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CRISPR/Cas9: A Review on Genome Editing Tool

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Abstract

Throughout the history of recombinant DNA technology various tools have been used for manipulating the genome of an organism. Recent discoveries have led to novel insights into the world of endonucleases which can guide researchers to achieve the skill of editing genome in an efficient and easy way. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/ Cas9 system is among the newest and most effective members of genome editing tool and has been accepted by discoverers around the world as one of the most precise method of targeting and removal of a genome sequence and further utilization of other techniques to even incorporate a gene of interest in the same position in order to develop a transgenic organism. The applicability in reverse genetics studies, animal model experiments and also in curing diseases caused by viruses like HIV makes CRISPR/Cas9 system a highly desirable technique with the ability to achieve quick plausible outcomes.

Key words: CRISPR/Cas9, endonuclease, Transgenesis.

Introduction

Transgenesis is the technique of genetic engineering where a stretch of DNA obtained from an animal or a plant of one species is introduced into the genome of some another animal or plant usually belonging to a different species, so that organisms with some important characteristics can be obtained which are typically not found in nature. The use of transgenic technology first became prominent in 1974 when a transgenic mouse was produced by introduction of viral DNA into the blastocysts by microinjection technology (Janeisch and Mintz, 1974). Since then scientists all over the world derived various techniques for introduction of foreign DNA into the genetic material of the host. Before going into any details on CRISPR /Cas9 system and how it is utilized we should just check in what different kinds of techniques have been used in transgenesis. The first of it was of course pronuclear microinjection of DNA into the oocytes of mouse which was later on explained in detail by Gordon *et al.* (1980). Another method in use have been sperm mediated transfer of gene (Hirabayashi and Hochi, 2010) and is quite useful as this technique is cheap and does not

require very high facilities for creation of a transgenic organism. The virus mediated gene transfer has been one of the common methods of choice in which modified retro virus package was used as vector (Bushman, 2007) which was later on replaced by more potent vectors such as Adeno viruses, Herpes simplex viruses, lenti viruses, Pox viruses and Epstein–Barr viruses systems (Nayerossadat *et al.,* 2012). Each type of viral vector had some advantages over its predecessor. Embryonic stem cell mediated gene transfer by transfection of male germ stem cells using in vivo (Zhang *et al.,* 2009) and in vitro (Brinster *et al.,* 2002) methods has also been in use. Certain specific modifications in the technique of transgenesis led to the development of Nuclear Transfer Mediated Cloning and are still being widely used as one of the successful methods of generating transgenic animals (Waghmare *et al.,* 2011). With the advent of time and demand for targeted genome modification approaches led to the discovery of ZFNs (zinc-finger nucleases) (Miller *et al.*, 2007) and TALENs (transcription-activator like effector nucleases) (Mussolino *et al.,* 2011) which helped in guiding the researchers in generating permanent mutations because they were able to make breaks in both the strands of DNA in order to activate repair pathways. However, use of these enzymes was costly, time consuming and highly demanding. Thus a cost effective way to ensure an efficient and better technology led to the development of CRISPR/Cas9 method which along with ZFNs and TALENs also acted as a molecular scissor for targeting and cutting away a DNA segments of interest.

What are ZFNs and TALENs?

ZFNs comprises of fusion proteins with an arrangement of site specific DNA binding domains which is attached to a restriction enzyme FokI of the bacteria (Gupta and Musunuru, 2017). Each of such domain can recognize a 3-4 bp long DNA sequence and tandem domains have the ability to bind an expanded DNA segment comprising of sequences that is multiple of 3 bp like 9, 12, 15 or 18 bp typical to genome of a cell. ZFNs are precisely designed as a pair in such a way that they cut flanking regions of the targeted site on both sides. Thus, ZFNs recognize the sequence on the forward strand and also on the reverse strand and bind on either side of the targeted site. This leads the pair of FokI domains to dimerize and cut at that particular site with the formation of DSBs (Double Stranded Breaks) with 52 overhangs (Urnov *et al.,* 2010). Cells then repair the cut sites with either non-homologous end joining (NHEJ) or homology directed repair (HDR).

Whereas, Transcription activator-like effectors (TALEs) are a group of proteins found only in a certain group of plant pathogens. The characterization and recognition of this DNA binding domain has been termed as TALE repeats. This naturally occurring repeats consists of tandem arrangements of 10 to 30 repeats that recognize and binds expanded DNA sequences (Bogdanove and Voytas, 2011). Each of such repeat is 33 to 35 amino acids long and is accompanied by two adjacent amino acids called as repeat-variable di-residue (RVD) which has the capability to confer specificity to one of the any four nucleotide bps (Streubel *et al.,* 2012; Cong *et al.,* 2012). Modifications of the RVD code introduces the possibility to create new type of site specific engineered domain of TALE repeats that combine with FokI endo-nuclease domain and are called TALENs (Miller *et al.,* 2011; Li *et al.,* 2011). A TALEN generates DSBs at the desired site of target in a way similar to ZFNs. When compared with ZFNs ,TALENs are comparatively easier to design (Reyon *et al.,* 2012; Cermak *et al.,* 2011) and produce very few off target mutations (Mussolino *et al.,* 2011). Furthermore, ZFNs has also been observed to be more toxic to the cells when compared with TALENs. However, ZFNs have an advantage over the TALENs when it comes to size where a cDNA required for encoding ZFNs are only 1 kb and that for TALENs it is 3 kb. The overall size and the highly repetitive nature of the TALENs makes them less appealing for therapeutic delivery where viral vectors are commonly used for efficient therapy of genetic diseases (Holkers *et al.,* 2013).

What is CRISPR/Cas9?

Clustered Regularly Interspaced Short Palindromic Repeat or CRISPR are a clustered family of short repeats of DNA that forms an integral component of the prokaryotic (bacteria and archaea) adaptive

immune system. The study on CRISPR/Cas9 system began with the findings of repetitive segments of DNA in *E. coli* in the year 1987 but their function was confirmed later on by Barrangou and coworkers in 2007. They are involved in protection of the bacteria or the archaea against the invading viruses (Gasiunas *et al.,* 2012). These CRISPR segments correspond to sequences of genome in the bacteriophage which tries to use the cellular mechanism of the unicellular bacteria or archaea. Each unit consists of a clustered set of CRISPR segment or Cas genes along with the peculiar CRISPR array that consists of a series of repeat sequences (direct repeats) which are broken or interspaced by variable sequences known as spacers that directly corresponds to segments within foreign genetic elements known as protospacers (Hsu *et al.,* 2014). Specific CRISPR sequences are first transcribed into primary RNA and then processed into shorter RNA segments which are known as small RNAs (crRNA – CRISPR RNA) that target the nucleic acid of the foreign organism by activating the Cas enzymes acting as nucleic acid scissors which are produced by Cas genes present in the bacterial genome (Hsu *et al.,* 2014).

The CRISPR-Cas system was originally divided into a total of eight subtypes (van der Oost *et al.,* 2009). However, with the advent of time a new system of classification was introduced because the original division did not include the distant relationships among various kinds of Cas proteins (Makarova *et al.,* 2011). The new classification included the division of CRISPR-Cas system into three categories. Among them the two categories i.e., Type I and Type III involve specialization of the Cas endonuclease capable of processing the pre-crRNAs and when the final product is formed the crRNA is constructed or gathered together to form a large Cas protein complex having the ability to recognize and cleave the complementary nucleic acid sequence (Jinek *et al.,* 2012). CRISPR-Cas9 system has also been classified into Type II system which is based on the immune system responses of the bacteria and archaea (Haft *et al.,* 2005). CRISPR-Cas9 system is the most recently developed technique commonly used as one of the genetic engineering tools for splicing of a targeted sequence as it is used through RNA-guided DNA nuclease enzyme which results in loss of function of the targeted gene (Hwang *et al.,* 2013). Cas9 cutting target is supervised by a double duplex of RNAs, one of which is the crRNA that identifies the foreign DNA by an approximately 20 base pair complementary region and the other being the tracrRNA that combines with the crRNA and is specific to the type II system (Deltcheva *et al.,* 2011; Jinek *et al*., 2012; Hsu *et al.,* 2014). Cas9 along with the crRNA–tracrRNA duplex molecule can be used as an efficient genomic editing tool (Jinek *et al.,* 2012; Cong *et al.,* 2013). Study to simplify the system has shown that the crRNA–tracrRNA duplex can be combined into a chimeric single guide RNA denoted as sgRNA (Jinek *et al.,* 2012). This Cas9–sgRNA system comprising of a single protein and a single RNA has been most commonly used for editing genes and also for some other Cas9-based applications. This endonuclease generates double stranded breaks (DSBs) at the region of the complementary nucleic acid segments in accordance with the crRNA sequence. After creation of breaks in the DNA double strand, localized DNA repair is started by either NHEJ or through HDR when an exogenous homologous donor sequence is present (Mali *et al.,* 2013a). The limitation that exists while designing a CRISPR/Cas9 guiding sequence is the essentiality of a protospacer adjacent motif (PAM) lying close to genomic target area (Hsu *et al.,* 2014). NHEJ results in random insertion and deletion mutations which are called indels at the DSBs target and may lead to knockout of a particular gene. This may be caused by a shift in the reading frame of the target gene or may even induce a mutation at an important region of the encoded protein (Lieber, 2010). Whereas HDR can be used to create the required sequence insertion at the DSBs through homologous recombination that is guided by a donor DNA template resulting in exact gene deletion causing mutagenesis and can be used for insertion or gene correction also (Choulika *et al.,* 1995). Thus, precise insertion of target sequences by HDR method makes HDR the method of choice for DNA repair in targeted insertion of a gene of interest at the site of DSBs. It is quite clear that the mechanism of CRISPR-Cas9 technology involves DNA cleavage followed by DNA repair. CRISPR-Cas9 is highly efficient, easy to design, very specific and is well suited for high-throughput as well as multiplexed gene editing which are used for variety of cell types and several different kinds of organisms (Shafie *et al.,* 2014). Using

Cas9 for a particular new DNA site can be easily achieved because it only requires the creation of a new contemporary sgRNA that can identify the new DNA targeting site arranged next to PAM.

CRISPR-Cas9 has several advantages over ZFNs and TALENs which has prerequisite demand for recoding of proteins through the use of large segments of proteins for every new target site whereas for CRISPR-Cas9 the only requirement is that of changing the 20 bp protospacer of the gRNA which is accomplished by introducing the required sequence of the protospacer into the gRNA of the plasmid backbone. The Cas9 protein component remains same for all the target sites and can be used to target various sites in a genome (Mali *et al.,* 2013b). This property is used to target several sites in the genome of the same cell (Cong *et al,* 2013; Mali *et al.,* 2013b). The PAM for CRISPR-Cas9 occurs once in every 8 bases in a genome which makes it highly suitable to target sites adjacent to the occurrence of PAM making almost every part of the genome to become a target according to sequence of the protospacer (Cong *et al.,* 2013).

Applications of CRISPR/Cas9 mediated genome editing

CRISPR-Cas9 system has been used as a tool for reverse genetics studies so that the function of specific genes can be studied especially when the genes are suspected to have some role in disease outcome and also for demonstration of some therapeutic values in animal disease models including cancer studies (Doudna and Charpentier 2014; Xiao-Jie *et al.,* 2015).

Cas9 system has been predicted to have the potential to treat many diseases, which includes HIV, several genetic diseases and also cancer (Xiao-Jie *et al.,* 2015; Barrangou and May*,* 2015). In HIV infection Cas9 along with sgRNAs having target sites on viral genomic elements can be introduced into cells infected with the viruses, so that it can cut and inactivate the viral genome which will help to cure the HIV infection (Hu *et al.,* 2014). The same procedure can also be applied for other viral diseases like hepatitis B virus (Liu *et al.,* 2015). Several studies have cited the use of Cas9 in editing genome for curing mutations causing diseases in animals which can be present only in the somatic cells (Yin *et al.,* 2014) or can be found in germ line cells (Wu *et al.,* 2015) or even in human stem cells (Schwank *et al.,* 2013) as well as in induced pluripotent stem cells (Li *et al.,* 2015).

Initially RNA interference (RNAi) through the use of small interfering RNA (siRNA) mechanism was used to control or regulate the expressions of various genes involved in the outcome of some kinds of disease. RNAi method does not interfere with the gene or DNA sequence, as its control is restricted to the messenger RNA that it inhibits (Taylor *et al.,* 2015). However as we have already discussed CRISPR/Cas9 platform can be easily used by creating a library of sgRNAs targeting gene coding regions. This mechanism has the potential to cause complete loss in the function of the targeted loci and will also help in easy identification of relevant genes under study as it induces changes directly on the gene sequence and may prove to be more efficient than RNAi method. But CRISPR/Cas9 method has one important limitation and that includes the presence of Protospacer Adjacent Motif (PAM) close to the target site (Sander and Keith, 2014). If any particular sequence does not have a PAM site it will not act at that particular target which is not the case when RNAi method is used which in principle can suppress the activity of any targeted mRNA.

Furthermore, for a very efficient knockout of gene by CRISPR/Cas9 it should be able to target all alleles of the same gene so that they can be mutated and this makes the screening difficult for cells carrying several alleles in their genetic material such as cancer cells (Taylor *et al.,* 2015). Also, CRISPR/Cas9 use as a knockout approach for studies involving essential genes is demanding because removal of a sequence from an essential gene or removing the whole gene itself may result into production of a lethal effect. In such cases it is better to use RNAi method as it does not produce any alteration of an essential gene.

Problems Associated with CRISPR technique.

CRISPR/Cas9 system is very efficient but this also increases the probability of its off-target effects

as the complementary target sequence is recognized by a short 20-bp sequence carried by the sgRNA along with the 3 bp PAM which makes it quite likely to be present somewhere else in the genome (Fu *et al.,* 2013; O'Geen *et al.,* 2015). This challenge is dealt with various adjustments which are made such as reducing the concentrations of the enzymes when the CRISPR/Cas9 method is used.

Another important challenge is to increase the use of HDR instead of NHEJ for substituting the removed DNA sequence. During natural repair of the DSBs NHEJ is more common in comparison to HDR but NHEJ repair results in unwanted changes in the structure of the genome and a more precise and targeted insertion can be only brought about by HDR (Maruyama *et al.,* 2015; Lin *et al.,* 2014). So it is important to modify the repair system according to HDR method so that a more precise integration of the gene of interest can be carried out. It would also be highly important to develop accurately regulated protocols for safe and efficient delivery methods to control the efficiency of Cas9 activity in a very precise manner as its uncontrolled use may prove to be havoc specially when used in treating genetic disorders.

Use of CRISPR/Cas9 for RNA sequences

CRISPR/Cas9 can be efficiently used for DNA sequences producing DSBs but Cas9 obtained from certain pathogenic bacteria like *Francisella novicida* which is a close relative of *F. tularensis* responsible for causing tularemia can precisely target and disintegrate mRNA transcripts for a bacterial lipoprotein which leads to the suppression of its host's immune response (Sampson *et al.,* 2013). Such Cas9 designated as Fn Cas9 along with specific tracrRNAs and a novel, small, CRISPR/Cas-associated RNA (scaRNA) can be used to target viral genome such as hepatitis C virus (HCV) in eukaryotic cells of multi-cellular organisms (Price *et al.,* 2015). Although the detailed mechanism of Fn Cas9-mediated RNA inhibition is unknown, it has been found to be nuclease independent and also does not require any PAM sequence for recognition of DNA sequence complementary to the RNA-targeting guide RNA (rgRNA) (Price *et al.,* 2015). This is an advantageous position where dependence on PAM is completely eliminated.

Conclusion

The use of ZFNs and TALENs are now being replaced by a more efficient, precise and easy to use CRISPR/cas9 technique for removal of targeted sequence from the genome of an organism and even dealing with more difficult situations like targeted insertion of a gene of interest. Use of this technology has enabled researchers to investigate more accurately about the function of a specific gene and to find alternatives for therapeutic control of various diseases. Further research, insight and advancement into the mechanism and use of this technology can lead to novel outcome on various problems related with human and animal life. There is no doubt that nature has provided us everything and one of its important gift is CRISPR/cas9 mechanism but to what extent we can use such hidden systems depends on our ability to investigate and direct its use for our own advancement and safety. Only further research in this technology can give us clue for newer discoveries and optimism.

Conflict of Interest: All authors express no conflict of interest.

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