Genome Editing: Precise and "CRISPER"

Annapurna Gupta and Meenal Agarwal

Department of Medical Genetics, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow

Email: meenal91@gmail.com

Introduction

Creation of a desired change in the genome either by gene addition/ gene insertion/ gene correction at targeted sites is known as genome editing. The various methods that are being conventionally used for genome editing typically include a nucleotide or amino acid sequence which identify the targeted genomic site and a nuclease which produces double stranded DNA breaks (DSBs). These DSBs are then repaired by using cellular intrinsic DNA repair machinery. If these broken DNA ends are joined end to end it leads to either insertion or deletion of a few nucleotides which often disrupts the reading frame and leads to frame shift mutation. This method of DNA repair which is called as nonhomologous end joining (NHEJ) is more commonly used but being error prone, it often causes the knocking down of a functioning gene. The other method for repair of DSBs, homology directed repair (HDR), wherein breaks are repaired by complementary pairing to a template strand, is more accurate and by providing an already synthesized DNA template strand, desired gene modifications are possible.[1,2]

Various Methods for Genome Editing

Zinc finger nucleases (ZFNs), Transcription activator like effector nucleases (TALENs) and Clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) are the three most common genome editing technologies.[1,3] All these methods are based on a combination of mechanisms to identify genome targets with great specificity and creating DSBs. They mainly differ in the mechanism of recognising target genomic sequences, multiplexing to various genomic targets, potential off-targeting effects and ease of in vitro genetic engineering. A comparison of the characteristics of these genome editing technologies is presented in table 1.

Potential Uses of Genome Editing Methods

These genome editing technologies have a vast range of utilities from research in understanding gene function to gene therapy and drug discovery. The potential uses include:

1) For gene functional studies- by producing gene disruption, can produce knock out models to study functions of genes. Conventionally these forward genetic screen methods used random chemical mutagenesis in model organisms like yeast, fruit fly, zebra fish, nematodes and rodents. However the process was labour intensive and random. Moreover the technique had few other limitations like incomplete knock down of the genes and identification of causative mutation was difficult. By use of genome editing technologies, specific genes can be targeted with minimal off targeting effects on global/ irrelevant genes and high efficiency, strong phenotypic effects and high validation rates. These technologies seem to be of immense value in the era of whole genome sequencing where establishing the functional significance of a genetic variant seems to be of utmost importance.[4]

2) For gene expression studies - nucleases which are used in genome editing methods can be made inactive by introducing a mutation in one of their functional domains which in turn can just tag at the desired genomic sites. These deactivated nucleases can control expression of endogenous genes and also are an important source to learn epigenetic modification of highly repetitive loci like telomeres and chromatin remodelling in live cells,

Character	Zinc finger nuclease (ZFN)	Transcription activator like effector-nucleases (TALEN)	Clustered regularly interspaced short palindromic repeats (CRISPR-Cas9)
Type of nuclease	FokI nuclease	FokI nuclease	Cas9
DNA identifying mechanism	Based on DNA- protein interaction. DNA binding domain is composed of repeats of 30 amino acids. 3-6 repeats per ZFN, each domain recognises 3 base pairs.	Based on protein DNA interaction. Repeats of 34 amino acids recognizing one amino acid.	Based on Watson crick base pairing between RNA and target DNA. 20 nucleotide long crRNA which typically consists of specific nucleotide sequences flanked by repetitive sequences. Target sequence should be preceded by NGG sequence called as protospace adjacent motif (PAM).
Mechanism of producing DNA double strands repair	Two ZFNs bind to opposite sides of the targeted DNA sequence with space of 5-7 nucleotides where Fok1 nucleases dimerise and produce double strand break in the space.	Same as ZFN	crRNA hybridizes with tracrRNA and activates cas9 nuclease which produces DSB.
Efficiency	0-12%	0-75%	0-80%
Off target effects	+	+	+++
Multiplexing	Difficult	Difficult	Easy
Ease of construction	Difficult	Difficult	Relatively easy

Table 1 Comparative features of the three important genome editing systems. [1,3]

a technique known as dynamic imaging.

3) For large scale high throughput gene disruption in drug discovery.

4) For gene therapy by using targeted gene disruption or gene correction methods.

5) For agricultural and live stock genomic modification.

CRISPR–cas9 System

CRISPR-Cas9 system is a Clustered, Regularly Interspaced, Short Palindromic Repeats-Cas9 (CRISPR-associated) system. This method of genome editing has been modified from the adaptive immune system of bacteria and archea against invasion of foreign viruses. Almost all bacterial genomes have CRISPR-Cas9 loci. As the name suggests these loci consist of clustered direct palindromic repeats which are spaced by interspersed nucleotide sequences. The repeats are typically 21-47 nucleotides long and identical in a single locus. The interspersed nucleotides sequences are called as spaces and are derived from foreign viruses. Spacer sequence is transcribed into Crispr RNA (crRNA). CRISPR locus also contains DNA sequences which code for a complementary transactivating Crispr RNA (tracrRNA) and various Crispr Associated genes (cas) which code for nucleases. These crRNA hybridize with complementary transactivating Crispr RNA (tracrRNA) and together, as a double strand, they recognise the complementary foreign nucleotide sequences. The diagrammatic representation of structure of CRISPR/Cas9 system is

given in fig 1.

There are 3 types of CRISPR-Cas immune systems. Out of them only the type II system (adapted from Streptococcus pyogenes), which uses Cas9 as a nuclease, is used as a method of genome editing, which is why it is known as the CRISPR-Cas9 system. Cas9 nuclease has HNH nuclease domain and the RuvC-like domain which generate DSBs.[1–3]

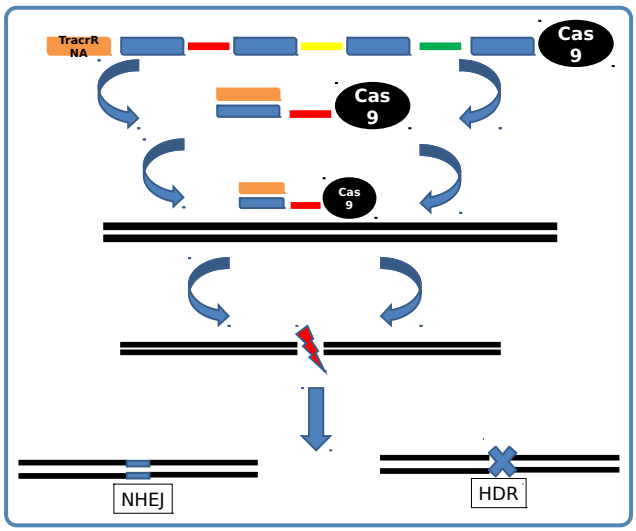

Figure 1 Schematic representation of the CRISPR-Cas9 system. Blue bars represent the array of identical repeats. Red, yellow and green bars represent the various spaces/interspersed elements. Black oval is Cas9 and blue bar is transactivating crRNA (tracrRNA). Mature crisper RNA (spacer with repeats) hybridises with tracrRNA and along with cas9 makes a ribonucleoprotein complex. This complex binds to targeted 20 nucleotide genomic sequence and produces double strand breaks (DSBs). These DSBs are repaired by either error prone nonhomologous end joining (NHEJ) or precise Homology directed repair (HDR).

CRISPR–Cas9 System Construction and Modifications

In the laboratory the original CRISPR-Cas9 system is slightly modified for use as a genome editing tool. crRNA and transcrRNA can be constructed together as a single guide RNA (gRNA). Multiple gRNA can be multiplexed in a single construct to target multiple genomic sequences in one experiment. Also these gRNA can be combined with another DNA template which can be used for homology directed repair to induce desired genetic changes. To reduce the off target effects of the CRISPR-Cas9 system instead of a single gRNA combination, a combination of 2 gRNAs can be produced which have modified nucleases. These nucleases can be modified at their functional domain so that they produce a nick only in a single strand (known as nickase). Two gRNAs bind to adjacent genomic regions on the sense and antisense strands and paired nickases produce independent breaks. Hence an off target effect break will be there only in one strand which will be repaired by the base excision repair method using the homologous strand as a template. It would be very rare and unlikely for two adjacent genomic regions to have off target effects. This modification thus decreases the likelihood of off target effects by 50-100 folds (Fig 1).

Various Applications of CRISPR–Cas9 System

Genome editing technology, especially the CRISPR-Cas9 system has emerged as the new platform to study correlation of DNA sequences with its functional significance i.e. reverse genetics. Apart from the research interest, it is also being used as a newer approach to treat human genetic disorders and for research in chromatin modification. Following are the few success stories of the CRISPR-Cas9 system.

- *Role of CRISPR/Cas9 in repairing of CFTR defect*: A recent study performed by Schwank *et al.* used the CRISPR-Cas9 genome editing approach to correct the d508 mutation in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*). They isolated and expanded adult intestinal stem cells from two patients affected with cystic fibrosis.[5] Genomic editing was done using CRISPR-Cas9 system and homology mediated repair of d508 mutation. The authors also demonstrated functionality of the gene in in vitro epithelial organoid system. This article provides proof of concept that the CRISPR-Cas9 system can be used as an effective gene therapy method in various

single gene disorders where no curative treatment is available.

- *Role of CRISPR in HIV therapy*: After the introduction of highly active antiretroviral therapy (HAART), human immunodeficiency viral (HIV) infection is now considered a chronic disease which requires prolonged treatment. However HAART is associated with relapse of disease as soon as the treatment is stopped, high cost of treatment, commitment of the family and treating clinicians and potential of emergence of HIV resistance. CCR5 is a co-receptor on CD4+ cells which is essential for HIV entry inside these cells. A 32 base pair deletion in single exon of CCR5 gene produces a frame shift and disrupts the gene function. The famous patient from Berlin who had HIV with lymphoma was cured by bone marrow transplantation from a person who was harbouring this homozygous deletion in CCR5 gene. Hence disruption of CCR5 gene by gene silencing methods appears to be an attractive model of gene therapy for HIV.[2]

The first reported genome editing method was based on using ZFN and then other methods such as TALEN and CRISPR-Cas9 system began to be used. Genome editing is heritable and therefore does not require repeated therapy like short interfering RNA (siRNA) and only one time therapy is sufficient. Trials are going on using gene editing of patient derived CD4+ /CD34 +/ hematopoietic stem cells and induced pluoripotent stem cells followed by autologous transplantation back in patients. Early results are positive in terms of persistence of genetically modified cells in the circulation and no serious side effects. However the true efficacy can only be determined after interruption of HAART and after the final results of these trials. Also genome editing system has been used to eliminate already integrated HIV proviral DNA. Ebina *et al.* in 2013 in their study targeted the long terminal repeats (LTR) of the integrated HIV viral genome using CRISPR-Cas9 and showed the blocked expression of genes.[6]

- *Role of CRISPR-Cas9 in curing liver disorder*: In a recent study published by Yin *et al.*, the CRISPR-Cas9 genome editing approach has been used to cure hereditary tyrosinemia in mouse models.[7] The genetic correction was observed in 1/250 liver cells. Strong positive selection and further expansion of genetically corrected cells also contributed to the success story.

- *Role of CRISPR-Cas9 in Duchenne muscular dystrophy*: Duchenne muscular dystrophy is one of the common and severe genetic disorders of muscles with onset in childhood. The condition is relentlessly progressive and there is no curative treatment available. Because of the large size of the causative gene (Dystrophin), delivery of a smaller gene coding for a small but functional protein (microdystrophin or microutrophin) has been tried but results are not very promising and in preclinical trials. The other methodologies which are being explored are exon skipping by using oligonucleotides which, by blocking expression of one or more exons, restores the reading frame of the coding part and produces a functional protein. The limitations of this approach are the requirement of repeated delivery of oligonucleotides and development of separate oligonucleotides for each type of deletion. Recently genome editing technologies have also been explored that insert the deleted exons, help in exon skipping or produce small indels, thereby producing a full/small transcript. Li *et al.* isolated fibroblasts from a patient with DMD having deletion in exon 44 of the dystrophin gene.[8] These fibroblasts were programmed into induced pluoripotent stem cells (iPSCs) and by using TALENs and CRISPR-Cas9 system, 3 gene correction methods were tried. These methods were exon skipping, insertion of exon 44 and inserting small indels and hence restoring the reading frame. The authors found minimal off -target effect and concluded that exon knock-in approach was the most promising. These experiments hold the promise for future patient-specific mutation correction and autologous ex vivo gene therapy using iPSCs.

- *CRISPR-Cas9 in treating beta Thalassemia major*: Hematopoietic stem cell transplantation from a HLA matched donor is the curative treatment for beta Thalassemia major. In absence of an HLA matched donor, hyper-transfusion and iron chelation therapy remain the mainstay of therapy for these patients. Recently, Song *et al.* have created iPSCs from patients suffering from beta thalassemia major and by using CRISPR-Cas9 system have corrected the mutation in the human beta globin gene (HBB).[9] The authors have shown that the differentiation capacity of these corrected iPSCs is great with minimal off-targeting effects. These experiments further raise the hope and potential for using CRISPR-Cas 9 technology in genetically modified iPSCs transplantation.

Conclusion

CRISPR-Cas9 system has revolutionised the area of genome editing mainly because of ease of laboratory construction, targeting multiple genomic sites simultaneously and application in broad areas. Future challenges involve invention of efficient delivery systems, reducing off-target effects and increase in efficient homology mediated repair.

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