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CRISPR-Cas9: Scientific Advances, Applications, and Ethical Challenges in Modern Genetics

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ABSTRACT

The advent of CRISPR-Cas9 represents a revolutionary advance in genome editing, offering unparalleled accuracy and efficiency in genetic modification. This review examines the fundamental ideas behind CRISPR-Cas9, its variants, its applications, and the effects it has had on a range of scientific fields. Since emerging as a groundbreaking biotechnological powerhouse, CRISPR-Cas9 has broadened its application to a variety of industries, including fundamental research, agriculture, and medicine. By carefully modifying disease-causing genes, a medical field has been developed with hitherto unheard-of possibilities for treating genetic illnesses. Through focused gene alterations, crop improvement in agriculture can be accelerated by improving yield, resilience, and nutritional content. It is impossible to overstate the tool's essential importance in comprehending gene activity and regulation in fundamental research. However, despite the enormous potential of CRISPR-Cas9, there are ethical concerns regarding its use, particularly concerning human germline editing. By integrating scientific advances with ethical and policy perspectives, this review highlights CRISPR-Cas9's innovative role in shaping the future of modern genetic engineering.

1. CRISPR AND ITS BACKGROUND

Harnessing an array of molecular techniques honed over recent decades, humanity has achieved a remarkable capability: precision microediting of DNA sequences and the ability to intricately shape the destiny of mRNA through sophisticated posttranscriptional modifications. This signifies a transformative leap in our mastery of genetic manipulation. The revolution in the field of genomic modifications was initiated by the discovery of clustered regularly interspaced short palindromic repeats (CRISPR) as a defense mechanism in prokaryotes at the beginning of the 21st century. The CRISPR system is a family of DNA direct repeat found in many bacterial genomes (Qiu *et al.*, 2019). Although

this finding initially appeared relevant only to microbiology, it has since transformed the field of genome editing in a variety of ways (Hsu *et al.*, 2014; Adli, 2018).

The CRISPR machinery was discovered in 1987 in DNA sequences from the bacterium *Escherichia coli* (Ishino *et al.*, 1987). Using the information encoded in CRISPR loci for medical research, scientists proposed genotyping different strains of bacteria, beginning with *Mycobacterium tuberculosis* (Groenen *et al.*, 1993) and later *Streptococcus pyogenes* (Hoe *et al.*, 1999). Scientists at the University of Alicante in Spain found comparable patterns in the archaeal genome of

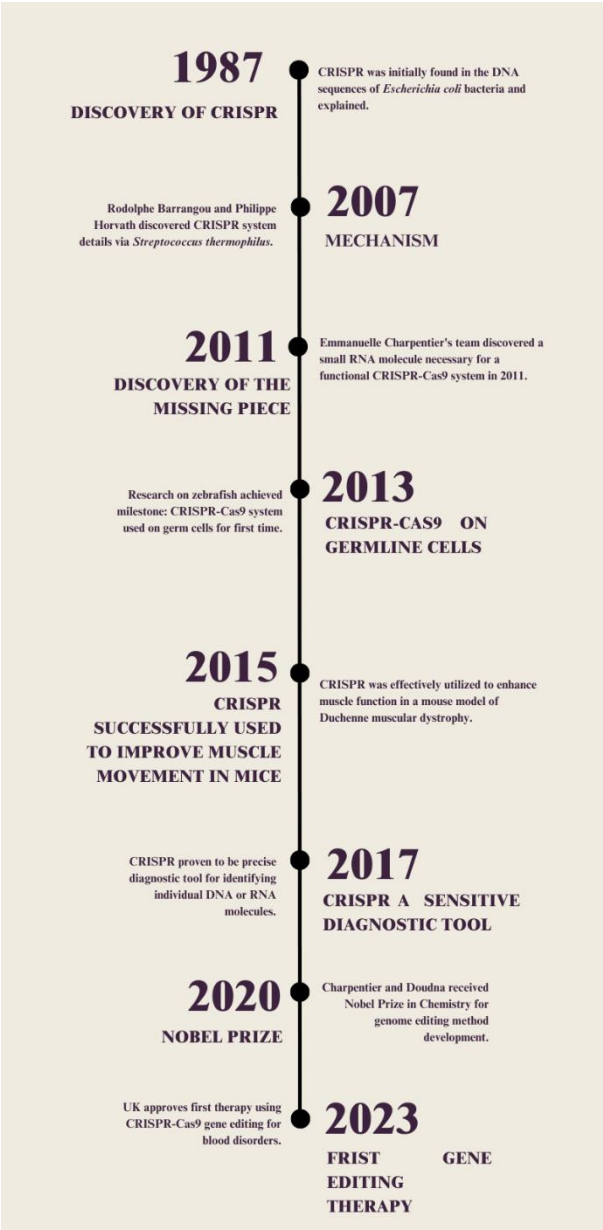


Figure 1. Timeline of CRISPR-Cas9 History and the details of Major Associated Events.

Haloferax mediterranei, and proposed that these unusual loci comprise segments of foreign DNA and are, in fact, components of

the adaptive immune systems of bacteria and archaea (Mojica *et al.*, 2005). In 2005, scientists discovered that the distinctive

CRISPR sequences originated from plasmids and bacteriophages—transmissible genetic components. Because spacer-matching sequences are usually absent in prokaryotic genomes, prokaryotes with these special sequences appear to be resistant to infection (Bolotin *et al.*, 2005). In the same year, the Cas9 protein was first described to be associated with CRISPR repeats and was originally named Cas5 (alternative names include Csn1 and Csx12) (Bolotin *et al.*, 2005). In 2007, the first functional understanding of the CRISPR system's mechanism emerged, focusing on Class 2, the Type-II CRISPR–Cas system in *Streptococcus thermophilus* and the associated human pathogen *Streptococcus pyogenes* (Barrangou *et al.*, 2007). At that time, it was believed that RNA interference was the mechanism by which this system operated. Nevertheless, it was experimentally shown for the first time in 2008 that foreign DNA, not mRNA, was the true target of the prokaryotic immune system (Marraffini and Sontheimer, 2008). The crucial element required for assembling a functional CRISPR–Cas9 system *in vitro* was revealed to be the short RNA molecule transactivating CRISPR RNA (tracrRNA), which was discovered by Emmanuelle Charpentier's group in 2011 (Deltcheva *et al.*, 2011). Then, in January 2013 the fundamental steps necessary for successful DNA editing in human cells were elucidated (Cong *et al.*, 2013; Mali *et al.*, 2013). In the same year, research conducted on zebrafish demonstrated, for the first time, the use of the CRISPR–Cas9 system on germline cells, marking a significant milestone in genetic editing (Hwang *et al.*, 2013). In February 2014, the first two crystallographic studies on Cas9 were conducted, and their structures were subsequently deposited into the PDBe (Protein Data Bank in Europe) database (Jinek *et al.*, 2014; Nishimasu *et al.*, 2014). In April 2014, the first patent for CRISPR was awarded to Cong *et al.* for groundbreaking work published in 2013 (Gostinskaya, 2022). The 2020 Nobel

Prize in Chemistry was awarded to Emmanuelle Charpentier and Jennifer Doudna, less than a decade after the discovery of all the principal molecular components of the system. The culmination of extensive research efforts resulted in a groundbreaking achievement: the first CRISPR-based gene editing treatment for sickle cell disease, Casgevy, secured FDA approval at a landmark moment on December 8, 2023 (F.D.A., 2023).

Prior endeavors to edit or modify the genome, employing homing endonucleases, nuclease technology, and specific chemical procedures, paved the way for the evolution of contemporary methodologies. The molecular methods initially developed for genome editing include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and meganucleases. These early technologies have a broader range of effects, including some that are not desired, which makes them less specific in their impact. Another major challenge was designing simple yet effective delivery systems for host cell penetration. On the other hand, RNA-guided CRISPR/Cas9 has greater effectiveness at producing specific genomic alterations *in vivo*. This approach is straightforward, dependable, and fast for inducing gene modifications in several living species, including humans (Khan, 2019). The skills and equipment required to use CRISPR are readily accessible in the majority of academic and commercial life sciences laboratories. Moreover, CRISPR components have been swiftly disseminated at a low cost through established channels for distributing biological reagents (Martin *et al.*, 2020). These factors collectively fueled the current global surge of interest and activity in gene editing through CRISPR–Cas9.

This review article aims to analyze the comprehensive transformative impact of CRISPR technology across various fields. Through a multidisciplinary approach, we aim to elucidate the potential benefits, challenges,



and ethical considerations associated with CRISPR, ultimately underscoring its pivotal role in shaping the future of genetic engineering and scientific innovation.

2. MECHANISM OF CRISPR-CAS9

The CRISPR-Cas9 system combines CRISPR and Cas9.

2.1 CRISPR Genes

Since the first CRISPR-associated genes were discovered, they have been found in a wide range of prokaryotic cells (Shmakov *et al.*, 2015). An array of repeated sequences separated by brief stretches of nonrepetitive sequences makes up a classic CRISPR locus in a type II CRISPR–Cas system, together with a collection of CRISPR-associated (*cas*) genes (Jiang and Doudna, 2017). This system has 4 *cas* genes, three of which play a role in spacer acquisition (*cas1*, *cas2*, and *csn2*), while the fourth, *cas9* (previously *cas5* and *csn1*), is required for interference (Bolotin *et al.*, 2005).

2.2 Cas Proteins

There are two types of proteins related to this mechanism: effector proteins (which are responsible for the recognition and cleavage of mobile genetic elements to destroy them) and adaptive proteins (which are involved in immune acquisition; Cas1 and Cas2) proteins. There is also evidence that some regulatory proteins are connected to the system (Shmakov *et al.*, 2015). Proteins are related to the system of directional breakage of foreign DNA or RNA, also called genetic scissors, in certain circumstances. Among all the Cas proteins that are known, the nuclease Cas9 is one of the most studied bacterial nuclease enzymes (Bolotin *et al.*, 2005) and is used both to cleave as well as to remember the foreign DNA sequences (Heler *et al.*, 2015; Khanzadi and Khan, 2020). The six domains that make up the Cas9 protein are the arginine-rich bridge helix, recognition lobes (REC I and REC II), protospacer adjacent

motif (PAM), HNH, and RuvC. The primary domain that binds to the gRNA is REC I; however, the function of REC II is unclear. When the arginine-rich bridge helix binds to a specific sequence, it starts to cleave. PAM specificity is achieved through Cas9 recognition of a conserved 2–5 bp sequence on the foreign DNA (Mojica *et al.*, 2005; Barrangou *et al.*, 2007), after which the nuclease domains HNH and RuvC cut the target sequence (Anders *et al.*, 2014).

2.3 Bacterial response to foreign DNA

The CRISPR system goes through several phases to develop an immune-like response to foreign DNA. The first stage is the acquisition stage, in which foreign DNA fragments are integrated into the host CRISPR locus with the help of acquisition machinery (*cas1*, *cas2*, and *csn2*). These integrated segments are known as spacer DNAs, and they generate cellular memory for past infections. The CRISPR array consists of repeated DNA sequences interspersed with these spacers (Deltcheva *et al.*, 2011). When fully incorporated, the new spacer is co-transcribed into a lengthy precursor CRISPR RNA (precrRNA) that contains repeats and spacers. The consequent transcription of the CRISPR array and the enzymatic cleavage of precrRNAs result in the production of short CRISPR RNAs (crRNAs) (Brouns *et al.*, 2008). The spacer derived crRNA is a short RNA segment located at the 5' end and complements a sequence present in foreign genetic material, whereas a segment of the CRISPR repeat is present at the 3' end (Gasiunas *et al.*, 2012; Doudna and Charpentier, 2014). The transactivating CRISPR RNA (tracrRNA) gene is expressed before the Cas operon and encodes a distinctive noncoding RNA that is homologous to the corresponding repeat sequence. To allow the precrRNA repeats to anneal to the tracrRNA for crRNA maturation, RNase III must first cleave the tracrRNA independently of the other transcripts (Garneau *et al.*, 2010). Unknown



nucleases further cut the 5' end of the crRNA, reducing the guide sequence to 20 nucleotides. When interference occurs, the mature crRNA-tracrRNA structure directs the Cas9 endonuclease to cut foreign DNA that has a 20-nt complementary crRNA sequence before the PAM region (Jiang and Doudna, 2017).

After a subsequent infection, the crRNA spacer hybridizes with a complementary foreign target sequence adjacent to the PAM motif, which guides Cas9 nucleases to specifically target foreign RNA or DNA (Heler *et al.*, 2015). Cas9 attaches to invasive DNA in the presence of the PAM sequence and cleaves the viral DNA at the target location, causing a double-strand break (DSB). After cleavage, the cellular repair machinery attempts to repair the break. Viral DNA is then frequently disrupted and rendered nonfunctional by the mistakes introduced by this repair process. Effective neutralization of viral DNA prevents the virus from spreading and shields the bacterium from infection (Hsu *et al.*, 2014). The assembly of mature crRNAs and Cas proteins to generate crRNA-effector complexes, which probe DNA targets and degrade matching sequences in foreign nucleic acids, is a unique characteristic of this system. Most CRISPR-Cas systems depend on this procedure for target DNA selection and destruction (Van Der Oost *et al.*, 2014).

In simple words, the CRISPR system provides immunity-like defense mechanism to its host against foreign DNA. The foreign DNA is integrated into the CRISPR array as spacers to create a memory of the previous infections. These spacers are processed into crRNAs to guide Cas9 to the foreign DNA, and upon recognition of PAM sequences, the foreign DNA is cleaved by Cas9.

3. GENOME EDITING VIA THE CRISPR-CAS9 SYSTEM

To facilitate genome editing in eukaryotic cells, the CRISPR-Cas system has been

designed as a two-part system: the active ribonucleoprotein (RNP) complex is composed of a Cas endonuclease and a single guide RNA (sgRNA). Two domains make up the sgRNA sequence: a backbone sequence that anchors the sgRNA to the Cas protein and a spacer region that targets the complex to DNA sequence using a 20 nucleotides spacer region (Wilbie *et al.*, 2019).

By inducing a DSB at the DNA locus, guided by the sgRNA, therapeutic gene editing is achieved. For the Cas protein to be activated in this process, a specific nucleotide sequence known as the PAM needs to be present on the target strand. Both the strands adjacent to PAM are cleaved by the active complex. PAM sequences are necessary for distinct Cas proteins but can also differ in their patterns of cleavage (Wilbie *et al.*, 2019). Modifying the 20-nt guide RNA sequence can cause a DSB to occur close to any PAM site that is unique to the selected Cas protein, which makes this approach preferable than other gene-editing approaches (Ou *et al.*, 2021) (Figure 2).

Cas9 protein and sgRNA can be introduced into the cell in several ways. (1) Cas9 and sgRNA sequences are cloned and inserted into a plasmid carrier and inserted into cells during the process of plasmid DNA transfection (Doudna and Charpentier, 2014). (2) RNA transfection involves the direct transfection of chemically synthesized Cas9 mRNA and sgRNA into cells (Gagnon *et al.*, 2014). (3) Viral vectors (lentiviruses, adenoviruses, etc.) are used to introduce Cas9 and sgRNA sequences into cells (Ran *et al.*, 2015). (4) In the process of delivering ribonucleoproteins (RNPs), sgRNA and the Cas9 protein bind *in vitro* to generate RNPs that are subsequently delivered into cells (Kim *et al.*, 2014). (5) In electroporation, Cas9 and sgRNA molecules enter cells when they are subjected to an electric field (Joung *et al.*, 2017). (6) In the lipid nanoparticle delivery mechanism, target cells are exposed to lipid nanoparticles that



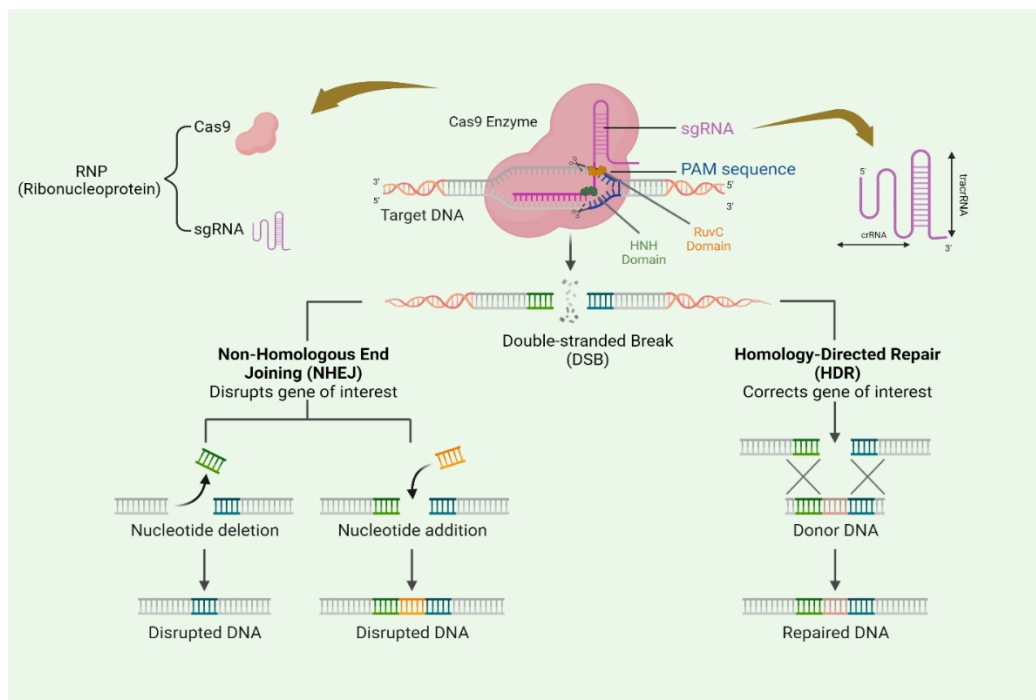


Figure 2. Mechanism of gene editing through CRISPR-Cas9 The sgRNA consists of two parts: crRNA, which binds to the target DNA sequence adjacent to the PAM, and tracrRNA, which provides stability and guidance. The sgRNA and Cas9 form a ribonucleoprotein (RNP) complex that cleave at the targeted DNA site near the PAM. After DSB formation, nonhomologous end joining (NHEJ) can occur by joining DNA ends, often leading to insertions or deletions that can disrupt the targeted gene; alternatively, homology-directed repair (HDR) can be used to establish a donor DNA template for the precise insertion of genetic material during gene repair.

contain Cas9 and sgRNA (Finn *et al.*, 2018). (7) The Cas9 protein is coupled with a peptide that penetrates cells and is delivered directly into cells via the protein transduction method (Ramakrishna *et al.*, 2014).

4. CRISPR VARIANTS AND DEVELOPMENTS

The landscape of CRISPR technology has evolved to encompass several sophisticated variations, each intended to improve a particular facet of gene editing accuracy, effectiveness, and adaptability. Severable notable variants include CRISPR-Cas9, CRISPR-Cas12, CRISPR-Cas13, CRISPR-Cas9n, CRISPR-Cas9-D10A, and CRISPR-

Cas9 Prime Editing. The details of these variants are summarized in Table 1.

In microbial communities, multiple varieties of CRISPR RNA-guided adaptive immune systems exist. The genes are classified into two major classes, five types, and sixteen subtypes. Class 1 includes three distinct types (Type I, Type III, and Type IV) that cleave DNA, recognizes targets, and bind to crRNA through multisubunit protein complexes. Type II, Type V, and Type VI are members of Class 2, and they are all characterized by the use of single effector proteins for the binding of target nucleic acids and crRNA. The Class 2 Type V system uses a single RuvC domain comprising



Table 1. Details of the CRISPR Variants- All of these variants were designed for different targeted edits

CRISPR Variants	Description	Reference
CRISPR-Cas9	Create targeted double-strand breaks in DNA	(Jinek <i>et al.</i> , 2014)
CRISPR-Cas12 (Cpf1)	For different PAM recognition and cleavage patterns	(Zetsche <i>et al.</i> , 2015)
CRISPR-Cas13	Targets RNA rather than DNA for cleavage	(Abudayyeh <i>et al.</i> , 2016)
CRISPR-Cas9n	For single-strand nicks in the DNA	(Ran <i>et al.</i> , 2013)
CRISPR-Cas9-D10A (Base Editing)	A Cas9 nickase fused with DNA deaminase enzyme that enables direct single-base conversions without introducing double stranded breaks	(Shin and Oh, 2020)
CRISPR-Cas9 Prime Editing	Enables precise insertion, deletion, or replacement	(Anzalone <i>et al.</i> , 2019)

effectors such as Cpf1, C2c1, or C2c3, whereas the most well-known Class 2 Type II system is defined by a single-component effector protein, Cas9, with RuvC and HNH nuclease domains (Shmakov *et al.*, 2015). However, Class 2 Type VI systems use a single C2c2 effector that has two higher eukaryotic and prokaryotic nucleotide-binding (HEPN) RNase domains (Abudayyeh *et al.*, 2016).

For exact gene expression modification, the CRISPR–Cas system is used in three distinct applications or methodologies: CRISPR knockout, CRISPRa, and CRISPRi. CRISPR-Cas9 knockout is a loss-of-function approach in which a specific gene in an organism's genome is knocked out or disrupted. This is accomplished by employing the Cas9 enzyme, which introduces specific gene mutations under the guidance of a specific gRNA. The innate mechanism of cell repair leads to DSBs caused by Cas9, resulting in insertions or deletions that interfere with the gene's open reading frame (ORF) and ultimately lead to its

knockout. By tracking the consequences of loss-of-function mutations in particular genes, this method can be used to investigate the function of genes. It is also used to identify novel biological pathways, such as signals important in cell survival (Cong *et al.*, 2013). The CRISPRa technique is employed to boost the expression of a certain gene. With CRISPRa, transcriptional activators are directed to the gene's promoter region rather than disrupting the gene itself. This is accomplished by combining transcriptional activators with dCas9, a catalytically inactive version of Cas9. Guided by a particular gRNA, the dCas9-activator complex enhances the transcription of target genes, thereby elevating mRNA levels. The function of genes can be investigated by monitoring the elevated expression of particular genes. Moreover, this approach can be applied to increase the expression of therapeutic targets (Cheng *et al.*, 2013). The CRISPRi technique is used for downregulating or inhibiting the expression of a gene without completely eradicating it. It also



uses the dCas9 protein, which is coupled to repressor domains such as the KRAB (Krüppel-associated box) domain but is not catalytically active. Transcription of the gene is either prevented or decreased by the dCas9 repressor complex, which is regulated by a specific gRNA and interferes with RNA polymerase binding. CRISPRi is a gene inhibition approach based on CRISPR that is used to screen the host for loss-of-functional phenotypes. It is also useful for researching genes that are necessary and cannot be completely eliminated (Martinez-Lage *et al.*, 2018).

5. APPLICATIONS

The genome engineering fields of biotechnology, medicine, and research benefited greatly from the precise application of CRISPR. From viruses and bacteria to simple model organisms, such as *Drosophila melanogaster* (fruit fly), to larger animals, such as pigs, cattle, monkeys, and even human zygotes, CRISPR experimentation has led to novel, important findings (Brokowski *et al.*, 2015).

5.1 Therapeutic Research

CRISPR-based therapeutic research is highly significant in revolutionizing medical treatment paradigms (Ou *et al.*, 2021). By enabling precise editing of disease-causing genes, CRISPR offers the potential to correct genetic mutations underlying a wide range of disorders, from genetic diseases such as cystic fibrosis and sickle cell anemia to complex conditions such as cancer and neurodegenerative diseases. This technology provides a promising avenue for developing targeted and personalized therapies, with the potential to mitigate symptoms, halt disease progression, and even cure previously untreatable conditions (Traylen *et al.*, 2011; Lange and Kappel, 2022). CRISPR-based approaches are marking a transformative shift

toward precision medicine in healthcare. For more details, see Table 2.

5.2 Livestock

Previous methods of genetic modification have proven effective for plants and smaller mammals, such as laboratory mice. However, these methods pose significant challenges when applied to larger mammals and birds commonly found in livestock agriculture (Perisse *et al.*, 2021). In contrast, the advent of CRISPR-Cas9 has ushered in a new era, enabling, for the first time, the large-scale genetic modification of livestock animals, including cows, pigs, and chickens. This breakthrough opens up novel editing targets, ranging from genes that mitigate disease vulnerabilities to those that do not. Researchers are also working to create animals that are resistant to diseases using CRISPR (Mehra and Kumar, 2022; Müller *et al.*, 2022) (Table 2).

5.3 Agriculture Traditional breeding methods for plants often require significant time investments, ranging from 7 to 12 years to achieve desired traits. Moreover, these methods typically enable the improvement of only one trait at a time. While traditional approaches can yield results, their prolonged timeline presents challenges, particularly in meeting the urgent need for measurable gains by the 2050. This underscores the pressing demand for more efficient and precise technologies such as CRISPR-Cas9, which offer accelerated breeding processes and simultaneous enhancement of multiple traits, thus allowing better alignment with the imperative timeline for agricultural innovation. To use CRISPR-Cas systems in a particular species for a given purpose, one must possess a thorough understanding of plant biology and gene functions. CRISPR has been used to produce bacterial and fungal-resistant crops and drought- and salt-tolerant plants (Camerlengo *et al.*, 2022) (Table 2).



Table 2. Applications of CRISPR-Cas9 Gene Editing in Various Fields

Applications	Targeted Cell/Gene	Reference
Therapeutic Research	Sickle Cell Disease (SCD)	β -globin gene (Frangoul <i>et al.</i> , 2021)
	Cardiovascular Disease (CVD)	PCSK9 gene (Wang <i>et al.</i> , 2016b)
	Cancer	T cells (Zych <i>et al.</i> , 2018)
	Cystic Fibrosis	Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene (Graham and Hart, 2021)
	Rheumatoid Arthritis (RA)	miR-155 gene (Jing <i>et al.</i> , 2015)
	Huntington's disease (HD)	Huntingtin HTT gene (Ekman <i>et al.</i> , 2019)
	Lymphoma and Leukemia	T immune cells (PTEN gene) (Song <i>et al.</i> , 2021)
	Hemophilia A and B	Blood Coagulation Genes Factor VIII (FVIII) (Stephens <i>et al.</i> , 2019)
	Hypertrophic Cardiomyopathy (HCM)	MYBPC3 gene (Stephens <i>et al.</i> , 2019)
	Cataracts	Cryg gene (Wu <i>et al.</i> , 2013)
	HIV/AIDS	CCR5, T cells, and CD34+ Hematopoietic Stem and Progenitor Cells (HSPCs) (Wu <i>et al.</i> , 2013; Soppe and Lebbink, 2017)
	Alzheimer's Disease	APP, PSEN1 and PSEN2 genes (Bhardwaj <i>et al.</i> , 2022)
	Duchenne Muscular Dystrophy	DMD gene (Paredes-Redondo <i>et al.</i> , 2021)
	Wolff Parkinson White Syndrome (WPW)	PRKAG2 gene (Xie <i>et al.</i> , 2016)
	β -thalassemia	BCL11A gene (Canver <i>et al.</i> , 2015)
	Dystrophic Epidermolysis bullosa	COL7A1 gene (Shinkuma <i>et al.</i> , 2016)
	X-linked chronic granulomatous disease (X-CGD)	CYBB gene (Shinkuma <i>et al.</i> , 2016)



Applications		Targeted Cell/Gene	Reference
Livestock	Meat Output in Cows	MSTN gene	(Zhao <i>et al.</i> , 2019)
	Tuberculosis Resistance in Cattle	NMRAP1 gene	(Gao <i>et al.</i> , 2017)
	Porcine reproductive and respiratory syndrome virus (PRRSV) Resistance in Pigs	CD163 gene	(Whitworth <i>et al.</i> , 2016)
	Enhance Muscle Mass in Goats	GDF8, FGF gene	(Ni <i>et al.</i> , 2014; Wang <i>et al.</i> , 2015)
	Genetically Engineered Pigs for Xenotransplantation	α 1,3GT gene	(Petersen <i>et al.</i> , 2016)
Agriculture	Improved grain yield under field drought stress conditions in <i>Zea mays</i>	ARGOS8 gene	(Shi <i>et al.</i> , 2017)
	Grain weight in <i>Oriza sativa</i>	GW2, GW5 and TGW6 genes	(Xu <i>et al.</i> , 2016)
	Blast (caused by <i>Magnaporthe oryzae</i>) tolerance in <i>Oriza sativa</i>	OsERF922 gene	(Wang <i>et al.</i> , 2016a)
	Herbicide resistance in <i>Oryza sativa</i>	C287 gene	(Shimatani <i>et al.</i> , 2017)
	Reduced susceptibility to ALS-inhibiting herbicides in <i>Solanum tuberosum</i>	Acetolactate synthase 1 (ALS1) gene	(Butler <i>et al.</i> , 2016)
	Salt stress tolerance in <i>Oryza sativa</i>	OsRAV2 gene	(Duan <i>et al.</i> , 2016)
	Powdery mildew resistance in <i>Solanum lycopersicum</i>	SIMlo gene	(Nekrasov <i>et al.</i> , 2017)
	Powdery mildew (<i>Blumeria graminis</i> f. sp. <i>Triticici</i>)	TaMLO-A1 allele	(Nekrasov <i>et al.</i> , 2017)

Applications	Targeted Cell/Gene	Reference
resistance in <i>Triticum aestivum</i>		
Citrus canker (caused by <i>Xanthomonas citri</i> subspecies <i>citri</i> (Xcc)) tolerance in <i>Citrus paradisi</i>	CsLOB1 gene	(Jia <i>et al.</i> , 2016)
Bacterial blight (caused by <i>Xanthomonas oryzae</i> pv. <i>oryzae</i>) tolerance in <i>Oryza sativa</i>	OsSWEET13 gene	(Zhou <i>et al.</i> , 2015)
Potyvirus resistance (TuMV) in <i>Arabidopsis thaliana</i>	eIF(iso)4E	(Pyott <i>et al.</i> , 2016)
Beet severe curly top virus (BSCTV) tolerance in <i>Arabidopsis thaliana</i> and <i>Nicotiana benthamiana</i>	43 candidate sites in coding or noncoding sequences of the BSCTV genome	(Ji <i>et al.</i> , 2015)
Tomato yellow leaf curl virus (TYLCV) resistance in <i>Nicotiana benthamiana</i>	AGO2 gene, other coding and noncoding sequences of TYLCV	(Ali <i>et al.</i> , 2015; Ludman <i>et al.</i> , 2017)
Cold, salt, and drought stress tolerance in <i>Arabidopsis thaliana</i>	UDP-glycosyltransferases UGT79B2 and UGT79B3	(Li <i>et al.</i> , 2017)

6. CHALLENGES AND LIMITATIONS

Although the CRISPR–Cas9 system is an extremely effective tool for editing the genome, it can also pose hazards in some situations. These include the genetic

engineering of plants and animals as well as the clinical study of hereditary illnesses in humans.

6.1 Off-target effects

A major challenge associated with the CRISPR–Cas9 system is off-target editing. These systems can occasionally alter unwanted



genetic regions, which can have off-target effects. These inadvertent alterations may have detrimental effects, such as cancer or other genetic disorders. Numerous studies have demonstrated the unintended consequences of CRISPR-Cas9-mediated genome editing in biological and therapeutic contexts (Zhang *et al.*, 2015; Saha *et al.*, 2019). It has also been shown to cause off-target effects in rat, mouse, and human cell lines. New research on additional CRISPR-Cas systems, such as RNA-guided base editing systems, has also revealed off-target effects in other animals (Fu *et al.*, 2013; Anderson *et al.*, 2018). Since off-target editing can result in loss-of-function mutations in normally functioning genes or inefficient repair of disease-causing genes by binding and cleaving at locations other than the target DNA sequence, this approach poses serious hurdles for therapeutic use (Cox *et al.*, 2015). In addition, this might result in chromosomal rearrangements and other modifications, such as DNA mismatches inserted into the PAM-distal location of the sgRNA sequence (Ghosh *et al.*, 2019).

6.2 Mosaicism

The emergence of genetic mosaicism in embryos is another consequence of CRISPR-mediated DNA editing in embryos. In particular, components of CRISPR-Cas9 are often introduced directly into fertilized zygotes as DNA, RNA, or protein molecules to generate knockout and transgenic animal models (Niu *et al.*, 2014). Simply put, the system can repeatedly identify and cleave genes while the embryo is developing at different stages, resulting in mosaic mutations (Xin *et al.*, 2016). The most evident adverse effect is the production of false-positive genotyping results. Furthermore, in people with chromosomal anomalies, mosaicism may complicate genotype analysis (Oliver *et al.*, 2015).

6.3 PAM Requirement

Another limitation of the CRISPR-Cas9 system is that a PAM needs to be located close to the target location. The bacterium *Streptococcus pyogenes* is the source of one of the most commonly used Cas9 variants, SpCas9. Its standard PAM recognition site is 5'NGG3' (N may represent any nucleotide). Unfortunately, loading SpCas9 into adeno-associated virus (AAV) vectors, the widely favored vehicle for gene therapy delivery, poses challenges owing to their size (Lau and Suh, 2017; Lino *et al.*, 2018). On the other hand, *Staphylococcus aureus* Cas9 (SaCas9) is a smaller and more compatible with AAV vectors but has a longer PAM sequence. This further limited therapeutic applications (Chatterjee *et al.*, 2018).

6.4 Apoptosis

Often, CRISPR-induced DSBs often fail to produce the intended gene alterations and instead trigger apoptosis (Hu *et al.*, 2014). Additional safety issues were brought up by employing this technique on human pluripotent stem cells (hPSCs) because p53 (important for the cell cycle, DNA repair, and apoptosis) activation often leads to cell death due to the damaging DSBs caused by CRISPR (Ihry *et al.*, 2018). Therefore, p53-suppressed cells are more likely to undergo effective CRISPR editing, which leads to bias in favor of oncogenic cell survival through selection (Haapaniemi *et al.*, 2018). A serious safety problem for clinical uses of CRISPR therapy involving the generation of DSBs is evidenced by the multiple occurrences of large deletions extending kilobases and complicated rearrangements, which have been described as unexpected results of on-target activity (Cullot *et al.*, 2019).

In short, despite its high efficiency, CRISPR-Cas9 faces several challenges, including unintended mutations, mosaicism, strict PAM requirements, and the chance of apoptosis. This



raises significant safety concerns for clinical applications.

7. ETHICAL CONSIDERATIONS

Researchers leveraging the CRISPR-Cas9 system to combat life-threatening diseases encounter ethical dilemmas, sparking debates on safety and ethical implications. These discussions encompass morality, the evolutionary role of genetics, clinical consent, religious considerations, and concerns about illegal germline alterations, as well as debates on cloning, designer babies, and the potential emergence of superhumans. Ethical concerns surrounding gene therapy became apparent soon after the first research showcasing the use of CRISPR in human cells was published (Shinwari *et al.*, 2018; Ye *et al.*, 2020).

A paper published in 2015 underscored ethical concerns by advocating against heritable alterations in human embryonic cells. Scientists have warned that such alterations could impede broader progress in gene therapy. A subsequent study by a group of scientists demonstrated the limitations of using CRISPR-Cas9 in human embryonic cells (Cyranoski and Reardon, 2015; Lanphier *et al.*, 2015).

In 2018, the researcher Jiankui He shocked the world by revealing the birth of CRISPR-modified babies whose genomes were altered during *in vitro* fertilization. This has led to widespread ethical debates, particularly regarding unintended mutations and the heritability of changes. His subsequent attempt to create HIV-resistant babies sparked another scandal, intensifying discussions on the ethics of human germline editing (Regalado, 2019a; b). Following this controversy, another report further fueled the debate by announcing genetically altered offspring with deafness-linked *GJB2* gene modifications, drawing strong and mostly negative reactions from the scientific community (Cyranoski and Reardon, 2015).

The birth of CRISPR-modified babies and subsequent controversial experiments highlights widespread concerns about the ethical implications of CRISPR, particularly in human germline editing. Although there are differing opinions, several authors emphasize the distinction between somatic and germline modifications since scientific and bioethical communities are particularly concerned about germline CRISPR gene therapy (Hofmann, 2018; Liao, 2019).

Another global risk is the possibility that allowing CRISPR germline editing could revive eugenics, which suggests that humans could be improved by carefully regulating breeding to increase the prevalence of desired, heritable traits (Brokowski *et al.*, 2015).

An additional ethical concern is that germline CRISPR gene therapy may be carried out without patient consent. This concern also applies to early-stage somatic cell therapy, and even if parents provide consent, there is only a limit to which they hold rights to their offspring's life (Ormond *et al.*, 2017).

Additionally, the current body of material allows for the possibility that CRISPR genome editing can be used as a weapon of war (Fraser and Dando, 2001). Technology abuse could result in the creation of extremely contagious or drug-resistant organisms, among other detrimental effects. This dual-use technology is widely distributed and developing at a rapid rate; therefore, purposeful or inadvertent misuse might have serious negative effects on world security (Willingham, 2018).

The potential effects of CRISPR gene therapy on social justice represent another ethical concern. If important stakeholders (such as patients) are not fairly involved in the decision-making process, CRISPR gene therapy may be procedurally biased (Jasanoff *et al.*, 2019). Various authors have also highlighted how CRISPR gene therapy may, due to cost, location, and other considerations, only benefit



wealthy groups (Hildebrandt and Marron, 2018).

8. REGULATIONS AND POLICY

Research indicates that discussions regarding the governance and regulation of CRISPR gene editing have extended across numerous disciplines and countries over time. Initially, discussions focused on the potential applications and benefits of gene editing, the discourse has progressively shifted toward advocating for stronger policy and regulatory interventions. Given the significance and contested nature of CRISPR technology, two international summits were also convened in 2015 and 2018 to address its implications (Meye et al., 2019).

In discussions surrounding human genome editing, an often overlooked aspect is the existing regulatory framework governing such research. This oversight is particularly notable in media coverage and scholarly literature,

where references to relevant policies tends to be vague or inaccurate (Kau et al., 2020). The World Health Organization (WHO) established an expert panel on the governance of human gene editing in 2018, reflecting the growing global concern in regulating the use of CRISPR in humans (Meyer and Vergnaud, 2021). Globally, the current stances range greatly, from outright banning the research to restrictions that prohibit the use of federal funds. In such countries, federal funding cannot be used to support research involving human embryos; however, private funding and the research itself remained permitted. Within the European Union, various institutions have addressed the concerns surrounding human genome editing, and several legal texts have contributed to establishing a regulatory framework applicable to human genome editing (Kuzma and Grieger, 2020). For the details of the regulations and policies regarding human genome editing in various countries, please see Table 3 (Baylis *et al.*, 2020).

Table 3. Regulatory framework for human CRISPR genome editing in different countries around the globe

Region	Country	Germline Genome Editing Research (other than reproduction)	Heritable Genome Editing Research (for reproduction)
Africa	Republic of Benin	No Relevant Information	Prohibits
	South Africa	No Relevant Information	No Relevant Information
	Central African Republic	No Relevant Information	No Relevant Information
	Burkina Faso	Intermediate	Intermediate
	Egypt	No Relevant Information	No Relevant Information
	Namibia	No Relevant Information	No Relevant Information
	Burundi	Permits	Prohibits
	Kenya	No Relevant Information	Prohibits
	Congo	Permits	Prohibits

Region	Country	Germline Genome Editing Research (other than reproduction)	Heritable Genome Editing Research (for reproduction)
	Tanzania	No Relevant Information	No Relevant Information
	Tunisia	Intermediate	Prohibits
	Guinea	No Relevant Information	No Relevant Information
	Uganda	No Relevant Information	No Relevant Information
	Nigeria	Intermediate	Prohibits
	Zambia	No Relevant Information	No Relevant Information
America	United States	Permits	Prohibits
	Argentina	No Relevant Information	Prohibits
	Colombia	Exception to prohibition	Exception to prohibition
	Chile	No Relevant Information	Prohibits
	Canada	Prohibits	Prohibits
	Costa Rica	Prohibits	Prohibits
	Guyana	No Relevant Information	No Relevant Information
	Peru	No Relevant Information	No Relevant Information
	Brazil	Prohibits	Prohibits
	Jamaica	No Relevant Information	No Relevant Information
	Antigua and Barbuda	No Relevant Information	No Relevant Information
	Panama	Exception to prohibition	Exception to prohibition
	Barbados	No Relevant Information	No Relevant Information
	Cuba	No Relevant Information	No Relevant Information
	Mexico	No Relevant Information	
	Trinidad and Tobago	No Relevant Information	No Relevant Information
	Uruguay	Prohibits	Prohibits
Europe	Albania	Prohibits	Prohibits



Region	Country	Germline Genome Editing Research (other than reproduction)	Heritable Genome Editing Research (for reproduction)
	Belgium	No Relevant Information	Exception to prohibition
	Belarus	Prohibits	Prohibits
	Bosnia and Herzegovina	No Relevant Information	Prohibits
	Bulgaria	No Relevant Information	Prohibits
	Czech Republic	No Relevant Information	Prohibits
	Croatia	Prohibits	Prohibits
	Estonia	No Relevant Information	Prohibits
	Denmark	No Relevant Information	Prohibits
	Austria	Prohibits	Prohibits
	Luxembourg	No Relevant Information	No Relevant Information
	Ireland	Permits	Prohibits
	Iceland	No Relevant Information	Prohibits
	Italy	Exception to prohibition	Exception to prohibition
	Latvia	No Relevant Information	Prohibits
	Lithuania	No Relevant Information	Prohibits
	Malta	Prohibits	Prohibits
	Moldova	No Relevant Information	Prohibits
	Montenegro	No Relevant Information	Prohibits
	Romania	No Relevant Information	Prohibits
	Serbia	No Relevant Information	Prohibits
	North Macedonia	No Relevant Information	Prohibits
	San Marino	No Relevant Information	Prohibits
	Slovak Republic	No Relevant Information	Prohibits

Region	Country	Germline Genome Editing Research (other than reproduction)	Heritable Genome Editing Research (for reproduction)
	Slovenia	No Relevant Information	Prohibits
	United Kingdom	Permits	Prohibits
	Netherlands	Intermediate	Prohibits
	Spain	No Relevant Information	Prohibits
	Switzerland	Prohibits	Prohibits
	Poland	No Relevant Information	Prohibits
	Vatican	Prohibits	Prohibits
	Portugal	Intermediate	Prohibits
	Norway	Permits	Prohibits
	France	No Relevant Information	Prohibits
	Sweden	Prohibits	Prohibits
	Finland	Exception to prohibition	Prohibits
	Georgia	No Relevant Information	Prohibits
	Germany	Prohibits	Prohibits
	Greece	Prohibits	Prohibits
	Hungary	No Relevant Information	Prohibits
	Ukraine	No Relevant Information	Intermediate
	Turkey	No Relevant Information	Prohibits
Asia	Armenia	No Relevant Information	No Relevant Information
	Japan	Permits	Prohibits
	South Korea	No Relevant Information	Prohibits
	Russia	No Relevant Information	Prohibits
	China	Permits	Prohibits
	Vietnam	No Relevant Information	No Relevant Information
	India	Permits	Prohibits
	Malaysia	Prohibits	Prohibits
	Thailand	Permits	Prohibits
	Pakistan	Prohibits	Prohibits



Region	Country	Germline Genome Editing Research (other than reproduction)	Heritable Genome Editing Research (for reproduction)
	Singapore	Intermediate	Intermediate
Eastern Mediterranean	United Arab Emirates	No Relevant Information	Exception to prohibition
	Qatar	No Relevant Information	Prohibits
	Cyprus	No Relevant Information	Prohibits
	Bahrain	Prohibits	Prohibits
	Israel	No Relevant Information	Prohibits
	Iran	Permits	Prohibits
	Lebanon	Prohibits	Prohibits
	Saudi Arabia	Prohibits	Prohibits
	Oman	No Relevant Information	Prohibits
Oceania	Australia	No Relevant Information	Prohibits
	New Zealand	No Relevant Information	Prohibits

**‘Prohibited with exceptions’ means where there are exceptions for so-called therapeutic purposes, ‘Indeterminate’ means it is not possible to determine from their policy documents to certainty whether the research in question is permitted or prohibited, and ‘No Relevant Information’ means where the policy documents do not explicitly refer to the human germline or heritable genome editing.*

Concerning the use of gene editing in agriculture, international conferences, such as those held at the OECD in 2018, have provided platforms for discussing key issues. Among these, the status and traceability of gene-edited organisms have emerged as hotly debated topics, underscoring the need for global dialogue and regulatory frameworks in this domain. The emergence of CRISPR-Cas9 gene editing has reignited discussions around genetically modified organisms (GMOs) from previous eras of genetic technology. A pivotal concern for many scientists and companies revolves around whether the new wave of gene-edited crops should be governed by existing legislation for GMO production and release. Diverse jurisdictions have adopted varying stances: the US Department of

Agriculture (USDA) does not regulate gene-edited crops further, provided that the gene-editing technique does not introduce "novel" DNA into the modified organism (Callaway, 2018). However, in 2020, the USDA implemented new regulations for genetically modified crops, which exempt many gene-edited crops from premarket oversight and do not mandate public disclosure upon their introduction into agricultural or food systems. Conversely, the European Union (EU) has mandated that all gene-edited plants and animals are regulated under its existing GMO directives (Wolt and Wolf, 2018). This decision has stirred considerable controversy and has prompted a range of proposals and demands to revise EU legislation (Ricroch and Hénard-Damave, 2016; Garland, 2021). To

address the worldwide concerns of food security, food safety, and sustainable agriculture, a new product-based, scalable regulatory framework for genome-edited crops is needed (Ahmad *et al.*, 2021).

Achieving the controlled implementation of CRISPR requires public consensus, expert deliberations, active engagement of biotechnologists, insights from bioethics experts, establishment of clear legislative frameworks, formulation of definitive rules, and vigilant oversight.

9. FUTURE PROSPECTS

In its brief existence, CRISPR-Cas9 has emerged as an unparalleled force, revolutionizing fundamental research through precise programmable DNA editing. Celebrated as a "*game-changing technology*," CRISPR-Cas has the potential to redefine nature at its most fundamental level.

As ongoing research indicates, patients who presently have no alternative treatment options may benefit from CRISPR, which addresses the underlying genetic abnormalities that cause hereditary disorders. By altering the genes of cancer cells to reduce their aggressiveness or eliminate them, CRISPR can treat or prevent cancer (Wang *et al.*, 2022). CRISPR could be used to create more crops with increased yields, improved nutritional value, and greater resistance to pests and illnesses. The problem of global food security can be addressed with the use of this technology (De Lange *et al.*, 2022). It may be possible to edit microbes with CRISPR to manufacture biofuels more effectively. Our dependency on fossil fuels can be reduced with the use of this technology (Lakhawat *et al.*, 2022). It may also be utilized to repair ecosystems that have been harmed by human activity or to manage invading species, such as disease-causing mosquitoes (Whitworth *et al.*, 2016).

The influence of CRISPR-Cas9 technology extends its impact across diverse realms, shaping not only the environment, agriculture, and medicine but also influencing the very fabric of future human generations.

10. CONCLUDING REMARKS

In conclusion, the revolutionary impact of CRISPR technology on genetic editing and its applications in medicine, agriculture, and environmental management is unmistakable. The sheer transformative power of CRISPR to precisely edit DNA has opened new frontiers in research. It holds promise for addressing hereditary diseases, enhancing crop resilience, and advancing our understanding of biological systems. The ongoing discourse on CRISPR reflects a delicate balance between innovation and ethical responsibility, emphasizing the need for continued dialogue, oversight, and collaborative efforts to harness its full potential for the greater good.

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The authors declare no conflict of interest.

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