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CRISPR-Cas in the clinic: The current state-of-the-art and what holds its clinical application back

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Abstract

CRISPR-Cas represents a rapidly evolving and highly promising tool for precise genome editing and the modulation of gene expression. There are various CRISPR-Cas systems which operate through a fundamentally similar mechanism but possess unique characteristics. Currently, the CRISPR-Cas technology is extensively implemented in plant and animal science as well as in biomedical research to establish animal models of human diseases. Nevertheless, this technique holds significant potential for offering novel therapeutic interventions for treating and curing a wide range of diseases in the future. This essay examines the current clinical applications of CRISPR-Cas, particularly its potential to treat monogenic blood disorders and specific cancers, and its role in the early detection of pathogens and in combating antibiotic resistance in bacteria. Once challenges such as off-target effects, immunogenicity, and ethical considerations are addressed, CRISPR-Cas holds the potential to be implemented as a revolutionary intervention in modern healthcare.

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Introduction

In healthcare, there is a continuous search for innovative treatments that can improve the efficacy of existing therapies and provide interventions for diseases that remain difficult or even impossible to treat. Within this context, the CRISPR-Cas technology has emerged as a highly promising tool, enabling precise genomic modifications and the modulation of gene expression levels for therapeutic applications, which has generated widespread interest among scientists and medical professionals. This innovative technique is now being studied in preclinical studies and clinical trials to assess its potential to treat and cure a wide range of diseases (Mahara et al., 2023).

Discovery of CRISPR-Cas in prokaryotes

In 1987, researchers discovered clustered regularly interspaced short palindromic repeats (CRISPR) in the genome of *Escherichia coli* and several years later, CRISPR loci were also found in archaea and other microorganisms (Ishino et al., 1987; Ishino et al., 2018). These loci, together with DNA repair proteins, known as Cas proteins, form the acquired immunity system protecting prokaryotes from bacteriophages and viral infections (Gostimskaya, 2022; Ishino et al., 1987). Although various CRISPR-Cas systems exist, they operate through a fundamentally similar mechanism involving three stages: adaptation, crRNA processing, and interference (Garneau et al., 2010; Makarova & Koonin, 2015). During adaptation, Cas proteins facilitate the site-specific insertion of a specific DNA fragment from intruding DNA into the CRISPR locus. The CRISPR locus is transcribed and processed into mature CRISPR RNA (crRNA) during crRNA processing (Makarova & Koonin, 2015; Newsom et al., 2020; Shabbir et al., 2016). In the final stage, the CRISPR-Cas system performs sequence-specific targeting and cleavage of reintroduced, foreign DNA (Newsom et al., 2020; Shabbir et al., 2016).

CRISPR-Cas9 for targeted genome modifications

Once scientists understood the function and working mechanism of the CRISPR-Cas system, they identified its potential for targeted DNA cleavage at specific genomic locations (D. Zhang et al., 2021). Class II CRISPR-Cas systems are especially attractive because of their simple effector complex structure, which consists of a single Cas protein. In contrast, class I CRISPR-Cas systems rely on multi-protein effector complexes (Ishino et al., 2018).

CRISPR-Cas9, classified as a class II system, is among the most extensively studied and applied CRISPR-Cas systems in scientific research (Xu & Li, 2020). The CRISPR-Cas9 system requires a guide RNA (gRNA), CRISPR-associated protein 9 (Cas9), and protospacer adjacent motif (PAM) sequence. The gRNA comprises a crRNA segment, an 18-20 nucleotide sequence complementary to the target DNA, and a trans-activating CRISPR RNA (tracrRNA), which functions as a binding scaffold for Cas9 (Asmamaw & Zawdie, 2021). The Cas9 effector protein contains two nuclease domains, HNH and RuvC, each responsible for cleaving a single strand of the double-stranded target DNA, resulting in a double-stranded break (DSB). Together, the gRNA and Cas9 protein form a ribonucleoprotein (RNP) complex, wherein the gRNA directs the complex to the target DNA through complementary base pairing, and Cas9 mediates the DSB (Xu & Li, 2020). Importantly, target recognition and effective cleavage requires a PAM sequence located three nucleotides downstream of the cleavage site. In the absence of this PAM sequence,

Cas9 is unable to bind the target DNA, thereby inhibiting its nuclease activity (Collias & Beisel, 2021; Shabbir et al., 2016).

In complex prokaryotic and eukaryotic organisms, the occurrence of a DSB triggers endogenous DNA repair pathways (Liu et al., 2021). By controlling and manipulating the specific repair pathways, targeted genome modifications such as substitutions, insertions, and deletions can be achieved (Xue & Greene, 2021). The two primary pathways for DSB repair are homology-directed repair (HDR) and non-homologous end joining (NHEJ). HDR, a highly accurate repair mechanism, uses a homologous donor template to repair the DSB. Combining CRISPR-Cas9 with HDR, allows for precise genomic modifications or insertions by providing a donor DNA template with sequence homology at the DSB site (Asmamaw & Zawdie, 2021, Redman et al., 2016). Conversely, NHEJ repairs DSBs by directly ligating DNA ends together without the need for a donor template, typically resulting in insertions or deletions (indels). These indels often lead to frameshift mutations which disrupts the reading frame of the coding sequence, generally introducing a premature stop codon producing truncated proteins that are typically non-functional, thereby resulting in a gene knockout (Lalonde et al., 2017; Stinson & Loparo, 2021). Notably, cells do not deliberately select between HDR and NHEJ. HDR predominantly occurs during the S and G2 phases of the cell cycle, while NHEJ is more active during the G1 phase (Yang et al., 2020). A schematic overview of the operational mechanism of CRISPR-Cas9 and its associated repair pathways is presented in Figure 1.

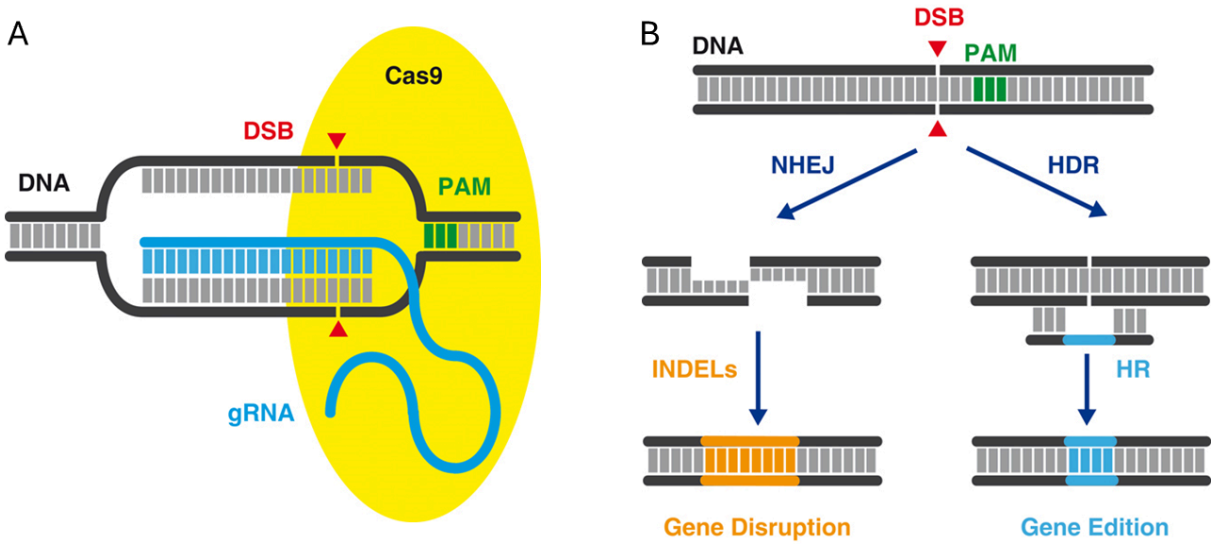

Figure 1: Schematic representation of the operational mechanism of CRISPR-Cas9 (A) and its associated repair pathways (B) (Montoliu, n.d.).

Prime- and base-editing

As previously discussed, HDR facilitates precise genomic modifications or gene insertions by introducing a corrective donor template (Asmamaw & Zawdie, 2021; Redman et al., 2016). Nonetheless, mammalian cells generally prefer NHEJ over HDR as NHEJ is more active throughout the cell cycle and operates faster. Consequently, the efficacy of genetic modifications or gene insertions is constrained by the predominance of NHEJ over HDR (Yang et al., 2020). Prime-editing, a novel CRISPR-Cas9 technique, offers a promising solution to this limitation (Ceglie et al., 2023). This technique allows for all 12 possible base-to-base conversions without relying on HDR, thereby enabling the correction of up to

89% of human monogenic diseases. Prime-editing involves the use of a prime-editing guide RNA (pegRNA) and a nickase Cas9 (nCas9) combined with reverse transcriptase. This pegRNA includes both the gRNA sequence and the corrective donor template for genetic modification. nCas9, unlike Cas9, has only one active nuclease domain, introducing solely single-stranded DNA breaks, following recognition and binding of the pegRNA. Reverse transcriptase then uses the pegRNA template to synthesize a new DNA strand, which is subsequently integrated into the genome (Zhao et al., 2023; W. Zhang et al., 2023).

Base-editing, like prime-editing, is a precise genome editing technology derived from the CRISPR-Cas9 system. In base-editing, nCas9 is fused with either a cytosine or adenine deaminase, referred to as base-editors. Cytosine deaminase facilitates the conversion of a cytosine to a thymine base, whereas adenine deaminase catalyzes the conversion of an adenine to a guanine base (Rees & Liu, 2018). The gRNA, similar to that used for CRISPR-Cas9, ensures targeted delivery of nCas9 and the base-editor to the specific mutation site, where the base-editor facilitates a transition (a purine-to-purine or pyrimidine-to-pyrimidine substitution) (Hiramoto et al., 2023). Importantly, while base-editing is limited to transition corrections, prime-editing enables both transition and transversion mutations, as well as small insertions (Kantor et al., 2020).

CRISPR activation and CRISPR interference

The CRISPR-Cas technology extends beyond genetic modification and is increasingly used to regulate endogenous gene expression. This is achieved by fusing transcriptional activators or repressors to a catalytically inactive Cas9 (dCas9), which lacks the ability to induce DNA breaks. In CRISPR activation (CRISPRa), dCas9 is fused to an activator domain and guided by the gRNA to the promoter or enhancer region of a gene to upregulate its expression (Bendixen et al., 2023; Heidersbach et al., 2023). Conversely, in CRISPR interference (CRISPRi), dCas9 is fused to a repressor domain, which, when directed to the promoter or enhancer region, suppresses gene expression (Bendixen et al., 2023). In both CRISPRa and CRISPRi, the nuclease-inactive dCas9 does not induce DNA breaks, thereby preventing permanent genomic modifications (Heidersbach et al., 2023).

CRISPR-Cas technology also enables the regulation of gene expression through targeted epigenetic modifications by fusing dCas9 with epigenetic modifiers. Examples of such epigenetic modifiers include histone acetyltransferases, which facilitate histone acetylation, and DNA demethylases, which remove methyl groups from cytosine residues (Pulecio et al., 2017).

Applications of the CRISPR-Cas technology

Nowadays, the CRISPR-Cas technology is extensively implemented in plant and animal science as well as in medical research to establish animal models of human diseases (Tavakoli et al., 2021; Xu & Li, 2020). In the field of plant biotechnology, CRISPR-Cas facilitates the genetic modification of plants to enhance their resistance to pathogens and environmental stressors, such as drought and frost, thereby aiming to increase crop yield. Similarly, in animal science, CRISPR-Cas is applied to genetically modify livestock to optimize meat production (Tavakoli et al., 2021). Furthermore, CRISPR-Cas has demonstrated exceptional efficacy in generating animal models of human diseases, which

are essential for understanding disease pathology and developing novel therapeutic interventions (Xu & Li, 2020).

Moreover, the applications of the CRISPR-Cas technology extended further as CRISPR-Cas is now being used for therapeutic interventions for genetic disorders and infectious diseases, as well as the innovation and enhancement of cancer therapies (Kang et al., 2022). Given the rapidly expanding use of CRISPR-Cas, particularly in therapeutic contexts, it is imperative to evaluate its current clinical implementations which raises the question: What is the current state-of-the-art of the CRISPR-Cas technology in clinical applications, and what are the major challenges and ethical issues that could impede its large-scale integration into healthcare?

As previously mentioned, various CRISPR-Cas systems exist that operate through a fundamentally similar mechanism while also having their unique functional characteristics (Garneau et al., 2010; Makarova & Koonin, 2015). This study addresses several therapeutic strategies utilizing various CRISPR-Cas approaches according to three pathologies: blood disorders, cancer, and pathogenic microorganisms (Morshedzadeh et al., 2024). These pathologies were selected due to their significant impact on overall patient well-being and the limited availability of effective therapeutic approaches (Bray et al., 2024; Inusa et al., 2019; Morshedzadeh et al., 2024). Current therapeutic approaches for blood disorders and cancer primarily focus on symptom alleviation and disease management, whereas the advent of the CRISPR-Cas technology offers the potential for curing these diseases (Bell et al., 2024; Rabaan et al., 2023). Furthermore, infectious diseases significantly contribute to the global mortality rates. The future application of CRISPR-Cas could provide novel approaches for managing infectious diseases, including their early detection and addressing challenges related to antibiotic resistance (Morshedzadeh et al., 2024).

Blood disorders

Monogenic blood disorders such as sickle cell disease, β-thalassemia, and hemophilia B continue to be challenging to treat with the available medical approaches. Recent developments in the CRISPR-Cas technology offer novel therapeutic avenues for these conditions by enabling targeted genetic modifications (Bell et al., 2024; Hu et al., 2024; Soroka et al., 2023). This chapter addresses several applications of CRISPR-Cas and its variants in potentially curing these blood disorders.

Sickle cell disease and β-thalassemia

Sickle cell disease (SCD) is classified as a hemoglobinopathy, a group of inherited genetic disorders characterized by either the production of abnormal hemoglobin or insufficient synthesis of hemoglobin chains (Feroze & Azevedo, 2024). The disease results from a point mutation in the β-globin gene, which replaces glutamic acid (a hydrophilic residue) with valine (a hydrophobic residue) at the sixth position of the β-globin chain and changing its protein structure. This causes the production of abnormal hemoglobin, hemoglobin S (HbS) instead of functional hemoglobin A (HbA). Consequently, the aggregation of HbS molecules results in the formation of sickle-shaped red blood cells (RBCs) (Elendu et al., 2023; Inusa et al., 2019). Patients with SCD typically experience pain, anemia, acute aplastic crisis (a condition in which the bone marrow fails to produce RBCs), and splenic sequestration crisis

(a significant decrease in circulating blood volume due to obstructed blood vessels), among other related complications (Conrad et al., 1988; Inusa et al., 2019; Kane et al., 2024). Current therapeutic approaches predominantly focus on disease management rather than curing it. Available treatments include blood transfusions, hydroxyurea (a chemotherapy drug that increases the production of HbA), and inhibitors of HbS polymerization. However, curing the disease requires correcting the underlying genetic mutation, as without such correction, the body will continue to produce the defective sickle-shaped RBCs (Bell et al., 2024). Future applications of the CRISPR-Cas technology holds promise as a potentially effective treatment for SCD, offering not only symptomatic relief but also the potential for a cure through two distinct approaches, both relying on the CRISPR-Cas9 system: (I) induction of fetal hemoglobin and (II) correction of the SCD mutation (Demirci et al., 2019; Park & Bao, 2021).

The induction of fetal hemoglobin (HbF) expression in patients with SCD offers a promising therapeutic approach as studies show that the presence of HbF inhibits the polymerization of HbS molecules, thereby preventing the formation of sickle-shaped RBSs (Demirci et al., 2019; Demirci et al., 2021; Park & Bao, 2021). As HbF is naturally produced in infants up until six months, babies are naturally protected from the complications associated with SCD. Around six months postnatally, HbF expression is markedly repressed by various transcription factors, with BCL11A identified as a key repressor (Demirci et al., 2019). Through the application of the CRISPR-Cas9 technology, genetic modifications can be introduced into the erythroid-specific enhancer of the BCL11A gene in hematopoietic stem and progenitor cells (HSPC), effectively downregulating BCL11A expression in erythroid lineage cells and thereby enhancing HbF production (Demirci et al., 2019; Frangoul et al., 2021; Park & Bao, 2021). In order to downregulate BCL11A, patient-derived HSPCs need to be genetically modified in vitro. A gRNA conjugated with Cas9, referred to as SPY101, is delivered as a RNP complex into the HSPCs via electroporation. The RNP complex then translocates to the nucleus, where the gRNA binds to the target DNA, and Cas9 induces a DSB in the erythroid-specific enhancer region. Repair of the DSB by NHEJ results in indels at the enhancer site, leading to a frameshift that subsequently reduces BCL11A expression. Consequently, HbF levels increase, which inhibits the polymerization of HbS molecules. The genetically modified HSPCs, still harboring the β-globin mutation but now expressing HbF, are finally reintroduced into the SCD patient (Frangoul et al., 2021). Current clinical trials are investigating the use of CRISPR-Cas9 as a therapeutic strategy for SCD, aiming to induce HbF expression by downregulating BCL11A (clinical trial ID: NCT03745287, NCT05477563). Further details on these trials are shown in Table 1.

Apart from enhancing HbF levels, SCD mutation correction holds promise for curing SCD patients. Similar to the process of HbF induction, patient-derived HSPCs carrying the SCD mutation are isolated and will be genetically modified using the CRISPR-Cas9 system. However, for SCD mutation correction, the DSB is introduced near the point mutation in the β-globin gene rather than in the erythroid-specific enhancer region of BCL11A and is repaired through HDR. A corrective donor template, which is introduced into the HSPCs through electroporation or adeno-associated viral vectors, is used by the DNA repair machinery to correct the mutation underlying SCD. Finally, the genetically modified HSPCs are reintroduced into the patient, where they differentiate into a population of healthy RBCs (Demirci et al., 2019; Park & Bao, 2021).

Despite its potential, gene editing of HSPCs encounters several challenges which are yet to be addressed. For instance, electroporation of RNP complexes is known for its efficiency and specificity, but while being non-cytotoxic to HSPCs, it appears to negatively impact their long-term repopulation capacity. Additionally, HSPCs predominantly repair DSBs by NHEJ rather than HDR. However, for the precise correction of the mutation underlying SCD, HDR is essential since NHEJ is ineffective in correcting a specific mutation (Park & Bao, 2021). Prime-editing, as described before, offers a solution to this limitation through allowing for precise base-into-base conversions without relying on HDR (Ceglie et al., 2023). This technique thus presents a promising approach for the effective treatment of SCD by enabling precise repair of the mutation in the β-globin gene.

The CRISPR-Cas9 technology offers potential future therapeutic interventions for not only SCD, but also for β-thalassemia through either HbF induction or mutation correction. While β-thalassemia is another type of hemoglobinopathy, it differs from SCD as it does not involve the production of HbS. Instead, β-thalassemia results from a reduced or absent β-globin chain production due to a point mutation or indels in the β-globin gene. The deficiency in β-globin chains results in the premature death of RBC precursors and ineffective erythropoiesis (production of RBCs) (Bajwa & Basit, 2024; Zeng et al., 2023). Induction of HbF production using CRISPR-Cas9, as described for SCD, can partially compensate for the β-globin chain deficiency in β-thalassemia (Frangoul et al., 2021). Moreover, current clinical trials are investigating the use of CRISPR-Cas9 to induce HbF expression as therapeutic intervention for β-thalassemia (clinical trial ID: NCT05477563, NCT03655678). Further details on these clinical trials are shown in Table 1. On the other hand, correcting the mutation causing β-thalassemia is more complex compared to SCD due to the existence of over 300 unique mutations associated with β-thalassemia (Cosenza et al., 2021). To address this complexity, a personalized medicine approach is particularly promising, as it tailors treatments to individual genetic profiles (Stefanicka-Wojtas & Kurpas, 2023).

Hemophilia B

Hemophilia B is a recessively inherited coagulation disorder characterized by a deficiency or complete absence of clotting factor IX. This deficiency results from mutations or indels in the F9 gene located on the X chromosome (Goodeve, 2015). Patients with hemophilia B frequently experience spontaneous bleeding, including hemorrhages in vital organs, potentially being life-threatening. The primary therapeutic approach involves the administration of clotting factor IX, with dosage adjusted according to the bleeding severity (Alshaikhli et al., 2024).

One of the mutations associated with hemophilia B is the c.947T>C; p.I316T mutation, which theoretically can be corrected through HDR, prime-editing, and base-editing. Research by Hiramoto et al. (2023) has successfully corrected this mutation in hemophilia B patient-derived cells in an in vitro setting using base-editing. Conventional nCas9 fused with cytosine deaminase was ineffective due to the lack of a PAM sequence at the mutation site. Therefore, the study introduced SpCas9-NG, a variant of Cas9 that recognizes NGN (a single guanine) as a PAM sequence, enabling the targeting of nearly any cytosine or adenine base with base-editing. The fusion of SpCas9-NG with cytosine deaminase allowed for the effective correction of the c.947T>C mutation, highlighting the potential of

SpCas9-NG to correct mutations associated with various genetic disorders that necessitate only a single guanine as the PAM sequence (Hiramoto et al., 2023).

The application of CRISPR-Cas9, prime-editing, and base-editing to correct mutations in monogenic blood disorders as discussed in this chapter, may also enable the treatment of other conditions, such as hereditary cancers, neurodegenerative diseases like Huntington's disease, and metabolic disorders such as familial hypercholesterolemia (Alkanli et al., 2023; Hoekstra & Van Eck, 2024).

Cancer

Cancer affects approximately 20% of individuals, with a mortality rate of about 8% (Bray et al., 2024). Despite the widespread use of traditional treatments such as surgery, chemotherapy, and radiotherapy, recent advances in the CRISPR-Cas technology offers new possibilities for targeting and treating specific cancers. (Debela et al., 2021; Rabaan et al., 2023; Stefanoudakis et al., 2023). Through this technique, it is possible to knock out or modulate gene expression of genes driving tumorigenesis, correct specific oncogenic mutations, and identify critical genes essential for tumor growth, viability, and drug resistance (Rabaan et al., 2023). Nonetheless, it is crucial to acknowledge that CRISPR-Cas is not applicable as a therapeutic intervention to all cancer types. Cancers with an unknown primary origin remain challenging as they continue to spread without addressing the primary site. Additionally, cancers with complex tumor microenvironments may limit the accessibility and efficacy of the CRISPR-Cas machinery (Garlisi et al., 2024; Qaseem et al., 2019). This chapter reviews the application of CRISPR-Cas for modulating gene expression of tumor suppressor genes, specifically focusing on melanoma due to the significant increase in melanoma patients and the urgent need for effective treatments. Additionally, it described the potential of CRISPR-Cas to improve chimeric antigen receptor (CAR)-T cell therapy.

Melanoma

Melanoma is a type of skin carcinoma originating from melanocytes, the pigment-producing cells of the epidermis responsible for skin coloration in response to ultraviolet (UV) radiation. Excessive UV exposure causes DNA damage within melanocytes, leading to impaired regulatory control of the cell division and the development of melanoma (PDQ Adult Treatment Editorial Board, 2002; Puckett et al., 2024). Early-stage melanoma can often be excised surgically. However, in more advanced stages, the malignancy may metastasize to distant organs such as the liver, lungs, or brain, requiring additional treatments including chemotherapy and radiotherapy (PDQ Adult Treatment Editorial Board, 2002).

Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene critical for various cellular processes, including cell survival, migration, proliferation, and metabolism (Y.-R. Lee et al., 2018). Notably, PTEN loss or its reduced expression is observed in approximately 65% of metastatic melanomas, where diminished PTEN levels are associated with more aggressive tumor progression (Yu et al., 2023). Multiple mechanisms cause loss of PTEN expression including genetic mutations, transcriptional repression, and epigenetic silencing (Moses et al., 2019). The CRISPRa technology presents a promising strategy for reactivating PTEN expression in cancers where loss of PTEN expression contributes to the disease progression. To induce PTEN expression in melanomas, dCas9-VPR, a fusion of

dCas9 with the transcriptional activator VP64-p65-Rta, has been used. A recent study successfully redirected dCas9-VPR to the PTEN proximal promoter in melanoma cells, significantly increasing PTEN expression and inhibiting downstream oncogenic signaling pathways (Moses et al., 2019).

The application of CRISPR-Cas described above is intended to activate tumor suppressor genes as part of a therapeutic approach for cancer. Similar to the activation of PTEN in melanoma, restoring the activity of other tumor suppressor genes may offer therapeutic potential for various cancers in which these genes are downregulated. Conversely, it is important to note that the CRISPR-Cas9 technology can also suppress or knockout specific oncogenes through indels following NHEJ, effectively inhibiting cancer progression (Kim et al., 2018).

Currently, no clinical trials are evaluating the use of the aforementioned CRISPR-Cas technologies as treatments for melanoma or other cancer types. Nonetheless, in vitro research indicates that CRISPR-mediated targeted activation of PTEN holds the potential to emerge as an alternative and effective therapeutic strategy for highly aggressive cancers characterized by PTEN loss which became resistant to existing treatments (Moses et al., 2019).

Chimeric antigen receptor T-cell therapy

CAR-T cell therapy is a type of immunotherapy in which T cells from cancer patients are genetically modified to target and eliminate cancer cells, predominantly in hematological malignancies such as leukemia and lymphoma. Its efficacy in solid tumors is limited, primarily due to the antigenic diversity and complex tumor microenvironment of those tumors. The CAR-T cells are engineered to express CAR proteins on their surface, allowing them to specifically recognize antigens present on the cell surface of cancer cells. Upon antigen recognition and binding, CAR-T cells become activated and start secreting cytokines that induce lysis or apoptosis of the target cell and activate additional immune responses (Sun et al., 2024). Despite its potential, the therapeutic efficacy of CAR-T cell therapy is often insufficient, with many patients facing relapse. The CRISPR-Cas technology has recently been identified as a promising approach to improve CAR-T cell therapy (Wei et al., 2023). Unlike other CRISPR-Cas applications that focus on genetic modifications or modulation of gene expression profiles as therapeutic strategies for patients, the use of CRISPR-Cas in CAR-T cell therapy does not involve altering the patient's genome. Instead, it aims to optimize CAR-T cells to improve their effectiveness in targeting and eliminating cancer cells (Song et al., 2024; Wei et al., 2023).

CRISPR-Cas presents numerous strategies to enhance CAR-T cell therapy, one of which is improving the precise insertion of the CAR cassette into T cells (Dimitri et al., 2022; Glaser et al., 2023). The conventional approach relies on viral vectors carrying the chimeric receptor sequence, a relatively safe method for stable integration. However, due to the semi-random integration of the CAR cassette into the genome, there is considerable variation in transcriptional activity, leading to inconsistent CAR expression (Dimitri et al., 2022). By using CRISPR-Cas9 to induce targeted DSBs and providing a donor template carrying the chimeric receptor sequence, HDR can direct the chimeric receptor sequence to a specific

genomic locus, allowing for controlled and stable CAR expression (Chen et al., 2024; Dimitri et al., 2022; Glaser et al., 2023).

Moreover, CRISPR-Cas9 is a powerful tool for creating off-the-shelf allogeneic CAR-T cells (Dimitri et al., 2022). Typically, CAR-T cells are developed on a patient-specific basis, a labor-intensive and costly process (Cliff et al., 2023). Off-the-shelf CAR-T cells are derived from healthy donors and are genetically engineered using CRISPR-Cas9 to prevent both autoimmune reactions and rejection of the CAR-T cells by the patient (Glaser et al., 2023; Wei et al., 2023). The generation of these specific CAR-T cells involves targeted integration of the CAR cassette into the T-cell receptor α constant (TRAC) locus using CRISPR-Cas9. This locus is involved in T-cell receptor (TCR) production, and its knockout via CAR cassette integration results in the absence of TCR expression, thereby preventing donor CAR-T cells from targeting the patient's healthy tissue through autoimmune mechanisms (Dimitri et al., 2022; Eyquem et al., 2017). To further avoid the patient's immune system from identifying donor CAR-T cells as foreign, CRISPR-Cas9 can introduce a Beta2-microglobulin (B2M) knockout in the CAR-T cells. The B2M gene encodes a subunit of human leukocyte antigen class-I (HLA-I), and the absence of B2M impedes the presentation of HLA-I peptides by the donor CAR-T cells, preventing immune rejection by the patient's immune system (O.-H. Lee et al., 2022). This B2M knockout is achieved through CRISPR-Cas9-mediated DSBs, followed by NHEJ, leading to indels that result in a frameshift and gene knockout. Donor CAR-T cells that possess both a TRAC and B2M knockout are classified as off-the-shelf allogeneic CAR-T cells (Dimitri et al., 2022). CRISPR-Cas-engineered CAR-T cells are currently being extensively applied in clinical trials as an innovative cancer treatment. Various clinical trials use CRISPR-Cas9 to integrate the CAR cassette into T cells and simultaneously knock-out TRAC and B2M to create off-the-shelf allogeneic CAR-T cells (clinical trial IDs: NCT04244656, NCT05643742, NCT04035434, NCT03166878). Further details on these trials are shown in Table 1. Thus, the use of CRISPR-Cas9 in the development of off-the-shelf allogeneic CAR-T cells holds significant promise for advancing current CAR-T cell therapies.

Despite the progress in CAR-T cell therapy, several significant challenges limit its effectiveness. One major issue is T-cell exhaustion, a state in which CAR-T cells become functionally impaired due to prolonged exposure to high levels of antigen. Exhausted T-cells exhibit diminished ability to proliferate and produce cytokines, reducing their overall therapeutic efficacy. Another challenge is the presence of negative regulators and immune checkpoints proteins which inhibit CAR-T cell activity and contribute to their dysfunction. Finally, suppressive cytokines in the tumor microenvironment negatively affect CAR-T cell function. To address these challenges, the CRISPR-Cas9 technology presents several innovative strategies. This includes disrupting immune checkpoints to reduce CAR-T cell exhaustion, deactivating negative regulators of CAR-T cells, and increasing CAR-T cell resistance to suppressive cytokines (Dimitri et al., 2022; Wei et al., 2023). Moreover, clinical trials are investigating the use of CRISPR-Cas9 to prevent CAR-T cell exhaustion (clinical trial IDs: NCT03545815, NCT03747965, NCT05812326). Table 1 provides more detailed information on these trials. The combination of the CRISPR-Cas technology with CAR-T cell therapy holds the potential for a significant breakthrough in the treatment of various cancers.

Pathogenic microorganisms

Infectious diseases are among the most prevalent and lethal global health challenges, characterized by frequent epidemics and growing resistance to antibiotics and vaccines. Major outbreaks, such as COVID-19, have been declared international emergencies by the World Health Organization, highlighting the urgent need for effective prevention and treatment strategies (Morshedzadeh et al., 2024). This chapter explores the role of the CRISPR-Cas technology in facilitating the early detection of pathogenic microorganisms and in preventing the emergence of antibiotic resistance among bacteria.

Detection of pathogenic microorganisms

Early and accurate detection of pathogenic microorganisms is crucial for the implementation of effective treatments. However, developing a detection method which is rapid, specific, sensitive, and cost-effective remains a significant challenge. Currently, the polymerase chain reaction (PCR) is the most commonly used method for pathogen identification, although it is known to be a time-consuming and expensive technique (T. Huang et al., 2023).

Recent studies have uncovered the potential of the CRISPR-Cas technology, specifically CRISPR-Cas13 and CRISPR-Cas12, for the rapid detection of pathogenic microorganisms, potentially replacing PCR. CRISPR-Cas13, in contrast to the DNA-targeting CRISPR-Cas9 system, targets single-stranded RNA (ssRNA) guided by a crRNA molecule and requires a protospacer flanking sequence (PFS) for activation. In Cas13-mediated targeting, crRNA binds to the complementary target RNA sequence, and upon recognition of the PFS, Cas13 is activated, initiating the degradation of both the target RNA and non-specific ssRNA through its collateral activity (Z. Huang et al., 2022; Y. Zhang et al., 2024).

For a specific and sensitive detection method of pathogens, the CRISPR-Cas13 system can be combined with a reporter RNA system. In this approach, a single-stranded reporter RNA molecule is designed to include a sequence that is cleaved by Cas13 upon its activation. When Cas13 is guided to the target RNA by the crRNA and activated after PFS recognition, it not only degrades the target RNA but also the reporter RNA. Cleavage of the reporter RNA releases a fluorescent signal, which can be easily detected and quantified. A clear fluorescent signal correlates with the presence of the target RNA, thereby indicating the presence of the pathogen. Conversely, the absence of such a fluorescent signal signifies that the target RNA, originating from the pathogen, is absent. This method offers a promising, real-time, approach for pathogen detection (T. Huang et al., 2023).

The CRISPR-Cas13 system is constrained by its ability to target only ssRNA, limiting its application to the detection of specific microorganisms. In contrast, the CRISPR-Cas12 system, which functions similarly to CRISPR-Cas13, can target both single-stranded and double-stranded DNA and also exhibits collateral activity. The combined use of these two CRISPR-Cas systems enables the efficient detection of a wide range of viral and bacterial pathogens. In conclusion, CRISPR-Cas12/13 represents a rapid and cost-effective method for detecting pathogenic microorganisms. Moreover, its heightened sensitivity enables earlier detection of pathogens compared to PCR (T. Huang et al., 2023; Selvam et al., 2022). Hence, replacing PCR with CRISPR-Cas12/13 is a critical step forward in addressing infectious diseases.

Antibiotic resistance bacteria

Antibiotics are the primary treatment for bacterial infections. However, due to the excessive and inappropriate use of antibiotics, along with the persistent adaptation of bacteria, the number of multi-drug resistant (MDR) bacterial strains is growing (Morshedzadeh et al., 2024). As a result, there is an increasing demand for alternative therapies to address infections caused by antibiotic-resistant strains, with CRISPR-Cas technologies emerging as an innovative solution (Kadkhoda et al., 2024; Tao et al., 2022).

The CRISPR-Cas9 system, as previously described, is capable of targeting antibiotic resistance genes (ARGs) (Kadkhoda et al., 2024; Tao et al., 2022). Through the application of a gRNA specifically designed to target these ARGs, Cas9 can effectively eliminate the resistance gene, thereby restoring the pathogen's sensitivity to this specific antibiotic. The use of multiple gRNAs simultaneously allows for the targeted disruption of various ARGs simultaneously. This strategy has been successfully applied to re-sensitize pathogens such as *Staphylococcus aureus* to antibiotics like methicillin and kanamycin (Kadkhoda et al., 2024). Additionally, ongoing research includes a clinical trial investigating the application of CRISPR-Cas9 for targeting ARGs in *Enterobacteriaceae* bacteria in mice (clinical trial ID: NCT05850871). Further details on this trial is shown in Table 1.

An alternative method for targeting antibiotic-resistant bacteria involves the direct targeting of genes essential for bacterial survival using CRISPR-Cas9, such as nuc (encoding staphylococcal thermostable nuclease) in *Staphylococcus aureus*. A phage or plasmid carrying a programmed CRISPR-Cas9 system is introduced into target bacteria for the killing of the bacteria (Kadkhoda et al., 2024). The introduction of the CRISPR-Cas9 machinery can be achieved through diverse delivery systems, including physical techniques like microinjection and electroporation, viral vector systems such as adeno-associated viruses or lentiviruses, as well as non-viral vectors like nanoparticles (J. Huang et al., 2022). Alternatively, the endogenous CRISPR-Cas system, which naturally occurs in bacteria, can also be used to target the bacteria through the delivery of self-targeting gRNAs. Nevertheless, relying on the endogenous CRISPR-Cas system is generally less effective compared to introducing a complete CRISPR-Cas system (Kadkhoda et al., 2024).

Table 1: Overview of ongoing and completed clinical trials using CRISPR-Cas as therapeutic strategy, including clinical trial ID, condition/focus, strategy, status, and estimated study completion.

Clinical trial ID	Condition/ focus	Intervention	Strategy	Status	Estimated study completion
NCT03745287	Sickle cell disease	HbF induction	CRISPR-Cas9	Active	October 2024
NCT05477563	Sickle cell disease, β-thalassemia	HbF induction	CRISPR-Cas9	Recruiting	February 2025
NCT03655678	β-thalassemia	HbF induction	CRISPR-Cas9	Active	August 2024
NCT04244656	CAR-T cell therapy	Generate off-the-shelf allogenic CAR-T cells	CRISPR-Cas9	Active	January 2027
NCT05643742	CAR-T cell therapy	Generate off-the-shelf allogenic CAR-T cells	CRISPR-Cas9	Recruiting	February 2030
NCT04035434	CAR-T cell therapy	Generate off-the-shelf allogenic CAR-T cells	CRISPR-Cas9	Active	August 2026
NCT03166878	CAR-T cell therapy	Generate off-the-shelf allogenic CAR-T cells	CRISPR-Cas9	Unknown status	May 2022
NCT03545815	CAR-T cell therapy	Prevent CAR-T cell exhaustion	CRISPR-Cas9	Unknown status	December 2020
NCT03747965	CAR-T cell therapy	Prevent CAR-T cell exhaustion	CRISPR-Cas9	Unknown status	May 2020
NCT05812326	CAR-T cell therapy	Prevent CAR-T cell exhaustion	CRISPR-Cas9	Completed	November 2022
NCT05850871	Combat antibiotic resistant *Entero-bacteriaceae*	Targeting antibiotic resistance genes	CRISPR-Cas9	Recruiting	January 2025

Discussion

The CRISPR-Cas technology is an advanced technique currently being investigated in both preclinical studies and clinical trials to explore its potential for treating monogenetic blood disorders, improve CAR-T cell therapy, early detection of pathogenic microorganisms, and preventing antibiotic resistance in bacterial strains (Mahara et al., 2023). This study investigated various therapeutic strategies employing different CRISPR-Cas approaches to treat specific blood disorders and cancer, and to address the management of pathogenic microorganisms.

Limitations of the CRISPR-Cas technology

Although the CRISPR-Cas technology holds significant potential for numerous therapeutic applications, it faces critical challenges. A major concern in its use in healthcare is the risk of off-target effects, which can result in unintended DNA breaks, mutations, or indels with unpredictable outcomes (Guo et al., 2023; Morshedzadeh et al., 2024). While off-target prediction tools can estimate the risk of such occurrences, their use does not eliminate the possibility of off-target effects (Vicente et al., 2021). To apply CRISPR-Cas safely and effectively in clinical settings, minimizing off-target effects is essential. Various strategies have been explored to prevent off-target effects, including optimizing the GC content of gRNAs between 40% and 60%, which improves on-target specificity by stabilizing the interaction between the gRNA and target DNA. Additionally, modified Cas9 variants, such as SpCas9-HF1, possess proofreading mechanisms that deactivate their nuclease domains upon detecting mismatches between the gRNA and target DNA (Asmamaw Mengstie et al., 2024). Moreover, longer gRNAs in HDR-based repair systems have been shown to enhance target specificity, thereby reducing off-target effects (Morshedzadeh et al., 2024).

Besides minimizing off-target effects, selecting an effective delivery method to introduce the CRISPR-Cas machinery into the cell is crucial. Viral vectors are commonly used for their high efficiency, but they present potential safety risks, such as inducing immune responses in patients and causing insertional mutagenesis, which may lead to unintended genetic modifications (Morshedzadeh et al., 2024; Sioson et al., 2021). Non-viral delivery methods, especially nanoparticle-based systems, offer promising alternatives with improved specificity for cell targeting and minimal immunogenic effects. Additionally, nanoparticles are advantageous due to their compact size (which enhances their cellular uptake), production efficiency, and cost-effectiveness. Different types of nanoparticles provide unique benefits. For instance, lipid nanoparticles are characterized by their high biocompatibility and biodegradability, whereas inorganic nanoparticles are known for their stability, making them more suitable for long-term applications (Sioson et al., 2021).

Immunogenicity, also independent of the delivery system, presents a significant challenge in the application of the CRISPR-Cas system. Cas proteins and gRNAs are often detected by the immune system as foreign molecules, thereby triggering an immune response (Morshedzadeh et al., 2024). Specifically, the two most commonly used Cas9 variants, derived from *Staphylococcus aureus* and *Streptococcus pyogenes*, are recognized as foreign by the human immune system, thereby activating the adaptive immune system. Additionally, gRNAs can trigger the innate immune system by interacting with pattern

recognition receptors (Crudele & Chamberlain, 2018; Ewaisha & Anderson, 2023). To address these challenges, novel Cas proteins are being developed with reduced immunogenic potential, and phosphatase treatment of gRNAs is being explored as a strategy to evade immune recognition (Morshedzadeh et al., 2024).

Ethics

In addition to the technical challenges associated with the CRISPR-Cas technology, ethical concerns emerge regarding its application in healthcare. One major dilemma is whether modifying the human genome is ethically justified. From one perspective, introducing genetic modifications using CRISPR-Cas can be considered as ethically justified when the overarching goal is to enhance the health prospects of individuals and future generations. On the other hand, the potential for unforeseen genetic changes and the risk of creating health disparities raises discussion. This debate intensifies when considering the potential application of CRISPR-Cas in editing human germline cells and embryos, which offers the possibility of correcting genetic disorders at their earliest stages. However, uncertainties persist regarding the long-term consequences of unintended modifications, as well as the ethical dilemma of who has the authority to consent to such modifications in embryos. Furthermore, the accessibility of the CRISPR-Cas technology is a matter of considerable debate (Ayanoğlu et al., 2020; Shinwari et al., 2018).

Current regulations regarding the genetic modification of the human genome exhibit considerable variation across different nations. In countries such as Australia, Canada, and Brazil, genetic modifications for therapeutic interventions are subject to stringent regulations, and modifications involving embryos are strictly prohibited. Meanwhile, in the United Kingdom, genetic modifications are regulated with prohibitions on those intended for reproductive therapies leading to pregnancy, but allowed under specific conditions if the modification will not be passed to future generations. Notably, several countries, including Russia and Israel, currently lack specific regulatory frameworks addressing genetic modification in humans. In the future, it is imperative to establish robust, uniform legislative frameworks to regulate the therapeutic use of CRISPR-Cas technology, ensuring equitable consideration of all stakeholders (Ayanoğlu et al., 2020; Shinwari et al., 2018).

Conclusion and future perspective

In summary, CRISPR-Cas represents a highly promising technology with significant potential for treating blood disorders and cancer, as well as for the early detection of microorganisms and the prevention of antibiotic resistance in bacteria. Yet, it is important to understand that the strategies discussed in this study represent just a subset of the diverse therapeutic applications of CRISPR-Cas. The current state-of-the-art CRISPR-Cas technology, as demonstrated through extensive preclinical studies and ongoing clinical trials, focuses on developing novel and effective treatments for a wide range of diseases that remain challenging or impossible to treat, with the goal of translating promising results into viable therapeutic interventions.

Projecting into the future, the CRISPR-Cas technology is expected to be increasingly implemented in clinical trials over the next 5 to 15 years. Initial applications are anticipated to focus on correcting monogenic disorders using CRISPR-Cas9, prime-editing, or

base-editing, as well as on improving CAR-T cell therapies for cancer. In the following years, the technology may progress to first-line treatments in patients and be investigated for its application in more complex disorders, including polygenic conditions. There is a possibility that, in around 25 years, CRISPR-Cas could play a significant role in personalized medicine and may extend to germline editing. However, for CRISPR-Cas to be implemented on a large scale in healthcare, it is essential to resolve its associated challenges and ethical issues. Once these are addressed, CRISPR-Cas has the potential to emerge as a revolutionary innovation in healthcare, offering precise genetic therapies and significantly improving existing therapeutic approaches.

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