

Gene Editing Tools in the Laboratory: CRISPR-Cas9 and Its Applications in Glioblastoma Research

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ABSTRACT

Glioblastoma is a highly aggressive and devastating type of brain tumor, recognized as the most prevalent and malignant amongst the primary brain tumors. Characterized by rapid growth, extensive invasiveness, and resistance to conventional therapies, glioblastoma presents significant challenges in clinical treatment. It has a high mortality rate, with most patients surviving for a short period of time after the diagnosis. Current treatments, which include surgical resection, radiation therapy, and chemotherapy, offer limited efficacy due to the tumor's location in the brain and its highly invasive nature. There is no current cure for glioblastoma. However, in recent years, gene-editing technologies have shown great promise for better understanding and treating genetic disorders, including cancers. Among these, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) systems have demonstrated particular promise due to their precision, efficiency, and versatility in targeting specific genetic sequences. This review paper will examine various gene editing tools (ZFNs, TALEN, and CRISPR), with an emphasis on CRISPR-Cas systems, for experimental and clinical uses for treating glioblastoma.

INTRODUCTION

1.1 Glioblastoma

The brain is the most complicated organ in the human body, and is considered by some to be the most complex object in the universe. It controls various essential functions such as allowing us to think, react to stimuli, store memories, and sense. It is made up of billions of nervous and glial cells that help with these functions. However, some diseases take root in these cells, impeding their function. One such disease is Glioma. Gliomas are mainly brain tumors that stem from glial cells but can also occur in the spinal cord (Weller et al., 2015). The rate of Gliomas is 6.6 per 100,000 people, with glioblastoma being one of the most malignant and prevalent forms (Weller et al., 2015; Davis, 2016).

There are multiple types of Gliomas, varying in malignancy (Weller et al., 2015). The World Health Organisation (WHO) uses a scale from I to IV, with I being the least malignant, to classify the different Gliomas. Some common examples include oligodendrogliomas; oligoastrocytomas; infiltrative astrocytomas, with various stages: diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO

December 2025

Vol 2. No 1.

grade III), and glioblastoma (WHO grade IV). Glioblastoma can start in the brain or spine, in a type of glial cell called astrocytes (*Glioblastoma - Symptoms and Causes, 2024; Glioblastoma (GBM), 2023*). Astrocytes are a type of non-neuronal cell that make up the majority of cells in the human central nervous system (CNS) (Wei & Morrison, 2023). Unlike neurons, they do not conduct electrical signals; instead, they aid in metabolic, structural, homeostatic, and neuroprotective tasks, including regulating the blood-brain barrier (BBB), clearing excess neurotransmitters, and encouraging synapse formation. In short, they provide support and protection to neurons (Wei & Morrison, 2023). When these astrocyte cells turn malignant, they give rise to glioblastoma. As it originates from the CNS, it has effects on CNS functions, causing symptoms such as memory loss, personality changes, speech difficulties, seizures, headaches, etc (*Glioblastoma (GBM), 2023*). There are two types of glioblastoma: glioblastoma-wild type and glioblastoma-isocitrate dehydrogenase (IDH) mutation (IDH-mutant) (Mohammed, 2022). Wild-type glioblastoma is considered primary glioblastoma as it develops directly from astrocytes without evidence of a preceding lower-grade tumor (Weller et al., 2015). It is more common in older adults and is more aggressive and therefore has a lower prognosis. On the other hand, IDH-mutant glioblastoma is a secondary glioblastoma as it typically develops from lower-grade Gliomas, usually astrocytomas. It is more common in young adults and is less aggressive and therefore has a higher prognosis compared to primary glioblastoma.

While the exact cause of glioblastoma is still unknown, researchers believe that DNA mutations are the cause of uncontrolled cell growth (*Glioblastoma - Symptoms and Causes, 2024*). Common risk factors include: radiation therapy to the head, exposure to chemicals in synthetic rubber, and genetic tumor-causing conditions.

Despite the severity, there is currently no cure for glioblastoma (*Glioblastoma (GBM), 2023*). However, several treatments are used to manage the disease progression. These treatments include surgery where the tumor is removed (craniotomy), radiation, and chemotherapy (*Glioblastoma (GBM), 2023*). That being said, even with a combination of these treatments, the median survival time remains at most 14 months (Mohammed, 2022). Only 3-5% of patients survive more than 3 years. Currently, new potential treatment strategies are starting to be explored, such as treating the cancer at its root through gene-editing tools such as CRISPR-Cas systems (Clustered Regularly Interspaced Short Palindromic Repeats). This new wave of research raises the question: How can CRISPR-Cas9 be used to study and treat glioblastoma? This paper will examine how CRISPR-Cas systems work, and how CRISPR-Cas is used for experimental and clinical uses for glioblastoma.

1.2 Gene Editing

Gene editing is the process of making targeted changes to the DNA of living organisms (Chen, 2019). The genome consists of gene sequences, each of which encodes instructions for protein synthesis via transcription and translation. While parts of DNA encode for proteins, the vast majority of human DNA is non-coding and likely has regulatory purposes. However, when cells are duplicating their genetic information in preparation for cell division, random errors may be introduced. These genetic mutations can lead to genetic disorders, including types of cancer. Glioblastoma is usually caused by a single cell

December 2025

Vol 2. No 1.

that has mutated to become cancerous and grows uncontrollably (Weller et al., 2015). Gliomas can develop from random gene mutations or genetic predisposition syndromes. Some of these syndromes include Li-Fraumeni syndrome, affecting the TP53 gene; Lynch syndrome, affecting MSH2, MLH1, MSH6, PMS2; Ollier disease/Maffucci syndrome, affecting IDH1/IDH2, etc (Rice et al., 2015). Researchers are exploring various gene-engineering approaches to better understand the genetic factors underlying glioblastoma and to develop novel treatments that target mutated cells.

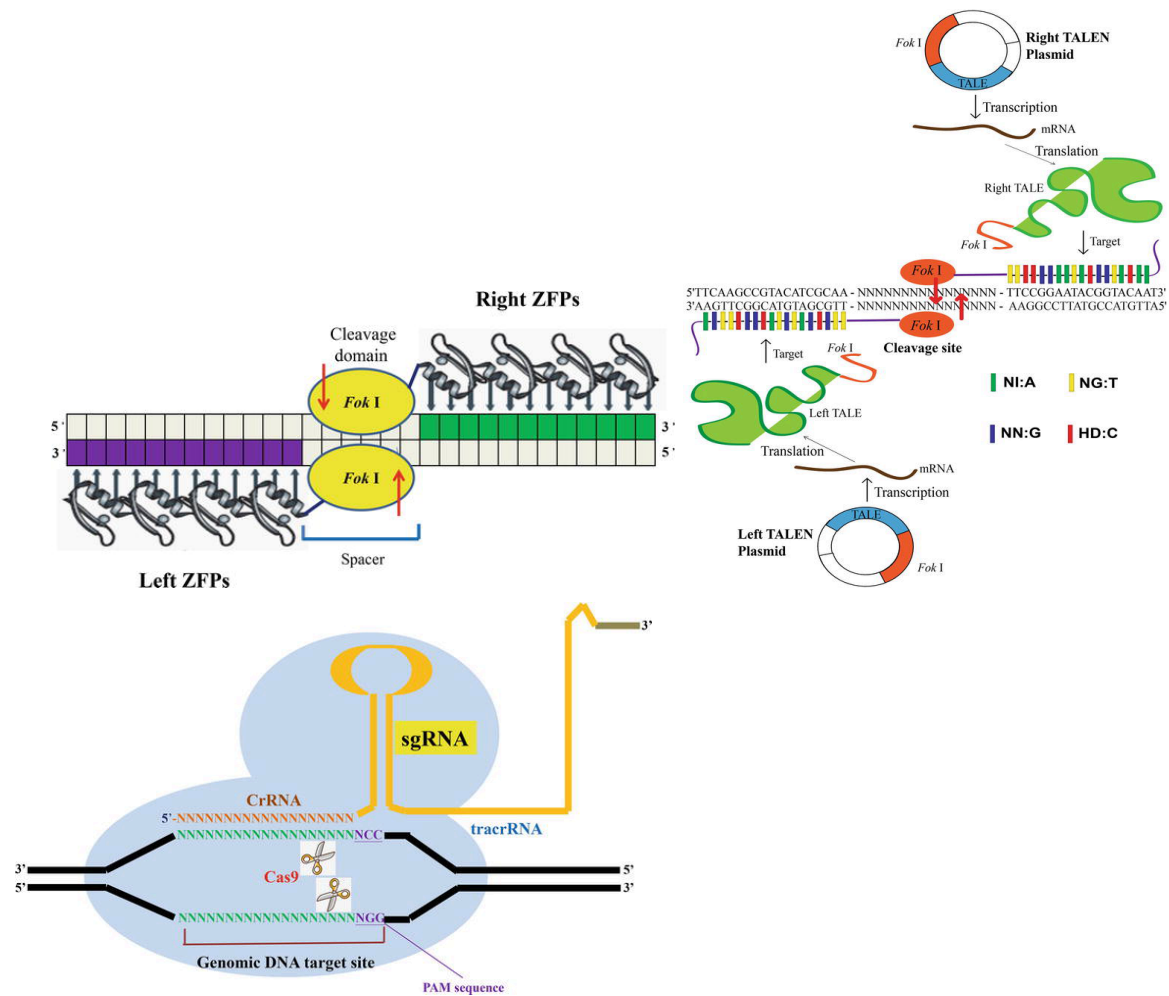


FIGURE 1. SCHEMATIC ILLUSTRATION OF GENE EDITING TOOLS. A) ZINC FINGER NUCLEASE (ZFN) INTERACTION WITH DNA. ZFN USES PAIRED ZINC-FINGER PROTEINS AND A FOKI NUCLEASE TO CREATE A DOUBLE-STRAND BREAK B) TALEN (TRANSCRIPTION ACTIVATOR-LIKE EFFECTORS NUCLEASE) INTERACTION WITH DNA. TALE REPEATS RECOGNIZE SPECIFIC NUCLEOTIDES AND FOKI DIMERIZES TO CLEAVE THE DNA. C) CRISPR (CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS) INTERACTION WITH DNA. CRISPR-Cas9 USES A SINGLE-GUIDE RNA (sgRNA) TO DIRECT Cas9 TO A TARGET SEQUENCE ADJACENT TO A PAM SITE, WHERE THE NUCLEASE INDUCES A DOUBLE-STRAND BREAK. ALL FIGURES TAKEN FROM CHEN, 2019.

December 2025

Vol 2. No 1.

Three tools are commonly used to edit DNA: Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and CRISPR-Cas systems (Chen, 2019). These tools can specifically find and modify specific sequences inside the genome to help scientists delete, modify, add, or replace the target genes. The gene scissors are used to create double-stranded breaks in the DNA at a specific spot. The break is then repaired by the cell's natural mechanisms (Harrison & Hart, 2018). There can either be small changes, by non-homologous end joining (NHEJ), or precise ones using a DNA template, by homology-directed repair (HDR).

The first programmable gene editing nucleases developed were ZFNs (Figure 1a), which utilise engineered nucleases created by fusing multiple zinc finger-specific DNA-binding domains to a nonspecific DNA cleavage domain of the Fok I restriction enzyme (Chen, 2019).

Another key gene editing tool is TALENs, which are very similar to ZFNs, but use a different protein for DNA binding. The transcription activator-like effectors (TALEs) are proteins released by the bacteria *Xanthomonas* when it infects plants (Chen, 2019). Each TALE has a DNA-binding domain with multiple tandem repeats that are made up of around 34 amino acids, with the 12th and 13th amino acids being variable (figure 1b). The Fok I enzymes then cut the DNA at the specific site, similar to ZFNs. This combination of TALEs with their tandem repeats and the Fok I enzyme is what makes TALENs more precise than ZFNs (Chen, 2019).

The last and most commonly used gene editing tool is CRISPR. It originates from the bacterial immune system, where it recognises and cuts viral DNA (Chen, 2019). It consists of repeated bacterial DNA sequences separated by fragments of viral DNA, which act as a genetic “memory” of past infections. This same technology is used to engineer CRISPR-Cas systems to recognise and cleave a specific DNA sequence for diagnostic or treatment purposes. Cas9 is the protein that cuts the DNA, and has two domains: one for recognising the DNA with a guide RNA, and the other for cutting it (figure 1c). It uses a guide RNA to recognise the target sequence and requires a short nucleotide sequence called protospacer adjacent motif (PAM), typically “NGG”. Cas9 first binds to the PAM site and then checks for sequence complementarity with the adjacent DNA; once matched, its nuclease domains cleave the DNA. In bacteria, targeting involves two RNAs: crRNA and tracrRNA. In research, both RNAs are combined into a singular synthetic guide RNA (sgRNA) for simplicity. When the Cas and guide RNA are added to the cell, they find the corresponding DNA and cut it.

All of these gene-editing tools (ZFNs, TALENs, and CRISPR-Cas9 systems) have been used to help cure and treat genetic diseases (Harrison & Hart, 2018). Genetic diseases are hereditary disorders or a result of mutations; common examples include Cystic Fibrosis, Huntington's disease, Color blindness, Sickle Cell Anaemia, and Down Syndrome (*Genetic Disorders*, 2021). CRISPR is the most commonly used gene editing tool because it is easy to use, has a low cost, high efficiency, and is less toxic than other options (Harrison & Hart, 2018). Scientists have started to look at how CRISPR can be used to help study and treat cancers, including glioblastoma. This includes creating precise models of the disease and disabling genes to find which ones are crucial for the tumor's survival and proliferation (Begagić et al., 2024).

December 2025

Vol 2. No 1.

LABORATORY AND CLINICAL USE OF CRISPR-Cas

2.1 Creating the Guide Complex

CRISPR-based gene engineering requires the Cas9 protein and the guide RNA to be in the target cell to reach the genome being targeted in the cell's nucleus. To do so, the guide RNA has to be designed to target the specific gene. For glioblastoma, guide RNAs are often designed to target oncogenes, tumor suppressor genes, or regulators of the glioblastoma stem-cell state. A key aspect is the location of the cut site. The cut site should be as close to the target site (the exact area in the gene where it is to be edited) as possible; the recommended distance being less than 10 nucleotides (Suresh, 2021). In order to improve the chances of success, 3-4 guides should be designed, and care must be taken to minimize off-target effects.

After the guide RNA is designed, a complex needs to be formed with the RNA and the Cas9 protein. There are three broad strategies to create the complex: plasmid-based CRISPR-Cas9 systems, the mixture of Cas9 mRNA and the sgRNA, and the Cas9/sgRNA ribonucleoprotein (RNP) (Liu et al., 2017). In the plasmid method, DNA sequences encoding Cas9 and the sgRNA are packaged into a circular DNA vector (plasmid). After the plasmid enters the target cell, the cell's transcription machinery produces the Cas9 mRNA and sgRNA. The mRNA is translated to express the Cas9 protein. The guide RNA and Cas9 protein then form a complex. This method is especially suitable for long inserts (>50 base pairs(bp)) and for replacing entire genes or adding reporter constructs, using homology arms (50–800 bp) for HDR. Plasmids are inexpensive and easy to prepare, but they pose a high risk for off-target integration into the genome. In the Cas9 mRNA and sgRNA delivery strategy, the Cas9 mRNA and sgRNA mixture is delivered directly into the cells (Liu et al., 2017). The mRNA is then translated into the Cas9 protein, resulting in a complex formation. Because there is no need for transcription, the process is faster. However, mRNA is less stable than plasmid DNA and requires careful handling to prevent degradation. In the third method (the Cas9/sgRNA or Ribonucleoprotein (RNP) strategy), a pre-assembled Cas9 protein already bound to sgRNA is introduced directly into the cells. While this approach is faster, RNPs are more expensive and more technically challenging to prepare and deliver.

2.2 Entering the Cell

Once the complex is ready, there are various ways for CRISPR to enter the cell. Which specific method is used is based on multiple factors, including the length of the edit, whether the edit is *in vitro* or *in vivo*, whether the edit is a knock-in or knock-out, etc. Genes that have been knocked out have been disabled, removed, or disrupted so they no longer work (Mansouri, 2018). This is often done in lab settings to see what happens to the body in the absence of a gene. On the other hand, knocking in involves inserting a new gene in that spot. This can include inserting a new gene, replacing one, and inserting mutations to study disease-related changes. Currently, scientists are investigating genes, such as PTEN, HSPB1, and CIS, that can be knocked out to reduce the malignancy of glioblastoma tumors (Feng, 2023; Bian et al., 2018; Nakazawa et al., 2023; Rodvold et al., 2020). While some of the methods of introducing CRISPR

into the cells (which are discussed below in more detail) are already being used for glioblastoma, other methods could hold future potential depending on the gene being affected.

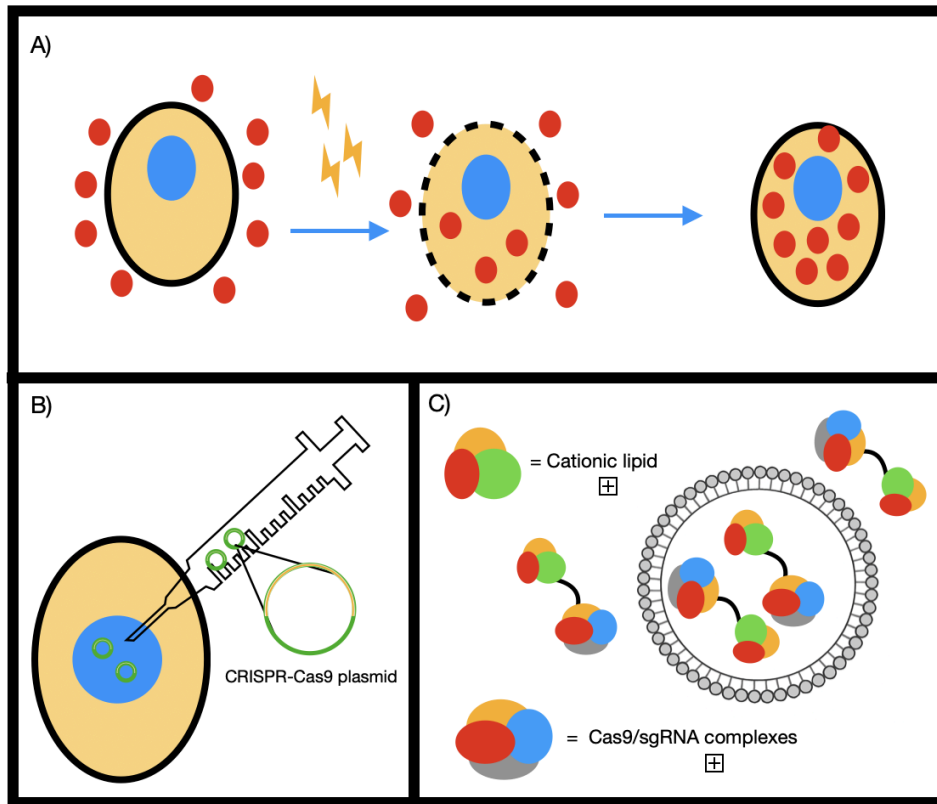


FIGURE 2. SCHEMATIC ILLUSTRATION OF NON-VIRAL METHODS OF CRISPR-CAS9 ENTERING THE CELL. A) ELECTROPORATION B) MICROINJECTION C) LIPID NANOPARTICLES. ILLUSTRATION WAS REDRAWN FROM/ADAPTED FROM LIU ET AL, 2017.

2.2.1 Non-viral Methods

One method commonly used for the complex delivery is electroporation (Liu et al., 2017). In this method, brief high voltage electric pulses are used to increase the permeability of the cell membrane, allowing for the Cas9 protein to enter (figure 2a). This method can be used on all three CRISPR-Cas strategies for creating the CRISPR-Cas9 complex, and both *in vitro* and *in vivo*. This was the method that has been used to deliver the gene engineering complex to correct the sickle cell mutation in hematopoietic stem cells (Frangoul et al., 2021). The scientists made the changes to the gene *ex vivo*. However, a limitation of electroporation is that plasmid DNA is only incorporated into roughly 0.01% of target cells. It also leads to significant cell death.

Another method used for the intracellular delivery of the gene editing complex is microinjection (Liu et al., 2017). This technique involves the direct injection of molecules into living cells using a micropipette (figure 2b). It is a simple and highly precise mechanical procedure, making it a commonly used technique. It can be used both *in vivo* and *in vitro*, and it has been used to edit cells of rabbits, zebrafish, and other

December 2025

Vol 2. No 1.

animals. Although it can be used in all three CRISPR-Cas assembly systems, it can induce cell damage and therefore requires skill, and only a single cell can be targeted with each injection.

Alternative delivery methods include lipid nanoparticles, which are usually used to deliver negatively charged nucleic acids into cells (Liu et al., 2017). The lipid particles can be designed to carry positive charges, which allows them to bind to DNA and RNA molecules, such as those encoding the CRISPR system (figure 2c). The lipid nanoparticles protect encapsulated CRISPR from nucleases and facilitate intracellular uptake by endocytosis or macropinocytosis. However, due to their positive charge, CRISPR proteins are difficult to encapsulate in cationic lip nanoparticles. This challenge is addressed by adding negatively charged proteins to neutralize it. Lipid nanoparticles have been effectively used *in vitro* for the delivery of gene editing tools. Although lipid nanoparticles work in both plasmids and RNPs, they are more effective with RNPs.

2.2.2 Viral Methods

While these methods are effective *in vitro*, viral methods are typically more effective *in vivo* (Liu et al., 2017). While they do have safety concerns, currently, viral systems are the most efficient and effective way for gene editing, both *in vivo* and *in vitro*. They typically involve plasmids for the delivery of genetic information into cells for the expression of proteins. Generally, there are two plasmids involved: one is a packaging plasmid with the information and materials to create the viral particles, and the other contains the foreign material, such as Cas9 and/or sgRNA cassettes (Liu et al., 2017). Adeno-associated viruses (AAVs) are non-pathogenic single-stranded viruses that have been modified to be non-pathogenic (Suresh, 2021). They are able to infect both non-dividing and dividing cells, have a low immunogenicity, and induce homologous recombination (Liu et al., 2017; Suresh, 2021). This has caused them to be one of the most commonly used viruses for gene transduction (Suresh, 2021). However, a drawback is that they are relatively difficult to produce, making them rather expensive. Another common viral vector used is Lentivirus (Suresh, 2021). They are a type of retrovirus and can therefore integrate into the genome of the host cell by penetrating through the nuclear envelope. They are highly useful as they lead to a low immune response, allowing for the long-term expression of transduced genes (Liu et al., 2017). Its biggest advantage is that it has a high infection efficiency in both dividing and non-dividing cells. This makes it crucial for editing genes in tissues such as the liver, brain, and muscle, making it a meaningful option for glioblastoma. Lentivirus has been successfully used in both *in vivo* and *in vitro*. Moreover, it has successfully been used to correct blood stem cells in Wiskott-Aldrich Syndrome patients, leading to stable and long-term results. It has achieved over 90% correction efficiency in patients with X-linked adrenoleukodystrophy. However, since Lentivirus integrates itself into the genome, it means that there is a higher risk of disrupting essential genes (Suresh, 2021).

Once an edit has been completed, scientists employ a variety of molecular techniques to confirm the success and accuracy of the gene edit (Thorarinn et al., 2020). The most common way is to use short polymerase chain reaction (PCR) amplicons surrounding the target site. The length is typically under 1000 bps. While this is an effective method for smaller edits, it is not able to detect larger or unwanted changes, especially when they only affect one allele. In order to address these limitations, more

December 2025

Vol 2. No 1.

sophisticated and advanced technologies have been developed. One such technology is Xdrop™ Indirect Sequence Capture, a microfluidic-based enrichment technology capable of isolating DNA fragments up to 100 kilobases (kbs) in size using only a standard primer set positioned several kbs from the editing site (Thorarinn et al., 2020). This enables both short and long-read sequencing; this broader analysis allows for the detection of complex genomic changes, such as large rearrangements or insertions that standard methods might overlook. In a study regarding the effectiveness of different methods, the Xdrop enrichment enabled the detection of an unintended 3.4 kbs plasmid insertion in a CRISPR-edited human cell line, which was missed in the PCR-based assays (Thorarinn et al., 2020).

2.3 Brain-specific Challenges

Delivering CRISPR therapeutics to patients with glioblastoma poses a unique challenge because the central nervous system (CNS) contains protective structures not found elsewhere in the body (Kaushik et al., 2019; Straehla et al., 2022). The most significant is the blood-brain barrier (BBB), a tightly regulated interface of endothelial cells, pericytes, and astrocytes that blocks nearly all large biomolecules from entering brain tissue. This barrier evolved to protect the brain from toxins and pathogens; however, it also prevents the entry of mRNA, viral vectors, RNP complexes, and other CRISPR systems from entering the brain.

Historically, glioblastoma was believed to disrupt the BBB and create “leaky” vasculature (Weller et al., 2015). This led researchers to assume that therapeutics, including gene-editing tools, might be able to penetrate tumor tissue more readily than normal brain tissue because the BBB would be compromised. However, in a study by Straehla and colleagues, only the necrotic core and enhancing rim of the tumor were found to display BBB disruption (Straehla et al., 2022). The majority of the infiltrative glioblastoma cells reside beyond this region, in areas where the BBB remains fully intact. Essentially, this means that CRISPR-based therapies cannot rely on passive leakage or BBB breakdown to reach glioblastoma cells. Because passive permeability is minimal, CRISPR delivery must occur through active, receptor-mediated transport pathways. Supporting this, Straehla et al. found that nanoparticle transport across the BBB depended entirely on LRP1-mediated transcytosis, with transport markedly reduced when this receptor was blocked. This suggests that future CRISPR delivery systems for glioblastoma will need to exploit similar molecular transport mechanisms.

The biology of glioblastoma poses an additional barrier: the tumor infiltrates extensively into healthy brain tissue and co-opts existing vasculature without altering its barrier properties (Straehla et al., 2022). As a result, cancer cells are widely dispersed behind an intact BBB. This makes *ex vivo* CRISPR approaches, such as those used for blood-borne diseases such as sickle cell anaemia, which modify hematopoietic stem cells outside the body before reinfusion, impossible, as glioblastoma cells cannot be fully extracted, edited, and reinfused (Frangoul et al., 2021; Weller et al., 2015).

Together, the intact BBB in infiltrative tumor regions, the need for reliance on receptor-mediated transport, and widespread tumor infiltration are the key obstacles preventing CRISPR-based therapies from reaching dispersed glioblastoma cells. These CNS-specific challenges remain the primary reason

why CRISPR treatments that have succeeded in hematologic and ocular diseases have not yet been translated into clinical glioblastoma trials.

APPLICATIONS OF CRISPR IN CANCERS

3.1 CRISPR in Relation to Cancers

CRISPR-Cas systems have shown promise for both therapeutic and diagnostic applications in cancer research (Huang et al., 2018). In treatment, CRISPR can be used to edit genes in order to disrupt oncogenes, restore the function of tumor suppressor genes, and reprogram immune cells to enhance their anti-tumor activity. For example, CRISPR has been used to edit T-cells to knock out immune checkpoint inhibitors or to introduce chimeric antigen receptors (CARs) (Huang et al., 2018). Furthermore, CRISPR systems have been used to develop better models for studying cancer. For instance, Lentivirus-mediated CRISPR-Cas9 genome editing has been used to create animal models of cancer (Liu et al., 2017). Scientists have successfully used a Lentivirus-mediated CRISPR-Cas9 system to edit cells in a mouse, generating a model of acute myeloid leukemia (AML). These models facilitate the study of tumorigenesis, metastasis, and resistance to therapy in a controlled manner (Huang et al., 2018). In addition to therapeutic purposes, CRISPR has also been pivotal to advancing cancer diagnostics. CRISPR-based detection platforms, especially those utilizing Cas12 and Cas13 enzymes, have been adapted to identify highly specific nucleic acid biomarkers associated with cancer (Huang et al., 2018). Cas12 and Cas13 enzymes are derived from different bacteria than Cas9, leading to distinct PAM sequences and functions (Hillary and Ceasar, 2022). Cas12 edits DNA and cleaves collateral ssDNA, which is helpful for diagnostics; meanwhile, Cas13 targets RNA. These systems can detect single-nucleotide mutations, gene fusions, and aberrant RNA transcripts from circulating tumor DNA or RNA in patient samples, allowing for faster and non-invasive cancer diagnostics (Huang et al., 2018).

3.2 CRISPR in Relation to Glioblastoma:

As research into both cancers and CRISPR continues, scientists are discovering ways to improve the treatment of glioblastoma by using CRISPR technology. Because glioblastoma is driven by complex interactions between tumor cells, stem-like subpopulations, and the immune microenvironment, CRISPR has been particularly valuable for identifying essential genes and mapping pathways that cannot be uncovered through sequencing alone (Feng, 2023). It has enabled three major applications: 1) the identification of targetable tumor genes, 2) the creation of realistic tumor models, and 3) the engineering of immune cells with enhanced anti-tumor activity.

3.2.1 Target Identification

Wang and colleagues conducted a study targeting the gene PDPN (Podoplanin) (Wang et al., 2021). PDPN is often overexpressed in glioblastoma and is linked to immune invasion via Neutrophil activation and Macrophage M2 polarization. The researchers then used CRISPR-Cas9 to knockdown (lower) the amounts of PDPN in glioblastoma cells, followed by an evaluation of apoptosis, proliferation, and tumor

immune microenvironment effects. Through this experiment, the researchers were able to determine that the knockdown of PDPN reduced proliferation and altered immune cell behavior. Furthermore, PDPN was found to promote an immunosuppressive microenvironment, pushing macrophages towards an M2 state and promoting neutrophil degranulation. This would mean that by targeting PDPN, researchers could reduce proliferation and reverse immune suppression.

In another study by Tang and colleagues, CRISPR was used to identify key genes and therapeutic targets in glioblastoma stem-like cells (GSCs) (Tang et al., 2020). The researchers performed a Cas9 genome-wide knockout screen using the GeCKO v2 sgRNA library in patient-derived GSCs; this allowed them to systematically inactivate genes and identify those essential for GSC survival and proliferation. They identified that the transcription factor SMARCC2 was a key regulator of GSCs, and that knocking it out impaired GSC self-renewal, tumor growth, and invasive properties. GSCs are the reason why glioblastoma is so resistant, as they drive recurrence. Identifying this factor has enabled the identification of potential drug targets and the development of more effective treatments.

Furthermore, in another study, Rodvold and colleagues focused on mechanisms that allow glioblastoma cells to survive under endoplasmic reticulum (ER) stress, a condition in which misfolded proteins accumulate within the cell (Rodvold et al., 2020). Glioblastoma cells are highly resistant to stress and treatment, contributing to poor patient outcomes. The researchers identified that HSPB1 (Heat Shock Protein Beta-1) was a crucial stress-protectant protein. They used CRISPR to knock it out of the cells. They found that cells lacking HSPB1 were much more sensitive to ER stress, especially when treated with 12ADT, a drug that induces ER stress. This means that the HSPB1 gene is another target for therapeutic use to make drugs more effective.

3.2.2 Modeling

As treatment plans and proposals continue, the study of the disease has also progressed. A novel way to study glioblastoma is through an organoid disease model (Feng, 2023). These models are made from stem cells and resemble real organs, and scientists are using gene editing to model glioblastoma. A group of researchers used CRISPR to deliver plasmids encoding CRISPR-Cas, gRNAs, and fluorescent reporters via electroporation (Bian et al., 2018). They introduced tumor-suppressant gene mutations such as PTEN, TP53, and CDKN2A, while simultaneously overexpressing oncogenes. This combination allowed them to induce glioblastoma-like tumors, creating the genetic and phenotypic features of human glioblastoma. Notably, the organoids exhibited glial identity markers, glioma-specific gene expression profiles, high proliferation, and invasive behavior. This has allowed researchers to test treatments on more realistic brain-like environments and perform functional screening of glioblastoma-related mutations.

In addition to organoids, researchers have used CRISPR in 3D bioprinted tumor models, which represent a controlled but still physiologically relevant system for studying glioblastoma behavior. Tang and colleagues fabricated a 3D bioprinted glioblastoma framework, which, besides patient-derived tumor cells, also includes brain tumor cells, the extracellular matrix, and immune components, all embedded in a hydrogel scaffold that resembles not only the mechanical properties but also the structure of human brain

tissue (Tang et al, 2020). This has allowed researchers to position different cell types precisely in defined spatial arrangements, enabling the study of tumor-microenvironment interactions in ways that 2D culture systems cannot capture. The bioprinted construct maintained the gradients of oxygen, nutrients, and stiffness found in real tumors, making it feasible to evaluate how gene knockouts influence behavior under realistic conditions. In this case, the use of CRISPR-Cas9 in the model allows for changes in genes such as SMARCC2 or PDPN, followed by examination of changes in invasion, proliferation, angiogenic signaling, and immune modulation in a milieu close to *in vivo* tissue. Tang et al. demonstrated that CRISPR-mediated gene disruptions in this system produced phenotypes that closely matched those observed in mouse models, underscoring the bioprinted platform's validity for preclinical testing. Tang et al. showed that gene changes with the help of CRISPR in the local system led to the development of specific characteristics or traits that, in fact, closely matched the ones they observed when they did experiments on mouse models, thus confirming the bioprinted platform as a valid tool for preclinical testing.

Patient-derived xenograft (PDX) models are among the most biologically accurate systems for studying glioblastoma, as they preserve the genetic heterogeneity, invasive behavior, and therapeutic resistance seen in human tumors. Recently, CRISPR has been incorporated into these models to enable functional interrogation of genetic dependencies within a living brain tumor environment. In a landmark study, Wirth and colleagues performed the first *in vivo* CRISPR/Cas9 dropout screens in orthotopic glioblastoma PDX models to systematically identify genes essential for tumor maintenance (Wirth et al., 2022). They successfully showed that simultaneous CRISPR editing within patient-derived tumors growing in mice, achieved using AAV-delivered sgRNA libraries targeting 125 DNA-damage and apoptosis-related genes, could reveal therapeutic vulnerabilities not detectable *in vitro*. In their screen, the team singled out BCL2, BRIP1, and COPS2 as mutually dependent survival genes: loss of any one gene impaired tumor growth, but combined disruption dramatically reduced tumor viability and clonal expansion. Notably, the PDX setting enabled the authors to observe selective pressure, clonal competition, and invasion patterns under real physiological constraints, yielding results far more predictive than those from cell-culture screens. This study highlights how CRISPR-based functional genomics in PDX models can identify glioblastoma dependencies that have clinical implications and, therefore, constitute a potent *in vivo* platform for therapeutic target validation, thus effectively linking the gap between mechanistic discovery and translational relevance.

3.2.3 Engineering of Immune Cells

In addition to detecting relevant genes and creating visual models, researchers have used CRISPR to modify immune cells to enhance their function. Nakazawa and colleagues conducted an experiment where they knocked out the Cytokine-inducible SH2-containing protein (CIS) gene in natural killer cells (NK cells) before introducing them into glioblastoma-bearing mice (Nakazawa et al., 2023). CIS is a negative regulator of cytokine signalling and therefore dampens NK cell activity. Moreover, oftentimes glioblastoma tumors create an “immunosuppressive environment” that blunts NK cell anti-tumor activity. By removing CIS, the NK cells could be more effective. The researchers found that the deletion of CIS led to stronger NK cell activation and more potent killing of tumor cells. In the mice, the median overall

survival improved as well, going from 41.0 days to 79.5 days. This means that removing CIS from NK cells could be a promising immunotherapy strategy to boost anti-glioblastoma immune responses.

Moreover, due to their ability to redirect T cells to tumor-specific antigens such as EGFRvIII, IL13R α 2, and HER2, Chimeric Antigen Receptor T-cell (CAR-T) therapy has become popular in glioblastoma. However, CAR-T therapy in glioblastoma is limited by multiple factors including antigen loss, functional exhaustion, limited trafficking across the BBB, and immunosuppressive signaling in the tumor microenvironment (Brown et al., 2016). CRISPR gene editing enables overcoming these barriers. In the case of T-cell receptors (TCRs), knocking them out reduces the possibility of the graft-versus-host reaction, while knocking out PD-1 or another inhibitory receptor increases T-cell persistence and decreases functional exhaustion. In a pioneering study, Ren et al. showed that multiplex CRISPR editing is a viable way to block PD-1 in primary human T cells, which resulted in more cytokine production (IFN- γ , TNF- α), cell proliferation, and tumor killing capacity (Ren et al., 2017). Furthermore, CRISPR can be used to knock-in CAR modules into safe-harbor genomic loci, such as the TRAC locus, thereby producing more uniform CAR expression and improved antitumor activity (Eyquem et al., 2017). These improvements address some of the core biological barriers that previously limited CAR-T efficacy in glioblastoma. Even though it is at the preclinical stage, CRISPR-edited CAR-T cells are a prominent immunotherapeutic platform for glioblastoma that combines gene-editing precision with antigen specificity to generate resistant T cells, and have a high functional capacity in the hostile glioblastoma microenvironment.

3.3 Comparison of Methods of Treatment of Glioblastoma

Currently, the most common way to treat glioblastoma is through a combination of surgery, radiotherapy, and a kind of chemotherapy (Wang, 2023). The surgery is a maximal safe resection, which aims for a ≥ 78 -98% removal rate (Lombardi & Assem, 2017). It is then followed by radiotherapy, with 60 Gy over six weeks (adjustments being made for those over 70). Simultaneously, the patient is given Temozolomide (TMZ), a form of oral chemotherapy, which continues afterwards for another six months. However, this treatment plan is not a cure; instead, it prolongs the patient's life. The median overall survival is around 14-16 months with the treatment, but without treatment, it is only 4-6 months. This is a critical limitation in the current treatment protocol. Furthermore, since the tumor is highly infiltrative, it is impossible to remove entirely, making recurrence practically inevitable, often within 6-9 months. The radiotherapy and TMZ have their own side effects, including cognitive decline and fatigue (Wang, 2023). The BBB also poses a challenge as it limits the delivery of drugs (Lombardi & Assem, 2017). These limitations make the exploration of CRISPR as a treatment even more crucial. CRISPR-based methodologies intend to remove or mitigate most of the above restrictions by directly targeting glioblastoma at the molecular level. Surgery and radiotherapy physically remove or kill cancer cells in the tumor mass. CRISPR can disrupt the functions of genes indispensable for tumor survival (e.g., PDPN, HSPB1, SMARCC2) or engineer T cells or NK cells to kill tumor cells. Regular therapies cannot target the tumor's genomic drivers or restore immune function. However, CRISPR has the potential to enable precise, programmable interventions that can directly alter the tumor or the immune system. Moreover, while TMZ is less effective in MGMT-unmethylated tumors, CRISPR-based strategies do not share this limitation (Wang,

December 2025

Vol 2, No 1.

2023). However, CRISPR is not yet a therapeutic alternative for glioblastoma, as it is confronted with serious obstacles such as delivery, off-target risks, and incomplete editing, but a promising avenue for research.

COMPARISON OF CRISPR APPLICATIONS IN GLIOBLASTOMA AND OTHER DISEASES

Understanding the factors that have led to the success of CRISPR therapies in several other human diseases provides crucial context for evaluating their feasibility in glioblastoma. CRISPR has been used or shows potential for disorders such as sickle cell disease, inherited retinal dystrophies, and certain hematologic cancers. However, these therapeutic successes rely on biological and technical factors that differ from the challenges posed by glioblastoma.

4.1 Approved and Advanced CRISPR Therapies in Other Diseases

Several CRISPR-based treatments have reached clinical trials or regulatory approval, demonstrating the technology's therapeutic potential. One of the most significant examples is Casgevy (exa-cel), approved in 2023 for sickle cell disease and β -thalassemia. This therapy utilizes *ex vivo* CRISPR-Cas9 editing to disrupt the BCL11A erythroid enhancer in patient-derived hematopoietic stem cells (Frangoul et al., 2021). This disruption leads to sustained reactivation of fetal hemoglobin and long-term clinical benefits. Because editing occurs outside the body, the challenges associated with *in vivo* delivery are entirely avoided.

Another landmark advancement was EDIT-101, an AAV5-mediated CRISPR therapy for Leber congenital amaurosis type 10 (LCA10), a genetic eye disease that causes severe vision impairment or blindness from birth (Maeder et al., 2019). EDIT-101 targets the CEP290 mutation directly in retinal photoreceptors and has been successful both *in vitro* and in non-human primates, demonstrating promise for the continued development of this therapy.

CRISPR has also been used to engineer T cells for hematological cancers, where disruption of PD-1 improves antitumor activity (Stadtmauer et al., 2020). The engineered cells administered to patients were well tolerated, with durable engraftment observed throughout the study. This study highlighted that pre-existing immune responses to Cas9 do not appear to present a barrier to the implementation of this promising technology.

The success of CRISPR therapies for these diseases is linked to a multitude of favorable biological and logistical factors. Diseases such as sickle cell allow for the removal of samples, which are then modified *ex vivo*, removing the need for *in vivo* delivery. Furthermore, diseases such as sickle cell and LCA10 emerge from well-defined mutations. Editing a single site is sufficient to produce benefits. The retina and bloodstream also lack the complex immunosuppressive barriers characteristic of glioblastoma, as well as the BBB, allowing for engineered immune cells to function effectively. These conditions collectively

create an environment favorable for CRISPR, one that contrasts sharply with the complex biology of glioblastoma.

4.2 Glioblastoma: Challenges in Implementing CRISPR Therapy Approaches Used in Other Diseases

Glioblastoma presents fundamentally different challenges that limit the applicability of strategies that have been successful for other diseases. Unlike monogenic disorders, glioblastoma is polygenic and highly heterogeneous, containing multiple subclones with distinct transcriptional and metabolic profiles (Patel et al., 2014). This makes targeting a single pathway insufficient. Furthermore, as mentioned earlier in this paper, both the BBB and the tumor's microenvironment create challenges. While CRISPR components can be directly infused in retinal tissue or delivered systemically for blood disorders, viral vectors, lipid nanoparticles, and Cas9 ribonucleoprotein complexes are prevented from reaching the glioblastoma tumor, since most infiltrative glioblastoma cells lie behind an intact BBB (Straehla et al., 2022). Furthermore, High levels of TGF- β , IL-10, regulatory T cells, and myeloid-derived suppressor cells create profound immunosuppression, limiting the effectiveness of CRISPR-edited T cells or NK cells, unlike in leukemias where edited immune cells circulate freely and readily engage malignant cells (Quail & Joyce, 2017; Park et al., 2018). Finally, glioblastoma cannot be treated with *ex vivo* editing, since the tumor cells cannot be removed and reinfused without compromising brain function. This means that all edits must occur *in vivo*, making it more complex and challenging.

4.3 Lessons From Other Diseases for Future Glioblastoma CRISPR Therapies

Despite the challenges unique to glioblastoma, successful CRISPR therapies in other diseases offer valuable lessons for glioblastoma research. The precision and durability attained in Casgevy highlight the importance of tools such as base editors and prime editors, which may be safer for brain applications. Similarly, the success of EDIT-101 suggests that localised delivery strategies, including the use of focused ultrasound to transiently open the BBB, could enable more targeted CRISPR administration in glioblastoma (Fishman and Fischell, 2021). Additionally, the stable benefit seen in monogenic diseases underscores the importance of identifying the core driver vulnerabilities in glioblastoma that could serve as future CRISPR targets. By integrating lessons learned from other successful CRISPR applications, researchers may develop next-generation gene-editing tools capable of overcoming the unique barriers posed by glioblastoma.

CRISPR TREATMENT DEVELOPMENT AND FUTURE DIRECTIONS FOR GLIOBLASTOMA

Although CRISPR technologies have advanced rapidly across medical research, their translation into effective glioblastoma therapies remains at an early stage and is primarily preclinical. Progress is driven by improvements in genome-editing precision, brain-targeted delivery technologies, and immunogenomic engineering. These developments provide potential pathways toward overcoming the anatomical and

biological barriers described earlier, while also highlighting the substantial innovation still required before CRISPR-based therapeutics can be considered for clinical trials in glioblastoma.

5.1 Current Stage of CRISPR Research in Glioblastoma

At present, CRISPR applications in glioblastoma remain limited to *in vitro* systems, patient-derived organoids, 3D bioprinted tumor constructs, and mouse xenografts. These platforms have enabled high-capacity gene-dependency screens and modeling of glioblastoma mutations (Feng, 2023), as well as testing of CRISPR-engineered immune cells (Feng, 2023). However, no CRISPR therapy has yet entered human trials for glioblastoma. In contrast, CRISPR therapies for sickle cell disease, LCA10, and hematologic cancers have all reached clinical testing or approval, highlighting the unique obstacles posed by brain tumors (Frangoul et al., 2021; Maeder et al., 2019; Stadtmauer et al., 2020).

5.2 Emerging Gene-Editing Tools for Safer and More Precise Glioblastoma Targeting

In addition to identifying crucial genes that permit the proliferation of glioblastoma, researchers have been focused on creating new CRISPR systems that aim to improve safety and precision. Base editors can convert single nucleotides without creating double-strand breaks, reducing risks of large deletions (Kosicki and Bradley, 2018). Prime editing expands this capability, enabling small insertions, deletions, and base substitutions with fewer off-target events (Anzalone et al., 2019). Although these tools have not yet been tested clinically in glioblastoma, their enhanced safety profiles make them strong candidates for eventual translation.

5.3 Innovations in Delivery Strategies for CNS and Tumor Penetration

Overcoming the blood–brain barrier (BBB) remains the central obstacle for CRISPR therapeutics in glioblastoma. Several delivery innovations are under active investigation. For one, focused ultrasound has been shown to transiently and safely open the BBB, allowing the passage of nanoparticles and viral vectors (Wei et al., 2021). Another innovation is Lipid nanoparticles (LNPs) and polymeric nanoparticles designed to exploit LRP1-mediated transcytosis, which show promise for penetrating the BBB (Straehla et al., 2022). Additionally, Engineered AAV capsids such as AAV-PHP.eB have demonstrated markedly enhanced central nervous system (CNS) transduction efficiency compared to earlier AAV serotypes, offering a potential route for more effective *in vivo* delivery of CRISPR components to brain tissue (Shaw and Suzuki, 2019).

5.4 Pathway Toward Clinical Translation and Outlook

For CRISPR therapies to proceed to human trials, several requirements must first be met. Firstly, safe *in vivo* delivery must be demonstrated in large-animal studies with predictable distribution and low immunogenicity. The therapies will need to include multiplex or combination editing, due to the tumor's heterogeneity (Patel et al., 2014). Another aspect that will need to be taken into consideration is how CRISPR therapies can be integrated with existing therapies, i.e., radiotherapy, TMZ, tumor-treating fields,

or CAR-based immunotherapies. Standardized biomarkers for patient selection and monitoring will also be necessary to design efficient first-in-human trials.

Although clinical translation is at an early stage, advances in editing precision, nanoparticle engineering, viral vector design, and immune cell modification suggest that the field is moving toward clinically viable CRISPR-based therapies for glioblastoma. Future success and progress will depend on combining factors such as tumor-specific delivery strategies, immune system engineering, and high-fidelity genome editing to overcome glioblastoma's resistance mechanisms. While CRISPR has already succeeded in diseases with simpler genetics or accessible tissues, the expanding toolkit of next-generation editing technologies provides a foundation for developing therapies tailored specifically to the complexities of glioblastoma.

CONCLUSION

Glioblastoma remains one of the most treatment-insensitive cancers, with none of the current therapies providing a cure or significant increase in survival period. The paper explains that CRISPR technologies offer a completely different approach: they do not remove or damage tumor tissue but instead change the genetic and immunologic environment, thereby cutting off the tumor's essential survival routes, lowering its resistance to treatment, and increasing anti-tumor immunity. CRISPR-based studies have identified vulnerabilities in PDPN, HSPB1, and SMARCC2, while advancements in organoid, 3D bioprinted, and PDX models have yielded experimental systems that better reflect human glioblastoma biology. Also, immune cells modified by CRISPR, such as CIS-knockout NK cells and next-generation CAR-T cells, are showing promise in preclinical studies for overcoming the tumor's severely immunosuppressive microenvironment.

Despite these advancements in CRISPR technologies and the success in sickle cell disease and retinal dystrophies, none of the CRISPR therapies for glioblastoma have reached clinical stage, which emphasizes the profound and unique challenges this cancer imposes. The preserved BBB in infiltrative areas, the extensive intratumoral heterogeneity, the diffuse invasion of normal brain tissue, and the need for safe and effective *in vivo* delivery are among the factors that hamper the clinical application of CRISPR.

Progress in the future will be primarily influenced by innovations in delivery methods, such as focused ultrasound-mediated BBB opening, receptor-mediated nanoparticles, and engineered AAV capsids, as well as by next-generation CRISPR systems comprising base editors, prime editors, and smaller Cas enzymes intended for safer and more precise genome modification. These advancements may, in fact, be the first steps towards developing tumor-specific, ultra-precise interventions that are capable of tackling the molecular complexity of glioblastoma.

In sum, while CRISPR is not yet a treatment for glioblastoma, it has the potential to evolve into one of the key tools for dissecting glioblastoma, uncovering therapeutic vulnerabilities, modeling the disease, and supporting immune-based strategies. The future of gene editing in terms of its accuracy, delivery, and tumor targeting will be what decides if CRISPR can eventually leap from being an experimental promise to a therapy that is clinically feasible for glioblastoma patients.

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December 2025

Vol 2. No 1.

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