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REVIEW ARTICLE

Applications of CRISPR-Cas9 Genome Editing Technology in the Treatment of Human Diseases

Elif Baser

Nesibe Aydin Yildizlar Science High School, Dumlupinar Bulvari Baglica Kavsagi, No: 1/A, Etimesgut, Ankara, 06890, Turkey; elifbaser2201@gmail.com

ABSTRACT: Genome editing (GED) technologies have advanced substantially and are currently being tested for the treatment of human diseases. Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) GED technology has advanced biotechnology by providing a relatively simple and cost-effective method for creating precise deoxyribonucleic acid edits compared to other GED technologies. This technology has become a promising method for treating diseases by enabling the targeted disruption, modification, or editing of pathogenic genes. However, several factors, including disease heterogeneity, off-target effects, and technical and ethical challenges in its application, limit the clinical use of CRISPR-Cas9. This article examines the therapeutic efficacy of CRISPR-Cas9 technologies in the treatment of numerous conditions, including cancer, neurodegenerative diseases, viral infections, allergic and immunological diseases, and hematologic diseases.

KEYWORDS: Cellular and Molecular Biology, Genetics, Biotechnology, CRISPR-Cas9, Genome Editing, Human Disease.

Introduction

In recent years, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins, which originated from bacterial and archaeal adaptive immune systems, have become widely used for genome editing (GED) in biotechnological and biomedical research.[1] GED technologies can enable the precise modification of deoxyribonucleic acid (DNA) sequences in living cells. In addition to CRISPR-Cas9, other advanced GED technologies include zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs).[2,3] ZFNs are composed of the zinc finger proteins and the non-specific FokI cleavage domain and are involved in protein-protein interactions and DNA transcription regulation in eukaryotes.[4] Notably, ZFNs can only identify nucleotide triplets in DNA, which limits their site selection.[5] TALENs also consist of FokI endonuclease and a complex of transcription activator-like effectors.[6] Although they are more specific than ZFNs because they can detect a single nucleotide, the larger size of TALENs when compared to ZFNs may cause difficulties in their packaging and delivery.[7]

CRISPR-Cas9 involves a single guide RNA (sgRNA) sequence and the Cas9 endonuclease. The sgRNA is formed by the composition of trans-activating crRNA (tracrRNA) and CRISPR-RNA (crRNA), enabling the Cas9/sgRNA ribonucleoprotein complex to recognize and bind to the target DNA sequence.[8] After DNA targeting occurs, Cas9 creates double-strand breaks (DSBs) in the target region of DNA. The DSBs are then repaired by non-homologous end joining (NHEJ) and homology-directed repair (HDR) mechanisms using template DNA. In the absence of homologous DNA sequences, the cell undergoes NHEJ, which creates small deletions or insertions around the cut site. Since there is no DNA template for repair, this process is somewhat random and thus highly error-prone. If donor DNA with homologous strands matching the genomic DNA is provided, a DNA molecule with high homology to the target region is inserted into the genome following a double-strand break.

The key difference between CRISPR-Cas9, ZFNs, and TALENs is that CRISPR-Cas9 uses an RNA-mediated sequence-specific cutting technique, while the others are protein-based DNA editing techniques. CRISPR-Cas9 provides several advantages over ZFNs and TALENs, such as improved cost-effectiveness, higher target specificity, and the relative ease of designing sgRNAs for diverse target DNAs. Moreover, CRISPR-Cas9 can enable direct genome editing in the embryo and the simultaneous induction of multiple mutations.[9] CRISPR-Cas systems are classified into class 1 and class 2, and the Cas 9 protein belongs to class 2.[10] CRISPR-Cas9 is preferred for GED due to its simplicity when compared to other systems.[11]

CRISPR-Cas9 GED technology is among the most promising and popular methods for treating multiple diseases through GED. This article will discuss various diseases for which CRISPR-Cas9 technology is being tested in treatment.

Discussion

CRISPR-Cas9 GED technology in human diseases:

Cancer:

Cancer is a critical disease resulting from genetic and epigenetic reasons, representing one of the predominant causes of death globally. Because of the complexity of its mechanisms, current cancer treatments (e.g., chemotherapy, surgery, radiotherapy, and targeted therapy) have shown limited effectiveness, highlighting the need for alternative treatment approaches. In recent years, GED has become one of the leading strategies in treating cancer. GED facilitates the manipulation of tumor-associated genes using CRISPR-Cas9 technology, with studies

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indicating that this technology can be effective in cancer treatment. Furthermore, CRISPR-Cas9 technologies continue to hold promise in areas such as tumor immunotherapy and overcoming resistance to chemotherapy drugs.[12] CRISPR-Cas9 GED-based therapy is being investigated in many cancers, such as colorectal cancer, breast cancer, and hepatocellular cancer.[13-15] A primary cause of tumors is the dysregulation of cell proliferation through the activation of proto-oncogenes and the inactivation of tumor suppressor genes.[16] Tumor development or growth can be prevented by knocking out oncogenes or restoring tumor suppressor genes and restoring their function with CRISPR-Cas9 technology.[17]

CRISPR-Cas9 technology can target specific disease-causing genes, as well as different types of genes, such as *epidermal growth factor receptor (EGFR), tumor protein P53 (p53), telomerase reverse transcriptase, v-Raf murine sarcoma viral oncogene homolog B, breast cancer gene (BRCA), human epidermal growth factor receptor 2*, and *Kirsten rat sarcoma viral oncogene homolog (KRAS)*. Liu *et al.* reported that editing genes, such as *E-cadherin, p21,* and *hBax*, via CRISPR-Cas9 decreased cell motility, inhibited cell proliferation, and initiated apoptosis in bladder cancer cells.[18] The *myeloid cell leukemia-1 (MCL-1)* gene plays a role in cell differentiation, proliferation, and tumorigenesis and is becoming a novel target for cancer therapy. It has been shown that the knockout of the MCL-1 gene via the lentiviral (LV) CRISPR-Cas9 system initiates apoptosis of Burkitt lymphoma (BL) cells and, through the repeated induction of sgRNA, impairs tumor development in a xenograft model.[19] CRISPR-Cas9 technology has also been reported to upregulate further sex combs-like 1 protein expression, which significantly reduces leukemia cell growth.[20] Besides, the CRISPR-mediated correction of protein kinase C mutations in a xenograft model has been shown to inhibit colon tumor growth.[21]

Carcinogenic viral infections are a crucial factor in cancer development. For example, hepatitis B virus (HBV) and hepatitis C virus (HCV) are associated with hepatocellular carcinoma (HCC), Epstein-Barr virus (EBV) with nasopharyngeal carcinoma, Hodgkin lymphoma, and Burkitt lymphoma, and human papillomavirus (HPV) with cervical cancer. By using viral genome-specific Cas9-sgRNA and targeting viral oncogenes, cancer cell death can be induced. In cervical cancer, the expression of HPV oncoproteins E6 and E7 causes malignant transformation in normal cells. Studies have shown that interfering with the *E6* and *E7* genes via CRISPR-Cas9 inhibits cervical cancer development and reverses the malignant phenotype.[22,23] Price *et al.* produced *Francisella novicida* Cas9 (FnCas9) to accurately interfere with the HCV RNA genome and observed that FnCas9 suppressed HCV infection by reducing viral protein production.[24] It is also possible to modify the EBV genome via CRISPR-Cas9. In one reported study, seven anti-EBV gRNAs were administered together via transfection into a B cell line obtained from a BL patient with latent EBV infection to disrupt the genome, which gave rise to the initiation of apoptosis, the inhibition of cell proliferation, and a reduction in viral load.[25]

CRISPR-Cas9 technology is increasingly being employed in cancer immunotherapy, which activates the innate or adaptive immune system through various methods rather than directly targeting the tumor. The goal is to mobilize T cells by acting on various control points such as cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death ligand 1 (PD-L1). Studies are currently underway to strengthen the response of T cells against cancer cells and their capability to successfully destroy cancer cells by knocking out the *PD-1* or *CTLA-4* gene, utilizing CRISPR-Cas9 systems.[26,27]

CRISPR-Cas9 can also be used to reprogram tumor stroma to achieve anticancer effects. A study comparing fibroblasts taken from normal ovarian tissue and ovarian cancer tissue found that cancer-associated fibroblasts expressed more genes involved in glutamine synthesis, and that inactivation of glutamine synthetase—essential for glutamine production—efficiently inhibited cancer cell growth.[28] It has been suggested that inactivation of the glutamine synthetase gene via CRISPR could be implemented to inhibit tumor growth.[29]

Drug resistance is a significant problem in cancer treatment since resistance to a drug can result in resistance to different drugs, resulting in a condition called multidrug resistance (MDR). Many mechanisms serve roles in the emergence of drug resistance, including the increased function of drug efflux pumps like the ATP-binding cassette (ABC), increased DNA repair capacity, increased drug detoxification, disruption of cellular signaling pathways, increased epithelial–mesenchymal transition, and decreased apoptosis.[30,31] Recently, studies have examined CRISPR-Cas9 technology to overcome drug resistance in different cancer treatments. A significant cause of drug resistance in cancer cells is the overexpression of ABC family transporters, which facilitate the excretion of cancer drugs. One study observed that intervention of the *ABCB1* gene via the CRISPR-Cas9 increased doxorubicin (DOX) accumulation in cancer cells, thereby enhancing chemosensitivity.[32] Another study indicated that targeting the *MDR1* gene via the CRISPR-Cas9 quadrupled drug uptake in drug-resistant breast cancer cells.[33] A similar phenomenon has been observed in ovarian cancer and osteosarcoma cells, demonstrating that the CRISPR-Cas9 can substantially reduce *ABCB1* gene expression and increase cancer cell sensitivity to DOX.[34,35]

Cyclin-dependent kinases (CDKs) are enzymes that serve roles in cell proliferation, DNA repair regulation, and the cell cycle. Increased levels of these enzymes in cancer cells suggest that inhibiting these enzymes may have promising results for cancer treatment. For instance, the inactivation of CDK11 in osteosarcoma cells utilizing the CRISPR-Cas9 system has been shown to increase cell death as well as reduce cancer cell invasiveness.[36] CDK6 was also found to be elevated in palbociclib-resistant breast cancer cells, while the CRISPR-Cas9-mediated knockout of CDK6 increased palbociclib sensitivity and induced cancer cell death.[37] Moreover, glutathione S-transferase may contribute to drug resistance by augmenting the detoxification of chemotherapy drugs, upregulating the conjugation of chemotherapy drugs with glutathione, and attenuating apoptosis. For example, disabling

glutathione S-transferase using the CRISPR-Cas9 leads to an increase in the cytotoxic effect of chemotherapeutics in colorectal cancer cells.[38]

Overexpression of *BRCA1* is associated with chemotherapy resistance.[39] Since *BRCA1* mutations can be variable and difficult to target, targeting PARP1, a synthetic lethal partner of *BRCA1*, is a more feasible approach. In breast cancer cells, intervention of *PARP1* with the CRISPR-Cas9 increased sensitivity to chemotherapeutics, resulting in lower drug doses being required to achieve therapeutic efficacy.[40]

Mutations affecting tumor suppressor genes and oncogenes, such as *P53* and *KRAS*, have been shown to serve important roles in drug resistance. In colorectal cancer, targeting *KRAS* via CRISPR-Cas9 technology markedly decreased tumor cell size and improved the efficacy of cetuximab in inducing apoptosis.[41] Moreover, inactivation of mutant *TP53* in osteosarcoma cells with CRISPR-Cas9 systems both increased sensitivity to DOX and reduced the overexpression of anti-apoptotic proteins.[42] CD44 is regarded as a surface marker of cancer stem cells and is also a marker of drug resistance. It has been shown that deactivating CD44 via the CRISPR-Cas9 system can prevent invasion and metastasis in osteosarcoma cells.[43]

Mutations in EGFR are found in various cancers.[44,45] While EGFR inhibitors are useful for treating non-small cell lung cancer (NSCLC) in which EGFR mutations are detected, there are problems with drug resistance.[46] It has been reported that disruption of mutant EGFR in NSCLC cells using the CRISPR-Cas9 leads to a reduction in tumor size, an increase in tumor cell death, and an extension of survival time.[47] RECQL4 helicase is a protein that serves roles in drug resistance and DNA repair, and can increase *MDR1* expression. It has been demonstrated that deactivating RECQL4 via the CRISPR-Cas9 technique in glioma cells can increase the toxic effect of temozolomide on glioma cells.[48]

Neurodegenerative diseases:

Alzheimer's disease (AD), Parkinson's disease (PD), spinocerebellar ataxia (SCA), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) are widespread neurodegenerative diseases, all of which involve a gradual deterioration of neuronal structure and function, leading to nerve loss over time.

AD affects memory, speech, behavior, and decision-making ability and can eventually lead to dementia.[49] In this disease, extracellular amyloid plaques composed of amyloid β-protein (Aβ) and neurofibrillary tangles containing hyperphosphorylated tau protein are found. To date, conventional treatments have not been successful in preventing Aβ formation or in clearing toxic proteins from the brain. Although AD is generally sporadic, a small number of cases are familial and result from autosomal dominant mutations in the *amyloid precursor protein (APP), presenilin-1 (PSEN1),* or *presenilin-2 (PSEN2)* genes, with *PSEN1* mutations often being the primary cause; these mutations increase the production of Aβ42, which is more prone to aggregation than Aβ40.[50] A study reported that CRISPR-Cas9 technology could selectively impair the *PSEN1M146L* allele and alter the Aβ42/40 ratio in carriers of this mutation.[51] Similarly, another study reported that CRISPR-Cas9 correction of neurons containing fibroblasts with *PSEN2N141I* mutation could correct the Aβ42/40 ratio.[52] One APP gene, known as the Swedish *KM670/671NL APP*, causes increased levels of Aβ protein.[53] Guyon *et al.* attempted to modify the APP gene using a CRISPR-Cas9-based approach and ultimately reduced Aβ protein accumulation.[54] The *apolipoprotein E (APOE)* gene is a predisposing factor for sporadic AD, with several variants, among which *APOE4* confers an increased risk for the disease. A study using CRISPR-Cas9 technology demonstrated that *APOE4* affects Aβ metabolism.[55] Additionally, Wadhwani *et al.* attempted to correct the *E4* allele to the *E3/E3* genotype in induced pluripotent stem cells (iPSCs) obtained from AD patients using the CRISPR-Cas9 method and observed that *E3* neurons were highly resistant to cytotoxicity and also exhibited decreased tau phosphorylation when compared to *E4* neurons.[56]

PD is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, resulting in decreased dopamine levels reaching the striatum. This results in impaired motor function and the emergence of symptoms such as rigidity, tremor, and bradykinesia. Intracytoplasmic Lewy bodies containing α-synuclein and ubiquitin are observed in this disease. Most patients with PD have idiopathic disease, and only a small number have mutations in various genes. In their studies utilizing the CRISPR-Cas9 technology to delete the *A53T-SNCA* gene, Yoon *et al.* observed significant improvement in various conditions associated with PD, such as motor symptoms, reactive microgliosis, α-synuclein overproduction, and dopaminergic neurodegeneration.[57] Furthermore, Chen *et al.* used CRISPR technology to correct neurons generated from iPSCs taken from PD patients with *A53T* and *SNCA* mutations and investigated the role of *SNCA* in the nucleus. Realizing that the absence of *SNCA* caused resistance to Lewy pattern formation, they reported that CRISPR-Cas9 technology could be a promising treatment for PD.[58] Moreover, other studies have been conducted to delete the *PRKN, PARK2, PINK1,* and *ATP13A2* genes using CRISPR-Cas9 technology.[59,60] Loss-of-function mutations in the *DNAJC6* gene have been observed in patients with early-onset PD, and CRISPR-Cas9 techniques were used in human embryonic stem cells to reveal these mutations.[61]

As the most common inherited neurodegenerative disorder, HD is an autosomal dominant disorder and is caused by the formation of a prolonged polyglutamine strand in the N-terminal area of the huntingtin protein due to CAG (cytosine-guanine-adenine) repeat expansions in the *huntingtin* gene (*HTT*). Accumulation of this protein in the brain affects molecular and cellular functions, causing a variety of symptoms, including decreased cognitive function, incoordination, chorea, or dystonia.[62] Since HD occurs due to a single mutation, it is a disease amenable to treatment with GED technology. Shin *et al.* utilized CRISPR-Cas9 technology to inactivate the mutant *HTT* allele selectively.[63] Additionally, Yang *et al.* reported that inactivating *HTT* in HD140Q-KI mice with CRISPR-Cas9 could eliminate the neuronal toxicity caused by polyglutamine accumulation in the brain and improve motor dysfunction.[64]

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Another study in R6/2 mice containing exon 1 of the human *HTT* gene showed that interfering with the mutant *HTT* gene by utilizing CRISPR-Cas9 reduced the formation of neurotoxic inclusions by twofold and improved motor deficits.[65]

ALS is a motor neuron disease characterized by degeneration in the motor neurons of the central nervous system. It can cause muscle weakness, muscle atrophy, paralysis, and in severe cases, respiratory failure and even death.[66] Sporadic cases account for approximately 90% of patients, while familial ALS, which is inherited, accounts for the remaining 10%. The pathogenic genes related to ALS are *C9orf72, SOD1, TARDBP,* and *FUS*.[67] Deng *et al.* demonstrated that CRISPR-Cas9-mediated editing was effective in targeting *hSOD1* in two different *hSOD1-G93A* transgenic mouse models of ALS, resulting in a disease-free state.[68] Yun *et al.* utilized the CRISPR-Cas9 to target gene correction associated with the *SOD1 E100G* mutation in iPSCs from a person with ALS, and these iPSCs were then differentiated into motor neurons.[69] In addition to these studies, Chen *et al.* investigated the CRISPR-Cas9 technique to modify specific point mutations associated with ALS, resulting in either correcting *I114T* mutations in *SOD1* in patient iPSCs or introducing new mutations such as *G94A* in *SOD1* or *H517Q* in *FUS* in iPSCs.[70]

SCA is an autosomal dominantly inherited neurodegenerative disease characterized by speech difficulties and disorders related to balance and coordination. There are more than 40 genetic variants of SCA, with the SCA1, SCA2, SCA3, and SCA6 subtypes constituting the majority of patients. He *et al.* reported that paired sgRNA/Cas9 and homologous recombination techniques could repair the 74 CAG expansion in exon 10 of *ATXN3* in SCA3-iPSCs, thereby reducing mutant ataxin-3 protein expression.[71] Song *et al.* also attempted to correct mutations in SCA3-iPSCs from SCA3 patients using homologous recombination and CRISPR-Cas9 technology.[72] In another study conducted on fibroblasts obtained from SCA1 patients, a reduction in the formation of both healthy and mutated *ATXN1* protein was observed using the G3 and G8 sgRNA/Cas9 complexes.[73]

Viral infections:

Acquired immunodeficiency syndrome (AIDS) is a serious illness caused by both human immunodeficiency virus (HIV)-1 and HIV-2. HIV-1 has higher pathogenicity, and *in vivo* active HIV-1 replication leads to CD4+ T cell depletion. Despite significant success with highly effective antiretroviral therapies, the persistent integration of HIV-1 into the host genome makes this disease difficult to treat. Numerous studies have been designed using CRISPR-Cas9 technology for the treatment of this infection, and in these studies, many genes have been targeted, especially long terminal repeats (LTRs).[74-76] LTRs are repetitive, identical sequences of DNA that aid in the insertion of retroviral DNA into the host chromosome and trigger HIV-1 gene expression. One study reported that CRISPR-Cas9 can cause the degradation of latent HIV-1 provirus by mutating the LTRs.[74]

HBV is a hepatotropic DNA virus and can cause liver cirrhosis and hepatocellular carcinoma. Studies have been conducted using CRISPR-Cas9 to target and cut various functional loci in HBV genomes, such as the surface antigen region and the reverse transcriptase gene.[77,78] The dead Cas9 (dCas9), a variant of Cas9, has also been shown to restrict HBV replication without causing fragmentation of the HBV genome.[79]

Infections with HCV, an RNA virus, can be treated effectively with appropriate pharmacological interventions; however, drug-resistant variants that are untreatable with current therapies may emerge. In this context, manipulation of the HCV genome using CRISPR-Cas9 may offer an alternative therapeutic approach. FnCas9, derived from the bacterium *Francisella novicida*, can target a bacterial mRNA, which can subsequently suppress a viral gene.[80] One study demonstrated that the CRISPR-FnCas9 system exhibits inhibitory activity against the HCV RNA genome in eukaryotic cells. It has been reported that the interaction of FnCas9 with the HCV RNA genome can suppress both the viral environment and genome replication.[24]

HPV infection can cause cervical cancer in women. To date, most studies have generally targeted the *E6* and *E7* regions of the HPV genome. Successful anti-HPV applications have been achieved by directly disrupting the HPV genome with the CRISPR-Cas9 system, and inhibition of tumor growth has been observed in most studies.[81,82]

Herpes simplex virus (HSV)-1, a neurotropic virus, can cause morbidity and even mortality in humans.[83] Studies have investigated the CRISPR-Cas9 targeting of viral proteins: one demonstrated that knockout of ICP0, a protein required for *HSV-1* gene expression, inhibited viral replication, while another reported that the disruption of UL7 using CRISPR-Cas9 reduced genome replication and attenuated neurovirulence.[84,85]

EBV causes infectious mononucleosis and is linked to some types of cancer.[86] Notably, interfering with the EBV nuclear antigen and latent membrane protein domains in the EBV genome with CRISPR-Cas9 has been reported to decrease proliferation and viral load in BL cells with latent EBV infection.[25] One study used two gRNAs to delete the promoter zone of BART, which encodes viral microRNAs, and this was shown to reduce miR-Bart3 expression and viral load in latently infected EBV models.[87] Another study by the same team reported that CRISPR-Cas9 technology reduced viral DNA loads and replication rates in nasopharyngeal carcinoma cells containing latent EBV.[88]

Cytomegalovirus (CMV) can cause serious infections in immunocompetent patients, particularly those undergoing organ transplantation. One study reported that using CRISPR-Cas9 technology to knock out UL122/123, a key CMV gene, disrupted CMV replication.[89] The paper reported that simultaneous targeting of different areas of the viral genome could disrupt multiple viral functions, and that a multiple-targeting strategy was superior to a single-targeting strategy.[89]

Allergic and immunological diseases:

Although CRISPR-Cas9 technologies have emerged relatively recently, they have made significant contributions to the field of allergy and immunology. Janus kinase 3 (JAK3) is a tyrosine kinase involved in signal transduction and leads to cy-

tokine production via T helper 2 cells. Its deficiency leads to a decrease in circulating natural killer (NK) cells and T cells, and disrupted B cell function. JAK3 is involved in the pathogenesis of various diseases, primarily allergic asthma.[90] Correcting the *JAK3* gene deficiency in patients with severe combined immunodeficiency via CRISPR-Cas9 regulated T cell improvement and normalized the number of NK and T cells.[91]

Mucine 18 (MUC18) is a transmembrane glycoprotein that is a marker of tumor progression in melanoma and is also expressed in the airway epithelial cells of patients with some pulmonary diseases, such as chronic obstructive pulmonary disease and asthma.[92] CRISPR-Cas9-mediated blocking of the *MUC18* gene in nasal airway epithelial cells leads to a significant decrease in IL-8, suggesting that *MUC18* serves a proinflammatory role.[93]

There has been growing interest in manipulating causative allergens in allergic diseases via GED to delete allergenic genes or minimize allergens. CYP11A1 is the first enzyme in the steroidogenic pathway that converts cholesterol to pregnenolone.[94] One study showed that children with peanut allergy had approximately 50-fold increased CYP11A1 gene expression in activated peripheral blood CD4+ T cells. Besides, significant increases in interleukin (IL)-4 and IL-13 produced by peanut-specific T cells were also demonstrated, and CYP11A1 mRNA levels were related to increased IL-13 production.[95] As a result of the study, targeting CYP11A1 via CRISPR-Cas9 technology decreased gene expression of CYP11A1 by over 50%, which in turn significantly reduced IL-13 production.[95]

The CRISPR-Cas9-mediated deletion of Fel d 1, the primary cat allergen, has been achieved in individuals with cat allergy.[96] Peanuts are a major cause of food allergy, and CRISPR-Cas9 has been tested in the development of non-allergenic peanuts. Ara h 2 is one of the peanut allergens, and it has been shown that disrupting the Ara h 2 gene in peanut protoplasts using CRISPR-Cas9 technology is possible.[97] Furthermore, CRISPR-Cas9-mediated β-lactoglobulin editing was performed in goat fibroblasts to prevent goat milk allergy.[98] Studies have also tested CRISPR-Cas9 technology to prevent egg allergy.[99]

Hematologic diseases:

CRISPR-Cas9 technology is also being investigated for the treatment of monogenic hematologic diseases, such as β-thalassemia, which result from mutations in the human *hemoglobin beta (HBB)* gene. This disease is characterized by hemolytic anemia and decreased β-hemoglobin production, leading to ineffective erythropoiesis. Using CRISPR-Cas9, it may be possible to repair the *HBB* mutation in β-thalassemia patients.[100] Another strategy for treating this disease is to reactivate the fetal hemoglobin gene by disrupting the *BCL11A* gene, a fetal hemoglobin silencer. It has been shown that the disruption of *BCL11A* by CRISPR-Cas9 may be beneficial in the treatment of β-hemoglobinopathies by facilitating the achievement of threshold levels of functional fetal hemoglobin.[101]

CRISPR-Cas9 technology can also correct the *HBB* gene mutation in sickle cell anemia.[102] Furthermore, Fanconi anemia is a genetic disorder in which most patients have mutations in the *FANCA, FANCC,* or *FANCG* genes, and this disease results in progressive bone marrow failure and a decrease in all blood cell types. CRISPR-Cas9 technology was used to correct a mutation in the *FANCC* gene in patients with Fanconi anemia.[103] X-linked chronic granulomatous disease is one of the primary immunodeficiencies arising from mutations in the *CYBB* gene and can cause fatal infections. Notably, homology-directed repair-based GED technologies have been used to adjust the *CYBB* mutation.[104] Wiskott-Aldrich syndrome (WAS) is a primary immunodeficiency disorder that exhibits X-linked inheritance and is caused by mutations in the WAS gene. Studies aimed to edit the WAS locus in a leukemia cell line using CRISPR-Cas9 technology.[105]

CRISPR-Cas9 GED technology in other diseases:

CRISPR-Cas9 is used for gene editing in cardiovascular disorders, particularly inherited lipid disorders. The *proprotein convertase subtilisin/kexin type 9 (PCSK9)* gene and the *angiopoietin-like 3 (ANGPTL3)* gene serve important roles in lipid hemostasis.[106] For example, the gain-of-function mutation in the *PCSK9* gene is associated with atherosclerosis and hypercholesterolemia.[107] In one study, a gRNA targeting a sequence in the mouse *PCSK9* gene and an adenoviral vector coding SpCas9 were implemented to knock out PCSK9 alleles in hepatocytes, resulting in an approximately 90% reduction in blood *PCSK9* protein levels and an approximately 40% reduction in blood cholesterol levels.[108] In another study, a chimeric liver-humanized mouse model was created, and it was shown that the *PCSK9* gene in hepatocytes was regulated by approximately 50%, and the *PCSK9* protein levels in the blood were decreased by approximately 50%.[109] In another mouse study using an adenoviral vector coding a cytosine base editor and a gRNA targeting the *ANGPTL3* glutamate-135 codon, 35% liver *ANGPTL3* editing was achieved, and approximately 50% decrease in blood *ANGPTL3* protein levels and 20% decrease in blood cholesterol levels were observed.[110]

Transthyretin (TTR) acts as a carrier for thyroxine and vitamin A by forming tetramers. However, unstable TTR monomers can accumulate in the heart, causing cardiomyopathy or polyneuropathy in the nerves. These accumulations are more likely to be caused by the mutant protein. By suppressing TTR expression in hepatocytes, it may be possible to treat diseases caused by the mutant protein. Studies in mice and primates have demonstrated that administering lipid nanoparticles containing a gRNA targeting the *Ttr* and *TTR* genes and SpCas9 messenger RNA resulted in liver remodeling in approximately 70% of all species, resulting in significant reductions in blood TTR protein levels.[111,112]

Duchenne muscular dystrophy (DMD) results from a mutation in the X-linked DMD gene. This gene is responsible for the production of dystrophin, a structural protein that fortifies myofibrils by binding cytoskeletal proteins. Characterized by progressive muscle weakness, this disease can lead to respiratory distress, swallowing problems, and cardiac complications over time. Four different preclinical studies using mouse models with mutations in exon 23 of the *DMD* gene have examined

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the effectiveness of CRISPR-CAS9 editing in the treatment of DMD.[113-116] Two of these studies used the systemic intravenous administration of adeno-associated viruses (AAVs), with one vector containing SaCas9 and the other containing a gRNA targeting the regions surrounding exon 23.[113-114] In another study, an AAV vector containing SaCas9 and two gRNAs was systemically administered to delete exons 21, 22 and 23.[115] In the latter, tandem AAVs were utilized, with one vector containing SpCas9 and the other containing a gRNA targeting the mutant sequence in exon 23.[116] In all of these studies, dystrophin expression was significantly restored in skeletal muscle and the heart, resulting in significant ameliorations in muscle function. In another study, a new rearrangement was induced in a dog model of DMD by the intravenous administration of an AAV vector expressing SpCas9 and a single gRNA targeting a region near the 5' end of exon 51, leading to partial reconstruction of dystrophin expression in heart and skeletal muscle.[117]

Inherited retinal diseases are a significant cause of blindness, yet there is currently no treatment available. However, gene therapy may be a potential treatment for these patients. Due to several distinct characteristics, the human eye could be a priority for gene therapy. Notably, the eye has immune privilege, meaning it has a higher tolerance to antigens.[118] Furthermore, the presence of a blood-retinal barrier reduces the likelihood of viral vectors administered to the eye during gene therapy migrating to other sites.[119] With current ophthalmic techniques, it is easier to reach the target area for gene therapy, resulting in a lower viral load. CRISPR-Cas9 technology has been utilized to target the allele-specific disruption of mutant genes in the treatment of some retinal diseases, such as wet age-related macular degeneration (AMD), Meesman epithelial corneal dystrophy, retinitis pigmentosa (RP), and Leber congenital amaurosis type 10 (LCA10).[120-122] Choroidal neovascularization (CNV) in wet AMD causes the deterioration of central vision. Researchers tested CRISPR-Cas9 to disrupt *Hif1a*, a gene involved in the development of CNV, in mice with laser-induced CNV and reported a significant decrease in CNV area when compared to controls in AAV-Hif1a-treated eyes without impaired cone function.[122] Two studies reported the use of CRISPR-Cas9 to reprogram mutation-susceptible rod cells into cone cells in an established RP model.[123,124] Notably, the transcription factors Nrl and Nr2e3 regulate rod cell differentiation, with the absence of either factor promoting rod-to-cone cell differentiation.[125] Researchers observed the downregulation of rod-specific genes and the upregulation of cone-specific genes following CRISPR-Cas9-mediated targeted gene deactivation of Nrl or Nr2e3 in mice.[123,124]

Cystic fibrosis (CF) is an autosomal recessive disease that affects the lungs and digestive system. CRISPR-Cas9 technology is also being tested to modify mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which causes CF, and may be a useful approach for treatment in the future. In one of the studies conducted for this purpose, CRISPR-Cas9 was successfully used to correct the CFTR mutation in iPSCs.[126] Another study attempted to correct the CFTR gene using CRISPR-Cas9 in cultured stem cells.[127]

Conclusion

Although CRISPR-Cas9 technology holds great promise for the treatment of diseases, it has certain shortcomings that must be addressed, the most significant of which are off-target effects. The effectiveness of the CRISPR-Cas9 technology depends on various factors, including Cas9 activity and delivery, sgRNA design, target site selection, and off-target effects.[128] Despite sgRNA being designed to target specific DNA sequences with high accuracy, the complexity of the genome, which contains numerous highly homologous sequences, can result in sgRNA binding to off-target sites and inducing unintended gene editing. As such, off-target effects of CRISPR-Cas9 have been demonstrated in some clinical applications, and these effects have been reported to be more common than anticipated.[129] Off-target effects can lead to experimental problems and even false results or phenotypes, which severely limit the application of CRISPR-Cas9 systems.

Accurate delivery of CRISPR-Cas9 cargo to the target tissue also represents a significant challenge. The most commonly used viral vectors for GED are AAVs and LVs.[130] While AAVs are less immunogenic, have serotype specificity, and have a good safety profile, mild toxicity has been demonstrated at high doses in animal models.[131] In addition to their non-immunogenic advantages, LVs also possess pseudotyping capabilities, allowing for changes in cellular tropism. Packaging limitations can also represent an important issue when using these vectors. Moreover, it is also probable that the generated CRISPR-Cas9 complexes can stimulate the host immune system.[132]

In addition to technical difficulties, ethical and legal issues are among the key challenges that could hinder the real-world applicability of the CRISPR-Cas9 systems. Specific deactivation of targeted genes with CRISPR-Cas9 systems may be possible via modifying the genome in the germline. While mutations induced by CRISPR-Cas9 GED in the embryonic period can produce new *in vivo* models and treat different diseases, there is concern that they could lead to ecological imbalances.[133,134] Embryonic genome editing can lead to the inheritance of undesirable changes, leading to irreversible impacts on future generations. Importantly, uncontrolled mutations can create organisms carrying modified genetic traits, potentially disrupting ecological systems.

In conclusion, CRISPR-Cas9 GED technology is a promising method for discovering important disease-associated genes and uncovering potential therapeutic targets. As the technical and ethical challenges encountered with CRISPR-Cas9 technology are overcome, the safety and effectiveness of treatment strategies will increase and continue to hold promise for the treatment of numerous diseases in the future.

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■ Author

Elif Baser is a high school student at Nesibe Aydin Yildizlar Science High School, Ankara. She has a special interest in computer science, chemistry, and molecular biology. She was named a mathematics finalist in a national research competition and aspires to be a scientist in the future.