CRISPR CAS9 SYSTEM: A NOVEL GENOME EDITING TOOL

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ABSTRACT: *In a recent era of modernity, a lot of trends have been set in Science and Technology. Despite of vast discoveries always some gaps exist. To bridge up them new trends are set which are commonly known as Emerging Trends. Although a number of emerging trends have been established but they are specific in their nature to fix the emerging or previous issues. Now a days, in the arena of Biological Sciences, CRISPR-Cas9 Technology an emerging approach has been agreed to accomplish the task associated with DNA. This is one of the best discoveries in Biological Sciences known to date which made the incorporation of desirable traits in eukaryotes more feasible. Moreover, these traits will be target specific rather than of random insertion of any desired gene in any organism's genome. This revolutionary technology is easy to handle, environment friendly and easily executable in every biological field including agriculture, health, virology and genetic disorders.*

Keywords*:* CRISPR/Cas9, DNA, Biological Sciences, HR, NHEJ, sgRNA, TALEN, ZFN

INTRODUCTION

Background of CRISPR Cas9 system

The word "CRISPR-Cas9"is abbreviated as Clustered Repetitive interspaced short Palindromic Repeats. Cas9 is a multi-domain nuclease protein [1].CRISPR-Cas9 technology is a genome editing approach involving the insertion, deletion and substitution of a specific targeted genome sequence [2]. It has a prokaryotic origin and initially it was reported in *Escherichia coli* (1987) and Streptococcus pyogenes (1991). Subsequently it is also recognized in archaea in 1993. In 2002, a group of Netherland "Francisco J.M. Mojica" coined the acronym "CRISPR". Finally, Jennifer Doudna introduced the CRISPR Cas9 system as a new efficient genome editing tool in 2013 in Streptococcus pyogenes. And in the same year first report on genome editing in mice by employing CRISPR Cas9 system was published [3-6]. For this purpose mouse embryonic stem cells were harvested. CRISPR/Cas9 system is used as a type II adaptive immune system [7] in Streptococcus pyogenes to mask themselves from bacteriophages.

Classification and Assembly units of CRISPR System

CRISPR Cas9 system is classified into three classes. However, class II has been well documented so far and others remain to be elucidated. CRISPR categorization depends on type's specific signature known as Cas9 protein [8]. Type I and III class possess some common attributes like maturation and assembly units. Due to these characteristics Type I and Type III systems are different from Type II system [9]. Type II system is easy to schematize as compared to type I and III system which makes it acceptable as worldwide. CRISPR Cas9 system comprises of (i) Cas9 enzyme (ii) CRISPR RNA (crRNA) (iii) trans-activating crRNA (tracrRNA). Cas9 endonuclease form complex with both RNA components. crRNA plays its role in target recognition. Whereas, tracrRNA plays its role in crRNA maturation and stabilization of Cas9 protein during complex formation[9].CRISPR and tracrRNA have non-coding ability and partially complement to each other [10]. Both RNA components form one chimeric RNA termed as single guided RNA (sgRNA) [11]. Now, CRISPR system components have been reduced to two (i) Cas9 endonuclease and (ii) sgRNA [9, 11, 12] whereas sgRNA recognizes target DNA sequence as well direct the endonuclease to cleave it. Cleaved DNA ends were then re-united to each other either through non homologous recombination (NHR) or homologous recombination (HR)[13].

Specific desired changes in target DNA can be accomplished through Homologus Recombination (HR).This is due to knock in of target DNA sequence in which desired DNA sequence replaces the parent sequence at the targeted region. In the same way Indels or frame shift mutations are induced by Non Homologus End joining (NHR) [14]. Indels or single nucleotide mutations are responsible for knock out of target DNA sequence in which gene expression is completely lost. One thing that make a DNA target specific for selection is presence of specific nucleotide sequence known as Protospacer Adjacent Motif (PAM) [9]. PAM motif must be present in targeted sequence because it helps CRISPR/Cas9 system to differentiate between self and non-self. PAM sequence is also reported to be involved in earlier association of CRSIPR/Cas9 complex with the target region and it is the place where separation of targeted DNA strands is initiated. Position and sequence of PAM motif is CRISPR/Cas9 type specific. PAM usually starts with 5′-NGG-3′ and it is present at downstream region of target sequence [15]. Cas9 endonuclease has two independent Ruvc and HNH domains. Both these domains were activated when CRISPR/Cas9 bind with PAM sequence. HNH nuclease is reported to be involved in cleavage of complementary strand while Ruvc cleaves the non-complementary strand resulting in the blunt end formation which after re-union gives frame shift mutations or indels [9]

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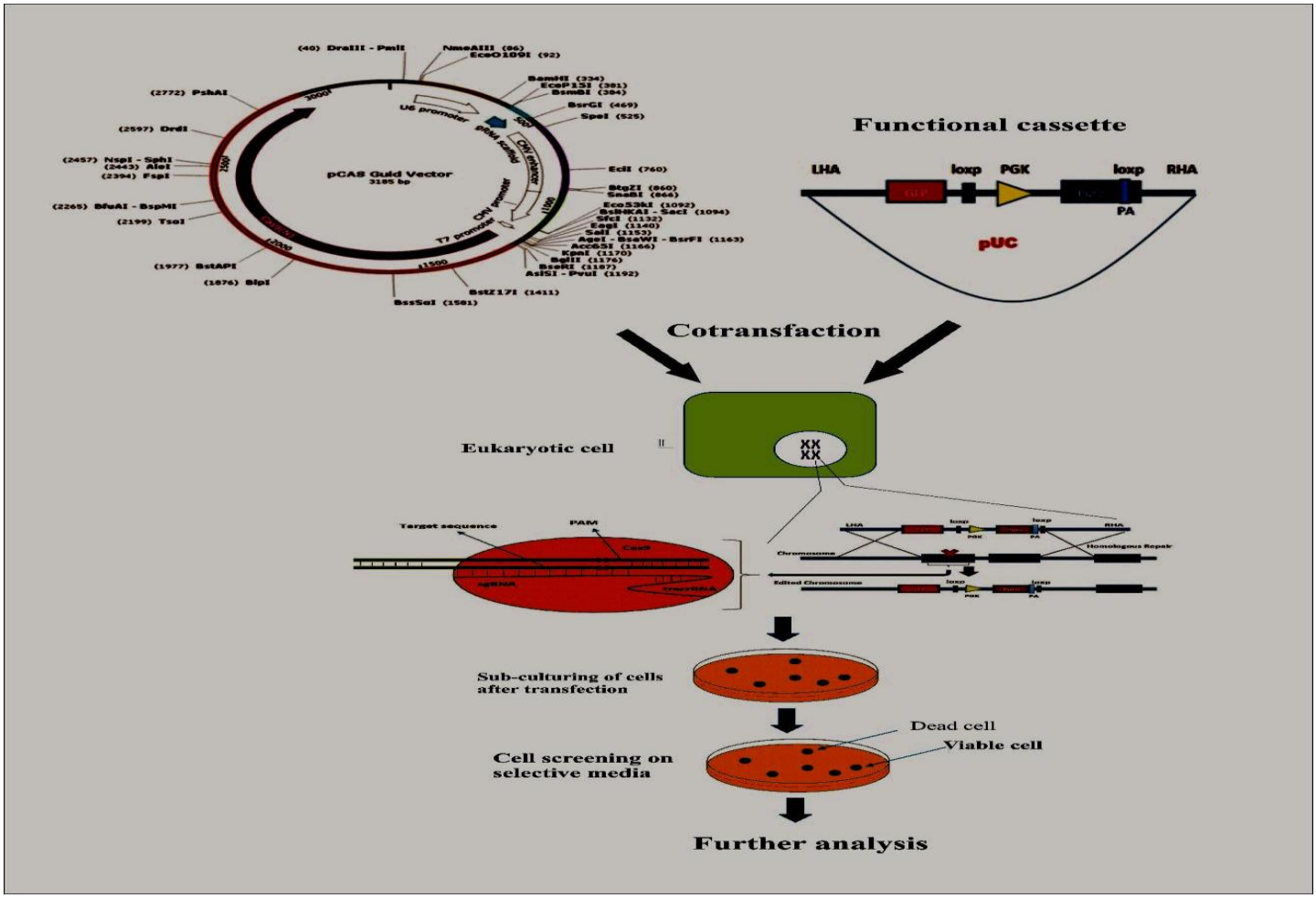

Figure 1. A general layout of CRISPR/Cas9 expression process and analysis

A bioinformatics approach is used to design sgRNA against a specific target region. This is done by using online software (http://tide.nki.nl/, http://crispr.mit.edu/). TIDE acronym abbreviates as Tracking of Indels by decomposition. TIDE was developed by Netherlands cancer institute used to assess genome editing by CRISPR-Cas9 system while crispr.mit.edu (Zhang Lab,MIT 2015) is used to design target guide RNA with high specificity and also highlights potential offtargets.sgRNA should be adjacent to PAM sequence. After that, sgRNA and Cas9 are ligated into a vector (pCas guide vector) under U6 and Cauliflower Mosaic Virus (CMV) promoter. Later this vector is transformed into embryonic stem cells. For transformation virus based delivery method (adenovirus, lentil viral vectors, and baculovirus), electroporation, nucleofection, agro-bacterium and liposomal delivery based methods are used. Consequently there will be hybridization sgRNA: target RNA and this will facilitate the nuclease protein to produce a double stranded break in targeted region. Broken ends will join to each other through non Homologus recombination and it will generate knock out. A number of model organisms have been employed for genome editing including Mouse, Zebra fish, Arabidopsis, Tobacco, Drosophila, Human, Monkey and *Xenopus tropicalis* [16].

Comparative Analysis of CRISPR Cas9 System to Obsolete genome editing Techniques.

CRISPR Cas9 system has a lot of merits as compared to other old fashioned genome editing techniques. *i.e.*, Zinc finger nucleases (ZFNs) and Transcription activator like effectors nucleases (TALENs). In ZFNs and TALENs chimeric proteins are programmed against target DNA sequence in every experiment and this was the major issue associated for the wide spread of these genome editing techniques. But in CRISPR/Cas9 system there is no need to design Cas9 protein to target DNA sequence. It employs its own RNA guided nuclease proteins to cleave the specific target site [17]. Additionally, success rate of CRISPR/Cas9 system is too high as compared to ZFNs and TALENs. CRISPR/Cas9 system has very low off target effects as compared to ZFNs and TALENs. Furthermore, CRISPR/Cas9 system can be used for multiple genome engineering. This concept is based on designing of many sgRNA which leads to the editing of multiple target sites simultaneously. Usually multiplexing is used for more complex target genes. Any genomic region in DNA can be targeted by CRISPR Technology by designing sgRNA and specific desired results can be achieved. A brief comparison of genome editing tools is given below in Table-1 [18].

Table-1. Comparison among genome editing tools

Characteristics	CRISPR-Cas9 System	ZFN	TALEN
Target region specifying unit	SgRNA	Zinc finger DNA binding domain	TALEN DNA binding domain
Enzyme	Cas9 protein	Folk	Folk
base pair length of target site	20-22	18-36	30-40
Success rate	High (>90%)	Low (24%)	High (>99%)
Modifications	More (20%)	Less (10%)	More (20%)
Restriction site	PAM region(NGG or NAG)	G-rich	A and T are the first and last bases

APPLICATIONS

Agriculture

There are numerous applications of CRISPR/Cas9 system in diverse fields like agriculture, health, virology as well as in curing of genetic disorders. In agriculture, virus resistant plants have been produced by employing this genome editing approach. This was carried out in *Nicotiana benthamiana* by gene knock down [19] as well as in cotton against cotton leaf curl kokhran virus. Furthermore, different traits have been modified by manipulating the genome. For example, different herbicide resistant plants have been synthesized such as *Oryza sativa.* In *O. sativa* it was done by knock in (gene replaced). *ALS (*Acetolactate synthase*)* was targeted through this experiment [20]. In the same crop, promoter was modified by using this novel approach to get disease resistant plants [21, 22]. Similarly, expression of Green Fluorescent Protein (GFP) was augmented in *Arabidopsis thaliana* [23]. This was acquired through target gene insertion (TFL1) by using donor DNA cassette. This technology was appreciated in enhancing the anthomycin pigment (kanamycin resistant pigment) in *Solanum lycopersicum* [24, 25]

Correctness of Genetic Disorders

First, CRISPR/Cas9 system was executed in the field of health. The results were so promised in curing the genetic disorders. Hemophilia B is an X-linked genetic disease in which blood fails to clot due to deficiency of coagulating factor IX. Researchers identified the mutation Y371D in F9 gene and they designed a sgRNA against a mutated region in F9 gene. After transformation through adenoviruses, mutated sequence was targeted by sgRNA and donor DNA template restored the mutation. Consequently, CRISPR/Cas9 system ameliorated the mouse carrying genetic disease [26]. By using the same model organism CRISPR Cas9 system was also used for the treatment of cataract- an eye disease which causes the blurred vision and it is due to mutation in crygc gene. CRISPR reverted the mutated sequence and resulting offspring of progeny were normal. Embryonic Stem Cells (ESC) were used for all these experiments. Similarly, β-thalassemia triggers due to variation in β-globin gene, the most prevalent genetic disorder worldwide [27]. Scientist harnessed CRISPR Cas technology to cure this abnormality. Mutations in β-globin gene (HBB) in induced pluripotent stem cells (iPSCs) were made error free using CRISPR/Cas9 system in association with *piggy Back* transposon without any complication. Edited iPSCs distinguished into erythroblasts with re-modeled expression level [28].

CRISPR Role in Virology

CRISPR Technology expands its wings to virology. Scientists have identified C-C chemokine receptor type 5 (CCR5) on the surface of white blood cells (leukocytes). Human immunodeficiency virus (HIV) recognizes these receptors to enter the host cells and infect them. Researchers have disrupted CCR5 gene by using CRISPR technology and consequently receptors lacking cells were produced. So HIV was unable to infect the host cells [29]. Similarly, bacteria having endogenous CRISPR System are naturally immune to phages. Such type of adaptive immunity exists due to presence of spacer region having a particular repeat array [30]. RNA guided system also showed its excellence in the control of Hepatitis B. Researchers, designed sgRNA against Hepatitis B surface antigen (HBsAg) region in a cell culture system. And quantitative PCR and immunohistochemistry revealed the reduced expression level of HB surface antigen.

Effectiveness in disease generation models

Modern genome editing tool and ZFNs both have a huge potential for the restoration of gene sequence, induction of target specific mutations and in disease generation models. sgRNA guided based genome editing system was executed in the disease models(heritable).For example, CRISPR Cas9 System was employed in the initiation of mouse models. This was done through lentiviral delivery method into stem cells (hematopoietic) of mouse [31]. Likewise, CRISPR was also harvested in the generation of cancer models by introducing mutations in cancer suppressing genes (*p53 and pten*) of liver cells [32]. A large number of modifications have been reported in genes (*runx1, ncor2 and klf6a)* having a role in the progress of blood cells of zebra fish. These modifications can be used study human disease models [33].

Barriers of CRISPR System

Although CRISPR system has proved to be best in the current scenario but still it is in its infancy. Most vital drawback is off-target effects in which specific designed sgRNA does not bind to target region in genome and creates non specificity [34]. But this can be overcome by employing online in silico analysis tools [35]. Several reports have shown some modifications to increase CRISPR/Cas9 target selection including changes in PAM recognizing domain to add other PAM motifs and incorporation of purines or pyrimidine's as a first nucleotide of sgRNA [36]. Immunosuppression is also another issue which limits its fidelity [37] but it can be resolved by genome optimization.

Advancement of CRISPR/Cas9 System

Cas9 Engineering

Until now, CRISPR Cas system has been most widely exploited in model organisms. To improve the proficiency and accuracy of CRISPR, various types of optimized cas9 nucleases have been created for mouse [38, 39], human [6] and zebrafish [39]. Usually, large size of Adeno-associated virus (AAV) is one of the main constraint for genome editing. Recently, researchers discovered smaller size cas9 protein from *streptococcus thermophilus* LMD9 strain (st1cas9) [5] and gram-possitive bacterium *staphylococcus aureus* (SaCas9). Moreover, cas9 protein was also engineered in combination with sgRNA into AAV against pcsk9 gene of mouse liver [15]. Newly designated nuclease is termed as Cas protein of PreFran subtype (cpf1) which shows proficient genome editing by targeting PAM (T-rich).

sgRNA Optimization

sgRNA guided genome editing system is also optimized through alterations in sgRNA. Now a days, CRISPR/Cas9 system efficiency has been improved for biallelic gene interference in F_0 population of zebrafish. In addition to this, both crRNA and tracrRNA have U bases at their 3´-end which have been changed to GGAUC [40]. If the sgRNA will be of smaller size than 20bp then it will minimize the off-target effects [41]. Currently, Ypest protein 4 (Csy4) which is subclass of CRISPR/Cas9 was exploited to enlarge targeting area in zebrafish and human cells [42]. Moreover, sgRNA also exhibited an increased efficacy to disrupt human T cells, CD34+ and progenitor cells in spite of any harmfulness [43].

Enhancement of Target specific cleavage

Single nucleotide mutation in two domains (RuvC, HNH) of Cas9 transforms it into Cas9 nickase [5, 9]. Cas9 nickase will cleave only a single strand of DNA while using both Cas9 nickases in association with sgRNA will cause double stranded DNA cleavage with very small off-target effects [44]. Quantitative studies showed that Cas9 nickase could lower the 50-1500 times off-target activity in different cell lines [44]. Further confirmation of CRISPR specificity to its target site was done by Skarnes group. They perceived not any mutation done through non homologus end joining (NHEJ) at non-specific region [45]. "Enhanced specificity" SpCas9 has been newly stated mutant well-known for its preciseness to targeted site and improved coherence [46].

Future Perspectives

The above facts draw an excellent image about CRISPR Cas9 as a forthcoming technology. In the field of health including virology and cancer biology this would be proven a miracle technology. As in the scenario of HIV this technology would probably be helpful in case of HCV. HCV is a major threat in the world including Pakistan. The similar concept would be applicable in cancer biology to knock out those factors which increases the probability of cancer. In the field of Agriculture, CRISPR Cas9 approach would be a weapon to combat with various insect pest in cotton (chewing and sucking insects) by targeting their female sterility genes to produce mutants unable to increase their generation. Likewise, efficiency and performance of Bt cotton would be improved by increasing cry protein expression. Genes which play negative roles in plant growth they would be silenced (zero expression) by gene knockin.

CONCLUDING REMARKS

Tool box CRISPR-Cas9 bears a huge potential to revolutionize the biological research and to expand the ability of genetic manipulation. This transformative tool has made possible to target any gene for required modifications in a more convenient way. It also reshaped the traditional techniques for discovering a particular gene function due to ease in execution, component designing flexibility and target specific mutations. Mostly, CRISPR Cas9 System is optimized in model organisms and now it is applicable in every field of Biological Sciences. This genome editing System has a huge capability for disturbing normal gene function, restoring errors and RNA based targeting against DNA sequence..

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