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Review Article

CRISPR Cas 9 – A New Era in Genome Editing and its Applications

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Abstract

The development of various tools to alter the targeted sequence of the genome of a living organism is necessary in the biomedical research. Recently, a new tool based on a bacterial CRISPR-associated protein-9 nuclease (Cas9) from Streptococcus pyogenes was developed. In this type of genome editing, the short RNA search string is used to guide a Cas9 protein which is an endonuclease, into a specific location within a complex genome. The modified RNA recognizes the DNA sequence, and the Cas9 enzyme cuts the DNA at the targeted location. Once the DNA is cut, researchers use the cell's own DNA repair machinery to add or delete pieces of genetic material, or to make changes to the DNA by replacing an existing segment with a customized DNA sequence. CRISPR Cas9 genome editing is used as an innovative tool in prevention and treatment of human diseases and animal diseases. Presently most of the research on genome editing is done to understand diseases using cells and animal models. This type of genome editing is used to target a protein domain for transcriptional regulations, epigenetic modification and microscopic visualization of specific genome loci. CRISPR cas9 has an advantage of introducing point mutations in a particular target gene using single guide RNA (gRNA). This enables rapid genome-wide interrogation of gene function by generating large guide RNA (gRNA) libraries for genomic screening. This system differs from the other method due to its simplicity, high efficiency, user friendly and versatility of the system. Any DNA sequence can be targeted by altering the first 20 nucleotides of the guide RNA (gRNA) for novel genome editing applications.

Keywords: CRISPR, Cas9 Proteins, Epigenetic Modification, Guiderna, Genome Editing, Point Mutation, Transcriptional Regulations

Introduction

Animals are extremely adaptive in nature; this characteristic has led to the worldwide distribution of the of the livestock species. Animals have been epigenetically and genetically adapted to varied climatic conditions. These adaptive mutations have been passed down to next generation, so that its genome can be redefined and improved. Recent advances in genome engineering modifies both the external environment and genetic adaptions in all the species. Scientists have devised many strategies to rewrite any genome after understanding the concept of central dogma. Gene editing is one of eminent technology which enables the researcher to alter the DNA of an organism. This technology allows the genetic material to be incorporated, deleted or altered at the specific locations in the genome. For several years' scientists have understood about genetics and function of a gene by studying the effects of changes in DNA. If a change in a gene is created either in a cell line or a whole organism, it is possible to then study the effect of that change to understand what the function of that gene is. Over the years geneticists are using chemicals or radiation to cause mutations. Scientists have devised a new technique called 'gene targeting' to introduce changes in specific places in the genome, by removing or adding either whole genes or single bases. An old method of gene targeting has been very valuable for studying genes and genetics; however, it takes a long time to create a mutation and is fairly expensive. Several 'gene editing' technologies have recently been developed to improve gene targeting methods, including CRISPR-Cas systems, transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs). The CRISPR-Cas9 system is the fastest, cheapest and most reliable method for editing genes. CRISPR-Cas9 stands for Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9. CRISPER- Cas9 is a distinct technology that allows geneticist and medical researches to edit parts of the genome by deleting, inserting or altering section of DNA sequence. It is presently the simplest, most versatile and precise method of genetic manipulation and hence it creates a high excitement in the world of science. The elegance and simplicity of Cas9 have sparked the imagination of scientists across many scientific disciplines.

CRISPR Cas9 System

CRISPR Array

Some bacteria have a similar, built-in, gene editing system to the CRISPR-Cas9 system that they use to respond to invading pathogens like viruses like an adaptive immune system. Using CRISPR the bacteria snip out parts of the virus DNA and keep a bit of it behind to help them recognise and defend against the virus next time it attacks. Scientists adapted this system so that it could be used in other cells from animals, including mice and humans. CRISPR stands for clustered regularly interspaced short palindromic repeats (Shalem *et al.,* 2014). These repeats were first noticed in *Escherichia Coli*. CRISPR repeat clusters are separated by non-repeating DNA sequence which are known as spacers. The CRISPR elements are located next to multiple well-conserved genes which are referred to as CRISPR-associated (Cas) genes (Sternberg *et al.,* 2014). These Cas genes are translated into proteins. CRISPR arrays are initially transcribed as single RNA and later it is processed into short CRISPR RNAs (crRNAs). The crRNAs along with a second transcript known as trans-activating crRNA (tracrRNA) guides Cas9 nuclease to degrade the target DNA (Jinek *et al.,* 2012).

Cas9 Protein (CRISPR Associated- Protein 9 Nuclease)

Cas9 is a multi-domain, multifunctional DNA endonuclease. Among the Cas proteins, Cas9 also referred to as Csn1 plays a vital role in CRISPR mechanisms specially type II CRISPR system. The mechanism of type II CRISPR system is different to other CRISPR system as Cas9 is the only protein that is required for gene silencing. Cas9 protein is a key player in processing crRNAs and in the destruction of the target DNA. There are two nuclease domains, a RuvC – like nuclease domain which is located at the amino terminus, and a HNH – like nuclease domain situated in the mid region of the protein (Jinek *et al.,* 2014).

For the destruction of target DNA, both HNH and RuvC – like nuclease domains cuts both DNA strands causing double stranded breaks (DSBs) within the crRNA transcript at sites that are defined by 20 nucleotide target sequence. RuvC domain cleaves the non-complementary strand whereas HNH domain cleaves the complementary strand. A short-conserved sequence about 2-5 nucleotides known as protospacer- associated motif (PAM) which is located 3' downstream of crRNA. Complementary sequence is essential for the double stranded endonuclease activity of Cas9 protein (Anders *et al.,* 2014).

Cas 9 binds with both the crRNA and a separate trans-activating crRNA (tracrRNA) for site specific DNA recognition and cleavage. Trans-activating cRNA is partially complementary to crRNA from a primary transcript which encodes multiple pre-crRNAs. Maturation is mainly due to the presence of RNAse III and Cas9 protein (Jaing *et al.,* 2015).

Mechanism of CRISPR Cas9 Genome Editing

Type II CRISPR loci mostly consist of the cas9, cas1, and cas2 genes for transcription, as well as a CRISPR array and tracrRNA. CRISPR array consists of repeated sequence and unique sequences. When the virus enters the bacterial cell, it injects its DNA into the bacterium. If this bacterium has a CRISPR array in its genome it can acquire the new pieces of DNA from the virus and incorporate them into the array keeping a pattern of repeats. The Cas genes are translated into proteins, most of the CRISPR arrays are first transcribed as a single RNA and then processed into shorter CRISPR RNAs (crRNAs) which, together with a trans-activating crRNAs forms the single guide RNA (sgRNA). This sgRNA forms a complex with the Cas9 protein. The sgRNA is the fusion of CRISPR RNA (crRNA) and trans-activating CRISPR RNAs (tracrRNA). Cas9 binds with sgRNA complex and recognizes Protospaces-adjacent motif (PAM) sequence. The complex slows down enabling sgRNA to bind the 5' end of the PAM sequence. When there is a perfect base pairing of sgRNA and the genomic sequence, sgRNA and its genomic complement enters the central channel of Cas9 protein. This causes the genomic DNA cleavage in the complementary genome by either of the nuclease domain located in Cas9 protein (Jinek *et al.,* 2014). Anti-complementary genomic DNA is also cleaved by entering into the second channel of Cas9 protein (Anders *et al.,* 2014). Cas9: sgRNA complex dissociates from the genomic DNA upon cleavage. The double stranded break caused by Cas9 protein leads to activation of DNA damage responses. The break is repaired either by homologous recombination (HR) or by non- homologous end joining (NHEJ).

Applications of CRISPR

Genome editing is one of the ways to bring about meaningful change in the genome and one among them is CRISPR Cas9. It has wide variety of applications in targeted genome engineering. The wild-type Cas9 nuclease has been efficient in targeted genome modification in many species that have been intractable using traditional genetic manipulation techniques (Hsu *et al.,* 2015). CRISPR has more potentials and applications compared to previous systems.

Antimicrobial and Antiviral Applications

Native or engineered CRISPR–Cas systems are associated with nucleases that generate the ds DNA break (DSB) (Sinkunas *et al.,* 2013) and can be programmed to target any bacterial species. The sequence-specific antibiotics generated can selectively transform bacterial populations and eliminate pathogens (Gomaa *et al.,* 2014). The main advantage of sequence-specific antimicrobials is that they can precisely target clinical genotypes or epidemiological isolates thereby enabling the survival of beneficial commensal bacteria, so has an advantage over broad-spectrum antibiotics. But the major challenge is the development of robust delivery options (Barrangou and Doudna, 2016).

In the area of antivirals, CRISPR-based therapies are being developed that can target human viruses, including herpes (Wang and Quake, 2014), papillomavirus (Yu *et al.,* 2014), HIV-1 (Wang *et al.,* 2014) and hepatitis B virus (Zhen *et al.,* 2015). For example, co-disruption of genes encoding the HIV cell-surface receptor proteins chemokine (C-X-C) motif receptor 4 (CXCR4) and chemokine (C-C motif) receptor 5 (CCR5) shows promising results in preventing HIV entry into CD4+ T cells, and for antiviral therapy (Wang *et al.,* 2014). The risk of porcine endogenous retroviruses (PERVs) viral spread during tissue transplantation can be prevented by the removal of PERVs from the entire pig genome using CRISPR-Cas9 technique (Yang *et al.,* 2015).

Agricultural Applications

Identification of quantitative trait loci (trait-associated chromosomal markers) and use of marker-assisted breeding has been followed recently to selectively advance valuable traits. This process will be accelerated using genome editing technologies, as shown in pigs to protect against viruses (Whitworth *et al.,* 2016) and dairy cattle to remove horns (Carlson *et al.,* 2016). This can be used engineer production of either medical products or tissues using knock in methods using transgenic animals (Peng *et al.,* 2015).

CRISPR-enabled engineering is being used in development of commercial and model crops in order to increase yield, improve drought tolerance and increase growth even in limited-nutrient conditions, and to breed crops with better nutritional properties (Ricroch and Hénard-Damave, 2016).

Applications in Food and Industrial Biotechnology

The success of CRISPR–Cas immune systems for the vaccination of *Streptococcus thermophilus* starter cultures used in dairy fermentations (yogurt and cheese) has tiled the way for CRISPRs in food (Barrangou and Horvath, 2012). Beneficial bacteria may also be generated that are immunized against the uptake and dissemination of genes that encode antibiotic resistance (Garneau *et al.,* 2010). CRISPR–Cas9 can also be used to engineer industrial bacteria, yeast and fungi with the intension to produce green chemicals, including biofuels (Ryan *et al.,* 2014) and biomaterials.

Resistance Against Malaria by Modification of Mosquito DNA

Using CRISPR, researchers have developed mosquitoes that transmit resistance to disease in the species of their own. Cas9 has been used to create gene drives in the mosquito vector for malaria, *Anopheles gambiae,* to drive a recessive female sterility genotype with transmission to progeny rates exceeding 90%. The inserted DNA encoded engineered antibodies attack the malaria parasites (Gantz *et al.,* 2015). Such approach suppresses the spread of malaria in humans (Hammond *et al.,* 2016).

Rapid Generation of Cellular and Animal Models

Cas9-mediated genome editing has enabled rapid generation of transgenic models (Sander and Joung, 2014). CRISPR-based editing could be used to recreate the genetic mutations found in patient population, thereby facilitating to acquire the causal roles of those genetic variations, instead of relying on disease models. This could be applied to develop novel transgenic animal models (Niu *et al.,* 2014). Using transient transfection of plasmids, Cas9 can be easily introduced into the target cells for generation of cellular models. Cas9 facilitates to study the effect of each individual variant or test the effect of manipulating each individual gene (Hsu *et al.,* 2014).

Cas9 protein and transcribed sgRNA can be directly injected into fertilized zygotes for generation of transgenic animal models such as rodents and monkeys (Wang *et al.,* 2013). The generation time for mutant mice and rats can be reduced significantly from more than a year to only several weeks. This facilitates cost-effective and large-scale *in vivo* studies in rodent models (Fu *et al.,* 2014). Further Cas9 could be used for direct modification of somatic tissue, enabling therapeutic use for gene therapy.

Personal Medical Applications of CRISPR

The CRISPR system could be used for editing embryonic stem cells, and then, re-injected into the patient in order to modify the faulty genes directly. By creating a mutation in the RuvC-NHN domains of CRISPR-Cas9, researchers plan to create a modified enzyme that does not have endonuclease activity and will be used merely to help target other enzymes (Driehuis and Clevers, 2017). The targeted mutations created in Cas9 make the protein lose its endonuclease property, but preserve its ability to identify its DNA target location and to bind it due to the presence of sgRNA sequences. The use of this system is to attach other enzymes to the Cas9 sequence so that it can bind to the target site with Cas9 and perform their specific enzymatic activity instead of cleaving the sequence (Hsu *et al.,* 2014).

CRISPR and Cancer

Presently, the CRISPR system has been used in the field of cancer (Yao *et al.,* 2015), by modulating the genes involved in cancer development, development of cancer models in which several genes are involved or cancers that are caused by chromosomal clutter and rearrangements, and identifying the genes involved in cancer development (Maresch *et al.,* 2016). In detection and the identification of factors involved in cancer the CRISPR system is expected to have a major role. The CRISPR system could potentially treat the disease by generating accurate mutations through turning off the oncogenes or turning on the tumour suppressor genes because generally cancer is generated either by the activation of oncogenes or suppression of tumour suppressor genes for various genetic and

epigenetic reasons (Khan *et al.,* 2016). In various cancers telomeres become activated. In this regard CRISPR has also been proposed as another potential method for cancer treatment by turning off telomerase (Harley, 2008). Presently, desired genetic mutations can be generated within four weeks using CRISPR technique, this helps in development of sexual and somatic cancer types can within a short duration for research purpose (Sanchez-Rivera and Jacks, 2015).

Imaging of Cellular Genome

The structure, function and the organisation of the cell is directly linked with the functional output of genome. However, the structural organisation of the genome is not clearly studied. Some of the traditional techniques like fluorescence in situ hybridisation and labelling DNA lacks live capturing of the genome. Cas 9 can be used as a live-cell imaging as an alternative to DNA FISH by fluorescently labelling Cas 9 to the specific DNA loci. This modified Cas 9 protein produces multiple colors at different loci enabling the study of nuclear organisation and complex chromosomal structure (Hsu *et al.,* 2014).

Application of Crispr in Improving Livestock Production Tuberculosis Resistant Cows

Homologous recombination (HR) is an important tool used for the exchange of DNA sequences between the target chromosomal locus and homologous template which consists of desired change. CRISPR-associated Cas9 protein is also an effective tool to target the genes at specific locus for cleavage using single guide RNA and stimulate gene insertion. Cas9n was used for *Natural resistance-associate macrophage protein-1* (*NRAMP1*) gene insertion at a specific locus in cattle. This technique had reduced off target effects. Somatic cell nuclear transfer technology was used to obtain transgenic cattle. Cas9n- mediate *NRAMP1* insertion produced the cattle with increased resistance to tuberculosis (Gao *et al.,* 2017).

Porcine Reproductive and Respiratory Syndrome (PRRS) – Resistant Pigs

Porcine Reproductive and Respiratory syndrome (PRRS) is highly contagious and panzootic disease which causes a huge economic loss to the swine industry. PRRS is caused by a positive strand RNA Virus (PRRSV) which can easily bind to CD163 receptor. CD163 is a scavenger receptor which contain nine scavenger receptor cysteine-rich (SRCR) domain 5 (SRCR5) which encodes exon 7 thereby causing PRRSV infection in vitro. The deletion of CD163 whole protein or SRCR5 region which is an interaction site for the virus results in resistance to infection in pigs. CD163 protein was knock down by using CRISPR / Cas9 gene targeting. Somatic cell nuclear transfer technology was used to produce PRRS resistant pigs (Chen *et al.,* 2019).

Cashmere Goats Production

Cashmere goats are characterized by the presence of double coat with outer hair produced by primary follicle and inner fine coat produced by secondary hair follicle. Goats are modified using CRISPR- Cas9 system by targeting *FGF5* (*Fibroblast growth factor 5*) gene thorough microinjection of Cas9 mRNA and sgRNAs. Cas9 system was used in disrupting *FGF5* gene. This increased the number of secondary hair follicles which in turn enhanced the fibre length (Wang *et al.,* 2016).

Xenotransplantation

Due to the shortage in tissues and organs of humans for xenotransplantation, a new promising strategy called Xenotransplantation is adapted. Organs of pigs are considered as a resource for Xenotransplantation not only as they are similar to human organs in function and size, but also due to its high prolificacy. Major disadvantage of using pig organs for transplantation is its risk of PERV (Porcine endogenous retro virus) transmission. CRISPR Cas9 can be used to inactivate PERV virus in primary cell line. PERV-inactivated pigs were produced through somatic cell nuclear transfer. Hence, these modified pigs can be used for xenotransplantation (Niu *et al.,* 2017).

CRISPR in Animal Welfare

Attempts have been made to avoid sufferings of animals and hence encouraging more compassion and respect

towards animals. Genome editing makes a huge contribution with this regard. Welfare-enhanced animals is a novel concept to avoid animal suffering. Many routine procedures used to mitigate some of the consequences of intensive livestock, such as calf dehorning, male castration, tail-docking in dairy cattle, mulesing and taildocking in sheep, abortion or offspring culling of not desired gender, results in both immediate and chronic pain. Some of these practices may be avoided with the application of CRISPR Cas9 genome editing (Menchaca *et al.,* 2020).

Conclusion

CRISPR Cas9 genome editing is a gift from mother nature. Nature is an inspiration to the researchers, but most power tools available to scientists are derived from natural products. The recent discovery on CRISPR Cas9 genome editing is the most convincing example of how scientists have been able to learn and adapt nature's invention. CRISPR Cas9 genome editing is the most convincing example of how scientists have been able to learn and adapt nature's invention. CRISPR Cas9 has various advantages and disadvantages from being known for its fastest, cheapest and most precise methods of genome editing to ethical concerns associated with manipulating the genome. However, this technology is able to eliminate many diseases very easily giving a ray of hope to billions of people. CRISPR has much success rate in genome editing compared to other nuclease technologies making this unique. CRISPR technology is like a software in a computer as we can program the genome easily by using a bit of RNA.

Conflict of Interests

There is no conflict of interest.

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