial-mesenchymal transition (EMT) process (Pi and Quarles, 2012). Significantly, the

prostate cancer cell line PC-3, in which GPRC6A has been effectively disrupted using CRISPR/Cas9 technology, exhibits significantly reduced growth and aggressiveness compared to non-manipulated cells both *in vitro* and *in vivo*. The editing of this gene resulted in the inhibition of osteocalcin activation of extracellular signal- regulated kinase (ERK), Akt strain transforming (AKT) and mammalian target of rapamycin (mTOR) signaling (Ye et al., 2017).

Applications of CRISPR/Cas9 in genetic diseases

CRISPR/Cas systems have been used in research to study and treat genetic diseases. Among the promising developments, the application of CRISPR/Cas to treat monogenic human genetic diseases has the potential to offer long-term therapy following a single treatment. We summarize in Table 4 the use of this new technology in some genetic diseases.

Limitations and challenges

Despite its enormous potential, CRISPR/Cas9 has a number of drawbacks and difficulties that must be resolved before it can be widely used in a secure manner (Li et al., 2023). Among these restrictions and difficulties are the following:

1. Off-target effects

Due to the possibility of off-target effects resulting from base mismatches between non-target sequences and the single-guide RNA (sgRNA), it is not desirable for unintended mutations to occur during the desired target's repair process. (Fu et al., 2013). Off-target effects can be found using techniques such as whole-genome sequencing and GUIDE-Seq (Zischewski, Fischer and Bortesi, 2017). To address this, scientists have been working to enhance

the specificity of sgRNAs and detach them from the DNA strand in the event of mismatches. To improve accuracy, Cas9 mutations in the REC3 domain have been studied, and sgRNA structure changes have been investigated to improve specificity and prevent off-target effects (Zhu et al., 2019).

2. Validity

The upregulation or knockdown of a gene may not be enough to produce the desired therapeutic effect when using CRISPR/dCas9 for gene regulation. The position of the sgRNA, the choice of the activating structural domain, and the particular cell and gene being targeted are all variables that can affect how effectively a gene is activated. To increase transcriptional activation and boost the efficiency of CRISPRa (CRISPR activation) systems, researchers have created synthetic systems and altered structural domains. (Li et al., 2023)

3. Applicability

Although CRISPR/Cas9 can theoretically target any position in the genome, it is limited by the requirement of a specific PAM sequence. This limitation prevents Cas9 from reaching certain positions in the genome. Efforts have been made to modify Cas9 to recognize a wider variety of PAM sequences, resulting in variants that are not restricted to the canonical PAM sequence (Collias and Beisel, 2021). Additionally, for base-editing tools such as cytidine base editors (CBEs) or adenine base editors (ABEs), the edited bases are located at specific relative positions to the PAM sites (Gaudelli et al., 2017). The Lack of suitable PAM sites can hinder the base-editing function of CBEs or ABEs.

4. Chromosomal disorganization

Double-stranded DNA cleavage by Cas9 can sometimes lead to unintended chromosomal structural translocations and deletions, which may have adverse effects such as the development of tumors (Yin et al., 2022). Researchers have explored approaches to reduce these mutations, such as combining Cas9 with exonuclease structural domains to limit the occurrence of chromosomal translocations. For example, the fusion of Cas9 with optimized three-prime repair exonuclease 2 (TREX2) has shown promise in suppressing chromosomal translocations (Yin et al., 2022).

5. Limitations of targeted delivery

Efficient and targeted delivery of CRISPR components to the desired cells or tissues is a challenge. The use of viral and nonviral vectors for systemic administration can result in the uptake of gene drugs by nontarget cells, potentially leading to unintended consequences. Improving delivery functionality by modifying carriers or designing nanoparticles responsive to specific microenvironments can enhance targeted delivery. Biocompatibility is another consideration, as the vector must be compatible with the target cells and avoid triggering immune responses. Modified Cas9 proteins and protective coatings on delivery vehicles are being explored to address immune responses and improve biocompatibility (Li et al., 2023).

Conclusion

The CRISPR/Cas9 system has revolutionized genome editing and holds great promise for therapeutic applications in model organisms and humans. It provides precise editing of target sequences and has the potential to treat malignancies, genetic abnormalities, and infectious

diseases. However, there remain multiple challenges that need to be addressed before its widespread clinical use.

There are initiatives to increase CRISPR/Cas9 specificity and lessen off-target effects. To maximize effectiveness and reduce immunological reactions, recent advancements have been made in eliminating undesirable mutations and creating effective delivery systems. For targeted delivery, Cas9 ribonuclear proteins (RNPs) are utilized instead of plasmid vectors, which increases effectiveness and prevents the insertion of vector sequences. However, challenges remain, such as the potential for chromosomal rearrangements.

Before its clinical utility can be fully realized, the efficacy, safety, and specificity of CRISPR/Cas9 must be optimized. Although the technology is still in its early stages, clinical trials are currently being conducted with a focus on safety and efficacy. Establishing laws and ethical standards for gene editing is crucial, particularly in regard to modifying the genome of human embryos.

Despite these difficulties, CRISPR/Cas9's journey is incredibly fascinating and offers hope for the treatment of many diseases. To improve the *in vivo* delivery of CRISPR components, nanocarriers and other delivery systems are promising. Additionally, gene editing of T cells holds promise for improving cell therapies and creating universal CAR-T cells, while CRISPR screening has the potential to find significant cancer genes and therapeutic targets.

In summary, the CRISPR/Cas9 system has already made a significant contribution to the life sciences and exhibits great potential for future applications in human therapy, although there are still challenges to be overcome.

Conflicts of Interest:

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