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CRISPR/Cas9: Genome Editing Applications, Ethical Concerns and Future Prospects

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Abstract: **The research applications associated with technological advances in genome editing based on clustered regularly interspaced short palindromic repeats (CRISPR) - associated proteins (Cas 9) system have revolutionized a variety of biological fields. Since it could target any genomic locus of interest by the ease principle of designing guide RNA with high specificity, versatility, and efficiency to introduce DNA double-stranded breaks as molecular scissors in genome have led to exciting applications in biological investigations. Studies on genetic disorders have recently used CRISPR/Cas9 to identify, modulate and correct, or delete the disease-causing alleles in vivo in animal models, or in vitro in somatic cells or in induced pluripotent stem cells, and provided potential therapeutic strategies using CRISPR/Cas9-based system in many different fields in biology, biotechnology and medicine. In this review, we will summarize the molecular basis of genome editing technology, and discuss the applications of CRISPR/Cas9 system in areas ranging from the Cas9-based treatment for genetic disorders, promoting resistance to infectious disease, combating cancer through research and treatment, optimizing the food production by creating infection-resistant crops in agriculture, enhancing biofuel resources, developing stem cell therapy for donor-specific tissues for transplantation, and bioengineering of new generation of drug development processes and medical therapeutics. Moreover, we have presented an account on the recent ethical concerns raised with application of this technology in human germline editing and the future perspectives associated with the wise usage of this remarkable genome editing technology.**

Keywords: **CRISPR/ Cas9; Genome editing; Guide RNA; Cas endonucleases; DNA; biotechnology; Genetic engineering; Human diseases.**

I. INTRODUCTION

The development of recombinant DNA technology almost five decades ago have marked the beginning of a new era by enabling the molecular biologists to manipulate DNA molecules, and to study genes to develop novel molecules and drugs through biotechnology [1]. Recent advances in genome engineering technologies are sparking a new revolution in biological research. With the advancement of new genetic engineering technology, researchers can now directly edit or modulate the function of DNA sequences in their endogenous context in virtually any organism of choice, rather than studying DNA taken out of the context of the genome [2]. Moreover, such advanced genome editing technologies enable them to elucidate the functional organization of the genome at the systems level, as well as identify causal genetic variations. Among the currently available genome editing technologies, a microbial adaptive immune system, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) using the RNA-guided endonucleases known as Cas9 has emerged as a powerful technology for genome editing and is now widely used in basic biomedical research to explore gene functions [3].

Since its first discovery by Ishino *et al.* [4] in 1987 within the genome of the bacteria *Escherichia coli*, these unusual DNA repeats of unknown function has been identified in many other microbes and termed as Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR by Mojica *et al.* in 2000 [5]. Soon, these sequences were found to be

involved in adaptive immune response in bacteria by resisting the invading viral pathogens by cutting their DNA with Cas nucleases **(Fig. 1)**. Due to the unique property of this pathogen-specific Cas endonuclease enzyme, which require an RNA guide sequence for both activating and selectively targeting the nuclease to complementary DNA sequences, it has been applied as a high-fidelity nuclease to produce DNA nicks at any location desired in the genomic DNA *in vivo* [3]. Among many Cas endonucleases, Cas9 from E. coli has become the most extensively studied and widely used endonuclease in basic research. Eventually, the CRISPR/Cas9 RNA-guided DNA editing technology has been exploited in a rapidly growing number of basic science experimental studies involving mammalian and invertebrate systems [6-9].

For the last few years, CRISPR/Cas9 technology was implemented as most efficient and advanced genome editing tool for various applications in Basic biology to Biotechnology and Medicine. This novel genome editing technology was used to unravel a variety of research or translational applications in clinical and therapeutic medicine **(Table 1)**. More recently, CRISPR/Cas9 technology has been increasingly applied to the study or treatment of many genetic disorders in humans, including Barth syndrome effects on the heart, Duchenne muscular dystrophy, Hemophilia, Thalassemia, and Cystic fibrosis [10]. This novel genome editing system has been used to correct disease-causing DNA mutations ranging from a single base pair to large deletions in a wide variety of animal and cell models, and to effectively implement them in treating human diseases. More importantly, it has also been applied in immunology-focused applications such as targeting CCR5 receptors of T cells for blocking the HIV infection [11, 1], and in manipulating the programmed death (PD-1) gene or creating chimeric antigen receptors in T cells for promoting anti-tumor immunotherapy [12, 13].

Furthermore, the use of this technology has been extended for the goal of generation of synthetic materials and renewable energy resource and for producing biologic medical materials, including molecules, cells or organs, on a large scale [3]. In biotechnology, precise manipulation of genetic building blocks and regulatory machinery also facilitates the reverse engineering or reconstruction of useful biological systems, for example, by enhancing biofuel production pathways in industrially relevant organisms or by creating infection-resistant crops in agriculture. Additionally, genome engineering is stimulating a new generation of drug development processes and medical therapeutics. Perturbation of multiple genes simultaneously could model the additive effects that underlie complex polygenic disorders, leading to new drug targets, while genome editing could directly correct harmful mutations in the context of human gene therapy [1]. Finally, CRISPR/Cas9 has been used as a platform technology in stem cell therapy after inducing pluripotent stem (iPS) cells to perform multiple tissue engineering tasks including the creation of disease models or the preparation of donor-specific tissues for transplantation [14, 15]. The wide application of this technology in different areas of studies are listed in TABLE 1.

In this review, we extensively discuss the development and applications of the CRISPR- associated endonuclease Cas9 as a broad technology for achieving targeted perturbation of endogenous genomic elements and also enlighten the challenges and future avenues for innovation.

II. REVIEW OF CRISPR/CAS9 IN GENOME ENGINEERING

A. CRISPR/Cas9 as Molecular Scissors in Genetic Engineering

CRISPR/Cas9 derived from RNA-guided adaptive immune system of bacteria and archaea against bacteriophages is a novel and highly efficient genome engineering tool that introduces alterations of the genetic code in molecular level. Introducing targeted modifications in the genome for functional studies and cancer modeling or for therapeutic purposes may require highly efficient and powerful systems with nucleases that are able to alter the existing DNA pattern with great precision.

Before CRISPR system, genomic editing tools that were commonly employed in genome editing were predominantly zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). However, engineering process is quite challenging for two reasons: 1) there are billions of the DNA bases in eukaryotic genomes, making it hard to target the specific site and manipulate; 2) ZNFs and TALENs are protein domain-based recognition tools to the targeted DNA sequence and require protein-domain modifications for specific recognition, thus giving it relatively lesser specificity and increased probability of off-target events, making it less efficient [16-18]. Designing suitable recognition domains are challenging and time-consuming. Due to these challenges, these tools are not applicable as much as expected, although the targeted genomic engineering have great potential to bring benefits upon biology and the society.

The CRISPR system surpasses other nuclease-based systems in terms of simplicity of design and versatility [18], and by 2013, the ability of the CRISPR/Cas9 system to engineer mammalian cell genomes has been experimentally validated, and the crystal structure of the Cas9 effector complex was resolved in 2014 [2] **(Fig. 1)**. Among the many CRISPR/Cas systems (I–VI) identified in both bacteria and archaea, the type II system from Streptococcus thermophilus or Streptococcus pyogenes is one among the versatile tool for genome engineering purposes [19].

During the immune response, with exposure to the invading genetic codes from the plasmids, short foreign DNA fragments are integrated into CRISPR repeat-spacer array as the new spacers, hence providing a genetic record for defensing the future invasion from the invader [20]. The subsequent transcription of CRISPR-array and the post transcriptional processing of the premature CRISPR transcripts *via* endonucleolytic cleavage yield the mature CRISPR RNAs (crRNAs). The repeat sequence in the crRNA, which is complementary to a strand of noncoding RNA known as the trans-activating RNA, pair with the tracrRNA via Watson-Crick base pairing to form a dual RNA structure, which can be synthesized artificially in the laboratory to function as a single-guided RNA (sgRNA) [21-23]. The assembly of the sgRNAs/dual-RNA and Cas9 enables this endonuclease to introduce a blunt-ended double-stranded DNA break (DSB) at specific site by directing Cas9 to the DNA sequence containing complementary target sequence and proto-spacer adjacent motif (PAM), triggering one of the two DNA repair mechanisms [24, 23]. One such mechanism is known as the non-homologous end joining (NHEJ) that results in random insertion or deletions of the DNA sequence (indels) usually associated with loss of function (knock-down/knock-out) [10]. The second one is Homology-directed repair, which occur in G2 phase of the cell cycle that allows precise genomic modification at which the DSB takes place using a single homologous DNA donor as the template resulting in gain of function (knock-in) [25]. These two mechanisms allow genetic editing with high specificity and efficiency **(Fig. 2)**. Unlike TALENs and ZFNs, DNA recognition of Cas9 is not specified by the protein instead by a programmable RNA sequence, giving a highly versatile genomic engineering platform that eliminate the need for tedious protein modifications of DNA-recognition domains [26].

Since the astonishing ability of CRISPR/Cas9 system to introduce site-specific DSBs is demonstrated in eukaryotic genomic editing, the toolbox of Cas9 is expanded to modify genomic sequences and also to introduce epigenetic and transcriptional modifications [6, 7]. This expanded CRISPR toolbox has been widely used in developing CRISPR-based therapies for genetic disorders, functional genomic screening via genome-wide modulation, generating animal models for cancer to identify cancer-specific vulnerabilities and studying mechanisms of the disease progression, and optimizing microorganisms and plant to improve yielding, or to augment human health. Further discussion is made on how the CRISPR system is applied in these fields, the ethical concerns regarding the use of this revolutionary genomic editing tool and also the future prospects of the CRISPR technology.

B. Applications of CRISPR/Cas9 in Cancer Biology

The remarkable versatility and efficiency of Cas9-mediated genomic editing enables a vast variety of applications in studying cancer and development of new drugs against cancers, including effective screening in functional cancer genomics for target discovery, examination of non-coding genome of cancer, modeling disease progression in animals *in vivo*, or producing engineered organoid to mimic tumor development *ex vivo* [27, 10].

The main goal of genetic screening practiced in clinical diagnosis is to identify cancer vulnerabilities. Some ‘essential’ genes can be druggable targets, as their depletion or loss of function could lead to cell death, providing a new approach for cancer treatment [28]. Screening of the genome in cancer models using CRISPR/Cas9 systems have greatly enabled the identification of these genes based on a concept known as ‘synthetic lethality’, which is a form of context-dependent gene essentiality [29]. To briefly explain, it is a principle that a cell is able to compensate the functional alterations of gene A or gene B, and the cell could not survive when the functional alterations of gene A and gene B occur simultaneously. In this way, if cells carry different sgRNAs cassettes, targeting genes to introduce alterations for each specific genomic sequence along with a group of cells that only carry the carriers acting as a control group may show a reduced viability, indicating that the gene specifically directed by the Cas9-sgRNA complex contained in cells is one of the potential drug targets. By relying this principle, some of the previously regarded non-essential genomic sequences are now considered as essential oncogenes or tumor suppressor genes, which could be potential drug targets in cancer therapeutics.

Another major application of CRISPR/Cas9 screening is the dissections of chemo-genetic interactions, revealing the insights of how cancer responds to drug treatments. This can reinforce the underlying mechanism of the apoptotic

pathways or of evasion from the immune recognition of the tumor cells. On treating the sample cells with sgRNAs targeting genes that confer drug resistance upon knockouts, an enhancement of cell growth can be noticed, while those targeting genes that gains susceptibilities to the drug will show a reduced viability. One study [30] underpins the possible mechanism that tumor response or resistance to a pro-survival protein MCL-1 inhibitor, S63845, using genome-wide and focused CRISPR/Cas9 screens in sample tumor cells. The authors confirmed that sgRNAs targeting BAK conferred resistance to S63845 (knockout of BAK alone does not confer resistance to S63845) and shows that this inhibitor works by disrupting MCL-1/BAK complexes. Kearney *et al.* have performed a genome-wide Cas9-based screening *in vitro* in two cell lines (MC38 colon adenocarcinoma and B16 melanoma cells) to uncover the genes and pathways regulating the sensitivity to T-cell mediated attack [31].

Recently, apart from the CRISPR-mediated screening, a system based on CRISPR-associated for non-coding RNA (ncRNAs) interrogation was developed, because the biological functions of most of the non-coding RNAs remain largely unknown. In cancer biology, expression of ncRNAs are dysregulated [32], which may exert an impact on a variety of cellular functions such as cell proliferation and evasion of tumor suppressors. Thus, a comprehensive understanding of ncRNAs may provide valuable insights into cancerous diseases. Cancer-associated regulators can be validated *via* CRISPR/Cas9-mediated deletion, functional rescue or CRISPR activation or interference which could block genes' functions by binding to the target site without introducing DSB.

Cancer modeling in animals, such as mice may provide more comprehensive understanding of the corresponding disease using CRISPR/Cas9-based targeted somatic multiplex-mutagenesis. Furthermore, mapping out a functional cancer genomic landscape in these models may enable the study of tumor progression and development *in vivo*. In addition, Cas9-induced organoid cancer model may open up new avenues to study various aspects of disease progression such as immune evasion, the genetic nature of cancer cells and its pathways conferring resistance to apoptosis and also for providing the necessary information on potential targets for future therapies. One study generated a mouse model with highly complex autochthonous liver tumors by intravenously injecting adeno-associated virus pools carrying 278 sgRNAs targeting a set of the most frequently mutated, known, or putative TSGs into Rosa-LSL-Cas9-EGFP knock-in mice *in vivo* [33]. Molecular inversion probe capture sequencing was performed to generate an illuminating mutational landscape of the tumor cells by direct readout of Cas9-generated variants at predicted sgRNA cut sites.

In another study, hepatocellular carcinoma and intrahepatic cholangiocarcinoma were induced *in vivo* in mice by Cas9-based somatic multiplex mutagenesis using hepatic sgRNAs delivery targeting large sets of genes [34]. This study demonstrated that animal models can be used in the characterization of tumor phenotypes at genetic levels, as these genes induced by CRISPR/Cas9 were successfully sequenced using next generation sequencing.

Genome editing using CRISPR/Cas9 holds promise for correcting pathogenic mutations, but off-target effects of this editing is difficult to determine due to single-nucleotide polymorphism in such individuals. Recently, a new method, GOTI (genome-wide off-target analysis by two-cell embryo injection) was introduced to detect off-target mutations by editing one blastomere of two-cell mouse embryos using either CRISPR-Cas9 or base editors, which showed that off-target single-nucleotide variants (SNVs) were rare in embryos edited by CRISPR-Cas9 or adenine base editor, with a frequency close to the spontaneous mutation rate [35].

C. CRISPR/Cas9 as a therapeutic tool in genetic disorders

A growing number of promising potential therapeutic strategies based on CRISPR system were developed after its powerful and versatile programmable RNA-guided editing ability were uncovered. Injection of CRISPR/Cas9 complex with sgRNAs *via* effective delivery methods can correct alleles in the genome of patients, by homology-directed repair (HDR) which uses a wild-type homologous DNA template, or NHEJ that creates indels [36]. Thus, this approach can pass on their offspring, achieving a permanent elimination of a particular type of genetic disease in the family. Min *et al.* used CRISPR/Cas9 system to create a patient-derived induced pluripotent stem cells (iPSCs) and a mouse model (DEx44) lacking exon 44 of the dystrophin gene that is responsible for stabilizing muscle membranes [37]. This study presented two strategies focused on the second most common mutational hotspot in Duchenne muscular dystrophin to restore the reading frame of dystrophin protein: i) skipping exon 43, which allows splicing between exon 42 and 45; ii) skipping exon 45, which allows splicing between exon 43 and 46, enabling the production of truncated dystrophin protein. In this study, the authors have intravenously injected the adeno-associated viruses 9 (AAV9) packaged with gene editing components: Streptococcus pyogenes Cas9 (spCas9) and sgRNAs into DEx44 mouse to correct the DMD exon 44 deletion.

No off-target cutting was detected in the top 10 potential off-target sites using T7E1 assays on the basis of CRIPSR design tools. In recent years, several studies in sickle cell diseases (SCD) also reported some potential CRISPR-based therapeutic strategies. One of these studies corrected the edited SCD cells (achieving up to 25% of alleles corrected to wild-type red blood cells at high dose of ribonuclease proteins) using ribonuclease Cas9 complex with sgRNAs via HDR *in vitro*, which conferred clinical benefits [38]. However, edited repopulating stem cells must engraft and repopulate within a recipient in order to manifest gene correction *in vivo*, where most of the cells are in G2 phase and the HDR pathway is active as they are in the checkpoint for DNA. Another study describing an approach for treating SCD and β-thalassemia used RNA-guided Staphylococcus aureus Cas9 nuclease (SaCas9) to delete 13kb of the β-globin locus to mimic the naturally occurring Sicilian HPFH mutation. This method conferred minimal hematological abnormalities and mild clinical manifestation via error-prone NHEJ pathway. T7 endonuclease 1 analysis showed that there were no off-target events in the edited cells. However, indels were found and it was up to 43%.

In a very recent study, the researchers used a Cas9 endonuclease along with bacterial recombinase A to increase homologous recombination to mediate HDR and then restored the expression of PDE6B in rod photoreceptor and enhanced the visual functions in a postnatal animal model carrying retinitis pigmentosa (RP) — rd1 mice [39]. They demonstrated that Cas9/RecA system efficiently enhanced HDR repair to improve photoreceptors viability, including postmitotic photoreceptors and Cas9/RecA system, and partially rescued the pupillary light reflex. Photoreceptors, which are sensory neurons for visual signal processing are rescued from its degenerations and regained their proliferation ability. It provided a precise therapeutic strategy for RP, and the evidence that Cas9/RecA system have the potential to be applicable to other genetic disorders in postnatal organisms including mammals as Cas9 introduced DSBs and RecA have shown increased rate of homologous recombination. This study ensured that the gene editing-based strategies could enhance safety and efficiency for genetic disorders.

D. CRISPR/Cas9 Genome Editing Tool in Promoting Resistance to Infectious Disease

The majority of currently used antibiotics act on microbes through either targeting DNA replication, synthesis of cell wall and protein synthesis, controlling pathogens and exerting a selective pressure to the colonies treated with these antibiotics, and thus confer resistance to these drugs. Several studies have reported that many different pathogens have acquired multiple drug resistance [40]. As a result, a growing need for new therapeutic strategies and explorations of new drug targets has emerged rapidly.

Recently, CRISPR/Cas9 endonuclease was put into use for studying mechanisms conferring drug resistance, identifying druggable genes and developing vaccines against infectious diseases. CRISPR-induced gene knockouts have greatly enhanced our versatility of strategies available in disease-associated genomic studies and treatments. A recent advancement in investigation of genes associated with susceptibility to human immunodeficiency (HIV) has been made using CRISPR/Cas9 system. Palstra *et al.* identified that rs4349147, an intergenic gene of HIV-1 located in a $CD4^+$ T cell-specific deoxyribonuclease 1 hypersensitive region (DNase I hypersensitive region) acting as a long-distance enhancer of Interleukin 32 (IL-32), a cytokine responsible for induction of cells of immune system to secrete inflammatory cytokines [41, 42]. Knockdown of this gene was achieved by the CRISPR/Cas9-mediated deletion of DNase I hypersensitive region containing rs4349147. This study also investigated isoforms of IL-32 derived from single nucleotide polymorphism alleles of rs4349147, which played distinct roles in immune systems [43]. They have found that the relative ratios of these IL-32 isoforms influences the susceptibility of primary $CD4^+$ T cells to HIV infections. The authors pushed the boundaries towards solving how exactly the resistance to HIV acquisition is evolved [42]. Moreover, a live attenuated cholera vaccine (HaitiV) based on 2010 Haitian cholera outbreak provided immunity to cholera in an infant model [44]. In particular, HaitiV encodes CRISPR system specifically targeting the toxin gene ctxA, which is the pathogen's principle virulence factor that encodes cholera toxin (CT) [45], providing rapid protection against cholera-like disease. In addition, a study conducted by Cowell *et al.* mapped the malaria parasites druggable genome *in vitro* [46], particularly, a Cas9-mediated gene insertion of the pfaat2, a gene encoding the amino acid transporter. Insertion of L903 stop codon into GNF179-sensitive Dd2 parasites confirmed that a stop mutation in pfaat2 with a splice acceptor intronic mutation in the gene encoding the acetyl coenzyme A transporter confers resistance to GNF179 on its own. GNF179 is one kind of imidazole-piperazine, an antimalarial drug [47]. These results demonstrated the applicability of CRISPR/Cas9 as a genomic editing tool in communicable disease-associated studies. CRISPR-based genome-wide screening and CRISPR-based gene knockouts have explored the role of disease-associated genes and genes that contribute to drug resistance.

As researchers are exploring the underlying principles of disease development and developing new vaccines to prevent, or new drugs to treat the infectious diseases, other groups of researchers started to focus on promoting resistance to those infectious diseases. The CRISPR/Cas9 system has been employed to promote the resistance against viral and bacterial diseases, followed by improving fungal resistance [48].

Recently, two strategies based on CRISPR/Cas9 was developed to defend viral infections. One of the strategies conferring resistance to virus infection made use of overexpression of CRISPR/Cas9 system in genome integrated with viral genome of the host cells, and thus target and cleave the viral genes during replication. Ali *et al.* developed sgRNAs targeting viral capsid protein, the RCRII motif of the replication protein and the intergenic region, which are specific for different coding and non-coding sequences of tomato yellow leaf curl virus. These sgRNAs caused a significant reduction of viral replication and accumulation [49]. This study provided evidence that the infection-immunity can be established *via* Cas9 endonuclease and sgRNAs, and the mechanism is similar to the natural indigenous adaptive immune system in bacteria. In addition, FnCas9 from *Francisella novicida*, which can bind to and cut RNA was reported to be defensing the invasion of RNA virus. Similar findings were also reported with FnCas9 and RNA-targeting sgRNAs specific for cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV) in *N. benthamiana* and Arabidopisis plants. CMV and TMV accumulation reduced by 40-80% in transgenic plants compared to the control group. FnCas9 endonuclease acts similar to CRISPR interference, where a Cas9 protein inactivates RNA by binding to RNA and preventing DSB. Another strategy is to modify genes that will generate virus resistance traits. For example, plant host factors are essential for RNA viruses to maintain their life cycles, such as eIF4E. Chandrasekaran *et al.* mutated independently two different sites of the host susceptibility genes using CRISPR/Cas9, obtaining potyviruses-resistant cucumber plants [50].

The safety issues associated with CRISPR/Cas9 based genome editing in the context of human gene therapy was addressed recently. The study showed that when CRISPR-edited CCR5-ablated hematopoietic stem and progenitor cells (HSPCs) was transplanted into a patient with HIV-1 infection and acute lymphoblastic leukemia, the acute lymphoblastic leukemia was in complete remission with full donor chimerism, and donor cells carrying the ablated CCR5 persisted for more than 19 months without gene editing-related adverse events. Although, transplantation and long-term engraftment of CRISPR-edited HSPCs was successful, the percentage of CCR5 disruption in lymphocytes was only approximately 5%, which question its reliability and safety in gene therapy [51].

As some researchers promoted resistance against viral diseases, others paid attention to fungal infections. CRISPR/Cas9 system can target potential genes and gene products involved in resistance against fungi. A group of researchers developed Cas9-induced mutagenesis targeting OsERF922 gene in rice, and the results showed a significantly increased resistance to blast lesions in the mutant lines [52].

CRISPR/Cas9 system was also utilized in promoting resistance and to fight against bacterial diseases. Jia *et al.* have reported the Cas9 endonuclease aimed for producing citrus plants resistant to citrus bacterial canker [53]. The authors edited the PthA4 effector binding elements in the promoter of the Lateral Organ Boundaries 1 gene in Duncan grapefruit to generate canker resistant mutants.

As many efforts are underway to use the CRISPR–Cas9 system to therapeutically correct human genetic diseases, a recent research study reported preexisting humoral and cell-mediated adaptive immune response towards the most widely used orthologs of Cas9 derived from Staphylococcus aureus (SaCas9) and Streptococcus pyogenes (SpCas9). By probing human serum for the presence of anti-Cas9 antibodies using an enzyme-linked immunosorbent assay, the authors detected antibodies against both SaCas9 and SpCas9 in 78 and 58% of donors and suggested that the antibody response should be taken into account as the CRISPR–Cas9 system moves toward clinical trials [54].

E. CRISPR/Cas9 as a tool for optimising food production

An increasing demand for food has emerged due to an ever-increasing population size, and to meet this huge demand, a large amount of food is required. Natural strains of indigenous plants such as crops and wheats fail to meet this need, hence optimization of food through alleviating biotic stress by improving resistance to plant pathogens is gaining popularity for improving yield and nutritional value of plants. CRISPR/Cas9 has been adopted in proof-of-concept studies to overcome the food shortage. The precise genome editing in rice, wheat and maize was demonstrated by a study carried out by Zong *et al.* [55].

Point mutation in rice generated by using a modified CRISPR/Cas9 system demonstrated successful application of base editing in rice. However, off-target events and transgenic integration of CRISPR cassettes are major associated concerns. Transient expression of Cas9 proteins drastically reduced the frequency of off-target events using biolistic delivery of Cas9 ribonucleoproteins (RNPs), allowing researchers to obtain transgene-free plant of wheat, which has been demonstrated by Liang *et al.* [56]. Moreover, in 2016 Qi *et al.* have demonstrated for the first time the applicability of multiplex genome editing using CRISPR/Cas9 in maize, elevating targeting efficiencies greatly. Their group selected maize glycine-tRNA to design multiple tRNA- gRNA unites allowing numerous productions of gRNAs with the control of one maize U6 promoter. They targeted three genes (MADS, MYBR and AP2) for multiplex editing [57].

CRISPR/Cas9 has also been adopted in functional studies in crop, and it can be used in crop improvement. Annexins in plants, which is essential to plant development and protecting plant against stress from the environment was examined for its roles in cold stress by CRISPR/Cas9 knockouts of OsAnn3 gene. A CRSIPR/Cas9 system is developed targeting annexin gene OsAnn3, that is essential for regulation of plant development and the protection from environmental stress. After the knockouts of OsAnn3 gene, the survival rate of obtained mutants decreased dramatically compared to the wild-type plant under 4~6˚C for 3days cold treatment, suggesting that OsAnn3 plays an important role in conferring cold tolerance, which may provide an aspect in developing plants that can survive in rapid climate change [58]. In addition, Shi *et al.* applied CRISPR/Cas9 to generate new variants of maize by replacing ARGOS 8 gene into maize native GO52 promoter, improving the yield of maize. ARGOS8, a negative regulator of ethylene responses is over expressed, reducing the ethylene sensitivity, and therefore improving crop yield under drought condition [59].

Microorganisms such as microalgae or cyanobacteria have the great potential of applications in biosynthesis and the development of bioenergy or biomaterial. Identifying suitable strains that have the potential to utilize these microorganisms in large scale industry was usually the starting point. However, there are no available strains that have most appreciated traits for 'ideal' situations to achieve industrial production. As a consequence, genomic engineering is required for the effective manipulation of microorganisms' genomes to perfect the conditions of industrial production. CRISPR/Cas9 system provides a versatile and powerful platform for this kind of practice.

Cyanobacteria, a division of microorganisms, growing in CO_2 and under sunlight alone can be utilized in carbon sequestration, photosynthetic production of fuels, or biosynthesis of other valuable chemicals [60, 61]. One example is succinate, which has numerous industrial applications [62]. Li and his colleagues increased the succinate production by 11-fold compared to that of wild-type cells with simultaneous CRISPR/Cas9 -induced glgc knock-out and gltA/ppc knock-in of *Synechococcus elongatus* PCC-7942 [63]. They made use of the toxicity of CRISPR/Cas9 protein as the selective pressure to accelerate the process of obtaining homogenous and stable desired strains as CRISPR/Cas9-induced cell death will enhance the chances of concomitant genes cassettes into all chromosomes of PCC-7942. This study employed CRISPR/Cas9 system for gene knockouts and knock-in into cyanobacteria, highlighting one possible strategy in genome editing of CRISPR/Cas9. Moreover, another strategy is the upregulation or downregulation of genes in cyanobacteria via CRISPR-interference (CRISPRi). CRISPRi systems are established in *Synechococcus elongatus* PCC-7942 to suppress the endogenous genes that are essential for glycogen accumulation and succinate conversion to fumarate for the increase in succinate production [64].

F. Ethical Concerns of CRISPR/Cas9 in Germline Editing

Germline editing using CRISPR/Cas9 has been performed in diverse model species to generate disease models or to study the molecular basis of specific gene functions. As this technology is being used widely, the simplistic engineering and efficiency of CRISPR/ Cas9 genome editing could create major ethical concerns, if it were used for wrong purposes. Injection of CRISPR/ Cas9 components (Cas9 messenger RNA or protein; sgRNA; HDR template) into the zygote or early stage embryo allows modifying the genome in all cells of the organism, including the germline [65]. Thus, this approach results in permanent changes that can be passed on to subsequent generations, offering the possibility to eliminate a genetic disease from an entire family.

In a very recent study, Liang *et al.* have also demonstrated the possibility of CRISPR/Cas9-mediated zygote editing in human embryos [66], generating controversy among scientists and in the public [67]. The ethical issues associated with CRISPR-Cas9 are mainly from the concern that this technology can be easily used to modify human embryos. Liang *et al.* have used tripronuclear human zygotes to modify the gene, hemoglobin beta responsible for beta-thalassemia and found that the procedure led to a high number of additional unwanted modifications (off- target effects), arguing against the use

of the current technique for clinical applications. Moreover, because existing methods in prenatal diagnostics such as genetic profiling after *in vitro* fertilization already offer a less risky alternative for selecting against offspring with inborn diseases, it was assumed that germline editing would mainly benefit parents who want to add nonmedically relevant traits to their children [27]. Because of these safety issues and ethical concerns, many researchers therefore do not support the legalization of CRISPR/Cas9-based genome editing studies in human zygotes.

A potentially problematic application of this technology is the editing of the human germline to adjust genes related to IQ [68], which has reached a stage where fiction is becoming a reality. Editing embryos, germ cells and the generation of designer babies is the subject of ethical debate, as a result of the implications in modifying genomic information in a heritable manner. Despite regulations set by individual countries' governing bodies, the absence of a standardized regulatory framework leads to frequent discourse in discussion of germline engineering among scientists, ethicists and the general public. Despite such concerns, however, scientists have gained license to use the CRISPR/Cas9 technology to edit human embryos and have reported the first "Designer baby" a reality in 2019 [69]. A designer baby's genetic makeup has been selected or altered, to include a particular gene or to remove genes associated with disease [70]. A process named PGD (pre-implantation genetic diagnosis) involves analyzing human embryos to identify genes associated with disease, and selecting embryos which have the desired genetic makeup. Other potential methods by which a baby's genetic information can be altered involve directly editing the genome - a person's genetic code - before birth. This process of germline engineering is not routinely performed and only one instance of this is reported, where Chinese twins Lulu and Nana were edited as embryos using CRIPR technology by the Chinese scientist He Jiankui [71], causing widespread criticism [72]. The twins are believed to be the first genetically modified babies. The clinical project involved IVF, PGD and genome editing procedures in an attempt to edit the gene CCR5, which encodes a co-receptor protein on T cells used by HIV to enter host cells. By introducing a specific mutation into the gene CCR5 Δ32, the researcher claimed that the process would confer innate resistance to HIV [73].

The research claims received significant criticism, and Chinese authorities suspended He's research activity. Following the event, scientists and government bodies have called for more stringent regulations to be imposed on the use of CRISPR technology in embryos, with some calling for a global moratorium on germline genetic engineering. While some scientists condone the use of this technology to treat disease, some have raised concerns that this could be translated into using the technology for cosmetic means and enhancement of human traits, with implications for the wider society [74]. Since genetic modification poses risk to any organism, researchers and medical professionals must give the prospect of germline engineering careful consideration, because these types of treatments will produce a change that can be passed down to future generations and therefore any error, known or unknown, will also be passed down and will affect the offspring [75]. Some bioethicists have raised concern that this could result in the accidental introduction of new diseases in future [76].

III. CONCLUSIONS AND FUTURE PERSPECTIVES

Since last few years, there has been an upsurge of interest with wide application of the CRISPR/Cas9 genome editing, because it is by far the most robust and efficient genome-editing technology. Recently, scientists from different parts of the globe have quickly realized its therapeutic potential for human patients. The first clinical trial using CRISPR/Cas9 was conducted in 2016, indicating the great promise of implementing CRISPR/Cas9 for treating genetic disorders. Even though, CRISPR/Cas9 displayed exceptionally outstanding attributes, when compared with the previously existing genome editing technologies, it may also face the same problems associated with gene therapy and other nucleic acid-based therapeutics [77]. Ethical issues, off-target effects, lack of safe and efficient delivery systems act as three major barriers in using CRISPR-Cas9 for therapeutic applications in humans.

One of the major draw-back of CRISPR/Cas9 mediated genome editing is off-target effects, especially among those studies using the plasmid-based system [78]. Off-target effects disrupt gene functions, induce genome instability, and induce epigenetic mutations, which are induced by off-target binding of the Cas9/sgRNA complex at sites distal from the PAM region. Particularly, off-target effect could be a potential hurdle for therapeutic applications in humans, because of short target DNA binding site of sgRNA with only 20 base-pairs and presence of many such potential off-target sites in the large human genome. Therefore, alternate strategies making use of a paired Cas9 nickase, rational designed sgRNA(s), and proper selection of the targeting site, can overcome this potential off-target binding effects of the CRISPR-Cas9 system. The ethical issues associated with using CRISPR-Cas9 for human embryo modification are similar to those of the

ethical challenges of CRISPR-Cas9 with genetic engineering and gene therapy. Hence, an International Summit on Human Gene Editing was held in 2015 and suggested to establish an international deliberative group to thoroughly evaluate the risks of using CRISPR-Cas9 in humans and to formulate regulations for genetic engineering and gene therapy [79].

The success of CRISPR/Cas9 technology is largely dependent on vectoral capacity of multiple delivery of genes encoding both the gRNA and Cas9 into cells and tissues. Hence, a safe and efficient DNA delivery system are crucial to guarantee the success of gene editing. To address this challenge, various strategies are being developed to introduce the CRISPR/Cas9 components including the direct delivery of mRNA and protein or the design of new viral vector and non-viral vectors [80-82]. Anc80, an adeno-associated virus vector provides a good example of a new delivery system for *in vivo* gene editing and has been tested for multiple tissue organs including liver, muscle, and retina [83]. Moreover, many other *in vivo* delivery systems, such as microinjection and hydrodynamic transfection have been successfully practiced in animals. However, a more efficient and specific *in vivo* gene replacement strategy remains to be developed.

The future use of CRISPR/Cas9 in translational medicine will largely depend on the ability to develop Cas9 variants with minimal or no off-target effect and novel methods to improve the yet inefficient engineering of precise genetic changes by homology directed repair. Furthermore, future improvements of viral and non-viral delivery methods will be necessary to improve the *in vivo* application of CRISPR/Cas9, laying the ground for the therapeutic use of CRISPR in the future. In summary, the intelligent use and appropriate application of the CRISPR/Cas9 based genome editing technology will greatly accelerate all basic and advanced research field associated with reinforcement of human life and well-being.

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APPENDICES - A

Table 1: CRISPR/Cas9 mediated genome engineering applications.

	Applications	Mechanism	Uses	Area of investigation
1	Animal models	Genetic mutations and Epigenetic variations	To study the therapeutic interventions of infectious diseases and malignancies	Biology &Medicine
2	Cellular models	Genetic and Epigenetic variations	Understand the mechanism of altered biological function or disease phenotypes	Biology & medicine
3	Generation of synthetic materials	Manipulating biological pathways	To make algae-derived, silica- based diatoms for oral drug delivery	Biotechnology
4	Agricultural crops	Genetic engineering methods	To confer resistance to environmental deprivation or pathogenic infection in plants; To improve the food security by avoiding foreign DNA in food	Biotechnology
5	Renewable energy resource	Efficient metabolic pathways for ethanol production in algae or corn (Fuel)	To manufacture sustainable and cost-effective biofuels	Biotechnology
6.	Gene therapy for genetic disorders	Direct *in vivo* correction of genetic or epigenetic defects in somatic tissue	Barth syndrome effects on the heart, Duchenne muscular dystrophy, hemophilia, b-Thalassemia, and cystic fibrosis	Clinical Medicine
7.	Tissue and Organ Transplantation	Multiple gene deletions by genetic engineering	Retrovirus free tissue and organs from pig for xenotransplantation; induction of pluripotent stem (iPS) cells	Transplantation Medicine
8.	Cancer therapy	Generation of PD-1 knockout T cells, genome editing of CAR-T cells	Treatment of different malignancies	Oncology Medicine
9.	Drug development	Genetic engineering of bacterial cells to optimize high yield	Generation of cost-effective therapeutic drugs and drug precursors	Therapeutic Medicine
10.	Stem cell therapy	Genetic engineering to induce pluripotent stem (iPS) cells	Creation of disease models or for the preparation of donor-specific tissues for transplantation	Regenerative medicine & Transplantation

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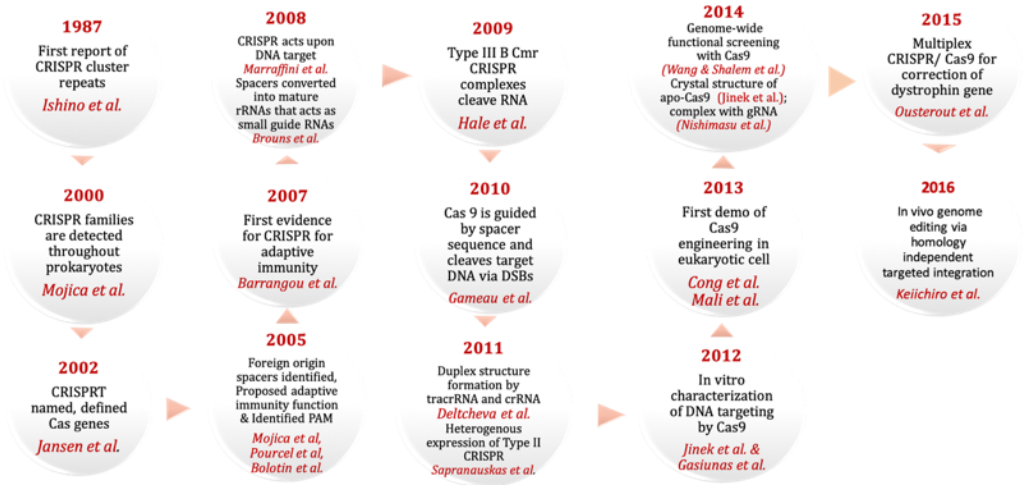

Fig. 1: CRISPR/Cas9: Timeline of development from mysterious repetitive elements to an excellent genome editing tool.

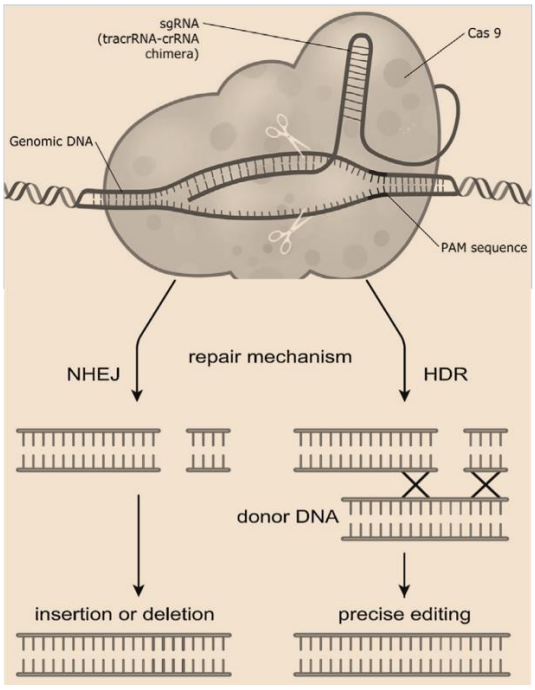

Fig. 2: CRISPR/Cas9 mechanism of action. The Cas9 nuclease is directed to the target DNA by complementary base-pairing with its bound sgRNA. The target site must be followed by a 3′ PAM sequence (NGG, NAG). Guide RNA hybridizes with 20bp genomic DNA sequence and directs Cas9 endonuclease to generate a double strand break in the target sequence (between 16th and 17th bp). When cleaved, the host DNA repair process can be restored through the non-homologous end-joining (NHEJ), where the hanging ends join together, creating small indels, or through the homology-directed repair (HDR) in the presence of a donor DNA. The final step could include insertion or deletion with several base pairs of DNA sequences (NHEJ pathway), or replacement with a particular DNA sequence used as a marker for further study (encoding for a fluorescence protein, tag protein, antibiotics, or the recognition sequence for a restriction enzyme digestion).