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The Recent Advances in the Use of Molecular Scissors (Gene Editing Tool) for the Treatment of Diseases

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Abstract

Sequencing of the human genome marked an important milestone in studying the genetic basis of disease states. Gene editing is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. This review seeks to shed more light on the recent advances in the use of molecular scissors (gene editing tool) for the treatment of diseases. Molecular scissors, also known as restriction enzymes or endonucleases, are a class of enzymes that play a crucial role in genetic engineering. Several nucleases genome editing strategies, including Zinc Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated proteins (CRISPR-Cas), are the most widely used gene editing techniques. The basic mechanism involved in genetic manipulations is the recognition of target genomic loci and binding of effector DNA-binding domain (DBD), double-strand breaks (DSBs) in target DNA by the restriction endonucleases (FokI and Cas), and the repair of DSBs through homology-directed recombination (HDR) or non-homologous end joining (NHEJ). With the continuous optimizations of these technologies, ZFNs, TALENs, and CRISPR-Cas9 have already entered human clinical trials. To date, the majority of clinical applications of these technologies are focused on ex vivo gene editing therapeutics, which are highly effective for many medical conditions, although with known limitations, the in vivo applications of CRISPR technologies are also challenged with issues. Moreover, the novel innovations, such as Base editing and Prime editing, are still at the pre-clinical stage. Therefore, more efforts are needed to address these limitations to accelerate the treatment of genetic and infectious diseases.

Keywords: CRISPR-Cas9; Gene Editing; Genome Engineering; Gene Therapy; Molecular Scissors.

1.0 Introduction

Sequencing the human genome was a significant step in studying the genetic basis of disease. Thanks to advancements in sequencing technology, the entire human genome can now be sequenced. The creation and analysis of disease models, as well as the functional characterization of genes, have been made easier by genome engineering, which involves single-base manipulation of the genetic material. The development of techniques to exchange DNA fragments in yeast through the homologous recombination system marked the beginning of genome engineering in the late 1970s (Ortega *et al*. 2018; Wu and Zhang, 2023). This review seeks to shed more light on the recent advances in the use of molecular scissors (gene editing tool) for the treatment of diseases.

The general term for techniques that allow scientists to precisely alter the genetic code of numerous organisms is "gene editing" (Khan, 2019). In gene editing, target genomic loci are recognized and the effector DNA-binding domain (DBD) is bound; restriction endonucleases cause double-strand breaks (DSBs) in target DNA; and DSBs are repaired by homology-directed recombination (HDR) or non-homologous end joining (NHEJ). This is the fundamental mechanism that underlies genetic manipulation using programmable nucleases. (Khan, 2019; Teboul *et al*. 2020).

Molecular scissors, sometimes referred to as restriction enzymes or endonucleases, are essential tools for working with and researching DNA because of their extraordinary capacity to cut DNA molecules at particular sequences (Makarova *et al.* 2018; Wu and Zhang, 2023).

Transcription Activator-like Effector Nucleases (TALENs), Zinc Finger Nucleases (ZFNs), and Clustered Regularly Interspaced Short Palindromic repeats-CRISPR-associated proteins (CRISPR-Cas) are among the most frequently employed nuclease genome editing strategies. With this exact cutting, scientists can make the desired modifications to the DNA, like fixing mutations or turning off genes that cause disease (Qasim *et al.* 2017; Sun *et al.* 2018; Inturi and Jemth, 2021).

2.0 DNA damage processes and repair system

In humans, 23 pairs of chromosomes—one paternal copy and one maternal copy—carry genetic information in the form of DNA. The conservation and transmission of genetic information depend on its double helix structure, which is made up of two complementary strands made up of a series of nitrogenous bases known as nucleotides. A codon, which is made up of three consecutive nucleotides, is equivalent to a particular amino acid in the assembled protein. DNA strands can sustain damage during divisions, including double-strand breaks (DSBs), which can compromise the viability of cells. When a DSB occurs, a cell can use either directed homologous recombination (HDR—homology-directed repair) or nonhomologous end joining (NHEJ) to fix the break and preserve its genetic integrity (Wang *et al*. 2018; Teboul *et al*. 2020).

3.0 Non-homologous End Joining (NHEJ)

Nucleotides that are no longer in multiples of three are randomly inserted or deleted during NHEJ correction, which most frequently results in the suppression of the repaired gene's expression (the creation of a knockout, or KO). Therefore, this kind of deletion or insertion most frequently causes a change in the codons' reading frame during translation into protein, which prevents translation into protein or results in a shortened protein that cannot perform its primary function.

4.0 Homologous Directed Repair (HDR)

On the other hand, the HDR approach fixes the break by homologously recombining an intact sister chromatin DNA strand (from the other chromosome) as a template strand for the creation of repaired DNA (Xue and Greene, 2021; Sauvagère and Siatka, 2023).

Since it enables the precise correction or even integration of a sequence that is determined and chosen by the experimenter at the level of the gene to be altered, the HDR strategy seems to be the preferred method in gene therapy. In practice, molecular scissors can be combined with single-strand oligonucleotide DNA (ssODN) in donor oligonucleotides that are homologous to the target sequence to be corrected (apart from the part that the

experimenter modified), which acts as a template for correction in the homologous recombination induced during HDR (Rivera-Torres *et al*. 2017; Sauvagère and Siatka, 2023).

5.0 Overview of molecular scissors (gene editing tools)

Molecular biology underwent a radical change with the discovery of molecular scissors. In the 1960s, researchers who were looking into the phenomenon of bacterial resistance to viral infections made the initial identification. Bacteria were shown to have a defense mechanism that involves snipping the DNA of invasive viruses at particular locations. Later research revealed that this method was mediated by restriction enzymes, which break DNA like molecular scissors (Jonlin, 2019; Fareh *et al.* 2021).

The fundamental purpose of molecular scissors is to recognize specific DNA sequences, known as restriction sites, and break the DNA at or near these places. Each restriction enzyme identifies an exact sequence of nucleotides and cuts the DNA at a specific point within that sequence. Because of its specificity, scientists can accurately target and work with particular DNA molecule regions (Barrangou and Horvath, 2017; Zhou *et al.* 2022).

Depending on the kind of enzyme and the precise sequence being targeted, molecular scissors can cut DNA in a variety of ways. The enzyme may occasionally cut the DNA in a way that produces "sticky ends," which are single-stranded extensions that readily attach to complementary sequences. This characteristic is frequently used to introduce foreign DNA into a plasmid or other DNA molecule through molecular cloning procedures. In other situations, the enzyme cuts the DNA in a way that produces "blunt ends," leaving no overhangs and cutting the DNA directly across both strands (Jonlin, 2019; Fareh *et al.* 2021).

Numerous applications in genetic engineering and biotechnology have been made possible by the ability to use molecular scissors to cut DNA at specified spots. Molecular scissors, specifically the CRISPR-Cas9 system, have transformed gene editing. Researchers can make precise modifications to the genome, such as repairing mutations that cause disease or adding advantageous features, by employing a guide RNA to guide the molecular scissors to a particular DNA sequence (Tycko *et al.* 2016; Zhang *et al.* 2021; Zhou *et al.* 2022).

Molecular scissors are crucial instruments for DNA analysis methods like DNA fingerprinting and restriction fragment length polymorphism (RFLP) analysis, which use the distinct patterns of DNA fragments produced by various restriction enzymes to identify people or examine genetic variations (Inturi and Jemth, 2021). Molecular scissors are employed in several diagnostic procedures to identify particular DNA sequences linked to illnesses or hereditary conditions. Researchers can determine if disease-related genetic markers are present or absent by snipping DNA at certain locations and examining the bits that are left over (Song *et al.* 2018; Revill e*t al.* 2019; Robb, 2019).

Owing to developments in genome editing, it is now feasible to effectively modify DNA by taking advantage of enzyme capabilities, which holds great promise for treating genetic illnesses in humans. To correct variations in genes, several nuclease genomes editing techniques have been developed, such as Zinc Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated proteins (CRISPR-Cas) (Qasim *et al.* 2017; Sun *et al.* 2018; Inturi and Jemth, 2021).

6.0 Types of molecular scissors

6.1 Zinc Finger Nucleases

Zinc Finger Proteins (ZFPs) are hybrid heterodimeric proteins that are produced artificially for site-specific genome editing. Zinc Finger (ZF) domains are a group of three to six peptides that are part of ZFPs. Each of these peptides binds to a certain base pair sequence for a particular attachment to a gene sequence (Lu *et al.* 2022). To generate a DSB at a particular genomic location, two ZFPs must fuse with Flavobacterium okeanokoites endonuclease I (~~FokI~~). ZFNs are commonly employed for the elimination and silencing of genes

thereby have become a flexible tool for treating inherited illnesses by targeting genes in a variety of mammalian cells, organisms and the development of disease models in animals (Sun *et al.* 2018; Lu *et al.* 2022).

6.1.1 The structure of ZFNS and their interaction with DNA

A site-specific DNA-binding domain loaded on the zinc finger is fused to a non-sequence-specific cleavage domain to create ZFNs (Smith *et al.* 2019). Xenopus oocytes' transcription factor IIIa was the primary source of the discovery of the zinc-finger protein with site-specific DNA binding capabilities in 1985 (Almeida and Matos, 2019). An array of Cys2His2 zinc fingers (ZFs) that are derived from highly conserved interactions of their zinc-finger domains make up the functional specificity of the designed zinc-finger domain. The zinc finger is composed of about 30 amino acids that form two anti-parallel β sheets that oppose an α-helix (Li *et al.* 2020). The most prevalent kind of DNA-binding motif in eukaryotic transcription factors is thought to be Cys2-His2-ZF, an adaptive DNA recognition domain.

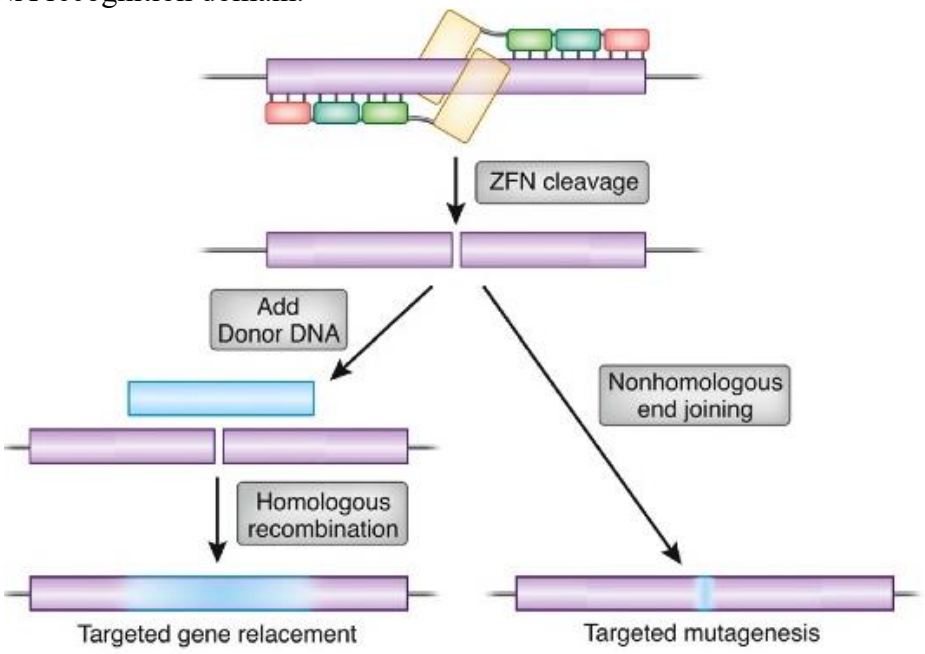

Figure 1: Mechanism of zinc finger nucleases (Carroll, 2011)

Figure 1 shows a pair of ZFNs, each with three zinc fingers binding to target DNA, introducing a double-strand break at the FokI domain, depicted in yellow. Subsequently, the double-strand break is shown as being repaired through either homology-directed repair or non-homologous end joining.

Through the interaction of its α-helix residues with the main groove of DNA, each zinc-finger unit selectivity may identify three base pairs (bp) of DNA and creates base-specific connections (Smith *et al.* 2019). The domain that cleaves DNA is formed by the FokI type II restriction endonuclease, and it can be used as a dimer to target specific genome sequences for efficient gene editing. Two ZFN molecules are often needed to attach to the target site in the proper orientation since the FokI nuclease must dimerize to cleave DNA, which doubles the number of base pairs that are particularly recognized. ZFN-induced DNA cleavage in eukaryotic cells triggers DSBs at a particular genomic location, causing the required changes in the body following NHEJ or HDR repair processes (Li *et al.* 2020).

Three primary aspects influence ZFNs' ability to recognize and specifically target sequences: (a) the amino acid sequence of each finger, (b) the number of fingers, and (c) the nuclease domain's interaction. Because of ZFNs' modular structure, scientists can optimize the DNA-binding and catalytic domains separately, creating new modular assemblies with adequate affinity and specificity for genome engineering. In preliminary research, ZFN dimers were able to specify 18–36 bp of DNA at each cleavage site by interacting with a 9–18 nucleotide target using individual ZFNs with three–six fingers (Li *et al.* 2020). This method made it easier to target particular regions in the human genome for the first time since the 18 bp DNA sequence can provide specificity within 68 billion bp of DNA. A more recent tactic called "selection-based methods" employed architectural diversification

to increase ZFN targeting accuracy (Paschon, 2019): this study increased the number of ZFN combinations available for targeting cleavage to any given base of DNA by 64 times by creating a novel linker option for spanning finger–finger and finger–FokI cleavage domain junctions (Paschon, 2019).

6.2 Transcription Activator-Like Effector Nucleases (TALENS)

The plant pathogen Xanthomonas has been found to include a class of naturally occurring DNA-binding proteins known as the Transcription Activator-Like Effector (TALE). The transcription of several host target genes is regulated by these TALEs (Bhardwaj and Nain, 2021). Artificially created proteins known as TALENs combine the DNA cleavage of the FokI endonuclease with the DNA binding capabilities of a TALE protein (Qasim *et al.* 2017). The 34 amino acid repeats that make up the core region of TALEs are divided into 32 constant and 2 variable repeat variable residues (RVDs). RVDs play a role in identifying DNA targets (Bhardwaj and Nain, 2021). To create a FokI dimer that causes a DSB, two TALENs target binding sequences must be present. Another kind of designed nuclease that outperforms ZFNs in terms of efficiency and specificity is TALENs (Li *et al.* 2020). It is because TALEs are compatible with a wide range of functional domains, they provide versatile applications in genetic engineering. TALE proteins can change from transcriptional modulators to genome editing tools depending on their relationships with transcriptional activators, repressors, or endonucleases (Thakore and Gersbach, 2016).

6.2.1 Talens and their interaction with DNA

A nuclear localization signal (NLS), an acidic domain for target gene transcription activation, a core DNA binding domain of 12–28 repetitions, and the Fok1 nuclease make up a typical TALEN unit (Juillerat *et al.* 2015; Benjamin *et al.* 2020). With polymorphic 12 and 13 repeat variable residues (RVDs), the DNA-interacting region is a sequential arrangement of 33–35 amino acids that has been conserved. Every repetition attaches itself specifically to one nucleotide on the target in the 5′ to 3′ orientation (Thakore and Gersbach, 2016). According to biochemical structure-function studies, a nucleotide on the DNA target major groove can be uniquely identified by the amino acid located at position 13 (Thakore and Gersbach, 2016; Qasim *et al.* 2017). The amino acid at position 12 stabilizes this DNA-protein interaction unit. There is a half-repeat of just 20 amino acids at the 3-end of the target locus that binds the DNA sequence. NN, NG, HD, and NI constitute the four most prevalent RVDs found by many experimental validations. They each have a distinct preferred binding affinity towards G/A, T, C, and A, respectively, providing target specificity. There are also reports of a remarkable screening of all 400 conceivable RVD combinations, which are regarded as non-conventional RVDs due to their uncommon occurrence in nature (Juillerat *et al.* 2015; Benjamin *et al.* 2020). The popularly used TALEN system comprises 2 units of DNA-binding domain (DBDs) from TALE proteins. Each unit is attached to a catalytic domain from the Folk1 restriction enzyme. Fok1 nuclease of the TALENs dimerizes, which generates a cleavage on both strands of the DNA double helix, activating the DNA repair machinery to fix the disruption (Bhardwaj and Nain, 2021).

TALENS can be produced with the necessary sequence precision by aligning repetition modules (RVDs) in a specific structure. However, the number of target locations that TALEN can choose from is limited. There must always be a thymine at position 0, or right before the TALE-repeat bound sequences (Benjamin *et al.* 2020). The modest van der Waal forces between the highly conserved tryptophan in the N-terminal and the C5 methyl group of thymine guarantee full gene activation. There have also been reports of more recent TALEs in nature that substitute cytosine for thymine at position 0 without affecting their function. These scaffolds do not require the 5′T requirement (Thakore and Gersbach, 2016). However, it is relatively easy and adaptable to modify any genome using TALE-based methods. Each repeating unit generates a V-shaped structure made up of two alpha helices joined to form a solenoid-like structure wrapped around the primary groove of DNA via the hypervariable 12 and 13 amino acids, according to the crystal structure of TALE proteins linked to target DNA (Bhardwaj and Nain, 2021).

6.3 Clustered Regularly Interspaced Short Palindromic Repeats—CRISPR-associated protein 9

The advanced gene editing technology known as the CRISPR-Cas9 system transformed the area of genome engineering and sparked interest in the possibility of developing new therapeutic strategies to cure human

illnesses (Makarova *et al.* 2018). For discovering the CRISPR-Cas nuclease system, Emmanuelle Charpentier, the director of the Max Planck Institute in Berlin, Germany, and Jennifer Doudna, a professor at the University of California, Berkeley in the United States, were granted the 2020 Nobel Prize in Chemistry (Sauvagère and Siatka, 2023).

The CRISPR-Cas system is divided into class I and class II (Sauvagère and Siatka, 2023). The class I uses multiprotein complexes for nucleic acid cleavage and is subdivided into CRISPR-Cas types I, III, and IV. The class II, which is further classified into CRISPR-Cas types II, V, and VI, cleaves using a single protein effector domain. CRISPR-Cas9 is a member of the type II system, which is the most commonly used tool for biological research and translational applications due to its ease of use (Robb, 2019).

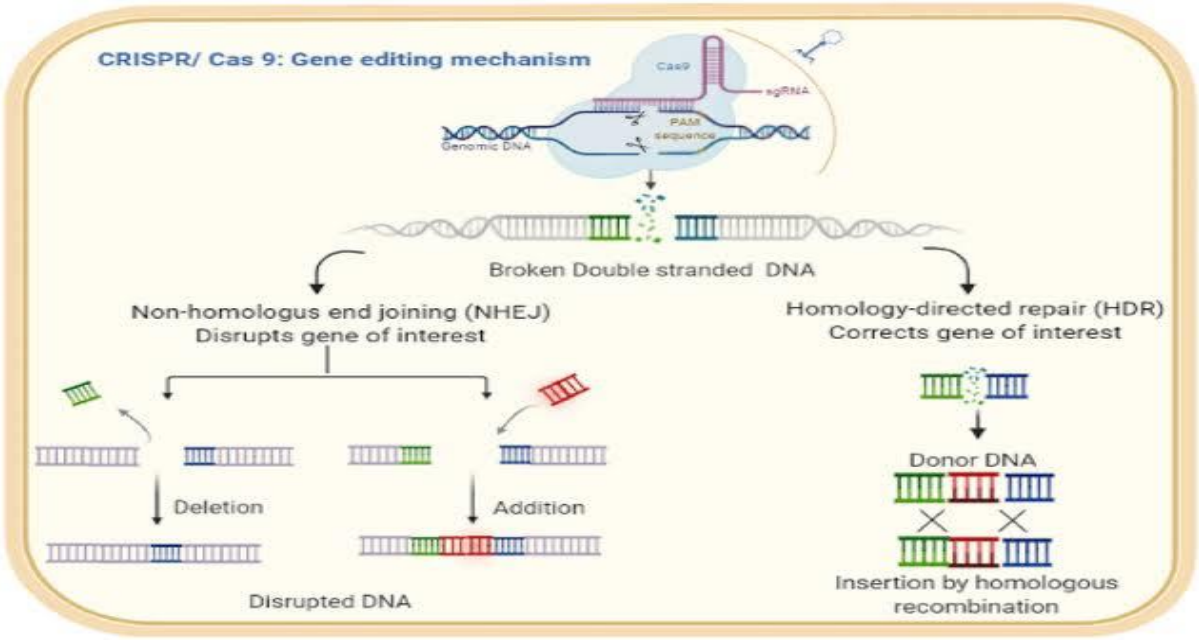

Figure 2: Mechanism of CRISPR (Robb, 2019)

A Cas9 nuclease and a single guide RNA (sgRNA) that is modified from CRISPR RNA (crRNA) are needed for the system. The sgRNA identifies the target location (the spacer sequence) and is fused with a trans-activating RNA (tracrRNA), which combines with the crRNA to form a complex (the scaffold sequence) (Barrangou and Horvath, 2017). The Cas9 nuclease first binds to a protospacer adjacent motif (PAM) to start the protein's early conformational alterations, and the sgRNA forms a stable ribonucleoprotein complex with it. Twenty nucleotides of RNA-DNA base pairing between the complementary RNA strand and the target DNA strand protospacer, as well as interactions between the non-target DNA strand PAM, are what drive the targeting activity, which mediates the protein's subsequent second conformational shift and causes it to become active. When Cas9 is activated, the RuvC domain cleaves at the PAM strand while the HNH domain cleaves the DNA strand that the sgRNA is bound to (Zhou *et al.* 2022). The fact that CRISPR/Cas9 just needs a sgRNA to identify the DNA sequence where a DSB has to be produced is one of its benefits (Sauvagère and Siatka, 2023).

6.3.1 CRISPR classification

Based on their flanking Cas genes and the target's placement on foreign DNA, the CRISPR/Cas system is divided into two categories, each of which has several subtypes (I–V) (Wu and Zhang, 2023), which have enhanced our knowledge of the phylogenetic categorization of bacteria and our capacity to create phage-resistant strains. CRISPR/Cas operates in three stages during phage/plasmid invasion: interference, expression, and adaptation. Specific traits that lead to antiviral or antiplasmid immunity are linked to each stage (Alagoz and Kherad, 2020). The process of adaptation involves integrating the invader-derived spacers, also referred to as the spacer sequence, with the CRISPR array. The spacer sequence is present in CRISPR-associated RNA (crRNA), which is produced by transcription of the CRISPR loci. After that, an endonuclease is created, which cleaves the invading genome using the spacer sequences as a guide (Rivera-Torres *et al.* 2017).

The traits of the Cas1 and Cas2 genes determine the functional features of the CRISPR/Cas system. Based on the specific marker proteins, taxonomic studies first divided the CRISPR/Cas system into three types: Cas3 (type I), Cas9 (type II), and Csm/Cmr (type III). Later, the CRISPR editing system's classes IV and V were introduced. The genes that encode the functional proteins and components (Cascade, Csm, Cmr complex, or Cas9) form the basis for the broad classification of the CRISPR system (Selle *et al.* 2020).

Class 1 CRISPR systems functions consist of multi-subunit crRNA-effector complexes, and include type I, III, and putative type IV (Tycko *et al.* 2016). All of the CRISPR system's actions are carried out by the single protein Cas9 in class 2, which is also seen in putative class V (Rivera-Torres *et al.* 2017). Type I, the importance of the Cascade complex, which is followed by the Cas3 nuclease, defines CRISPR. Cas6e cleaves pre-crRNA, the outcome of a transcribed CRISPR array, to produce crRNA. The Cascade-related gene crRNA is in charge of identifying the protospacer in the target DNA (Li *et al.* 2020).

Additionally, the protospacer adjacent motif (PAM), a brief sequence that is close to the target sequence, is recognized and found by Cas8, another Cascade subunit. For type I cascade-C to operate as an immunological defense mechanism, the PAM sequence is essential; when it malfunctions, proteins are unable to (Chen *et al*., 2017), prevents the establishment of the R-loop between target DNA and crRNA, which ultimately leads to viral evasion of CRISPR screening. When a fully working CRISPR system is present, recognition triggers the Cas3 nuclease, which nicks the target's (virus or plasmid) single-stranded DNA and causes it to degrade (Alagoz and Kherad, 2020).

Unlike type I, type II depends on two RNAs, RNase III and tracer RNA, a PAM that is found downstream of the protospacer sequence in target DNA, and that the Cas9 protein recognizes, and Cas9 as the only Cas protein (Rubin *et al*., 2020).

When one strand is cleaved by the HNH nuclease domain and another by the RuvC nuclease domain, a DSB is created. However, cleavage necessitates the discovery of a PAM sequence by Cas9, which causes dsDNA to dissociate and an R-loop to develop between the DNA and crRNA. Because of this structural alteration, the tracer RNA binds to the cleavage target sequence (Rubin *et al*., 2020).

Cas6 is the only one with type III, whose endoribonuclease mechanism cleaves pre-crRNA to create crRNA. In contrast to other CRISPR system models, this one presents the outcome of Cas6-induced crRNA cleavage. After maturing, the crRNA tag, which is downstream of the spacer sequence, becomes six nucleotides (Chen *et al.* 2017). In type III-A and Cas10-C mr complex III-B systems, the crRNA complex grows in size and generates Cas10-C sm. Unlike types I and II, type III targets both DNA and RNA, resulting in co-transcriptional cleavage of the target DNA guided by crRNA. Csm3 (type III-A) and Cmr4 (type III-B) cleave RNA transcripts, while the palm domain of Cas10 cleaves DNA strands. The PAM is not always necessary for the system to start immunological processes, which is another significant distinction of type III (Alagoz and Kherad, 2020).

6.3.2 Specificity of the CRISPR-CAS9 tool

The two components of this tool are essential for genome modification: the Cas9 enzyme, which functions as an endonuclease with two cleavage sites, allowing a break in the double strands of DNA; and guide RNA (gRNA), which condenses the crRNA and tracrRNA into a single RNA and is selected to precisely direct Cas9 to the cleavage site. This RNA is made up of a fixed 42-nucleotide sequence that is used to connect to Cas9 and a variable 20-nucleotide sequence that is complementary to the targeted gene's sequence (Song *et al.* 2018). Since Cas9 self-inhibits in the free state and cannot bind to the target sequence to carry out its endonuclease function, the 20 nucleotides are selected in proximity to the PAM motif (three nucleotides upstream). After the guide RNA binds to the target sequence, Cas9 physically rearranges and adopts a conformation that allows it to bind to the target DNA (Tycko *et al*., 2016). The PAM motif is required for Cas9 binding. Each DNA strand is cleaved by the RuvC and HNH catalytic sites of Cas9 once the Cas9/gRNA complex has attached to the DNA. This sets off a chain of events that eventually attracts the cellular machinery to start the DNA repair pathways. Protein complexes first identify the break, which then attracts enzymes like glycosylases, endonucleases, and exonucleases. The nucleotide rearrangement required for DNA repair is made possible by this multiple complex (Chen *et al.* 2017). After that, a DNA polymerase is enlisted to either synthesize a DNA strand from a template strand (complementary DNA strand or exogenous ssODN single-strand donor) in the event of the HDR

technique or to add nucleotides at random in the case of the NHEJ strategy (Charpentier *et al*., 2015). A gene may be declared invalid or a harmful mutation corrected through the induction of repair through guided homologous recombination. In this case, nucleotide sequences homologous to those before and after the cleavage site must be present in the exogenously synthesized ssODN donor strand. The donor strand may have a nucleotide sequence between these sequences that allows for the modification of the target gene, specifically the correction of a mutation, the introduction of missing exons, the reduction of gene expression, or its inhibition (Sauvagère and Siatka, 2023).

Table 1**:** Key features of each technology (ZFN, TALEN, and CRISPR/Cas9).

Key features of each technology	**ZFN**	**TALEN**	**CRISPR/Cas9**
Enzyme	Fok1-nuclease	Fok1-nuclease	Cas9 nuclease
Target site	Zinc-finger binding sites	RVD tandem repeat region of TALE protein	PAM/spacer sequence
Recognition sequence size	9 – 18 bp	14 -20 bp	3 – 8 bp/20 bp
Targeting limitations	Difficult to target non-G-rich sites	5’ targeted base must be a T for each TALEN monomer	The targeted site must precede a PAM sequence
Advantage	1. Small protein size 2. Relatively easy in vivo delivery	1) High specificity 2) Relatively easy to engineer	1) Easy to engineer 2) Easy to multiplex
Disadvantage	1) Expensive 2) Time-consuming 3) Difficult to select the target sequence 4) All the ZF domains should be active	1) Difficult to multiplex 2) Not applicable for methyl cytosine DNA 3) Limited in vivo delivery 4) All the TALEs should be active	1) Lower specificity 2) Limited in vivo delivery

7.0 Applications in disease treatment of infectious diseases

7.1 Human immunodeficiency virus

AIDS, which is caused by the human immunodeficiency virus-1 (HIV-1), poses a serious threat to human health worldwide. There are an estimated 38.6 million HIV-1-positive individuals worldwide (Olson *et al*., 2020). It is a retrovirus that uses the envelope surface protein gp120 to target human cells. By attaching itself to the CD4+ receptor on the cell membrane, the virus engages with either C-X-C motif chemokine receptor 4 (CXCR4) or co-receptor chemokine receptor 5 (CCR5). HIV-1 uses RNA reverse transcriptase to transform the viral genome into double-stranded DNA once it has entered the cell. The host genome incorporates the viral DNA. It then develops into a provirus, which can either enter a latent state without producing any virus or actively transcribe RNA to become a descendent virus. We examine two HIV treatment approaches that focus on either host or viral genes (Liu *et al*., 2020).

7.2 Targeting viral genes

CRISPR-Cas9 was utilized in a 2015 study to disrupt ten locations in the HIV-1 genome in JLat10.HIV-1 was latently present in six cells. All target sites were effectively exposed to mutation by the CRISPR-Cas9 system. The most disrupted exon of Rev was the second one, designated T10. The expression of HIV-1 and virus production were significantly diminished (Zhu *et al*., 2015). Furthermore, gRNAs for efficient and sustained protection against HIV-1 infection in primary human T cells and HIV reservoir cell types generated from human

pluripotent stem cells (hPSCs) were screened and discovered in a study. Their findings demonstrated that CRISPRCas9-mediated mutagenesis significantly suppressed viral genes at the target sites within the LTR sequence, especially in the R region (Liao *et al*., 2015).
Alongside the aforementioned research, another researcher used a smaller Cas9 from *Staphylococcus aureus* (Sa) and the CRISPR-Cas9 system from *Streptococcus pyogenes* (Sp) to create gRNAs targeted at the HIV-1 genome. According to the in vitro experiments, gRNAs/SaCas9 may effectively lower the expression of provirus genes in infected Jurkat C11 cells and lower the production of viruses in TZM-bl and Jurkat T cells (Wang *et al*. 2018).
Similarly, in 2020, a study developed a dCas9 that uses a transcriptional repressor domain derived from the Kruppel-associated box (KRAB) to epigenetically silence the HIV-1 provirus DNA. Through chromatin modifications such as increased histone H3 lysine 9 trimethylation and decreased H3 histone acetylation, the gRNAs/dCas9-KRAB selectively reactivate latent HIV-1 provirus and suppress HIV-1 transcription. This makes the shock-and-kill method of HIV-1 antiviral treatment more effective (Olson *et al*. 2020).

7.3 Targeting host genes

A ZFN-mediated method for rupturing CCR5 genomic sequences in HSPCs was devised. They caused 72.9% biallelic CCR5 excision by nucleofecting HSPCs with CCR5-specific ZFN mRNA. The ZFN-treated CCR5−/− HSPCs preserved lineage potential in immunodeficient NSG mice, according to the in vivo test. This indicates that the therapeutic management of HIV-1 infection could be greatly enhanced by a transplant of virus-resistant HSPCs (DiGiusto *et al*. 2016).
A study effectively knocked out the CCR5 gene in human CD34+ HSPCs using CRISPR-Cas9. The in vivo findings demonstrated a strong resistance to HIV-1 and substantial CCR5 excision in immunodeficient NPG mice (Xu *et al*. 2017).
A study conducted in 2020 used viral vectors and the CRISPR/AsCpf1 technology to effectively alter the endogenous CCR5 gene in vitro. In comparison to the sgRNAs employed with CRISPR-Cas9, the identified sgRNAs for CRISPR/AsCpf1-mediated CCR5-targeting excision showed little off-target effects at the expected sites. Compared to the wild-type, the CCR5−/− cells exhibited a selection advantage and demonstrated considerable resistance against CCR5-tropic HIV-1 infection. Additionally, they demonstrated that the CRISPR/AsCpf1 system hardly ever impacted CCR5−/− cells' ability to proliferate and undergo apoptosis (Liu *et al*. 2020).
Furthermore, a study used CRISPR/SaCas9 to target CXCR4 in human CD4+ T cell lines to render these cells resistant to HIV-1 infection that is X4-tropic. They created resistance to HIV-1 infection and effective CXCR4 excision in primary CD4+ T cells using the AAV-SaCas9/sgRNA system without compromising cell viability or proliferation (Wang *et al*. 2017).

7.4 Hepatitis B Virus (HBV)

Frequent hepatic irritation, which can result in fatal liver disease or neoplasm, are a common feature of hepatitis, mostly brought via a viral infection called HBV (Revill *et al.* 2019). Every year, 1.4 million individuals pass away from issues related to hepatitis, and there are over two billion cases of HBV infections globally (Revill *et al.* 2019). The Hepatitis B virus (HBV) represents a circular deoxyribonucleic acid (DNA) virus, characterized by a genome that encodes several critical components, including the hepatitis B core antigen (HBcAg), the e antigen (HBeAg), DNA polymerase, the surface antigen (HBsAg), and a transcriptional transactivating protein known as HBx (Chuang *et al.* 2022). The virus infiltrates hepatocytes through its interaction with the HBV receptor, sodium taurocholate co-transport polypeptide (NTCP). Upon its entry into the nucleus, the viral DNA is converted into closed covalent circular DNA (cccDNA), which possesses the capability to integrate into the host genome and transcribe ribonucleic acid (RNA) molecules, ultimately facilitating the expression of viral proteins. Pregenomic RNA (pgRNA), one of the RNA species discovered, is essential to the formation of viral particles (Chuang *et al.* 2022). Because it confers the templates required for viral replication and pgRNA synthesis, the cccDNA of HBV has become the most solid option for genome - editing. Additionally, it shows a strong association with both the progression of Viral hepatitis after

antiretroviral therapy and the tenacity inside liver cells (Chuang *et al.* 2022). Therefore, in addition to reducing the Viral infection, current treatment approaches focus on reducing cccDNA levels in liver tissues (Revill *et al.* 2019; Chuang *et al.* 2022).

Researchers have used the CRISPR-Cas9 technology to study anti-HBV. CRISPR-Cas9 was used in one research to identify and cause particular gene mutations in the conserved regions of the Viral genome. Strong viral genomic expression studies and replication reduction were the results of this, both in vivo and in vitro Similarly, CRISPR-Cas9 can be used to break the Nucleotide sequence of HBV and prevent the virus from replicating (Ramanan *et al.* 2015).

A study defined a CRISPR-Cas9 system (gRNA-S4) that was targeting the gene that is encoding HBsAg and suppressed viral replication with little off-target activity and impact on cellular viability. In a murine infection model of HBV, the gRNA-S4 system reduced serum levels of HBsAg by 99.91%±0.05% and suppressed serum levels of HBV DNA to below the negative cut-off (Li *et al.* 2015).
A study designed gRNAs to target the preS1/preS2/S open reading frames of the HBV genome and generated HBsAg knockout hepatocellular carcinoma (HCC) cell lines with the aid of CRISPR-Cas9. The outcome was that reducing HBsAg levels in the HCC cell lines strongly suppressed in vitro HCC growth and in vivo tumorigenicity. Additionally, knocking out HBsAg suppressed the production of interleukin (IL)-6 by the HCC cells and blocked the activity of the STAT3 pathway. In contrast, the overexpression of HBsAg produced the accumulation of the intracellular pY-STAT3, which revealed the tumorigenic potential of HBsAg of the HBV-related HCC (Song *et al.* 2018).
The DSB induced by CRISPR-Cas9 is associated with host genome destabilization and is inefficient at editing the genome, with a limitation to its usage (Khalil, 2020). The CRISPR cytidine base editors (CBEs) are capable of gene silencing by inducing premature stop codons (Gaudelli *et al.* 2017).
A study also took a CBE approach to silence the gene expression of HBV by replacing a nucleotide within the viral genome. gRNA/CBE was targeted to the 30th codon of the gene S to mediate the exchange of the native CAG to a premature stop codon of TAG. It resulted in the induction of premature stop codons at the target site by about 71% of cultured cells. Expectedly, the levels of the HBV mRNA were significantly less while the secreted HBsAg was downregulated by 92%, with the intracellular HBsAg being downregulated by 84% of cultured cells. Besides this, off-target effects were rarely observed within predicted off-target sites within the HBV genome (Zhou *et al.* 2022).

7.5 Severe Acute Respiratory Syndrome (SARS) CoV-2

The SARS-CoV-2 is a linear single-stranded RNA coronavirus (Monteil *et al.* 2020). Upon receptor binding, SARS-CoV-2 attaches to the cellular membrane and inserts the genome inside the cytoplasm to produce viral proteins. Once the viral particles are produced, the SARS-CoV-2 is transported to the cellular membrane by the action of the vesicles and is expelled by the action of the exocytosis (Monteil *et al.* 2020). Since SARS-CoV-2 is an RNA coronavirus, the RNA-targeting CRISPR-Cas13 is a highly promising drug to combat COVID-19. The Cas13 protein uses a designerable spacer sequence of a CRISPR RNA (crRNA) that can guide the Cas13 protein to excise RNA molecules with an exact cut (Zhang *et al.* 2018).
In 2020, a treatment method called prophylactic antiviral CRISPR in human cells (PAC-MAN) was established by a research work. It is used to combat infection by targeting highly conserved viral genome regions and eliminating the sequences by way of CRISPR-Cas13d. In the case of the human lung epithelial cells, the crRNA is directed to degrade the fragments of the synthetic SARS-CoV-2 by Cas13d and significantly limits viral infection. Bioinformatics analysis demonstrated that the rationally designed crRNAs target greater than 91% of the sequenced regions of the SARS-CoV-2, showing that the viral sequence excision by way of the CRISPR-Cas13d could be a prospective antiviral solution to the treatment of the SARS-CoV-2 (Abbott *et al.* 2020). In addition, research used a reprogrammed CRISPR-Cas13b to effectively silence the replicase gene of the genotypes of various SARS-CoV-2 types within cultured models of monkeys and humans. In their results, they also demonstrated that Cas13 is able to associate with single-nucleotide mismatches with the guide crRNA while

retaining the activity of the catalyst. It means that the gene editing tool of the CRISPR-Cas13 is a potential treatment of SARS-CoV-2 (Fareh *et al.* 2021).

7.6 Human Papilloma Virus

The HPV is a DNA papillomavirus with a double helix structure with a total of about 150 documented subtypes (Zheng *et al.* 2022). It is differentiated into low-risk types that produce genital warts and the high-risk types that produce cancers (e.g., cervical cancer) (Zheng *et al.* 2022). Of the best-studied subtypes are HPV-16 and HPV-18 that are highly infectious causing sexually transmitted diseases that are associated with cervical cancer (Zheng *et al.* 2022). HPV E6 and E7 are the genes that are the oncogenes that are required to activate derived malignant cells (Zheng *et al.* 2022). Gene knockout targeting the E6 and E7 is a potential treatment approach to HPV infection-derived cancers of the cervix (Zheng *et al.* 2022).

In 2015, a research made the use of TALENs to target the genes E6 and E7. The disruption of the genes by the action of TALENs reduced the viral DNA copy number. It recovered the activity of the tumor suppressors retinoblastoma 1 (RB1) and p53, thus reversing the malignant signs of HPV-16 infection within a murine transgenic model (Hu *et al.* 2015).

A study yielded a cervical cancer model that contained HPV E6 and E7 proteins inside them, and they applied gene therapy with the assistance of CRISPR-Cas9 by way of PEGylated liposomes. It yielded notably small tumors (Jubair *et al.* 2019).

In addition, a research revealed that the CRISPR Cas9-mediated deletion of the genes E6 and E7 initiates cellular senescence of immortal HPV-18 infected cells. They demonstrated that the specific downregulation of HPV-18 E6 triggers the tumor-suppressing pathway of p53/p21 and pRb/p21. In addition to that, deletion of the gene E7 reduces the expression of E6 and initiates the pathway of pRb/p21 (Inturi and Jemth, 2021).

7.7 Gene editing and non-viral infection

In addition to viral infection, gene editing can also provide treatment to other kinds of infection that are not viral, e.g., bacterial infection, parasitic infection, infection by fungi.

7.7.1 Bacteria

Clostridium difficile is a nosocomially acquired pathogen that results in half a million *C. difficile* infections (CDI) and a corresponding thirty thousand deaths yearly within the United States. Abuse of antibiotics is a predisposing factor of CDI due to broad-spectrum antimicrobials disrupting the native microbiota of the gut and compromising the colonization resistance to C. difficile. For this reason, a novel treatment that can regulate CDI while clearing *C. difficile* with a specific effect without harming the microbiota of the gut is a priority that must be addressed with all due speed. An experiment incorporated a self-targeting system within the anti-*C. difficile* agent and then induced the CRISPR-Cas3 gene that targets the bacterial chromosome. The results indicated that the bacteriophage-mediated CRISPR-Cas system is a robust inhibitor of *C. difficile* replication within the mouse model. It is a sign that treatment with CRISPR-Cas is a potential approach to the treatment of CDI, in vivo (Selle *et al*. 2020).

7.7.2 Fungi

The CRISPR-Cas9 complex can be optimized and modulated to target fungi by the usage of gene elements of fungi (e.g., the usage of a fungal promoter) to provide highly efficient delivery of the CRISPR-Cas9 complex. Candida albicans is a pathogen that is diploid and causes the majority of the cases of fungal infection. Nevertheless, genetic manipulation challenges to Cas9-mediated gene editing of C. albicans are present, as will be elaborated below. Nevertheless, a research work established a gene drive array (GDA) approach to monitor genome manipulation and successfully introduce biallelic mutations into C. albicans. With the usage of the GDA technology, they researched certain potential targets of C. albicans to treat with drug pumps and biofilm adhesins.

Various barriers to genetic manipulation are present within the species of Candida. These include the inability to preserve the engineered plasmids, the choice of specific codons, and inefficient homologous recombination

(Shapiro *et al.* 2018). An article provided a speedy CRISPR-Cas9-based solution to the C. albicans genome to overcome the challenges and manipulate the genome within a time frame of about a month. It provided a practical solution to the transformation by way of the fungus haploids and provided a handy approach to crossing the edited Candida to generate the biallelic mutant fungi. Scientists can adapt this approach to develop genetic manipulation to other infectious, mating-proficient, haploid fungi (Halder *et al.* 2019).

7.7.3 Parasite

Toxoplasma gondii is a diet-borne pathogen that causes toxoplasmosis, a potentially fatal disease among immunocompromised individuals or individuals with a congenital infection. Another study also made use of Cas9 to conduct a genome-wide CRISPR analysis of Toxoplasma to discover potential gene-editing targets. It revealed that the claudin-like apicomplexan microneme protein (CLAMP) is a key component of P. falciparum infection (Sidik *et al.* 2016). The protein is a key player of the asexual stages of the parasite. Inhibiting the CLAMP gene specifically significantly impairs the asexual cycle of P. falciparum. In addition to this, since the CLAMP homologues are present in nearly all the apicomplexan genomes, CLAMP can potentially serve as a potential gene editing target to treat toxoplasmosis Sidik*et al.* 2016). Another research carried out the gene editing of the TgCPSF3 gene by the aid of CRISPR-Cas9. The gene encodes the endonuclease that is a key component of eukaryotic mRNA processing and is a potential target to develop anti-T. gondii medicines. The in vivo assay demonstrated that the murine models that were infected by the gene-edited T. gondii together with oral treatment with the drug AN3661 (which is a commonly applied benzoxaborole drug to inhibit the Toxoplasma growth) had no observable illness while the groups that were not treated had a fatal infection (Palencia *et al.* 2017).

In America, more than ten million individuals and many animals are afflicted by the parasite Trypanosoma cruzi (Wu and Zhang, 2023). In one work, the TcGP72 and paraflagellar rod proteins 1 and 2 genes were disrupted using CRISPR-Cas9. Both flagellar adhesion and flagellum development depend on them. According to the findings, T. cruzi genes can be effectively edited by CRISPR-Cas9 with negligible host damage (Lander *et al.* 2015). Additionally, PFR1, PFR2, and GP72 mutagenesis dramatically impairs flagellar attachment and boosts the parasites' motility (Lander *et al*. 2015).

A previous study made the targeting of the Leishmania parasite genes that lead to the fatal leishmaniasis disease in humans by the use of the CRISPR-Cas9. It utilized a dihydrofolate reductase-thymidylate synthase (DHFR-TS) to regulate the Cas9 protein, while a U6 was utilized to regulate the gRNA. Using this approach, they were successful in knocking out the paraflagellar rod-2 locus (Sollelis *et al.* 2015).

7.8 Cancer treatment

Oncogenes and mutant tumor suppressor genes present exceptional opportunities to exploit the applications of genome-modulating strategies. Genome editing technology has achieved key target-directed cleavage events in various ground-breaking studies, ranging from its earlier proofs of highly efficient gene editing of eukaryotes to its current applications within the hematopoietic stem cell (HSC) and the targeting of tumors with T cells; this technology has introduced novel gene modification concepts and has broadened to a border area of research on cancers (Li *et al.* 2020). Scientists have made the usage of ZFN to enhance the therapy of tumors by T cells. For instance, by importing a chimeric TCR that includes a zetakine-containing extracellular IL-13 domain and a cytoplasmic domain of CD3 into the CD8 + T cells, glioblastoma-specific cytolytic T lymphocytes (CTLs) can be produced. To this end, the modified CTLs were silenced with the glucocorticoid receptor by the usage of ZFNs. Therefore, the cytolytic activity of “zetakine” CTLs that are transgenic was maintained regardless of the administration of the treatment with glucocorticoid. It was possible to knock out the genes of glucose transport-related genes (MCT4 or BSG) by this technology with effectiveness within two models of the glycolytic tumors of the types of the colon adenocarcinoma and the glioblastoma (Marchiq *et al.* 2015).

It was demonstrated that specific cleavage of the BCR-ABL fusion gene by highly specific ZFNs abolished the translation of the BCR-ABL protein and induced apoptosis of the imatinib-resistant chronic myeloid leukemia (CML) cells (Huang *et al.* 2018). CRISPR/Cas9 gene editing was also used to produce organoid models of tumors. Under the direction of colonoscopy, by mucosal injection, a study established the CRISPR-engineered mouse tumor organoids by injecting viral vectors that carry the elements of the CRISPR/Cas9 into the distal

colon of the mice (Roper *et al*. 2018). An approach of this kind was also used to a research mimicking the sequence of the adenoma-carcinoma-metastasis of a tumor (Li *et al*. 2020).

7.9 Hereditary disorders

TALENs have been successfully applied in editing T-cells in treating leukemia (Qasim *et al.* 2017; Benjamin *et* al., 2020). UCART19, a CAR-T-cell therapy with a TALEN engineered design, was investigated in phase I trials in adults and children (NCT02808442 and NCT02746952) in treating advanced lymphoid malignancies and treatment-refractory B-cell acute lymphoblastic leukemia (B-ALL). The trials demonstrated the potential UCART19 in aggressive leukemia patients but significant adverse events in the guise of cytokine release syndrome, graft-versus-host and infection-related complications have been observed (Benjamin *et al*. 2020).
Sickle anaemia is the first genetic disease with a pathogenesis distinctly elucidated. An A to T mutation in a sole nucleotide in the first exon of the human β-globin creates a lesion. In 2016, a paper was reported on a research on the usage of CRISPR/Cas9 technology in editing mutations in patient-derived HSCs in a laboratory. After differentiation of edited iPSCs into red blood cells, normal HBB mRNA was traceable. The preclinical trial provided a theoretical foundation for the use of gene editing technology in treating sickle anemia. The system based on CRISPR/Cas was also utilized in editing mutations in the HBB, resulting in β thalassemia. By harnessing the usage of the CRISPR/Cas technology in directing the targeted DNA sequence to the location where the mutation in the HBB is situated, editing two independent mutations in the beta thalassemia in the patient's HBB gene was enabled via HR (Martin *et al*. 2019).

8.0 Difficulties and restrictions

Among the largest issues with gene editing yet are efficiency, specificity, and delivery. The most appropriate strategy for delivering gene-editing treatment would depend on the cell-targeting strategy that is most appropriate in treating a specific illness. The ex vivo methods are viable in a contained environment, are available in efficient means with a variety of methods, and could be a relatively uncomplicated way of getting precision gene-editing medicine to the clinic (Khalil, 2020).

8.1 The ZFN associated constraints

Despite ZFNs revealing their promise in editing a specified gene in mammalian cells, the process is met with three major limitations.
1) Cannot handle random gene sequences (Lu *et al.* 2022).
2) A ZFN coding gene is engineered for every specific target site (Porter *et al.* 2019).
3) The danger of off-target gene editing is yet another constraint in ZFN technology. The targeted sequence would originally need to contain 5′-GNN, 5′-ANN, 5′-CNN, or 5′TNN. This technology is consequently labor-intensive, time-consuming, and costly, and involves highly qualified scientists in protein engineering (Porter *et al*. 2019).

8.2 The TALENS associated constraints

In relation to ZFNs, TALENs are efficient, exhibit low off-target effects, and are proven to home in on the DNA in mitochondria. Despite a few limitations preventing a wider implementation, there are.
1) The repeated elements in TALEs are challenging to synthesize with polymerase chain reaction (PCR)
2) Due to the effect caused by cytosine methylation, which may prevent binding and change recognition by its usual RVD, TALENs are unable to attach to methylated DNA (Lu *et al*., 2022).

Various approaches have been proposed to overcome the challenges associated with the TALEs' repetitive sequences, including: (i) creating cloning techniques that do not require ligation, (ii) high levels of throughput solid-phase assembly, (iii) the Golden Gate Cloning and (iv) alternative one-day TALE assembly (Lu *et al.* 2022).

8.3 Restrictions on the use OF CRISPR technology

Despite their numerous uses, these designer nucleases are not yet considered accurate and safe enough for specific treatments, e.g., in gene therapy. While there are off-target effects in every genome editing system, high frequencies (≥ 50%) of unforeseen off-targets in the technology of CRISPR/Cas9 are a major setback (Bhardwaj and Nain, 2021). Researchers have made efforts in trying to eliminate the problem of CRISPR off-targets with the design of new variants of Cas9 and the structure optimization of the gRNA (Jonlin, 2019), but these efforts have not been particularly effective. Another major setback with the technology is the requirement for a PAM close to where editing is intended. With a brief PAM recognition sequence of 5′ NGG3′, where N is a variable nucleotide, Cas9 originating in the bacterium Streptococcus pyogenes (SpCas9) is among the widely used Cas9s. Additionally, spCas9 is hard to fit in the most widely used gene therapy vehicle, the AAV vectors, due to its large structure (Bhardwaj and Nain, 2021). The resulting DSBs in CRISPR induce apoptosis and DNA damage, and cellular cytotoxicity (Robb, 2019). All conventional gene editing systems are technically and functionally flawed, and there are reservations about their viability in reality (Bhardwaj and Nain, 2021).

Table 2: Limitations of ZFNs, TALENs, and CRISPR-Cas9.

Molecular Scissors	**Limitations**
Zinc–Finger Nucleases (ZFNs)	1. It can have off-target effects, leading to unintended mutations. 2. The competition between NHEJ and HR repair pathways can complicate precise editing. 3. ZFNs are larger compared to TALENs.
Transcription Activator-Like Effector Nucleases (TALENs)	1. Similar to ZFNs, TALENs can have off-target effects. 2. NHEJ and HR repair pathways can compete, hindering precise editing. 3. TALENs are larger than ZFNs.
CRISPR-Cas9	1. Off-target effects can occur, leading to unintended mutations. 2. Mosaicism (variation in gene edits) can occur. 3. Achieving complex genome modifications can be challenging.
General Limitations	1. Delivery of the editing machinery to target cells and tissues can be a challenge. 2. Potential risks include cancer, allergic reactions or tissue damage.

9.0 New developments in gene editing for the treatment of diseases

9.1 Summary of the main advances

Until the introduction of CRISPR technologies in genome editing, ZFN and TALEN approaches were suitable alternatives to gene replacement treatments owing to their precision and efficiency in DNA cutting and editing (Gaudelli *et al*. 2017). However, they possess a variety of disadvantages: These two approaches need the production of a protein specific to the targeted site, a process that is labor-intensive and costly. The ZFN method is not capable of targeting all existing sequences of nucleic acids, and the construction of the chimeric protein permitting the scission is not something a researcher is capable of doing and involves a labor-intensive process provided by external service companies. The TALEN, on the other hand, is extremely bulky (3 kb), and encapsulation within a transfection vehicle is extremely difficult. The fact restricts its usage in therapy in comparison with ZFN (1 kb) (Porter *et al.* 2019; Bhardwaj and Nain, 2021). In contrast, the CRISPR-Cas9 system has the advantage of having the ability to design itself to identify any DNA sequence in the genome, owing to the reduced design complexity in guide RNAs. The second has the ability to construct a multiplex for the system to identify and modify multiple places in a sequence. One injection could consequently modify multiple genes in a cell in a simultaneous process (Zhang *et al.* 2021).

9.2 Gene-engineering technologies based on nickase

Double-strand breaks (DSBs) in a targeted genomic site would be accompanied by undesirable consequences such as activation of p53, translocations, off-target mutation, and complex undesirable products. Furthermore, half of all identified disease-associated gene variants are point mutations (Robb, 2019). The Cas9 nickases with a targetable feature arose consequently. The Cas9n D10A and Cas9n H840A are variants of Cas9 mediating the incision in a single DNA strand, respectively, in the complementary or non-complementary DNA strand in the presence of a guide RNA. The Cas9 nickases have been fused with other enzymes to engineer newer gene editing technologies such as Cytidine base editor (CBE), Adenosine base editor (ABE), and Prime editor (PE) (Zhao *et al.* 2021; Lu *et al.* 2022). The optimal setup for SpyCas9 D10A nickases in human cell lines is to use guide RNAs that are positioned outside the nicking sites, with the nicking sites 40–70 bp apart. SpyCas9 D10A nickases can also be used to create insertion-type genome edits using repair templates that are exogenously supplied.

9.3 The base editor

Base editors are mostly used in a way that aims at targeting a point mutation that would cause a changed DNA sequence with a new and enhanced function and inactivating a gene (Gaudelli *et al.* 2017). DNA base editing is briefly characterized by having two major elements: a fused Cas9n and a deaminase, and a targeted guide RNA (sgRNA) targeted to a specific DNA sequence. There are three (3) types of base editors, such as Cytidine Base Editor (CBE), Adenosine Base Editor (ABE), and Glycosylase Base Editors (GBE) (Gaudelli *et al.* 2017; Zhao *et al.* 2021).

In theory, base editors are useful in dividing and non-dividing cells to cure a variety of single-nucleotide polymorphisms (SNPs) in human diseases.

Base editing is applied in research and treatment of hereditary diseases in a variety of cell types (Tekel *et al.* 2021) and in a variety of organisms (Kim *et al.* 2021), e.g., in models of hereditary diseases in humans (Caso and Davies, 2022). One such research applied mRNA with the Base editor in the treatment of hematopoietic stem and progenitor cells (HSPCs) in patients with SCD, resulting in a high percentage conversion of an allele of SCD (HBBS) to Makassar ß-globin (HBBG), a non-pathogenic allele (Newby *et al.* 2021). It was proven that CAR T-cells edited with base editing facilitated the enhancement of molecular remission preceding allo-HSCT in the treatment of malignancies in the T-cells (Georgiadis *et al.* 2021).

Base editing, unlike HDR, does not necessitate a DSB, which can cause indel mutations and, in rare cases, chromosome rearrangements. Additionally, base editing does not necessitate an exogenous repair template, as the strand containing the deaminated base serves as the repair template. Future versions of base editors may become more adaptable as they are being developed. Taken together, these factors increase the efficiency of making single-base changes with base editors (Gaudelli *et al.* 2017; Komor *et al.* 2016). The drawback is that the choice of base-editable sites as targets may be limited by base-editing windows when compared to targets that can be cleaved by Cas9 nuclease and potentially repaired with HDR to make a single-base change.

9.4 Genome editing

Base editing efficiently brought about C→T, G→A, A→G, T→C, C→G, and C→A substitutions in the genome without inducing DSB (Gaudelli *et al.* 2017; Zhao *et al.* 2021). They are not, however, capable of correcting other variants apart from these six transition mutations, or other modes such as DNA insertions and deletions, efficiently brought about by Prime editing (PE). PE makes use of a fusion protein composed of engineered Cas9 nickase and a reverse transcriptase (RT) enzyme and a prime editing guide RNA (pegRNA), a modified guide RNA (Lu *et al.* 2022). The efficiency in the PE system was sequentially improved in recent research to PEmax system and the engineer pegRNA referred to as epegRNA. The precision in the insertion is made with a length of up to 1 kb, and the removal with a length of up to 10 kb, DNA fragment (Choi *et al.* 2022; Happi Mbakam *et al.* 2022).

As base editing, prime editing remains in a phase yet to reach clinical trials owing to its underdevelopment. Notwithstanding, the promise in PE has been demonstrated in the past 2 years in vitro (Habib *et al.* 2022; Happi Mbakam *et al.* 2022; Petri *et al.* 2022) and in animal models in animals (Jang *et al.* 2022; Zhi *et al.* 2022).

Efficiency in gene editing is low in a few models. The cause could be that split viruses are used to avoid the size limitation in PE, making them incapable of fitting in a single viral vehicle. The promise in prime editing is in augmenting the safety and increasing the reach in genome editing in T-cells, showcasing method is adaptable in order to augment the efficiency in CAR T-cell therapy by performing other complex gene edits in combination with CAR in T-cells (Petri *et al.* 2022).

10.0 Ethical aspects and the regulatory structure

Despite the worldwide expansion of genetic engineering, a variety of problems are encountered in utilizing it (Wu and Zhang, 2023). The germline has been edited in a variety of model organisms to obtain models of diseases or investigate the molecular basis of specific gene functions. More recent information is available on germline engineering in primates. One such potential misuse of the technology is editing the germline in humans to modify genes with a connection with IQ (Kim *et al.* 2021). Various ethical implications particularly hinder its usage in gene therapy. The Oviedo Convention, adopted in 1999 by Member states of the European Council, prohibits every genetic modification transmitted in turn to future generations; care is taken not to commit such in the process of medical interventions (Tsang *et al.* 2018). Other non-member states of the European Council, such as Canada, the United States of America, Japan, and the Holy See, contributed to preparing the above-mentioned convention. The usage of CRISPR-Cas9 on embryos in particular, to repair genetic disorders transmitted by the parents and resulting in extreme pathologies, is monitored in fundamental and medical research in order not to risk eugenics (Sauvagère and Siatka, 2023). Scientists are conscious that there could be a need for legislation, but research and provision of research funds need not stop. Policymakers are conscious of the potential and possibilities the technology has in store for humanity. Accordingly, efforts are made to obtain enabling legislation such that research and generation of knowledge are not hindered and impaired (Sauvagère and Siatka, 2023; Wu and Zhang, 2023).

11.0 Potential guidelines and perspectives

Different approaches have been reported in order not to endure the process difficulties in repeated sequences in the TALEs, and these are the following: (i) design ligation-independent approaches, (ii) high-throughput solid-phase assembly, (iii) the Golden gate, and (iv) other one-day TALE assembly (Zhang *et al.* 2020; Lu *et al.* 2022). To boost the efficiency in gene editing, a novel bicistronic TALE termed T2A, based on classical TALEN coding sequences fused with a variety of molecules with the assistance of the 2A "self-cleaving peptide," has been engineered. The enhancement would cause every TALEN monomer to transcribe in the same reading frame to boost the efficiency in gene editing (Martín-Fernández *et al.* 2020; Lu *et al.* 2022).

As shown, genome editing tools hold promise in cell-based therapy and deciphering many genetic diseases (Sauvagère and Siatka, 2023). Effort over a tenner is paying in generating targeted and permanent multiple-hit pluripotent cell (PC) lines for reprogrammation into cell types with diseases. Despite such efforts, however, there are no properly developed protocols in such approaches. Refinement of such methodology is needed, and efficient gene alteration and biallelic gene editing need to be accomplished before such approaches are taken to clinics. Comparative efficiency in gene editing was examined in a few reports among available tools such as ZFNs, TALENs, and CRISPR/Cas9. It was demonstrated in recent times in human pluripotent stem cells (hPSCs) that the CRISPR-Cas9 system is superior in efficiency over gene editing in TALEN (Lu *et al.* 2022).

Effective extension of genome profiling in a way that facilitates deep characterization of patient-derived tumors is creating thorough roadmaps in designing cell- and whole-animal-based empirical approaches. Single and multi-plex nuclease- and/or small-molecule-based approaches are going to drive the design of personalized genome editing, applicable in the revelation of genotype-specific susceptibilities and noxious synthetic interactions at a high pace. These personalized approaches are capable of facilitating the revelation of death-causing pathways and designing respective treatments at a high pace (Sauvagère and Siatka, 2023).

The field of genome editing has been revolutionised with a sudden scientific revolution and is going to revolutionise basic biological and biomedical research. Genome editing is useful in a vast array of iPSC-based therapy and cancer therapy and promises exciting possibilities in enhanced evaluation of disease progression

and treatment design (Wu and Zhang, 2023). In gene therapy in humans, despite the potential issues with implementation in the field of technology and ethics, the technology promises a vast array of possibilities in a future generation in treating genetic diseases with a hereditary basis that have been, in the majority, incurable (Wu and Zhang, 2023).

Application of editing technology for treating infection with fungi is still at a phase where editing efficacy and delivery modes have to be improved. In the future, these must be resolved for gene editing therapy in treating infection with fungi to progress to clinical studies in a timely fashion (Halder *et al.* 2019).

In the future, the use of CRISPR/Cas9 technology in generating accurate models of cancer is going to drive research in functional genomics in cancer and revolutionize the treatment of cancer in a significant way (Zheng *et al.* 2022).

12.0 Conclusions

During the past ten years, gene editing technologies made enormous progress in uses and optimizations. With continual optimizations of these technologies ZFNs, TALENs and CRISPR-Cas9 are already in human clinical trials. Most of these technologies are currently in their clinical uses on ex vivo gene editing therapeutics. The editing is highly efficient in treating numerous medical disorders such as sickle cell disease, but ideally genome editing would be in diseases where in vivo cell editing is required. In spite of what is stated above, the in vivo uses of CRISPR technologies are constrained with issues such as low efficiency, induction of counterproductive immune responses and off-target editing. The research in overcoming these issues could pave the way for future directions in these technologies' clinical uses. Moreover, recent innovations such as Base editing and Prime editing are still in pre-clinical phase. All these approaches require DNA strand break inducing DNA damage responses. More efforts are still required in order to address these drawbacks such as wide off-target events, genome stability, transcription-activation systems and cell proliferation in order to speed up the treatment of genetic and infectious diseases. To overcome the limits of the gene editing tools currently in use, more work is required. More work is required to resolve the technological and ethical issues surrounding human gene therapy. There is still room for improvement in the application of editing technologies to the treatment of cancer and fungal infections.

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