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Review article

Clustered Regularly Interspaced Short Palindromic Repeat Paired Associated Protein 9 (CRISPR-Cas9) System and Its Opportunity in Medical Science - A Narrative Review

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Abstract

The clustered regularly interspaced short palindromic repeat paired associated protein 9 (CRISPR-Cas9) is a site-specific genome editing tool that enables scientists to edit or introduce genetic mutation at will. CRISPR-Cas9 consists of two essential key players; a programmable RNA called single guide RNA (sgRNA) and the Cas9 protein which functions as a molecular scissors that does the cutting. Since its discovery, CRISPR-Cas9 has received vast attention due to its simplicity, convenience, and superior precision of use. Its application extends into various fields including the health sciences where it has been used to enhance the understanding of pathogenesis and help in therapeutic intervention. Despite the promising potentials and applications of CRISPR-Cas9, there are several aspects that need to be addressed including the method of delivery, off-target cutting and ethical issues in human germline modification. The purposes of this review are to perform a comprehensive literature search of publications on the CRISPR-Cas9 system and to highlight potential applications of CRISPR-Cas9 in the field of medical sciences. In this present review, we discuss the background of CRISPR-Cas9, its mechanisms of genome modification and its applications in the medical field including its use in the study of animal model production, genetics, multifactorial and complex diseases. In addition, we also discuss the limitations associated with CRISPR-Cas9 application. CRISPR-Cas9 has accelerated medical studies and facillitate the collection of vast amounts of information. However, its limitations should be further studied in order to reap its greatest benefits.

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1. Introduction

Understanding of deoxyribonucleic acid (DNA) has allowed substantial progress in various scientific fields including the areas of biomedical and pharmacological research. DNA is the hereditary material which stores information as a code characterized by an arrangement of four chemical bases: adenine (A), guanine (G), cytosine (C) and thymine (T). Nowadays, enormous data collected from the Human Genome Project and DNA sequencing experiments has improved the understanding and the correlation of diseases to their underlying genetic variation. According to the National Human Research Institute in the United States of America, abnormal DNA sequence mutation may be partial or whole contributors to genetic disorders. Mutational effects can be detrimental, beneficial, or neutral, depending on their context or location.

Realizing the role of gene sequences in body function, genome modification techniques were developed to explore the causes, effects, and potential treatment of gene-related diseases. Besides the discovery of an endogenous DNA strand break-repair mechanism, Zinc Finger Nucleases (ZFNs) and Transcription Activator-like Effector Nucleases (TALENs) were developed and optimized to allow site-specific double strands breaks (DSBs). Despite providing the solution for site-specificity problem to a certain extent, the use of ZFNs and TALENs were limited due to their complexity, low flexibility, high cost and extensive labor requirement [1-4]. In 2012, the CRISPR-Cas9, short for clustered regularly interspaced short palindromic repeat (CRISPR) paired with CRISPR-associated protein 9 (Cas9), was introduced as a site-specific genome-editing tool. CRISPR-Cas9 technology enables scientists to introduce or edit any genetic mutation at will, hence curing gene-related disorders or creating animal models by deleting, adding or changing the targeted DNA sequence [2, 4].

Comparing to TALENs and ZFNs, CRISPR-Cas9 is a simple technique and provides significantly higher targeting efficiency, depending on the species and method of delivery [1]. The CRISPR system has greater potential and broader application in biological science. Numerous studies have proven the clinical importance and applicability of CRISPR-Cas9 in animal model production and in the therapeutic intervention. For instance, genomic modification using CRISPR-Cas9 was used to successfully produce a hepatic cancer mice model and the first ever insulin-deficient piglet model which mimic the human pathological condition [5, 6]. In a cystic fibrosis (CF) study, the mutated gene which is responsible for regulator protein malfunction was corrected successfully using CRISPR-Cas9, resulting in normal gene expression and function [7, 8]. Similarly, normal cell karyotypes were also achieved in patient-derived induced pluripotent stem (iPSC) cells through CRISPR-Cas9 mediated modification of the mutated β-globin gene (HBB) and alteration of HBA2 gene which helped to correct the imbalance between α and β subunits [9-11]. Another successful story was the disruption of the growth of oncogenic cells such as Burkitt lymphoma cells, HPV-positive SiHa and Caski cells via CRISPR-Cas9 mediated alteration that induced suppression and apoptotic events [12]. This genomic tool also helps in determining the role of genes and reverting the drug-resistance causative genes [13, 14].

Despite all these advantages, some aspects including the efficiency of delivery method, off-target cleavage and ethical issues are major challenges in ensuring CRISPR-Cas9 specificity and efficiency [7]. The delivery of the CRISPR complex is a crucial step that influences the mutation outcome [15]. The limitations of the delivery vehicle may affect CRISPR-Cas9 performance and restrict its clinical use. Besides, the occurrence of cleavage at unintended sites will cause an undesired mutation and may cause major adverse events [2]. Moreover, ethical issues concerning the safety and efficiency of human germline cells and embryo alteration prevents the extensive use of CRISPR-Cas9 as editing tool. According to the National Library of Medicine in the United States, many countries have banned genome editing involving germline cells and embryos.

The aims of this review are to perform a comprehensive literature search of publications on the CRISPR-Cas9 system and to highlight potential applications of CRISPR-Cas9 in the field of medicine. This will enhance an understanding of the latest advancement in CRISPR-Cas9 technology and its potential use, especially in the field of medical sciences. Besides, the search should also help in identification of which studies should be explored in the future and increases the chances of discovering new solutions with better efficacy and therapeutic use to cure the diseases.

The literature search for this narrative review focused on the opportunity for use and previous applications of CRISPR-Cas9 in medicinal and pharmacological fields. We looked for English articles published between 2015 until 2021 in Medline, PubMed, Embase and Google scholar. The search keywords include "CRISPR-Cas9 AND therapy", "CRISPR-Cas9 AND genome editing", "CRISPR-Cas9 AND applications", and "CRISPR-Cas9 AND diseases". Articles were selected according to their relevance towards the study and their potential to meet predefined inclusion and exclusion criteria.

2. CRISPR-Cas9

Genome editing allows scientists to manipulate DNA by introducing breakage and hijacking the repair system to give the desired alteration. ZFNs and TALENs were introduced as alternatives to the classical method which lacked efficiency, and was time consuming and laborious [4]. Both artificial proteins consist of DNA-binding and DNA-cleavage domains that need to be dimerized in order to execute the effect [4, 7]. DNA cutting by ZFNs usually forms 5' overhangs while TALENs can be varied [16]. Even though ZFNs and TALENs provided better specificity and precision at the time, their applications were limited due to complexity, expensiveness, and laborious manufacturing steps [1-3].

A simpler method named CRISPR-Cas9 was introduced and has received a lot of attention due to its simplicity, better precision and convenience compared to the previous methods. Unlike ZFNs and TALENs, the DNA recognition in CRISPR-Cas9 system relies on a programmable single guide RNA (sgRNA) instead of a protein design. The sgRNA interacts with the target site on the basis of Watson-Crick Base Pairing for site recognition and specificity. The designation or construction process of CRISPR-Cas9 is simple and less tedious than ZFNs and TALENs. It involves encoding of sgRNA, Cas 9 and if genome knock needs to be performed, the construction of a donor template is added to direct homology directed repair. The CRISPR-Cas9 technique is capable of inducing one or more mutations simultaneously, which means its application in research requires considerably less time and resources compared to ZFNs and TALENs. CRISPR was said to be one of the best genome editing techniques [17]. This technology has been widely used to study human diseases and treatment in cells as well as in animal models.

Initially, CRISPR was discovered in 1987 as the unique sequences of *Escherichia coli* comprising 5 repeated segments where each segment was made of 29 nucleotides (nt) and separated by non-identical 32-nt fragments known as spacers [18, 19]. CRISPR is said to be an adaptive immune system in bacteria and archaea against invading bacteriophages. Later in 2012, these alternate spacer nucleotide sequences were officially defined as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). In the same year, CRISPR-Cas9 was introduced as a site-specific genome-editing tool. Due to vigorous study, the CRISPR key components was successfully identified along with their biological functions, which subsequently contributed to CRISPR categorization or classification. In 2015, a CRISPR-Cas system was categorized into 5 types and 16 subtypes. The combination of computational and experimental studies further increased the discovery of CRISPR-Cas diversity, which consisted of 2 classes, 6 types (I, II, III, IV, V, VI) and 33 subtypes [20]. CRISPR-Cas9 falls under type II CRISPR system which is characterized by one

effector protein and its capability in gene screening and editing activities [21]. Discussion on programmable guiders in CRISPR-Cas9 DSBs activity and their potential as genomic editing tools produced a great breakthrough at that moment.

2.1 Mechanism of CRISPR-CAS9 system

Cas9 protein and programmable RNA called single guide RNA (sgRNA) are the key molecules in the CRISPR-Cas9 system. Figure 1 shows a schematic representation of CRISPR-Cas9 along with the site of cleavage. Cas9 derived from *Streptococcus* pyogenes consists of 6 domains; REC I, REC II, Bridge Helix, PAM Interacting, HNH and RuvC that are responsible for binding and cleavage actions. The Cas9 specific cutting action is closely related to its linkage with sgRNA [4,22]. The sgRNA is a single RNA strand that is made up of two parts: CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). In nature, crRNA and tracrRNA exist as two separated structures. But for clinical and experimental use, crRNA and tracrRNA are chemically bound together into a single RNA structure (sgRNA). This avoids the need to express several molecular structures during an experiment.

The crRNA is a 20 nucleotide (nt) sequence obtained by transcription of alternate repeat-spacer unit of the CRISPR array that is generated from invading nucleic acid. In CRISPR-Cas9 system, the crRNA sequence is designed according to the desired DNA target site. The crRNA recognizes the DNA target sequence that is complementary to its sequence (spacer), while the tracrRNA assists Cas9 to specifically cut the intended DNA strand site effectively by linking the Cas9 with crRNA, promoting crRNA maturation and facilitating Cas9 activation [23]. Basically, tracrRNA is the binding scaffold for Cas9.

Besides crRNA, target recognition is also greatly influenced by the presence of a short DNA sequence called a protospacer adjacent motif (PAM). PAM helps to differentiate between the self and non-self-DNA. Once Cas9 binds to the sgRNA, it undergoes conformation rearrangement and become activated. An activated Cas9 will bind to sequence matches to its PAM, melt the bases, pair with a complementary sequence of sgRNA, and cleave 3-4 nucleotides upstream to the PAM sequence using Ruv C and HNH domain [4]. To completely cut double strand of DNA, the HNH domain is responsible for cutting the target DNA strand, while the RuvC domain helps to cut the other strand located opposite to target strand [22].

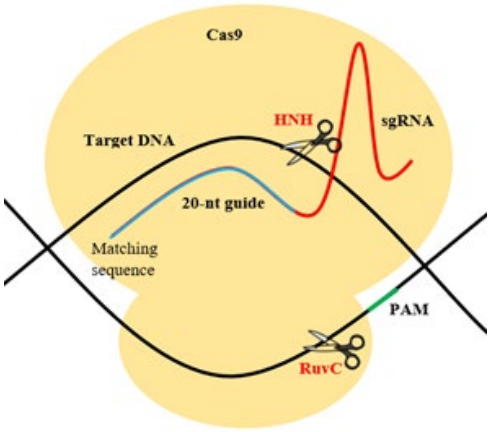

Figure 1. Schematic representation of CRISPR-Cas9

Figure 2 explains the outcomes possible from the endogenous DNA repair mechanism. The breakage will be fixed by the host natural repair mechanism either via Homology directed repair (HDR) or Non-homologous end joining (NHEJ). By controlling these repair machineries, genomic modification can be done through addition, deletion or substitution at the target DNA sequence [24]. NHEJ is an error prone process that frequently leads to disruption of gene function. The end ligation by NHEJ is often associated with random insertions or deletions (indels) and even substitution at DSB site [22]. Unlike NHEJ, HDR requires the presence of a DNA donor template containing a sequence of interest flanked by homology arms to guide its repairing process. The desired mutation can be obtained by HDR that supports precise gene modification involving gene deletion, knock in, correction, or mutagenesis [22]. However, due to the low prevalence of HDR, difficulty in preparing donor DNA and resistance to cellular assault are the common challenges for HDR [25].

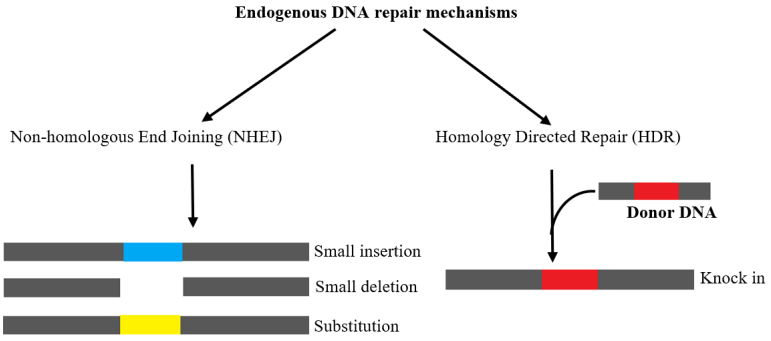

Figure 2. An illustration of outcomes from endogenous DNA repair mechanisms

2.2 Applications

The efficient and site-specific action of CRISPR-Cas9 enables its application in various experimental procedures with different subjects. Numerous studies have employed CRISPR-Cas9 and proved the excellent capability of this system to overcome the issues related to somatic and germline mutation [13]. One of them was in creating animal models that mimic human pathological conditions [26]. The production of animal models is crucial in biomedical research as it supports further understanding of the diseases and directs further research for potential treatments. CRISPR-Cas9 facilitates animal model production and its single step approach discards the need for previous labor intensive and time-consuming procedures [5].

One of the methods available for genomic alteration is by using direct injection of sgRNAs and Cas9 mRNA into single cell embryos. This approach was proven to be effective in generating the monkey, rat and mouse models [27-29]. Besides, CRISPR-Cas9 was also used to knock out INS in pig somatic cells, a process which successfully generated the first ever insulin-deficient piglet model with typical diabetes symptoms since birth [6]. Analyzation confirmed that INS mutations in 21 cell colonies and 5 stillborn piglets derived from reconstructed embryos bearing mutated INS. All these experimental results confirmed the ability of CRISPR-Cas9 as an efficient and rapid genome editing tool in introducing one or several mutations simultaneously.

Another remarkable study that demonstrated the CRISPR-Cas9 potential in constructing animal models involved a sheep model bearing cystic fibrosis disease. CRISPR-Cas9 which was designated to target exon 2 and 11 sequences was used to edit the primary fetal fibroblasts of sheep (*Ovis aries*). Then, the clone embryos were transferred using the somatic cell nuclear transfer

method into 73 estrus-synchronized recipients. The reported result stated that the new born CFTR–/– sheep exhibited a severe pathological state similar to CF disease in humans [30].

In the generation of a mouse cancer model, the Cre-Lox recombinant system that targets the loxP fragment sequence (Cre-LoxP) was widely used to generate models [31]. The Cre-LoxP system consists of the enzyme Cre recombinase and its recognition site, LoxP. It is a site-specific recombinase method used for genetic excision [31]. Unfortunately, due to complexity of genetic events in cancer, it is difficult to determine the role and effect of a specific mutation. In comparison to CRISPR-Cas9, this conventional method is relatively more expensive and also time-consuming [32]. By using the CRISPR-Cas9 system, these limitations have become trivial [13]. In the development of a mouse model sarcoma, CRISPR-Cas9 was reported to be able to generate multiple subtypes of tissue sarcomas in mice [32]. Comparing to the mice model generated by the Cre recombinase system, the whole exome sequencing showed that both produced models had similar characteristics. However, CRISPR-Cas9 is a better option due to its rapid and efficient performance. Another study that employed CRISPR-Cas9 to alter the tumor suppressor genes, Pten and p53, successfully induced concurrent knockout of these genes. As a result, bi-allelic mutations of both genes occurred and resulted in the development of hepatic cancer which possessed great similarities to the one mediated by Cre-LoxP technology [33]. An alteration of Pten alone increased Akt phosphorylation and lipid accumulation in liver cells. All those mentioned studies revealed the opportunity for the CRISPR-Cas9 system to be used in facilitating the production of animal models that involved states similar to human pathological states.

Apart from applications in creating animal model, the current state of gene editing technologies and their usage in the treatments of human disease is very possible. CRISPR technology is quickly transforming life science research around the world and speeding up the progression into clinical trials. An example of the promising progress is in genetically transmitted diseases, multifactorial diseases and complex disease.

2.2.1 Cystic fibrosis

Cystic fibrosis is an autosomal recessive disease characterized by the mutated cystic fibrosis transmembrane conductance regulator (CFTR). CFTR encodes for the cAMP-regulated anion channel and its mutation leads to dysfunctional protein, causing disruption of multiple organ system. Most fatalities result from progressive lung infection and inflammation with the onset being during early childhood. There are numerous types of CFTR mutations which are responsible for the disease development and progression. However, the most common occurence is an alteration of ΔF508 [34]. The homozygous deletion of F508 (CFTR F508 del) erases phenylalanine located at 508, causing CFTR disruption, and consequently leads to protein misfolding, premature degradation and reduction of CFTR transfer and stability [35-37].

Genomic alteration by editing tools such as ZFNs, TALENs and CRISPR-Cas9 has the potential to recover the function of an endogenous CFTR gene [34]. In two different studies involving patient derived intestinal stem cells and the lung epithelial cells, CRISPR-Cas9 was successfully used to correct the mutation and produce the desired outcome. In the cultured intestinal stem cells of organoids isolated from CF patients, CRISPR-Cas9 mediated homologous recombination at exon or intron 11 of CFTR amended the F508 del [38]. The findings showed successful restoration of the normal expression and phenotype of CFTR. This study highlighted the opportunity of using CRISPR-Cas9 in editing stem cells ex-vivo that can be transferred to cystic fibrosis patient in-vivo.

A similar positive result was also obtained in the study of lung epithelial cells derived from patient induced pluripotent stem cells (iPSCs). The customized CRISPR system and excisable selection system was utilized to correct the mutation. An efficient result of genomic correction was obtained with an absence of the local genomic footprint, and scar-free cells were subsequently

produced [8]. Later, mature airway epithelial that originated from the corrected iPSC cells showed normal CFTR expression and functionality [8]. Following the result, the possibility of off-target mutation was studied to detect its occurrence. The outcome showed no random integration or *indels* occurred in the predicted off-target site, thus proving the site-specific action and safety of CRISPR-Cas9.

Another parallel study was done by employing the CRISPR-Cas9 system along with a single stranded oligodeoxynucleotide (ssODN) to observe the efficiency of CRISPR-Cas9 corrective action in two cell cultures bearing p. F508del mutation; CFTE29o- and iPSCs [39]. Though the CRISPR-Cas9 technique was able to modify the causative gene of cystic fibrosis, its application required additional processes such as cell selection and cultivation as the frequency of the corrected cells event was rare or low. The findings concluded that genome editing in iPSCs followed by another clinical process can be beneficial for cystic fibrosis treatment. The autologous transplantation of corrected cells helps to avoid cell rejection and graft versus host-disease problems. All studies discussed above have demonstrated the opportunity of CRISPR-Cas9 application in the study of the pathological and treatment of CF.

2.2.2 Thalassemia

Thalassemia, specifically β-thalassemia, is a haematological disorder caused by a mutated β-globin gene (HBB). This abnormality consequently leads to reduction or absence of haemoglobin (HB) subunit β (HB β chain) [11]. β-thalassemia is one of the most common thalassaemic syndromes in Thailand and Southeast Asia, while thalassemia generally is the most common genetic disease worldwide [40, 41]. Currently, no medications are available to cure this disease and continuous transfusion is the regular treatment which help patients to survive [42]. Unfortunately, frequent transfusions eventually lead to multi-organs impairment. Allogeneic hematopoietic stem cell transplantation, which is the only curative procedure for beta thalassemia, is limited due to the lack of HLA-matched donors and the risk of several drawbacks/complications after transplantation [41, 43]. Therefore, another alternative must be discovered or developed as an effective strategy to prevent or correct the mutation, and this hopefully will greatly help reducing the morbidity and mortality associated with thalassemia.

CRISPR-Cas9 has been used in several studies to correct the mutation in HBB, and it is considered as a promising novel therapeutic approach for effective haemoglobin production [42]. A study involving Beta thalassemia (β-Thal) patient-specific induced pluripotent stem cells (iPSCs) reported that utilization of the CRISPR-Cas9 system in correcting homozygous CD17 (A-T) point mutation of HBB have greatly improved the differentiation efficacy of the corrected cells. The successful restoration of HBB expression produces cell lines with normal karyotypes and full pluripotency as human embryonic stem cells (hESCs) [10]. The result of this study was in agreement with the study of the CRISPR-Cas9 mediated correction of 4-bp deletion (-CTTT) at codon 41/42 (CD41/42) [9]. Both studies demonstrated the capability of CRISPR-Cas9 system in correcting mutation. In the CD41/42 mutation study, Lenti-CRISPR V2 vector was used to transfect β-Thal iPSCs. It was reported that the cells exhibited normal karyotyping and retained full pluripotency. The corrected CD41/42 cell clones also underwent the whole-exome sequencing process, which further confirmed the correction of mutation and revealed only minimal mutation load associated with gene editing present in the exome [9].

The CRISPR-Cas9 system was also programmed to target the mutated haemoglobin E in the HBB gene and induce HDR process with a single-stranded DNA oligonucleotide as a donor template [41]. Sanger sequencing confirmed the CRISPR-Cas9 corrective action of HbE/β-thalassemia iPSCs. The corrected clones successfully differentiated into progenitor cells and erythroid cells that were capable of becoming mature HBB gene and protein.

In CD34+ hematopoietic stem cells and progenitor cells (HSPCs), CRISPR-Cas9 was used to modify the erythroid-specific enhancer region of BCL11A and to increase production of foetal haemoglobin [44]. An elevated level of foetal haemoglobin helps to reduce the morbidity and mortality related to transfusion-dependent β-thalassemia (TDT). Intravenous infusion of CTX001 (autologous CRISPR-Cas9 edited CD34+ HSPCs) was successfully performed in a TDT patient but it was associated with several adverse events. A year later, the patient had an increase in fetal hemoglobin expression and independency toward transfusion. This indicates that the CRISPR-Cas9 mediated mutation was durably maintained and helped to improve TDT. The long-term corrective effect by CRISPR-Cas9 was once again reported in recent study that involved α-globin locus alteration in human hematopoietic stem/progenitor cells (HSPCs). Both β and α subunits are responsible for the development of the main oxygen carriers in erythrocytes (HbA, $\alpha_2\beta_2$) [11]. The lack of a β subunit causes free α-globins to precipitate and disrupt the cell membrane, consequently cause hemolysis and ineffective erythropoiesis. CRISPR-Cas9 was used to edit the HBA2 gene which resulted in downregulation of α-globin and upregulation of β-globin. The edited HSPCs were reported to have maintained their long-term repopulation capacity and multipotency in xenotransplant mice [11]. The imbalance between α and β was also corrected successfully. Both previous and recent studies are in agreement that CRISPR-Cas9 is beneficial for thalassemia gene therapy.

2.2.3 Cancer

Cancer is a multifactorial disease that may develop as a result of multiple genetic modifications and environmental factors [45]. It is characterized by an alteration of epigenetic regulation, abnormal cell growth and eventually carcinogenesis. The opportunity and benefits of CRISPR-Cas9 application seems to be boundless, and its advancement may also contribute to improve cancer treatment. CRISPR-Cas9 helps to suppress progression and induce apoptosis of the cancer cells by activating and increasing cancer suppressors gene activity. In cellular models of diseases such as Burkitt lymphoma (BL), bladder, colon, and liver cancer cells, remarkable tumour growth reduction and apoptosis have been observed, suggesting the potential of CRISPR-Cas9 as cancer therapeutic tool [7, 46]. In addition, CRISPR-Cas9 can also be employed in breast cancer disease to determine the role of genes, target and revert the drug resistance genes into sensitive ones.

Several studies which employed CRISPR-Cas9 guided by HPV16-E7 sgRNA successfully interrupted the gene responsible for malignant phenotype (HPV16-E7 DNA) in human papillomavirus (HPV) positive cells. The oncogenes E6 and E7 commonly disrupt the tumour suppressor genes including retinoblastoma (pRb) and p53 in order to promote tumorigenesis and maintain the malignancy nature of cancer [7, 12]. CRISPR-Cas9 interfered with this process and suppressed the action of E7 protein. It also increased the production of retinoblastoma (pRb) tumour suppressor protein. Therefore, it inhibited the progression and induced death in HPV positive SiHa and Caski cell lines, but not in HEK293 and HPV-negative C33A cells [12]. In concurrent year, another study which also used CRISPR-Cas9 as an editing tool also reported similar findings whereby the growth inhibition of HPV cancer cells was obtained. There was notable increase of tumour suppressor protein (p53 and p21) in SiHa cell and the mice cancer model [47] thus slowing down the tumorigenesis process.

Another remarkable use of CRISPR-Cas9 is in the study of breast cancer. Breast cancer is a prevalent disease affecting people all around the world and is the leading cause of death for women. Mutation in one of the genes that responsible for normal biological process and cellular function results in formation of tumour [48]. MicroRNAs (miRNAs), specifically miR-23b and miR-27b, were reported to have both tumour promoting and suppressing function in various cancer types, including breast cancer [14, 49-51]. CRISPR-Cas9 was employed to determine the specific roles of miR-23b and miR-27b by knockout of these genes in breast cancer cells, MCF-7 [14]. In-

vivo study was also done by inoculating the mutated MCF-7 into the mammary fat pads of athymic nude mice. The findings showed that miR-23b and miR-27b were oncogenic and they supported breast cancer progression. However, miR-27b was less oncogenic than miR-23b and had some tumour suppressive activity in certain circumstances.

In breast cancer treatment, drug resistance issue is a common obstacle which eventually causes uncontrollable disease progression and high mortality rate [13]. A report discussed the potential of the CRISPR-Cas9 system to identify the potential target responsible for drug resistance and also its application in reversing the gene mutation in breast cancer. It was reported that an application of the CRISPR-Cas9 system can disrupt and inactivate those causative genes. This was achieved through an alteration of the functional domains of estrogenic receptor (ER) or the human epidermal growth factor receptor 2 (HER2). Both of these receptors are important for tumorigenesis as well as acquired drug resistance [13].

The mutated ER and HER2 specifically contribute towards endocrine therapy resistance. The clinical evidence further explained an example of conditions that play a part in drug resistance including the loss of Erβ, ERα expression and ERα mutations. This statement was supported by a cellular study which reported that ER mutation cause development of antioestrogen resistance. The mutated ERs, especially Y537S and D538G, recruit coactivators and cause conformational changes that result in ligand-binding dysfunction. It is characterized by the maintenance of an agonist state and an altered antagonist state that resists inhibition [52]. Some researchers believed that CRISPR-Cas9 has the potential to alter and repair the mutated exon of ER or HER2. In addition, this robust tool can also be designed to disrupt the specific functional domains of ER or HER2, which are responsible for the development of cancer [52].

Fusion oncogenes (FOs) are commonly and exclusively present in various types of cancer cells. FOs comprised of two genes which are fused together at a coding region that is important for chromosomal rearrangement. A study which used CRISPR-Cas9 to modify FO reported that tumour burden reduction and cancer cell elimination was achieved successfully in both in-vitro and in-vivo study [53]. CRISPR-Cas9 mediated FO deletion in patient-derived xenograft (PDX) and Ewing sarcoma cell lines showed an effective disruption on cancer cell. Human primary mesenchymal stem cells (hMSC) with mutated FO (EWSR1-FLI1) were injected into immunosuppressed mice, confirming that CRISPR-Cas9 FO targeting system was a specific and safe procedure as it did not impair genomic stability in healthy cells. In addition, the study also showed that the combination of chemotherapy agents with CRISPR-Cas9 mediated FO deletion significantly potentiated cell viability in xenograft models.

2.2.4 HIV/AIDS

Human Immunodeficiency Virus (HIV) is a virus that attacks the body's immune system, specifically the CD4 cells. HIV-1 is the common type of HIV which accounts for most infection cases whereas HIV-2 is relatively uncommon and less infectious. Inefficient HIV management and control will lead to the severe and life-threatening condition known as acquired immunodeficiency syndrome (AIDS). AIDS is the end-stage of HIV disease which renders the body totally vulnerable to other infections.

Several studies on HIV/AIDS have utilised CRISPR-Cas9 to eliminate the virus including those constitute to the latent viral reservoir [54, 55]. The main challenge in treating this infectious disease is to efficiently target and eradicate the reservoir [56]. However, CRISPR-Cas9 was reported to be helpful in overcoming this obstacle through its alteration of the HIV-1 genome or by targeting the cellular co-factors [54]. Compared to the commonly used Highly Active Antiretroviral therapy (HAART), CISPR-Cas9 gives a better outcome as it can better target latently infected cells [55, 57]. HAART is able to control HIV-1 replication but it cannot completely eliminate provirus present

in CD4+T cells. As a result, those viruses remain stable for many years or even decades, and capable of reactivation later [56, 58].

CRISPR-Cas9 was used in a study to target 10 different sites of HIV-1 DNA that latently infected T cell lines [59]. Modification of these site results in an interference with viral protein production that is essential for viral replication and invasive activity. As a consequence, it reduces viral gene expression and viral production. In the same year, a study showed that CRISPR-Cas9 was effective in different targeting strategies and at various stages of the infection lifecycle. It is believed that the inhibitory effect on viral replication in CRISPR-Cas9 harnessed cells resulted from modification of HIV DNA before and after the integration process [55]. In human-induced pluripotent stem cells, CRISPR/Cas9 not only induced the disruption of the latent viral genome, it also enabled cell to effectively target and maintain long term adaptive defenses against HIV-1 [59].

Another study examined the inhibitory action of CRISPR-Cas9 on HIV-1 targeting the viral long terminal repeat (LTR) and the gene coding region. LTR is an important structure for HIV-1 nucleic acid integration and gene expression. The study reported that the use of CRISPR-Cas9 that had been specifically designed to target LTR in HIV-1 LTR expression dormant and inducible T cells showed reduction in intracellular proviral load and LTR-driven expression [60]. This resulted from CRISPR-Cas9 mediated cleavage and modification action on LTR sequence. A similar study which also targeted LTR and the gene coding region, described o an inhibitory action on HIV-1 infection that occurred along with significant reduction of the late viral DNA products and decrease of the integrated viral DNA [61]. Besides, this study also suggested that CRISPR-Cas9 can target and edit HIV-1 DNA located either in the cytoplasm or in the nucleus. Cytoplasmic Cas9 can suppress HIV-1 in a similar manner to nuclear Cas9, but it is ineffective towards latent provirus.

Despite the effectiveness of CRISPR-Cas9 in targeting and inhibiting HIV-1 provirus, there are several issues that affect its efficiency in HIV/AIDS treatment including HIV-1 quasispecies and the occurrence of viral escape. The diversity of quasispecies requires targeting of multiple sites and personalized engineering of CRISPR/Cas9, which are challenging to achieve [58]. However, viral escape may be solved through several potential solutions including reprogramming Cas9, modifying gRNA and even suppression of the NHEJ activity [56].

HIV-1 develops resistance towards single gRNA either by mutagenic reverse transcription or NHEJ. NHEJ repair machinery can cause mutations in CRISPR-Cas9 cleavage site that result in two opposite effects; suppression of HIV-1 replication and acceleration of viral escape [62]. This was demonstrated in an animal model study which showed both the positive and negative aspect of CRISPR-Cas9 application towards HIV-1. It was reported that CRISPR-Cas9 mediated mutations successfully occurred in different parts of mice, proving the effectiveness of CRISPR-Cas9 in suppressing and eliminating HIV-1 [63]. However, the subsequent long-term culture T-cells showed that the virus is capable of escaping from this inhibitory effect. This is due to DNA repair mechanism which prevents further Cas9 cleavage action, and the fact that some mutations may still be compatible for viral replication, and thus facilitate virus escape action [55, 58].

2.3 Limitations

Upon its discovery, CRISPR-Cas9 received tremendous attention and intrigued researchers all around the globe due to its robustness, flexibility and versatility. Despite the CRISPR-Cas9 promising potentials and applications, there are several aspects that need to be addressed. The method of delivery, off-target cutting and ethical issues in human germline modification are the examples of limitations to the application of CRISPR-Cas9 as a gene editing tool [15]. By addressing these challenges, we can find better solutions or alternatives to overcome these limitations, and then the benefits of CRISPR-Cas9 can be fully reaped and its potential can be maximized to support medical field advancement.

The main delivery vehicles for CRISPR-Cas9 can be categorized into three types; physical delivery, viral vectors, and non-viral vectors [64]. For each delivery vehicle, there are certain limitations that restrict its application. Physical delivery for instance, is relatively safer than viral vector, but it is less efficient for in-vivo application [15, 65]. Microinjection, which involves cell membrane piercing, is a technically challenging and laborious process [64]. Electroporation, on the other hand, is not efficient to transfect mammalian cells due to sensitivity of these cells towards precise voltage and current application time. Besides, electroporation also possesses toxic effects, which leads to cell death and also inefficiency in primary cell cultures [1].

For viral delivery vectors, adeno-associated virus (AAV) is known as the most efficient to deliver plasmid for both in vivo and in vitro studies. Unfortunately, it is restricted by limited packing size and infection risk when multiple admission was conducted [15, 54]. AAV also may cause organ toxicity due to the long-term durability of these vectors in animal model production [1]. In comparison to AAV , adenovirus (AdV) and lentivirus (LV) have larger diameters, and thus can tolerate greater insertion [65]. However, AdVs and LVs have the tendency to be integrated into the host and also cause off-target effects [65]. Non-viral vehicle such as lipid nanoparticles/liposomes may degrade or fail to translocate into nucleus. In addition, delivery of CRISPR-Cas9 using nanoparticles is less likely to produce high efficacy results [64].

Chemical method such as lipid nanoparticles (LNP)/liposomes has been extensively studied and improved over time. Delivery of Cas9/sgRNA using lipoplex (cationic lipid) involves formation of a stable nanocomplex resulting from electrostatic interaction between cationic lipids and negatively charged Cas9/sgRNA [66, 67]. This system increases the likelihood of entry into the nucleus and decrease of enzymatic degradation [66]. However, LNPs are toxic for phagocytic cells, cause strong immune responses and are rapidly removed from circulation [68]. A novel modifiable LNP platform called selective organ targeting (SORT) LNP offers better genome editing efficiency and target delivery. Its effectiveness was successfully demonstrated in CRISPR/Cas gene editing of tdTomato (tdTom) transgenic mice [69]. Despite its efficient performance, SORT LNP application is limited to certain organs [69, 70]. Further development is required to improve LNP efficiency and safe delivery to other non-liver tissues.

Another challenge is the off-site mutation of CRISPR-Cas9 itself. Off-site mutation can be defined as unintended cleavage that occurs at non-target sites due to similarities with the target site as well as tolerance of mismatch [71]. Some mismatches between sgRNA and target DNA sequences are well tolerated, especially those that occur at the 5′ upstream region [72, 73]. Several studies also reported that the mismatches up to 5nt can result in off-site mutation in human cells studies, even with higher frequency than intended on-site [65, 74, 75]. For CRISPR-Cas9 associated study of human embryo, whole-genome sequencing detects an off-target event in one of the embryos. However, this finding was not reported due to insignificance number (n=1) and on-target locus of 1 mismatch with the sgRNA [76]. Another in-vivo study on mouse embryos also concluded that high-efficiency off-target occurred at the site with three mismatches, and with higher tendency when the site was ≤3 mismatches away from the seed region [77]. Nowadays, researchers try to reduce and overcome these off-target issues by developing and testing numerous types of approaches such as Cas9 paired nickase, dCas9, biased or unbiased off-target detection, ribonucleoprotein (RNP) delivery and truncated gRNAs [78]. Hopefully, the ongoing studies will provide better solutions for the off-target issue.

Ethical issues are major barriers that hinder CRISPR-Cas9 alteration in germline cells. Even though the application of CRISPR-Cas9 in somatic cells is widely accepted, when it comes to germline cells, various debates, opinions and responses are involved. This is due to the uncertainty of the effect, off-target events and lack of understanding on the association between genetic makeup and phenotype which restrains the use of CRISPR-Cas9 in human germline cells [7, 79]. The possibility of generating permanent changes that causing multigenerational implications is one of the major concerns which draws debate in the community [26, 80]. Due to those possibilities,

germline modification by genome editing tools including CRISPR-Cas9 is not fully supported and continued to ignite ethical debates. Also, it is difficult to construct informed consent when there is the risk for genetic alteration that can be passed down to the future generations [81]. The uncertainty associated with the long-term effects of CRISPR-Cas9 mediated mutation is another worrisome issue as it can cause unintended drawbacks in the future [7].

3. Conclusions

CRISPR-Cas9 is a simple, robust and versatile genome editing tool that allows scientists to alter DNA sequence via two series of process; the introduction of DSB to DNA, and the hijacking of the cellular repair mechanism. The functional molecules in CRISPR-Cas9 system are single guide RNA (sgRNA) and Cas9 protein which work hand in hand to complete genome alteration. The fact that certain diseases are greatly influenced by genetic variation makes the use of CRISPR-Cas9 as manipulation tool very possible in enhancing pathological understanding and as a therapeutic intervention technique.

Numerous studies have proven the clinical importance and vast applications of CRISPR-Cas9 in pharmacological field mainly in animal model production and therapeutic intervention. This simple-to-design genome editing tool has accelerated the studies and allowed discovery of enormous information on animal model construction as well as genomic modification in genetic, multifactorial and complex diseases. Despite its advantages that are greatly discussed in the research, there are some challenges to the current CRISPR-Cas9 system including the method of delivery, off-target cutting and ethical issues in human germline modification.

As most of the previous research was done only on somatic cells, hence more studies need to be carried out on germline cells, that is once ethical issues have been resolved. It is our hope that this report, which contains cumulative information about CRISPR-Cas9 opportunities in the medical and pharmacological fields, will increase understanding of the latest advancement and the potentials for use of CRISPR-Cas9 as well as enlightening researchers as to which medical aspects should be further explored. Therefore, the chances and possibility of discovering new and more efficacy solutions and therapeutic use for better health quality will be increased.

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References

[1] Zarei, A., Razban, V., Hosseini, S.E. and Tabei, S., 2019. Creating cell and animal models of human disease by genome editing using CRISPR/Cas9. *The Journal of Gene Medicine*, 21(4), e3082, https://doi.org/10.1002/jgm.3082.

[2] Peng, R., Lin, G. and Li, J., 2016. Potential pitfalls of CRISPR/Cas9-mediated genome editing. *The FEBS Journal*, 283(7), 1218-1231.

[3] Zhao, J., Fang, H. and Zhang, D., 2020. Expanding application of CRISPR-Cas9 system in microorganisms. *Synthetic and Systems Biotechnology*, 5(4), 269-276.

[4] Gupta, D., Bhattacharjee, O., Mandal, D., Sen, M.K., Dey, D., Dasgupta, A., Kazi, T.A., Gupta, R., Sinharoy, S., Acharya, K., Chattopadhyay, D., Ravichandiran, V., Roy, S. and

Ghosh, D., 2019. CRISPR-Cas9 system: A new-fangled dawn in gene editing. *Life Sciences*, 232, 116636, https://doi.org/10.1016/j.lfs.2019.116636.

[5] Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F. and Jaenisch, R., 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*, 153(4), 910-918.

[6] Cho, B., Kim, S.J., Lee, E.J., Ahn, S.M., Lee, J.S., Ji, D.Y., Lee, K. and Kang, J.T., 2018. Generation of insulin-deficient piglets by disrupting INS gene using CRISPR/Cas9 system. *Transgenic Research*, 27(3), 289-300.

[7] Kotagama, O.W., Jayasinghe, C.D. and Abeysinghe, T., 2019. Era of genomic medicine: A narrative review on CRISPR technology as a potential therapeutic tool for human diseases. *BioMed Research International*, 2019, 1369682, https://doi.org/10.1155/2019/1369682.

[8] Firth, A.L., Menon, T., Parker, G.S., Qualls, S.J., Lewis, B.M., Ke, E., Dargitz, C.T., Wright, R., Khanna, A., Gage, F.H. and Verma, I.M., 2015. Functional gene correction for cystic fibrosis in lung epithelial cells generated from patient iPSCs. *Cell Reports*, 12(9), 1385-1390.

[9] Niu, X., He, W., Song, B., Ou, Z., Fan, D., Chen, Y., Fan, Y. and Sun, X., 2016. Combining single strand oligodeoxynucleotides and CRISPR/Cas9 to correct gene mutations in β-thalassemia-induced pluripotent stem cells. *The Journal of Biological Chemistry*, 291(32), 16576-16585.

[10] Song, B., Fan, Y., He, W., Zhu, D., Niu, X., Wang, D., Ou, Z., Luo, M. and Sun, X., 2015. Improved hematopoietic differentiation efficiency of gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system. *Stem Cells and Development*, 24(9), 1053-1065.

[11] Pavani, G., Fabiano, A., Laurent, M., Amor, F., Cantelli, E., Chalumeau, A., Maule, G., Tachtsidi, A., Concordet, J.P., Cereseto, A., Mavilio, F., Ferrari, G., Miccio, A. and Amendola, M., 2021. Correction of β-thalassemia by CRISPR/Cas9 editing of the α-globin locus in human hematopoietic stem cells. *Blood Advances*, 5(5), 1137-1153.

[12] Hu, Z., Yu, L., Zhu, D., Ding, W., Wang, X., Zhang, C., Wang, L., Jiang, X., Shen, H., He, D., Li, K., Xi, L., Ma, D. and Wang, H., 2014. Disruption of HPV16-E7 by CRISPR/Cas system induces apoptosis and growth inhibition in HPV16 positive human cervical cancer cells. *BioMed Research International*, 2014, 612823, https://doi.org/10.1155/2014/612823.

[13] Chen, Y. and Zhang, Y., 2018. Application of the CRISPR/Cas9 system to drug resistance in breast cancer. *Advanced Science*, 5(6), 1700964, https://doi.org/10.1002/advs.201700964.

[14] Hannafon, B.N., Cai, A., Calloway, C.L., Xu, Y.F., Zhang, R., Fung, K.M. and Ding, W.Q., 2019. miR-23b and miR-27b are oncogenic microRNAs in breast cancer: evidence from a CRISPR/Cas9 deletion study. *BMC Cancer*, 19(1), 642, https://doi.org/10.1186/s12885-019-5839-2.

[15] Liu, C., Zhang, L., Liu, H. and Cheng, K., 2017. Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. *Journal of Controlled Release*, 266, 17-26.

[16] Porteus M. H., 2015. Towards a new era in medicine: therapeutic genome editing. *Genome Biology*, 16, 286, https://doi.org/10.1186/s13059-015-0859-y.

[17] Cai, L., Fisher, A.L., Huang, H. and Xie, Z., 2016. CRISPR-mediated genome editing and human diseases. *Genes and Diseases*, 3(4), 244-251.

[18] Ishino, Y., Krupovic, M. and Forterre, P., 2018. History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology. *Journal of Bacteriology*, 200(7), e00580-17, https://doi.org/10.1128/JB.00580-17.

[19] Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. and Nakata, A., 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *Journal of bacteriology*, 169(12), 5429-5433.

[20] Makarova, K.S., Wolf, Y.I., Iranzo, J., Shmakov, S.A., Alkhnbashi, O.S., Brouns, S., Charpentier, E., Cheng, D., Haft, D.H., Horvath, P., Moineau, S., Mojica, F., Scott, D., Shah, S.A., Siksnys, V., Terns, M.P., Venclovas, Č., White, M.F., Yakunin, A.F., Yan, W. and Koonin, E.V., 2020. Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. *Nature Reviews Microbiology*, 18(2), 67-83.

[21] Tang, Y. and Fu, Y., 2018. Class 2 CRISPR/Cas: an expanding biotechnology toolbox for and beyond genome editing. *Cell and Bioscience*, 8, 59, https://doi.org/10.1186/s13578-018-0255-x.

[22] Jiang, F. and Doudna, J.A., 2017. CRISPR-Cas9 structures and mechanisms. *Annual Review of Biophysics*, 46, 505-529.

[23] Cao, J., Xiao, Q. and Yan, Q., 2018. The multiplexed CRISPR targeting platforms. *Drug Discovery Today: Technologies*, 28, 53-61.

[24] Porteus, M., 2016. Genome editing: A new approach to human therapeutics. *Annual Review of Pharmacology and Toxicology*, 56, 163-190.

[25] Cubbon, A., Ivancic-Bace, I. and Bolt, E.L., 2018. CRISPR-Cas immunity, DNA repair and genome stability. *Bioscience Reports*, 38(5), https://doi.org/10.1042/BSR20180457.

[26] Rodriguez, E., 2016. Ethical issues in genome editing using Crispr/Cas9 system. *Journal of Clinical Research and Bioethics*, 7(2), https://doi.org/10.4172/2155-9627.1000266.

[27] Shen, B., Zhang, J., Wu, H., Wang, J., Ma, K., Li, Z., Zhang, X., Zhang, P. and Huang, X., 2013. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Research*, 23(5), 720-723.

[28] Shao, Y., Guan, Y., Wang, L., Qiu, Z., Liu, M., Chen, Y., Wu, L., Li, Y., Ma, X., Liu, M. and Li, D., 2014. CRISPR/Cas-mediated genome editing in the rat via direct injection of one-cell embryos. *Nature Protocols*, 9(10), 2493-2512.

[29] Niu, Y., Shen, B., Cui, Y., Chen, Y., Wang, J., Wang, L., Kang, Y., Zhao, X., Si, W., Li, W., Xiang, A.P., Zhou, J., Guo, X., Bi, Y., Si, C., Hu, B., Dong, G., Wang, H., Zhou, Z., Li, T. and Sha, J., 2014. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell*, 156(4), 836-843.

[30] Fan, Z., Perisse, I.V., Cotton, C.U., Regouski, M., Meng, Q., Domb, C., Van Wettere, A.J., Wang, Z., Harris, A., White, K.L. and Polejaeva, I.A., 2018. A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption of the CFTR gene. *JCI Insight*, 3(19), e123529, https://doi.org/10.1172/jci.insight.123529.

[31] Kim, H., Kim, M., Im, S.K. and Fang, S., 2018. Mouse Cre-LoxP system: general principles to determine tissue-specific roles of target genes. *Laboratory Animal Research*, 34(4), 147-159.

[32] Huang, J., Chen, M., Whitley, M.J., Kuo, H.C., Xu, E.S., Walens, A., Mowery, Y.M., Van Mater, D., Eward, W.C., Cardona, D.M., Luo, L., Ma, Y., Lopez, O.M., Nelson, C.E., Robinson-Hamm, J.N., Reddy, A., Dave, S.S., Gersbach, C.A., Dodd, R.D. and Kirsch, D.G., 2017. Generation and comparison of CRISPR-Cas9 and Cre-mediated genetically engineered mouse models of sarcoma. *Nature Communications*, 8, 15999, https://doi.org/10.1038/ncomms15999.

[33] Xue, W., Chen, S., Yin, H., Tammela, T., Papagiannakopoulos, T., Joshi, N.S., Cai, W., Yang, G., Bronson, R., Crowley, D.G., Zhang, F., Anderson, D.G., Sharp, P.A. and Jacks, T., 2014. CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature*, 514(7522), 380-384.

[34] Cooney, A.L., McCray, P.B. Jr. and Sinn, P.L., 2018. Cystic fibrosis gene therapy: Looking back, looking forward. *Genes*, 9(11), 538, https://doi.org/10.3390/genes9110538.

[35] Mall, M.A. and Hartl, D., 2014. CFTR: cystic fibrosis and beyond. *The European Respiratory Journal*, 44(4), 1042-1054.

[36] Odera, M., Furuta, T., Sohma, Y. and Sakurai, M., 2018. Molecular dynamics simulation study on the structural instability of the most common cystic fibrosis-associated mutant ΔF508-CFTR. *Biophysics and Physicobiology*, 15, 33-44.

[37] Sondo, E., Falchi, F., Caci, E., Ferrera, L., Giacomini, E., Pesce, E., Tomati, V., Mandrup Bertozzi, S., Goldoni, L., Armirotti, A., Ravazzolo, R., Cavalli, A. and Pedemonte, N., 2018. Pharmacological inhibition of the ubiquitin ligase RNF5 rescues F508del-CFTR in cystic fibrosis airway epithelia. *Cell Chemical Biology*, 25(7), 891-905.

[38] Schwank, G., Koo, B.K., Sasselli, V., Dekkers, J.F., Heo, I., Demircan, T., Sasaki, N., Boymans, S., Cuppen, E., van der Ent, C.K., Nieuwenhuis, E.E., Beekman, J.M. and Clevers, H., 2013. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell*, 13(6), 653-658.

[39] Smirnikhina, S.A., Kondrateva, E.V., Adilgereeva, E.P., Anuchina, A.A., Zaynitdinova, M.I., Slesarenko, Y.S., Ershova, A.S., Ustinov, K.D., Yasinovsky, M.I., Amelina, E.L., Voronina, E.S., Yakushina, V.D., Tabakov, V.Y. and Lavrov, A.V., 2020. P.F508del editing in cells from cystic fibrosis patients. *PloS One*, 15(11), e0242094, https://doi.org/10.1371/journal.pone.0242094.

[40] Wattanapanitch M., 2021. Correction of hemoglobin E/beta-thalassemia patient-derived iPSCs using CRISPR/Cas9. *Methods in Molecular Biology*, 2211, 193-211.

[41] Wattanapanitch, M., Damkham, N., Potirat, P., Trakarnsanga, K., Janan, M., U-Pratya, Y., Kheolamai, P., Klincumhom, N. and Issaragrisil, S., 2018. One-step genetic correction of hemoglobin E/beta-thalassemia patient-derived iPSCs by the CRISPR/Cas9 system. *Stem Cell Research & Therapy*, 9(1), 46, https://doi.org/10.1186/s13287-018-0779-3.

[42] Ali, G., Tariq, M.A., Shahid, K., Ahmad, F.J. and Akram, J., 2021. Advances in genome editing: the technology of choice for precise and efficient β-thalassemia treatment. *Gene Therapy*, 28(1-2), 6-15.

[43] Srivastava, A. and Shaji, R.V., 2017. Cure for thalassemia major - from allogeneic hematopoietic stem cell transplantation to gene therapy. *Haematologica*, 102(2), 214-223.

[44] Frangoul, H., Altshuler, D., Cappellini, M.D., Chen, Y.S., Domm, J., Eustace, B.K., Foell, J., de la Fuente, J., Grupp, S., Handgretinger, R., Ho, T.W., Kattamis, A., Kernytsky, A., Lekstrom-Himes, J., Li, A.M., Locatelli, F., Mapara, M.Y., de Montalembert, M., Rondelli, D., Sharma, A. and Corbacioglu, S., 2021. CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. *The New England Journal of Medicine*, 384(3), 252-260.

[45] Fialkowski, A., Beasley, T.M. and Tiwari, H.K., 2019. Multifactorial inheritance and complex diseases. In: R.E. Pyeritz, B.R. Korf and W.W. Grody, eds. *Emery and Rimoin's Principles and Practice of Medical Genetics and Genomics*: *Foundations*. 7th ed. London: Academic Press, pp. 323-358.

[46] Chen, M., Mao, A., Xu, M., Weng, Q., Mao, J. and Ji, J., 2019. CRISPR-Cas9 for cancer therapy: Opportunities and challenges. *Cancer Letters*, 447, 48-55.

[47] Zhen, S., Hua, L., Takahashi, Y., Narita, S., Liu, Y.H. and Li, Y., 2014. In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9. *Biochemical and Biophysical Research Communications*, 450(4), 1422-1426.

[48] Ahmed, M., Daoud, G.H., Mohamed, A. and Harati, R., 2021. New Insights into the therapeutic applications of CRISPR/Cas9 genome editing in breast cancer. *Genes*, 12(5), 723, https://doi.org/10.3390/genes12050723.

[49] An, Y., Zhang, Z., Shang, Y., Jiang, X., Dong, J., Yu, P., Nie, Y. and Zhao, Q., 2015. miR-23b-3p regulates the chemoresistance of gastric cancer cells by targeting ATG12 and HMGB2. *Cell Death and Disease*, 6(5), e1766, https://doi.org/10.1038/cddis.2015.123.

[50] Qi, P., Xu, M.D., Shen, X.H., Ni, S.J., Huang, D., Tan, C., Weng, W.W., Sheng, W.Q., Zhou, X.Y. and Du, X., 2015. Reciprocal repression between TUSC7 and miR-23b in gastric cancer. *International Journal of Cancer*, 137(6), 1269-1278.

[51] Chen, L., Li, H., Han, L., Zhang, K., Wang, G., Wang, Y., Liu, Y., Zheng, Y., Jiang, T., Pu, P., Jiang, C. and Kang, C., 2011. Expression and function of miR-27b in human glioma. *Oncology Reports*, 26(6), 1617-1621.

[52] Fanning, S.W., Mayne, C.G., Dharmarajan, V., Carlson, K.E., Martin, T.A., Novick, S.J., Toy, W., Green, B., Panchamukhi, S., Katzenellenbogen, B.S., Tajkhorshid, E., Griffin, P. R., Shen, Y., Chandarlapaty, S., Katzenellenbogen, J.A. and Greene, G.L., 2016. Estrogen receptor alpha somatic mutations Y537S and D538G confer breast cancer endocrine resistance by stabilizing the activating function-2 binding conformation. *ELife*, 5, e12792, https://doi.org/10.7554/eLife.12792.

[53] Martinez-Lage, M., Torres-Ruiz, R., Puig-Serra, P., Moreno-Gaona, P., Martin, M.C., Moya, F.J., Quintana-Bustamante, O., Garcia-Silva, S., Carcaboso, A.M., Petazzi, P., Bueno, C., Mora, J., Peinado, H., Segovia, J.C., Menendez, P. and Rodriguez-Perales, S., 2020. In vivo CRISPR/Cas9 targeting of fusion oncogenes for selective elimination of cancer cells. *Nature Communications*, 11(1), 5060, https://doi.org/10.1038/s41467-020-18875-x.

[54] Xiao, Q., Guo, D. and Chen, S., 2019. Application of CRISPR/Cas9-based gene editing in HIV-1/AIDS therapy. *Frontiers in Cellular and Infection Microbiology*, 9, 69, https://doi.org/10.3389/fcimb.2019.00069.

[55] Das, A.T., Binda, C.S. and Berkhout, B., 2019. Elimination of infectious HIV DNA by CRISPR-Cas9. *Current Opinion in Virology*, 38, 81-88.

[56] Liang, C., Wainberg, M.A., Das, A.T. and Berkhout, B., 2016. CRISPR/Cas9: a double-edged sword when used to combat HIV infection. *Retrovirology*, 13(1), 37, https://doi.org/10.1186/s12977-016-0270-0.

[57] Huang, S.H., Ren, Y., Thomas, A.S., Chan, D., Mueller, S., Ward, A.R., Patel, S., Bollard, C.M., Cruz, C.R., Karandish, S., Truong, R., Macedo, A.B., Bosque, A., Kovacs, C., Benko, E., Piechocka-Trocha, A., Wong, H., Jeng, E., Nixon, D.F., Ho, Y.C. and Jones, R.B., 2018. Latent HIV reservoirs exhibit inherent resistance to elimination by CD8+ T cells. *The Journal of Clinical Investigation*, 128(2), 876-889.

[58] Panfil, A.R., London, J.A., Green, P.L. and Yoder, K.E., 2018. CRISPR/Cas9 genome editing to disable the latent HIV-1 provirus. *Frontiers in Microbiology*, 9, 3107, https://doi.org/10.3389/fmicb.2018.03107.

[59] Zhu, W., Lei, R., Le Duff, Y., Li, J., Guo, F., Wainberg, M.A. and Liang, C., 2015. The CRISPR/Cas9 system inactivates latent HIV-1 proviral DNA. *Retrovirology*, 12, 22, https://doi.org/10.1186/s12977-015-0150-z.

[60] Ebina, H., Misawa, N., Kanemura, Y. and Koyanagi, Y., 2013. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. *Scientific Reports*, 3, 2510, https://doi.org/10.1038/srep02510.

[61] Yin, L., Hu, S., Mei, S., Sun, H., Xu, F., Li, J., Zhu, W., Liu, X., Zhao, F., Zhang, D., Cen, S., Liang, C. and Guo, F., 2018. CRISPR/Cas9 inhibits multiple steps of HIV-1 infection. *Human Gene Therapy*, 29(11), 1264-1276.

[62] Wang, Z., Pan, Q., Gendron, P., Zhu, W., Guo, F., Cen, S., Wainberg, M.A. and Liang, C., 2016. CRISPR/Cas9-derived mutations both inhibit HIV-1 replication and accelerate viral escape. *Cell Reports*, 15(3), 481-489.

[63] Yin, C., Zhang, T., Qu, X., Zhang, Y., Putatunda, R., Xiao, X., Li, F., Xiao, W., Zhao, H., Dai, S., Qin, X., Mo, X., Young, W.B., Khalili, K. and Hu, W., 2017. In vivo excision of HIV-1 provirus by saCas9 and multiplex single-guide RNAs in animal models. *Molecular Therapy*, 25(5), 1168-1186.

[64] Lino, C.A., Harper, J.C., Carney, J.P. and Timlin, J.A., 2018. Delivering CRISPR: a review of the challenges and approaches. *Drug Delivery*, 25(1), 1234-1257.

[65] Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A. and Liu, D.R., 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature Biotechnology*, 31(9), 839-843.

[66] Bessodes, M., Dhotel, H. and Mignet, N., 2019. Lipids for nucleic acid delivery: Cationic or neutral lipoplexes, synthesis, and particle formation. *Methods in Molecular Biology,* 1943, 123-139.

[67] Nelson, C.E. and Gersbach, C.A., 2016. Engineering delivery vehicles for genome editing. *Annual Review of Chemical and Biomolecular Engineering,* 7, 637-662.

[68] Moss, K.H., Popova, P., Hadrup, S.R., Astakhova, K. and Taskova, M., 2019. Lipid nanoparticles for delivery of therapeutic RNA oligonucleotides. *Molecular Pharmaceutics* 16(6), 2265-2277.

[69] Cheng, Q., Wei, T., Farbiak, L., Johnson, L.T., Dilliard, S.A. and Siegwart, D.J., 2020. Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR-Cas gene editing. *Natural Nanotechnology*, 15(4), 313-20.

[70] Behr, M., Zhou, J., Xu, B. and Zhang, H., 2021. *In vivo* delivery of CRISPR-Cas9 therapeutics: Progress and challenges. *Acta Pharmaceutica Sinica B*, 11(8), 2150-2171.

[71] Modrzejewski, D., Hartung, F., Lehnert, H., Sprink, T., Kohl, C., Keilwagen, J. and Wilhelm, R., 2020. Which factors affect the occurrence of off-target effects caused by the use of CRISPR/Cas: A systematic review in plants. *Frontiers in Plant Science*, 11, 574959, https://doi.org/10.3389/fpls.2020.574959.

[72] Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A. and Zhang, F., 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science*, 339(6121), 819-823.

[73] Jiang, W., Bikard, D., Cox, D., Zhang, F. and Marraffini, L.A., 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature Biotechnology*, 31(3), 233-239.

[74] Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K. and Sander, J.D., 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology*, 31(9), 822-826.

[75] Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., Cradick, T.J., Marraffini, L.A., Bao, G. and Zhang, F., 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology*, 31(9), 827-832.

[76] Tang, L., Zeng, Y., Du, H., Gong, M., Peng, J., Zhang, B., Lei, M., Zhao, F., Wang, W., Li, X. and Liu, J., 2017. CRISPR/Cas9-mediated gene editing in human zygotes using Cas9 protein. *Molecular Genetics and Genomics*, 292(3), 525-533.

[77] Aryal, N.K., Wasylishen, A.R. and Lozano, G., 2018. CRISPR/Cas9 can mediate high-efficiency off-target mutations in mice *in vivo*. *Cell Death and Disease*, 9(11), 1099, https://doi.org/10.1038/s41419-018-1146-0.

[78] Naeem, M., Majeed, S., Hoque, M.Z. and Ahmad, I., 2020. Latest developed strategies to minimize the off-target effects in CRISPR-Cas-mediated genome editing. *Cells*, 9(7), 1608, httos://doi.org/10.3390/cells9071608.

[79] Brokowski, C. and Adli, M., 2019. CRISPR ethics: Moral considerations for applications of a powerful tool. *Journal of Molecular Biology*, 431(1), 88-101.

[80] Vassena, R., Heindryckx, B., Peco, R., Pennings, G., Raya, A., Sermon, K. and Veiga, A., 2016. Genome engineering through CRISPR/Cas9 technology in the human germline and pluripotent stem cells. *Human Reproduction Update*, 22(4), 411-419.

[81] Billings, P.R., Hubbard, R. and Newman, S.A., 1999. Human germline gene modification: a dissent. *Lancet*, 353(9167), 1873-1875.