Review Article

Overview of CRISPR-Cas mediated Genome Engineering

ABSTRACT

Genetic diseases are prevailing in 3-5% of new-born worldwide and estimated to cause around 50% of child deaths. The development of efficient and regulated system for customizing genome modifications to treat such disorders has been the zone of interest. Over time, different editing techniques have emerged based on programmable nucleases such as ZFN or TALENS but among all, CRISPR-Cas has significantly progressed as alteration tool from bench to clinical practices. CRISPR-Cas is an immune system discovered in prokaryotes that enables organism to recognize and destroy any invading genetic elements. This functional property of CRISPR has opened up plethora of applications across different disciplines such as diagnostics, agriculture and therapeutics. So here, the review attempt to discuss origin, mechanisms pertinent to CRISPR and applications along with challenges concerning them.

Key words: Genome editing; CRISPR-Cas; Genetic disorder; Disease model; Agriculture; Bioenergy; Therapeutics.

ABBREVIATION

ZFN: Zinc finger nuclease
TALENS: Transcription-activator like effector nucleases
CRISPR-Cas: Clustered regularly interspaced short palindromic repeats
NHEJ: Non-homologous end joining
HDR: Homology-directed repair
ALP: Alkaline phosphatase
PAM: Protospacer adjacent motif
GOI: Gene of interest
iPSC: Induced-pluripotent stem cell
CFTR: cystic fibrosis transmembrane conductance regulator
CAR T: Chimeric antigen receptor cell
SCD: Sickle cell disease
CF: Cystic fibrosis

1. INTRODUCTION

Genetic engineering is an approach in which nucleic acid sequences are deliberately knocked out, inserted or altered in living cells. But it came into spotlight with the discovery of CRISPR in prokaryotic genome. In nature, bacteria are the most predominant organisms on planet and are constantly being threatened by viruses or bacteriophages. To tackle this, prokaryotes have developed an advanced defense strategy termed as CRISPR Cas (clustered regularly interspaced short palindromic repeats).[1,2] This system mainly involves a genomic locus referred as CRISPR that has array of repeats separated by unique 'spacers' sequences derived from bacteriophages, and Cas proteins encoded by end sequence. The short 'spacer' regions of infecting elements that are integrated in bacterial genome are expressed as guide

RNA. So, during re-occurring infection, Cas enzyme utilize CRISPR repeats to deploys guide RNA targeting specific-sequence and terminate the phage genetic material.[3,4] This way CRISPR system play crucial role in antiphage defense mechanism of prokaryotes, by providing a kind of protection. These observations within the bacterial system gained enormous attention in the scientific research for its adaptive nature as well as for its biomedical applications.

Since the discovery (CRISPR), researchers are exploring this mechanism to edit genetic information's that is significant for understanding biological processes. Globally, scientists are artificially manipulating CRISPR by reprogramming endonuclease Cas9 proteins with guide RNA to the target genes.[5] The basic principle behind editing includes a guide RNA and Cas9 protein, which identifies particular bit of DNA within the cell genome and creates a gap in the strand. Further, these gaps are repaired by body via non-homologous end joining (NHEJ) or homology-directed repair (HDR), leading to mutations that usually cause gene to be disabled.[6] This editing approach is demonstrated to be beneficial and is growing exponentially as powerful tool for genome perturbation. It has opened plethora of applications spanning from unravelling novel gene functions and biological pathways to developing advanced therapies for genetic disorders.

However, before CRISPR-Cas system, researchers looked upon two other editing approaches that use restriction enzymes are, zinc finger nucleases (ZFN) and transcription-activator like effector nucleases (TALEN).[7] The ZFN technique involves artificial enzyme formed by fusing zinc finger DNA-binding site to cleavage-region of restriction endonuclease enzyme. This allows the ZFN to bind specific sequence and cleave it off which in turn provokes repair process leading to modifications. TALENs also constitute similar structure and mechanism but these tend to have more potential than ZFN.[6,7] But both methods include various challenges such as high cost, more time consumption and difficulties in engineering proteins. Thus, scientists explored techniques to engineer biological system, that is effective and reliable as well as hold enormous benefits across biotechnology and medicine. One such breakthrough was CRISPR which subsequently has revolutionized bioscience. So, this review intends to provide a comprehensive understanding on mechanisms involved in CRISPR system, current applications of CRISPR-Cas across different fields, and summarizing the future prospects along with boundaries of this technology.

2. CRISPR-CAS SYSTEM

2.1 History and evolution

Looking into the history, in 1987, a researcher Ishino and his group from Japan serendipitously recognized short repetitive sequences in E coli while identifying certain genes liable for conversion of ALP isozyme.[8] But they did not unveil any of its biological significance. Later in 1990, corresponding sequences were found in other prokaryotic species by Francisco Mojica and he coined the term CRISPR. Following this, the significant functions of short sequences in immunity mechanism were proposed but the nature of that importance was explained recently. There are various independent studies conducted to show the connection between spacer sequence origin with bacterio-phage and observed the functions of CRISPR associated with Cas as a system. After 2011, there was continuous bloom regarding CRISPR across the world and the potential ability of this system was declared as breakthrough in 2015.[9] However, in 2020, CRISPR sequences along with Cas protein role was elucidated in detail by two scientists Jennifer Doudna and Emmanuelle Charpentier, who were awarded with Nobel prize.[9,10]

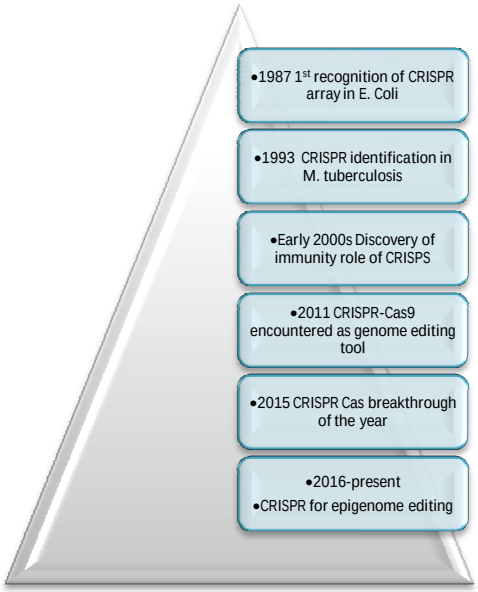

Figure 1: Milestones of CRISPR-Cas system

2.2 Structure and classification of CRISPR

The CRISPR system is mainly categorized into two classes depending on organization pattern and Cas genes. The Class 1 CRISPR system targets nucleic acid mainly by employing effectors with multiple subunits (proteins) and is further subdivided into I, III, IV type based on structure and function of Cas protein, whereas Class 2 CRISPR system comprise effectors with single protein and sub-divisions include type II, V, VI.[11,12] Altogether, six types of CRISPR systems with at least 29 sub-types are reported till now and list is growing rapidly. Among all, Class 2 type II comprising cas9 nuclease and guide RNA is widely applied in genome engineering because of its simplicity and accuracy in creating DNA double strand break.[11] Generally, this cas9 nuclease is extracted from *streptococcus pyrogenes* (*spycas9*) but it's use is limited due to large amino acid sequence which can cause high off target events. Thus, leading to alternative candidates for Cas protein, where cas9 from *Neisseria meningitidis* (*Nmecas9*) has gained interest over time because of its smaller amino acid chain and also exhibit extreme target-specificity perhaps due to longer PAM (protospacer adjacent motif).[11,13] These properties have facilitated NmeCas9 as potential tool for gene editing.

2.3 Functions of CRISPR system

The CRISPR Cas system was recognized to follow a specific sequence to defend invading viruses in bacteria. The functional stages of this immune mechanism are divided into three steps: adaption, expression and target-interference.

Adaption is the first stage of the mechanism, where a short sequence of the invading virus termed as PAM is inserted into the CRISPR sequence. This process allows the host to remember the genetic information of the intruders and indicates the adaptive nature of the system. The entire process of acquiring spacer is not completely understood. However, some recent studies have demonstrated the mechanism of PAM integration. It has been described that two proteins Cas1 and 2 of *E. coli*, tends to form a hetero-hexameric complex which supports the incorporation of spacers in the CRISPR array. These two proteins, Cas1 and Cas 2 which are found in all CRISPR system except III and IV.[14,15]

Expression is considered as the second stage of the immune mechanism and it's also termed as biogenesis. In this process, the CRISPR sequence undergoes transcription and results in long pre-crRNA (precursor CRISPR RNA). The crRNA is further processed by cleaving it into shorter and mature guide-crRNA that containing PAM viral recognizing sequences.[1] In addition, there are various enzymes involved in this trimming process, which differ with classes of CRISPR system.

Interference is third stage, where mature CRISPR-RNA are employed as guide to precisely interfere with external genetic element. This stage involves the formation of multi-protein or single effector protein complex. Usually, for class I system, multi-protein complex consisting of several Cas proteins and RNA is used to attain target degradation. Whereas Class II system employs only a single protein (Cas with

guide RNA), which is efficient enough for interference.[1,15]

2.4 Mechanisms to repair double stranded breaks

Generally, there are two pathways namely NHEJ (Non homologous end joining) and HDR (homology directed repair), which facilitate this process of repairing cleavage sites generated by Cas enzyme in CRISPR system.[3] NHEJ is a most active mechanism present in all the stages of cell cycle. This pathway promotes repairing of the strand by directly joining broken-ends of DNA via enzymes without any need of homologous DNA template. It is most pre-dominant cellular mechanism but then it has high error rates which results in random insertions or deletions at the gap region leading to pre-mature stop codons or framing error. On other hand, HRD is mostly active in two stages of cell cycle, S and G2 phase. This pathway repairs the DNA strand by referring to donor template strand with sequence of interest.[16] This is highly accurate and need huge amount of donor strand. HRD can insert or replace specific gene by adding template at predicted cleavage region.

3. DIFFERENT METHODS OF UTILISING CRISPR-CAS AS EDITING TOOL

The CRISPR-Cas approach is emerging as powerful tool to edit specific gene within genome of an organism. There are multiple ways this system can be effectively implemented such as gene knockout, knock-in, CRISPR-modulation, base and prime editing.

3.1. Gene knock-out and knock-in

In the CRISPR technique, Cas enzyme generates a double strand break which is mostly mended by non-homologous end joining mechanism. But this is error prone and results in indels (deletions) or insertions in the repaired sequences. These changes within the coding regions of gene sequence leads to frame-shift mutation, which eventually cause functional loss. This process of disabling gene is termed as knockout.[17]

On other hand, cells are capable of repairing the double stranded break themselves through homology directed repair. This pathway allows an individual to insert a specific gene sequence into the repaired position. The process is called as gene knock in.[18] However, for this a donor DNA strand is necessary as template and should contain the GOI with regions that are complimentary to the area on both side of cut. Then this DNA along with Cas protein and gRNA is delivered to the modified cells. Besides, gene knock-in is more challenging than gene knockout. This is due to repairing pathways, HRD is less common compared to NHEJ as it occurs at certain stages of cell cycle. As a result of less frequency of HRD, efficiency of knock in process is lowered. [18,19] However, researchers are trying to address this obstacle including experimental optimization and treatments that accelerate HDR pathways for knock-in. Gene knock-in are considered as breakthrough in biotechnology especially for generation of recombinant proteins, improving the viability of immortal cells and accurate disease modelling. In addition, this approach can be useful in cell and gene therapies to correct genetic mutations leading to human disorders.

3.2 CRISPR modulation - CRISPRa and CRISPRi

It is already known that CRISPR Cas can either remove or introduce specific sequences but with miniscule of alterations, it is possible to expand its use to control gene expressions. This method is termed as CRISPR-activation (CRISPRa) and CRISPR-interference (CRISPRi). The mechanism of these approaches mainly involves attachment of a modified Cas variant which is catalytically-dead Cas protein to transcriptional effectors and regulate targeted expression of gene. Here,

the Cas protein is deactivated and thus cannot create gaps in the DNA sequences even after binding to target region. The CRISPRa approach can enhance the gene expression, whereas CRISPRi can down-regulate it.[19,20]

3.3 Base editing and Prime editing

The base and prime editing are recently developed novel CRISPR methods. The principle behind both editing tools remains same, which is enabling programmable nucleotide substitution at precise scale without any need of donor DNA strand.
The base editing method involves two main key molecules, catalytically inactive Cas enzyme and DNA modifiable enzyme for specific substitutions. At present, two categories of base editors are developed which are cytosine and adenine base editors that are capable of inducing various alterations.[21] In base editing, either inactive-Cas 9, which cannot cut DNA or nCas9 (nickase cas9) that create nicks or single strand break are used and any one is fused with modifying enzyme to produce required nucleotide substitution. However, one of main drawback of this editing technology is that it cannot change all nucleotides and thus lead to the development of prime editing.[22]

Whereas prime editing utilizes nickase Cas9 attached to an engineered reverse transcriptase and a prime-editing guide RNA (peg RNA). This RNA is different from conventional CRISPR guide RNA, and it consist of two sequence, one that guides to targeted region of gene and other section has required change for repairing after single strand breaks are produced.[22] So, when prime editor modifies one strand then subsequently, the complimentary strand can be revised by utilizing previously altered strand as template. In addition, prime editing has lower rates of error and can create all possible point mutations.[22,23] However, both techniques can be used to insert, delete, or substitute nucleotide sequence and are gaining attention for treating genetic mutations.

4. APPLICATIONS OF CRISPR

The programable CRISPR-Cas system is gaining enormous scope as powerful gene editing tool and has inspired myriad of potential applications. Few applications of CRISPR system are described below.

4.1 Developing disease models

This editing system has facilitated rapid as well as affordable production of disease cell and animal models compared to conventional gene targeting approaches. Stem cell research is one of the fastest emerging fields in biology and CRISPR is revolutionizing this science. Generally, stem cells are specialized cells present in humans from the early developmental stage and through adulthood. These are extraordinarily pluripotent which means they can proliferate and differentiate into any other form of body cells. Due this incredible ability, they are considered as exceptionally valuable in medicinal research.[24] But use of embryonic stem cells is still controversial and thus lead to the development of iPSCs (induced-pluripotent stem cells). Interestingly, iPSCs are adult body cells for example skin or epithelial cells which are reprogrammed back to pluripotent stage, and they do not involve ethical concerns as embryonic stem cells. But it is difficult to genetically modify them, and such obstacles can be resolved by CRISPR. Various studies have demonstrated efficient results after engineering iPSCs using CRISPR in contrast to old editing techniques.[24,25]

Recently, researchers differentiated blood derived iPSCs into cardiomyocytes and induced specific mutations to study cardiac diseases.[26] Similarly, next advanced version of models are organoids, are also developed using CRISPR edited iPSCs. Organoids are 3D structures representing *in vivo* organs and provide more relevant environment to study disease than cell cultures.[27] These models are beneficial as they resemble complex nature of organs and can better mimic the disease progression as well as to drug treatment. In addition, iPSCs are continuously used to

create brain, liver, and other human organoids to study their evolution. [24,27]

At the same time, CRISPR method has improved the efficiency in primary cells for example synchronization of cell-cycle, developing and optimizing delivery complexes depending on cell type. All these developments are leading towards advanced cell therapies.[28]

4.2 Diagnostics

The CRISPR tool is employed in developing diagnostic tools that can improve the accuracy of detecting the diseases. During COVID-19 pandemic, various rapid viral detection tools are manufactured based on CRISPR Cas system. For example, CRISPR SARS-CoV-2 kit by Sherlock Science, DETECTR by mammoth Bioscience and another recently released STOPCOVID by Sherlock.[29] They main principle behind them includes use of Cas enzymes (nucleases) such as Cas12 and Cas13 to identify the viral genetic element within the patient derived DNA and readout are revealed via plate-readers. Furthermore, these applications of Cas are expanding to detect other infectious or genetic conditions. In 2021, a scientist namely Dr Kiana Aran published an approach to identify SNPs (single nucleotide polymorphism) that are major disease-causing mutations, by combining graphene and transistors along with CRISPR into small chip.[29,30] This study is one of the major breakthroughs in biomedical field.

4.3 Bioenergy

This is renewable energy which is usually produced using natural biomass which could be agriculture, animals, or human waste. Generally, the biomass which is typically an organic matter contains energy and when burnt, releases it in the form of heat. Bioenergy is one the enormously researched concept and an alternative to conventional fuels. But there are various obstacles while manufacturing biofuels at larger scale and it's exciting to know how CRISPR can be used to address these problems. Recently, researchers used knock out strategy to inhibit several transcription factors involved in regulating production of lipids in algae.[31,32] This resulted in high generation of lipids for manufacturing bio diesel at industrial level. Likewise, few reports have shown to enhance the tolerance nature of yeast under harsh environment during generation of biofuels.[32] It is important to protect yeast as it plays crucial role for fermenting sugars to biofuel. In addition, the efficiency of ethanol production via bacterial species have been demonstrated to increase by employing CRISPR editing.[31,33]

4.4 Agriculture

As the global population of humans is enormously growing, the scarcity of agricultural resources tends to rise. Thus, new ideas and techniques are required to increase natural-food production. Here, CRISPR-Cas which is an existing system, is being used to modify crops and foods to enhance their various properties such as nutritional values, shelf-life, disease resistance and drought-tolerance.[8,34] Overall, CRISPR can help to solve food crisis in three common ways. This can be by restoring food supply, assisting plants to sustain harsh environment, and improving plants health. In addition, this editing method can reduce food waste as well as allow people to get benefits from healthy food at affordable cost.[35] Some examples of revolutionizing CRISPR edited crops include ground cherry tomato (*physalis pruinosa*) by Lemmon and team from USA, modified domestic characters such as plant size, fruit size and fruit number.[36] Researchers from Berkeley along with Mars, Inc are developing disease resistant cacao plants and similarly mushrooms, rice and gluten free wheat are being researched.[35,36] Most of these crops are on the verge of entering commercial market.

4.5 Optogenetics

Researchers are working on improving precision of CRISPR approach by

conditional activation of Cas protein function using external factors such as temperature or chemical triggers, which has been reported for cas9 protein.[37] At the same time, another field namely 'optogenetics' is gaining attention for its potential to spatially control cellular functions by applying light. Here, it combines optics and genetics to remarkably turn 'on and off' individual cells or regions within them. Notably, in 2015 a group of researchers combined optogenetics method with CRISPR Cas and generated light-mediated gene activation tool.[38] A ground-breaking tool that facilitates fine tuning of genes to control specific behaviours by switching them on and off.

This combinational approach could accelerate development of advanced delivery systems and one such noteworthy instance is optogenetic plasmids which express proteins such as opsin, controlled by light. Other two recent remarkable efficacies include silencing or activation of gene transcription in different types of microbes as well as plants with high precision.[39,40] Another one is light-induced neuronal differentiation of human iPSCs in culture. In addition, apart from applications in basic research, optogenetic CRISPR encourages spatially confined remedy for malignancies.[37] However, the field is continuously expanding, and more excited innovations are awaited. The simplicity and flexibility of photoactivable CRISPR system has generated enthusiasm for broad spectrum of research with promise to cure variety of human defects.

4.6 Therapeutics

CRISPR editing strategy has revolutionised bioscience with its ability to correct a broad spectrum of genetic conditions such as cancer, blood diseases (sickle cell, thalassemia), neuro degenerative disorders and even ocular related condition.[41]

At present, around 6000 genetic mutations leading to disease are known. But for majority of them, there is no specific cure or treatment.[41] For such condition, gene therapy is explored, which is a process of altering a mutated gene at its original position and replacing the flawed gene using an exogenous nucleic acid sequence. From the time of CRISPR discovery, it is being studied to cure various genetic diseases like sickle cell (SCD), muscular dystrophy, cystic fibrosis (CF), and thalassemia.[42] The first clinical trial of CRISPR-Cas was carried out in 2019 for treating sickle cell patients. The results were promising as they were able to restore haemoglobin. Generally, SCD is a genetic disease related to red blood cells, which is caused due to point mutation in globin chain resulting into sickle haemoglobin. This is autosomal recessive and involves complications such as haemolytic anaemia. So, for this disease, CRISPR is applied through two different ways: one by directly repairing the sequences of haemoglobin S or by boosting gamma-globin.[41,43]

Further, researchers are investigating CRISPR approach to treat cystic fibrosis. A genetic mutation has been recognized in CFTR (cystic fibrosis transmembrane conductance regulator) gene, which is known to disturb structural integrity as well as functions of the encoding protein, eventually resulting in diseased condition.[44] At present there is no cure for this disorder, but certain modulating drugs are available for symptoms and to reduce complications. Interestingly, in 2013 scientists were able to correct CFTR mutation in intestinal stem cells obtained from CF patients and results showed normal protein expression.[44,45] Since then, CRISPR-Cas has been explored for its potential application as gene therapy to treat cystic fibrosis.

Another notable application is cancer, a disease involving high mortality due to marginal interventions and prevention strategies.[46] Over years, researchers have gained better understanding of tumour biology and mechanism involved in cancer progression. With this, CRISPR-Cas is also explored to modify T-cells, which are immune cells that are capable of targeting

any infections. Based on this concept, CRISPR is used to develop an immunotherapy involving production of CAR T cells (Chimeric antigen receptor cells) to target cancer.[47,48] The mechanism behind this is T cells obtained from affected individuals are modified to express chimeric-antigen receptors and reinjected back into patient. These receptors are expected to improve the activity of T cells to efficiently identify and fight against specific cancer cells in patients. The results showed no side-effects and the presence of engineered cells post-infusion were detected up to 9 months.[49] However, the clinical trials are going on and more study is required. CRISPR is emerging rapidly in this field for its potential not only to dissect disease process but also to identify targets for drug development and help in cell therapies.[47,49] Some of the clinical trials are listed in table1.

Table 1: List of registered clinical trials based on CRISPR gene-editing technique[41,50]

Registered number	Disease type	Target-gene and consequence	Interventions	Clinical phase	Country
NCT03745287	Sickle-cell disease	Disrupting erythroid enhancer and haemoglobin regulator genes	Engineered hematopoietic stem cells	Phase II, III	USA, Canada, France
NCT03655678	β-Thalassemia	Disrupting erythroid enhancer to haemoglobin genes.	Engineered hematopoietic stem cells	Phase I (Early stage)	USA, Canada, France
NCT04819841	Sickle cell disease	Correction of Beta-globulin gene	Haematopoietic stem cells (altered ex-vivo)	Phase I, II	USA
NCT03398967	B cell leukaemia and lymphoma	Generation of Cas mediated CAR-T cells	CAR-T cells (CD19, 20 or CD22)	Phase I, II	China
NCT04557436	B-cell leukemia	Modification of CD52 and TRAC	Revised CAR-T cells and Crispr modified TRAC (loci)	Phase I	United Kingdom
NCT05144386	HIV	Targeting HIV DNA (genome)	Removing viral genome	Phase I	USA
NCT05210530	Diabetes (Type-1)	CRISPR engineered allogenic-pancreatic cells	Allogenic beta-cell replacement therapy	Phase I	Canada
NCT04601051	Amyloidosis	Disrupting TTR	Disrupting genes (in vivo)	Phase I	UK, NZ, Sweden
NCT03690011	T-cell malignancy	Knocking out CD-7 in CAR-T cells	CAR-T cells	Phase I	USA

**[PD-1, programmed cell death protein 1; CAR, chimeric antigen receptor; TCR, T-cell receptor: HPV, human papillomavirus; TRAC, T-cell receptor a constant; HIV, human immunodeficiency virus; NZ, New Zealand]*

Furthermore, this technology would be useful to treat pathogenic diseases. One such area of research is HIV, a virus causing AIDS (Acquired immune-deficiency syndrome). Notably, in 2017 a group of scientists from Temple University showed the possibility to shut down the replication of HIV and eliminated virus from infected cells via excision of its genome using CRISPR within *in vivo* models.[51] Apart from targeting viral genome, researchers are also using CRISPR to block the viral entry by engineering certain chemokine genes in the host organism.

5. FUTURE PROSPECTS AND CONCLUSION

CRISPR-Cas technique is widely useful for modulating and engineering biological mechanisms. At present time, CRISPR has revolutionized biomedical research combined with cell and gene therapies. Nevertheless, its significant to address the drawbacks of a system to unbar its maximum capacity. In complex genomes, there are high possibilities that CRISPR can produce off-target effects as by-product. However, it's not limited to this, there are several questions such as spacer integration, link between adaptation and interference as well as evolution of CRISPR which are partially understood. Along this, other concepts which need further attention are CRISPR function in non-immunity process, counter measures for this system and its ecological functions. It is necessary to investigate all these aspects of the technique at higher extent and conduct clinical studies for better knowledge.

Furthermore, a CRISPR-Cas system with precise spatiotemporal control, lack of side effects, simple optimization, fast response and efficient *invitro* and *in vivo delivery* would provide ultimate benefits. New innovative techniques are being developed to increase CRISPR function such optogenetics as discussed in the paper. Along with this, more of these studies focus on mammals but variety of other species like plants and fungi would be beneficial for biotechnological applications. In addition, both Cas9 and guide RNA are being engineered to improve efficiency of CRISPR. It is possible to have tailor made Cas enzymes for large scale of genetic engineering domains. Overall, for its simplicity and flexibility, CRISPR-Cas is now routinely applied in research across the world and its popularity is exponentially growing. In future, more excited innovations are awaited and it's not far from encountering different medicinal treatments based on this technique.

REFERENCES

1. Hille F, Charpentier E. CRISPR-cas: Biology, mechanisms and relevance. Philosophical Transactions of the Royal Society B: Biological Sciences. 2016.
2. Gupta D, Bhattacharjee O, Mandal D, Sen MK, Dey D, Dasgupta A, et al. CRISPR-Cas9 system: A new-fangled dawn in gene editing. Life Sci. Elsevier Inc.; 2019.
3. Mei Y, Wang Y, Chen H, Sun ZS, Ju X Da. Recent Progress in CRISPR/Cas9 Technology. Journal of Genetics and Genomics. Elsevier; 2016. p. 63–75.
4. Rath D, Amlinger L, Rath A, Lundgren M. The CRISPR-Cas immune system: Biology, mechanisms and applications. Biochimie. Elsevier; 2015; 117:119–28.
5. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013; 8:2281–308.
6. Moon S Bin, Kim DY, Ko JH, Kim YS. Recent advances in the CRISPR genome editing tool set. Exp Mol Med. Springer Nature; 2019;51.
7. Gaj T, Sirk SJ, Shui SL, Liu J. Genome-Editing Technologies: Principles and Applications. Cold Spring Harb Perspect Biol. Cold Spring Harbor Laboratory Press; 2016;8.

8. Mengstie MA, Wondimu BZ. Mechanism and Applications of CRISPR/ Cas-9-Mediated Genome Editing. 2021;
9. Kozovska Z, Rajcaniova S, Munteanu P, Dzacovska S, Demkova L. CRISPR: History and perspectives to the future. Biomedicine and Pharmacotherapy. Elsevier Masson s.r.l.; 2021;141.
10. Han W, She Q. CRISPR History: Discovery, Characterization, and Prosperity. Prog Mol Biol Transl Sci. Academic Press; 2017; 152:1–21.
11. Makarova KS, Haft DH, Barrangou R, J Brouns SJ, Charpentier E, Horvath P, et al. Evolution and classification of the CRISPR–Cas systems. 2011.
12. Koonin E V., Makarova KS, Zhang F. Diversity, classification and evolution of CRISPR-Cas systems. Curr Opin Microbiol. Elsevier Current Trends; 2017; 37:67–78.
13. Makarova KS, Wolf YI, Koonin E V. Classification and Nomenclature of CRISPR-Cas Systems: Where from Here? CRISPR J. Mary Ann Liebert Inc; 2018; 1:325–36.
14. Horvath P, Barrangou R. CRISPR/Cas, the immune system of Bacteria and Archaea. Science (1979). American Association for the Advancement of Science; 2010; 327:167–70.
15. Nussenzweig PM, Marraffini LA. Molecular Mechanisms of CRISPR-Cas Immunity in Bacteria. Annu Rev Genet. Annual Reviews Inc.; 2020; 54:93–120.
16. Yang H, Ren S, Yu S, Pan H, Li T, Ge S, et al. Methods Favoring Homology-Directed Repair Choice in Response to CRISPR/Cas9 Induced-Double Strand Breaks. Int J Mol Sci. Int J Mol Sci; 2020; 21:1–20.
17. Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, et al. An updated evolutionary classification of CRISPR–Cas systems. 2015;
18. Mohr SE, Hu Y, Ewen-Campen B, Housden BE, Viswanatha R, Perrimon N. CRISPR guide RNA design for research applications. FEBS Journal. Blackwell Publishing Ltd; 2016. p. 3232–8.
19. Anton T, Karg E, Bultmann S. Applications of the CRISPR/Cas system beyond gene editing. Biol Methods Protoc [Internet]. Oxford University Press; 2018 [cited 2022 Oct 6];3. Available from: /pmc/articles/PMC6994046/
20. Liu Z, Dong H, Cui Y, Cong L, Zhang D. Application of different types of CRISPR/Cas-based systems in bacteria. Microbial Cell Factories 2020 19:1. BioMed Central; 2020;19:1–14.
21. Antoniou P, Miccio A, Brusson M. Base and Prime Editing Technologies for Blood Disorders. Front Genome Ed. Frontiers; 2021; 0:1.
22. Cohen J. New 'prime' genome editor could surpass CRISPR. Science (1979). American Association for the Advancement of Science (AAAS); 2019;
23. Anzalone A V, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. 2019; 576:149.
24. Yang G, Huang X. Methods and applications of CRISPR/Cas system for genome editing in stem cells. Cell Regeneration. KeAi Communications Co.; 2019. p. 33–41.
25. Waddington SN, Privolizzi R, Karda R, O'Neill HC. A Broad Overview and Review of CRISPR-Cas Technology and Stem Cells. Curr Stem Cell Rep. 2016. p. 9–20.
26. Lin H, McBride KL, Garg V, Zhao MT. Decoding Genetics of Congenital Heart Disease Using Patient-Derived Induced Pluripotent Stem Cells (iPSCs). Front Cell Dev Biol. Frontiers Media S.A.; 2021;9.
27. Bai X. Stem Cell-Based Disease Modeling and Cell Therapy.
28. Li H, Yang Y, Hong W, Huang M, Wu M, Zhao X. Applications of genome editing technology in the targeted therapy of human diseases:

mechanisms, advances and prospects.
29. 1∗ SE, Khanbabaei H, Abbasi S, Fani M, Soltani S, Zandi M, et al. CRISPR-Cas System: A Promising Diagnostic Tool for Covid-19. Avicenna J Med Biotechnol. 14:3–9.
30. Berber B, Aydin C, Kocabas F, Guney-Esken G, Yilancioglu K, Karadag-Alpaslan M, et al. Gene editing and RNAi approaches for COVID-19 diagnostics and therapeutics. Gene Therapy 2020 28:6. Nature Publishing Group; 2020;28:290–305.
31. Javed MR, Noman M, Shahid M, Ahmed T, Khurshid M, Rashid MH, et al. Current situation of biofuel production and its enhancement by CRISPR/Cas9-mediated genome engineering of microbial cells. Microbiol Res. Microbiol Res; 2019; 219:1–11.
32. Umar Ibrahim A. Application of Crispr Technology for the Generation of Biofuels: A Review. Journal of Fundamentals of Renewable Energy and Applications Article in Journal of Fundamentals of Renewable Energy and Applications · [Internet]. 2019 [cited 2022 Oct 8]; Available from: https://www.longdom.org/open-access/application-of-crispr-technology-for-the-generation-of-biofuels-a-review.pdf
33. Estrela R, Cate JHD. Energy biotechnology in the CRISPR-Cas9 era. Curr Opin Biotechnol. Elsevier Ltd; 2016. p. 79–84.
34. Hsu PD, Lander ES, Zhang F. Development and Applications of CRISPR-Cas9 for Genome Engineering. Cell. NIH Public Access; 2014; 157:1262.
35. Zhu H, Li C, Gao C. Applications of CRISPR–Cas in agriculture and plant biotechnology. Nat Rev Mol Cell Biol. Nature Research; 2020. p. 661–77.
36. Zaidi SS e. A, Mahas A, Vanderschuren H, Mahfouz MM. Engineering crops of the future: CRISPR approaches to develop climate-resilient and disease-resistant plants. Genome Biology 2020 21:1. BioMed Central; 2020;21:1–19.
37. Hemphill J, Borchardt EK, Brown K, Asokan A, Deiters A. Optical control of CRISPR/Cas9 gene editing. J Am Chem Soc. American Chemical Society; 2015; 137:5642–5.
38. Hoffmann MD, Mathony J, Upmeier Zu Belzen J, Harteveld Z, Aschenbrenner S, Stengl C, et al. Optogenetic control of Neisseria meningitidis Cas9 genome editing using an engineered, light-switchable anti-CRISPR protein. Nucleic Acids Res. Oxford University Press; 2021; 49:1–11.
39. Nihongaki Y, Kawano F, Nakajima T, Sato M. Photoactivatable CRISPR-Cas9 for optogenetic genome editing. Nat Biotechnol. Nature Publishing Group; 2015; 33:755–60.
40. Polstein LR, Gersbach CA. A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. Nat Chem Biol. Nature Publishing Group; 2015; 11:198–200.
41. Tavakoli K, Pour-Aboughadareh A, Kianersi F, Poczai P, Etminan A, Shooshtari L. Applications of CRISPR-Cas9 as an Advanced Genome Editing System in Life Sciences. BioTech 2021, Vol 10, Page 14. Multidisciplinary Digital Publishing Institute; 2021; 10:14.
42. Demirci S, Leonard A, Haro-Mora JJ, Uchida N, Tisdale JF. CRISPR/Cas9 for Sickle Cell Disease: Applications, Future Possibilities, and Challenges. Adv Exp Med Biol. Springer New York LLC; 2019. p. 37–52.
43. Frangoul H, Altshuler D, Cappellini MD, Chen Y-S, Domm J, Eustace BK, et al. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. New England Journal of Medicine. 2021; 384:252–60.
44. Pranke I, Golec A, Hinzpeter A, Edelman A, Sermet-Gaudelus I. Emerging Therapeutic Approaches for Cystic Fibrosis. From Gene Editing to Personalized Medicine. Front Pharmacol. Frontiers Media SA; 2019;10.

45. Bergeron C, Cantin AM. New Therapies to Correct the Cystic Fibrosis Basic Defect. Int J Mol Sci. Int J Mol Sci; 2021;22.
46. Sánchez-Rivera FJ, Jacks T. Applications of the CRISPR-Cas9 system in cancer biology. Nat Rev Cancer. Nature Publishing Group; 2015; 15:387–95.
47. Zhan T, Rindtorff N, Betge J, Ebert MP, Boutros M. CRISPR/Cas9 for cancer research and therapy. Semin Cancer Biol. Semin Cancer Biol; 2019; 55:106–19.
48. Yin H, Xue W, Anderson DG. CRISPR–Cas: a tool for cancer research and therapeutics. Nat Rev Clin Oncol. Nature Publishing Group; 2019. p. 281–95.
49. Stadtmauer EA, Fraietta JA, Davis MM, Cohen AD, Weber KL, Lancaster E, et al. CRISPR-engineered T cells in patients with refractory cancer. Science (1979). American Association for the Advancement of Science; 2020;367.
50. Anliker B, Childs L, Rau J, Renner M, Schüle S, Schuessler-Lenz M, et al. Regulatory Considerations for Clinical Trial Applications with CRISPR-Based Medicinal Products. NY 10801 USA; 2022; 5:364–76.
51. Strich JR, Chertow DS. CRISPR-Cas Biology and Its Application to Infectious Diseases. J Clin Microbiol. J Clin Microbiol; 2019;57.