

Application of CRISPR/Cas9 based therapy in SCD, DMD and HIV

Mia Shen

Westminster School, London, UK

mia.shen@westminster.org.uk

Abstract. Gene editing has revolutionised the possibilities of molecular biology, and the emergence of CRISPR as the novel groundbreaking genome engineering tool has since proved superior to its predecessors such as TALENs and ZFNs owing to its cost-effectiveness, simplicity of design, flexibility and high specificity. Under the guidance of gRNA, the endonuclease Cas9 could achieve manipulation at nearly any location. This review concentrates on the application of CRISPR/Cas9 based therapies in three diseases-SCD, DMD and HIV. By taking a corresponding drug or frontier research as an example, its mechanism and efficiency are clearly illustrated. For instance, Casgevy, a CRISPR/Cas9 based drug, used to treat SCD, which could modify the mutation in HBB and rescue the aberrant production of foetal haemoglobin, has proven to be in high efficiency. In the therapy of DMD, a similar strategy is adopted, except taking AAV as the delivery vector. For HIV treatment regimen, instead of targeting host genome, the Cas9 aims to engineer the promoter of pathogen, inhibiting the pathogen activity and clearing the latent infected HIV virus permanently. According to the clinical data, all of these methods tend to be therapeutic alternatives. However, despite CRISPR/Cas9's plethora of merits, its challenges require further in-depth research and strategies of prevention before its routine application in disease treatment. This review briefly discusses its most significant shortcoming of off-targets effects, which can lead to fatal genetic mutations, and the potential interventions.

Keywords: CRISPR/Cas9, SCD, DMD, HIV.

1. Introduction

In 1987, Japanese scientist Ishino and his team were performing gene analysis on *Escherichia coli* when they discovered previously unrecognised short repeats of the viral DNA which were interrupted by spacers. Not long after in 1990, similar sequences were spotted in other prokaryotes by Francisco Mojica. He then coined the term CRISPR. Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) is a series of short, partially repeated DNA sequences found in prokaryote genomes. Cas-9 is CRISPR's associated protein encoded by the Cas gene, which is most commonly found adjacent to CRISPR in the DNA sequence. In 2007, CRISPR was concluded to be a natural mechanism of adaptive immunity in prokaryote organisms in order to protect themselves against invading viruses and bacteriophages. This is done by the CRISPR associated (Cas) protein, which cuts and inserts spacers into the CRISPR array. Short pieces of RNA with copies of these viral codes bind with the Cas-9 protein. These complexes recognise and destroy the virus in case of reinfection, thus protecting the bacterium. In 2012, scientists Doudna and Charpentier were able to redesign CRISPR so that it can target any, not just viral DNA. It

was ever since this significant discovery that CRISPR has become the most successful procedure of genome modification so far.

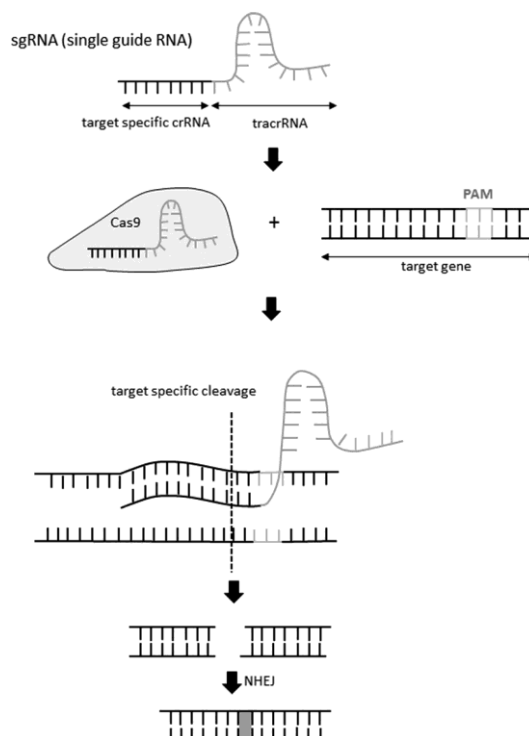


Figure 1. Mechanism of CRISPR/Cas9 as gene editing tool

The CRISPR/ Cas system is divided into two classes. Class II involves a single Cas-protein, and this is the system studied extensively for gene engineering. The system's two vital constituents are the Cas9 protein and the guide RNA (gRNA). The guide RNA which targets viral DNA in prokaryotes is synthetically modified in laboratories, forming the tracrRNA-crRNA chimeric, which is essentially a single guide RNA (sgRNA) that has the ability to identify any specific gene for modification. The Cas-9 protein is obtained from bacteria *Streptococcus pyogenes* and has two regions: the recognition (REC) lobe which has REC1 and REC2 domains, and the nuclear (NUC) lobe which has domains RuvC and HNH. CRISPR/Cas-9 as a gene editing tool works in 3 steps: recognition, cleavage, and lastly repair. First, the sgRNA guides the Cas-9 protein to the target gene sequence. The Cas-9 protein is subsequently activated DNA excision. The RuvC domain cleaves the non-complementary strand, whereas the HNH cleaves the complementary, creating double strand breaks (DSBs) in the DNA. The cell will attempt to repair the cleaved DNA through nonhomologous end joining (NHEJ). However, this fixing process is prone to errors and often introduces mutations that cause the resulting gene to be unusable. This makes CRISPR useful for 'turning off' undesired genes (Figure 1).

Moreover, CRISPR is able to repair and even introduce new genes through homology recombination repair, the most common form of homology directed repair (HDR), where a homologous template DNA is used to guide the repair process and incorporate new genes into the DNA sequence [1]. Unlike NHEJ, it does not introduce indels and is a far more accurate and precise method of DNA repair, possessing the ability to alter even singular nucleotides.

2. Application of CRISPR/Cas 9 as genetic engineering tool in Disease Treatment

Since the identification of CRISPR/Cas9, scientists have recognized its huge potential as a tool for genome editing which can be used extensively in various industries including agriculture and medicine. CRISPR/Cas9 is currently the preferred gene manipulation tool over conventional technologies, ZFNs

and TALENs, due to its simplicity of design, scalability, efficiency and also affordability [2]. CRISPR/Cas9 has the potential to cure and even eradicate infectious diseases such as HIV and develop genetically superior crops; this review will be focusing on the former.

2.1. Sick Cell Disease (SCD)

Sickle Cell Disease (SCD) is a heterogeneous genetic disorders as a result of the point mutation in the haemoglobin beta gene (HBB), which codes for beta haemoglobin, a protein subunit of haemoglobin. This mutation results in the sickle Hb (HbS) allele. The most common SCD genotype is sickle cell anaemia (SCA), when patients are homozygous for the HbS allele. Health complications, such as haemolytic anaemia, occur due to the rapid polymerisation of deoxygenated abnormal haemoglobin, which have two mutated HbS subunits instead of the regular β -globin subunits, causing the red blood cells to become crescent shaped. The deformation of the erythrocytes to assume this irregular shape leads to the blockage of blood flow, especially in smaller blood vessels, causing vaso-occlusions which result in widespread and irreversible organ damage [3]. On top of that, HbS polymerisation is exacerbated by HbS lower affinity to oxygen compared with normal adult haemoglobin (HbA) [4].

Despite our knowledge of it for over a century, so far there are only four FDA approved medicines, with the first one being hydroxyurea (HU) in 1998 and later voxelotor, L-glutamine, and crizanlizumab-tmca [5]. Each of these medications help to alleviate different symptoms of the illness, but there are still no definitive cures. Here, gene editing becomes an attractive treatment option for research as it is able to completely, once and for all alter or erase the genetic mutation. CRISPR/Cas9 achieves this through first creating DSBs at the target site, inducing either NHEJ or HDR, with the former usually resulting in the gene being unusable, and the latter being manipulated to either correct the mutated gene or introduce synthesis of foetal haemoglobin (HbF), which is the most common method conducted in clinical trials, since research shows association between heightened levels of HbF and lessened severity of disease [6]. Studies have shown that newborn babies with SCD often do not display very little, or no symptoms at all of disease due to the increased levels of HbF [7]. Furthermore, individuals with SCD who have inherited conditions where HbF production persists into adulthood often also experience little or no disease symptoms [8].

CTX001 is an autologous drug, edited using CRISPR/Cas9, that targets the enhancer segment of the B-cell lymphoma 11A(BCL11A) gene which is in charge of deactivating production of HbF 6 months after birth and gradually replacing it with HbA. In the case of CTX001, CRISPR/Cas 9 is used ex vivo as it is introduced in the BCL11A gene in hematopoietic stem and progenitor cells (HSPCs) extracted from the bone marrow of the individual patients themselves. It then makes an edit that hinders the region's ability to drive BCL11A expression. These engineered cells are subsequently reinfused into the patient, allowing it to engraft within the bone marrow, and since the BCL11A gene is effectively disabled, the levels of HbF is elevated which in turn ameliorates the severity of the disorder.

A phase 1/2 CLIMB SCD-121 trial involving SCD patients and over two vaso-occlusive episodes annually for the previous two years demonstrated high levels of efficacy of CTX001. Overall, patients experienced high levels of HbF and the eradication of vaso-occlusive episodes after infusion. CTX001 also provides transfusion independence to its patients. A 33 year old female patient with total Hb level being 7.2g per deciliter of which 9% was HbF had increased to 12g per deciliter and 43.2% was HbF within 15 months of the CTX001 infusion. She had previously 7 vaso-occlusive episodes a year but none have been experienced in the 16.6 months after infusion. Three serious adverse events were encountered out of 114, which were cholelithiasis, abdominal pain and sepsis, but all were treated and resolved. However, this trial has a very limited sample size and thus the analysis of CTX001 in additional patients with lengthier follow up periods are required to generalise the success of these preliminary results [9].

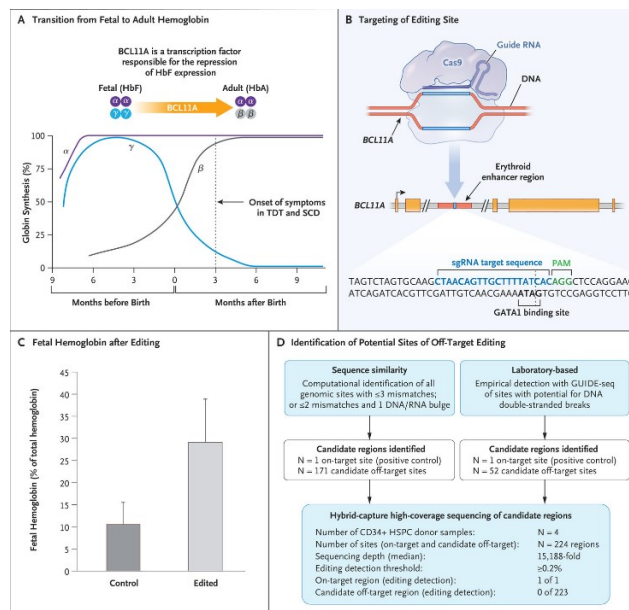


Figure 2. Summary of study. A: decline of HbF after birth; B: CRISPR/Cas9 in genetically modifying HSPCs; C: results of preclinical studies; D: evaluation result showing no evidence of off-target editing.

Furthermore, Casgevy, the first FDA approved CRISPR-based therapy in 2023 produced by Vertex Pharmaceuticals for the treatment of SCD, has also shown impressive data in phase 3 clinical trials. Casgevy is a one-time treatment which increases the production of HbF in patients aged 12 and over. 16 out of 17 patients receiving Casgevy ceased to experience vaso-occlusive crises, and the remaining patient has been free of hospitalisation due to complications caused by these episodes [10]. There has also been no off-target effects observed so far in people receiving Casgevy, although this possibility cannot be disregarded. However, Casgevy has proven difficult to manufacture and relatively inaccessible since only few locations globally possess the level of technology and expertise required to carry out this treatment. Moreover, its accessibility is further compromised by its sky-rocketing cost of \$2 million dollars per patient. Although this is not a direct limitation of the CRISPR technology itself, the chemotherapy required before initiating the CRISPR treatment can also have potential adverse effects that can be detrimental on patients.

2.2. Duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD) is a severe disorder characterised by progressive muscle degeneration. This condition almost exclusively affects males as it is caused by a mutation of the DMD gene on the X chromosome. The DMD gene encodes for dystrophin, and the mutation causes truncation during protein translation and thus produces unreliable or completely useless dystrophin. Dystrophin deficiency results in disconnection between the cytoskeletal F-actin and the extracellular matrix, leading to a wide range of muscular dysfunction including loss of muscle integrity. Even with optimal care, most suffering from DMD die between 20 and 40 years old, often due to heart failure. However, life expectancy for patients with DMD has improved as shown by a study in France in which the median life expectancy with the disease has increased by 15.18 years for those born after 1970 compared to before [11].

Therapeutic approaches to DMD require restoring skeletal and cardiac muscle function and structure, but many have failed and there remains no curative treatment. The CRISPR/Cas9 tool provides the opportunity to correct the gene mutation with high precision and potentially permanently restore dystrophin expression. Experiments have been conducted on muscular dystrophy X-linked (mdx) mice, which have a nonsense mutation in exon 23 of the DMD gene, therefore acting as a model for the disease. The study objective was aimed at correcting the defective gene using the CRISPR/Cas9 system in vivo

with HDR. The Cas9 endonuclease, specifically designed sgRNA, and a single-stranded oligodeoxynucleotide (ssODN) as the exogenous DNA template for HDR were injected into mouse zygotes in hopes of correcting the point mutation during embryonic development. The results concluded demonstrated a 2 to 100% correction of the gene mutation throughout the mice sample, while an even higher rate of phenotypic success of muscle function was recorded suggesting the benefit of the rectified cells in restoring healthy muscle tissue [12]. However, the translation of this into human germline editing is still under vast debate [13], and the process of promoting HDR in postmitotic cells, for instance in muscle tissue, is extremely inefficient and not yet a feasible task, requiring further technological research into the means of triggering the process or finding a suitable alternative [14].

NHEJ is used as an alternative mean to rectify the DMD mutation since it is the dominant repair process in cells. In this case, CRISPR/Cas9 is able to induce NHEJ in the cell and thus deactivate the dysfunctional genes and add deletions and inserts (indels), making this method another research potential for DMD treatment.

The sheer size of the dystrophin gene, the largest human gene containing 79 exons [15], further complicates gene delivery. Adeno Associated Viruses (AAVs) at the moment appears to be the most efficient way of delivering all the components required for HDR in vivo. AAVs prove superior to other viral vectors owing to their low immunogenicity, and in one study when it was used as a vector for a CRISPR/Cas9 based technology used to treat DMD, it showed no sign of toxicity [16]. Despite this, there were indications of unprecedented gene alterations, therefore further research is required in order to ensure the minimum level of off-target effects.

Instead of the traditional exon skipping where defective exons are effectively bypassed, gene sequence removal by CRISPR/Cas9 proves to be a more advantageous method, applicable to 83% of patients suffering from DMD [17] as it takes advantage of NHEJ repair, which is effective in all cells, in order to restore reading frame and create a shortened but more functional dystrophin protein. Moreover, this treatment has a long-term effect in altering the gene mutation and therefore leads to reduction of complications as well as lowered costs. This concept was explored in vitro where exons 21-23 in mdx mice were excised using the CRISPR/Cas9 system; the results proved a successful restoration of dystrophin reading frame [18], as well as substantial recovery of muscular function. Although DMD progression in mdx mice does not flawlessly mirror disease progression in humans, the rodent model still has many common pathological features.

Another study concerns the therapeutic treatment of DMD using CRISPR/Cas9 to correct patient-derived induced pluripotent stem cells (iPSCs) [19]. Exon knock-in proved to be more effective than exon skipping and frameshifting as it was able to produce an entire length of a dystrophin protein. The ability of iPSCs to differentiate could be capitalised on for replacing dysfunctional muscle cells in patients affected by DMD. There is the potential for immunogenic reactions, but these could be stifled due to the immunosuppressive effects of the treatment. CRISPR/Cas9 has other general limitations that apply to its involvement in all therapeutic treatments which impact both its safety and efficacy. The sgRNA could deviate from target site and cause off-target cutting resulting in unintended, arbitrary genetic modifications [20]. Various modifications to optimise the CRISPR/Cas9 system have been investigated to minimise possibility of off-target effects. Most importantly, designing suitable sgRNA is vital as target specificity is majorly reliant on the sgRNA and PAM sites [21].

2.3. HIV

Nowadays, the acquired immunodeficiency syndrome (AIDS) caused by HIV poses significant challenges for the medical realm, and despite considerable efforts, there is still no absolutely effective vaccine nor cure. HIV affects 39.9 million people globally and 630,000 had died from AIDS related complications as of 2023. [22] While HIV is a chronic condition that is manageable with effective treatment, AIDS represents an advanced stage of HIV and occurs when HIV has already severely impaired the immune system and the body has become highly vulnerable due to the critically low number of CD4 cells. HIV belongs to a collection of lentiviruses characterised by long incubation periods; many people live unknowingly with HIV for years before symptoms of the disease manifest in

the body [23]. Current therapies include highly active antiretroviral therapy (HAART) which serves to minimise virus reproduction. This has significantly reduced morbidity and mortality in those infected with HIV, however, this has many limitations, most importantly it cannot eradicate latent viral reservoirs meaning that HIV remains an incurable condition. Moreover, not only does HIV have the ability to develop resistance against these antiretrovirals (ARVs), the issue of long-term, frequent treatment doses and the costs that come with it are most dire in countries where the disease is the most rampant. As a result, only 59% of HIV patients are receiving this treatment despite it being first line therapy for HIV-1 patients [24].

HIV takes over the body by first entering CD4 cells and making copies of itself, resulting in cell death. The immune system combats this by rapidly generating CD4 cells, but once it cannot supersede the depletion of CD4 cells by HIV, it is severely weakened, and the body becomes predisposed to even the most common diseases [25].

The exploration of the CRISPR/Cas9 system for the treatment of HIV is hugely important as unlike antiretroviral therapy (ART), it possesses the key potential to purge the latent viral reservoirs, meaning that the virus could be entirely eliminated in the body and terminate the need for lifelong treatment. [26] The latent viral reservoirs are found in the resting memory CD4 cells and degrade at an excruciatingly slow pace, which makes it almost impossible to expunge [27]. One study used two Jurkat clone cell lines to mimic the latent state of HIV infection. The CRISPR/Cas9 system was then introduced into these cell lines in an attempt to disrupt the latent HIV provirus; results showed a 92.56% and 98.48% HIV latency expression in the two cell lines without CRISPR/Cas9, and an impressive 68.78% and 66.95% in the two cell lines which had been transfected with the CRISPR/Cas9 tool targeting HIV. This experiment conveyed the success of CRISPR/Cas9 in reducing the number of cells with latent HIV, therefore indicating its potential in treating and preventing latent HIV infection [28].

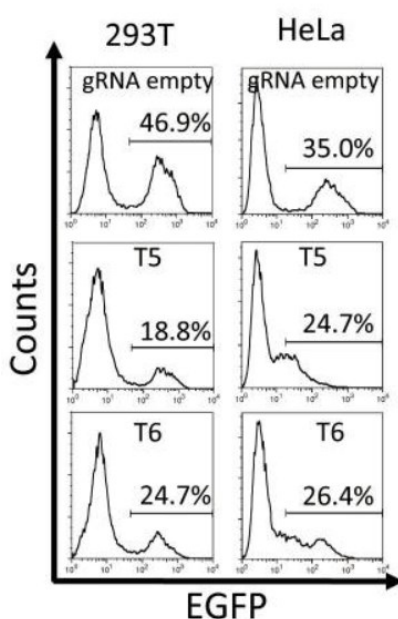


Figure 3. Histograms showing GFP expression after CRISPR/Cas9 treatment [28].

Furthermore, HIV viral genome lies in the chromosomes of the infected patient as a provirus with two long terminal repeat (LTR) regions on either side, which is an essential component for HIV replication. Two specific sgRNAs were designed to target two regions within HIV-1 LTR- TAR sequence and NF- κ B binding site in order to inhibit HIV replication. This was tested using a mimic of provirus-integrated human cells- 239T and HeLa cells infected with an LTIG HIV vector which allows the activity of the virus to be assessed through the level of production of green fluorescent protein (GFP).

Results of flow cytometry analysis showed a considerable reduction in GFP of 25.6% in 293 T cells, suggesting the successful hinderance of HIV-1 gene expression. Less pronounced effect was observed in the HeLa cells possibly due to lower efficiency of CRISPR/Cas9 and/or lower levels of GFP expression (Figure 3).

Moreover, when the protocol of triple transfection was carried out on 293 T cells, an even greater percentage reduction of 38.7% cells expressing GFP of was detected. Sequence analysis was performed on the plasmid DNA and it showed the success of CRISPR/Cas9 in producing mutations in the desired TAR region of proviral DNA as a result of cleavage and subsequent repair by NHEJ (Figure 4).

A. Deletion

```

WT  GGGTCTCTCTGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCA
                                     WT: 9.1%(2/22)
GGGTCTCTCTGGTTAGACCAGATCTGAG---TGGGAGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTGACACAGATCTGAG---GGGAGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAG---CTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGATCTGAG---GGGAGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGATCTGA-CCTGGGAGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGATCTG---GGGAGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGATCT---GAGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGATCTGAG---TCTCTGGCTAACTAGGGAACCCA
GGG-----AGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGATC---CTGGGAGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGA-----GCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGATCTGAG---AGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGA-----TCTGGCTAACTAGGGAACCCA
                                     Deletion: 81.8%(18/22)

```

B. Deletion and Insertion

```

WT  GGGTCTCTCTGTTAGACCAGATCTGAG-----CCTGGGAGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGATCTGAA-----AGCTCTCTGGCTAACTAGGGAACCCA
GGGTCTCTCTGGTTAGACCAGAGATGTCTCAGCAGAGAGATGGGAGCTCTCTGGCTAACTAGGGAACCCA
                                     Insertion: 9.0%(2/22)

```

Figure 4. Sequence analysis of target cleavage site. 18 out of 22 DNA clones having deletion mutants (A) and 2 out of 22 clones having insertion and deletion mutants (B) Orange indicates deletion target site of CRISPR/Cas9; blue indicates unexpected mutation; red indicates insertions.

Moreover, another method to reduce HIV activity utilised LTRs as the ideal target to be excised simultaneously by the CRISPR/Cas9 tool in order to extract the proviral DNA integrated in the host cell genome. 293 T cells were used which were infected with a lentivirus vector which lacked the U3 region of LTR but an additional EF promoter driving GFP expression, this was to ensure that GFP expression was not impacted by CRISPR/Cas9's effect on the LTR but rather exclusively the result of successful lentivirus DNA excision. Results of this experiment concluded a successful reduction in cells expressing GFP, and double TF demonstrated further decrease of around 10%.

3. Discussion

This review discussed the application of CRISPR/Cas9 as a genome editing tool in three diseases, namely SCA, DMD, and HIV. In SCA, CRISPR/Cas9 is applied ex vivo in the patient's own hematopoietic stem cells where the BCL11A gene is edited and suppressed in order to increase the production of HbF. They are transplanted back into the patient and engraft within the bone marrow. Currently, the first and only FDA approved CRISPR/Cas9 based therapy, Casgevy, is used to treat patients with SCD with this mechanism of action. In DMD, CRISPR/Cas9 has been applied in various ways in attempts to ascertain the most suitable therapy for patients. CRISPR/Cas9 was introduced directly into murine zygotes in order to correct the defective DMD gene, but the ethicality of this practice in translation to human zygotes is still subject to questioning. CRISPR/Cas9 editing was also applied to iPSCs in order to replace dysfunctional muscle cells, and lastly its ability to extract mutated exons was also investigated in mdx mice. Furthermore, CRISPR/Cas9's application in HIV therapy has targeted regions of the LTR for two purposes- to inhibit viral replication, and to excise the integrated proviral

DNA as a whole. Importantly, it also has been manipulated to target and eliminate latent viral reservoirs, which are crucial hurdles to overcome in the journey to tackling HIV.

CRISPR/Cas9 is efficient, convenient, and versatile, not only providing huge insight into interactions between the genetic organisation and subsequent effects on biological phenotype, but also drastically improving our hope of curing previously tenacious and debilitating diseases. Despite its vast potential, a main concern is its off-target effects, which can result in unprecedented genetic mutations thus the apprehension of its implementation in the medical realm. These effects occur due to the mistaken binding of the Cas9 protein to sequences similar to the target site.

CRISPR/Cas9 is primarily guided to the target site by the sgRNA as discussed previously and the proto-spacer adjacent motif (PAM) sequence. PAM is a short sequence of DNA that signals the cleavage site; it is present adjacent to the target DNA sequence and serves to prevent the CRISPR/Cas9 system from excising host DNA and introducing undesired mutations.

Studies have demonstrated that a 10-12 base pair sequence of the sgRNA which is next to the PAM, referred to as the seed sequence, is vital in ensuring Cas9 specificity. It has also been concluded that uracil-rich seeds have the potential to ameliorate target specificity [29]. However, further experiments were carried out which revealed that shorter seeds, only one to five base pairs in length, are identified to be the seed region [30]. The reason for its importance lies in its composition. The sequence determines not only the frequency of the target site appearance in the genome but also the likeliness of these target sites to be open and accessible. Off-target effects are made more prominent when the seed sequence is more common and when potential cleavage sites are easily accessible.

Methods of off-target detection can be separated into biased and unbiased. Biased detection relies on the intricate designing of guide RNA for target precision and the prediction of certain conditions which may induce off-target effects [31]. In silico tools have been utilised to minimise these effects through the data generated in predictive models. Unbiased off-target detection involves monitoring unprecedented cleavage sites through in vitro and in vivo detection and aims to do so in an unrestricted manner [32].

Although studies have suggested the use of several sgRNAs for the most effective gene activation [33], and in order to reduce off-target binding, it often also increases the number of possible off-target binding sites. Moreover, ChIP-seq assays only determine the binding site of DNA and provides no insight into the location of cleavage, thus leading to overassessment of potential off-target sites. Some studies have suggested that the direct delivery of the sgRNA and purified Cas9 protein into cells could reduce off-target effects due to the inclination of this complex to degrade swiftly in cells. The methods of eliminating off-target effects of CRISPR/Cas9 are still largely uncertain, but it is certainly necessary to carefully design the seed sequence and PAM as they are integral aspects of the system.

References

- [1] Asmamaw M and Zawdie, B 2021 Mechanism and Applications of CRISPR/Cas-9-Mediated Genome Editing. *Biologics: Targets and Therapy*, 15, 353–361. <https://doi.org/10.2147/BTT.S326422>
- [2] Huang X, Yang D, Zhang J, Xu J and Chen, Y. E. (2022). Recent Advances in Improving Gene-Editing Specificity through CRISPR-Cas9 Nuclease Engineering. *Cells*, 11(14), 2186. <https://doi.org/10.3390/cells11142186>
- [3] Kamal S M, Fouad A M, (2018) Chapter 4.5 - Hepatitis C Infection in Patients With Hemoglobinopathies <https://doi.org/10.1016/B978-0-12-803233-6.00014-X>
- [4] Iqbal, Z., Li, M., McKendry, R., Horton, M., & Caruana, D. J. (2013). Investigation of sickle-cell haemoglobin polymerisation under electrochemical control. *Chemphyschem : a European journal of chemical physics and physical chemistry*, 14(10), 2143–2148. <https://doi.org/10.1002/cphc.201300203>
- [5] National Heart, Lung, and Blood Institute; National Institutes of Health; U.S. Department of Health and Human Services <https://www.nhlbi.nih.gov/health/sickle-cell-disease/treatment#>:

- ~:text=Four%20medicines%20have%20been%20approved,different%20aspects%20of%20the%20disease.
- [6] Rochette, J., Craig, J. E., & Thein, S. L. (1994). Fetal hemoglobin levels in adults. *Blood reviews*, 8(4), 213–224. [https://doi.org/10.1016/0268-960x\(94\)90109-0](https://doi.org/10.1016/0268-960x(94)90109-0)
 - [7] Watson, J., Starman, A.W., & Bilello, F.P. (1948). THE SIGNIFICANCE OF THE PAUCITY OF SICKLE CELLS IN NEWBORN NEGRO INFANTS. *The American Journal of the Medical Sciences*, 215, 419-423.
 - [8] Forget B. G. (1998). Molecular basis of hereditary persistence of fetal hemoglobin. *Annals of the New York Academy of Sciences*, 850, 38–44. <https://doi.org/10.1111/j.1749-6632.1998.tb10460.x>
 - [9] Frangoul H, Altshuler D, Cappellini MD, Chen YS, Domm J, Eustace BK, et al. 2021 CRISPR-Cas9 gene editing for sickle cell disease and β -thalassemia. *New England Journal of Medicine*. 384(3):252-260. DOI: 10.1056/NEJMoa2031054
 - [10] Henderson, H (2024) CRISPR Clinical Trials: A 2024 update <https://innovativegenomics.org/news/crispr-clinical-trials-2024/#>
 - [11] Duan, D., Goemans, N., Takeda, S. et al. Duchenne muscular dystrophy. *Nat Rev Dis Primers* 7, 13 (2021). <https://doi.org/10.1038/s41572-021-00248-3>
 - [12] Chengzu Long et al. , Prevention of muscular dystrophy in mice by CRISPR/Cas9–mediated editing of germline DNA.*Science*345, 1184-1188(2014).DOI:10.1126/science.1254445
 - [13] Bosley, K., Botchan, M., Bredenoord, A. et al. CRISPR germline engineering—the community speaks. *Nat Biotechnol* 33, 478–486 (2015). <https://doi.org/10.1038/nbt.3227>
 - [14] Hsu P. D., Lander E. S., and Zhang F., Development and applications of CRISPR-Cas9 for genome engineering (2014). <https://doi.org/10.1016/j.cell.2014.05.010>
 - [15] Gao, Q. Q., & McNally, E. M. (2015). The Dystrophin Complex: Structure, Function, and Implications for Therapy. *Comprehensive Physiology*, 5(3), 1223–1239. <https://doi.org/10.1002/cphy.c140048>
 - [16] Mollanoori H, Rahmati Y, Hassani B, Havasi Mehr M, Teimourian S. Promising therapeutic approaches using CRISPR/Cas9 genome editing technology in the treatment of Duchenne muscular dystrophy. *Genes Dis*. 2021;8(2):146–156. doi: 10.1016/j.gendis.2019.12.007
 - [17] Christopher E. Nelson et al. , In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy (2016).DOI:10.1126/science.aad5143
 - [18] Xu, L., Park, K. H., Zhao, L., Xu, J., El Refaey, M., Gao, Y., Zhu, H., Ma, J., & Han, R. (2016). CRISPR-mediated Genome Editing Restores Dystrophin Expression and Function in mdx Mice. *Molecular therapy : the journal of the American Society of Gene Therapy*, 24(3), 564–569. <https://doi.org/10.1038/mt.2015.192>
 - [19] Li HL, Fujimoto N, Sasakawa N, et al. Precise correction of the dystrophin gene in Duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Reports*. 2015;4(1):143–154. doi: 10.1016/j.stemcr.2014.10.013
 - [20] Lino CA, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: a review of the challenges and approaches. *Drug Deliv*. 2018;25(1):1234–1257. doi: 10.1080/10717544.2018.1474964
 - [21] Manghwar H, Li B, Ding X, Hussain A, Lindsey K, Zhang X, Jin S. 2020. CRISPR/Cas systems in genome editing: methodologies and tools for sgRNA design, off-target evaluation, and strategies to mitigate off-target effects. *Advanced Science* 7:1902312 DOI 10.1002/adv.201902312
 - [22] UNAIDS Global HIV & AIDS statistics- Fact sheet 2024 <https://www.unaids.org/en/resources/fact-sheet>
 - [23] Klatt E C 2022 Pathology of HIV/AIDS. The Mercer University of Medicine Savannah
 - [24] 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>

- [25] New York State Department of Health HIV: The Basics https://www.health.ny.gov/diseases/aids/general/resources/child/docs/chapter_1.pdf
- [26] Saayman, S., Ali, S. A., Morris, K. V., & Weinberg, M. S. (2015). The therapeutic application of CRISPR/Cas9 technologies for HIV. *Expert Opinion on Biological Therapy*, 15(6), 819–830. <https://doi.org/10.1517/14712598.2015.1036736>
- [27] Siliciano, J. D., Kajdas, J., Finzi, D., Quinn, T. C., Chadwick, K., Margolick, J. B., Kovacs, C., Gange, S. J., & Siliciano, R. F. (2003). Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4⁺ T cells. *Nature medicine*, 9(6), 727–728. <https://doi.org/10.1038/nm880>
- [28] Ebina, H., Misawa, N., Kanemura, Y., & Koyanagi, Y. (2013). Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. *Scientific reports*, 3, 2510. <https://doi.org/10.1038/srep02510>
- [29] Xiao, H. Z., Tee, L. Y., Xiao, G. W., Qun, S. H., & Shi, H. Y. (2015). Off-target effects in CRISPR/cas9-mediated genome engineering: Molecular therapy - nucleic acids. *Off-target Effects in CRISPR/Cas9-mediated Genome Engineering*. <https://doi.org/10.1038/mtna.2015.37>
- [30] Wu X, Scott D A, Kriz A J, Chiu A C, Hsu P D, Dadon D B, Cheng A W; Trevino, Alexandro E; Konermann, Silvana; Chen, Sidi; Jaenisch, Rudolf; Zhang, Feng; Sharp, Phillip A . (2014). Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nature Biotechnology*, 32(7), 670–676. doi:10.1038/nbt.2889
- [31] Naeem M, Majeed S, Hoque MZ, Ahmad I. 2020 Latest Developed Strategies to Minimize the Off-Target Effects in CRISPR-Cas-Mediated Genome Editing. *Cells*. ; 9(7):1608. <https://doi.org/10.3390/cells9071608>
- [32] Martin F, Sánchez-Hernández S, Gutiérrez-Guerrero A, Pinedo-Gomez J, Benabdellah K. 2016 Biased and Unbiased Methods for the Detection of Off-Target Cleavage by CRISPR/Cas9: An Overview. *International Journal of Molecular Sciences*. 17(9):1507. <https://doi.org/10.3390/ijms17091507>
- [33] Cheng, A. W., Wang, H., Yang, H., Shi, L., Katz, Y., Theunissen, T. W., Rangarajan, S., Shivalila, C. S., Dadon, D. B., & Jaenisch, R. (2013). Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell research*, 23(10), 1163–1171. <https://doi.org/10.1038/cr.2013.122>