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CRISPR-Cas9 Unleashed: Gene-Slicing Adventures in the Cancer Battlefield

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ABSTRACT

Cancer, a global health menace, continues to pose significant challenges in terms of incidence and mortality, necessitating innovative therapeutic strategies. Despite existing treatments, the limitations persist, prompting a quest for novel approaches. The emergence of immunotherapy marked a transformative era in solid tumor treatments, yet its efficacy is constrained by adverse effects. Concurrently, the integration of advanced technologies into cancer treatment explores the vast potential residing at the molecular level through gene analysis and manipulation. This review articulates the role of state-of-the-art genome editing technology, notably clustered regularly interspaced short palindromic repeats (CRISPR-Cas9), in overcoming the constraints of immunotherapy for cancers. Unveiling the intricacies of CRISPR-Cas9-mediated genome editing, the review introduces the formidable CRISPR toolbox. A spotlight is cast on the transformative impact of CRISPR-induced double-strand breaks (DSBs) on cancer immunotherapy, encompassing knockout and knock-in strategies. The utilization of CRISPR/Cas9 technology in pre-clinical cancer research has demonstrated notable success; however, its transition to the clinical setting remains in the nascent stages of development. This review aims to elucidate the fundamental aspects of CRISPR technology and offer a comprehensive survey of its existing applications while outlining its prospective role in the realm of cancer therapies. Through an exploration of CRISPR's mechanisms, current applications, and anticipated future potentials, this review provides valuable insights into the evolving landscape of CRISPR-based cancer treatment strategies.

KEYWORDS

Cancer; CRISPR; Cas9; Clinical trials; Preclinical studies

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1. Introduction

Cancer is a multifaceted and intricate ailment, fundamentally rooted in the intricate landscape of our genetic makeup. It takes its initial form through mutations in DNA, activating oncogenes while disabling tumor suppressors (Siegel et al. 2022). Simultaneously, it disrupts the epigenome, orchestrating the orchestra of gene expression. On another front, it is a disease of the cell, exploiting changes in metabolism, cell architecture, and motility to sustain growth even in the most challenging environments (Phan et al. 2021). At its core, cancer extends its grasp to the entire organism, manipulating normal cell types and tissue functions while cunningly outwitting the host's defense mechanisms. Unraveling the complex interplay of genomic transformations, cellular adaptations, and microenvironmental shifts driving cancer's onset, progression, and response to treatment is paramount. Such insights are instrumental in enhancing therapeutic options and elevating outcomes for the countless individuals grappling with cancer each year.

Cancer stands as either the foremost or second most significant contributor to global mortality, posing a substantial impediment to the augmentation of life expectancy on a global scale (Mohan et al. 2016). The development of tumors is intricately entangled with a myriad of genetic and epigenetic alterations. Comprehensive sequencing of cancer genomes has substantiated the presence of a multitude of genetic and epigenetic mutations within human tumors.

Between 1975 and 2018, the advancement of novel treatments is estimated to have prevented over 2 million cancer-related deaths among men and approximately 1 million among women(Siegel et al. 2021). Although this represents a remarkable reduction in mortality, cancer continues to assert itself as the second leading cause of death, with cardiovascular disease being the sole contender ahead. In the United States alone, the year 2021 saw approximately 2 million new cancer cases emerging. Notably, within the demographic of women aged 40–60 and across all individuals aged 60–80, cancer retains its unwelcome status as the primary cause of mortality (Siegel et al. 2021).

Currently, cancer treatment encompasses a spectrum of approaches, each with its unique advantages and challenges. Conventional cancer therapies, which include surgical resection, chemotherapy, and radiotherapy, have long been the cornerstone of cancer treatment. However, they present significant challenges, often affecting patients' tolerability and adherence due to the toxicity associated with these treatments (Wyld, Audisio and Poston 2015).

In recent years, molecular targeted therapy has emerged as a game-changer in the field of oncology. These drugs offer high specificity and efficacy, replacing traditional chemotherapeutic agents in many cases. This shift has marked revolutionary progress in the treatment of malignant tumors. However, in clinical practice, some challenges persist, including dramatic but short-lived tumor regressions and the financial burden of these therapies.

Immunotherapy has ushered in a new era in cancer treatment. It aims to harness the body's immune system to actively or passively suppress cancer. Key approaches within immunotherapy include immune checkpoint blockade (ICBs), adoptive cell transfer (ACT), and tumor-specific vaccines (Stefanoudakis et al. 2023). While immunotherapy has shown remarkable promise, it is important to note that it is effective in only a subset of cancers, and not all patients with cancer respond to these treatments (Al-Ogaili et al. 2020). Additionally, immune escape mechanisms within tumors can reduce the expected therapeutic effect.

Gene therapy is another avenue in cancer treatment, involving innovative techniques such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 system (Baghini et al. 2022, Katti et al. 2022). These approaches are employed to knock out, insert, or mutate specific genes to combat cancer. While ZFNs and TALENs have been used for gene editing, they are often regarded as time-consuming and complex (Table 1) (Lino et al. 2018, Cui et al. 2021, Gaj, Gersbach and Barbas 2013). In contrast, the CRISPR/Cas9 system has gained prominence due to its simplicity, versatility, and wide-ranging applications. In this

landscape, CRISPR, having been adapted for mammalian cells, emerges as a versatile powerhouse, profoundly impacting our comprehension of cancer biology and continuously providing new revelations poised to expedite the diagnosis and management of this formidable disease.

Table 1. Comparative Analysis of Genome Editing Tools: Mechanism, Target Specificity, Efficiency, and Applications.

Genome Editing Tool	Mechanism	Target Specificity	Efficiency	Applications
CRISPR/Cas9	RNA-guided endonuclease	High (depends on guide RNA design)	High	Gene knockout, knock-in, gene regulation, and more
Zinc-Finger Nucleases (ZFNs)	Protein-guided endonuclease	Moderate to High	Moderate	Gene knockout, gene addition
Transcription Activator-Like Effector Nucleases (TALENs)	Protein-guided endonuclease	Moderate to High	Moderate	Gene knockout, gene addition
CRISPR/Cas12	RNA-guided endonuclease	High (depends on guide RNA design)	High	Gene editing, diagnostic applications
CRISPR/Cas13	RNA-guided endonuclease	High (depends on guide RNA design)	High	RNA editing, diagnostic applications
Homing Endonucleases	Protein-guided endonuclease	High	Low to Moderate	Gene knockout, gene addition
MegaTALs	Protein-guided endonuclease	High	Moderate	Gene knockout, gene addition
Prime Editing	RNA-guided endonuclease	High	Moderate	Precise base editing
Base Editors	Protein-guided base editors	High	High	Base pair substitutions
CRISPR/Cas9-Cas12 Dual System	RNA-guided endonuclease	High	High	Multiplex genome editing
RNA-guided FokI Nucleases	RNA-guided endonuclease	High	Moderate	Gene knockout, gene addition

2. Introduction to CRISPR-Cas9

The CRISPR-Cas9 system, short for Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9, is a groundbreaking genome editing tool that has transformed the field of molecular biology (Agrawal et al. 2023). It is a revolutionary technology that allows precise modification of DNA in a targeted manner, offering a versatile and highly effective approach to genetic manipulation (Akram et al. 2020, Barrangou et al. 2007). CRISPR-Cas9 was initially discovered as part of the bacterial immune system, a mechanism that bacteria employ to defend themselves against viral infections (Watters et al. 2020). Researchers recognized its potential to be repurposed for gene editing in a wide range of organisms, including humans (Marraffini 2015). The system relies on a two-component mechanism: the guide RNA (gRNA) and the Cas9 protein (Figure 1). The gRNA is designed to be complementary to a specific DNA sequence in the target genome, providing a precise "address" for the Cas9 protein to find and bind to the DNA (Shola et al. 2020). Once bound to the target DNA, the Cas9 protein introduces double-stranded breaks, initiating DNA repair processes that can result in gene knockout, gene addition, or precise gene editing.

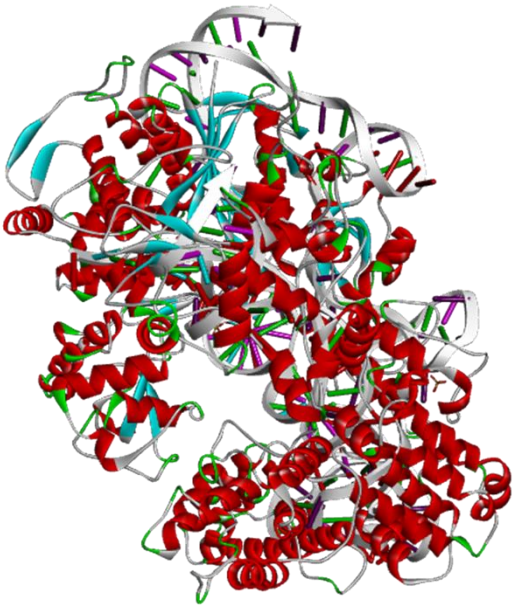

Figure 1. Crystallographic depiction unveiling the three-dimensional organization of the Streptococcus Pyogenes (Sp) Cas9 protein within the complex, intricately intertwined with "guide" RNA (sgRNA) and substrate (Target double-stranded DNA) (PDB ID: 5F9R). This structural insight provides a visual representation of the molecular arrangement critical to the CRISPR/Cas9 genome editing system for primed target DNA cleavage.

What makes CRISPR-Cas9 particularly powerful is its simplicity and versatility. It offers an efficient way to modify genes, allowing scientists to investigate gene function, create disease models, and potentially develop novel therapeutic approaches for genetic disorders and other diseases(Chen et al. 2020, Jeong 2016, Wang 2019). This technology has opened up new possibilities in biotechnology, agriculture, and medicine, and its impact on the scientific community has been profound(Padmaswari et al. 2023). In the following sections, we will delve deeper into the mechanism, applications, and ethical considerations surrounding CRISPR-Cas9.

2.1. Mechanism of CRISPR/Cas9

The CRISPR/Cas9 system is a revolutionary genome editing tool that relies on a two-component mechanism: the guide RNA (gRNA) and the Cas9 protein (Zhu et al. 2023, Jiang and Doudna 2017). Here's a detailed explanation of its mechanism (Figure 2).

2.1.1. Creation of Guide RNA (gRNA)

The process begins with the design and synthesis of a small piece of RNA called the guide RNA (gRNA) (Table 2). The gRNA is a synthetic molecule composed of two essential parts:

(a) CRISPR RNA (crRNA): This portion is derived from the CRISPR region of the bacterial genome. It contains a sequence that matches the target DNA to be edited.

(b) Trans-activating CRISPR RNA (tracrRNA): The tracrRNA acts as a scaffold that binds to the Cas9 protein, facilitating its interaction with the crRNA.

2.1.2. Formation of the gRNA-Cas9 Complex

The gRNA and the Cas9 protein are then combined to form a ribonucleoprotein complex (RNP). The Cas9 protein is an endonuclease enzyme that has two nuclease domains responsible for cutting DNA.

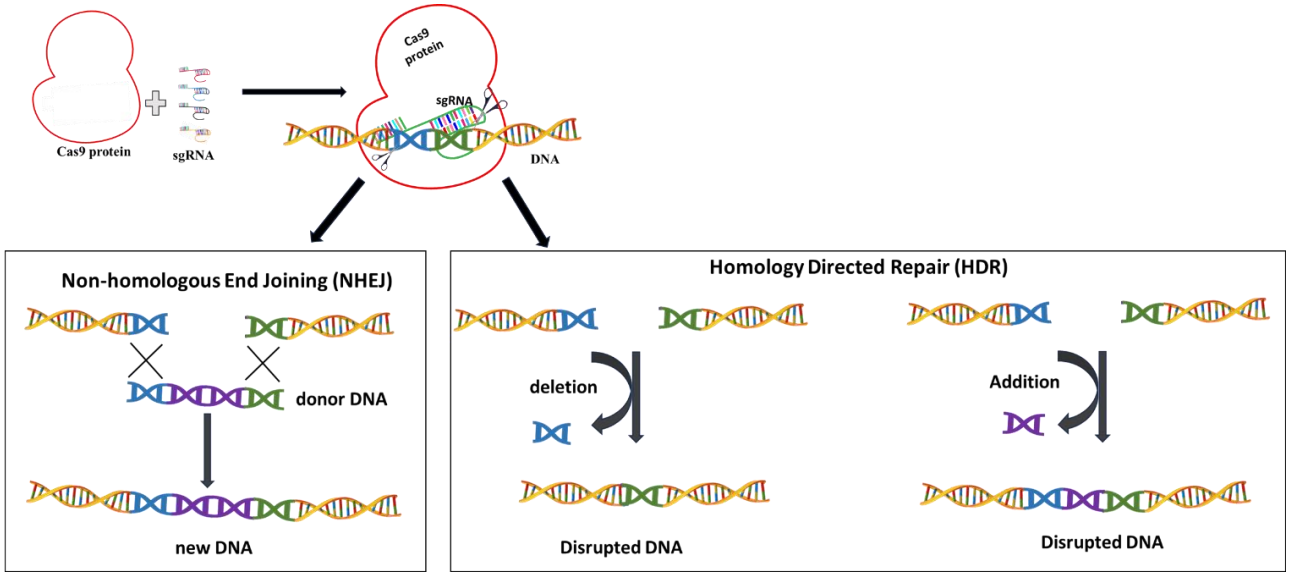

Figure 2. Overview of the CRISPR/Cas9 genome editing mechanism.

Notes: The process involves a single guide (sg) RNA guiding the Cas9 endonuclease to a precise genomic DNA location, inducing a double-strand break. Introduction of donor DNA facilitates the creation of transgenic DNA. In the absence of donor DNA, the host cell repairs the break, potentially leading to an insertion or deletion and perturbation of the gene's open reading frame.

2.1.3. Guided DNA Recognition and Binding

The gRNA in the RNP complex is designed to be complementary to a specific DNA sequence in the target genome. It provides the "address" for the Cas9 protein to find and bind to the DNA. The Cas9 protein, guided by the gRNA, scans the host DNA until it locates a DNA sequence matching the one specified in the gRNA.

2.1.4. DNA Cleavage

Once the Cas9 protein locates the target DNA sequence, it forms a complex with the DNA through base-pairing interactions. The Cas9 protein introduces double-stranded breaks in the target DNA. It does this by creating a cut at a specific site on each DNA strand, resulting in a break. The cell's natural repair machinery attempts to fix the DNA break. This repair process can introduce changes (mutations) in the target gene, allowing researchers to disable, modify, or replace the gene.

2.2. Cellular Repair Mechanisms

There are two primary DNA repair pathways that the cell can employ to fix the Cas9-induced DNA breaks (Figure 2):

(a) Non-Homologous End Joining (NHEJ): This mechanism often results in small insertions or deletions (indels) at the site of the DNA break. These indels can disrupt the reading frame of the gene, leading to a non-functional protein (Chu et al. 2015)

(b) Homology-Directed Repair (HDR): In cases where a template DNA sequence is provided, the cell may use HDR to repair the DNA break. This allows for precise gene editing by inserting, deleting, or modifying specific DNA sequences (Chu et al. 2015, Yang et al. 2020).

Table 2. Tabular representation of some tools for gRNA design (Kulishova et al. 2023, Khan et al. 2018).

Resource/Tool Name	Description	Features	Website/Link	Availability
Benchling gRNA Design	Online platform for designing gRNAs with customizable parameters and target selection.	Customizable gRNA design options- Integration with other molecular biology tools- Off-target prediction	Benchling	Web-based, Registration Required
CHOPCHOP	Web-based tool that provides gRNA design for CRISPR-Cas9 and TALEN-based genome editing.	Multi-species support- Visualizes gRNA target sites- Predicts potential off-target sites	CHOPCHOP	Web-based, No registration required
CRISPR Design	A web tool that assists in designing gRNAs for CRISPR-Cas9 experiments.	Simplified gRNA design process- Predicts off-target effects	CRISPR Design	Web-based, No registration required
E-CRISP	An online tool for designing gRNAs with an emphasis on off-target prediction and minimization.	Focuses on reducing off-target effects- Allows optimization for gRNA specificity	E-CRISP	Web-based, Registration Required
sgRNAcas9	A software tool for the design of single guide RNAs (sgRNAs) for CRISPR-Cas9 gene editing.	Command-line interface for gRNA design- Flexibility for local use	sgRNAcas9	Downloadable, Open-source
Benchling Gene Design	Comprehensive platform for designing gRNAs, primers, and other molecular biology tools.	Integrated design for gRNAs, primers, and more- Visualization of gRNA binding sites	Benchling	Web-based, Registration Required
MIT CRISPR Design Tool	A web-based tool for designing gRNAs and assessing their potential off-target effects.	Efficient gRNA design- Off-target prediction and minimization	MIT CRISPR Design Tool	Web-based, No registration required
CCTop	A CRISPR-Cas9 target online predictor that incorporates user-defined parameters for gRNA design.	Customizable gRNA design- Visualization of gRNA targets and off-targets	CCTop	Web-based, No registration required
CRISPOR	A web tool for gRNA design with options for off-target analysis and ranking of gRNA efficiency.	User-friendly interface- Efficiency and specificity ranking	CRISPOR	Web-based, No registration required
Benchling Inference	Part of Benchling's suite of molecular biology tools, this is for gRNA design and gene knockout predictions.	Integrated gene knockout predictions- Customizable gRNA design	Benchling	Web-based, Registration Required

The specificity of the CRISPR/Cas9 system is determined by the design of the gRNA, as it dictates where the Cas9 protein will bind to the target DNA. This remarkable precision makes CRISPR/Cas9 a powerful tool for genome editing in a wide range of organisms, from bacteria to plants, animals, and even human cells.

3. "Unlocking Genetic Potential: The Power of CRISPRa and CRISPRi"

While CRISPR-Cas9 is widely recognized for its ability to induce gene knockout or modification, there is a powerful duo of CRISPR techniques that has been quietly reshaping the way we explore and manipulate gene expression. These techniques are CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi).

3.1. CRISPR Activation (CRISPRa)

CRISPRa, an exciting addition to the CRISPR toolbox, empowers researchers to enhance gene expression without altering the DNA sequence. It works by guiding a deactivated Cas9 (dCas9) protein to a specific gene's promoter region. This dCas9 is fused with transcriptional activators, which, when brought to the target gene, initiate the transcription process, resulting in increased gene expression (Dai et al. 2021). CRISPRa is a game-changer for studying gene function and has potential applications in therapeutics, biotechnology, and beyond. One of its primary applications lies in gene function studies. By enhancing the expression of specific genes, CRISPRa allows researchers to delve deeper into their roles in development, disease, and other biological processes. In disease modeling, CRISPRa enables scientists to mimic the overexpression of genes associated with certain diseases, providing more accurate disease models for the study of disease mechanisms and potential treatments (Jia et al. 2020). In biotechnology, CRISPRa is harnessed to boost the production of proteins, enzymes, or metabolites, with applications in biofuel production, pharmaceuticals, and industrial enzyme production. Moreover, in agriculture, CRISPRa is used to elevate the expression of genes associated with desirable crop traits, contributing to the development of improved crop varieties. In regenerative medicine, it holds promise for the activation of genes related to tissue regeneration and repair, potentially advancing regenerative therapies (Wagnon 2020). Additionally, CRISPRa offers therapeutic potential by enhancing the expression of missing or defective genes in genetic disorders.

3.2. CRISPR Interference (CRISPRi)

On the flip side, CRISPRi is another dynamic technique that allows for the specific inhibition of gene expression. CRISPRi employs dCas9, this time coupled with transcriptional repressors, to target a gene's promoter region. This prevents RNA polymerase from initiating transcription, effectively silencing the gene. CRISPRi is a valuable tool for investigating gene function and has applications in gene regulation studies and the development of therapeutic interventions. Gene function studies are greatly advanced by CRISPRi, allowing researchers to selectively inhibit the expression of specific genes, providing insights into their roles in biological processes (Jang et al. 2018, Backstrom et al. 2020). Functional genomics benefits from CRISPRi in large-scale studies to identify essential genes, pathways, and interactions. In the realm of therapeutics, CRISPRi can be employed to silence oncogenes in cancer research, presenting a potential avenue for cancer treatment. It plays a crucial role in antibiotic discovery by silencing genes in bacteria, aiding in the identification of antibiotic targets and the development of new antibiotics. In the field of metabolic engineering, CRISPRi fine-tunes the expression of genes involved in metabolic pathways, optimizing the production of bio-based chemicals, biofuels, and other valuable products. Additionally, CRISPRi can explore the functions of non-coding RNAs, shedding light on their roles in gene regulation and disease. It also aids in epigenetic research by investigating the influence of epigenetic modifications on gene expression, providing valuable insights into various biological processes and diseases (Nishiga et al. 2021, Qu et al. 2019). These applications underscore the versatility and transformative potential of CRISPRi in advancing our understanding of genetics, fostering innovation in biotechnology, and offering promising avenues for therapeutic interventions in diverse fields.

4. Classes of CRISPR/Cas9

The CRISPR system can be categorized into two distinct classes: Class I and Class II. These two classes represent different types of CRISPR systems with unique structural and functional characteristics.

(a) Class I CRISPR Systems: Class I CRISPR systems are relatively complex and consist of multiple effector complexes. These complexes are composed of multiple Cas proteins, which work together to perform their functions. Class I systems are typically larger and more diverse, with different subtypes and variations. One notable feature of Class I systems is their ability to target multiple sites in the genome simultaneously (Liu and Doudna 2020). This

class includes subtypes such as Type I, Type III, and Type IV CRISPR systems. Class I systems are known for their complexity and are involved in various functions, including interference with invading genetic elements.

(b) Class II CRISPR Systems: Class II CRISPR systems are characterized by their simplicity and are often associated with a single, large effector protein. The most well-known and widely used CRISPR system, CRISPR-Cas9, falls into the Class II category. In Class II systems, a single protein, such as the Cas9 protein in the case of CRISPR-Cas9, is responsible for both DNA recognition and cleavage (Zhang and Ye 2017, Shmakov et al. 2017). Class II systems are known for their efficiency and ease of use in genome editing applications (Shmakov et al. 2015).

While Class I systems are more diverse and complex, Class II systems like CRISPR-Cas9 have gained immense popularity due to their simplicity and versatility, making them a widely adopted tool for genome editing and gene regulation (Sakuma 2021). Understanding the distinction between Class I and Class II CRISPR systems is crucial for researchers and scientists working with these technologies.

5. Preclinical use of CRISPR

Preclinical use of CRISPR in treating cancer represents a dynamic and promising frontier in cancer research and therapy with a success rate of 5-60% (Yarnall et al. 2023). This innovative technology has opened up new avenues for understanding cancer biology and developing potential treatments. Here are some key aspects of the preclinical use of CRISPR in treating cancer:

(a) Identification of Oncogenic Mutations: CRISPR technology has played a crucial role in identifying and characterizing oncogenic mutations in various cancer types. By selectively targeting and modifying specific genes associated with cancer development, researchers can pinpoint key drivers of the disease. For instance, some of the most commonly identified and well-described tumor suppressor genes include BRCA1, BRCA2, and TP53, which are capable of controlling different stages of the cell cycle (Malkin 1993). This knowledge is invaluable for understanding the underlying mechanisms and potential therapeutic targets.

(b) Gene Editing for Therapeutic Development: Preclinical studies have harnessed CRISPR's gene-editing capabilities to develop potential cancer therapies. By modifying genes responsible for tumor growth, researchers aim to disrupt cancer's ability to proliferate and evade the immune system (Gonzalez et al. 2018, Davis et al. 2018). CRISPR offers a precise and customizable tool for altering gene expression in cancer cells.

(c) Functional Validation of Genes: CRISPR technology serves as a powerful tool for in-depth investigations into the functional significance of individual genes in cancer. By precisely knocking down or modifying genes in preclinical models, researchers can reveal the pivotal roles of specific genes in processes such as tumor growth, metastasis, and drug resistance. These findings not only expand our understanding of cancer biology but also lay the foundation for the development of targeted therapies.

One notable success in lung cancer research achieved through CRISPR technology involves the targeting of mutated versions of the EGFR gene (Agrawal et al. 2021a, Kumar et al. , Kerr et al. 2019, Cheung et al. 2018). CRISPR has effectively reduced cell proliferation in both in vitro and in vivo settings, resulting in cancer cell death and a noticeable reduction in tumor size. Additionally, CRISPR has played a crucial role in blocking the tumor suppressor phosphatase and homologous tensin (PTEN) in non-small cell lung cancer (NSCLC), promoting cancer growth through the Akt pathway (Elumalai et al. 2019). In colorectal cancer, especially in cases associated with KRAS or BRAF mutations, preclinical studies employing genome-wide CRISPR screening have identified novel pathways that offer potential targets for future clinical applications (Yau et al. 2017).

Beyond functional validation, gene knockout represents one of the simplest yet highly effective approaches offered by CRISPR technology, with immense potential in clinical trials, particularly for pathogenic genes. The ability of Cas9 to programmatically cut various DNA sites in vitro has opened doors to a range of applications. For instance, gene editing of CCR5 in CD4+ T cells from individuals infected with HIV has demonstrated promise in combating

HIV infection (Tebas et al. 2014). Similarly, the disruption of the intronic erythroid-specific enhancer for the BCL11A gene has shown potential in increasing HbF protein expression, holding promise for addressing conditions like sickle-cell anemia (SCD) and β-thalassemia (Psatha et al. 2018). These advances underscore the versatility and clinical significance of CRISPR technology in precision medicine and the development of novel therapies.

(d) Immunotherapy Advancements: One of the most thrilling applications of CRISPR technology in cancer preclinical studies revolves around the advancement of immunotherapy approaches. A prime example of this is how CRISPR is employed to enhance the effectiveness of CAR-T cells, addressing a significant hurdle - T cell exhaustion, which not only hampers endogenous antitumor responses but also substantially limits the efficacy of CAR-T cells, particularly within immunosuppressive microenvironments, as highlighted by Fraietta et al. In their work on patients with relapsed/refractory chronic lymphocytic leukemia (CLL) treated with CD19+ chimeric antigen receptor T (CAR-T) cells, the presence or absence of T cell exhaustion signatures at the apheresis stage was found to be a pivotal determinant of clinical outcomes (Fraietta et al. 2021). Utilizing CRISPR/Cas9, it becomes possible to eliminate the negative regulators of T cell function and persistence in CAR-T cells, thus holding theoretical potential for significantly improving clinical responses. This exemplifies how CRISPR is revolutionizing the landscape of immunotherapy in cancer.

(e) Drug Resistance Studies: CRISPR-based preclinical studies are essential for understanding drug resistance mechanisms in cancer. By genetically modifying cancer cells to mimic drug resistance, researchers can explore ways to overcome this challenge and develop more effective treatment strategies.

(f) Personalized Cancer Therapies: CRISPR technology has opened the door to highly personalized cancer therapies, which can be tailored to an individual's unique genetic profile. Preclinical studies utilizing patient-derived samples have paved the way for the development of customized treatments that optimize therapeutic outcomes. Since 2018, researchers have harnessed CRISPR/Cas9 technology to identify potential individualized treatment approaches based on specific cancer subtypes. This approach holds enormous promise for addressing various cancer types. For instance, Ebright et al. employed CRISPR/Cas9 as a powerful genome screening tool to pinpoint genes responsible for metastases, such as the overexpression of RPL15, a component of the large ribosomal subunit implicated in breast cancer metastasis (Ebright et al. 2020). Other research endeavors have explored the potential of CRISPR/Cas9 to disrupt the FASN gene, linked to estrogen receptor signaling, effectively reducing the proliferation and migration of breast cancer cells. Furthermore, while triple negative breast cancer (TNBC) poses a unique challenge, given its aggressive nature, it's worth noting that CRISPR/Cas applications could potentially target the poly (ADP-ribose) polymerase 1 (PARP1) gene (Faraoni and Graziani 2018). This gene plays a crucial role in synthetic lethality, particularly in BRCA1-deficient cells. These developments represent a significant step towards the future application of CRISPR/Cas9 technology in devising tailored treatments for a range of breast cancer subtypes.

(g) Exploration of Novel Targets: CRISPR screens in preclinical models have led to the discovery of novel targets for cancer therapy. These innovative findings expand the range of potential interventions and therapies in the fight against cancer.

(h) Leveraging Animal Tumor Models: Animal tumor models have historically served as foundational tools in cancer research, offering insights into the molecular mechanisms of tumorigenesis and cancer development. These models bridge the gap between fundamental science and clinical cancer research, underpinning many cancer investigations. They are indispensable for drug development and therapeutic strategies, providing an in vivo platform to test novel treatments and therapies. Animal tumor models can be classified into four categories: carcinogen-induced models (CIMs), spontaneous and induced models, genetically engineered models, and transplant models (Onaciu et al. 2020). Genetically engineered models, particularly gene knockout animal tumor models, have become increasingly significant for understanding the role of specific genes in tumorigenesis.

(i) CRISPR-Enabled Animal Tumor Models: Traditional methods of establishing transgenic animal models often relied on embryonic stem cells (ESCs) and homology-directed repair (HDR) techniques, which were time-consuming, typically taking at least a year to create a model. In stark contrast, CRISPR/Cas9 technology has revolutionized this process, offering advantages in terms of cost, efficiency, and time savings. Researchers have leveraged CRISPR to create multiple animal tumor models across various species, including rats, goats, rabbits, dogs, monkeys, pigs, C. elegans, and zebrafish (Jin and Li 2016). The introduction of CRISPR technology has offered three distinct methods for creating these models:

- Editing Genes in Embryos: This involves obtaining zygotes, delivering sgRNA and Cas9 mRNA into the zygote, and subsequently transferring the edited embryos into animals to produce the desired generation.
- Editing Haploid Embryonic Stem Cells (ESCs): The use of haploid ESCs simplifies the production of homozygous mutants for transgenic model generation.
- Gene Editing in Spermatogonia Stem Cells (SSCs): This method allows for autologous transplantation into pseudo-pregnant animals, maintaining the paternal imprinting pattern while holding promise for therapeutic applications.

Compared to traditional embryo microinjections, which can be time-consuming and require specialized skills, CRISPR technology presents a more efficient and versatile alternative. Notably, editing in ESCs provides a high-efficiency platform capable of generating multiple knockouts and large deletions. In the context of animal research, CRISPR/Cas9-mediated genome editing accelerates the development of transgenic animals.

Expanding Beyond Animals and into Insects: CRISPR/Cas9 technology's applications have extended beyond animals to include insects. The recent use of "direct parental" CRISPR (DIPA-CRISPR) in cockroaches and Tribolium castaneum is a notable breakthrough, expanding the horizons of gene editing in these unique organisms (Shirai et al. 2022). This achievement underscores the immense potential of CRISPR technology to enhance comprehensive cancer research and shape the future of cancer therapeutics.

The use of CRISPR-Cas9 involves the delivery of the Cas9 protein along with guide RNA (gRNA) molecules into cells to induce targeted modifications in the genome (Agrawal, Padmaswari and Nelson 2022). Several vectors and delivery methods have been employed for this purpose in preclinical studies. Commonly used vectors for delivering CRISPR-Cas9 components include:

- Plasmid Vectors: Cas9 and gRNA sequences can be incorporated into plasmid vectors, which are then transfected into target cells. Plasmids offer simplicity and ease of use in various cell types.
- Viral Vectors: Viral vectors, such as lentivirus or adeno-associated virus (AAV), have been extensively used for delivering CRISPR-Cas9 components. Viral vectors enable efficient delivery into a wide range of cells, including non-dividing cells (Agrawal, Padmaswari and Nelson 2022).
- Ribonucleoprotein (RNP) Delivery: Direct delivery of Cas9 protein and gRNA as an RNP complex is another strategy. This method allows for transient Cas9 expression, minimizing off-target effects and potential immune responses (Agrawal, Bryan and Nelson 2023).
- Electroporation and Lipid Nanoparticles: Physical methods, such as electroporation, and chemical methods, like lipid nanoparticles, have been used for direct delivery of CRISPR-Cas9 components into cells (Agrawal, Bryan and Nelson 2023) (Figure 3).

In summary, the preclinical use of CRISPR in treating cancer is a rapidly evolving field that holds tremendous promise for advancing our understanding of cancer biology and developing more effective therapies. By harnessing the precision and versatility of CRISPR technology, researchers are making significant strides in the quest to combat this complex disease.

6. CRISPR use for Clinical Cancer Treatment

CRISPR/Cas9 technology has emerged as a groundbreaking frontier in clinical trials, offering new hope in the treatment of previously incurable diseases, with a notable focus on cancer. This technology, which was recognized as the "breakthrough of the year" in 2012, 2013, and 2015, has been harnessed to advance medical science, particularly in the realm of cancer therapy. Importantly, CRISPR/Cas9 has progressed to phase I clinical trials, demonstrating its immense potential in oncology (Agrawal et al. 2021) (Table 3).

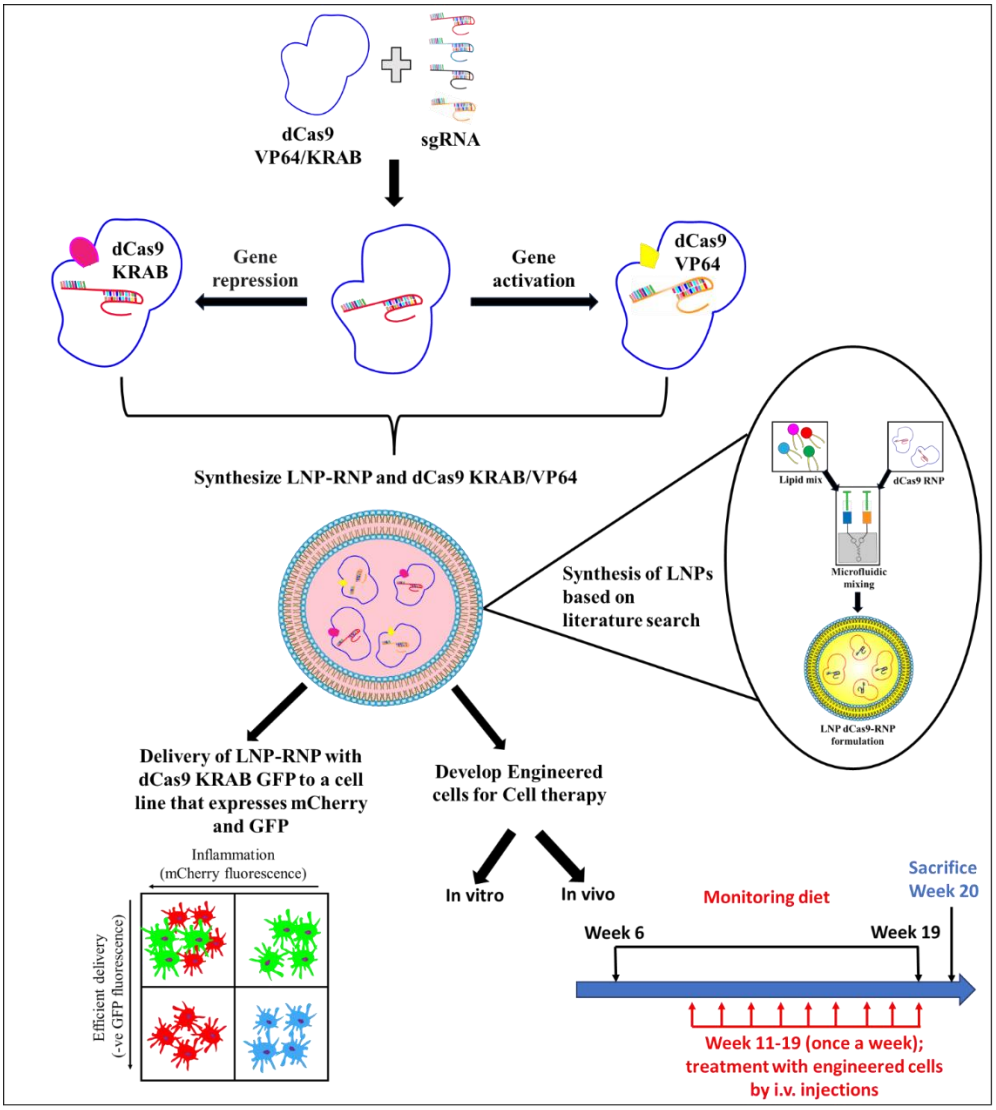

Figure 3. Schematic representation of the research workflow encompassing the in vitro and in vivo CRISPR screening.

Notes: Beginning with the synthesis of dCas9 RNPs through gRNA transcription and Cas9 protein complexation, the approach advances to crafting LNPs with reduced immunogenicity by leveraging literature and computational models. The workflow extends to encompass in vitro assays probing LNPs and RNPs functionality and progresses to in vivo assays gauging their impact within mice.

The initial phase I trial, conducted by Stadtmauer et al., assessed the safety and feasibility of CRISPR-Cas9 for engineering T cells. The study enrolled three patients with refractory cancers and utilized CRISPR/Cas9 to delete two genes responsible for endogenous T cell receptor (TCR) genes, thereby reducing TCR mispairing. Additionally,

a gene encoding programmed cell death protein 1 (PD-1) was removed to enhance antitumor immunity (Stadtmauer et al. 2020). The outcome was promising, with all three T cell transfers proving successful and persisting for up to nine months, highlighting the potential of CRISPR/Cas9 for gene editing in immunotherapies.

Table 3. CRISPR in clinical practice.

Trial Phase	Cancer Type	Targeted Genes	CRISPR Approach	Status	Main Objectives
Phase 1/2/3 NCT03655678	β-thalassemia	BCL11A	CRISPR/Cas9	Ongoing	The study will evaluate the safety and efficacy of autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (hHSPCs) using CTX001
Phase 1/2 NCT04035434	Non-Hodgkin lymphoma	N/A	Ex-vivo genome editing	Ongoing	This study is evaluating the safety and efficacy of CTX110 in subjects with relapsed or refractory B-cell malignancies
Phase 1 NCT04244656	Multiple Myeloma	N/A	Ex-vivo genome editing	Ongoing	This study is evaluating the safety and efficacy of CTX120 in subjects with relapsed or refractory multiple cell myeloma
Phase 1 NCT04037566	Haematopoietic Malignancies	CD19, HPK1	Downregulate gene expression	Ongoing	This study is to test CD19-specific CAR-T cells with edited endogenous HPK1 (XYF19 CAR-T cells) in patients with relapsed or refractory CD19+ leukemia or lymphoma
Phase 1 NCT00842634	HIV	CCR5	ZFN	Completed	This study is carried out to study a new way to treat HIV using Zinc finger nuclease
Phase 1/2/3 NCT03745287	Sickle cell disease	BCL11A	CRISPR-Cas9	Ongoing	The study will evaluate the safety and efficacy of autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (hHSPCs) using CTX001

Within the context of lung cancer, two separate phase I trials have demonstrated the safety and efficacy of CRISPR/Cas9 T cell editing. In one trial led by Lu et al., 22 patients with advanced non-small cell lung cancer (NSCLC) participated, and 12 of them received T cells edited with CRISPR/Cas9 to target PD-1 (Table 3). The edited T cells remained detectable in peripheral blood post-infusion, and no severe adverse events were observed. The median progression-free survival was 7.7 weeks, and overall survival reached 42.6 weeks. This study's use of next-generation sequencing to assess off-target events showed a median mutation frequency of 0.05%, underscoring the safety and feasibility of CRISPR/Cas9-edited T cells (Lu et al. 2020). Most recently, Wang et al. recruited 15 patients with mesothelin-positive solid tumors and used CRISPR/Cas9 to create PD-1 and TCR-deficient CAR-T cells specific to mesothelin, evaluating their responses to dose escalation. Encouragingly, two patients achieved stable disease, with circulating edited T cells peaking at days 7-14 before becoming undetectable after one month, all without severe adverse effects or toxicities (Wang et al. 2021).

Tumor evolution is a complex process involving various genetic mutations, including proto-oncogenes and tumor-suppressor genes. The advent of genome-sequencing technology has led to the identification of numerous mutated genes associated with cancer. CRISPR/Cas9's efficient and precise gene editing capabilities provide the opportunity to directly target the mutated genes responsible for cancer development. For instance, the epidermal growth factor receptor (EGFR) gene is mutated in approximately 10%~15% of NSCLC cases, playing a critical role

in tumor progression. While EGFR inhibitors are the standard treatment for EGFR-mutated lung cancer, they face limitations in terms of drug resistance and efficacy. CRISPR/Cas9 gene editing technology shows promise in correcting cancer-driven mutations in EGFR-mutated NSCLC. Experiments have demonstrated that knocking out the EGFR mutant allele (L858R) in lung cells resulted in cancer cell death and reduced tumor volume (Cheung et al. 2018).

Cervical cancer is closely linked to the human papilloma virus (HPV). By targeting E6 and E7 oncogenes using CRISPR/Cas9, tumor growth can be suppressed. Additionally, CRISPR/Cas9 technology has shown potential in targeting specific genes involved in malignancies. For example, the nuclear receptor binding SET domain-containing protein 1 (NSD1) is associated with various malignancies, including human hepatocellular carcinoma (HCC) (Zhang et al. 2019). Knocking down the NSD1 gene in HCC cells has been found to suppress cell proliferation, migration, and invasion.

Ultimately, the CRISPR/Cas9 system has the potential to provide individualized targeted therapy, offering exciting possibilities for cancer therapy at the genetic level. The success of CRISPR-based gene editing therapy in other genetic diseases, such as Leber's congenital amaurosis type 10 (LCA10), where vision was successfully restored, demonstrates the feasibility of this approach in treating cancer and other genetic diseases (Chen et al. 2022).

In addition to these developments, Liao et al. demonstrated that PD-L1 is a viable target for knockout by CRISPR/Cas9 in patients with osteosarcoma. These successful results represent significant strides in establishing the safety and efficacy of CRISPR/Cas9 in treating conditions like NSCLC and sarcoma, with implications for other malignancies given the critical role of the PD-1/PD-L1 axis in cancer immunotherapy (Liao et al. 2017).

The groundwork laid by preclinical studies, such as those by Inturi and Jemth, has paved the way for clinical trials focused on the efficacy and safety of CRISPR/Cas9 in targeting HPV E6/E7 for the treatment of persistent HPV and HPV-related cervical intraepithelial neoplasia I (Kanter et al. 2022, Stefanoudakis et al. 2023). In this particular trial, Cas9 and guide RNAs are encoded on a plasmid, which is then delivered to cervical epithelial cells via a topical gel applied locally to the HPV-infected cervix.

Most recently, Foy et al. developed an approach using CRISPR/Cas9 technology to knockout two T cell receptor genes. This approach was employed to treat 16 patients with various refractory solid cancers in a phase I trial. Each patient received up to three edited TCR products as part of a dose-escalation clinical trial, with only two patients experiencing cytokine release syndrome or neurotoxicity (Foy et al. 2023). Encouragingly, five patients achieved stable disease, thereby demonstrating the feasibility of isolating endogenous T cell receptors and employing simultaneous knock-out and knock-in technology with CRISPR/Cas9.

As the potential of CRISPR/Cas9 in cancer treatment becomes increasingly evident, numerous phase I and II clinical trials are ongoing to explore its utility further. Addressing a long-standing challenge in T cell therapy, several trials are now employing T cells donated by healthy individuals and modified to express CRISPR/Cas9-engineered CAR cells. In addition, other phase I trials are evaluating autologous T cells engineered to target CD19 and employing CRISPR gene editing to eliminate endogenous HPK1 in CD19+ leukemia or lymphoma (XYF19 CAR-T cells) (NCT04037566).

The unique capabilities of CRISPR/Cas9 enable site-specific, consistent integration, mitigating the risk of heterogenic transgene expression commonly seen in retro- or lentiviral transduction. Several clinical trials are currently underway, targeting specific antigens and genetic modifications for cancer therapy. Examples include CD19-targeted CTX110 and CTX112 for relapsed or refractory B-cell malignancies, BCMA-targeted CTX120 for relapsed or refractory multiple myeloma, and CD70-targeted CTX130 for advanced, relapsed, or refractory renal cell carcinoma. Moreover, various clinical trials encompass phase I trials for PD-1 targets in EBV-associated malignancies and phase II trials in leukemia, lymphoma, and esophageal cancer, all contributing to the diverse range

of studies reflecting the remarkable potential of CRISPR/Cas9 technology in advancing cancer treatments (Sorkhabi et al. 2023). The fusion of CRISPR/Cas9 technology and oncology heralds a new era of precision medicine and innovative therapies for cancer patients. While challenges remain, the promise of precise gene editing in cancer treatment is a significant leap forward in our ongoing battle against this complex disease.

Future challenges: As for next-generation CRISPR technologies, researchers are actively working on improving the precision, efficiency, and safety of genome editing (Dai et al. 2021). Some advancements include:

- Base Editing: Base editors enable the conversion of one DNA base pair into another without causing double-strand breaks, reducing the risk of unintended mutations.
- Prime Editing: Prime editing is a highly precise method that allows for the introduction of specific edits without the need for donor DNA templates. This technique offers enhanced accuracy and reduces the potential for off-target effects (Wagnon 2020).
- CRISPR Interference (CRISPRi) and CRISPR Activation (CRISPRa): These technologies enable the modulation of gene expression without inducing permanent changes to the genome. CRISPRi suppresses gene expression, while CRISPRa enhances it (Dai et al. 2021).
- Epigenome Editing: CRISPR-based tools for targeted epigenome modification are being developed. These technologies aim to regulate gene expression without altering the underlying DNA sequence.

Researchers are continually working to refine CRISPR technologies for more precise and controlled genome editing applications, both in preclinical research and potential therapeutic interventions (Wagnon 2020).

7. Navigating CRISPR Challenges: Paving the Way for Safe and Effective Cancer Therapy

As CRISPR/Cas-based genome editing strategies advance in efficiency and specificity, holding promise for revolutionary strides in cancer prevention and treatment, there are critical considerations hindering its seamless integration into clinical practice. The paramount concern lies in unraveling the long-term safety implications of in vivo CRISPR use, which profoundly influences its viability for primary cancer prevention or therapeutic intervention. The foremost limitation stems from the potential off-target activities, harboring the risk of unintended mutations and posing a substantial threat to the recipients. Despite evidence showcasing the rarity of off-target effects, it remains a significant impediment to the widespread application of CRISPR technology. Beyond off-target concerns, the landscape of CRISPR applications faces challenges related to immunogenic toxicity. This arises from pre-existing antibodies against commonly used bacterial nucleases, introducing an additional layer of caution in therapeutic implementation. Ethical considerations also loom large, especially concerning germ-line gene editing studies, raising important societal and moral questions. Another facet to ponder is the economic viability of manufacturing and delivering CRISPR/Cas9-based therapies, potentially impacting accessibility on a broader scale. The application of CRISPR-based technologies in cancer treatment also raises several ethical concerns due to the profound impact it can have on the human genome. The ethical implications of germline editing, where changes are heritable, need careful consideration. Many ethical guidelines currently discourage germline editing due to unknown long-term consequences and the potential for unintended effects on future generations. Ultimately, the availability of these cutting-edge therapies outside academic settings may prove pivotal in determining the practicality of CRISPR technology as a therapeutic option.

8. Conclusions

In summary, CRISPR/Cas9 emerges as a groundbreaking genome editing tool, firmly establishing its efficacy in preclinical settings for cancer treatment and various other diseases. Ongoing Phase 1 and 2 trials mark its transition to clinical applications, signaling a new frontier in immune oncology and beyond. While formidable challenges

persist, the evolving landscape of CRISPR heralds an exciting era with the potential to reshape the paradigms of cancer therapy, laying the groundwork for transformative advancements in the realm of medicine.

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Conflict of interest

All the authors claim that the manuscript is completely original. The authors also declare no conflict of interest.

Author contributions

Conceptualization, S.A.; Writing-original draft preparation, S.A, S.S.; Writing-review and editing, S.A. All authors have read and agreed to the published version of the manuscript.

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