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A Review

CRISPR/Cas systems in therapeutics: Transforming gene editing into medical solutions

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The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR- associated (Cas) systems are known for their innate ability to provide adaptive immunity against viruses and plasmids in bacteria and archaea. With research and development, versatility of CRISPR/Cas systems has been employed in gene editing technology enabling precise and efficient gene manipulation, and has been harnessed extensively in both diagnostics and therapeutics. CRISPR/Cas system is useful whether in correcting monogenic disorders caused by point mutations, cancer-associated gene alterations, or countering viral diseases. While disease treatment is primary, these systems are also being implemented for designing animal models for research purposes. This comprehensive review aims to provide researchers with an in-depth understanding of various CRISPR/Cas systems, highlighting their applications in the treatment and diagnostics of a range of diseases and disorders.

Keywords: CRISPR, Disease genomics, Gene editing, Medicine, Therapeutics

Introduction

Back in 1987, a unique repeating sequence in prokaryotes first came into light. It consisted of clusters of short repeated palindromic sequences of 24-47 bp separated by a unique intervening spacer of 26-72 bp sequence. These repeating interspaced sequences were termed clustered regularly interspaced short palindromic repeats or CRISPR and the sequences were found near a family of DNA repair genes that later called Cas (CRISPR-associated). These sequences served a vital role in providing immunity against phages in prokaryotes as the spacer region in the CRISPRs shows a higher similarity to the prophage DNA or foreign genetic material than the chromosomal DNA[1].

The CRISPR sequence incorporates the foreign DNA into the spacer region through different Cas proteins which when transcribed, act to silence the foreign DNA by cleaving it with complementarity. This ability showed the potential of the CRISPR/Cas system as a tool for gene editing. It was demonstrated in the year 2012 that CRISPR/Cas9 can be used as a programmable RNA-guided tool for site-specific DNA cleavage[2]. Soon, CRISPR/Cas9 became highly studied while providing better-targeted gene editing.

In this review, we will focus on how the CRISPR/Cas systems are being used in the field of therapeutics and how it is modified to achieve better-targeted gene editing capabilities whether for any monogenic disorders, viral disease or even treating oncogenic transformation in cancer cells. While also diving into the current clinical studies that are being carried out on various model organisms.

CRISPR/Cas systems

In prokaryotes, there are several defence mechanisms present for dealing with phages and foreign DNA, yet only the CRISPR/Cas serves as a system that has memory. It incorporates the foreign DNA in its spacer region of the CRISPR array, which leads to not only the evolution of CRISPR over time but also diversifying it further[3].

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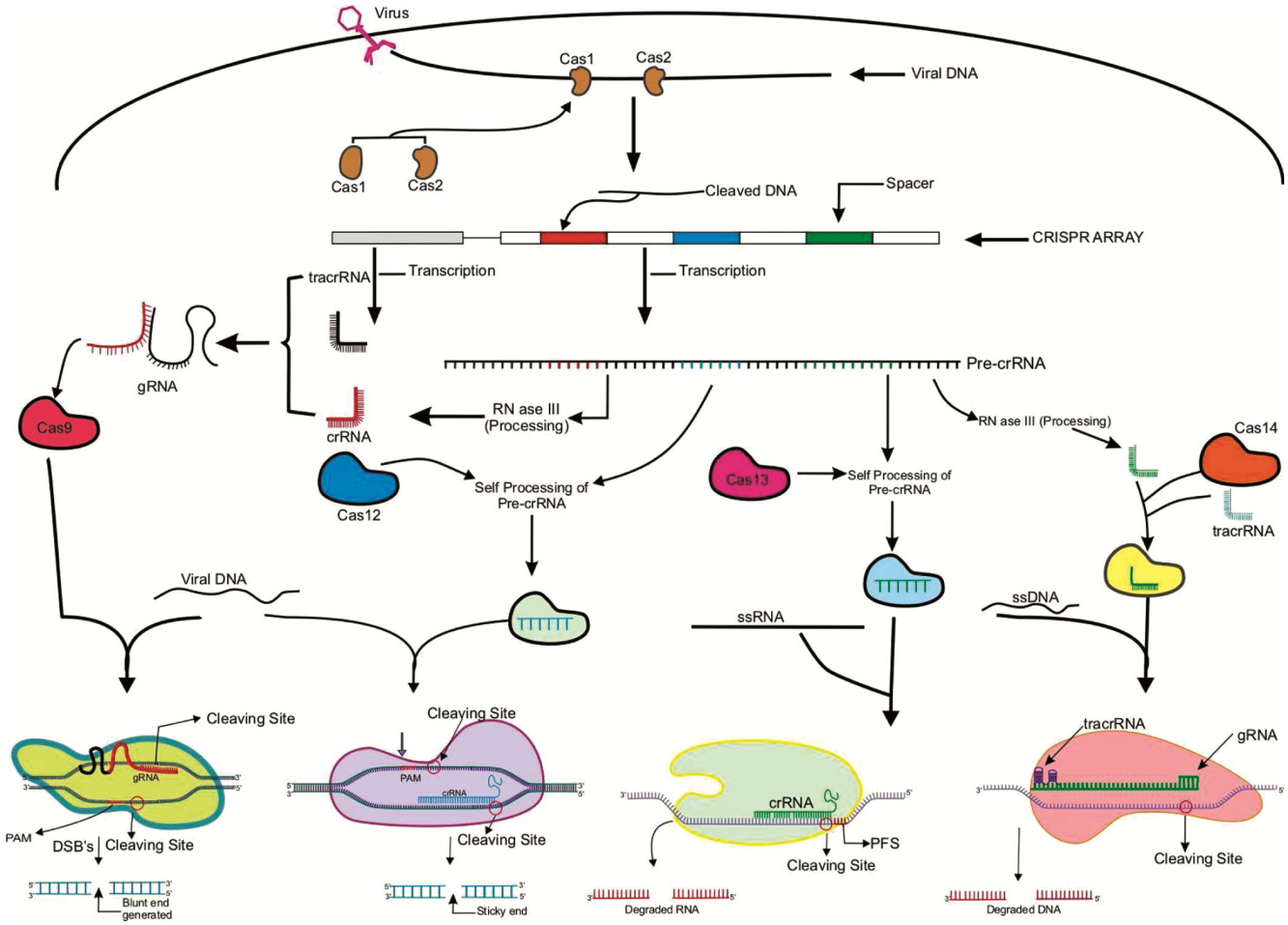

Fig. 1 — Mechanisms of CRISPR-Cas Systems: The mechanisms of various Cas proteins in CRISPR-Cas adaptive immune system are schematically illustrated. Upon viral infection, the viral DNA triggers Cas1 and Cas2, they cleave and incorporate a segment of viral DNA into the CRISPR-array. The array when transcribed produces pre-crRNA. The pre-crRNA is processed to mature crRNA differently depending on the Cas protein, Cas9 and Cas14 require RNase3 while Cas12 and Cas13 self-process the pre-crRNA. The crRNA combines with tracrRNA forming gRNA that assembles with Cas protein to target and cleave specific sequence. Where Cas9 and Cas12 target dsDNA , Cas14 targets ssDNA AND Cas13 selectively targets RNA.

The mechanism comprises three stages, where in the 1st stage the viral DNA or foreign genetic material is incorporated in the spacer region of the CRISPR array by the help of Cas 1 and Cas 2 proteins. In the 2nd stage, the so-formed CRISPR array is transcribed producing a precursor of crRNA referred as pre-crRNA. The pre-crRNA is processed into a mature crRNA by the help of Cas protein or RNase III. In the 3rd stage, the Cas uses mature crRNA to recognize the target sequence and cleave it by complementarity[4] (Fig. 1). Adjacent to the CRISPR array is trans-activating RNA coding region which is transcribed to produce the tracrRNA. Where some Cas proteins use the tracrRNA along with the crRNA (mature) forming guide RNA (gRNA) for target recognition. In some CRISPR/Cas systems, a specific region called PAM (protospacer adjacent motif) is present on the target which is recognized by the Cas complex to distinguish between self and target DNA[4].

Classifications

The classification of the CRISPR/Cas system is a very complicated task even with today's technology and equipment as the defence systems tend to evolve and diversify further with time. The Cas proteins cannot serve as a phylogenetic marker for the classification as even the phylogeny of the Cas1 protein which is the most conserved of all fails to elaborate on the relationship between CRISPR/Cas systems. So existing classification employs multiple criteria including the organization of Cas operons, phylogenies of conserved Cas proteins, and signature Cas genes[3,5].

Class 1

The class 1 CRISPR/Cas systems are most commonly found in archaea and bacteria (including all hyperthermophiles) comprising approximately 90% of all identified CRISPR/Cas loci. Class 1 is subdivided into three types namely 1, 3, and 4, as per their unique signature proteins and their distinct architecture[5].

In the class 1 system, the maturation phase of pre-crRNA is done by a protein complex cascade which further recruits Cas proteins (Cas6 or, on rare occasions Cas5) having nuclease properties responsible for processing[5].

Class 2

The class 2 CRISPR/Cas system comprises only 10% of the remaining CRISPR/Cas loci exclusively in bacteria. Similarly, class 2 is also further subdivided into three distinct types based on unique signature proteins type namely 2, 5, and 6[5].

Class 2 in comparison to class 1 is rather simpler, as it involves a single multidomain protein instead of a cascade of signature proteins in class 1 systems[4,5].

Cas proteins in CRISPR/Cas

The CRISPR-associated proteins or Cas proteins are near the CRISPR loci and are also gaining popularity over time due to their programmability hence enhancing the precision of CRISPR/Cas-mediated genome editing.

Cas 9

The Cas9 protein is responsible for introducing double-strand breaks in the target DNA molecule. Cas9 is a six-domain protein comprising of [1] Recognition lobe (REC I), [2] REC II, [3] Arginine-rich bridge helix, [4] PAM Interacting, [5] HNH, and [6] RuvC. The double-strand break is achieved by a complementary guide RNA (gRNA) which is covalently linked to the enzyme Cas. The gRNA comprises a duplex of crRNA produced by the CRISPR array and a partial complementary trans-activating crRNA (tracrRNA)[2,4,5].

The gRNA also recognizes the PAM region adjacent to the target sequence, this enables it to distinguish between self and target sequences. Cas9 protein cuts 3bp upstream of the PAM by its HNH and RuvC domain[2,4].

Cas 12

The Cas12 protein belongs to class 2 of the CRISPR/Cas system[4]. While it shares the same class as that of Cas 9 protein, the mechanism of action is far different. It not only cleaves the dsDNA but also ssDNA and unlike Cas9, which requires both crRNA and tracrRNA, the former only requires the crRNA. The processing step of the pre-crRNA is carried out by itself without the RNase3[2,4].

The RuvC and NUC (nuclease lobe) domains are present in Cas12 for cleaving activity to generate the double-strand breaks in the target sequence. The cut so generated is adjacent to the PAM sequence on the non-target strand. Hence overhangs are generated in the non-target strand resulting in sticky end formation. When Cas12 matches the target sequence of <17 bp it forms a unique R loop in the crRNA after it binds to the target sequence. This R loop results in the activation of the RuvC and NUC domain to cleave the target[4].

Cas 13

Cas13 is also a part of class 2 CRISPR/Cas systems alongside Cas9 and Cas12 but unlike them, it only cleaves ssRNA and not the DNA. Cas13 is of two subtypes Cas13a also known as C2c2 and Cas13b[4,5].

In Cas13a there is crRNA alongside a NUC lobe and HEPN (RNase) for cleaving the target ssRNA. Contrary to Cas9 or Cas12 which has PAM, Cas13 has PFS (proto-spacer flanking site). Similar to Cas 12 it doesn't require a tracrRNA for activation and cleaving of the target ssRNA[2,4,5].

Cas13b similar to Cas13a only cleaves ssRNA but it is more precise as protein flanks 5` end with PFS and 3` end with PAM on the target ssRNA[4].

Cas 14

Cas14 is the lowest molecular weight protein with approximately 400-700 amino acids. It belongs to class 2 of CRISPR/Cas system, which cleaves only ssDNA. Cas14 is similar in functioning to Cas9 as Cas14 also requires a tracrRNA alongside the crRNA, but unlike Cas9 it doesn't require a PAM sequence for cleaving the target[4,6].

Cas14 is far more precise when compared to Cas9, Cas12, and Cas13. This could be suggested to be a reliable tool for high fidelity genome-editing. The Cas14 itself is unique to specific sequence recognition, yet the reason for this is unknown[4,6].

Monogenic disorders

These are rare genetic disorders that follow the Mendelian laws of inheritance. Where a single gene is affected by either an insertion or deletion. These defective genes are either received from maternal or paternal parents while in some cases from both[7].

Table 1 — Summary of CRISPR/CAS Applications in Monogenic Disorders

Disorder	Type	Mutation	CRISPR/Cas System	Target	Function	References
Sickle Cell Anemia	Autosomal Recessive	Beta-globin gene: Glutamic acid to valine at position 6	*CRISPR/Cas9*	HBG1, HBG2 promoters	Increases fetalhemoglobin production	*Sharma et al. (2018) [8] - 16 December 2024*
Thalassemia (Beta)	Autosomal Recessive	Beta-globin gene mutations	*CRISPR/Cas9*	BCL11A, HBB	Increases fetalhemoglobin production	*Frangoul et al. (2021) [9] - 16 December 2024*
Thalassemia (Alpha & Beta)	Autosomal Recessive	HBB gene deletion; HBA2 HB-Ws mutation	*CRISPR/Cas9*	HBB, HBA2	Corrects mutations in both alpha and beta chains	*Li et al. (2022) [10] - 16 December 2024*
Cystic Fibrosis	Autosomal Recessive	CFTR gene mutation, e.g., ΔF508	*CRISPR/Cas9*	CFTR gene	Restores CFTR function	*Vaidyanathan et al. (2022) [11] - 16 December 2024*
Duchenne Muscular Dystrophy	X-linked Recessive	DMD gene mutations causing frameshifts	*CRISPR/Cas12a*	DMD gene (exon 51)	Restores dystrophin production	*Zhang et al. (2017) [12] - 16 December 2024*
Duchenne Muscular Dystrophy	X-linked Recessive	DMD gene mutations causing frameshifts	*CRISPR/Cas9*	DMD gene (exon 51)	Corrects frameshift mutation	*Xiang et al. (2021) [13] - 16 December 2024*
Huntington's Disease	Autosomal Dominant	HTT gene CAG repeat expansion (≥40 repeats)	*CRISPR/Cas9*	HTT gene	Silences mutant HTT allele	*Shin et al. (2021) [14] - 16 December 2024*
Phenylketonuria	Autosomal Recessive	PAH gene mutation, e.g., Pahenu2	*CRISPR/Cas9*	PAH gene	Corrects PAH gene mutation	*Richards et al. (2020) [15] - 16 December 2024*
Phenylketonuria	Autosomal Recessive	PAH gene mutation, e.g., P28L1	*CRISPR/Cas9*	PAH gene	Corrects PAH gene mutation	*Brooks et al. (2020) [16] - 16 December 2024*

Monogenic disorders are of two types: autosomal disorders (where genes on the autosomes are affected) and sex-linked disorders (where genes on the sex chromosomes are affected). Both types can have dominant forms, where expression occurs even in the heterozygous condition, and recessive forms, where expression occurs only in the homozygous condition[7].

Here are selected few monogenic disorders being treated by the use of CRISPR/Cas systems (Table 1).

Sickle cell anemia

This is an example of an autosomal recessive disorder where the beta-globin chain of haemoglobin is affected. At the 6th position of the beta-globin chain glutamic acid is substituted by valine. Resulting in the polymerization in low oxygen conditions and taking a sickle-like shape becoming rigid and fragile leading to hemolysis and inflammation. The affected individuals tend to face progressive multi organ damage, recurrent pain , chronic pain and higher chances of early death[7,8].

A modulated form of CRISPR/Cas9 was used to target the promoter regions HBG1 and HBG2 (promoter element of the gamma chain in fetalhemoglobin) in hematopoietic stem cells (HSC). This targeted gene editing with gRNA resulted in disruption of the promoter region (HBG1 and HBG2), which led to production of the fetalhemoglobin resulting in decreased manifestation of sickle cell disease[8].

Thalassemia

A type of inheritable genetic hematological disorder affecting either the alpha or beta chain of haemoglobin.The genes on either chromosome 11 for beta or chromosome 16 for alpha are affected. The change in the gene can occur at a single site or multiple sites, hence the severity can vary accordingly[9,10]. Alpha thalassemia is caused when there is a deficient or null synthesis of the alpha chain while increasing the percentage of the beta chain. In the case of beta-thalassemia, the beta chains are affected, while the alpha chain percentage increases. It's reported that commonly detected α-thalassemia and β-thalassemia mutations was in SEA/αα (31.53%) and CD41/42 (30.27%) in Hainan province, southernmost China. Treatment for thalassemia mainly comprise of transfusion and iron chelators in TDT7 affected patients, while luspatercept and crizanlizumab mediated therapies have reduced transfusion requirement in patients. Similar to sickle cell disorder, beta thalassemia is also being treated with a

similar approach of CRISPR/Cas system. By increasing the production of fetalhemoglobin hence inhibiting the repression of the gamma chain by gene editing[9,10].

CRISPR/Cas9 was used for gene editing in hematopoietic stem cells and progenitor cells (HSPCs). Whereas in BCL11A (responsible for switching the production of fetalhemoglobin to adult hemoglobin), an erythroid-specific enhancer region is edited to reduce its expression. This resulted in the production of the gamma chain leading to fetalhemoglobin formation[9]. In 2022 concurrent alpha and beta thalassemia was being treated by CRISPR/Cas9. The patient had a deletion at position 41-42 in the HBB gene and an HB-Ws mutation in the HBA2 gene (hemoglobin alpha). The human induced pluripotent stem cells (hiPSCs) were derived from the patient's amniotic cells and corrected by specific sgRNA. These mutations were corrected by homology-directed repair with the help of linearized donor DNA. The hiPSCs were able to differentiate into hematopoietic progenitor cells *in vitro*, proving their multi-lineage differentiation potential[10].

Cystic fibrosis

It is an autosomal recessive disorder that results in multiorgan dysfunction affecting the lung airways, pancreas, and sweat glands. The gene that is affected is called the CFTR gene (cystic fibrosis transmembrane conductance regulator). Which affects the transmembrane chloride channel leading to a decreased level of chloride ion conductance. The mucus produced in airways thus gets dehydrated by transepithelial and paracellular uptake of water[11].

In 2022 a gene therapy was performed where CFTR cDNA was inserted at the CFTR locus, which enabled a durable cure for cystic fibrosis patients irrespective of the type of mutation. Upper airway basal stem cells and human bronchial epithelial cells were used. They employed CRISPR/Cas9 along with two adeno-associated viruses (containing halves of the cDNA in each) and tunicated CD19, all inserted in upper airway basal stem cells (UABCs) and human bronchial epithelial cells (HBECs). This insertion corrected the expression of the CFTR gene, restoring it to around >70% of the non-cystic fibrosis control. Their results showed that this method could potentially treat around >99.5% of cystic fibrosis patients[11].

Duchenne muscular dystrophy

An X-linked recessive disorder where the muscle in an individual degenerates due to mutations in the DMD gene. The gene is responsible for the production of dystrophin protein that maintains muscle integrity. The females in such X-linked recessive disorders are most of the time carriers and mostly asymptomatic[12,13].

CRISPR-Cas12a also called Cpf1 was used in the year 2017 to treat DMD by treating fibroblast-derived induced pluripotent stem cells (iPSCs). As exon 51 skipping could overcome the 13% DMD mutation, it became a target for gene editing. This restored dystrophin production after differentiation to cardiomyocytes[12]. This was the first finding to be carried out of Cpf1 mediated correction of a genetic mutation in human cells.

Similarly, to treat the exon 51 frame shift, CRISPR/Cas9 was also implemented in 2021. Where spCas9 (Cas9 from Streptococcus pyogenes) along with dual gRNA (two distinct types of gRNA) was used to perform a non-homologous blunt end joining (NHBEJ). This enabled the in-frame deletion of a part of exon 51 resulting in corrected frameshift mutation in human DMD. Restoration of dystrophin was achieved at mRNA and protein levels[13].

Huntington's disease

This is a neurodegenerative disorder following autosomal dominant inheritance. The CAG repeats present in the 1st Exon of the huntingtin gene (HTT) are affected. The repeats are supposed to be 1-25 in a normal individual, while the symptomatic individuals tend to have 40 or more repeats in exon 1[14]. The cause of Huntington's disease is known for like 25 years yet no effective treatments are developed on ground of complicated disease biology[14].

An allele-specific CRISPR/Cas9, along with an SNP to generate a PAM site, is used to target the specific allele with the mutant HTT gene. Silencing this mutant gene through nonsense mediated decay (NMD) resulted in no mRNA expression and no protein translation of the mutant HTT gene[14].

Phenylketonuria

It is an autosomal recessive disorder affecting phenylalanine metabolism. The PAH gene encodes the enzyme phenylalanine hydroxylase, which converts the amino acid phenylalanine to tyrosine. The mutation in the PAH gene impairs the gene expression leading to the accumulation of phenylalanine. This causes brain dysfunction in affected individuals[15,16]. The untreated patients have approximately 10 time higher blood Phe levels as compared to normal levels (normal level is less

than 120 μmol/L, while in patients is more than 1200 μmol/L). The current treatment involves sapropterin, which improves mutant PAH activity and pegvaliase that directly catabolized Phe, where both are approved medical therapies[15].

CRISPR/Cas was used in a study conducted on mice for gene addition therapy in 2019 alongside AAV's. The Cas9 was used to generate a double-strand break near Pahenu2 mutation for homologous recombination. To enhance the homologous recombination of the repair template, a non-homologous end joining (NHEJ) inhibitor was co-administered. This combination led to a permanent treatment in the Pahenu2 mice mutant. This study revealed partial restoration of PAH activity in mice liver[15].

In a recent study, to treat a specific PAH variant (P28L1) CRISPR/Cas9 was employed. Adenine base editing *via* gRNA in a cellular model and in an *in vivo* humanized mouse model was used. The delivery of this combination was done *via* lipid nanoparticles (LNPs) in the liver. The Phenylalanine level went back to normal within 48 h of the treatment. The result of this study showed around 100% corrective editing efficiency at higher doses *in vitro*. In the in-vivo model, the PKU and non-PKU mice became indistinguishable after 1 week of treatment. The mean Phe was 103 μmol/l in PKU while in non-PKU it was 96 μmol/l[16].

Cancer

It is a genetic disease, in which the normal cell undergoes unregulated growth. The cells tend to grow without contact inhibition and divide rapidly, forming a cluster of cells called a tumor. The cells can be either benign or malignant, depending on their evasiveness. The benign is localized to the location of proliferation. While the malignant tends to break off and move to other parts of the body and proliferate[17].

Under normal conditions, tumor suppressor genes and proto-oncogenes are associated with proper cell growth and division. The proto-oncogenes function as growth factors that influence normal cell differentiation and proliferation[17]. The tumor-suppressor genes are responsible for normal cell growth and cell differentiation. It blocks the development of cancer. However, under abnormal conditions, the cells experience certain changes at the molecular level and become cancerous[17].

Diagnosis

Diagnostics play a very critical role in cancer treatment as early detection gives a better chance to prepare the treatment process. Different types of CRISPR/Cas systems are being used for the diagnosis of cancer. These systems tend to have a higher specificity when compared to other diagnostic processes (Table 2).

Table 2 — Summary of CRISPR/Cas Applications in Cancer Diagnostics and Treatment

Aspect	Type	Target	Function	Technology	Outcome	Reference
Diagnostics	*CRISPR/Cas12a*	HPV16 DNA	Detects HPV16 DNA	Fluorescent Detection	Confirms presence of HPV16 by color change	*Dai et al. (2019) [18] - 16 December 2024*
Diagnostics	*CRISPR/Cas13a*	Viral nucleic acids	Detects single base mismatches	SHERLOCK System	Identifies specific viral nucleic acids	*Gootenberg et al. (2017) [19] - 16 December 2024*
Diagnostics	*CRISPR/Cas12a*	ctDNA (Breast cancer gene-1)	Detects ctDNA using gold nanoparticles	Fluorescent Detection	Indicates presence of breast cancer	*Choi et al. (2021) [20] - 16 December 2024*
Diagnostics	*CRISPR/Cas13a*	miRNA (e.g., miRNA-17)	Directly detects miRNA	Fluorescent Detection	Provides fluorescence signal upon cleavage	*Shan et al. (2019) [21] - 16 December 2024*
Treatment	*CRISPR/Cas9*	EGFR (Lung cancer)	Targets EGFR mutations	Gene Editing	Leads to tumor regression in NSCLC	*Koo et al. (2020) [24] - 16 December 2024*
Treatment	*CRISPR/Cas12a*	EGFR (Lung cancer)	Disrupts EGFR mutations	Gene Editing	Results in tumor regression in NSCLC	*Yoon et al. (2021) [23] - 16 December 2024*
Treatment	*CRISPR/Cas9*	KRAS (Cancer)	Targets KRAS mutations	Gene Editing	Inhibits cancer cell proliferation	*Kim et al. (2020) [26] - 16 December 2024*
Treatment	*CRISPR/Cas9 & dCas9-KRAB*	KRAS G12S	Edits and represses KRAS G12S mutant	Gene Editing & Transcriptional Repression	Leads to tumor regression	*Gao et al. (2020) [25]- 16 December 2024*
Treatment	*CRISPR/Cas9 Base Editing*	KRAS & TP53	Corrects KRAS and TP53 mutations	Base Editing	Improves treatment efficacy and prevents escape variants	*Sayed et al. (2020) [27] - 16 December 2024*

In diagnostics, nucleic acids, miRNA, ctDNA, and even proteins are used. CRISPR/Cas12a and Cas13a are being extensively used for the detection of specific viral nucleic acids as viruses are one of the main factors for carcinogenesis[18,19]. Cas12a is being used for the detection of HPV16 DNA. The method involves a methylene blue (MB) labeled ssDNA reporter on the surface of gold nanoparticles, which would be cleaved in the presence of HPV16 DNA by Cas12a. Released MB from the reporter ssDNA leads to a change in the current signal that is later detected, hence confirming the presence of HPV16 DNA [18]. Similarly, the SHERLOCK system was constructed for single-base mismatch detection with the help of CRISPR/Cas13 combined with isothermal amplification for viral nucleic acid[19].

The circulating tumor DNA (ctDNA) being released from a necrotic or cancerous cell that circulates in the bloodstream is known as circulating tumor DNA (ctDNA). Hence ctDNA is a crucial and selective biomarker for cancer cells. In a special strategy for breast cancer, gene-1 detection CRISPR/Cas12a was used. Here, ssDNA linked to a fluorophore to be quenched was hybridized onto gold nanoparticles. When ctDNA was present Cas12a would cleave the ssDNA leading to dissociation and color change from purple to red-purple[20].

miRNA is non-coding RNA regulating the expression of mRNA. Where the specific miRNA can be used for early diagnosis as they are involved in biological processes their abnormal expression can direct towards various diseases especially cancer. CRISPR/Cas13a is usually used for the detection of such miRNA as it targets RNA[21]. Researchers conducted a study where Leptotrichiabuccalis Cas13a (LbuCas13a) directly detected 4.5 amol of the target miRNA-17 in mild incubation conditions. This resulted in the cleaving of the ssRNA acting as a reporter recovering fluorescent signal detected in just 30 min[21].

Treatment

Cancer, being a genetic disease, can be treated by a gene editing tool, as such CRISPR/Cas systems fill the role perfectly (Table 2). It serves the role of a gene editing tool to alter the defective gene in the cancerous cell.

Lung cancer is the most diagnosed cancer type with an almost 42% mortality rate. The majority of lung cancers are caused by a mutation in the epidermal growth factor receptor (EGFR) gene, as EGFR overexpression/dysregulation leads to activation of the oncogenic signaling pathway[24]. In 2017 CRISPR/Cas9 system was demonstrated to differentiate between oncogenic and non-oncogenic cells for the treatment of non-small cell lung cancer (NSCLC). The Cas9 system was delivered by the use of o-AD (oncogenic adenovirus) along with a single gRNA. They targeted a single nucleotide (CTG > CGG) missense mutation, which generated PAM from the spCas9 recognition site. The delivery of spCas9 with mutation-specific gRNA led to enhanced cancer cell killing and tumor regression[24]. Similarly, in 2020 a study for the treatment of lung cancer (NSCLC) with the help of Cas12a was done. They constructed an o-AD (oncogenic adenovirus), which coexpressed Cas12a and crRNA against EGFR. When delivered, the targeted gene was disrupted leading to tumor regression[23].

The mutations caused in the proto-oncogene RAS, develop oncogenic transformation in cells. The oncogene RAS has three types KRAS, HRAS, and NRAS[25]. Lentiviral infection was used to introduce Cas9 and sgRNA for treating specific mutations of KRAS in 2018, where specific gRNA was screened by the reporter system. The expression of gRNA was doxycycline-inducible in KRAS mutant cancer cells, transduced by lentiviral vector encoding Cas9. The Cas9 disrupted the mutant KRAS gene inhibiting cancer cell proliferation both *in vitro* and *in vivo*[26].

Similarly in the year 2020, spCas9 and a modulated CRISPR/Cas system called dCas9-KRAB were used to treat cancer under mutation KRAS. The spCas9 was used for genome editing along with a sgRNA, and dCas9-KRAB was used for the transcription regulation approach. Both targeted the KRAS G12S mutant allele, cleaving the gene by spCas9 resulted in gene depletion. While dCas9 downregulated the mRNA transcription of the mutant allele using KRAB acting as a transcriptional repressor. The net result of the study was the finding of two ways to treat KRAS G12S mutants either by downregulation or by gene silencing leading to significant tumor regression. The gene silencing method performs more effectively than transcription suppression[25].

Similar to oncogenes responsible for cancer, the mutation in tumor-suppressor genes can also make cells cancerous[17]. In 2022 a study implemented base editing CRISPR/Cas9 to target not only the oncogene KRAS but also the TP53 (a tumor-suppressor gene) mutation for cancer treatment[27]. They depicted how

cleaving of KRAS might result in tumor regression but some KRAS variants might escape and later proliferate. So, moving to base editing with Cas9 suggested that not only did it treat the tumor but also no escape variants were observed. The cas9 and sgRNA were introduced by lentiviral infection in patient-derived organoids. But unlike in other studies, the Cas9 variant was less restrictive, and enhanced base editor AbE8e was also present for effective base editing in the KRAS-G12 mutant. Similarly, they proceeded with another mutation of TP53 in pancreatic cancer cells. The base editing led to the restoration of the p53 function (MDM4 and GADD45A). In both cases, base editing approaches were proven to be more effective than cleaving the mutant gene by CRISPR/Cas system[27].

Viral diseases

There are several diseases caused by viruses, whether they are DNA viruses or RNA viruses. The virus uses the host cell for its replication and multiplication. Some of these viral diseases, which are diagnosed and treated with the help of CRISPR/Cas systems, are mentioned below (Table 3).

SARS-CoV-2

Severe acute respiratory syndrome coronavirus-2 is the virus responsible for the COVID-19 pandemic, causing widespread illness and death globally. It's an RNA virus that causes infection in the airways of an individual. The use of CRISPR-based lateral flow technique was implemented to detect specific RNA associated with the virus. The same strategy was used in the detection of HIV-1 in the year 2023[28]. The study details the use of CRISPR/Cas13a along with crRNA targeting the conserved region of the SARS-CoV-2 N gene. MIRA products of the N gene were used for screening ten crRNAs, with crRNAs N8 and N10, exhibiting the highest fluorescence due to vigorous nuclease activity. COVID-19-negative clinical samples were tested using CRISPR immunochromatographic detection that showed 100% consistency with RT-PCR results[29]. To target the viral RNA for treatment, a study in 2023 was conducted

Table 3 — Summary of CRISPR/CAS Application in viral diseases Diagnostics and Treatment

Disease	Causative Agent	Aspect	Type	Target	Function	Technology	Outcome	Reference
COVID-19	SARS-CoV-2	Diagnostics	*Cas13a*	SARS-CoV -2 N gene	Detects specific RNA of SARS-CoV-2	Lateral flow CRISPR-based detection	100% consistency with RT-PCR results	*Hongbo Liu et al. (2023) [29] - 16 December 2024*
COVID-`19	SARS-CoV-2	Treatment	*Cas13d*	SARS-CoV-2 +RNA and – RNA	Silences viral RNA	CRISPR-based antiviral strategy	Effective silencing of target sequence	*Mouraya Hussein et al. (2023) [30] - 16 December 2024*
AIDS	HIV-1	Diagnostics	*Cas13a*	HIV-1 variant	Detects specific RNA sequence of HIV-1	Lateral flow CRISPR-based detection	Highly specific, does not cross-react with other pathogens	*Xiaohui Li et al. (2023) [28] - 16 December 2024*
AIDS	HIV-1	Treatment	*Cas13a*	Long terminal repeats (LTR) , gag, rev	Degrades viral RNA from both free and integrated forms	CRISPR-based antiviral strategy	Effective inhibition of HIV-1 RNA	*Lijuan Yin et al. (2023) [31] - 16 December 2024*
Hepatitis B	HBV	Diagnostics	*Cas12b*	HBV genotypes B and C	Detects HBV DNA	Isothermal amplification with CRISPR	Highly effective and precise diagnostics of HBV	*Xu Chen et al. (2022) [32] - 16 December 2024*
Hepatitis B	HBV	Treatment	*SaCas9*	HBV genotype C	Gene editing of HBV DNA	CRISPR/Cas9-based gene editing	Successful gene editing in 5 out of 8 mice	*Daniel Stone et al. (2020) [33] - 16 December 2024*
Dengue	DENV	Diagnostics	*Cas12*	Dengue virus RNA	Detects viral RNA by signal amplification	CRISPR-based electrochemical biosensor	Allows detection of viral RNA	*Yeonju Lee et al. (2021) [34] - 16 December 2024*
Dengue	DENV	Treatment	*Cas13a*	NS3 gene	Inhibits viral replication	CRISPR-based antiviral strategy	45.54% ± 5.82% inhibition of viral replication	*Hao Li et al. (2019) [35]- 16 December 2024*

where Cas13d was used for such a role. The reason is that it is smaller in size for gene delivery by viral vectors. Moreover, it lacks the sequence constraints that enable it to attack any viral RNA sequence. The conserved target sequence was selected with the help of the alignment of 31,576 viral genomes through which 29 cr-RNA were synthesized specific to such conserved regions. The Cas13d with the cr-RNA targeting the +RNA demonstrated an effective silencing of the target sequence. The cr-RNA for the +RNA resulted in a high antiviral activity as compared to the one targeting –RNA. The cr-RNAs that could inhibit the SARS-Cov2 were also able to suppress the SARS-CoV-2 hence demonstrating the strength of this antiviral strategy[30].

HIV

Human immunodeficiency virus (HIV) is a single-stranded RNA virus. It uses reverse transcriptase to synthesize DNA from its genomic RNA, which is then incorporated into the host genome. This viral infection leads to acquired immunodeficiency syndrome (AIDS). It has infected over 75 million people, while 37 million currently live with the virus. The main target of HIV is CD4+ T cells, hence reducing their number over time[31]. In a recent study, CRISPR/Cas13 was employed to detect specific RNA sequences of HIV-1 variants, where a special lateral flow strip of Cas13, combined with reverse transcriptase recombinase-aided technology was employed. The results can be observed by the naked eye. This technology provides a very high specificity that doesn't cross-react with other pathogens[28]. One study involved CRISPR/Cas13a to treat infection caused by HIV-1 variant, which used the cleaving activity of Leptotrichiabuccalis (Lbu) Cas13a targeting single-stranded RNA. The Lbu Cas13a variant was able to degrade not only the viral RNA that enters the cell but also the viral RNA being transcribed by the viral DNA which is integrated into the host genome. The target of the Lbu Cas13a was the long terminal repeats (LTR), gag, tav, and rev regions of HIV-1. Where rev targeted cr-RNA inhibits the HIV-1 RNA most effectively of all[31].

Hepatitis B VIRUS

Hepatitis B is caused by the HBV that infects the hepatocytes, and the HBV infection in an individual can lead to either acute or sometimes chronic liver infection. The infection caused by HBV is quite difficult to target as its DNA is covalently closed circular DNA[32,33]. For diagnostics of the HBV, a specialized platform called CRISPR/HBV was synthesized. The platform targeted two major genotypes of HBV, the HBV-B and HBV-C. For the amplification of the target DNA, MCDA amplification, an innovative nucleic acid isothermal amplification technique, was used. CRISPR/Cas12b-lateral flow biosensor is employed for the detection of the target sequence. The target after being amplified when detected by Cas12b results in cleaving of a reporter ssDNA giving fluorescence. This study gives highly effective and precise diagnostics of the HBV infection [32]. The CRISPR/Cas systems are not only used in the detection but also to obtain an antiviral effect for HBV infection. In 2020 *Staphylococcus aureus* Cas9 (SaCas9) technology was used to target the HBV genotype C in humanized mice. The humanized FRG mice were having chronic HBV infection. AAVs were used to deliver the SaCas9 along with the gRNA that targets the viral DNA. The study depicted that five mice out of eight were having successful gene editing. The treatment depicted the survival of human hepatocytes, while it was also observed the better the delivery of the SpCas9 in the target cell the better the chance of gene editing. Hence this study suggested that Cas9-associated base editing could resolve the HBV infection in hepatocytes and can even be considered safe[33].

Dengue virus

Dengue virus (DENV) is a single-stranded positive-sense RNA virus that causes dengue fever. The World Health Organization (WHO) has reported 50-100 million people infected with dengue fever worldwide. DENV has 4 serotypes DENV-1, DENV-2, DENV-3, and DENV-4. Sometimes it causes more severe diseases, dengue hemorrhagic fever and dengue shock syndrome[34]. A novel detection of DENV, performed in 2021, involved a mechanism that bypasses the nucleic acid amplification step, instead amplifying the signal using methylene blue conjugated with gold nanoparticles (MB-AuNPs). The CRISPR/Cas12 system (formerly known as Cpf1) targets the single-stranded DNA (ssDNA) linked to biotin and MB-AuNPs. In the presence of DENV-4 RNA, the Cas12 is activated, resulting in the trans-cleavage of the ssDNA. This cleavage leads to a decrease in the electrochemical signal produced by the MB-AuNPs. The alteration in the signal allows for the detection of the viral RNA's presence [34]. CRISPR technology not only helps in the diagnostics of the

DENV but also the treatment. In 2019, CRISPR/Cas13a was used in a study for inhibiting DENV replication by cleaving the NS3 gene. They identified 10 conserved regions in the different serotypes that would serve as target regions for Cas13a. The various types were then delivered to the vero cells using Lipofectamine CRISPRMAX. After 3 days the supernatant was collected from the cells and reverse transcription PCR was performed to check anti-viral activity. Where NS3 gene targeted cr-RNA gave the highest effective inhibition (45.5% ± 5.82%) of the viral replication when compared to the other 9 targets. So, it was suggested as an effective and precise programmable method for treatment and should be developed further[35].

Animal models

The various CRISPR/Cas systems are not only used for diagnostics of various diseases or treatments but also used to construct various animal models.

Mouse models

To develop conditional knockout mice, a new method called Easi-CRISPR (Efficient additions with ssDNA inserts-CRISPR) was employed. It is a targeting strategy where long single-stranded DNA along with crRNA, tracrRNA, and Cas9 ribonucleoprotein (pre-assembled) is injected into the mice zygote. Two guide RNA were designed to immediately cut the genome adjacent to each homology arm. The Easi-CRISPR generates correctly targeted insertional and conditional alleles in 8.5-100% of the resulting live offspring[36]. The Easi-CRISPR technology overcomes the problem that other gene editing technologies face in animal model development which is the inefficiency of targeted DNA cassette insertion. It's a highly effective strategy as it treats on average 50 zygotes to produce one or more correctly targeted animals, per gene-targeting project[36]. In 2023 CRISPR/Cas9 technology was used for constructing a C57BL/6J-SLC23A2 em1 (flox) Smoc mouse model. The model was further used to develop a conditional knockout mouse model of SLC23A2 (SVCT2) gene in the mice brain. The Cas9 mRNA and a gRNA obtained by *in vitro* transcription were injected with a donor vector in the fertilized egg of the C57BL/6J. The recombinant vector (donor vector) was constructed containing a 3.0 kb 50 homology arm, a 0.7 kb flox region, and a 3.0 kb 30 homology arm by In-Fusion cloning. The mice were crossed first with C57BL/6J mice to obtain positive F1 generation mice then selfed to obtain a homozygous knockout SLC23A2 flox/ flox mice[37].

Pig model

The pig model is the closest animal model to humans except for non-human primates[38]. In 2019, miniature pig models were prepared for type 2 diabetes by the use of CRISPR technology. IAPP is a polypeptide playing a very vital role in glucose and lipids metabolism was the chosen target to develop the type 2 Diabetes model. The vector backbone included U6-sgRNA and Cas9 expression elements, while first the complementary sgRNA was synthesized and ligated to BbsI sites of the backbone vector to construct a plasmid. A donor vector containing the hIAPP gene was constructed and inserted between homologous left and right arms (500 bp long), while porcine fetal fibroblast (PFF) cells were isolated and cultured from the Bama miniature pig fetus. Both vectors were introduced into the PFFs by electroporation, to check the cutting efficiency of the Cas9 PCR was used. The positive gene-edited PFFs underwent somatic cell nuclear transfer and embryo transfer. 300 embryos were transplanted per surrogate sow and a total of 5 were transplanted. After 120 days of gestation, 28 piglets were obtained, further by Sanger sequencing and HaeII digestion 24 piglets were found to be positive for hIAPP. These models can now be used for further studies and research experiments[39]. Taking advantage of the relative size of the pig model, they can be used to better grasp congenital heart disease in humans. A study used the Cas9 system with gRNA, which was directly injected into the cytoplasm of the oocyte by microinjection. The gRNA targets the end of exon 10 into the downstream intron of porcine SAP130. The oocyte was *in vitro* fertilized and cultured for 4-6 days. Genetically edited pigs generated with a mutation in chromatin modifier SAP130 exhibited tricuspid dysplasia, with tricuspid atresia associated with early embryonic lethality. The major events of cardiac morphogenesis were largely complete in 30 days. Where the development profile is similar to that of humans suggesting the new opportunities this model offers[40].

Monkey model

Non-human primates are important models for study and research due to their physiological similarities to that of humans. Generating a biallelic mutant for a loss of function study is difficult as they

have slow reproduction rates. Research in 2015 produced the first live p53 biallelic mutant. It was achieved by Cas9 gene editing technology and sgRNA. A total of six sgRNA were designed to target different sites of the p53 gene in monkeys. The Cas9 mRNA/gRNA was directly injected into the monkey zygote with varying concentrations. The mutations were observed with the help of Sanger sequencing. The strategy led to the development of a biallelic mutant in monkeys and achieved homology-directed repair for precise gene editing in monkeys[41]. Similarly, to generate mutation for Hutchinson-Gilford Progeria Syndrome (HGPS) in a monkey model gene editing was implemented by CRISPR/Cas9. This syndrome is mainly responsible for premature aging due to a rare point mutation in the LMNA gene. The researchers fused cytidine deaminase with CRISPR/Cas9 forming base editor. These base editors along with specific gRNA to target the LMNA gene were injected into the monkey zygote directly. As a result five out of six newborn monkeys carried the specific desired mutation. The characteristics observed phenotypically such as vascular abnormality, growth retardation, and bone alteration, suggest that developed mutants were genetically and clinically mimicking the HGPS[42]. So one can say that CRISPR-based gene editing technology, especially Cas9, can be highly precise and effective in generating animal models in a rapid one-step manner.

Clinical studies

The various CRISPR/Cas systems are being extensively researched and explored as time goes by. While many treatments are in the pre-clinical phase some studies have made it through. Here in this section, we will look into a few studies which are underway in human trials.

In 2020 NSCLC (non-small cell lung cancer) was treated with the help of spCas9 targeting the PD-1 gene. The T-cells were gene-edited and reinfused into the patients demonstrating the technique's feasibility and safety. Twenty-two patients underwent the treatment process and five patients didn't receive the infusion due to lack of T cell expansion. The median time to prepare the T cells for reinfusion based on 17 patients was 25 days. A pair of single gRNA was selected specifically for the EXON-2 of the PD-1 gene. Electroporation was used to introduce the Cas9 and sgRNA plasmid into the T cells. By cytometric analysis, it was observed that the PD-1 expression in the T cell was decreased with the median distribution of 46.3% (range, 33.7–67.0%) in edited T cells. To check the editing efficiency, NGS-targeted sequencing was done of the PD-1 gene. Where 12 patients' median editing efficiency was 5.81% (range, 0.42–24.85%). To check the off-target editing by Cas9, NGS with cSMART (circulating single-molecule amplification and resequencing technology) was performed. The median mutation frequency of all off-target sites was 0.05% (range, 0–0.25%), which was much lower than that of the on-target site. After the cell fusion, the *in vivo* editing of the T-cell NGS was done to detect the presence of the PD-1 gene in the blood sample extracted DNA of the patients. The median progression-free survival was 7.7 weeks (95% confidence interval, 6.9 to 8.5 weeks) and the median overall survival was 42.6 weeks (95% confidence interval, 10.3 to 74.9 weeks). The study demonstrated the gene-editing strategy by CRISPR is a safe approach as treatment-related adverse events were very few. Only one patient faced grade 1 arrhythmia, possibly due to lymphocyte infiltration, a rare complication of immune checkpoint inhibitors. Also, the efficiency is high as there is very minimal off-target editing[43]. The strategy for using the highly effective and precise nature of CRISPR for gene editing is also being studied for viral disease as mentioned in section 4 of this review. In the year 2019 CRISPR system was executed on a patient with HIV and Acute Lymphocytic Leukemia. The study is based on the approach of inducing mutation in the CCR5 of the hematopoietic stem cells (HSPCs). That is a key core receptor for entry of HIV [73].The CCR5-edited HPSCs were transplanted in a patient having HIV-1 infection, who had developed acute lymphoblastic leukemia. To culture the HSCs in HLA matched donor-mobilized peripheral blood cells were separated based on CD34 (a cell surface protein used for the detection of HSCs) expression. The HSPCs were cultured on a serum-free medium for 48 h before the transfection. The transfection was carried out with a ribonucleoprotein complex comprising two gRNAs and a Cas9 protein. The edited cells were transplanted along with the CD34-depleted cells from the donor-mobilized peripheral blood. To check the efficiency of the editing by Cas9 Sanger sequencing of the CD4+ cells from the bone marrow, peripheral blood and peripheral blood cells were done. As for the off-target editing, whole genome sequencing was carried out of the edited cell after gene editing and engraftment. Neutrophil and platelet engraftment

occurred on 13 and 27 days of the post transplantation and full donor chimerism was attained by 4 weeks. The acute lymphoblastic leukemia was under complete remission at week 4 while continued remission was still observed till 19 months of transplantation. The edited CD4+ cells persisted for over 19 months in the patient without any gene-editing-related adverse effects. But the gene-editing therapy wasn't sufficient to control HIV on its own. Even though the patient received a successful transplantation and long-term engraftment, the percentage of ablated CCR5 HPSCs was only 5%. Hence, further research is needed to enhance this strategy[44]. We have already studied how the production of the HBF is used to treat beta-thalassemia and sickle cell anemia in section 3 of this review. A similar strategy is adopted for the clinical studies of patients suffering from beta-thalassemia and sickle cell disease. The transcription factor BCL11A is targeted in the HSCs. Patients (aged 18 to 35 years) with severe sickle cell disease, defined as ≥2 VOCs/year (vaso-occlusive crises) requiring medical care in the previous 2 years and those with TDT receiving packed red blood cell (RBC) transfusions of ≥100 mL/kg/year or ≥10 units/year in the previous 2 years were selected for treatment. HPSCs were collected from the patients by apheresis after mobilization with G-CSF (filgrastim) and plerixafor. The erythroid enhancer region of BCL11A was edited by the specific gRNA and Cas9 in the CD4+ cells. After the CTX001 infusion on day 1, the patients were then followed by observation for stem cell engraftment and hematopoietic recovery. The median neutrophil engraftment occurred on day 32+ (range: +27 to +36) for beta thalassemia, day 30+ and 22+ for sickle cell. The platelets engraftment was observed on day 37+ (range: +34 to +52) for beta-thalassemia and 30+ for sickle cell. All the patients demonstrated a total increase in HB and HBF over time. Where patients with sickle cell disease had no VOC and all patients of beta thalassemia ceased to receive pRBC transfusions since CTX001 infusion. These findings suggest that even in clinical trials the production of the HBF by the CRISPR/Cas9 technology could provide a treatment for both diseases[45].

Limitations of CRISPR/Cas

CRISPR/Cas systems are a very effective and precise strategy that has been highly studied and modified for better understanding and development of gene editing. But even with all these applications in therapeutics, there are certain challenges that people face when working with this technology for base editing. Firstly, off-target-based editing is a phenomenon that has been mentioned in the previous section[43,44]. In some cases, it can be of lower percentage while in some it can vary and result in a higher percentage. This could further lead to hampering the efficiency of CRISPR/Cas technology. The CRISPR/Cas9 that is highly used in diagnostics and treatment also requires a PAM region to cleave the target genome, making it restrictive[2,4]. Hence for cleaving a particular target, one must devise the gRNA such that the PAM region is also in association. If the PAM is not there or not recognized the cleaving of the genome won't take place even if gRNA is complementary to the target region[2,4,6]. The cleaving action of CRISPR/Cas9 generates a double-strand break (DSB) in the target DNA. Which can sometimes lead to apoptosis rather than the gene editing of the cell. But this can be overcome by dCas9 which can transiently manipulate the expression of specific genes without introducing DSBs through the fusion of transcriptional activating or repressing domains or proteins to the DNA-binding effector (refer to figure.1 for the mechanism of Cas9)[27]. It's also observed in some cases that individuals tend to get an immunogenic effect against this therapy while some even have pre-existing antibodies against the most commonly used orthologs SaCas9 and SpCas9. Even with all these limitations one cannot turn a blind eye to the benefits we obtain by performing the gene editing with the help of CRISPR/Cas. Whether its precise nature for the gene editing or the programmability one gains from it.

Conclusion

CRISPR/Cas system, originally discovered as an adaptive immune system mechanism in bacteria, is now extensively used for gene editing in therapeutics. The system offers unmatched programmability, along with high precision in cleaving action[2]. The various types and subtypes so present allow the researchers to not only use a diverse mechanism but also target diverse nucleic acids. In the context of diagnostics and treatment, the CRISPR/Cas gene editing technology is significantly more precise even when sometimes it gives off-target editing. The most highly anticipated and utilized Cas system is Cas9 for its programmability, but the requirement of the PAM region hinders the usage to some degree. Whereas

Cas12 and Cas13 don't require tracrRNA the possibility of their usage. While Cas14 is also more effective as of smaller size for easy delivery and no PAM requirements, the target is ssDNA. In cancer treatment and diagnostics one can say it's an efficient strategy as it offers to detect the tumorigenesis and even treatment leads to tumour regression over time[18-27]. While being harnessed in monogenic disorders to produce HBF *in vivo* models to overcome sickle cell disease or beta thalassemia[2-16]. The development of animal models, mice to that of monkeys is being produced by the use of gene editing by CRISPR/Cas[36-42]. The system, when being highly precise and effective, can sometimes lead to off-target gene editing which could in the end hamper the efficiency of the CRISPR/Cas. But further modulated forms and highly complementary gRNA can help overcome this issue.

Hence CRISPR /CAS systems not only provide a tool for basic genetic engineering but also show its possibility of being used as an irreplaceable tool in therapeutics.

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Conflict of interest

All authors declare no conflicts of interest.

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