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Advantages, Disadvantages and Risks of CRISPR/ Cas9 Technique for Gene Therapy

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Abstract

The CRISPR/Cas9 system has gained significant attention as a gene editing method in recent years because of its simple design, cost-effectiveness, high efficiency, and ease of use. Additionally, it allows for the simultaneous editing of many locations. Additionally, it may be performed without the use of plasmids, so avoiding the many complications associated with plasmids. CRISPR/Cas9 has shown significant promise in the investigation of genes and genomic activities in microbes, plants, animals, and humans. Transfusion-dependent β-thalassemia (TDT) and sickle cell disease (SCD) are genetic disorders characterized by severe and possibly life-threatening symptoms. In this article, we discuss the use of CRISPR/Cas9 technology in the treatment of these two diseases and FDA-approved drugs based on CRISPR/Cas9. In addition, we address the most important challenges of gene therapy using this technology.

Keywords: CRISPR/Cas9, Gene therapy, β-thalassemia, Sickle cell disease.

Introduction

CRISPR/Cas9, also known as Clustered regularly interspaced short palindromic repeats, is a rapidly expanding tool that has a wide range of applications in therapeutics. It is particularly useful for making modifications to genes, to repair or eliminate any defective genes that cause diseases such as cancer and AIDS (Acquired immunodeficiency syndrome). Additionally, it involves the identification of the target sequence using sgRNA and then replacing a defective gene with a functional one. It provides a high level of efficiency, specificity, and effectiveness after gene editing. However, it also has some unintended effects on non-target areas and may trigger immune responses (1). The efficacy of CRISPR/Cas9 has been predominantly shown in vitro utilizing animal germ cell lines. However, its use in in vivo models is currently limited owing to ethical constraints. Recent developments include research and clinical trials that specifically target the treatment of diverse genetic illnesses. An example of the use of the CRISPR gene knock-in technology is its use in treating Leber Congenital Amaurosis 10 in vivo. CRISPR/Cas is often used to develop disease models for conditions such as Alzheimer's disease, osteogenesis imperfecta, X-linked adrenoleukodystrophy, and

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aniridia-related keratopathy. Furthermore, CRISPR/Cas has shown its role in the treatment of monogenic human genetic diseases, which is a captivating and very hopeful advancement in this technology. The main emphasis of this topic is on the intersection of scientific advances and ethical debates, which are crucial for the continued development and acceptance of CRISPR-based drugs (2, 3).

Function of the CRISPR/Cas9 technology

The mechanism by which bacteria resist the invasion of foreign nucleic acids primarily involves three stages: adaption, expression, and interference. Upon the first invasion of a virus into a bacterium, the bacterium will enzymatically break down the viral DNA into spacer sequences of appropriate lengths. This process occurs by identifying certain PAM sequences and integrating them into the bacterium's CRISPR spacer region. As a result, the bacterium gains the ability to remember and recognize the invading virus in the future. Upon subsequent invasion by the same kind of virus, the bacteria possess the ability to identify and convert the spacer sequences into pre-crRN (4). The pre-crRNA molecule will undergo pairing with tracrRNA and subsequent processing by CnsI and RNase III enzymes to produce mature crRNA. The crRNA identifies and attaches to the foreign DNA using complementary sequences, which is why it is also referred to as guide RNA (gRNA). Cas nuclease cannot independently break foreign DNA. However, when it is joined with a mature tracrRNA and crRNA to create the ribonucleoprotein complex, the Cas protein may be directed by the crRNA to cleave the invading DNA. This is accomplished by detecting the PAM site on the DNA, destroying the foreign DNA and the achievement of self-defence (5).

The CRISPR/Cas system has the potential to edit genes because of the particular target sequence identification capacity of crRNA, the DNA cleavage activity of Cas nuclease, and the DNA repair processes of cells. Cells will initiate their intrinsic repair systems to mend DNA damage and prevent cellular demise when double-strand breaks (DSBs) occur. There are two ways of repairing: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (6). If there are homologous sequences present, cells often use the HDR method to repair DSBs by homologous recombination, which involves incorporating homologous segments into the DNA. By using this repair process, we may deliberately create a repair template (donor) DNA fragment with matching arms and introduce it along with a gene-editing vector into the cell. This enables the integration of the donor DNA into the genomic DNA of the cell, resulting in the successful insertion of the desired gene. In the absence of homologous DNA, cells often activate the NHEJ process, which directly connects the damaged DNA. This repair method is susceptible to base pair insertions or deletions (indels) that may cause gene frameshift alterations, leading to gene knockout (7, 8).

For gene editing purposes, Cas9 and gRNA may be combined into a single vector or separated into two independent vectors. The artificially engineered gRNA is a hybrid RNA molecule that incorporates all the necessary crRNA and tracrRNA elements (9). The anterior segment of the gRNA is referred to as single guide RNA (sgRNA), and its primary function is to identify and bind to the specific target site. This section serves as a framework for attaching to the Cas9 protein. Modification of sgRNA is necessary for each identification of distinct targets. The optimal length of the single guide RNA (sgRNA) is 20 nucleotides, which facilitates the construction of the CRISPR/Cas9 vector. Given that, on average, there is a possible target present in every 8 bp DNA sequence, there exists a considerable number of potential targets that are suited for CRISPR/Cas9. The CRISPR/Cas9 system's popularity in gene editing is due to these features. Upon transformation of CRISPR/Cas9 vectors into target cells, the cells will produce Cas9 and gRNA, enabling them to carry out gene editing.

FDA-approved diagnostics and clinical interventions

Sickle cell disease and β-thalassemia

SCD and TDT are prevalent monogenic illnesses that result from mutations in the hemoglobin β component gene, making them two of the most common genetic disorders globally. SCD is defined by a disruption in the hemoglobin chain and the occurrence of hemolytic anemia. Typically, this condition is managed with blood transfusions and iron-chelation treatment (10). Individuals diagnosed with TDT have sickle-shaped red blood cells that have reduced oxygen-carrying capacity, leading to frequent discomfort. Consequently, these patients often need treatment in a clinical setting, which includes the administration of hydroxyurea, pain medications, and blood transfusions. Both disorders have also been treated via bone marrow transplantation, however finding a suitable match is challenging. Upon genetic analysis of the pathogenesis of these two hematological diseases, the transcription factor BCL11A was discovered to inhibit the production of fetal hemoglobin and γ-bead protein. Sustaining elevated levels of these proteins proved to alleviate the symptoms of SCD and TDT (11).

In 2019, Wu et al. used CRISPR/Cas9 to precisely cut the BCL11A enhancer region in hematopoietic stem cells (HSCs) and effectively reduced its expression without causing notable adverse consequences. In December 2020, clinical data was

disclosed for CTX001, a gene therapy developed by CRISPR Therapeutics in collaboration with Vertex Pharmaceuticals. CTX001 is a single-dose medication designed for the treatment of SCD and TDT. In this clinical experiment, Cas9 was used to precisely cut the BCL11A enhancer region in hematopoietic stem and progenitor cells (HSPCs), resulting in the loss of enhancer function in these cells. This method decreased the level of BCL11A and reinstated the synthesis of γ-hemoglobin and fetal hemoglobin. Afterwards, the researchers performed a transplantation of the altered Hematopoietic Stem and Progenitor Cells (HSPCs) into two patients who were diagnosed with SCD and TDT. The subsequent investigation revealed a significant increase in fetal hemoglobin levels in both individuals 12 months after the treatment. During the last follow-up visits at 18 and 15 months, both patients successfully attained normal levels of fetal hemoglobin. The subsequent treatment of eight patients had comparable outcomes to the first two patients, demonstrating the overall suitability and effectiveness of this approach. Nevertheless, this approach is not entirely innocuous, since both of the first patients encountered different levels of detrimental consequences, which were not life-threatening and subsided after receiving therapy. In a separate clinical trial aimed at treating SCD a method using RNA interference (RNAi) was used to suppress the expression of BCL11A. A lentiviral vector containing short hairpin RNA (shRNA) was created and used to introduce the lentivirus into CD34+cells derived from individuals with SCD. This approach resulted in successful therapeutic outcomes (12, 13).

Transthyretin amyloidosis

Transthyretin (TTR) amyloidosis is a genetic ailment that is primarily caused by the accumulation of amyloid fibrils surrounding cells. This condition mostly affects the human nervous system and heart. TTR monomers are produced in the liver to form tetrameric complexes in their normal condition, which play a role in the transportation of thyroid hormones. Mutant TTRs fail to maintain the tetrameric structure and disassemble before reassembling into amyloid fibrils (14). Nevertheless, individuals suffering from TTR amyloidosis do not exhibit notable symptoms of thyroid hormone insufficiency, indicating that TTR could not play a large role in transporting thyroid hormones. Consequently, lowering TTR expression might be a potential strategy for treating this condition. The primary therapeutic choices are liver transplantation and the use of the tiny chemical tafamidis to stabilize tetramers. However, the latter strategy is not stable and successful.31 Patisiran, a siRNA medication, has been authorized by the FDA to treat this condition. Patisiran inhibits the translation of TTR to decelerate the progression of the illness, but, several injections are necessary on an ongoing basis for the duration of the patient's life. Given the absence of notable adverse consequences from reduced TTR expression, it may be possible to eradicate TTR amyloidosis by using a method that permanently destroys the mutant TTR gene. Gillmore et al. published the findings of clinical studies conducted in June 2021, which examined the in vivo administration of a CRISPR-based gene-editing medication called NTLA-2001. NTLA-2001 is composed of a liver-targeted lipid nanoparticle (LNP) that contains sgRNA targeting the TTR gene and messenger for SpCas9. The LNP has been used several times as a carrier for gene medicines to be delivered to the liver (12, 15).

During initial trials conducted on animal models, NTLA-2001 demonstrated very effective and long-lasting suppression. A total of six individuals were chosen to participate in this experiment, and each patient had the medication injection without experiencing any negative effects during the treatment period. By the seventh day of medicine administration, the patient's blood indicators and liver function indicators had returned to normal levels. Three patients were administered a dosage of 0.1 mg per kg, while the other three patients were given a dosage of 0.3 mg per kg to assess the effectiveness of NTLA-2001. On the 28th day, the blood TTR concentrations of the three patients who got the low dosage were reduced by 47%, 52%, and 56%. The three patients who received the high dose had decreases of 80%, 84%, and 96% in their blood TTR concentrations. This discovery suggests that the effectiveness of NTLA-2001 is directly influenced by the dosage and is very effective. Several months later, the FDA awarded orphan drug status to the approach, acknowledging both NTLA-2001 and the in vivo administration of CRISPR-based gene therapy (16, 17).

Limitations and challenges

Precisely delivering CRISPR/Cas gene medicines to the body has promise for treating illnesses in both laboratory and clinical settings. The great specificity, efficacy, and simplicity of handling this technology make it very desirable for future applications. Nevertheless, scientists have unearthed unforeseen circumstances when using CRISPR technology for gene editing.

Genomic integrity, off-target risk, on-target unwanted editing

Although CRISPR is an effective gene editing technique, it has several drawbacks, including off-target effects, off-target binding, and editing that can lead to dangerous problems. Human cells are

more susceptible to off-target mutations than mice or zebrafish. Gene editing with CRISPR assistance may result in massive deletions, ineffective DEAD spot repair, and inadvertent complicated rearrangements that have a deleterious effect and cause cell death. In recent years, several techniques such as IDLVs (integrase-defective lentiviral vectors), DISCOVER-seq (discovery of in-situ Cas off-targets and verification by sequencing), CIRCLE-seq (Circularization for in vitro reporting of cleavage effects by sequencing), digenome-seq etc., have been employed to identify off-target genome modifications. Several techniques can be employed to achieve this, such as the manipulation of Cas9 nucleases, the use of dimeric Cas9 nuclease, the combination of Cas9 with artificial inhibitory domains, the design of optimized sgRNA, the "hit-and-run" approach involving the transfer of Cas9 protein instead of the Cas9 gene, and the utilization of non-viral delivery methods to reduce off-target effects. dCas9, or dead Cas9, is more effective in therapeutic applications because it can alter the expression of target genes without causing double-stranded breaks (DSBs). On the other hand, Cas9n exclusively induces single-stranded breaks (SSBs) (18).

Chromosomal disorganization

Researchers have expressed significant worry regarding the safety of CRISPR-based gene-editing technologies. Double-stranded DNA is typically cleaved by Cas9, leading to the activation of NHEJ repair. As a consequence, the resulting repaired DNA strands often exhibit a little deficiency or excess of base pairs, which aligns with the anticipated outcome. Nevertheless, throughout the process of validating editing efficiency, researchers discovered instances of significant base losses and chromosomal structural translocations. These errors can result in conditions such as malignant tumors and are unacceptable in clinical settings, notwithstanding their low likelihood of occurring. CRISPR/Cas9's repetitive cleavage of target genes is a significant factor in the occurrence of chromosomal translocations and deletions. Yin et al. integrated an exonuclease structural domain with Cas9 to mitigate the frequency of these alterations. This structure executes post-processing shortly after cleavage, hence minimizing the probability of generating intact ends. This method efficiently inhibits the complete restoration of the DNA strand, hence preventing the replication of the genome by Cas9. The researchers combined spCas9 with improved three-prime repair exonuclease 2 (TREX2) to create a modified version of Cas9 called Cas9TX. Through research involving modified T cells and other cells, it was evident that Cas9TX effectively inhibited chromosomal translocations in comparison to the high-precision SpCas9 version (19).

Stimulation of the immune system

The required treatment area or cell count varies considerably based on the specific target organ or tissue, the kind of disease, and its severity. For instance, when aiming at a small organ with a restricted number of cells, it is generally quite simple to reach a therapeutic threshold with a single injection. Nevertheless, tissues and organs of considerable size, INCLUDING skin, liver OR lung contain a significant number of cells (20). Hence, the capacity for recurrent administration is a crucial characteristic for effectively treating illnesses that impact extensive tissues or organs (20). The delivery technique must provide sufficient safety and be capable of evading the host immune system to facilitate multiple administrations. As mentioned before, AAV is rendered ineffective by an anti-capsid antibody, which is why the second injection does not work. The number of potential administrations significantly affects the extent of the treatable region for genome editing therapy. Repeated dose for local injection enables gradual extension of the treated area over a while. Systemic infusion can lead to the accumulation and enhancement of both the treated region and the efficacy through multiple treatments. It is crucial to understand that the idea of administering many doses is only relevant when the effects of genome editing build up over a significant period (21).

The potential worry of immunogenicity to CRISPR-Cas9 protein has been explored for therapeutic applications due to the presence of preexisting anti-Cas9 antibodies in over 50% of the human population. These antibodies are specifically targeted against the routinely utilized SaCas9 and SpCas9 bacterial orthologs. To reduce the problem of immunogenicity, the Cas9 protein can be modified to eliminate immunogenic epitopes. Transient immunosuppression can be used as a countermeasure to reduce immunity to both Cas9 and the delivery payload. When Cas9 RNP is directly delivered with CPP, the presence of the anti-Cas9 antibody can greatly impact the efficacy of delivery. This is because the antibody's binding may conceal the CPP portion, hindering its function. However, it is worth noting that the majority of alternative delivery systems do not externally expose the Cas9 protein. Therefore, the impact of the anti-Cas9 antibody in serum should be negligible, particularly throughout the delivery process. Utilizing a nanocarrier technology to encapsulate the Cas9 enzyme could potentially offer a temporary safeguard against neutralizing antibodies and degradation caused by nuclease/protease activity (22).

Limitations of targeted delivery

Deviation from the desired position

Viral and nonviral vectors are typically

administered systemically to animals, effectively protecting CRISPR gene medicines from degradation in the blood and tissues. Nevertheless, unaltered vectors are vulnerable to being taken up by metabolic organs within the body. The CRISPR/Cas system retains its functionality while entering nontarget cells but genetically alters healthy cells, perhaps resulting in unforeseeable ramifications. Enhanced vehicles are required to minimize the infiltration of gene medications into non-targeted cells (23). Methods to enhance delivery functionality involve applying a biofilm to the carriers or including peptides that are recognized by receptors on the target cells. The design of nanoparticles that are responsive to the specific microenvironment of the target organ can boost the concentration of gene drugs. This can be achieved by considering factors such as pH fluctuations, reactive oxygen species (ROS), and adenosine triphosphate (ATP) levels. The nanomaterial shell undergoes disintegration in a particular environment, so revealing the core, which subsequently penetrates the cell via endocytosis. Nevertheless, if the microenvironment in specific diseased tissues is not substantially distinct from that of other tissues, it is possible to create a nanoparticle that can be triggered by various situations to release its contents, so enabling targeted treatment for the disease. Furthermore, there have been advancements in the development of CRISPR/Cas9 delivery systems that are responsive to light, magnetic fields, and ultrasound. These systems are designed to ensure accurate and targeted delivery (23).

Biocompatibility

It is necessary to create appropriate vectors for candidate cells to minimize the risk of negative reactions resulting from off-target CRISPR/Cas9 systems. For the vector to effectively perform its role, it must possess biocompatibility, a strong encapsulation capacity, and the ability to penetrate the cell membrane. Three hundred thirty-six When building the system, it is important to take into account the immune reaction that occurs when the material is delivered into the body. It has been observed that Cas9 proteins often obtained from *S. pyogenes* and *S. aureus* can cause an immunological response in humans. To address this difficulty, a modified version of Cas9 that does not contain exons that trigger a response was administered to juvenile and adult mice via AAV, resulting in the successful prevention of both humoral and cellular immune responses. Furthermore, the modified Cas9 must also be carried in a vector specifically engineered to prevent activation of the host immune response. Within living organisms, viruses, lipids, and exosomes can evade the immune system, while synthetic chemical nanoparticles need to be coated with a protective layer on their surface. This coating not only stabilizes the polymer in the bloodstream but also requires the presence of modified CD47 protein. Moreover, plant exosomes have a higher probability of evading immune system recognition due to their inherent source. Plant exosomes are considered a safer option for delivering CRISPR/dCas9 systems due to the significant distinctions between plant and mammalian diseases. Nevertheless, the study of administering gene medicines through plant exosomes is still in its early stages, particularly due to the variability in properties among exosomes produced by various plants (12, 24).

Potential risk of cancer

Recently, two separate study teams discovered that CRISPR-Cas-induced DSBs could trigger the p53 signaling pathway. Genetically modified cells have the potential to become cancer-initiating cells, meaning they could start the development of cancer. Therefore, using CRISPR-Cas systems for treatment could unintentionally raise the risk of cancer. While there is yet no conclusive evidence to establish a direct link between CRISPR-Cas-mediated genome editing and the development of cancer, this research serve as a cautionary reminder regarding the use of CRISPR-Cas systems in gene therapy. It serves as a reminder that there is still a considerable distance to cover before CRISPR-Cas technologies can be effectively utilized in humans (25).

Application and advantages of CRISPR-Cas systems in the study of human diseases

Utilizing CRISPR-Cas systems to create animal and cell models

Animal models play a vital role in comprehending gene function, investigating the development of human diseases, and advancing the creation of new medications. Nevertheless, conventional techniques for producing animal models are intricate, expensive, and time-consuming, significantly restricting the use of animal models in fundamental medical research and preclinical investigations. Following the identification of CRISPR-Cas systems, a sequence of genetically altered animal models has been created with remarkable efficiency. Mice are extensively utilized as model animals in scientific studies and are considered the primary model animals for research on human diseases. Researchers have successfully created numerous genetically engineered mice models. However, because of significant disparities in species characteristics between humans and rats, they are unable to offer reliable evaluation and sustained monitoring for the study and management of human diseases (26).

Research has shown that the effectiveness of CRISPR-Cas mediated genome editing is greater in

laboratory conditions (in vitro) compared to living organisms (in vivo). Therefore, the utilization of genetically modified cell models can significantly reduce the time required for medical research. Previously, scientists have utilized CRISPR-Cas systems to conduct genetic alterations on many types of cell lines, including tumor cells, adult cells, and stem cells, to replicate a range of human disorders (26).

Uses of CRISPR-Cas systems in the diagnosis of diseases

CRISPR-based molecular diagnostic technology is advancing at a rapid pace, and it has been distinguished as one of the world's top ten science and technology advancements in 2018 due to the discovery of novel Cas enzymes (Cas12, Cas13, etc.) and the growth of CRISPR-Cas platforms (27). The 'collateral cleavage' activity of Cas13 enzymes is distinct from that of Cas9. This activity can result in the cleavage of nearby non-target RNAs following the cleavage of the target sequence (28). Feng Zhang et al. created SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing), an in vitro nucleic acid detection platform that uses Cas13a and is based on the "collateral cleavage" activity of Cas13. It consists of fluorescent RNA reporters, sgRNA that targets specific RNA sequences, and Cas13a. When the Cas13a protein recognizes and cleaves the target RNA, it will cut the report RNA and release the detectable fluorescence signal to achieve the diagnostic objective. Researchers have employed this approach to identify tumor DNA mutations, genotype human DNA, distinguish pathogenic microbes, and detect viruses. Subsequently, Feng Zhang et al. enhanced the SHERLOCK system and rebranded it as SHERLOCKv2, which is capable of simultaneously detecting four viruses. Collateral cleavage activity is also observed in Cas12 enzymes, in addition to Cas13 CRISPR-Cas systems: Overview, innovations and applications in human disease research and gene therapy (29).

Conclusions

CRISPR-Cas-mediated genome editing technologies are considered a significant milestone in the field of molecular biology in the 21st century due to their ability to provide a flexible and accessible method for modifying, regulating, and visualizing a genome. The extensive application of CRISPR-Cas systems in gene function analysis, human gene therapy, targeted medication development, animal model construction, and livestock breeding has thoroughly demonstrated their significant potential for further development. Nevertheless, there are still some constraints that must be surmounted in the practical applications of CRISPR-Cas systems, and significant efforts must be made to assess their long-term safety and efficacy.

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Shabnam Radbakhsh: data curation; editing and review. Hoda Namdari Moghaddam: investigation and writing. All authors read and confirmed the final manuscript.

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