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CRISPR/CAS SYSTEM: A REVOLUTION IN GENOME EDITING

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ABSTRACT
A major revolution in the field of biological research and translational applications has started with the advent of recent advances in genome engineering. The unique prokaryotic defence mechanism CRISPR/Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) system and its variants have proved to be a major technological breakthrough enabling diverse manipulation of genome functions. It has come forward as a more efficient and faster tool for effective sequence specific DNA targeting and genetic modification than the previous genome-editing methods. The current review highlights the development of various CRISPR engineering tools and their applications in functional genomics, cell biology research, crop development, creation of animal models and human therapeutic approach. With their innovative applications from basic biology to biotechnology, these tools are paving way towards future horizons. Offering ground breaking technologies, many CRISPR systems hidden in the genomes of different organisms and species around us may open doors in the future with their potential functions and mechanisms.

KEYWORDS: Clustered Regularly Interspaced Short Palindromic Repeats, Nonhomology End Joining, Homology Directed Repair.

INTRODUCTION
Eukaryotic cells manipulate unique networks of gene regulatory elements for carrying out functions involved in organelle formation and development, cell growth and death, microenvironment sensing and metabolite production. To understand dynamic and complex cellular processes, it is essential to have precise genome manipulation ability. In a broad perspective, genetic engineering explains different strategic approaches for altering genomic DNA sequence, transcriptional regulation, epigenetic editing and structural manipulation in the form of chromosomal structural reorganization. For carrying out any of these functions, a designer molecule, toolkit is needed to be conveniently constructed and delivered into the target cells to achieve the goals (Harrison et al., 2014).

Naturally occurring pathways and systems have come up as a great tool building resource. To modify DNA sequence at a targeted gene locus targeted, the discovery of homology directed repair (HDR) mechanism has proved to be a useful mean. This pathway enables the designing of DNA template with flanked homologous sequences which precisely recombine with the target gene locus. However, in mammalian tissues and cells, this mechanism is usually an inefficient process. By contrast, the mechanism of a double stranded break (DSB) can increase efficiency. Moreover, alternatively process of nonhomology end joining (NHEJ) offers another targeted gene elimination approach for eukaryotes by inducing random insertions or deletions at DSB site in the absence of template DNA (Belhaj et al., 2013).

Synthetically Engineered Endonucleases
The main concern in the field of genome editing was to make it possible to induce target specific DSBs for initiating repair mechanism of DNA. The molecules which allowed sequence specific DNA binding were of prime interest. One of these molecules were synthetically engineered endonucleases such as transcription activator-like effectors (TALENs) and zinc finger proteins (ZFNs). The mechanism used for the recognition of DNA-proteins could be used for designing peptide domains of these proteins. However, the utilization of this approach was obstructed by expensive construction process and the context dependency issue in the designing of protein (Gaj et al., 2013). However, recent work showed that these DNA binding synthetic proteins could be linked to nuclease domains, epigenetic modifiers, transcriptional activators and repressors enabling diverse forms of genomic manipulation. Nevertheless, even a simpler mechanism like Watson-Crick base pairing couldn't

explain the way to precisely target specific DNA sequence of interest (Fu et al., 2014).

CRISPR/Cas System: An Advanced Approach

Due to recent development in genome engineering and site-specific nuclease technologies, a mechanism has come forward as a game changer which is truly a gift from nature and performs all such functions, it is the CRISPR/Cas system. During 1980s, this system was discovered very first time in *Escherichia coli* (Ishino et al., 1987) but its elaborative function remained unknown until 2007. Bacterium *Streptococcus thermophilus* (Deveau et al., 2008) involved in yogurt production demonstrated that using the host CRISPR locus to encode the bacteriophage sequence confers acquired resistance against the same bacteriophage. Later researches showed that this novel system uses small RNAs like CRISPR-associated RNAs (crRNAs) for guiding Cas protein nuclease activity in *E. coli* (Ishino et al., 1987). Collectively, these researches revealed RNA guided nuclease activity of CRISPR system that suggests an efficient and highly specific genetic system of DNA binding and cleavage (Wang & Qi, 2016).

In the current review, a brief outline of the mechanism and function of CRISPR–Cas system in different organisms are elaborated. The role of CRISPR–Cas9 technology in gene editing and other important applications have been highlighted along with examples. The consideration to design an active CRISPR–Cas system in crop plants, animal models and human therapeutic approach has also been mentioned. In the end future perspective of this versatile technology has been briefly outlined.

An Overview of the advanced Technology

CRISPR/Cas system discovered as an immune system in bacteria *Escherichia coli* (Ishino et al., 1987), helps them in protecting against foreign DNA particles such as bacteriophages (Mojica, & Rodriguez-Valera, 2016). The Cas system consists of CRISPR locus and a Cas protein in the genome. A genomic locus of protospacer and direct tandem repeat sequences are derivatives of invading elements (Zhang, et al, 2014). This locus also contains sequences of trans activating CRISPR RNA, i.e., (tracrRNA) and non-coding RNA known as CRISPR RNA (crRNA). A complex of these two sequences with RNA sequences make up a guide RNA. It then directs the cleavage of site-specific nucleic acid sequences which is placed adjacent to a 50 -NGG sequence called Protospacer Adjacent Motif (PAM). The cleavage of double stranded DNA target sequence occurs close to protospacer. An endonuclease protein Cas9 associated with CRISPR loci causes the double strand breakage at target site under the direction of guide RNA (Xiong et al., 2015).

In 2012, the vast applications of molecular mechanism of the CRISPR/Cas system emerged as a potential candidate tool for genome editing (Jinek, et al, 2012). Later it was coined as RNA-guided engineered nucleases (RGENs) which used sequence specific nucleases for precise genetic modification. They are programmable nucleases that are further composed of two components, an engineered single guide RNA (sgRNA) and Cas9 nuclease that must be expressed in cells in order to perform genome editing. The sgRNA comprising of 20 nucleotides that direct Cas9 at the complementary target site. For novel genome editing applications, by altering the first 20 nucleotides of sgRNA, any N20-NGG form of DNA sequence can be targeted. This genome editing approach is utilized for the development of desired endogenous modifications such as gene addition, disruption, correction or deletion at one or more specific site by incorporation of nuclease mediated DNA cleavage using synthetic nucleases (Khandagale & Nadaf, 2016).

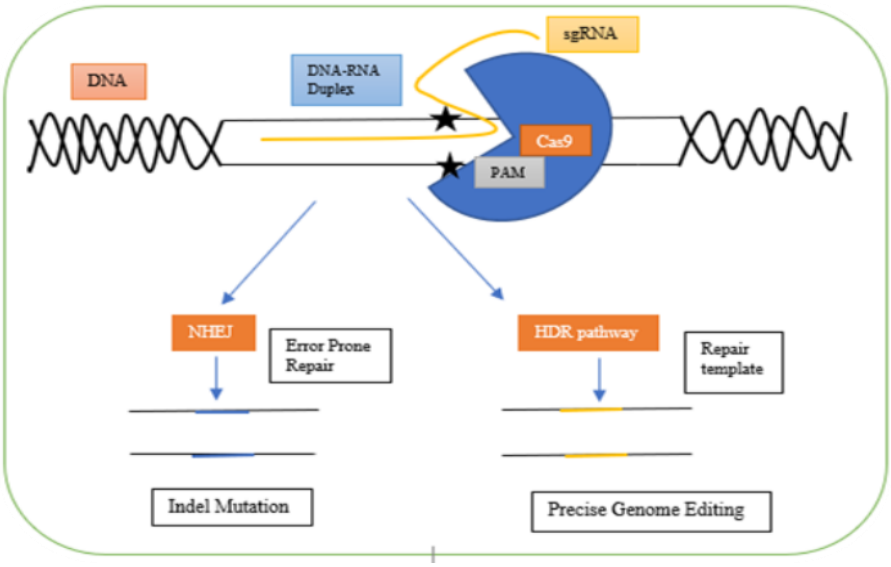

Figure 1: **sgRNA guided Cas9 endonuclease activity (A complex of gRNA and Cas9 binds with the DNA site keeping a distance close to PAM site. As a result, a DSB is formed which could be repaired via HDR or NHEJ**

repair mechanisms. (A) The DNA repair using NHEJ mechanism causes insertions or deletions or frameshift which results in gene knockout by interruption. (B) If in the end, a DNA donor with homology is provided, it can modify the gene on target site by adding nucleotides and leading to insertion of cDNA.) (gRNA: guide RNA; DSB: double-strand break, PAM: protospacer adjacent motif; HDR: homology-directed repair, NHE: non-homologous end joining)

The desired modification in a broad range of cell types and organisms can be done using cellular recombination repair mechanism after double strand break. Many fundamental discoveries in biology have been originated by the elucidation of CRISPR-Cas9 mechanism. As a fledgeling technology, the CRISPR genome editing technique owing to its ease of design and simplicity has reinvented the molecular and genetic biology research. There are available many sgRNA and Cas9 variants which could serve for future novel applications especially in the domain of plant biotechnology (Feng *et al.*, 2013).

Mechanism of Action

The pathogen's genetic material is recognized by type II CRISPR-Cas system in three stepwise stages that are acquisition, expression and interference. The process of "Acquisition" includes recognition and incorporation of foreign genetic material as protospacer within the locus of CRISPR. Generally, it consists of 2-5bp short stretch of conserved nucleotides PAMs that serves as a motif of recognition for incorporation of DNA fragment. Approximately 30bp long single copy of spacer is inserted and duplicated at the leader side of CRISPR. CRISPR mediated immunity can be prevented against pathogenic attack if mutations are caused in the viral genomic PAMS (Sander & Joung, 2014).

The second step includes the Expression phenomena in which CRISPR locus actively transcribes long pre–crRNA and with the help of Cas proteins (Cas1, 2, 9 and 4/n2) and the tracrRNA molecule, process them into mature crRNA. Recently, the role of tracrRNA in pre-crRNA processing was reported in Streptococcus pyogenes. With the repeat regions of crRNA, the tracrRNA pairs up via base complementarity and speed up the pre-crRNA to crRNA processing. This processed mature crRNA makes an entry into the complex of CRISPR for antiviral defence and helps in recognizing and pairing up with the target site of foreign DNA (Jore *et al.*, 2009).

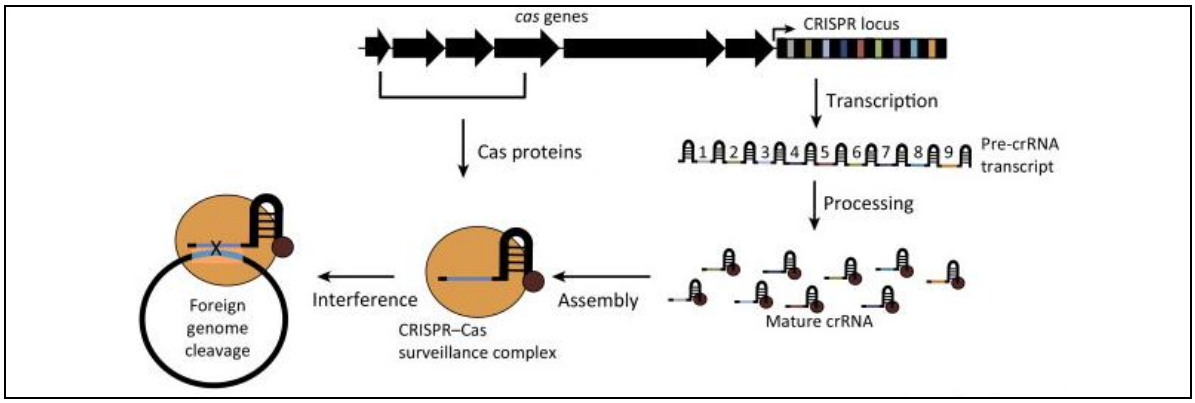

Figure 2: Mechanism of action of CRISPR/Cas system (Joseph B., & Alan R., 2014).

In the third step called "Interference", the Cas protein complex is guided by the crRNA to the target site of foreign DNA for breakage and confers immunity against pathogenic attacks (Deveau et al., 2010). For the purpose of genome editing in different organisms, the type II CRISPR-Cas system has been manipulated. The construction and design of CRISPR-Cas9 complex is cheap, straightforward and devoid of barriers of intellectual property. The components of this complex i.e., trcrRNA and crRNA can be fused into guide RNA to lead Cas9 for inducing site specific DSBs (Horvath & Barrangou, 2010).

The single guide RNA designing is also simple so it is preferred for gene editing. Initially, the type II CRISPR-Cas system was designed for inducing cleavage at different DNA sites in vitro. Recently this technology has been used for genome editing in yeast, bacteria and other organisms for efficient site-specific mutagenesis. The efficiency of target mutation of this system has found to be similar to TALENs and ZFNs in vivo (Kumar & Jain, 2015).

Comparison of Cas System with Conventional Techniques

Previously used genome editing techniques like engineered transcription activator-like effector nucleases (TALENs) and as zinc finger nucleases (ZFNs), both of them depends for DNA double strand break on the nuclease domain of *Fok* I endonucleases. Comparative to TALENs and ZFN, CRISPR/Cas9 technology is easier and simpler to manipulate and has a vast range of applications. For example, ZFN comprised of an array of Cys2–His2 domains of ZF, each finger binds to specific

PAMs that results in difficulty in selection of target sequence. To locate a unique DNA sequence of 18-24 bp region, two ZFNs form a dimer while working. The applications of TALENS and ZFN remain restricted, owing to the risks of off-target, context-dependent binding requirements and difficulty in DNA-binding proteins engineering modular. (Song *et al.*, 2016).

CRISPR Engineering Tools

Cas9 Nuclease and its variants

The endonuclease Cas9 comprised of two different domains that include a large globular recognition functional domain (REC) linked to a smaller (NUC) nuclease domain. The latter domain further consists of two nuclease sites HNH and RuvC, also a PAM-interacting site. Upon the loading of guide RNA, Cas9 protein is activated which further undergoes a conformational rearrangement forming a central channel for canonical PAM motifs recognition and RNA-DNA heteroduplex binding (Belhaj et al., 2015) The crystal structure study of Cas9 protein, elucidated the mechanism of nuclease activity of this protein in a complex with partially duplex site-specific DNA containing sgRNA and PAM motif. This facilitates the insight into different strategies of creating variants of Cas9 proteins with novel PAM specificities (Fauser et al., 2014). Many variants of this protein are still available today that can be used for silencing, high throughput genome editing and transcriptional control along with decreased off target effects and enhanced specificity in various systems from Drosophila (Yu et al., 2013), yeast (DiCarlo et al., 2013), bacteria (Oh et al., 2014), HEK293T in humans (Pattanayak et al., 2013), zebrafish (Xiao et al., 2013) and wheat (Shan et al., 2013). There are various other organisms and cell types modified by Cas9 nuclease such as embryonic cells in Rabbit (Yang et al., 2014), Rat (Li et al., 2013), Frog (Nakayama et al., 2013), silkworm (Wang et al., 2013) and Sorghum (Jiang et al., 2013).

Native Cas9

The native Cas9 creates DSB that can be repaired by either Non-homologous ends joining or Homologous repair methods. Specific point mutations such as nucleotide substitution or insertion of desired sequence through target locus recombination with exogenously supplied DNA strands can be created using HR mediated repair. The efficient deletion or insertion mutation known as indels, can cause frame shift in translational coding sequence or the binding sites of transcription factors in enhancers or promoters. The high alterations induced by Cas9 mediated DSB makes the identification of desired mutations easy without using drug-resistance marker selection (Xing et al., 2014).

Cas9 Nickase

A Cas9 nickase variant (Cas9n) was developed by inducing mutation (by substituting aspartate to alanine) in native Cas9 by (Cong et al., 2013). With the HNH or RuvC mutation, the Cas9 nickase is capable of creating a nick instead of causing DSB. Using high fidelity homology directed repair (HDR), the individual nicks can be repaired. Along with two different gRNA, this protein has been used in a paired nickase system to increase the number of specifically recognized bases for target breakage that has helped reduced off target phenomena and improved specificity. In human cells, this system has increased the efficiency of HDR with decreased off target cleavages by 50 – 15000 folds without affecting the efficiency of on-target cleavage (Khatodia et al., 2016). There are several examples of Cas9 Nickase gene modification in HEK293FT locus in humans (Mali et al., 2013) and in mice embryonic cells (Ran et al., 2013). Alternatively, one can transfect or microinject in vitro engineered gRNA or Cas9 mRNA into plant protoplast or animal embryos. This DNA free gene editing strategy has stimulated advanced breeding technologies based on CRISPR/Cas system. However, the protoplast regeneration capacity for most of the plants is very low so the direct injection method is applicable to a few plants only. This technology is a potential platform to manipulate genome for different purposes in addition to the genome editing tool (Ding et al., 2016).

CRISPR Interference as an Editing Tool

Another strategy known as CRISPR interference has been developed in plants for RNA guided efficient and stable target gene transcription modulation by fusing effector domains and inactivated dCas9 proteins. In functional genetics, the dCas9 has been used for novel synthetic biology applications and regulation of gene expression (Khatodia et al., 2016). With the repressor or activation domain of TF, as a fusion protein, the dCas9 has been directed to specific DNA sequences by gRNAs. In *Nicotiana benthamiana* (Li et al., 2013) transcription of both the endogenous PDS gene and a reporter construct have been formulated by the fusion of the SRDX domain as a repressor and the dCas9 C-terminus as a transcription activator to the EDLL domain. For efficient sgRNA dependent reversible and inducible gene expression inhibition, the recognition complex of sgRNA/dCas9/effector interferes with transcriptional regulation. Gilbert et al. (2015) observed that the CRISPRi effective target site should lie from −50 to +300 bp relative to the Transcription Start Site (TSS) of a gene. Gene expression is modulated over a ∼1000-fold range using CRISPR activator (CRISPRa) system with one binding site by the expression of a single sgRNA. In plants, for mapping complex stress-related signalling pathways, generation of CRISPRa libraries and genome-scale CRISPRi would come out to be a powerful tool for analysis of functional genomics (Khatodia et al., 2016).

Multiplex Genome Editing

CRISPR/Cas9 in plants have been adapted for the fast assembly of plasmids that encode genome editing system components. For *Agrobacterium* based expression, the construction of CRISPR/Cas9 plasmids in plants was first streamlined by Xing et al., (2014). To combine

gRNA targeting sequence amplified by PCR into a single vector, Golden Gate cloning technology can be used. The final vector consists of two gRNA behind promoters U6 and a codon optimized sequence of Cas9 of maize behind and ubiquitin promoter (Cong *et al.*, 2012). It has been demonstrated that DNA can be efficiently edited in maize and Arabidopsis plants by this tool. Mutations were heritable till the T2 generation in case of Arabidopsis. Another toolkit that has a feature of PCR based protocol with construction of all components by Golden Gate cloning in one step has been reported (Lowder et al., 2015). It is possible to carry out high efficiency mutagenesis in rice and heritable mutations in Arabidopsis by using this kit. A tool box of CRISPR/Cas9 system devoid of the need for PCR amplification was demonstrated in plants and it can also regulate transcriptional expression with dCas9 fusion (Xing *et al.*, 2014). CRISPR/Cas9 systems assembly with conventional cloning approaches was recently demonstrated by two publications. In one case, a single vector was used for constructing six gRNA expression cassette with Cas9 in three steps. Although its efficiency was not so high, still it managed to simultaneously edit six target sites. In another research, an iso-caudamer technique along with restriction enzymes of high compatibility was also developed. In rice, three genes were targeted simultaneously using this approach. This system allows for countless gRNA expression cassette stacking although it's laborious and time taking (Shan et al., 2015).

Cpf1 Genome Editing tool

Similar to the type II CRISPR/Cas system, Cpf1 (CRISPR originated from Prevoltella and Francisella) has emerged as a new gene editing tool. This protein functions in target site recognition, crRNA processing and DNA cleavage, however, it differs from Cas9. It recognizes T-rich PAM sequences, PAM sequence is located upstream of protospacer sequence, it's a ribonuclease guided by crRNA only. A type V CRISPR effector Cpf1 recognizes T-rich protospacer adjacent motif and using crRNA introduces DSBs at target sites. In a study, it has been demonstrated that DNA free editing of plant genome can be carried out using Cpf1 (Tang et al., 2017). Using intro-transcribed or chemically synthesized target specific crRNA, recombinant Cpf1 has been delivered into protoplast isolated from wild soybean and tobacco. Engineered crRNA appear to be unique and show no similar sequences in the entire reference genome of soybean. Deep sequence analysis of targeted sequence showed that mutations occur successfully in AOC in wild tobacco and FAD2 soybean paralogues. Unlike Cas9, this novel protein introduces target site deletions with no potential off-target mutations in soybean genome. These results strengthen Cpf1–crRNA complex, a candidate gene editing tool for DNA free genome modification (Kim et al., 2017). The Table 1 shows the application of different variants of CRISPR/Cas system in various organisms and their cell types modified using this advance approach.

APPLICATIONS OF CRISPR GENE EDITING IN CELL BIOLOGY

CRISPR in Crop Improvement

Plants provide us with food, medicine, biofuels, animal feed, chemicals and renewable materials. Efforts are being made to develop strategies for crop improvement and modifying their properties. Existing natural genetic variation is the basis of conventional breeding and to modify the selected traits into an elite form, extensive back crossing strategies are needed. Therefore, the limitations to the extreme potential of this approach are due to the unavailability of beneficial alleles in nature. By random mutagenesis, new alleles can be introduced but this includes time consuming screening of desirable traits into the large population of mutants. Plant breeding, crop improvement and introduction of new properties can be accelerated by genome editing and allowing the incorporation of predictable and precise modifications directly into the target site. Due to the ease of simultaneous modification of multiple traits, this technique is of great importance (Brook *et al.*, 2014).

CRISPR/Cas mediated Gene knockout

Knocking out genes that negatively affect the quality of food using the simplest form of targeted modifications conferring susceptibility to invading pathogens or to distract metabolic flux from important end products (Gaj *et al.*, 2013). For example, targeting the mildew-resistance locus (MLO) genes of wheat using CRISPR/Cas and TALEN technologies, all the three *MLO* homoalleles were successfully knocked out producing powdery mildew disease resistant plants. (Bortesi & Fischer, 2015).

The basic step in plant genetic analysis is the functional knock out of target gene. Indels could be readily induced into the coding region using Cas9/gRNA system which will result in translational disruption. Indeed, this system has been very successfully used for knocking out gene in tobacco (Mercx et al., 2016), rice (Miao et al., 2013), sorghum and Arabidopsis (Fauser et al., 2014). The study of these early proof of concept proved that Cas9/gRNA using variable efficiency functionally knocks out target genes by creating Indels at specific sites. Further analysis of rice and Arabidopsis showed that the modified gene is passed onto the next generation. CRISPR/Cas9-mediated genome editing has been carried out in the past 3 years in many valuable crops like wheat (Zhang et al., 2016), tomato (Pan et al., 2016), soybean (Cai et al., 2015), sweet orange (Jia & Wang 2014), poplar (Fan et al., 2015) and petunia (Zhang et al., 2016). This strategy has become the primary choice for developing plant knockout mutants (Ding et al., 2016). In a study, expression of Cas9/sgRNA system was demonstrated in two dicot species like tobacco and Arabidopsis and two monocots crop species like sorghum and rice. The results showed successful Cas9/sgRNA mediated mutagenesis at the target sites. The successful demonstration of this system in model crops shows its potential use in genetic

engineering for agricultural and scientific applications (Jiang et al., 2013).

Table 1: Organisms and cell types modified by CRISPR-Cas9 system.

Organism	Cell type	DNA Repair	CRISPR Variants	Reference
Mammals				
Vertebrates				
Humans	HEK293FT	NHEJ/HDR	Cas9 nuclease	(Pattanayak et al., 2013)
	HEK293T	NHEJ/HDR	Cas9 nuclease	(Mali et al., 2013)
	HUES1	NHEJ/HDR	Cas9 nuclease	(Mali et al., 2013)
	BJ-RiPS	NHEJ/HDR	Cas9 nuclease	(Fu et al., 2013)
	Jurkat	NHEJ/HDR	Cas9 nuclease	Ebina et al., 2013)
	HEK293	NHEJ/HDR	Cas9 nuclease	(Cong et al., 2013)
	U2OS	NHEJ/HDR	Cas9 nuclease	(Cho et al., 2013)
	HUES9	NHEJ/HDR	Cas9 nuclease	(Sander & Joung, 2014)
	HeLa	NHEJ/HDR	Cas9 nuclease	(Ding et al., 2013).
	iPSC	NHEJ/HDR	Cas9 nuclease	(Ding et al., 2013)
	K562	NHEJ/HDR	Cas9 nuclease	(Mali et al., 2013)
	Embryos	NHEJ/HDR	Cas9 nuclease	(Ran et al., 2013)
	HEK293T	HDR	Cas9 nickase	(Mali et al., 2013)
	HEK293FT	HDR	Cas9 nickase	(Cong et al., 2013)
	HEK293T	HDR	dCas9 (imaging)	(Chen et al., 2013)
	HeLa	HDR	dCas9 (imaging)	(Chen et al., 2013)
	UMUC3	HDR	dCas9 (imaging)	(Chen et al., 2013)
Mouse	Embryos	NHEJ/HDR	Cas9 nickase	(Ran et al., 2013)
Rat	Embryos	NHEJ/HDR	Cas9 nuclease	(Li et al., 2013)
Zebrafish	Embryos	NHEJ/HDR	Cas9 nuclease	(Hwang et al., 2013)
Rabbit	Embryos	NHEJ	Cas9 nuclease	(Yang et al., 2014)
Frog	Embryos	NHEJ	Cas9 nuclease	Nakayama et al., 2013)
Invertebrates				
Fruitfly	Embryos	NHEJ/HDR	Cas9 nuclease	(Bassett et al., 2013)
Silkworm	Embryos	NHEJ	Cas9 nuclease	(Wang et al., 2013)
Plants				
Wheat	Protoplast	NHEJ	Cas9 nuclease	(Shan et al., 2013)
Tobacco	protoplasts,leaf tissue	NHEJ/HDR	Cas9 nuclease	(Nekrasov et al., 2013)
Sorghum	Embryos	NHEJ	Cas9 nuclease	(Jiang et al., 2013)
Thale Cress	protoplasts, seedlings	NHEJ/HDR	Cas9 nuclease	(Li et al., 2013)
Rice	protoplasts, callus cells	NHEJ	Cas9 nuclease	(Shan et al., 2013)
Sweet Orange	Leaf tissues	NHEJ	Cas9 nuclease	(Jia & Wang 2014)
Corn	protoplasts	NHEJ	Cas9 nuclease	(Liang et al., 2014)
Liver wort	Embryo	NHEJ	Cas9 nuclease	(Sugano et al., 2014).
Prokaryotes				
Bacteria	*E. coli/ Streptococcus pneumoniae*	HDR	dCas9 (gene regulation)/Cas9 nuclease	(Jiang et al., 2013)
Eukaryotes				
Yeast	*Saccharomyces cerevisiae*	HDR	Cas9 nuclease	(DiCarlo et al., 2013)
Fungi	*Pichia pastoris*	NHEJ	Cas9 nuclease	(Weninger et al. 2016)
Algae	*Chlamydomonas reinhardtii*	NHEJ	Cas9 nuclease	(Jiang et al., 2014).

Generating Animal Models with CRISPR

Synthesizing genetically modified animal models is very crucial for studying complex physiological and cellular processes. Although the genetic editing approach using CRISPR/Cas system has been established in many animal models but the mouse models are most widely produced using this advanced approach to test the effect of mutations. Using animal models other than mice can accelerate the development of novel therapeutic strategies and consequently prove advantageous to biomedical research (Le *et al*., 2013).

In-Vivo Approach in Mice Model

Compared to conventional gene-editing technologies, CRISPR has emerged as an easier approach for creating animal models. Moreover, target specific guide RNAs and Cas9 proteins can be more conveniently inserted into embryos for generating transgenic mice with the applications of multiple gene knocked out, defined gene mutations or introduction of peptide tags or fluorescence reporters to endogenous genes. For instance, co-injection of sgRNA and mRNA targeting two genes into zygote created mice with mutations of biallelic nature in both genes showing an efficiency of 80%. Furthermore, the applications of Cas 9 in vivo have been broadened by the development of Cre-conditional Cas9 knock-in mouse. This model is a great resource for rapid generation of mutations in sub-populations of in-vivo cells and test the effect of mutations on disease phenotype. In another research, a Cre-dependent Cas9 knock-in mouse was generated in vivo as well as ex vivo using lentivirus, adeno-associated virus (AAV), or particle mediated delivery of gRNA in immune cells, neurons and endothelial cells targeting three genes in lung adenocarcinoma. The results suggested that this mice model give a wide range of disease modelling applications (Platt et al., 2014).

In another approach, a single injection of AAV9-Cas9/sgRNA at postnatal day 4 and 42, it was observed that the function and morphology of the heart were restored in H530R PRKAG2 knock-in and transgenic mice. It also showed that by selective disruption of disease-causing mutations, the in-vivo CRISPR/Cas9 gene editing technique is an important tool for PRKAG2 cardiac syndrome treatment (Xie et al., 2016). To probe gene function, viral vectors encoding sgRNA and Cas9 other than Cas9 knock-in mice model, can be directly inserted into Cre/loxP mice model or wild type models. In another research for studying cancer genes in the liver of mouse, a plasmid DNA expressing sgRNA and Cas9 was delivered using hydrodynamic injection that precisely targeted the tumour suppressor genes. From these results, it is proven that this strategy can be used for studying liver cancer disease (Wang & Qi, 2016).

In-vivo approach in Primate Models

For establishing transgenic mice models, transcribed sgRNA and Cas9 protein can be directly inserted into the fertilized zygote in monkey and rodent models for achieving heritable gene modification. The generation time can be decreased from a year to only few weeks of mutant rats and mice by by-passing the ES cell targeting stage in the process of producing genetically engineered lines. Such advancements will not only facilitate large scale in vivo and cost-effective mutagenesis in rodent models but can also be associated with highly precise editing to avoid off-target mutations. In cynomolgus monkey models, successful multiplex targeting was recently reported showing the potential for creating primate models for more complex human diseases including neuropsychiatric abnormalities. Furthermore, precluding the need for therapeutic use of gene therapy and embryonic manipulation, Cas9 can be used in direct somatic tissue modifications (Hsu et al., 2014). In another study, using genome editing technique for gene correction appeared to be a promising technique for the treatment of Duchenne muscular dystrophy (DMD) using adeno-associated virus mediated CRISPR/Cas9 mouse model with the aim of improving histology and function of DMD (Mendell et al., 2016).

In-Vivo Approaches in Other Organisms

Not only animal models but many other organisms have been used as a candidate models for studying the intervention of CRISPR/Cas system and consequent attributes of gene editing techniques. In a recent study, RNA guided nucleases have been used in vivo in zebrafish embryos to introduce specific genetic modifications that were comparable to those obtained from conventional techniques (TALENs and ZFNs) for the same genes. This technique enabled gene editing at 9 out of 11 targeted sites even including those that were failed to show alterations in TALENs. This system offers a rapid, simple and highly scalable method for gene alteration in-vivo making RNA guided nucleases a useful tool in a wide range of organisms for gene modification (Hwang et al., 2013).

It has been reported that Genome modifications through Cas9 system are efficiently transmitted through germline when Drosophila genome was precisely engineered using prokaryotic CRISPR RNA/Cas9 system. For probing gene function in Drosophila, this system can be reprogrammed for generating targeted alleles (Gratz et al., 2013).

With single-guide RNAs, CRISPR/Cas system is reported to be used for precise gene conversion and disruption in various biological systems. The use of endonuclease Cas9 for targeting genome sequence using single-guide RNAs has been reported in the *C. elegans* germline, obtained from U6 promoter of a small nuclear RNA promoter. It shows, targeted genetic modifications in *C. elegans* offers an effective and convenient approach for producing mutants with function loss (Friedland et al., 2013).

CRISPR in the treatment of Genetic Disorders

In a study, a group of researchers from China used CRISPR genome editing technology in germline of human embryo for eradicating human β-globulin gene, a major cause of β-thalassaemia. This research was abandoned and led to serious debate on the ethical implications of this novel technique in human germline modification (Mo, 2015). In another report on using Cas9 mediated gene editing for the prevention of genetic disorders in next generation offspring by targeting human embryos raised serious social concerns over the misuse of this technique for human enhancement (Ishii, 2015; Baltimore et al., 2015).

Another major success in the field of therapeutics through genome editing is by using Cas9 ribonucleoproteins that decreases the integrational mutagenesis and unwanted gene targets through the common strategies of gene delivery. In a study the combination of carrier nanoparticles and Cas9 proteins were used for direct cytoplasmic delivery with highly efficient (~90%) construct showing (~30%) gene editing efficiency gives a platform for genome dynamic study (Mout et al., 2017) (Feng et al., 2013) (Mahfouz et al., 2014) (Reis et al., 2014). Using appropriately designed sgRNA and plasmids carrying Cas9, the cellular models can be generated by transfecting target cells. Moreover, the multiplex genome editing attributes of Cas9 promises a potential platform for studying different polygenic human diseases that includes heart diseases, diabetes, autism and schizophrenia (Cai et al., 2016).

Efforts have been made to use a structurally optimized sgRNA and EGFP labelled endonuclease-deficient Cas9 to show genomic images of repetitive elements in coding genes and telomeres of living cells. Additionally, in living human cells, CRISPR imaging tool enables to improve the ability to study dynamics and conformation of native chromosomes (Chen et al., 2013). For Genome editing, 29-nucleotide based chemically modified synthetic crRNA developed in combination with unmodified trans RNA proved to be an alternative to the natural gRNA through CRISPR/Cas system. These promising results open a door for therapeutic applications through engineered crRNA to suppress/activate Cas9 endonuclease activity for nervous system disorders (Rahdar et al., 2015). Haematological diseases can be treated using CRISPR/Cas9 mediated gene therapy by removing or correcting mutated genes (Zhang & McCarty, 2016). This technology also promises epigenetic regulation through RNA guided CRISPR /Cas toolkit advancements (Vora et al., 2016; Nissim et al., 2014).

CONCLUSION

The importance of basic scientific research has been highlighted by the revelation of a mysterious bacterial defence system which has emerged as one of the most versatile and powerful platforms for genetic engineering. Just like recombinant DNA technology gets benefits from restriction enzymes which is critical to the warfare between bacteria and phage, the CRISPR genome editing technology also relies on the components of bacterial anti-phage defence system. The accessibility and simplicity of CRISPR technology give advantages over conventional genome editing techniques. It shows that it is now affordably possible on a genomic scale to screen out function loss. Further advancements in our knowledge of this technology are likely to show up rapidly, leading to the discovery of new generation of genome editing technologies. Offering groundbreaking technologies, many CRISPR systems hidden in the genomes of different organisms and species around us may open doors in the future with their potential functions and mechanisms. It will allow the increasing amount of genomic biology data to be manipulated more comprehensively, fasting up both gene discovery and trait development in plant species. It has a wide range of applications in eukaryotes, vertebrates, invertebrates, mammals and humans. Looking towards the futuristic approach, CRISPR/Cas9 technology envisions to be bringing advances to the basic clinicians and bioresearches promising approaches.

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