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Possibilities of Using CRISPR-Based Genome Editing Technologies in Livestock

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ABSTRACT

Genome editing technologies are promising to improve the genetic structure of farm animals against diseases as well as increase economically important yields. Among these technologies, Clustered Regularly Interspaced Palindromic Repeats (CRISPR) has revolutionized the science of genome editing due to its numerous advantages such as accuracy, usefulness, and time-efficiency. Genome editing in large animals has always been complicated due to many technical and other complicated limitations. On the other hand, CRISPR may provide several alternatives to carry out the difficult task with more feasibility and better results. Many of the disease-promoting genes in animals have been knocked down using the CRISPR gene-editing technique thereby helping the animals to become resistant to the particular diseases. Moreover, genetic defects can be treated by knocking down the genes in animals by using CRISPR technology. Knocking down the defective gene in the embryo at an early stage helps the animals to grow and develop without any genetic diseases. Though the CRISPR technology is relevantly new, and it has not been used more comprehensively for genome editing in large animals, but still there are numerous studies making use of the CRISPR technology in chicken and pig genome editing. Furthermore, CRISPR-adopted gene editing has been used very extensively and it has generated favorable results to improve plant production to cope with the food security problem in the world. In this review, we discuss the methodology and feasibility of some of the CRISPR-based gene-editing technology and the success stories in animal science. Moreover, we will also discuss the future application of CRISPR genome editing in developing disease-resistant farm animals with more yield than before.

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INTRODUCTION

Human food preferences make them dependent on animal products like milk, meat, and eggs. These livestock products are a daily need for humankind because humans tend to like animal products more than plant-based food products. The increase in the demand for livestock products needs to be met with the growing population by increasing livestock production. Gene alterations and gene editing technologies provide a big opportunity to increase livestock production by knocking in or knocking out specific genes. Therefore, livestock genetic engineering is focused on producing disease-resistant animals, livestock products without allergens, animal sexing, and introducing new valuable traits in the major livestock animals (Singh and Ali, 2021).

Nucleases dependent genome editing techniques offer a unique process to cut and paste a gene or a specific sequence in the genome with more precision. These techniques are considered to be the most efficient in knocking in/out a specific gene and making accurate genome modification in the genome of an organism. The four major techniques that are based on nucleases: Transcription Activator-Like Effector nuclease (TALENs), Zinc Finger Nucleases (ZFNs), Clustered Regularly Interspaced Short Palindromic Repeat-Associated Nuclease cas9-cas12 (CRISPR, cas9 and cas12), and Mega nucleases. After recognizing the specific sequences in the DNA, the nuclease will cut the DNA molecules by making Double-Strand Breaks (DSBs). And if the repairing enzymes are not inserted along with the nucleases, the endogenous repair mechanisms will be activated by the DSB. The endogenous repair mechanism that joins a double-stranded DNA cut is known as Non-Homologous End Joining (NHEJ). NHEJ repair mechanism after activation will rejoin the two open ends of DNA that can lead to deletion or insertion at the site. When this mutation happens in the DNA sequence it causes frameshift mutation which will then lead to knockout of the gene. Therefore, this mutation in the gene will lead to a loss of function of the targeted gene in the desired cells or organism. Furthermore, introducing two simultaneous DSB breaks in the targeted DNA sequence can cause inversion, deletion, duplication, or translocation of the DNA part and it can cause rearrangement of the chromosome at the cleavage site (Cox et al., 2015).

In case, one is trying to achieve the desired and predictable deletion, insertion, or substitution the repair template is made available with the nucleases that can help direct the rejoining of the DNA ends. If a repair template is available, then homologous directed repair (HDR) which is slower will occur instead of non-homologous end joining (NHEJ). Although HDR is slower than NHEJ but with HDR one can predict the results. Furthermore, if the NHEJ (DNA ligase IV) promoting molecules are inhibited the HDR repair can be promoted (Osakabe and Osakabe, 2015; Weber et al., 2015). In the recent applications of ZFNs, CRISPR, meganucleases, and TALENS have been used to conduct site-specific genome editing by taking advantage of their ability. ZFNs, TALENs, and meganuclease perform their cleavage by using DNA and protein

interaction (Maruyama et al., 2015). Which needs more engineering and diverse proteins if different targets are to be cleaved in the different experiments, consuming more time and capital.

On the other hand, CRISPR-Cas dependent gene editing occurs through a different method. The CRISPR-Cas method of genome editing technology includes a guided RNA (gRNA) for base pairing with the specific target site, and cas9 or cas12 proteins which will cut the DNA at the specific sites (Maruyama et al., 2015).

The CRISPR-Cas system is an innate immune pathway system found in prokaryotes to protect them against foreign plasmids, viruses, and DNA molecules (Maruyama et al., 2015). The CRISPR system is divided into different mechanisms. The type I and III CRISPR mechanisms include various CRISPR proteins that are required for recognition and cutting of the specific sequence on the gene. The type II mechanism is CRISPR-Cas9 which requires lesser proteins, but it includes the guided RNA (gRNA) (Hsu et al., 2014). The type V mechanism involves CRISPR-Cas12, which has a specific multiplex ability to recognize and cut the gene without the gRNA. The type IV mechanism includes CRISPR-Cas13 protein and is different from others because it cuts the RNA instead of DNA.

The CRISPR-Cas9 system is comprised of three parts, the CRISPR RNA (crRNA), trans-activating RNA (tracrRNA), and the Cas9protein which has the endonuclease activity (Cho et al., 2013). The tracrRNA and the crRNA make double-stranded RNA on the base pairing principle because both have complementary sequences to each other. After the formation, the formed complex with Cas9 protein and is ready to be delivered to the target site for activity. The recognition ability of crRNA is dependent on the specific region found the downstream of 3'end of the target sequence known as the protospacer adjacent motifs (PAM) (Li et al., 2020).

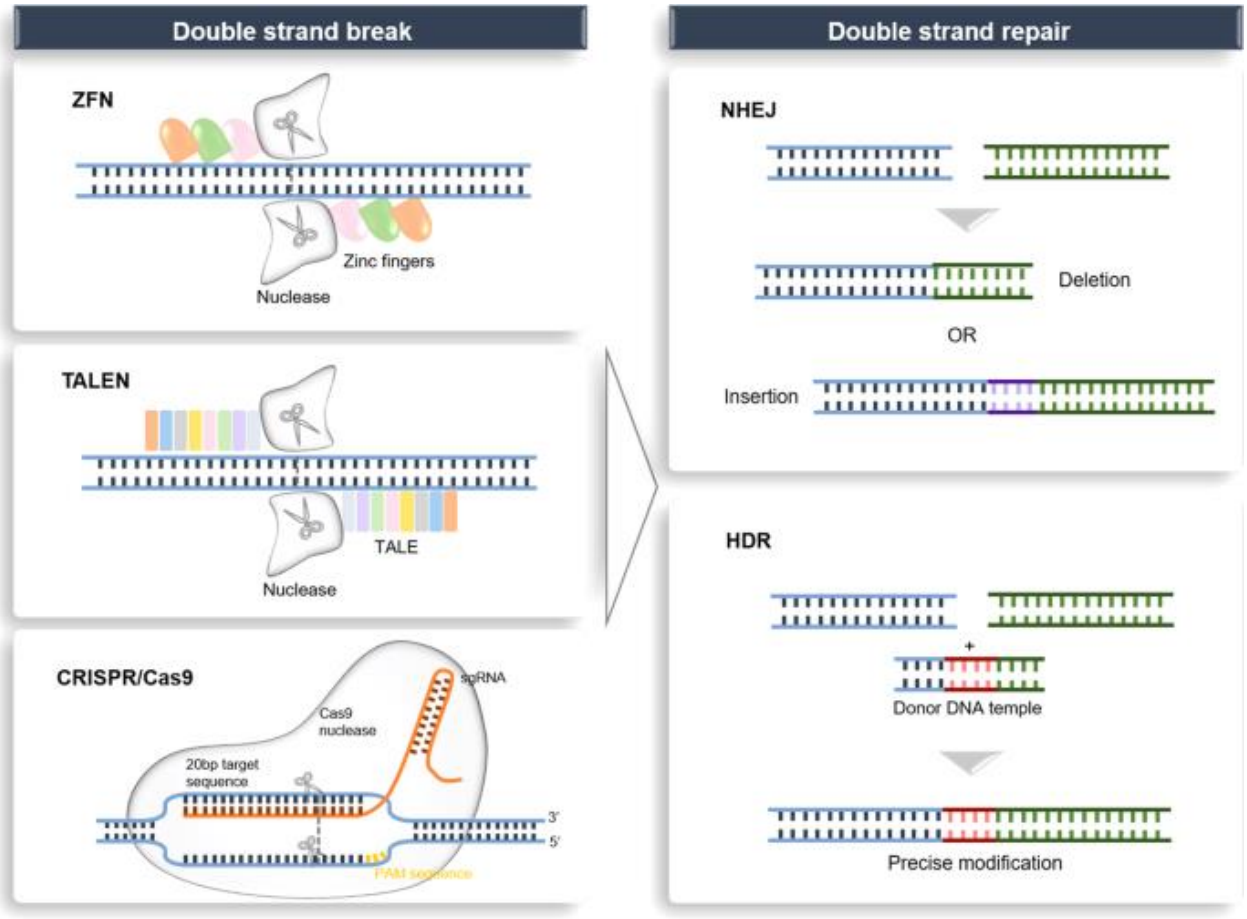

Figure 1. Genome editing exploits endogenous DNA repair mechanisms. A) Genome editing nucleases (ZFNs, TALENs, and CRISPR-Cas9) induce DSBs at targeted sites. DSBs are repaired by NHEJ or, in the presence of donor template, HDR. NHEJ will induce indels at the editing site and HDR could insert predicted DNA fragments. The inhibition of NHEJ key enzyme DNA_ligase IV could increase the efficiency of HDR. B) NHEJ and HDR would occur in different stages of the cell cycle.

Furthermore, Cas12a belongs to the V-A system and needs lesser components to start working than that of Cas9, in Cas12a tracrRNA is not required only crRNA is enough for endonuclease activity. fnCas12a recognizes a 5′-TTN-3′ protospacer adjacent motifs PAM upstream region and begins its cutting activity. In the case of similarities Cas12a and Cas9 both get a structural modification after recognizing and loading on the target DNA, this structural modification makes a cleft in these proteins to accommodate the RNA/DNA complex formed. Furthermore, the fnCas12a creates double-stranded break DSB catalyzed by its RuvC domain, unlike Cas9 the fnCas12a makes a staggered DSB break with 5-nt overhang outside upstream to the PAM (Fonfara et al., 2016; Zetsche et al., 2016). However, the exact mechanism of cleavage by Cas12a is still not understood thoroughly and is being studied further.

The other important member of the CRISPR family is Cas13a nuclease which unlike Cas9 and Cas12a cleaves RNA rather than DNA. Cas13a nuclease belongs to the CRISPR-Cas VI system (Abudayyeh et al., 2016; Abudayyeh et al., 2017). Moreover, Cas13a cleaves RNA by its RNase activity and requires guided RNA for recognition of

specific RNA targets. In addition, Cas13a not only cuts the pre-crRNA but also cleaves the guided RNA as well. It was seen in the structural study of Cas13a that Helical-1 and HEPN domain are involved in the cleavage of pre-crRNA and target RNA respectively (Liu et al., 2017). The in-vitro studies showed that after the cutting of pre-crRNA Cas13a also cuts the collateral RNA which does not show any complementation with crRNA. This undefined and unnecessary cutting can be lethal for cell division and cell growth. Although this nontargeted cleavage was seen in prokaryotes when Cas13a was introduced in Human cells it showed no off-target cleaving activity, but it cleaved the designated crRNA. In E. Coli cells the off target cutting activity of Cas13a showed some side effects like cell growth retardation and cellular toxicity (Abudayyeh et al., 2017).

Methodologies involved in CRISPR-cas based gene editing

NHEJ and HDR dependent DNA repairing

The DSB (double-stranded break) created by CRISPR nuclease can be repaired by two major pathways i.e., NHEJ (nonhomologous end joining) and HDR (Homology-Directed Repair) repair mechanisms. NHEJ based repair of the DSB will lead to a point mutation in the DNA sequence of the chromosome. This point mutation can either be an insertion or deletion of a single nucleotide or insertion or deletion of a few hundred nucleotides. Hence the NHEJ DNA repair mechanism is prone to errors in the DNA structure of the chromosome. Although sometimes if the two DNA overhangs do not join immediately, they will ultimately base pair after the addition or deletion of a few nucleotides by exonucleases (Lieber et al., 2003). This DNA damage is present in the initial exon of the gene will lead to a frameshift mutation which will intern result in a complete loss of the protein code and hence in the end the knockout of the gene will occur. Moreover, this approach through CRISPR-cas can be applied to silence any defaulted gene to prevent the hereditary disease to be progressed from the start.

Furthermore, multiple gene knockouts can be achieved through this approach by triggering the NHEJ at different locations simultaneously. The designing of several sgRNA and endogenous injection of this sgRNA in several locations will lead to cleavage of mRNA by cas9 protein will lead to a multiplex knocking down of genes in animals. One such study in which they engineered the mice by knocking down 5 genes simultaneously at different loci by triggering cellular NHEJ after cleavage by cas9 nuclease. CRISPR-cas based genome engineering is preferred over TALENs and ZFN while working with mice because it requires small sgRNA preparation as compared to other nucleases complex and laborious requirements (Wang et al., 2013). Moreover, multiplex genome editing while working through TALEN and ZFN will require more amount of RNA concentrations to activate the editing by nucleases. This much-increased amount of RNA will lead to cell toxicity and hence not applicable for editing.

Hence multiplex genome editing using TALEN and ZFN has not been reported in research studies.

Ma et al. (2014) knocked down fur genes simultaneously in rats by introducing multiple sgRNA four the targeted genes. Although genome editing in complex genomes of large animals has been faced with different problems. However, this CRISPR-cas mediated gene-editing approach can offer genome editing in complex animals efficiently. The above-mentioned mice examples and many other genome editing successes in animals have been reported till this year, and it can be said that the larger animals can be engineered by using the CRISPR-cas mechanism. Furthermore, to create such editing possibilities the larger chromosomal arrangements can be targeted by different sgRNA simultaneously. Hence deletion or insertion can be promoted by targeting chromosomes with multiple sgRNA for the larger gene sequences. Deletion as large as 10kb and 15kb has been reported in mice (Fujii et al., 2013), in another example, a 40kb deletion has been reported in zebrafish (Xiao et al., 2013).

In a similar study, CRISPR-cas technology has been used to genetically engineer monkeys, a species that has been technically difficult to achieve and has many similarities to the human genome. A mixture of CRISPR machinery which included sgRNA mix, Ppar-c (two sgRNA), Rag1(one sgRNA), and cas9 mRNA was injected intracytoplasmic into fertilized eggs. Despite being in the gestation period during publication, in the newborn two of three targeted genes were edited as a result of the experiment. Several other cleavages were also detected during different embryonic stages in the same animals because of CRISPR-cas mediated engineering which further supported the feasibility of the technology to be used (Niu et al., 2014).

In addition, this technology can be adapted to delete the intronic region in the genes in order to study their regulatory roles in gene expression. In the recent past, similar research ENCODE project (ENCODE, 2012) studied the use of the CRISPR-cas system to delete the noncoding region of the gene to check their role in gene regulation. This study showed that the NHEJ repair pathway can be used to integrate transgene into the target chromosome without the requirement of designing a large vector with homology arms (Auer et al., 2014). However, the knocking in the vector must be provided with a similar sequence as the endogenous targeted gene sequence on a circular plasmid. Moreover, while working with gene replacement, one must keep in mind that only 1/3rd of the edited targets will retain the replaced fragment in their open reading frame.

Homology dependent repair mechanism for CRISPR-cas methodology

The general conventional gene-editing methods are although valuable, but they cannot sometimes create the desired results for the geneticist. Conditional gene disruptions create an opportunity to study the effect of mutation on organs and tissues at different

developmental stages. This conditional mutagenesis is often used to disrupt a lethal gene in order to protect the toxic gene results (Mastracci et al., 2013). The complex conventional methods are used for producing gene knockout in mice by targeting the ES (embryonic stem cells) by Cre-loxp technique, as a result, the new genetically altered mice are then bred to the Cre-driver line of choice. But while using this it is to be insured the expression pattern of both targeted tissue and the Cre lines must be compatible for developmental studies, and also it requires more resources and better training. Due to this complex methodology and handling the creation of all gene knockouts for mice is handled by the international knockout mouse Consortium (IKMS) (Skarnes et al., 2011). Even though this is handled by such a big organization still cannot always fulfill the desired targeted deletion and insertion by the researcher worldwide.

However, this can be achieved more efficiently and precisely by using the CRISPR-cas system for creating targeted mutagenesis without the need for ES cell work (Shen et al., 2014). As explained above we can create a targeted DSB and activate homologous recombination by inducing the HDR mechanism (Rouet et al., 1994). Wang et al. induced a targeted mutation in the mouse zygotes by introducing a combination of ssDNA donor and sgRNA in mouse which activated homologous recombination (Wang et al., 2013). This was achieved by targeting the exon with two sgRNA and two oligonucleotide-bearing loxP sites, which resulted in a single allele in a one-step embryo injection (Yang et al., 2013). These impressive results illustrate the efficiency of CRISPR-cas methodology, which helped to create a mouse that carried floxed allele in just two months. On the contrary, the conventional method of ES will take 9-10 months to complete the constructed building, transfection, screening, and validating and detecting the germline transmitting chimera.

Furthermore, the CRISPR-cas technology can help generate animal models for human disease studies with more preciseness and efficiency. With simplicity, this technology can be used to insert a single long strand of template donor DNA in the desired site by just creating DSB through nucleases. However, in order to create such results first, certain parameters need optimization (Yang et al., 2013). These parameters include DNA quality, length of DNA oligonucleotide, mutation and position between the cleavage sites, concentration, and purity of DNA template.

Application of CRISPR-cas in Farm Animals

The CRISPR-cas gene-editing technology has created the possibility of transgenesis in livestock and other large animals. For instance, the genome of cattle and pigs was altered by using this technology in the recent past (Tan et al., 2013). The classical breeding of different animals is carried to transfer the desired genes from one animal into another in livestock production. However, this process can take a very long time to transfer the desired characters from one animal to another. Moreover, certain characters show hindrance for breeding because of the natural less value to

heritability. Hence some very important characters cannot be transferred from one animal to another due to time and natural resistance. But this technology can help us to target certain character genes for creating important necessary mutations into the animal genomes. Furthermore, the lethal genes which can be harmful to the survival of the animal can be knocked down by CRISP-cas technology. For instance, the Angus polled allelic variant in the bulls can be created by using these nucleases and dehorned bulls can be produced (Tan et al., 2013).

Genome engineering of cattle, pigs, and other mammalian animals is done by delivering the desired gene through somatic cell nuclear transfer SCNT technique or microinjection inside the nucleus (Li et al., 2015; Gao et al., 2017). The methodology of somatic cell nuclear transfer involves the transfer of transgene into the nucleus of the primary cell in vitro and after that transferring the nucleus into the enucleated cells of oocytes for embryo production (Verma et al., 2015). The transfer of these genetically engineered oocytes into the surrogate mother is done at the blastocyst stage to complete the process. The production of transgenic pigs can also be achieved by transferring the CRISPR-cas reagents directly through microinjection in the zygote (Wang et al., 2016).

The CRISPR-cas genome editing technologies are being used to produce pathogen-resistant pigs in order to fight the economic loss to the industry. Furthermore, this technology has been used to produce Porcine Reproductive and Respiratory Syndrome Virus resistant pigs. This pathogen has been responsible for a large number of stillbirths and abortions in pigs. The gene responsible for the production of the receptor for the PRRS virus CD163 was knocked down by using the CRISPR-cas9 (Burkard et al., 2017) Moreover, the technology has also been recently used to introduce a gene in the cattle genome to provide resistance against tuberculosis (Gao et al., 2017).

The use of gene-editing technology particularly CRISPR has provided enormous opportunities for scientists to create more resistant animals against different diseases. For example, in 2015 three labs in the USA collaborated on a project to make new mice that were partially resistant against muscle dystrophy by using CRISPR-cas9 protein (Long et al., 2014; Ousterout et al., 2015; Nelson et al., 2016; Tabebordbar et al., 2016). This creates the possibility of raising the Aberdeen cattle in the warmer climate regions in the world which can help to diversify the availability of Angus beef worldwide. In another effort, the private enterprise of AgGwntics genetically engineered a cow Aberdeen breed to produce short white hair which is thought to be more appropriate for warmer climates (Patel, 2017). Furthermore, the Recomibinetics have genetically engineered a cow that is hornless, which means there is no need to manually dehorn the cattle anymore (Carlson et al., 2016).

The chicken industry has also a lot to gain by using modern gene-editing technologies. For example, the egg has very high nutritional value but because of the presence of

some allergens, some individuals cannot consume eggs (Dhanapala et al., 2016). Moreover, eggs not only provide us with great nutritional contents, but they can also serve as an efficient carrier for delivering the vaccines indirectly to humans. But because of the presence of allergens, this cannot be achieved for everyone. CRISPR-cas9 technology has been used to produce genetically engineered chickens that lack the allergens like ovalbumin and ovomucoid hence making the eggs safe for sensitive people (Oishi et al., 2016).

Disadvantages and Ethical Issues with CRISPR-cas

CRISPR-cas mediated gene editing technologies stand out in performing the desired task better than traditional methods, but there are some limitations and ethical issues attached with technologies that make the technology face criticism from the certain scientific community. Off targe cleavage of the DNA in the genome is one of these problems. Because in some cases after cleaving the desired DNA fragment the CRISPR machinery may interact with the resembling off-target DNA fragment and starts cleaving which creates unwanted and non-healthy mutations in the organism genome. Moreover, some ethicists and researchers present at International Summit on Human Gene Editing raised some serious ethical questions about the use of technology. Scientists are concerned that until germline genome editing is considered safe and accepted by the people and scientific community, the CRISPR technology should not be used for reproductive purposes (Lanphier et al., 2015). However, some scientists believe that even though reproductive genome editing should not be carried out, but the use of the technology for conducting safe genome therapy research should continue (Hinxton Group, 2015; Briere and Resnick, 2017). Furthermore, some bioethicists argue that allowing the use of technology for therapeutic and non-therapeutical enhancement will put us on a slippery slope which can lead to some dangerous experiments. However, there is another scientific community that believes that if proven safe the technology should be used for curing the genetic disease (Savulescu et al., 2015). The use of technology for removing the homozygous recessive genetic defective genes can be a field where the use of technology can be accepted in near future. Because knocking down defective genes in the target individuals can help save lives and help the people live normal lives.

CONCLUSION

Gene-editing technologies can offer a lot to animal science. For example, the modern gene-editing tools CRISPR-cas technology have made it possible to genetically engineer farm animals the first time. Furthermore, these gene-editing technologies not only provide the opportunities to produce disease-resistant animals but can also be used to increase the production of farm animals in different fields. The increased production and better yield from the animals is the need of the hour with the growing population and increasing food requirements. CRISPR-cas technology can also be used

for gene therapy and help remove the defective genes and replacing with normal genes. This can allow the target organisms to live a normal life. However, certain ethical issues and safety concerns from the scientific community and general public should be addressed first before allowing the use of technology to be used at a large scale for production and therapeutic purposes.

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Conflict of Interest

The authors have declared that that there are no competing interests.

Authors' Contributions

All authors have equally contributed.

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