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CRISPR/Cas9 System: A Breakthrough in Genome Editing

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Abstract

Clustered Regularly Interspaced Short Palindromic Repeats is a new and advance gene editing tool using specific nuclease enzyme for specific cleavage. It is the most efficient technique, commonly used for many purposes like gene therapy, production of desired plants and transgenic animals. But it has some limitations like off-target issue and this issue can be minimized by the production of specific sequence of guide RNA. In the future, it can be used for the treatment of many human genetic diseases.

Keywords: Gene expression; Double-strand break; Gene therapy

Abbreviations: TALEN: Transcription Activator-Like Effector Nucleases; ZFN: Zinc Finger Nuclease; ZFP: Zinc Finger Peptide; PAM: Protospacer Adjacent Motif; HDR: Homologous Directed Repair; NHEJ: Non-Homologous End Joining; CRISPR: Clustered Regularly Interspaced Palindromic Repeats

Introduction

Genome editing is an insertion, deletion or replacement of a gene for eliminating or inducing specific and desired characters in genome. In the past, it's totally a dream that specific or mutated gene can be deleted from genome and foreign or desired gene can be inserted into genome. But this dream was true in 1960 when the cell lines developed [1]. In the Mid 1960s, researchers work on SV40 transformed cells of viral DNA and reported that virus also has the ability to transfer genes into any type of target cell [2,3]. Stanfield et al. [4] used virus for first time to transfer gene for the treatment of a girl who was suffered with hyperargininemia but they failed to accomplish any result.

Now days, genome editing is easiest and most accurate due to knowing the sequence and functions of DNA and nucleases [5]. Genome editing technique was first use in 1990 by the chemist, Kim et al. [6]. Three genome editing techniques are present that are commonly used. First is Zinger Finger Nuclease (ZFNs) second is Transcription Activator-Like Effector Nucleases (TALENs) and the third is Clustered Regularly Interspaced Short Palindromic Repeats with Cas9 Nuclease (CRISPR/Cas9). All these techniques have two domains for perfect and accurate genome editing. Specifically, the first is specific DNA binding domains that are complementary to the target sequence and second is nuclease domain that produces the Double Strands Break (DSBs). ZFN and TALEN use chimeric protein as a DNA binding domains whereas CRISPR/Cas system uses specific sequence of RNA molecule for DNA binding instead of chimeric protein [7].

ZFN is a first technique for genome editing. It contains two domains as write earlier. First is Zinc Finger Peptide (ZFP) domain for DNA binding and second is FokI restriction enzyme for cleaving DNA strands. Binding domain binds to the complementary sequence of target DNA and then FokI restriction enzyme cleaves the double strand of DNA on site where ZFP and DNA bind [8]. But the problem is its specificity is very low due to the presence of the tandem array of Cys2-His2 zinc fingers in ZFP region because each finger recognizes only 3 bp [6,8]. ZFNs are used to correct the mutations of genes that cause sickle cell anemia and Hemophilia B [9].

TALEN is the second technique that is commonly used for genome editing. It comes from the bacterium Xanthomonas a pathogenic plant. First it is detected in 2010 and it also has two domains. First is transcription activator-like effector domain for binding with DNA. It is a conserved region of 30-35 repeated amino acids. And second domain is FokI restriction enzyme for DNA Cleavage [7,10,11]. TALEN is economical and their manufacturing is easy as compared to ZFNs [12]. TALENs also have the ability to do disease modeling [13].

CRISPR-Cas technique is the most common and advanced technique for genome editing. It is an accurate method for gene modification and it also has the ability to edit more than one gene at a time [14,15]. This system is based on adaptive immune system of bacteria. It was initially noticed in bacteria in 1987, found in 40% of the sequenced bacterial genome and almost 80% of the sequenced genome of archaea [16,17]. Researchers use this system first time in 2002 [17,18]. CRISPR-Cas system also has two domains like others, first is Single Guide RNA (sgRNA) that is used for specific binding purpose and second is Cas9 enzyme that is used for cleavage purpose. sgRNA contains two types of RNAs, CRISPR RNA (crRNA) and Trans-activating crRNA (tracrRNA). So, those gRNA that have both RNA (crRNA and tracrRNA) are sufficient for perfect genome editing [15]. sgRNA have a specific sequence that is complementary to target sequence so, specificity depends on the sequence of gRNA [19]. Cas9 is an enzyme that has a properties of helicases and polymerases and it is used for the Double Strand Breaks (DSBs) [16,20]. Cas9 make a complex with sgRNA (crRNA and tracrRNA) and cleaves the DNA sequence that is complementary to the sgRNA sequence and it was first identified in 2001 [21,22].

CRISPR locus is basically a repeated sequence of conserved region interspaced by a spacer sequence that is non-repeated sequence. Spacer is a viral DNA that is cleaved by a Cas9 enzyme and it is joined with a CRISPR locus as a spacer [15,23]. Downstream of a spacer called Protospacer that have a specific sequence called Protospacer Adjacent Motif (PAM). PAM is simple and 2-5 bp long. Cas9 recognizes the PAM sequence on viral DNA and if PAM is not found then it not cleaved the DNA. Usually the sequence of PAM is 5'-NGG-3'. 'N' is any nucleotide base and 'G' mean Guanine. Cleavage occurs only on that side where PAM sequence present [14,24]. In 2005, researcher have successfully constructed the spacer sequence from phage genome [25,26]. Almost 40 types of Cas proteins discovered but the most common is Cas9 that is produced in *Streptococcus pyogenes* [23,27,28].

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CRISPR/Cas system is divided into 8 subtypes [20]. But according to modern classification, it is divided into three types [29]. In type I and III, first pre-crRNA produce and when this pre-crRNA mature then it fuse with Cas9 enzyme to form a complex. Then this complex cleaves the sequence of DNA that is complementary to the sequence of crRNA. Type I and III are not commonly use [22]. While type II is the most famous and it is commonly use in gene editing. In this type, gRNA guide the Cas9 enzyme for producing DSBs [15,30].

Comparing CRISPR/Cas9 system with ZFNs and TALENs, ZFNs and TALENs are time consuming and have more off target effects. While CRISPR/Cas9 system has very less off-target effects because it uses a specific sequence of guide RNA that binds with the specific sequence of DNA (target DNA) and then Cas9 enzyme cleaves the strands where binding occur. This system is able to produce transgenic animal and edit more than one gene (multiplexes gene editing) at a time [31,32] (Table 1).

CRISPR-Cas System of Immunity

CRISPR-Cas system is based on immune system of bacteria and it has three stages to work as an immune system in bacteria. First is adaptation, second is expression and final and third is interference. During Adaptation stage that is also called recognition stage, viruses or phages attack the bacteria and introduce their own DNA into bacteria. Cas gene recognizes this DNA fragment as a foreign particle and breaks it into small fragments. Then these small fragments of invading DNA are incorporated into CRISPR locus as spacers [23]. At Expression stage, spacer produces a pre-crRNA by acting as a transcriptional template. When pre-crRNA produce then Cas gene perform a series of action and convert pre-crRNA into mature crRNA. In Interference stage, mature crRNA guides the enzyme to the target site and enzyme destroys the invading or foreign DNA [23,33,34].

CRISPR/Cas9 System in Genome Editing

In gene editing, it is most common because it is efficient in gene editing and has very less off-target effects. So, production of desired characters can be very easy by using this system.

Cas9 Structure and Working Mechanism

CRISPR/Cas9 is highly efficient, precise, accurate and specific due to Cas9 enzyme. Cas9 is basically a bi-lobed structure having active sites and two grooves for binding of nucleic acid that is REC (large recognition lobe) and NUC (small nuclease lobe). Both are connected by a helix bridge [35-37]. Specificity of Cas9 depends on REC whereas NUC has further two domains. First, RuvC and HNH and second is PAM interacting domain (PI). In natural conditions, Cas9 is inactive and it is active only when REC lobe of Cas9 binds with sgRNA. A complex of Cas9 and sgRNA forms and then this complex scan the target DNA sequence and recognize the target site easily because target sequence is complementary to sgRNA sequence and PAM sequence also present on downstream of target site. Then sgRNA bind with target sequence and after binding, target sequence strand are cleave by HNH and the opposite strand of the target sequence are cleave by RuvC and DSBs are forms [30,38-41]. Researchers have developed an advanced type of CRISPR/Cas9 that can identify 20-24 nucleotides sequence of target site that is complementary to the sgRNA and 2-5 nucleotides of PAM sequence. As a result, CRISPR/Cas9 able to target a sequence which is 22-29 nucleotides long [42,43].

Repair Processes

When HNH and RuvC of Cas9 form DSBs then CRISPR/Cas9 system activates the repair mechanism that fixed the DSBs [41]. Two types of repair mechanisms present that play an important role in the fixing of DSBs. First is Homologous Directed Repair (HDR) and second is Non-Homologous End Joining (NHEJ). HDR is an error free method and it joins only homologous templates. Commonly used in plants for gene knock-in, however, it is more complicated as compared to NHEJ [44-46]. While NHEJ is an error prone method, it applies DNA ligase to rejoin the ends. It insert or delete the specific gene sequence and causes Indels mutation. So, it is commonly used for gene knock-out [44,47] and for working of CRISPR/Cas9 system (Figure 1) [48].

Delivery Methods

Delivery of CRISPR/Cas9 is very critical problem. Accurate delivery can produce an efficient and desired genome editing. Both viral and non-viral based systems are used for the delivery of CRISPR/Cas9 to a target site.

Viral Delivery Method

Viral systems have been preferred over the past few decades to deliver the gene like lentiviruses, adeno-associated virus (AAV), retroviruses and adenoviruses. Viral delivery systems are divided into two categories. First, those viruses whose genetic material can be integrated into host genetic material, like lentiviruses or retroviruses. Those viruses that remains inside the nucleus and cytoplasm of host like AAV or adenoviruses [49]. Viral delivery has both advantages and disadvantages like retrovirus integrates into the host and activates the oncogene that increases the frequency of haphazard mutations. But the advantage is retroviral delivery system produces a long term expression [50].

Factors	ZFN	TALEN	CRISPR/Cas9
Nuclease Assembly	Significant	Significant	Simple
Choose Target Site	Limited	Limited	Unlimited
Gene Mutation	Limited	Limited	Unlimited
Target efficiency	High	Low	Low
Components	ZFP + FokI Fusion Protein	TALE + FokI Fusion Protein	guide RNA + Cas9 Protein
Enzymes	FokI Restriction Enzyme	FokI restriction enzyme	Cas9 Protein
Time	7-15 days	5-7 days	1-3 days
Cost	High	High	Low
Recognition Site	9-18 bp	14-20 bp	22 bp (20 gRNA sequence + 2 bp of PAM sequence)
Immunogenicity	Low	Unknown (protein derived from *Xanthomonas* sp.	Unknown (protein Derived from Different Bacteria)
***Ex vivo* Delivery**	Easy	Easy	Easy
Specificity	Low	Moderate	Highly

Table 1: Comparing CRISPR/Cas9 system with ZFNs and TALENs, ZFNs and TALENs.

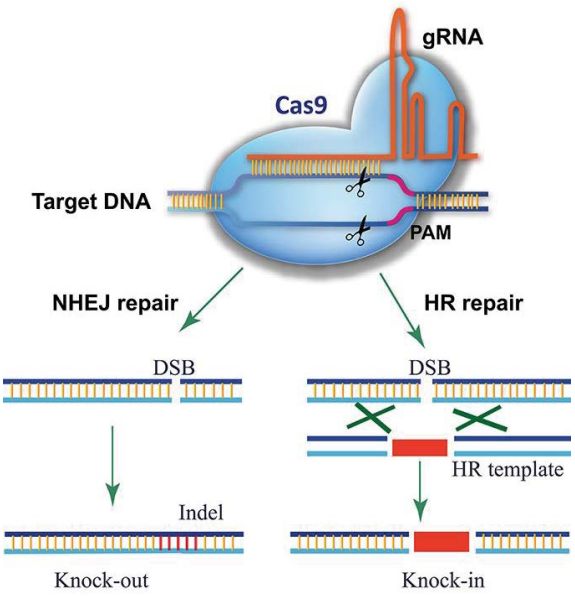

Figure 1: Working of CRISPR/Cas9 system.

AAV is the approved method for gene therapy because it is safe, produces long term expression and the immune response is mild [51]. Nevertheless, some limitations also present like poor packaging ability (4.7 kb) while size of Cas9 of Streptococcus pyogenes is 4.2 kb. So, it creates a challenging situation to deliver SpCas9, sgRNA and additional necessary components by using AAV delivery method [51,52]. This problem is minimized by using small orthologs of Cas9 like SaCas9 (~3.3 kb) or StCas9 (~3.3 kb). Still a major problem using these small orthologs of Cas9 is that they need large PAM sequences like SaCas9 identify 5'-NNGRRT-3' while StCas9 identify 5'-NNAGAAW-3' sequences [53-55].

Lentivirus is also use to deliver genes. Researchers modify the HIV-1 and make Lentivirus capable to deliver gene into any type of host. Its packaging capacity is high as compared to others (9.7 kb). So, it easily delivers Cas9, sgRNA and other additional necessary components into host [56].

Non-Viral Delivery Approach

Non-viral delivery is also applicable for delivery CRISPR/Cas9 to the target sites. In this approach, liposomes, nano-particles and hybrodynamic injections are commonly used [56].

This approach has many advantages:

- It is safe
- Packaging capacity is large
- Immune response is low
- Production is easy

Hybrodynamic injection is the recently use approach for delivery of CRISPR/Cas9. It is high volume injection (approximately 8-10% of mice body weight). This injection delivers directly into the vascular system of the body of host. Delivery speed is very high (approximately 5-7 s). Researchers use this method or injection and deliver the CRISPR/Cas9 into the liver for study purpose [57,58]. But the problem is it damages the heart and liver tissues so, it is not clinically applicable [59] (Table 2).

New Developments

The fact that CRISPR/Cas9 is the preferred method used for genetic editing makes it difficult to expand on new developments. The history is short and the system is widely used because of its precision and efficiency in genome editing [60].

Cas9 Modifications

Qi et al. [61] produced a dCas9 (dcas9 or CRISPRi) that is inactive by producing point mutation in HNH and RuvC domains of Cas9. dCas9 lacks cleavage activity, so it cannot be able to produce DSBs. This dCas9 is used for the activation and repression of specific gene. dCas9 first time developed in 2013 at UCSF [60]. Both (dCas9 and sgRNA) are co-expressed in transcriptional elongation so, transcription can be control by CRISPRi and it is able to express or repress any target gene as shown in Figure 2 [62].

Cas9 Combines with Proteins

dCas9 fuse with epigenetics factors (methylation) and produce desired changes in the epigenomes of host. Cas9 also combined with some fluorescent proteins for labeling purpose. It is also used to study the complex organization of nuclear material [63].

PAM Developments

New Cas9 have been discovered that able to identify variety of PAM sequences. Like StCas9 recognizes "NNAGAA" while SaCas9 recognizes three different PAM sequences: "NNGAGT" "NNGGGT"

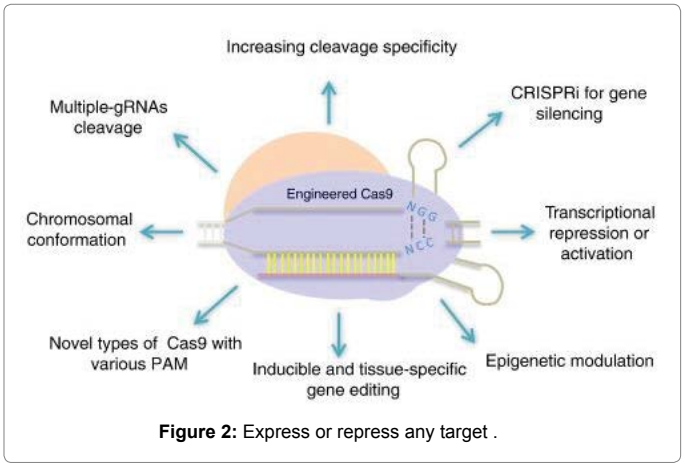

Figure 2: Express or repress any target .

Mode of Delivery	Immunogenicity	Duration of Expression	Genomic Integration Risk
Retro-virus	Low	Long term	High (activate oncogene)
Adenovirus	Low	Medium	Low
Lenti-virus	Low	Long term	Low (Low oncogenecity)
Nano-particles	Under Evaluation	Transient	Low
Proteins	Under Evaluation	Transient	No
RNA	Low	Transient	No

Table 2: Hybrodynamic injection process for delivery of CRISPR/Cas9.

and "NNGAAT" [54,55]. A new enzyme (Cpf1) has been discovered that recognizes T-rich PAM sequences [64].

Cpf1 Proteins

In 2015, Chen et al. [63] and Zetche et al. [64] discovered a new class of CRISPR protein name Cpf1. It is discovered from Prevotella and Francisella and it reduces the current limitation of Cas9. 16 Cpf1 proteins have been identified, but only two are appropriate for human. CRISPR/Cpf1 system has some advantages over CRISPR/Cas9 system for example, it is small in size and easy to handle. Compare with Cas9, Cpf1 requires a single guide RNA while Cas9 requires two guide RNA molecules. It produces sticky ends after cleavage that improves its accuracy by producing 5'overhang while Cas9 produces blunt ends and Cpf1 doesn't needs tracrRNA for its working while Cas9 needs tracrRNA [14,65].

Applications of CRISPR/Cas9 System

CRISPR/Cas9 is the modern technique for genome editing and it is very simple, efficient and precise method. This system has the ability to do multiplexed gene editing [66]. It also has the ability to change chromosomal target by insertion or deletion (Indels) [67]. This system has a variety of applications like ability to remove internal viral genes from host that causes diseases [68].

Gene Therapy

CRISPR/Cas9 system has the ability to treat many genetic and viral diseases like Cystic Fibrosis. For cystic fibrosis treatment, first isolate intestinal stem cell and develop it in culture in laboratory and then remove the target allele (F508) and placed back to the human [69,70]. Huntington is also treated by CRISPR/Cas9. In this disease treatment, CAG repetitions present in HTT gene that can be removed by CRISPR/CAS9 [71]. Rett Syndrome and Schizophrenia are also treated by CRISPR/Cas9 system [72,73]. Nowadays, researchers use the CRISPR/CAS9 system to mutate the oncogene for treating cancer, but it is on trial basis. Edina et al. disturbed the long terminal repeats of HIV-1 that cause AIDS by CRISPR/Cas9 so, the concentration of AIDS in that patients is decreased [74,75].

Biotechnology

CRISPR/Cas system has also been used in biotechnology and pharmacology. It plays important role in making vaccines for Pseudorabies virus [76]. Many pharmaceutical companies started investing in this field and the aim of the private companies may be achieving high profit.

Bill Lundberg said; "Interest in the gene editing forces of CRISPR-Cas9 by pharmaceutical companies literally exploded in the last year" [77].

Now a day, CRISPR-Cas9 can able to treat HIV patients. Hepatitis B is also treated by CRISPR-Cas9 system. In this disease, covalently closed circular DNA (cccDNA) present that produces a new type of antigen and it can cause hepatitis B. Researchers removes cccDNA from hepatitis B patient's genome by using CRISPR/Cas9 system [78].

In Plants

This diverse system is also used in plants for many purposes like gene mutation, gene knock-in, gene knock-out, epigenomes modifications, etc.

In Arabidopsis, several genes (AtPDS3, AtFLS2, AtADH, AtFT, AtSPL4, AtBRI1) are targeted by using mutational efficiencies and these mutations are transferred to their offspring successfully [79]. Some other successful examples have been reported in other crops like *Sorghum bicolor*, *Zea mays*, *Citrus sinensis*, *Populus tricocarpa*, *Solanum esculentum* and *Triticum aestivum* [80].

Genome Editing

Insertion, deletion or replacement of gene from genome is called genome editing. Introduce the desired modifications in genome by mixing multiple gRNA with Cas9, this produces multiple mutations in mammalian genome [30]. CRISPR/Cas9 also show its editing ability in zebra fish [81], mice [31], drosophila [44,82] and bacteria [83]. Bassett et al. [82] apply CRISPR/Cas9 injections directly into the embryo of Drosophila for constructing desired mutagenesis and the success rate is 88%. These mutations can be transferred to the next generation through germ line and their success rate is 33%. This system has the ability to produce desired phenotypes and eliminate disease causing mutations from animal [84].

Transcriptional Regulations

Researchers produce catalytically inactive Cas9 protein; name dead Case or dCas9, by point mutation in HNH and RuvC domains of Cas9 enzyme. dCas9 forms a complex with CRISPRi and disturbs the functional sites of transcription that controls the transcription process. Then this complex interferes in the elongation steps of transcription and express or repress the target sequence [85,86]. Brief application of CRISPR/Cas9 system [87] (Figure 3).

Limitations of CRISPR/Cas9 system

CRISPR/Cas9 has following limitations.

Off-Target Effects

Off-target effects are major problem in genome editing. ZFNs and TALENs also have this problem. CRISPR/Cas9 also faces this off-target issue, but in this system the quantity of off-target effects are less compared to ZFNs and TALENs. Large genomes have large and complex sequences and this complex genome has some identical or homologous sequences. CRISPR/Cas9 cleaves these identical sequences instead of target sequence and produced un-desired mutations that are harmful to individual health [30,40,42].

Some Reasons for Undesired Mutations are Identified as Followed

First, Cas9 and sgRNA ratio is not accurate and high ratio of Cas9 and sgRNA produces more off-target effects [88,89].

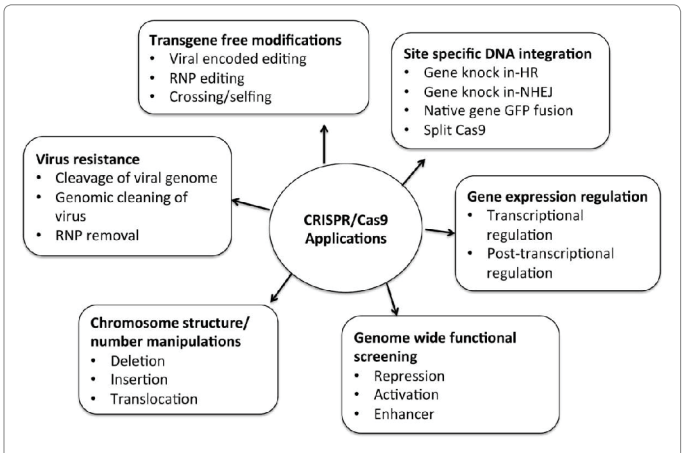

Figure 3: Brief application of CRISPR/Cas9 system.

Second, indiscriminate PAM sites also cause off-target effects. So, use bioinformatics tools like E-CRISPR for designing gRNA to overcome this problem [90,91].

Many systems and promoters are present for the expression of Cas9 and sgRNA. Select optimal promoter vectors for the expression of Cas9 and sgRNA. In eudicots, U6 promoter use for sgRNA expression while 35S CaMV promoter for Cas9 expression. In monocots different promoters are used for the expression of sgRNA and 35S and Ubi promoters are used for the expression of Cas9 [92]. Some researchers reported an abrupt mutation when using CRISPR/Cas9 system [40].

PAM Dependence

PAM is a specific sequence present on the downstream of the target sequence. Cas9 recognizes the PAM sequence and when it recognized the PAM sequence then Cas9 cleaves the double strands of DNA. Cas9 is totally depending on PAM sequences because if PAM sequence is not recognized by Cas9 enzyme then it will not able to cleave the DNA double strands. PAM sequence are different for different Cas9 like "NGGNG" and "NNAGAAW" from Streptococcus thermophiles [93,94], "NGG" from Streptococcus pyogenes and "NNNNGATT" from Neisseria meningitides [95,96].

Conclusion

Good gene editing tools has ability to target any sequence without any off-target effects and the CRISPR/Cas9 is the one. It is simplest, precise and economical method of gene editing as compared to previous ones. It uses a specific sequence of RNA called guide RNA whose sequence is complementary to the target DNA sequence and Cas9 enzyme that act as helicases and polymerases. Specificity is depending on gRNA and Cas9. It has some off-target effects but this problem is in less quantity as compared to previous ones and this issue can be minimized by the production of specific sequence of gRNA. CRISPR/Cas9 uses for many purposes like production of desired, high yield and diseases resistance plants. In animals, it is used to treat animal diseases and produces transgenic animals. In human, use for gene therapy, treatment of genetic and viral diseases, production of vaccines and active or silence any gene sequence.

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