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**Review Article**

# Remodelling of a bacterial immune system as the simple gene editing tool, Crispr-Cas, for food security and human health

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**ABSTRACT**

The CRISPR system consists of a guide RNA (gRNA) complementary to a target editable DNA sequence and a CRISPR-associated endonuclease (Cas). The gRNA and Cas together form a ribonucleoprotein (RNP) complex. The gRNA guides the Cas enzyme to the precise site for cutting the target DNA. In bacteria, the gRNA leads the endonuclease to the viral DNA for destruction. This ingenious bacterial immune principle has been used to design gRNA to target an organism's genome at precise locations for gene editing purposes. Gene edits may include deletion or insertion, repression or activation, base, and epigenome editing, or nucleotide replacement. Therefore the CRISPR-Cas system has created the potential for altering genomes of microbes, plants, and animals. The CRISPR-Cas system has now been developed for use in many applications like finding functions of genes, searching for drug targets, diagnostics, crop improvement, and gene therapy.

## Introduction

Bacteria have an ingenious immune system to stop specific viruses called bacteriophages from invading and destroying them. Scientists noticed that bacterial DNA contained repetitive palindromic sequences (that read the same backward as forward), interspersed with other fragments of genetic material (Barrangou et al., 2007). The repetitive segments of DNA were named CRISPR or Clustered Regularly Interspaced Short Palindromic Repeats. However, the DNA interspersed among the repeats was the intriguing part of the immune system. When bacteriophages invade bacteria, the latter uses a CRISPR-associated nuclease called Cas to snip off a piece of viral DNA after creating double-stranded breaks in a targeted sequence, thus inactivating the virus. The question is, how does the nuclease know where to cut? This is where the intriguing DNA sequences come in. These sequences are the remnants of previous viral infections and are homologous to viral DNA (Barrangou et al., 2007).

So how did the sequence homologous to viral DNA become a part of CRISPR? When the nuclease Cas targets viral DNA for cleavage, it not only destroys the viral DNA but also retains a part of it referred to as 'spacer' within the palindromic sequences of the CRISPR arrays. Thus if the virus re-invaded, it would be quickly targeted and destroyed. The Cas enzyme recognises the homologous target sequences with the help of guide RNA (gRNA) associated with it. The guide RNA is actually transcribed from the bacterial DNA containing the CRISPR repeats.

The CRISPR system, therefore, consists of 1. A guide RNA (gRNA) specific to the target DNA sequence and 2. A CRISPR-associated endonuclease (Cas). The guide RNA and Cas endonuclease together form a ribonucleoprotein (RNP) complex for conducting CRISPR gene editing experiments.

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The Cas enzyme cuts double-stranded DNA, while the gRNA guides it to the specific site for cutting. In bacteria, the gRNA leads the nuclease to the viral DNA (Hille and Charpentier, 2016). However, the same principle can be used to design a gRNA to target an organism's genome at precise locations for gene editing purposes (Qi et al., 2013). Therefore, CRISPR-Cas system has created the potential for engineering the genomes of the full range of organisms, like microbes, plants, and animals. The CRISPR-Cas system is now used for many applications like finding functions of genes, searching for drug targets, diagnostics, crop improvement, and gene therapy (Adli, 2018).

**Crispr-Cas9 system of gene editing**

The most common tools for Crispr-based gene editing applications, the Cas9 protein (specific to *Streptococcus pyogenes*) is used. The binding of the gRNA to the genomic target is dependent on the existence of a short protospacer adjacent motif (PAM) directly downstream of the target (on the reverse DNA strand ) (Fig 1).

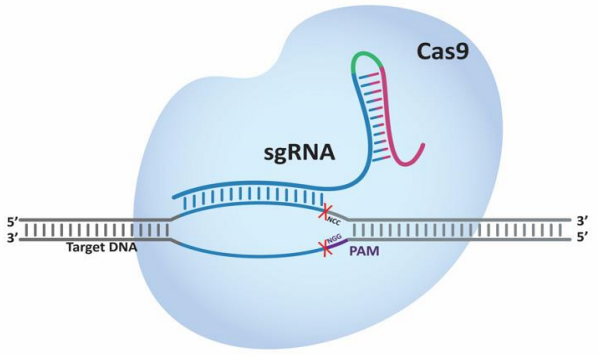


**Fig. 1. The Crispr-Cas9 system consists of a gRNA and a Cas9 endonuclease and exists as a ribonucleoprotein complex called RNP. If a specific PAM motif is present 5'NGG3 on the reverse strand of genomic DNA, then Cas9 will make a double-stranded break in the DNA denoted by a cross on both strands. The cell's internal repair mechanism caused by the break can cause some bases to be deleted and DNA re-joining, resulting in a frame-shift mutation. This is referred to as non-homologous end-joining or NHEJ. On the other hand, if a DNA template is present in the cell, it can direct the addition of homologous DNA bases into the genome at that precise location. This is called homology-directed repair.**

The Cas proteins from different bacteria recognize variant PAM motifs. The PAM for Cas9 was identified to be 5′-NGG-3′; N denoting any of the 4 possible nucleotides. If the correct PAM is located, the gRNA will successfully bind to the target, and Cas9 will cleave both DNA strands 3-4 nucleotides upstream of the PAM site (Le Rhun et al., 2019). This short genetic element is only present in marauding viruses (and not the bacterial genome) in real life. This ensures that Cas9 does not cleave its CRISPR locus.

In nature, gRNA exists in two parts: CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). The crRNA is an 18-20 bp sequence that targets genomic DNA by binding to it. The tracr RNA acts as a base for the crRNA-Cas9 interaction. Thus guide RNAs form a duplex molecule consisting of crRNA and tracr RNA. However, guide RNAs are now synthesized together with crRNA and tracr RNA connected by non-specific linker RNA, called single guide RNA (sgRNA) (Fig. 2).

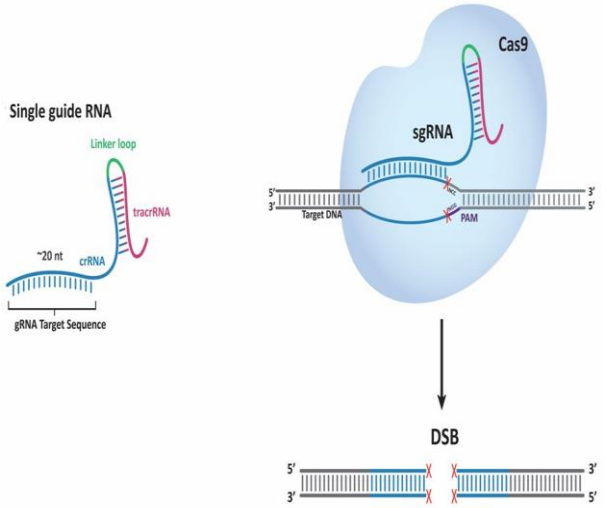


**Fig. 2. Targeting genomic DNA for editing: The crRNA (blue) and tracrRNA (purple) are connected by a linker RNA (green) to form a continuous molecule single guide RNA or sgRNA. The sgRNA and Cas9 complex forms a ribonucleoprotein. The sgRNA binds to the genomic target, and Cas9 makes a double-strand break (DSB) in the DNA if a PAM motif is present 3-4 bases downstream on the reverse genomic DNA strand.**

Cas9 initiates gene editing by producing a double-stranded break (DSB) in the DNA (Fig 2). DSB triggers the cell to induce its DNA repair system. Using this endogenous repair system to advantage, a scientist can plan two different kinds of repair. The first is referred to as non-homologous end joining (NHEJ), which usually causes a frame-shift mutation in DNA. The second repair mechanism is called homology-directed repair (HDR), where one has to provide a DNA template which the cell will use to edit the genomic DNA (Zaboikin et al., 2017).

Often a scientist may want to disrupt a gene permanently. For example, if they want to find the function of an unknown gene-this is called functional genomics. Or they may need to silence disease-causing genes, like the ones in cancer or a gene used by a virus to gain entry into a cell (Hsu et al., 2014). Therefore, if we desire that no functional protein be made (a knockout), then the non-homologous end joining (NHEJ) repair mechanism is designed (Fig 3, left). NHEJ re-joins the two cut ends of the DNA together, but causes the insertion or deletion of a few nucleotides (called indels). If the number of nucleotides inserted or deleted is not in factors of three, it will induce a frame-shift mutation and disrupt the coded protein's function.

Sometimes we want to change one to several nucleotides, referred to as a knock-in, making the protein better. The cell, can be induced to do a homology-directed repair (HDR). In this case, a DNA donor template will have to be introduced into the cell, with the desired sequence having regions of homology with the DNA on both sides of the intended cutting site. With the other Crispr components introduced previously into the cell, the template will be used to repair the broken sequence via homologous recombination, introducing the desired changes into the target region (Fig 3, right) (Miyaoka et al., 2016).

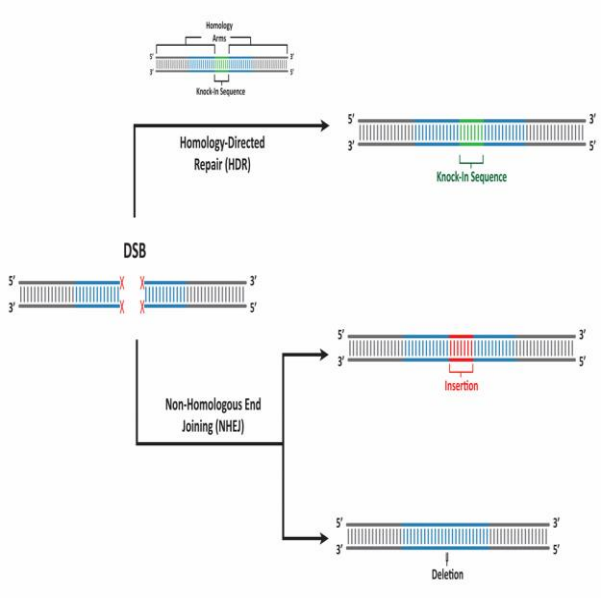


**Fig. 3. Crispr-Cas 9 gene-editing involves two different types of repair mechanisms. Left: Non-Homologous End Joining (NHEJ). The cell's endogenous repair system causes the deletion or insertion of a few nucleotides to repair the double-stranded DNA break (DSB). Right: Homology-Dependent repair (HDR) causes repair by using a DNA template to incorporate homologous DNA sequences in the target genome.**

***Transcriptional regulation with dead Cas9***

The CRISPR system can also be used for transcriptional regulation. A Cas9 variant was produced by mutation of certain domains so that the enzyme lost its nuclease activity. This is referred to as dead Cas9 or dCas9. Scientists use dCas9 in the CRISPR complex to bind to its DNA target with a transcriptional activator or repressor attached to it. Fusing a transcriptional repressor domain (such as KRAB) to dCas9 allows a reversible and fine-tuned reduction in gene expression (Qi et al., 2013). This is referred to as CRISPRi (Fig 4). RNAi was previously used to repress genes by destroying RNA transcripts. However, CRISPRi represses genes much more efficiently because it does it at the DNA level. If a transcriptional activator (such as VP64) is fused to dCas9, scientists can overexpress genes of interest (Fig 4). Similarly, if the effector is

a fluorescent protein-like GFP, you can tag your DNA. It is also possible to do epigenetic modification if the effector is DNA methylase (Khatodia et al., 2016). One system was created whereby light-inducible proteins were fused to mutate Cas9, causing gene expression to be activated in the presence of blue light and repressed in its absence (Polstein and Gersbach, 2015).

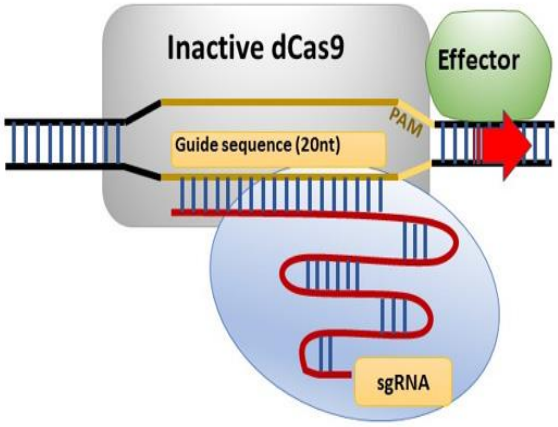


**Fig. 4. CRISPRi or CRISPRa for transcriptional regulation. Transcription will be silenced or reduced if the effector is a repressor such as KRAB. Transcription will be enhanced if the effector is a transcriptional activator such as VP64. Gene action can be seen by a confocal microscope, if the effector is a green fluorescent protein. Epigenetic modification can be done, if the effector is DNA methylase.**

***Novel applications with Cas9 as a Nickase***

The Cas9 endonuclease has 2 main domains, a recognition (REC) specific functional domain and a nuclease (NUC) domain. The NUC domain has 2 nuclease sites called RuvC and HNH. It also has a PAM-interacting site (Doudna and Charpentier, 2014). The Cas9 nickase (nCas9) with a RuvC or HNH mutation can create a nick, instead of a DSB at the target site (Cong et al., 2013). When Cas9 functions as a nickase, Base Editing as well as Prime editing can be used to insert desired nucleotides without the need for a DNA template, as explained below.

***Base Editing or Prime Editing for template-free desired nucleotide alteration***

As explained above, HDR can be harnessed to insert a specific DNA template to alter a DNA sequence. However, the process is inefficient and many random nucleotide insertion or deletions (indel) mutations may occur. DNA base editors consist of a Cas9 nickase (nCas9) and a single-stranded DNA modifying enzyme for targeted nucleotide alteration. The two most common classes of base editors are cytosine and adenine base-editors attached to the nCas9. For example, cytosine deaminase produces uracil at the target site, behaving like thymidine. Adenine deaminase produces inosine which acts like Guanosine when forming base pairs (Fig. 5). Therefore these two base editors can produce the following transitions: C to T, T to C, A to G, and G to A (Kantor et al., 2020).

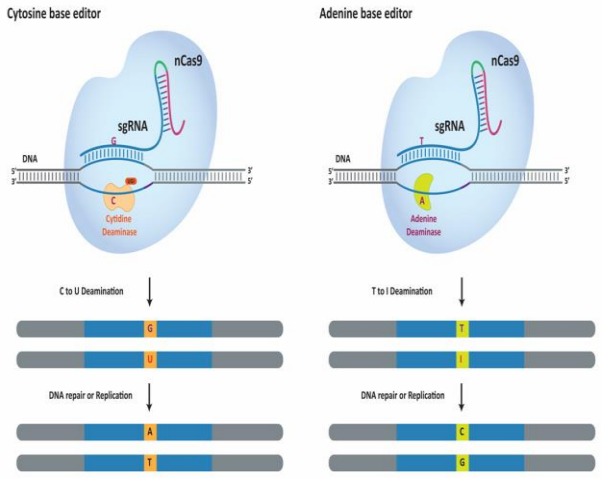


**Fig. 5. Base editing with nCas9: Cytosine deaminase produces Uracil at the target site, behaving like thymidine. Adenine deaminase produces Inosine, which acts like Guanosine when forming base pairs. Therefore, these two base editors can produce the following transitions: C to T, T to C, A to G, and G to A.**

On the other hand, prime editors have a cloned modified reverse transcriptase associated with nCas9 and a prime-editing gRNA with two arms. The 5' end of the arm contains the normal 20-bp guide RNA, while the 3' end has an edit-containing RNA template as a contiguous extension of the

guide RNA. This 2-armed special guide RNA is called pegRNA. The nCas9 simply nicks the non-complementary strand of the genomic DNA 3 nucleotides upstream of the PAM site. This produces a 3' OH group, which can attach to the primer binding site of the RNA template having the desired edit. The RNA template with the edit then serves as a primer for reverse transcriptase, which extends the 3' OH group by copying the edit sequence of the pegRNA. The edited 3' extension displaces the variant unedited 5' end, which is removed by an endogenous cellular nuclease (Fig. 6) (Scholefield and Harrison, 2021). Prime Editors expand the scope of DNA editing to all transitions and transversions as well as small insertions and deletions.

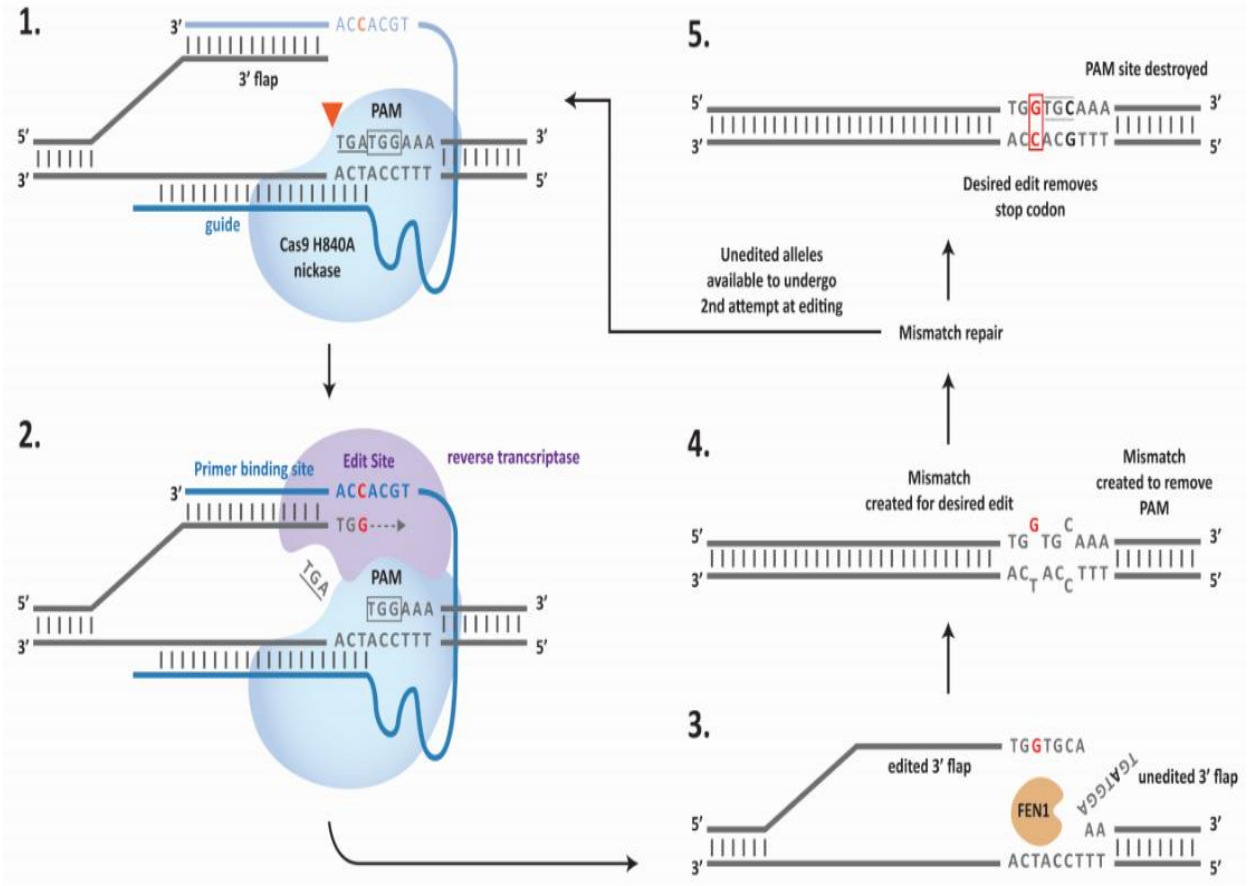


**Fig. 6. Prime editing: Cas9 nickase fused to a modified reverse-transcriptase (nCas9) and a multifunctional prime editing guide RNA (pegRNA). 1. nCas9/pegRNA complex targets the desired region and produces nick 3 nucleotides upstream of the PAM site. The nick must be upstream of the first edit-target site (in this case, a TGA stop codon) and occurs on the same strand as the PAM sequence, and generates a 3' OH-end. 2. This 3' OH group pairs in a sequence-specific manner with a 15bp primer binding site at the 3' end of the pegRNA. This RNA/DNA hybrid serves as the site for DNA synthesis using the RNA as a template. The modified Reverse Transcriptase copies the template, thereby extending the 3'OH end. 3 The edited 3' extension displaces the variant unedited 5' end, which is removed by an endogenous cellular nuclease called FEN1. 4 Two MisMatches remain to be resolved, one in the edited codon (G≠T), and one in the modified PAM (C≠C). A modified PAM can serve to prevent further editing. 5 MisMatch Repair occurs, and the resulting DNA is either precisely edited (with no indels) or the original sequence remains. In the latter case where the PAM sequence has not been modified, the nCas9/pegRNA complex can bind to the edit-target sequence again and have another attempt at PRIME editing. Key: nCas9 (Cas9nickase) is shown in light blue, Reverse Transcriptase in purple. The PAM site is highlighted in a grey box; the pegRNA in light blue; the 3' edit site in red; the edited PAM site in bold; FEN1 in light orange.**

***Applications of Crispr-Cas9 Editing for Crop Improvement*:** Crispr-Cas9 has already been used to improve food, feed, fiber, oil, and ornamental crops, as well as those used for industrial purposes (Liu et al., 2021).

***Increase in rice yield:*** Grain weight (GW) in rice has been increased by mutation of multiple grain weight negative regulators referred to as GW2, GW5, and GW6 (Xu et al. , 2016).

***Increase in Tomato shelf-life:*** Targeted control of tomato softening using Crispr-Cas9 silencing of the enzyme Pectate Lyase. This enzyme causes softening during ripening (Uluisik et al., 2016).

***Fragrant Rice:*** Aroma has been introduced into rice by knocking out Betaine Aldehyde Dehydrogenase, which leads to the production of excess amounts of 2-acetyl-1-pyrroline (2AP) compound. CRISPR/Cas9 tool to create novel alleles of *OsBADH2,* leading to the introduction of aroma into an elite non-aromatic rice variety ASD16 (Ashokkumar et al., 2020).

***Crop antioxidant enhancement:*** Lycopene is an antioxidant having anti-cancer properties. A 5-fold increase in lycopene was produced in tomatoes by simultaneously multiplexing the Crispr-Cas9 knock-out of genes converting lycopene into downstream products. This was done by disruption of five carotenoid metabolic pathway enzymes. These are *SGR1* (GenBank accession no. DQ100158), lycopene ε-cyclase (GenBank accession no., EU533951), beta-lycopene cyclase (GenBank accession no., XM_010313794), lycopene β-cyclase 1 (GenBank accession no., EF650013), and *LCY-B2* (GenBank accession no., AF254793) (SGR1, LCYe, BLC, LCY-B1, and LCY-B2) (Li et al., 2018).

***Increase in rice iron content:*** Disrupting the vacuolar iron transporter OsVIT2 has been shown to increase iron in the rice grain. Therefore there is a potential to knock-down the OsVIT2 gene by Crispr-Cas9 (Che et al., 2021).

***Increasing the desirable Oleic Acid content in Soybean:*** Two homologous genes in the soybean of the Fatty Acid Desaturase 2 (GmFAD2) gene were targeted for disruption for this purpose. This converts the monounsaturated oleic acid (C18:1) to the polyunsaturated linoleic acid (C18:2). The fatty acid content of the $T_1$ seeds from the CRISPR-edited plants showed an increase in oleic acid content to over 80%, which is similar to that found in Olive Oil (Do et al., 2019).

***Disease resistance:*** Novel alleles of rice eIF4G, which were generated by CRISPR/Cas9-targeted mutagenesis, confer resistance to rice tungro spherical viruses in IR64 rice. The mutations which resulted in in-frame mutations in the SVLFPNLAGKS residues (mainly NL), adjacent to the YVV residues, were shown to confer resistance (Macovei et al., 2018). The Banana Streak virus was inactivated by editing the virus sequences incorporated into the Banana genome, producing functional viral proteins. Seventy-five percent of the edited events remained asymptomatic compared to the non-edited control plants under water stress conditions, confirming the inactivation of eBSV into infectious viral particles (Tripathi et al., 2019).

***Domestication of wild rice containing abiotic and biotic stress resistance:*** Sixagronomically important traits were improved by Crispr-Cas9 down-regulation of O. Alta homologs of the genes in diploid rice. Such domestication showed that agronomic traits and resistance genes could be edited into elite rice (Yu et al., 2021).

***Application of CRISPR/Cas in medical research***

Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-associated protein 9 (CRISPR-Cas9) has revolutionized numerous medical research sectors, including disease models, therapeutic exploration, genetic screens, and more, since its introduction into mammalian cells and animals (Wang et al., 2013) in 2013. The charm of CRISPR-Cas9 lies in its multiplexing nature and ease of use. Although much attention has focused on the potential of CRISPR–Cas to cure Mendelian diseases, the technology also holds promise to transform the development of therapies to treat complex heritable

and somatic disorders (Fellmann et al., 2017) along with biomedical diagnostics (Wang and Cui, 2020) and next-generation drug developments (Luo, 2016).

***Applications of CRISPR in gene therapy***

Cancers: CRISPR is proved to be more effective for treating multigenic disease than conventional therapeutics. Cancer, for example, is a disease rooted in multiple genetic mutations. Mutations in the genome of individuals are taking place more frequently due to the excessive progress of xenobiotics and industrialization in the present world. There are minimal prevention and cure strategies available for the disease. However, with the advent of molecular biology, effective therapies based on targeted gene editing are becoming available. One emerging and exciting CRISPR-based tool for cancer treatment is based editing where catalytically deactivated Cas9 (dCas9) is fused with cytidine or adenine deaminase that can create stop codon by base conversion to correct cancer-associated mutations with higher efficiency and fewer undesired products (Huang et al., 2018). This method doesn't require any double-stranded break formation for gene disruption (Komor et al., 2016)). Another similar approach is delivering functional RNA using site-specific gRNA and dCas9 for targeted gene activation in a genome-wide fashion (Shechner et al., 2015).

Recent advances and success in the field of cancer immunotherapy highlight the therapeutic potential of engineering chimeric antigen receptor (CAR) T cells (Kalos et al., 2011). To further enhance the efficacy and safety of T cell therapeuticsCRISPR-Cas9, has been recently used to modify primary human T cells genetically. Programmed cell death protein 1 (PD-1) is responsible for inhibiting T-cell activation, which ultimately causes the immune escape of cancers. So, patient-derived T cells are isolated and genetically engineered with CRISPR-Cas9 to endogenous knockout PD-1 to allow rapid T-cell manufacture and boost T-cell efficacy. The first clinical trial for this therapy was started in 2016 in China to treat metastatic non-small-cell lung cancer patients (Men et al., 2018). Several similar trials by knocking down PD-1 to treat of esophageal cancer, prostate cancer, bladder cancer, metastatic renal cell carcinoma, and Epstein-Barr virus-associated malignancies have also been initiated in China (Huang et al., 2018).

CRISPR/Cas has the potential to grow in the field of cancer not only for its therapeutic approaches but also for identifying the mechanism behind tumor genesis and diagnosing the stage and mutation type of cancer.

***Duchenne muscular dystrophy (DMD):*** DMD is an X-linked recessive muscular disorder caused by mutations in the *DMD* gene located on chromosome 21. Due to this mutation, a lower level of dystrophin protein leads to decreased muscle membrane integrity, which increases susceptibility to degeneration (Lu et al., 2017). Haematopoietic therapy has been tried to regenerate muscle tissue. However, as myoblasts lose their ability to engraft into muscle tissues, this technique didn't bring much good news. To find effective treatment options for DMD, scientists planned to use CRISPR/Cas9 technology due to its potential for permanent exon skipping/reframing/deletion, which can restore the disrupted *DMD* reading frame in DMD and lead to dystrophin restoration (Lim et al., 2018). CRISPR/Cas9 mediated *In vitro* removal of exons 45-55 in the *DMD* gene resulted in the restoration of dystrophin protein synthesis (Young et al., 2016). *In vivo* researchers have used CRISPR to treat muscular dystrophy in mice by targeting exon 23 and removing the defective part of the gene, thus allowing the animals to produce one of the major proteins in the muscles (Bengtsson et al., 2017). This success of the CRISPR method in the treatment of adult animals with a genetic disease is promising towards finding proper treatments for Duchenne muscular dystrophy in the future.

***Huntington disease (HD):*** This is an autosomal neurodegenerative disorder caused by CAG repeat expansions in the huntingtin (*HTT*) gene, which produces an abnormal HTT protein. Although there are several therapeutic options for HD, there is no

cure yet. Gene therapy is a promising approach to treat HD disease that is currently under investigation and targets DNA transcription aiming to reduce the abnormal HTT protein level or utilize non-coding RNAs to decrease RNA translation (Karimian et al., 2020). The application of CRISPR therapeutics has led to the successful depletion of *HTT* aggregates and reduces or weakens early neuropathology (Luthra et al., 2021). It has been shown that CRISPR/Cas9 -mediated inactivation of endogenous mutant HTT (mHTT) expression in the striatum of mHTT-expressing mice can reduce the production of mHTT, effectively ultimately attenuating early neuropathology (Yang et al., 2017).

***Alzheimer's disease (AD):*** Alzheimer's disease (AD) is an irreversible and progressive neurological disorder that leads to cognitive decline. It is the leading cause of dementia, and some of the associated symptoms are memory and orientation loss combined with other cognitive impairments. With traditional AD drug treatments succeeding in offering only symptomatic relief and not being able to modify the disease, scientists have started looking at alternate strategies using CRISPR-Cas9 (Karimian et al., 2020). Researchers from Laval University in Canada recently used CRISPR to edit genes in brain cells to prevent Alzheimer's. The team led by Jacques Tremblay identified a genetic variant called A673T, which can reduce Alzheimer's biomarker beta-amyloid and has also been found to reduce Alzheimer's likelihood by a factor of four (Guyon et al., 2021).

Sickle cell anemia: Sickle cell disease is a group of inherited red blood cell disorders that affects hemoglobin, the protein that carries oxygen through the body, by only one replacement on the 11th chromosome. Although the disease was characterized long back, the only cure currently available for sickle cell disorder is bone marrow transplantation. This approach is difficult and presents significant challenges (Luthra et al., 2021). However, CRISPR has opened the door to the efficient treatment for SCD. Researchers reported that gene editing modified stem cells' DNA by deleting the gene *BCL11A*, the gene responsible for suppressing fetal hemoglobin production. By doing so, stem cells start producing fetal hemoglobin so that patients with congenital hemoglobin defects (beta-thalassemia or sickle cell disease) make enough fetal hemoglobin to overcome the effect of the defective hemoglobin that causes their disease (Foell et al., 2020).

***Haemophilia:*** Haemophilia is an X-linked genetic disease characterized by a mutation on the coagulation factor IX (FIX). To restore the function of the F9 gene in patients with haemophilia, scientists transfected hepatocytes of haemophilia B mice with codon-optimized SaCas9 cDNA and sgRNA to create DSBs in the exon near the F9 gene (exon 2-8) and insert cDNA into an intron of the gene (Ohmori et al., 2017). Some interesting results have been reported for *in vitro* and *in vivo* CRISPR studies using human iPSCs from patients with haemophilia B. Scientists prepared iPSC cell lines from peripheral blood mononuclear cells from patients with haemophilia B and modified them by inserting the complete F9 human cDNA using CRISPR/Cas9. Analysis of mice following transplantation of those modified lines revealed secretion of human FIX (Lyu et al., 2018), indicating a promising result that may serve as the basis for future studies.

***Genetic lung diseases:*** Cystic Fibrosis (CF) is a genetic disease caused by a mutation that occurs in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The defective CFTR protein leads to an imbalance of water and ion flow in and out of cells, resulting in thick mucus that obstructs airways and traps bacteria (Maule et al., 2020). Researchers utilized CRISPR/Cas9 to inactivate the expression of the mutant human SP gene in an embryonic mouse model. This gene modification reduced pulmonary pathogenesis due to immunohis-tology, and enhanced lung development and life expectancy by 22.8% (Alapati et al., 2019). In another study, CRISPR-Cas9 was proven valid to correct CFTR-bearing homozygous F508 deletions (F508del) in advanced cellular models, such as intestinal

organoids derived from patients, where CFTR channel function could be restored (Schwank et al., 2013).

***Genetic liver disease:*** Patients with Hereditary tyrosinemia type I (HTI) accumulate toxic metabolites that cause liver damage due to loss of function *FAH* mutation (Vanlith et al., 2019). Researchers have tried to use CRISPR technology in the HTI mouse model to correct $FAH^{mut/mut}$ by hydrodynamic injection of plasmids encoding CRISPR/Cas components or by combined delivery of AAV carrying HDR template and sgRNA and of nanoparticles with Cas9 mRNA. The second type of delivery showed initial expression of the wild-type Fah protein in ~1/250 liver cells. The study indicates that CRISPR/Cas9 can be used in adult animals and humans to correct genetic diseases (Yin et al., 2014).

***Acquired immunodeficiency syndrome (AIDS):*** Acquired immunodeficiency syndrome (AIDS) is a chronic, potentially life-threatening condition caused by the human immunodeficiency virus (HIV). By damaging the immune system, HIV interferes with the body's ability to fight infection and disease. The existing treatment facility has improved the quality of life of the patients. However, it failed to eradicate the HIV-1 virus from the body, resulting in high morbidity and mortality rates. Therefore, scientists are trying to treat HIV by reducing HIV-1 infection and clearing proviruses by targeting cellular cofactors. In a study of 2013, when a group of researchers used CRISPR/Cas technology to target HIV-1 LTR, they observed a significant loss of LTR-driven expression of this virus, and also this system was being able to remove embedded viral genes from chromosome indicating the significance of CRISPR/Cas technology in the treatment of deadly AIDS (Ebina et al., 2013). In another study, it has been shown that targeting HIV co-receptors CCR5 and CXCR4 by using CRISPR-Cas9 to restrain the attachment of the virus with CD4 cells can also be a safe and effective treatment opportunity (Liu et al., 2017).

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the coronavirus that causes COVID-19 (coronavirus disease, 2019), the respiratory illness responsible for the ongoing COVID-19 pandemic. Specific drugs for SARS-CoV-2 are not available. In the quest for effective antiviral therapy, scientists are trying to manage covid infection using CRISPR technology. As CRISPR/Cas13d is an RNA-guided, RNA-targeting CRISPR system, a group of researchers proposed targeting ORF1ab and S, which represent the replicase-transcriptase (ORF1ab) and the spike (S) of the virus to restrict COVID-19 infection (Nguyen et al., 2020). In another study, RNA-dependent RNA-polymerase (RdRp) has been proposed as a target for viral RNA degradation mediated by CRISPR/Cas13 (Buonaguro et al., 2020). All the studies show a lot of prospects as a treatment option for covid-19 and future virus outbreaks.

### *Application of CRISPR in disease diagnosis*

Along with the gene therapy property, CRISPR has attracted increasing interest in the *in vitro* diagnostic field because of its inherent allele specificity, which is one of the critical factors for the successful application of this technology in developing high-precision treatment and diagnosis.

Usually, Cas protein, along with a guide sequence, binds to its complementary sequences and cuts the matching nucleic acid. However, there are some Cas proteins that chop up nearby pieces, DNA or RNA, including reporter molecules. Scientists are trying to discover those Cas protein variants and unveil their functions to open up possibilities for novel applications such as nucleic acid detection (Huang et al., 2018). Cas13a (formerly C2c2) was computationally identified due to its genomic proximity to the highly conserved cas1 gene. This is an RNA-guided, RNA-targeting, single-component enzyme and have the ability to cleave single-stranded RNA when it becomes activated by binding to a complementary ssRNA (Abudayyeh et al., 2016). Based on this biochemical property of Cas13a, scientists develop specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) for sensitive and specific detection of nucleic acid. SHERLOCK

includes recombinase polymerase amplification (RPA), T7 RNA polymerase transcription from DNA to RNA and Cas13a general RNase activity. In this method, reporters sensitive to Cas13 are added to the amplified RNA sample from the patients. Cas13, along with specific guide RNA, binds only with targeted RNA and becomes activated. Then it randomly slices nearby RNA, including the reporter probes, and the fluorescence is measured (Kellner et al., 2019). SHERLOCK can detect viral nucleic acids conveniently from body fluids. Most of the time, the existing viral detection systems fail to discriminate between associated viruses, which share a similar phenotype (Myhrvold et al., 2018). However, a study demonstrated that SHERLOCK could reliably distinguish between Zika and a closely-related virus, Dengue, from multiple sample sources (Abudayyeh et al., 2016). Recently, a protocol for using the SHERLOCK method to distinguish COVID-19 target sequences in a range between 20 and 200 mmol/L validated by 10-100 copies per microliter (Wang and Cui, 2020). This method doesn't require elaborative instrumentation and a dipstick can display results. Although this protocol is not approved for clinical use, it is hoped that this will make viral particle detection easier in the future. In addition, this method can detect low-frequency cancer mutations like EGFR L858R and BRAF V600E from cell-free DNA (cfDNA) fragments (Gootenberg et al., 2017). In another study, Gootenberg et al. detected two bacteria, *E. coli* and *P. aeruginosa,* using a CRISPR-Cas13a based molecular detection platform, indicating CRISPR can also be used in the diagnosis of bacterial disease (Gootenberg et al., 2017).

After 2017, when SHERLOCK was first introduced, scientists tried to improve this detection system due to its few limitations by doing some modifications and named it SHERLOCKv2. The enhanced SHERLOCKv2 detection system uses less primer concentration to make the readout signals quantitative. Moreover, multiple targets can be detected in a single reaction due to variations of Cas13 and Cas12a in combination with different fluorescent reporters for each enzyme. The detection signal was also enhanced by using a CRISPR-associated enzyme, Csm6, which can significantly amplify the detection signal for a single target. Lastly and most importantly, SHERLOCKv2 was adapted to detect a cleaved reporter on commercial lateral flow strips, similar to pregnancy tests. This detection system can be transported easily and provide reliable results within an hour (Gootenberg et al., 2018). Same as the previous one, SHERLOCKv2 can detect EGFR L858R mutation or the exon 19 deletion from non-small cell lung cancer patients (Gootenberg et al., 2018).

Like SHERLOCK, another detection system is DETECTOR (DNA Endonuclease Targeted CRISPR Trans Reporter). The Doudna lab discovered Cas12a's ability to cleave non-specific (trans) ssDNAon a bound dsDNA substrate. It harnessed this ability to create a DNA detection platform called DETECTOR. In this method, the DNA is first amplified through isothermal amplification by RPA. Binding to the crRNA-complementary DNA sequence DNase activity of Cas12a is initiated and triggers cleavage of an unrelated ssDNA-fluorophore-quencher (ssDNA-FQ) reporter, generating a fluorescent signal (Chen et al., 2018).

Apart from these, CRISPR can also be used to detect tuberculosis, a deadly disease all over the world. Ai et al. (2019) developed a rapid assay named CRISPR-MTB where a polymerase amplification reaction was combined with CRISPR-Cas12a for target identification. This test picked up 46 out of 51 pulmonary TB cases, indicating a high sensitivity for pulmonary TB detection (Wang and Cui, 2020).

***Application of CRISPR in drug discovery***

Although the major focus is on CRISPR/Cas based treatment of diseases, this tool also has a big part in drug discovery, which could prove to be as important as its therapeutic use. The major steps for drug discovery are target identification and validation, high-throughput compound screening, hit validation, and lead drug candidate optimization. Each step of drug development can be notoriously long and costly and,

most of the time, span more than a decade and exceed a billion dollars. Genome editing tools like CRISPR can be used to expand drug discovery by removing all the hurdles.

With the advent of CRISPR, high-throughput genetic screening has been easier than ever which can help to identify drug targets effectively. Before discovering this technology, loss-of-function (LOF) and gain-of-function (GOF) screens in mammalian cells were made possible by RNAi-based gene knockdown libraries and cDNA-based gene overexpression libraries, respectively. However, due to high false-positive and negative rates, off-target effect, time consumption and significant increase in cost scientists started to search for newer and efficient drug target identification methods. (Fellmann et al., 2011). Due to shorter and uniform size of sgRNA in CRISPR, this can be used to generate array-based oligonucleotide without facing the problems of previous methods. CRISPR-based screens are commonly used to systematically knockout (CRISPRn), inhibit (CRISPRi), or activate (CRISPRa) large numbers of candidate genes (Luo, 2016). CRISPRi screens identify both the essential genes for cell viability and disease lethal genes (Wang, 2014), while CRISPRa based positive selection assesses gene targets whose overexpression leads to a given phenotype (Konermann et al., 2015). The screening information can provide valuable mechanistic insight for developing new therapeutic approaches.

In addition to its utility in high-throughput screening, CRISPR/Cas9 enables the rapid construction of cell line and animal models for drug target validation and compound screening. For example, three forms of chromosomal rearrangement (EML4–ALK, KIF5B–RET and CD74–ROS1) found in lung adenocarcinoma were engineered in somatic cells lines by using CRISPR/Cas, which led to in situ tumor initiation (Choi and Meyerson, 2014). This proves that multiple genes can be targeted in a single step using CRISPR/Cas, which is laborious and time-consuming through the traditional embryonic stem (ES) cell manipulation (Wang et al., 2016). Another example of CRISPR/Cas mediated genome editing directly to the somatic tissue of adult animals is the transfection of plasmids encoding Cas9 and sgRNA targeting *Pten* and *Tp53* led to the deletion of both genes in hepatocytes *in vivo (*Xue et al., 2014). Thus, various types of oncogenic lesions, or disease genotypes can be generated using CRISPR/Cas without the need for germline mice. Again, these models can assess the potential drugs more effectively and within a short period.

Finally, CRISPR holds tremendous potential in advancing pharmacological research as CRISPR-based gene editing is more tractable and precise, making the identification of drug targets faster and the generation of disease models less laborious.

## Conclusion

CRISPR/Cas9 is a widely adopted genome editing tool that has been extended for fine-tuning, regulating, and monitoring multiple genes in plants and humans as well as microbes for a wide range of purposes. These include improvement of agronomic traits for ensuring crop security, development of efficient treatment for human disease, therapeutic drug development, functional gene screening, and the effects of gene mutation. However, CRISPR is associated with some risk and ethical and regulatory issues. One of them is off-target effects which still need further optimization, innovative genome editing complexes, and specific nanostructured complexes for correct action at the target site. Another fear is the unintended changes to organism's genome that can get carried through to the next generation. This would be particularly difficult to control if it has a harmful effect. However, to minimize the issue so that the modern world can benefit from this wondrous invention of science, the regulatory authorities, and the scientists are working to make sure that international scientific regulation and cooperation developments continue safely. The challenges and ethical issues are potentially easy to resolve. It is greatly hoped that the technology will be strengthened and open up newer avenues for precise gene editing in different prokaryotic and eukaryotic organisms for the welfare of mankind.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest regarding the publication of this article.

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