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Development and Applications of CRISPR/Cas Based Genome Editing Technology

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Abstract

The discovery of an ancient adaptive immune system in bacteria called the CRISPR/Cas system has revolutionized modern science. The bacterial proteins that identify and target viral DNA for degradation have been characterized and manipulated for a variety of uses in agriculture, molecular biology and medicine. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-Associated (Cas) Protein 9 system provides a robust and multiplex genome editing tool, enabling researchers to precisely manipulate specific genomic elements, and facilitating the elucidation of target gene function in biology and diseases. CRISPR/Cas9 gene-editing technique has set the stage for remarkable developments using this technology to modify, regulate, or mark genomic loci in a wide variety of cells and organisms from all three domains of life. These results highlight a new era in which genomic manipulation is no longer a bottleneck to experiments, paving the way towards fundamental discoveries in biology with applications in all branches of biotechnology as well as strategies for human therapeutics.

Keywords: Applications, CRISPR/Cas9, Genome Editing, Genetic Engineering.

1. Introduction

The development of recombinant DNA technology in the 1970s marked the beginning of a new era for biology [1]. For the first time, molecular biologists gained the ability to manipulate DNA molecules, making it possible to study genes and harness them to develop novel medicine and biotechnology [2]. Recent advances in genome engineering technologies are sparking a new revolution in biological research [3]. Rather than studying DNA taken out of the context of the genome, the researcher can now directly edit or modulate the function of DNA sequences in their endogenous context in virtually and organism of choice, enabling them to elucidate the functional organization of the genome at the systems level as well as identify causal genetic variations [4].

Broadly speaking, genome engineering refers to the process of making targeted modifications to the genome, its context (e.g., epigenetic marks), or its outputs (e.g., transcripts). The ability to do so easily and efficiently in eukaryotic and especially mammalian cells holds immense promise to transform basic science, biotechnology, and

medicine [5]. Precise modification of specific sites within a gene of interest is considered to be a standard approach to elucidate gene function, to create disease animal models, and to improve desired characteristics of animals and plants [6]. Targeted gene modification also provides the potential for therapeutic applications. In the past decades, strategies for precise genome modifications using embryonic stem cell-mediated modification by homologous recombination were limited to certain organisms [7]. Recently, engineered nucleases, including zinc finger nucleases, transcription activator-like effector nucleases, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-Associated Protein (Cas) 9 has provided a much simpler and more economical method for gene-targeted modification [8, 13]. These engineered nucleases generate a DNA double-strand break (DSB) at the targeted genome locus. The break activates repair through error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR). In the absence of a template, NHEJ is activated, resulting in insertions and/or deletions that disrupt the target loci [9, 14]. In the presence of a donor template with homology to the targeted locus, the HDR pathway operates, allowing for precise mutations to be made. The CRISPR/Cas system was first described as an adaptive immune system in bacteria and archaea and has now been engineered as RNA-guided endonucleases (RGENs) for genome editing [10, 15].

2. Genome Engineering

Ever since the discovery of the DNA double helix, researchers and clinicians have been contemplating the possibility of making site-specific changes to the genomes of cells and organisms [12]. Many of the earliest approaches to what has been referred to as genome editing relied on the principle of site-specific recognition of DNA sequences. The study of natural DNA repair pathways in bacteria and yeasts as well as the mechanisms of DNA recombination [97, 101], revealed that cells have endogenous machinery to repair double-strand DNA breaks (DSBs) [89, 98]. Thus, methods for introducing precise breaks in the DNA at sites where changes are to be introduced were recognized as a valuable strategy for targeted genomic engineering.

Early approaches to such targeted DNA cleavage took advantages of DNA base pair recognition by oligonucleotides or small molecules. Building on the original description of triple helix formation by Rich and colleagues in the late 1950s [28, 117], oligonucleotides coupled to chemical cleavage or crosslinking reagents such as bleomycin and psoralen were shown to be useful for site-specific chromosome modification in chemical recognition of DNA sequences, such as peptide nucleic acids (PNAs) and polyamides were shown to enable targeted binding of chromosomal loci that could be modified if the chemical recognition agent was coupled to a cleavage reagent such as bleomycin [11, 29]. Another strategy that relied on nucleic acid-base pairing was the use of self-splicing intron to change sequences at the DNA [128, 131] or RNA [108] level.

Although these approaches did not lead to robust methods, they demonstrated the utility of base pairing for site-specific genome modification. The use of self-splicing introns for genome editing also suggested the possibility of using intron-encoded nucleases homing endonucleases that are capable of site-specific DNA cleavage and integration of the intron sequence [111]. By inserting desired sequences into the intron first, the researcher could incorporate selected genetic information into a genome at sites recognized by the homing endonucleases [16, 51]. At around the same time, the initial reports of zinc finger mediated DNA binding [76, 85] led to the creation of modular DNA, recognition proteins that, when

coupled to the sequence-independent nucleases domain of the restriction enzyme FokI, could function as site-specific nucleases [59]. When designed to recognize a chromosomal sequence, such zinc finger nucleases were found to be effective at inducing genomic sequence changes in *Drosophila* and mammalian cells [4, 5].

Although ZFNs are effective genome editing reagents for some experiments, they were not widely adopted because of the difficulty inherent in designing and validating such proteins for a specific DNA locus of interest. Thus, the field was primed for the first reports of transcription activator-like (TAL) effectors, which occur naturally in bacteria that infect plants, enabling rapid creation of FokI coupled versions that could be used similarly to ZFNs for site-directed genome editing [7, 17, 80]. Such TAL effector nucleases (TALENs) were easier than ZFNs to produce and validate, generating widespread excitement about the possibility of superficial genome editing that would be fast and inexpensive. But difficulties of protein design, synthesis, and validation remained a barrier to widespread adoption of these engineered nucleases for routine use.

3. Timeline of CRISPR/Cas System

The CRISPR story began in 1987. While studying the iap enzyme involved in isozyme conversion of alkaline phosphatase in *E. coli*, Nakata and colleagues reported a curious set of 19 nt repeats downstream of the iap gene [48]. Unlike most repetitive elements, which typically take the form of tandem repeats like TALE repeat monomers, these 29 nt repeats were interspaced by five intervening 32 nt non-repetitive sequences. These early findings began to stimulate interest in such microbial repeat elements. By 2002, Jansen and Mojica coined the acronym CRISPR to unify the description of microbial genomic loci consisting of an interspaced repeat array [52, 115].

At the same time, several clusters of signature CRISPR-Associated (Cas) genes were identified to be well conserved and typically adjacent to the repeat elements [52], serving as a basis for the eventual classification of three different types of CRISPR [40]. A key turning point came in 2005 when systematic analysis of the spacer sequences separating the individual direct repeats suggested their extra-chromosomal and phage-associated origins [79, 90]. This insight was tremendously exciting, especially given previous studies showing that CRISPR loci are transcribed [109] and that viruses are unable to infect archaeal cells carrying spacers corresponding to their genomes [79]. Together, these findings led to the speculation that CRISPR arrays serve as an immune memory and defense mechanism, and individual spacers facilitate defense against bacteriophage infection by exploiting Watson-Crick base-pairing between nucleic acids [79, 90].

Despite these compelling realizations that CRISPR loci might be involved in microbial immunity, the specific mechanism of how the spacers act to mediate viral defense remained a challenging puzzle. Several hypotheses were raised, including thoughts that CRISPR spacers act as small RNA guides to degrade viral transcripts in an RNAi-like mechanism [70] or that CRISPR spacer's direct Cas enzyme to cleave viral DNA at spacer-matching regions [18-20]. By 2010, just 3 years after the first experimental evidence for CRISPR in bacterial immunity, the basic function and mechanisms of CRISPR systems were becoming clear. A variety of groups had begun to harness the natural CRISPR system for various biotechnological applications after that time; the race to harness Cas9 for genome editing was on.

4. History and Biology of CRISPR/Cas System

In a parallel but completely separate area of research, a few microbiology and bioinformatics laboratories in the

mid-2000s began investigating CRISPRs (Clustered Regularly Interspaced Palindromic Repeats), which had been described in 1987 by Japanese researchers as a series of short direct repeats interspaced with short sequences in the genome of *Escherichia coli* [48, 49]. CRISPRs were later detected in numerous bacteria and archaea [78], and predictions were made about their possible roles in DNA repair or gene regulation [38, 71, 72]. A key insight came in 2005 with the observation that many spacer sequences within CRISPRs derive from plasmid and viral origins [78, 90].

Together with the finding that CRISPR loci are transcribed [109] and the observation that Cas (CRISPR-Associated) and helicase domains [39, 52], it was proposed that CRISPR/Cas is an adaptive defense system that might use antisense RNAs as memory signatures of past invasions [70]. In 2007, infection experiments of the lactic acid bacterium *Streptococcus thermophilus* with lytic phages provided the first experimental evidence of CRISPR/Cas mediated adaptive immunity [21-25]. This finding led to the idea that natural CRISPR/Cas systems can also be used as a gene-editing method.

Genome editing with site-specific nucleases allows reverse genetics, genome engineering and targeted transgene integration experiments to be carried out in an efficient and precise manner. It involves the introduction of targeted DNA double-strand breaks (DSBs) using an engineered nuclease, stimulating cellular DNA repair mechanisms. Different genome modifications can be achieved depending on the repair pathway and the availability of a repair template. Two different DSB repair pathways have been defined: non-homologous end joining (NHEJ) and homologous recombination (HR). In most cases, NHEJ causes random insertions or deletions (indels), which can result in frame shift mutations if they occur in the coding region of a gene, effectively creating a gene knock-out [26, 27].

Alternatively, when the DSB generates overhangs, NHEJ can mediate the targeted introduction of a double-stranded DNA template with compatible overhangs [67-69]. When a template with regions of homology to the sequence surrounding the DSB is available, the DNA damage can be repaired by HR, and this mechanism can be exploited to achieve precise gene modifications or gene insertions. Even though the generation of breaks in both DNA strands induces recombination at specific genomic loci, NHEJ is by far the most common DSB repair mechanism in most organisms, including higher plants, and the frequency of targeted integration by HR remains much lower than random integration [91-93]. Strategies such as the over-expression of proteins involved in HR or the use of negative selection markers outside the homology regions of the insertion Cassette to prevent the survival of random integration events can achieve moderate improvements in gene targeting efficiency [92-95].

5. CRISPR/Cas System

The CRISPR system provides a potential platform for targeted gene regulation [30-35]. About 40% of bacteria and 90% of archaea possess CRISPR/CRISPR-Associated (Cas) systems to confer resistance to foreign DNA elements [73-75]. CRISPR systems use small base-pairing RNAs to target and cleave foreign DNA elements in a sequence-specific manner [124]. There are diverse CRISPR systems in different organisms, and one of the simplest is the type II CRISPR system from *Streptococcus pyogenes*: only a single gene encoding the Cas9 protein and two RNAs, a mature CRISPR RNA (crRNA) and a partially complementary trans-acting RNA (tracrRNA), are necessary and sufficient for RNA-guided silencing of foreign DNAs [56-58, 66].

The CRISPR is an array of short repeated sequences separated by spacers with unique sequences. The CRISPR can be found on both Chromosomal and Plasmid DNA. The spacers are often derived from the nucleic acid of viruses and plasmids, an observation that gave rise to the idea that CRISPRs are part of an anti-viral system [41-47]. By adding new spacers, new viruses can be recognized. The spacers are used as recognition elements to find matching virus genomes and destroy them [36, 37].

CRISPR activity requires the presence of a set of CRISPR-Associated (Cas) genes, usually found adjacent to the CRISPR that code for proteins essential to the immune response [50, 53-55]. Since the genome is modified in the process of spacer acquisition, off-spring inherits the protection. New spacers are usually added at one side of CRISPR, making CRISPR a chronological record of viruses, the cell and its ancestors have acquired.

6. CRISPR Mechanism

There are three main types of CRISPR systems, each utilize slightly different strategies.

In types I and III CRISPR, the pre-crRNA transcript is cleaved within the repeats by CRISPR-Associated ribonucleases, releasing multiple small crRNAs. Type III crRNA intermediates are further processed at the 3′ end by yet to be identified RNases to produce the fully mature transcript. In type II CRISPR, an associated trans-activating CRISPR RNA (tracrRNA) hybridizes with the direct repeats, forming an RNA duplex that is cleaved and processed by endogenous RNase III and other unknown nucleases. Maturated crRNAs from type I and III CRISPR systems are then loaded onto effector protein complexes for target recognition and degradation. In type II systems, crRNA-tracrRNA hybrids complex with Cas9 to mediate interference [60-65].

Both type I and III CRISPR systems use multi-protein interference modules to facilitate target recognition. In type I CRISPR, the Cascade complex is loaded with a crRNA molecule, constituting a catalytically inert surveillance complex that recognizes target DNA. The Cas3 nuclease is then recruited to the Cascade-bound R loop, mediating target degradation. In type III CRISPR, crRNAs associate either with Csm or Cmr complexes that bind and cleave DNA and RNA substrates, respectively. In contrast, the type II system requires only the Cas9 nuclease to degrade DNA matching its dual guide RNA consisting of a crRNA-tracrRNA hybrid [77].

6.1: Mechanism of Type II CRISPR System

Type II CRISPR systems are most commonly used in gene editing applications. CRISPRs act in immunity through a multistep mechanism that begins with the integration of new spacers into CRISPR loci. To function, CRISPR RNAs (crRNAs) are then transcribed which leads to a process called CRISPR interference.

The CRISPR/Cas9 type II system is carried out in three steps given as follows:

6.1.1: Spacer Integration

New spacers are inserted at the leader end of the CRISPR array when bacteria are infected with foreign DNA [96]. However, this mechanism does not cause CRISPRs to expand indefinitely; when a new spacer is inserted, a different spacer is generally deleted [99, 100]. New spacer integration allows for organisms to adapt immunity based on the variety of phages present in their current environment while retaining relevant ancestral spacers. CRISPR prokaryotic adaptive immunity is heritable and based on nucleic acids which interestingly contrast eukaryotic adaptive immunity which is structured around amino acids and therefore is not inherited.

6.1.2: crRNA Transcription

In CRISPR Type II systems, Cas genes encode four Cas proteins, including Cas1 and Cas2 proteins which are universal in all CRISPR systems. Type II systems also

express either Cas4 or Csn2 which are involved in spacer integration. Finally, all Type II CRISPR systems encode a highly conserved Cas9 gene [116]. Long primary pre-crRNA is transcribed from the CRISPR loci. Pre-crRNA pairs with trans-activating CRISPR RNA (tracrRNA) to be processed by RNase III [81, 82]. This creates shorter mature crRNAs. Cas9 catalyzes the formation of a crRNA-tracrRNA complex [83, 84].

6.1.3: CRISPR Gene Editing

After transcription and processing, mature crRNAs complex with Cas9 and tracrRNA. This complex binds to a proto-spacer sequence of extra-chromosomal double-stranded DNA. The process is dependent on a proto-spacer adjacent motif (PAM) [86, 87]. When the Cas9/tracrRNA/crRNA complex binds the target sequence of the dsDNA, R-loop forms and one DNA strand pairs with crRNA and the other disassociates. Both strands of DNA are cut near the PAM sequence. The crRNA acts as a guide while Cas9 acts as the endonuclease to cleave the DNA. The presence of double-stranded breaks (DSB) in the DNA leads to activation of the DSB repair machinery either Non-homologous end joining (NHEJ) or homology-directed repair (HDR) [86-88]. NHEJ causes insertions or deletions (indels) at the break [102, 103], which leads to gene silencing of the invading DNA, the method through which CRISPR mediated immunity in Type II systems functions.

7. CRISPR Interference (CRISPRi)

In 2013, Qi *et al*., created a catalytically dead Cas9 (dCas9), lacking endonuclease activity, to function in gene silencing as opposed to gene editing through DSBs. This method, called CRISPR interference (CRISPRi) halts mRNA synthesis by blocking RNA polymerase at the promoter region of the DNA. CRISPRi can silence multiple genes at the same time. This method can be compared to RNAi. However, CRISPRi has the possible advantage of working earlier than RNAi by silencing the gene before mRNA is created rather than simply degrading the mRNA. CRISPRi gene knock-down is inducible and reversible unlike gene knock-out methods; one advantage of using CRISPRi-based knock-down of gene expression is the fact that this perturbation should be reversible [104, 105].

7.1: CRISPRi in Manipulation of Stem Cell Differentiation

CRISPRi has many possible applications in medical research. For example, Kearns *et al*., (2014) researched the ability of dCas9 to influence the differentiation state of human pluripotent stem cells. The authors' research determined that dCas9 could be used to positively or negatively regulate the expression of particular target genes that influence cell differentiation. CRISPRi could, therefore, be useful to investigate stem cell differentiation pathways [58].

8. CRISPR/Cas9 in the Generation of Animal Models

Gene targeting based on homologous recombination and embryonic stem cells has been used as the typical approach for animal genome modification, which has played indispensable roles in making a causal link between genomic mutations and phenotypes during development and in disease. However, gene targeting has limited applications in some organisms due to time-consuming procedures and the lack of available embryonic stem cells. Many recent studies have shown that CRISPR/Cas9 technology could be used for rapidly generating targeted genome modifications in the germ lines of various model organisms [106, 107], which will significantly advance the functional genomics. Microinjection of Cas9 encoding mRNA and customizable sgRNA into one-cell stage zebrafish embryos can efficiently modify the target genes *in-vivo* in a simple, rapid and scalable manner [110]. Co-injection of

Cas9 mRNA and sgRNAs targeting different genes into mouse zygotes generate mutant mice with biallelic mutations, confirming that CRISPR/Cas mediated gene editing could be used for the simultaneous disruption of multiple genes with high efficiency [112].

Gene knock-in mice carrying precise point mutations of two genes can be obtained by co-injection of Cas9 mRNA/sgRNAs together with mutant oligos [113]. The following study demonstrates that reporter and conditional mutant mice can also be generated in one step by co-inject in mouse zygotes with Cas9 mRNA and different sgRNAs, as well as DNA vectors of different sizes. Additionally, mice with the predicted deletions have been generated using sgRNAs targeting two separate sites in the gene [120]. Multiplexed activation of endogenous genes can be achieved by injecting a two-component transcriptional activator including a nuclease-dead Cas9 protein fused with a transcriptional activation domain and sgRNAs targeting gene promoters [12, 15]. These previous studies have demonstrated that CRISPR/Cas9 technology can be used for efficient one-step generation of various sophisticated mutant mice, including mice carrying gene insertions, deletions, conditional alleles and endogenous reporters at different loci.

CRISPR/Cas9 technology has been used for efficient genome engineering in many other model organisms, including *Drosophila* [37], *Caenorhabditis elegans* [32], *Xenopus tropicalis* [6, 82], *Rattus rattus* [46], and *Sus domesticus* [123]. Significantly, the CRISPR/Cas9 system is an efficient and reliable approach for targeted modification of *Cynomolgus* monkey genomes *Macaca fascicularis* [86]. The application of CRISPR/Cas9 technology for genome editing in a wide range of organisms will promote understanding of development and disease and help develop animal models and therapeutic strategies for human diseases.

8.1: CRISPR/Cas9 in Somatic Genome Editing

Rapid progress in genome engineering based on the CRISPR/Cas9 system enables fast functional characterization of putative disease genes in various mouse models via somatic genome editing [66, 99, 126]. A CRISPR plasmid DNA expressing Cas9 and sgRNAs can be delivered to the liver through hydrodynamic injection, and CRISPR mediated PTEN mutation with or without p53 mutation phenocopies the effects of PTEN and p53 gene knock-out using Cre-LoxP technology [126]. Previous studies have also shown that an activated mutant β-catenin gene could be delivered into hepatocytes by co-injection of Cas9 plasmids expressing sgRNAs targeting the β-catenin gene and a DNA oligonucleotide donor carrying β-catenin activating point mutations [126]. This previous study demonstrated that the CRISPR/Cas system could be used for directly mutating tumor suppressor genes and oncogenes in somatic tissues, providing a new approach for developing new types of disease models. The CRISPR/Cas9 system has also been used to induce a specific chromosomal rearrangement, the EML4-ALK inversion, in somatic cells of adult animals to generate a mouse model of EML4-ALK driven lung cancer [66]. The resulting tumors exhibit the typical histopathological and molecular features of ALK (+) human non-small cell lung cancer (NSCLC), which is sensitive to ALK inhibitors [66]. Interestingly, using a Lentiviral-based delivery system, a recent study demonstrated that CRISPR-induced genome editing of tumor suppressor genes together with Cre-dependent somatic activation of oncogenic KRAS (G12D) causes lung adenocarcinomas with different histopathological and molecular features [99]. Using the Cas9 gene knock-in mice, lung adenocarcinoma models can be generated by simultaneously introducing a single AAV vector carrying loss-of function mutations in p53, LKB1 and KRAS

(G12D) mutations in the lung [88], suggesting that Cas9 gene knock-in mice could be widely used for somatic genome editing. The rapid somatic genome engineering approach will greatly help to systematically identify critical genes underlying disease initiation and progression in many well-established disease mouse models.

8.2: CRISPR/Cas9 in Functional Genomic Screening

Functional genomics screening is largely used for identifying the essential genes for a specific cellular process. The RNA interference (RNAi) has been dominantly applied for genome-wide screening; however, the off-target effects of RNAi have limited its applications [1, 49, 106]. Besides, RNAi could not be used for silencing RNAs located in the nucleus. The CRISPR/Cas9 system has been successfully used in various genome-scale loss of function screening [121]. Using a genome-scale Lentiviral sgRNA library, all expected genes of the DNA mismatch repair pathway have been identified in screening for resistance to the nucleotide analog 6-thioguanine, and numerous genes corresponding to fundamental processes have been obtained with a negative selection screening for essential genes [121]. A genome-scale CRISPR/Cas9 knock-out (GeCKO) library has been developed and successfully used for screening genes essential for cell viability in cancer and pluripotent stem cells and genes associated with the resistance to vemurafenib, a drug for late-stage melanoma [104]. A CRISPR/Cas based knock-out library has been applied to identify the host genes mediating the cellular responses to anthrax and diphtheria toxins [131]. A recent study has shown that saturation editing of genomic regions could be achieved by coupling CRISPR/Cas9 technology with multiplex homology-directed repair using a complex library of donor templates, facilitating the high-resolution functional screening of both cis-regulatory elements and trans-acting factors in the genome [31]. A series of studies have demonstrated that CRISPR-mediated repression (CRISPRi) and CRISPR-mediated activation (CRISPRa) are powerful tools for functional genomics screening. A CRISPRi system consisting of a catalytically inactive Cas9 and a guide RNA has been shown to specifically and efficiently repress the transcription of target genes in *Escherichia coli* and mammalian cells [36, 94], whereas a catalytically inactive Cas9 fused with a transcriptional activation domain has been used to activate the expression of specific endogenous genes [65-70].

Genome-scale CRISPRi and CRISPRa libraries that specifically target transcriptional repressors or activators to endogenous genes have been successfully used for screening essential genes for growth, tumor suppression, differentiation regulation, and cellular sensitivity to a cholera-diphtheria toxin, suggesting that CRISPRi and CRISPRa are valuable tools for mapping complex pathways [114]. A very recent study has shown that CRISPR/Cas9 complexes with synergistic activation mediators can achieve robust, single sgRNA-mediated gene up-regulation at endogenous genomic loci. When used with a sgRNA library, the engineered Cas9 activation complexes can activate multiple genes simultaneously, up-regulate long intergenic non-coding RNA transcripts and identify genes conferring resistance to a BRAF inhibitor through a genome-wide dCas9 based transcription activation screening in a melanoma model [61]. These results demonstrate that CRISPR/Cas9 technology can be a promising functional genomic screening tool for discovering essential genes in various biological processes.

8.3: CRISPR/Cas9 in Correction of Genetic Disorders

One of the most exciting applications of CRISPR/Cas9 is the possibility of curing genetic diseases. The CRISPR/Cas9 system has been shown to efficiently correct a dominant *Crygc* gene mutation in a cataracts

mouse model by co-injecting Cas9 mRNA and sgRNA targeting the mutant *Crygc* allele into zygotes [125]. A very recent study has shown that the CRISPR/Cas9 system can be used to modify an EGFP transgene or the endogenous *Crygc* gene in spermatogonial stem cells (SSCs). The modified SSCs carrying a corrected *Crygc* mutation can undergo spermatogenesis and produce off-spring with the corrected phenotype at an efficiency of 100% [125]. The injection of Cas9, sgRNA and homology-directed repair template into mouse zygotes has been shown to correct the dystrophin gene mutation responsible for muscular dystrophy in the germline and prevent the development of muscular dystrophy in mutant mice [122]. Interestingly, a similar strategy using the CRISPR/Cas9 technology has successfully corrected the cystic fibrosis transmembrane conductor receptor (CFTR) locus by homologous recombination in cultured intestinal stem cells of cystic fibrosis human patients [118], demonstrating that primary adult stem cells derived from patients with a single-gene hereditary defect could be corrected by CRISPR/Cas9 mediated homologous recombination, suggesting a promising strategy for gene therapy in human patients.

8.4: CRISPR/Cas9 in the Treatment of Infectious Diseases:

Considering that the CRISPR/Cas system originally functions as an antiviral adaptive immune system in bacteria, this system could be used for treating infectious diseases by eradicating pathogen genomes from infected individuals. Recently, studies have shown that the CRISPR/Cas9 system can eliminate the HIV-1 genome and prevent new HIV infection [27, 45]. When transfected into HIV-1 provirus-integrated human cells, a sgRNA expression vector targeting the long terminal repeats (LTR) of HIV-1 efficiently cleaves and mutates LTR target sites and suppresses LTR-driven viral gene expression. Also, this system has been shown to delete viral genes from the host cell chromosome [27]. The high specificity of Cas9/sgRNAs in editing the HIV-1 target genome has also been recently demonstrated [45]. Cas9/sgRNAs efficiently inactivate HIV gene expression and replication in latently infected cells, including microglial, promonocytic and T cells. Significantly, Cas9/sgRNA mediated genome editing has been shown to immunize cells to prevent HIV-1 infection [45]. These results indicate that the CRISPR/Cas9 technology can serve as a potential tool for clinical applications to cure infectious diseases.

9. RNA-Guided Genome Editing in Plants Using CRISPR/Cas System

Most recently, a new gene-targeting tool has been developed in microbial and mammalian systems based on the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Associated nuclease system. The CRISPR-Associated nuclease (Cas) is part of adaptive immunity in bacteria and archaea [119]. The Cas9 endonuclease, a component of the *Streptococcus pyogenes* type II CRISPR/Cas system, forms a complex with two short RNA molecules called CRISPR RNA (crRNA) and trans activating crRNA (transcrRNA), which guide the nuclease to cleave non-self DNA on both strands at a specific site [120]. The crRNA–transcr RNA heteroduplex could be replaced by a chimeric RNA (so-called guide RNA (gRNA)) and the gRNA could be programmed to target specific sites [46]. The CRISPR/Cas system has been demonstrated for genome editing in human [23, 37], mice [34], zebrafish [22], yeast [35], and bacteria [44].

Due to the significant differences between animals and plants, however, it is important to test the functionality and utility of the CRISPR/Cas system for genome editing and gene targeting in plants. Here, the establishment of RNA-guided genome editing in plants using the

CRISPR/Cas9 system is described. As a proof of concept, targeted gene mutation was successfully achieved in three specific sites of a mitogen-activated protein kinase gene in the rice genome. Furthermore, the mutation efficiency and off-target effect have been assessed for the RNA guided genome editing in plants. This study demonstrates that the CRISPR/Cas9 system is functional in plants and can be exploited for gene targeting and genome editing in crop species.

To adapt the CRISPR/Cas9 system for plant genome editing, two RNA-guided genome editing vectors (pRGE3 and pRGE6) was created for expressing engineered gRNA and Cas9 in plant cells. In both vectors, CaMV 35S promoter was used to control the expression of Cas9 which was fused with a nuclear localization signal and a FLAG tag.

The pRGE3 and pRGE6 vectors contains: (1) a DNA-dependent RNA polymerase III (Pol III) promoter (rice snoRNA U3 or U6 promoter, respectively) to control the expression of engineered gRNA molecules in the plant cell, where the transcription was terminated by a Pol III terminator (Pol III Term), (2) a DNA-dependent RNA polymerase II (Pol II) promoter (e.g. CaMV 35S promoter) to control the expression of Cas9 protein, (3) a multiple cloning site (MCS) located between the Pol III promoter and gRNA scaffold, which is used to insert a 15-30 bp DNA sequence as gRNA seed for producing an engineered gRNA [127].

Rice protoplast transient expression system was used to test the engineered gRNA/Cas9 constructs. The efficient transformation of rice protoplasts was demonstrated with a plasmid construct carrying the *green fluorescence protein* (GFP) marker gene. Fluorescence microscopic analyses indicate that GFP expression was found in approximately 60% of the protoplasts at 18 h after transformation and in about 90% of the protoplasts at 36-72 h after transformation. Following the transformation of empty pRGE3 vector and the pRGE3–PS1/2/3 gRNA constructs into rice protoplasts, the Cas9 nuclease was successfully expressed as revealed by the immunoblot analysis [87].

10. Efficient Ablation of Genes in Human Hematopoietic Stem Cells and Effector Cells Using CRISPR/Cas9

The hematopoietic system is at the forefront of cell-based gene therapies because the cells can be readily obtained, manipulated, and reintroduced into patients. The development of genome editing methodologies such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) [67-70] have enabled site-specific gene repair or ablation and raised the possibility of treating a broad range of diseases at the genetic level [76]. Despite many promises, limitations associated with these technologies, including low targeting efficacy and *de-novo* engineering of proteins for each target has precluded wide-spread adoption of these technologies for therapeutic use [65]. The recent emergence of the Clustered Regularly Interspaced Palindromic Repeats (CRISPR) system for gene editing has the potential to overcome these limitations [98].

The CRISPR technology utilizes a fixed nuclease, often the CRISPR-Associated protein 9 (Cas9) from *Streptococcus pyogenes*, in combination with a short guide RNA (gRNA) to target the nuclease to a specific DNA sequence [15-20. CRISPR/Cas9 relies on simple base-pairing rules between the targets DNA and the engineered gRNA rather than protein-DNA interactions required by ZFNs and TALENs [43, 47]. As a result, the CRISPR/Cas9 system has proven extremely simple and flexible. Perhaps most important, this system has achieved highly efficacious alteration of the genome in several cell types and organisms [33-39]. Given the importance of the

hematopoietic system in cell-based gene therapies, we tested the CRISPR/Cas9 system in primary human $CD4^+$ T cells and $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) targeting two clinically relevant genes, beta-2 microglobulin (B2M) and chemokine receptor 5 (CCR5). B2M encodes the accessory chain of major histocompatibility complex (MHC) class I molecules and is required for their surface expression [22-27]. Deletion of B2M is a well-established strategy to ablate MHC class I surface expression [32] and could be used to generate hypo immunogenic cells for transplantation and adoptive immunotherapy. CCR5 is the main co-receptor used by CCR5-tropic strains of HIV-1 [55] and a validated target for gene ablation, as mutations resulting in loss of protein expression against HIV infection.

11. *Drosophila* CRISPR System

Several groups have used the CRISPR/Cas9 system to induce targeted mutations in *Drosophila* [25-34], but differ in their approach to supplying the Cas9 protein and sgRNA components of the system. The first description of mutagenesis with CRISPR/Cas9 involved co-injection of two plasmids into syncytial blastoderm stage *Drosophila* embryos [23]. One plasmid expresses the Cas9 gene under the Hsp70 promoter, and the second produces the sgRNA, driven by a pol III promoter from the U6 gene. This was tested at the yellow gene and resulted in mutagenesis of the gene that was capable of being transmitted to subsequent generations. The efficiency of mutagenesis due to inefficient NHEJ was fairly low, with 5.9% of the injected flies giving rise to at least one mutant offspring [24]. However, the authors further demonstrated that if two sgRNAs are supplied, targeting either end of the yellow gene, this can result in deletion of the intervening sequence, and that integration of short sequences at the cleavage site is possible by co-injection with a short single-stranded oligonucleotide donor sequence [22].

12. Applications of CRISPR/Cas9 Technology

Genetic and epigenetic control of cells with genome engineering technologies is enabling a broad range of applications from basic biology to biotechnology and medicine. Causal genetic mutations or epigenetic variants associated with altered biological function or disease phenotypes can now be rapidly and efficiently recapitulated in animal or cellular models (Animal models, Genetic variation). Manipulating biological circuits could also facilitate the generation of useful synthetic materials, such as algae-derived, silica-based diatoms for oral drug delivery (Materials). Additionally, precise genetic engineering of important crops could confer resistance to environmental deprivation or pathogenic infection, improving food security while avoiding the introduction of foreign DNA (Food).

Sustainable and cost-effective biofuels are attractive sources for renewable energy, which could be achieved by creating efficient metabolic pathways for ethanol production in algae or corn (Fuel). Direct *in-vivo* correction of genetic or epigenetic defects in somatic tissue would be permanent genetic solutions that address the root cause of genetically encoded disorders (Gene surgery). Finally, engineering cells to optimize high yield generation of drug precursors in bacterial factories could significantly reduce the cost and accessibility of useful therapeutics (Drug development).

12.1: Application of CRISPR in Eukaryotic Organisms

Recent work has proven that the CRISPR/Cas9 system can be utilized for gene editing in a plethora of systems including yeast, mice, zebrafish, mouse, and even humans [73, 74]. Based on the type II CRISPR/Cas9 mechanism, researchers have engineered RNA chimera of tracrRNA-crRNA called single guide RNA (sgRNA) which can cause sequence-specific binding to dsDNA [30]. Cas9 nuclease is capable of interacting with this engineered

sgRNA to specifically cleave dsDNA in regions complementary to the introduced sgRNA. The presence of double-stranded breaks (DSB) in the DNA leads to activation of the DSB repair machinery for either NHEJ or the Homology Directed Repair (HDR) pathway which requires the presence of a repair template. The HDR mechanism copies the sequence of the template into the cut target sequence to repair the DSB. This method has been found to work at high efficiency for genome editing in most eukaryotic model systems [73, 74]. Moreover, modified versions of this system can be used to knock-out genes, insert new exogenous DNA into the host genome, and to block RNA transcription for a variety of applications.

12.2: Gene Knock-out System

One can imagine many potential ways in which knocking out a specific gene could be useful. CRISPR could be used to delete harmful disease-causing mutations in the human genome, especially in IVF embryos. Alternatively, the system could be used to knock-out specific genes in model organisms to study diseases. The applications of CRISPR/Cas9 knock-out for HIV therapy and multiple simultaneous knock-outs in model organisms will be discussed.

12.3: Application of CRISPR Gene Knock-out System as Therapy for HIV:

Human immunodeficiency virus (HIV) is a Lentivirus that causes acquired immunodeficiency syndrome (AIDS). HIV infection systematically destroys the human immune system. Recently, zinc finger nucleases have been utilized to disrupt CCR5, a protein necessary for HIV to enter target cells [42]. However, the CRISPR/Cas9 system could also be employed to combat HIV in a slightly different manner. Instead of targeting CCR5, CRISPRs can be used to disrupt the long terminal repeat (LTR) promoter of HIV [27]. This LTR is necessary for viral genomic RNA transcription. Ebina *et al.,* (2013) showed that disruption of the LTR region by a CRISPR system could be accomplished in HIV-1 provirus integrated human cell lines [27]. During the course of infection, HIV integrates itself into the host genome, so while retroviral therapies can control HIV, the dormant virus still exists in the host genome. The CRISPR system has the unique potential to target integrated genomic HIV. LTR regions exist on both sides of the integrated HIV genome. Due to this, the CRISPR system can remove the sequence of integrated HIV DNA from the host genomes by cleaving at both LTRs [27]. This appears, potentially, to be very promising form of therapy, though it is still at very early stage. Future challenges include determining potential off-target effects, as well developing safe and effective delivery system.

12.4: Application of CRISPR Gene Knock-out System for the Creation of Multiple Gene Knock-out Model Organisms:

One interesting aspect of the CRISPR/Cas9 system is that multiple sgRNAs can be inserted into cells making it possible to knock-out more than one gene [129, 130]. This ability is unique to the CRISPR system. Wang *et al.*, (2013) describe the efficient creation of mouse stem cells with five genes disrupted at the same time using the CRISPR system. Normally, to create mice with multiple mutations, it would be necessary to cross different mice or complete other time-consuming procedures. Niu *et al.*, (2014) describe the simultaneous disruption of two genes in single-cell monkey embryos which were then inserted into surrogate mothers. This resulted in monkeys being born with mutations in the two genes targeted by the CRISPR/Cas9 system without off-target effects. The ability to edit the genomes of model animals, especially primates, is extremely important to medical research and will assist in the development of new treatments in future.

12.5: Gene Knock-in System:

Cong *et al.*, (2013) studied Cas9 nickase, a variation of Cas9, which nicks target DNA to induce homology-directed repair (HDR). Cas9 nickase can be utilized as a tool for gene insertion as well as gene knock-out because the HDR pathway results in repair template substitutions rather than the deletions (or less commonly, insertions) caused by non-homologous end joining. This approach is particularly interesting because it decreases the probability of off-target mutagenesis. There are many applications for gene insertion via the CRISPR/Cas nickase system.

12.6: Application of CRISPR/Cas Nickase Gene Knock-in System in Stem Cell Therapies:

Many genetic diseases could be cured by modifying genomic sequences of pluripotent stem cells of patients to express wild-type copies of the disease-causing genes. This would allow for autologous stem cell therapies which reduce the risk of graft-host disease compared to allogeneic treatments. For example, Schwank *et al.*, (2013) studied the use of CRISPR gene knock-in as a therapy for cystic fibrosis. They succeeded in correcting mutant CFTR Delta-F508 alleles (alleles with mutation that causes cystic fibrosis) using the CRISPR/Cas9 mediated homologous recombination in intestinal stem cells. Schwank *et al.*, (2013) showed that corrected genes could function normally in an organoid system. Other studies revealed that mouse organoids grown *in-vitro* can be successfully transplanted into living mice [123]. Systems like this could eventually use for human stem cell therapy. However, this technique does give rise to risk of endogenous gene disruption and activation of nearby oncogenes. More work must be done to accurately determine and reduce the risks of this technique.

12.7: Applications in Gene Therapy:

Precisely genome editing has the potential to permanently cure diseases through disrupting endogenous disease-causing genes, correcting disease-causing mutations or inserting new protective genes [115-120]. Using ZFNs-induced HDR, Urnov *et al.*, (2005) corrected disease-causing gene mutation in a human cell for the first time. Subsequently, ZFNs were used to correct the gene mutations causing sickle-cell disease [131] and Haemophilia B [63]. Through disabling virulence genes or inserting protective genes, ZFNs have been used to induce resistance to virus infection in human cells [125-130] and enhance the efficiency of immunotherapies [120-123]. As the newest engineered nucleases, CRISPR/Cas9 provides a novel highly efficient genome editing tool for gene therapy studies. For instance, Ebina *et al.*, (2013) disrupted the long-terminal repeat promoter of HIV-1 genome using CRISPR/Cas9, which significantly decreased HIV-1 expression in infected human cells. The integrated pro-viral virus genes in host cell genomes can also be removed by CRISPR/Cas9. With the rapid development of induced pluripotent stem (iPS) cell technology, engineered nucleases are applied to genome manipulation of iPS cells.

The unlimited self-renewing and multi-potential differentiation capacity of iPS cells make them very useful in disease modelling and gene therapy. Using CRISPR/Cas9, Horii *et al.*, (2013) created an iPS cell model for immunodeficiency, centromeric region instability, facial anomalies syndrome (ICF) causing by DNMT3B gene mutation. In this study, iPS cells were transfected with plasmids expressing Cas9 and gRNA, which disrupted the function of DNMT3B in transfected iPS cells. Using the same hPSC lines and delivery method, Ding *et al.*, (2013) compared the efficiencies of CRISPR/Cas9 and TALENs for genome editing of iPS cells. They observed that CRISPR/Cas9 was more efficient than TALENs. However, it is still a long road to clinically applying CRISPR/Cas9 for gene therapy. We

must ensure the high specificity of CRISPR/Cas9 for target sites and eliminate possible off-target mutations with negative effects. Careful selection of target sites, delicate gRNA design and genome-wide search of potential off-target sites are mostly required.

12.8: Applications of the CRISPR/Cas9 System in Cancer Biology:

In the current era of cancer genomics, several large-scale cancer genome sequencing efforts have produced an expanding catalogue of the genetic alterations present in human tumors [119]. Amongst a background of so-called passenger mutations, which are presumed not to directly affect the tumorigenic process, driver mutations directly or indirectly promote the transformation of normal cells to cancer cells through mutational activation of oncogenes and/or inactivation of tumor suppressor genes. Oncogenes are typically activated via gain-of function mutations whereas tumor suppressor genes are usually inactivated via loss-of function mutations. Moderate to large-scale functional genetic studies aimed at dissecting the role of putative oncogenes and tumor suppressor genes in cell culture, xenografts, allografts and in some cases, transgenic mouse models have traditionally relied on cDNA-based over-expression and RNA interference (RNAi)-mediated knock-down approaches. While these approaches have led to many important discoveries in cancer biology over the last several years, they have several important limitations. First, cDNA-based expression systems can lead to supra-physiological levels of gene expression [24], which might cause aberrant and artifactual effects on signaling pathways and cell biological processes. RNAi-based inactivation approaches are limited by the uncertainty of the degree of gene silencing and the stability of the inhibition. This is not problematic for some targets or experimental protocols, but for others, complete and permanent inactivation is required to obtain consistent results. RNAi-based approaches can also suffer from substantial off-target effects. The deployment of the CRISPR/Cas9 system for targeted modification of endogenous loci offers a rapid method for overcoming these limitations. In addition to simplifying the study of oncogenes and tumor suppressor genes, the CRISPR/Cas9 system also allows for rapid discrimination between driver and passenger mutations.

Permanent Cas9-mediated modification of single or multiple endogenous loci can be achieved via transient or stable delivery of the CRISPR components. Several groups have reported successful editing of endogenous genes in cells in culture via transient transfection of plasmid DNA encoding Cas9 and sgRNAs 19–22 or Cas9-sgRNA ribonucleoprotein complexes (RNPs). Alternatively, CRISPR components can be stably delivered into cells through the use of retroviruses or Lentiviruses [67-70].

To engineer loss-of function mutations, one relies on NHEJ, which often results in the generation of indels near the Cas9 cleavage site that frequently lead to frameshift mutations. Engineering gain-of function mutations requires the inclusion of an HDR template in the form of single-stranded or double-stranded DNA carrying the desired mutation. Transient expression of the CRISPR components offers the advantage of a hit-and-run strategy, which should allow for unlimited serial editing of endogenous genes without the need for multiple viral integrations or continuous expression of CRISPR components. Cell lines carrying one or more targeted mutations can then be tested using a battery of cell-based and *in-vivo* assays to examine the effects of the mutation(s) on cancer-associated phenotypes. This approach can be used on established cancer cell lines, primary cell lines obtained from mouse or human origins, as well as patient-derived xenografts and organoid

cultures, among others. Moreover, this technology should allow for systematic analysis of epistatic interactions and comprehensive dissection of oncogenic signaling pathways via sequential or multiplex gene editing. In addition to allowing the functional characterization of true cancer genes, such studies can also help rule out a functional effect of a passenger mutation on cancer initiation and progression. Several review articles Hsu PD, *et al*., (2014); Doudna JA, *et al*., (2014) have recently described most applications of the CRISPR/Cas9 system in detail for genome engineering.

12.9: Live Imaging of the Cellular Genome:

The spatial organization of functional and structural elements within the cell contributes to the functional output of genomes, which can be amplified or suppressed dynamically. However, the way that genomes are modified and how their structural organization *in-vivo* modulates functional output remains unclear. Studying the interactions of specific genes given changing chromatin states would require a robust method to visualize DNA in living cells. Traditional techniques for labeling DNA, such as fluorescence *in-situ* hybridization (FISH), require sample fixation and are thus unable to capture live processes. Fluorescently tagged Cas9 labeling of specific DNA loci was recently developed as a powerful live-cell imaging alternative to DNA FISH. Advances in orthogonal Cas9 proteins or modified sgRNAs will build out multi-colour and multi-locus capabilities to enhance the utility of CRISPR based imaging for studying complex chromosomal architecture and nuclear organization.

12.10: Future Applications in Biomedicine and Biotechnology:

The CRISPR/Cas9 system of genome editing and manipulation has proved to be an exciting new development for the fields of molecular biology and translational medicine. The ability to use RNA guided endonucleases to target virtually any area of an organism's genome has led to significant improvements in our ability to study various aspects of the genome, including the importance and function of the genes themselves, as well as the regulatory components that control them. This technology has shown the potential to bring about a new age of gene therapy that could lead to the treatment of diseases that were previously thought untreatable. The ease of design and testing of Cas9 may also facilitate the treatment of highly rare genetic variants through personalized medicine. Supporting these tremendous possibilities are several animal model studies as well as clinical trials using programmable nucleases that already provide important insights into the future development of Cas9-based therapeutics.

13. Advantages of CRISPR/Cas9 System:

Everything that can be achieved with the CRISPR/Cas9 system principle also be achieved using either ZFNs or TALENs. Nevertheless, the appearance of such a large number of publications based on the CRISPR/Cas9 technology in such a short time, including virgin reports of genome editing in species such as sweet orange. Jia and Wang, *et al.*, (2014), highlights the clear advantages of CRISPR/Cas9 in terms of simplicity, accessibility, cost and versatility.

Unlike its predecessors, the CRISPR/Cas9 system does not require any protein engineering steps, making it much more straight forward to test multiple gRNAs for each target gene. Furthermore, only 20 nt in the gRNA sequence need to be changed to confer a different target specificity, which means that cloning is also unnecessary. Any number of gRNAs can be produced by *in-vitro* transcription using two complementary annealed oligonucleotides. This allows the inexpensive assembly of large gRNA libraries so that the CRISPR/Cas9 system can be used for high-throughput functional genomics

applications, bringing genome editing within the budget of any molecular biology laboratory. Unlike ZFNs and TALENs, the CRISPR/Cas9 system can cleave methylated DNA in human cells as described in Hsu *et al*., (2013), allowing genomic modifications that are beyond the reach of the other nucleases presented in Ding *et al*., (2013). Although this aspect has not been specifically explored in plants, it is reasonable to assume that the ability to cleave methylated DNA is intrinsic to the CRISPR/Cas9 system and not dependent on the target genome.

Approximately 70% of CpG/CpNpG sites are methylated in plants, particularly the CpG islands found in promoters and proximal exons (Vanyushin and Ashapkin, 2011). The CRISPR/Cas9 technology is, therefore, more versatile for genome editing in plants generally but particularly suitable for monocots with high genomic GC content such as rice as described in Miao *et al*., (2013). Conventional TALENs cannot cleave DNA containing 5-methylcytosine but methylated cytosine is indistinguishable from thymidine in the major groove. Therefore, the repeat that recognizes cytosine can be replaced with a repeat which recognizes thymidine, generating TALENs that can cleave methylated DNA albeit at the expense of target specificity [128-131]. The main practical advantage of CRISPR/Cas9 compared to ZFNs and TALENs is the ease of multiplexing. The simultaneous introduction of DSBs at multiple sites can be used to edit several genes at the same time [120-123] and can be particularly useful to knock out redundant genes or parallel pathways. The same strategy can also be used to engineer large genomic deletions or inversions by targeting two widely spaced cleavage sites on the same chromosome [121-131]. Multiplex editing with the CRISPR/Cas9 system simply requires the monomeric Cas9 protein and any number of different sequence-specific gRNAs. In contrast, multiplex editing with ZFNs or TALENs requires separate dimeric proteins specific for each target site.

Finally, the open-access policy of the CRISPR research community has promoted the widespread uptake and use of this technology in contrast, for example, to the proprietary nature of the ZFN platform. The community provides access to plasmids (e.g., via the non-profit repository Addgene). These facilities have encouraged newcomers to adopt the technology and contributed to the rapid progress in our understanding of the system and its practical applications.

14. Limitations of the CRISPR/Cas9 system

A limitation of the CRISPR/Cas9 system is its relatively low targeting specificity, which is determined by a 20 nt recognition site and the requirement for the neighboring PAM sequence (NGG) as described in Wei *et al*., (2013). This is further confounded by the fact that several mismatches within the target sequence can be tolerated, whilst still directing efficient cleavage [89-94]. Another issue with sgRNA design is that the efficiency of cleavage varies considerably at different target sites. This could be due to many reasons such as secondary structures within the sgRNA, the thermodynamic stability of the sgRNA-DNA duplex or accessibility of the target sequence within the context of chromatin. Rigorous studies of such effects have not yet been performed, and it is, therefore, important to design multiple sgRNAs for each desired target to maximize the chances of successful mutagenesis. Recent observations have also suggested that overexpression of Cas9 alone with the active GAL4 driver can result in toxicity. This suggests that even in the absence of sgRNA, there may be a degree of non-specific off-target mutagenesis, which should be borne in mind when analyzing Cas9-induced mutations. As the technique becomes more widely adopted, all of these problems will

be better understood, allowing us to minimize their effects.

15. Future Directions of CRISPR technology

Though CRISPR technology has come a long way in a very short amount of time, there are still many challenges that must be overcome for its value in applications to be fully realized. The foremost problems that must be overcome are addressing CRISPR specificity and developing effective and safe delivery systems.

The era of straight-forward genome editing raises ethical questions that will need to be addressed by scientists and society at large. How can we use this powerful tool in such a way as to ensure maximum benefit while minimizing risks? It will be imperative that non-scientists understand the basics of this technology sufficiently well to facilitate rational public discourse. Regulatory agencies will also need to consider how best to foster responsible use of CRISPR/Cas9 technology without inhibiting appropriate research and development. Despite the tremendous potential for CRISPR applications outside of research, there needs to be a dialogue to develop rules and protocols that protect against rash use of CRISPR that could irreversibly alter ecosystems. Nonetheless, the discovery of CRISPR is an immediate step-change improvement for researchers, with long-term implications that are promising, potentially risky, but currently undetermined.

16. Conclusion

CRISPR/Cas9 is a novel technique with a bright future in genomic editing. It has the potential to be useful in a broad range of applications from simplifying research to acting as a new form of gene therapy for patients with HIV and genetic diseases.

CRISPR is still a young system and more research must be completed to rectify its problems. While there are many challenges ahead before CRISPR/Cas9 can be utilized as safe and reliable gene therapy, these challenges do not seem insurmountable. Research in the area of CRISPR/Cas9 is gaining speed and this system could very well be the solution to many medical issues we face today.

17. References

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