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The Overview of CRISPR Technology and its Application on Farm Animals

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ABSTRACT

Livestock animals are important for agriculture economy and biomedical research. They are sources of mike, meat, egg, leather and other products. The development of genome editing technologies, especially CRISPR-Cas have revolutionized the generation of gene edited farm animals. In this review, we briefly introduce the CRISPR-Cas technology and highlight its application on livestock such as human disease modeling, disease resistant animal, alteration of milk composition, animal welfare and other agricultural and biomedical related traits which enhance the livestock production in order to meet the increasing demand of food worldwide. Besides the several benefits of CRISPR-Cas technology, the risk factors and ethics issues related to this technology should be reconsidered before enter into the CRISPR era.

Keywords: CRISPR; Genome editing; Livestock; Disease model; Cow milk allergy

INTRODUCTION

Large animals play important roles in biomedical research. Animal models are crucial for understanding disease pathogenesis and developing novel therapeutic drug and treatments. Livestock animals are important sources of milk, meat, leather and other products. The recently developed genome editing techniques have been used for generation of gene-modified large animals that are used for biological, biomedical and agricultural research and increase the performance of the animals in terms of both qualitative and quantitative. The gene-edited livestock animal has been used as a bioreactor for the production of human biological products such as transgenic pig with human albumin in their blood [1].

Genome engineering is defined as direct manipulation into the genome that can make specific edition/deletion to the genome by engineered nucleases, which offer a perfect platform to knock out/in and replace the particular DNA fragment, and make accurate genome editing on the genome level. There are three major types of programmed nucleases: Zinc Finger Nucleases (ZFNs), Transcription Activator Like Effector Nucleases (TALENs) and the Clustered Regularly Interspaced Short Palindromic Repeat-associated nuclease Cas9 (CRISPR-Cas9). These nucleases produced DNA Double-Strand Breaks (DSBs) at specific sites in the genome by targeted recognition and cleavage. Out of these CRISPR/Cas9 system is the latest genome editing technology, which are most commonly used nowadays. CRISPR is part of the bacterial genome system, which makes the bacterial cells immune to virus [2]. The CRISPR was first observed in *Escherichia coli* and served as an adaptive immune system in bacteria against bacteriophages. CRISPR systems, found in 90% of archaeal and 40% of bacterial genomes are highly

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diverse, with variation in Protospacer Adjacent Motif (PAM) sequences and the number and type of Cas proteins. The CRISPR system has three types of mechanisms *i.e* type I, II and III. In type I and type III CRISPR, various types of Cas proteins are participated in the recognition and destruction of target. However, in Type II CRISPR/Cas9 system low number of Cas proteins are involved, so thereby engineering of type II CRISPR system much simpler [3].

The main question is that why genome editing technology should be used in livestock. Although conventional methods, such as management of animal health, nutrition and reproduction, make an important contribution to improving the productivity of the animals. However, increasing the demand of food allover growing world, these approaches are not enough to meet the demands, so CRISPR/Cas9 technology emerges as a powerful tool to overcome this problem globally. So the main aim of this review paper is to describe the CRISPR technology and its application in livestock animals [4].

LITERATURE REVIEW

The CRISPR/Cas9 System

The CRISPR/Cas9 system is a part of adaptive immune system of the bacteria. For the first time showed that this system could be designed as genome editing tool *in vitro* and in 2013 the CRISPR/cas9 genome editing tool demonstrated on the human and mice cells [5]. The CRISPR/Cas9 system contains three main components: Endonuclease (Cas9), CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The Cas9 endonuclease which belongs to Type II CRISPR/Cas9 system, cut the target sites with the help of two RNAs: The crRNA which assign the genomic target for Cas9 and the tracrRNA which serve as a scaffold linking the crRNA to Cas9 and help in processing of maturation of crRNAs into a small single RNA sequence known as the guide RNA (gRNA) or single guide RNA (sgRNA). The specific recognition of target site by sgRNA depends on the Protospacer Adjacentmotifs (PAM), which is located downstream of 3' end of the target sequence and after recognition of target site by guide RNA, the Cas9 produces a Double Stand Break (DSB) on the complementary locus [6]. The generated DSBs will be repaired by two pathways, Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR) (**Figure 1**).

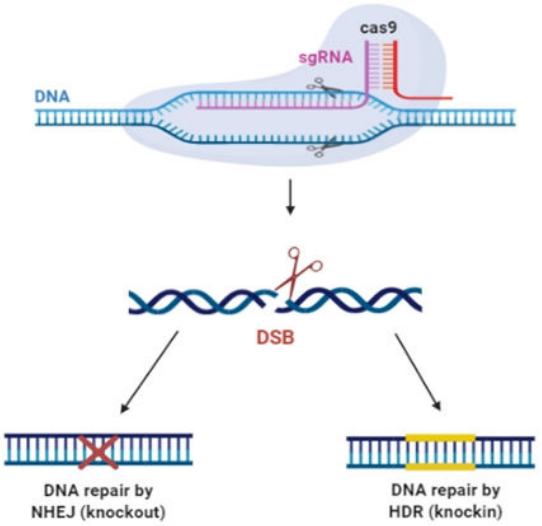

Figure 1: CRISPR-Cas system, consisting of a single guide RNA (sgRNA) designed to direct Cas9 to the desired DNA site and induce Double Strand Breaks (DSB). DNA cleavage results in different gene repair mechanisms as Nonhomologous End joining (NHEJ) or Homology Directed Repair (HDR).

The NHEJ mechanism caused small indels or chromosomal rearrangements and disrupts open reading frames, which leads to knockout of the gene. In case of HDR pathway DSB is repaired by the through sequence homology. Compare to HDR, the NHEJ is more active since HDR mechanism needs a homologous template and is mainly restricted to S and G2 phases of the cell cycle [7,8].

The Effec ive Endonuclease

For any CRISPR experiment one of the main things is to determine the suitable endonuclease [9]. The spCas9 (*Streptococcus pyogenes*) nuclease is most commonly used but several other type of nucleases such as Cas12a or CasX are also present ,which have some unique properties and can be used depending on the objective of the project. There are two domains in the Cas9 nuclease: HNH and RuvC-like domain. The HNH nuclease domain cut the target strand of the nucleotide and RuvC-like domain cut the non-complementary strand. To increase the specificity and adaptability of the Cas9, researcher reprogrammed the Cas9 into dCas9 nuclease (dead Cas9). The dCas9 do not have the DNA cleaves activity but can still bind to the target site with the help of guide RNA (gRNA) [10]. However the CRISPR/dCas9 system cannot insert or delete the gene, instead it silences the target gene by blocking the transcription process, while CRISPR/Cas9 system produced DSB on the target site. So the CRISPR/dCas9 tool can be used for genetic screening, gene regulation and epigenetic regulation. Additionally, the newly RNP based CRISPR tools have also been developed, in which different Cas RNA with sgRNA used for genome editing [11].

The single guide RNA (sgRNA)

The second important component of CRISPR/Cas9 system to edit the genome is selection and design of sgRNA [12]. Generally three rules considered during designing of sgRNA; the sgRNA should have

- Fewest potential off-target matches.
- Highest predicted cleavage efficiency.
- Target early exons these rules increase the probability of frameshift and nonsense mutations that disrupt gene expression.

The target sites for sgRNA are generally 18 bp-20 bp long and designed immediately to upstream of a Protospacer Adjacent Motif (PAM). The PAM sequence varies depending upon the type of cas nuclease. The PAM for spCas9 is 5'-NGG-3' and the PAM for saCas9 is 5'-NNGRRT-3. There are many online CRISPR tools and resources are available to design the sgRNA, some of this are list in **Table 1**.

Table 1: List of CRISPR guide RNA designing tool.

Name	URL
Addgene	http://www.addgene.org/crispr/
Benchling	https://benchling.com/crispr
Breakingcas	http://bioinfogp.cnb.csic.es/tools/breakingcas/
Broad institute GPP	https://portals.broadinstitute.org/gpp/public/
Chopchop	http://chopchop.cbu.uib.no/
Crispor	http://crispor.net/
Crisflash	https://github.com/crisflash/crisflash
Deskgen	https://www.deskgen.com
E-crisp	http://www.e-crisp.org/E-CRISP/
Microsoft research crispr	https://crispr.ml
RGEN tools	http://www.rgenome.net/
Synthego	http://design.synthego.com
WTSI genome editing	https://www.sanger.ac.uk/htgt/wge/

These softwares analyze the target sequence and identify all possible 18 bp-20 bp sequences which are immediately after the PAM sequence (5'-NGG) and provide the essential information require for selection of sgRNAs. In livestock, the gestation length is long, so after selecting the desired gRNA for target sequence, it is highly recommended to confirm them before producing the edited animals [13]. This analysis can be done in *in vitro* using cultured cells of the same species or directly in embryos.

Delivery of CRISPR System

There are mainly three approaches to deliver the CRISPR system *in vitro* or *in vivo*

- Plasmid encoding both the Cas9 protein and the guide RNA.
- Cas9 mRNA with guide RNA.
- Cas9 protein with guide RNA.

The vehicles used for delivery of CRISPR/Cas9 system are divided into three groups: Physical delivery, viral vectors and non-viral vectors. The most common physical delivery methods are microinjection and electroporation.

In case of viral vectors delivery method, specifically engineered Adeno-Associated Virus (AAV), and lenti-virus are used as vehicles. The microinjection delivery method has highest efficiency compare to other CRISPR/Cas9 delivery methods [14]. The mail problem for generation of edited livestock animal is the *in vivo* zygote production. *In vitro* Embryo Production (IVEP) is the preferable method for microinjection delivery of the CRISPR component. In microinjection delivery method CRISPR system is directly injected into the cytoplasm of the zygote, thus this method is fast and more efficient and preferable method in large animals. The second most preferable method for CRISPR system delivery in livestock is the SCNT (somatic cell nuclear transfer) approach; however the efficiency is low compare to microinjection [15].

CRISPR applications on livestock: Most of the biological and biomedical research is carrying out on rodents but validation and preclinical assessment are performed on large animals. In the last few years, the nuclease-mediated gene editing technologies has been used in livestock breeding; however these approaches are more complicated. The development of CRISPR technology makes the genome editing in large animal

easier. Various livestock animal have been produced by using CRISPR technology during the last few years [16]. The major applications of CRISPR technology in farm animals are to increase the productivity like milk, meat and egg, produce disease resistant animal, production of therapeutic proteins into the milk and production of human organs for transplantation which can be describes as follows (**Figure 2**).

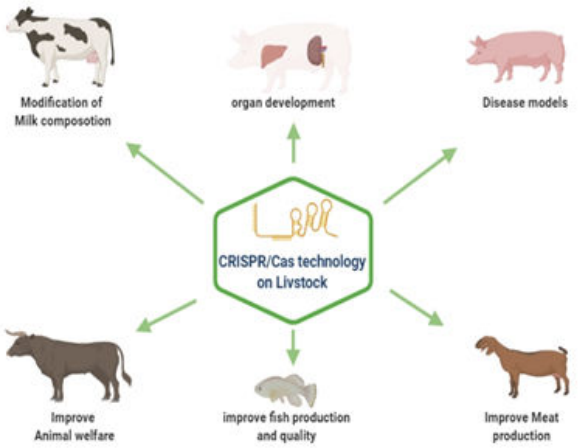

Figure 2: CRISPR/Cas mediated genome editing in major livestock species, enabling improvement of the genetics underlying traits associated with welfare, production, disease resistance and xenotransplantation.

Modification of Milk Composition

β-Lactoglobulin (BLG) is a major whey protein of ruminant milk and is considered as allergens when it is devoid of iron in the milk and responsible for cow milk allergy disease. Several methods have been used to diminish the allergenicity of the BLG, including heat treatment, fermentation, hydrolyzed protein desensitization, glycation, however these methods are not so effective. A possible option have been attempted to disrupt the β-lactoglobulin gene expression by targeting BLG gene. In 2014 Ni have successfully edited the β-lactoglobulin gene by using CRISPR/Cas9 system in goats [17, 18]. Similarly also knock out the β-lactoglobulin gene in goat through CRISPR/Cas9 technique and reduced the expression of β-lactoglobulin in milk. The BLG knock out cattle has also been produce by using Zinc-Fnger Nuclease (ZFNs) mRNA but till now no CRISPR edited BLG knock out cattle has been produced. In a study human Lactoferrin (hLF) gene was knocked in at BLG locus through TALEN approach in goat. These edited goats produce the human lactoferrin, a glycoprotein involved in iron adsorption and in non-specific immune reactions in the intestinal tract (**Table 2**).

Table 2: List of CRISPR/Cas9 edited livestock animals for the different purposes.

Species	Gene	Modifications	Applications
Pig	Npc1l1	KO	Disease model for cardiovascular and metabolic diseases
Pig	ApoE/LDLR	KO	Disease model for cardiovascular diseases
Pig	vWF	KO	Disease model for vWD
Pig	TP53/PTEN/APC	KO	Disease model for lung cancer
Pig	TPH2	KO	Disease model for 5-HT deficiency induced behavior abnormality
Pig	Huntingtin	KI	Disease model for HD
Pig	ApoE	KO	Disease model for cardiovascular disease
Dog	MSTN	KO	Improve muscle growth, new strains
Dog	GGTA1/CMAH	KO	Xenotransplantation
Pig	PERV	KO	Xenotransplantation
Goat	MSTN	KO	Goat meat production, composition and quality
Goat	MSTN (fat-1)	KI	Goat meat production, composition and quality

Pig	UCP1 (mouse UCP1)	KI	Pig meat production, composition and quality
Pig	CD163	KO	Disease resistance to PRRSV
Cattle	NRAMP1	KO	Disease resistance to tuberculosis

Note: KO-Knock Out, KI-Knock In

DISCUSSION

Improvement of Meat Production and Reproduction Performance

The well-known example related to meat production is knockout of the Myostatin gene (MSTN). This gene secreted a myostatin protein in muscle tissues, which act as a negatively regulator of muscle growth [19]. The mutation in MSTN gene leads to double-muscling phenotype in the animals and this was, first reported in cattle and then in sheep, dogs and humans. The cattle Belgian blue and piedmontese or Texel sheep are the well-known example of natural mutation in NSTN gene. So making double-muscling phenotype is an attractive target for genome editing to increase the meat production in livestock. In MSTN-mutant Meishan pigs through Zinc Finger Nucleases (ZFN) technology that showed an increase in muscle mass by 100% and a decrease in fat deposition compared to wild-type. In 2018 A CRISPR edited MSTN knock out goad has been produce, which showed significantly higher weight gain than that of Wild-Type (WT) goats. In the same year Zhang et al., knock out the MSTN gene and then knock-in the fat-1 gene into the goat MSTN locus by using CRISPR technology. The fat-1 gene product converts n-6 PUFA (n-6 Polyunsaturated Fatty Acid) into n-3 PUFA, so this CRISPR edited goat showed improved muscle growth and also produced nutritious meat by decreasing the ratio of n-6 PUFA to n-3 PUFA, which has been reported as a risk factor for many human diseases.

The CRISPR technology has also been used for improvement of thermoregulation in livestock. Uncoupling Protein 1 (UCP1) play an important role in thermoregulation. The functional UCP1 protein is absent in pig, which makes them liable to cold and prone to fat deposition and results in increase the mortality rate neonates and decreased production efficiency. Knock-in the UCP1 gene in pig through CRISPR technology which showed an improved ability to maintain body temperature during acute cold exposure and also showed reduced fat deposition, which make them valuable resource for the pig industry that can improve pig welfare and reduce economic losses. In 2017, knock out the NANOS2 gene in domestic pigs by using CRISPR/Cas9 technology to generate offspring with monoallelic and biallelic mutations. They found that NANOS2 knockout boar lack a germ line phenotype but other form of testicular development were normal. These NANOS2-null boars may provide a good environment to host germ cells from a genetically superior male, and thus broaden his genetic potential.

Disease Resistance

Mycobacterium bovis which caused tuberculosis in livestock animal especially in cattle is becoming a major risk to the agricultural and public health which causes significant economic loss to the farmers. The NRAMP-1 (Natural Resistance-Associated Macrophage Protein-1) gene provided innate resistance to intracellular pathogens such as *Mycobacterium*, *Leishmania*, *Salmonella* and *Brucella*. This NRAMP-1 gene has been inserted into the cow genome through CRISPR technology and makes the cow resistant to tuberculosis. Porcine Reproductive and Respiratory Syndrome (PRRS) is the most economically important disease of swine all over the world, which is cause by Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). The vaccines have been developed for this disease but unable to control the disease. The CD163 is the receptor for entry of PRRSV into cells. So in pig the CD163 protein encoding gene has been knock out through CRISPR technique, results in no clinical signs of PRRS were observed in edited pigs which demonstrated that a single gene deletion creates PRRSV resistant pigs. Niemann-PickC1-Like-1 (NPC1L1) protein is potent cholesterol absorption inhibitor that lowers blood cholesterol in humans and highly expressed in human liver. The NPC1L1 gene has been introduced into the pig genome to produce animal mode for understanding of cardiovascular and metabolic diseases.

Biomedical Application

The most crucial issue for the patients awaiting organ for transplantation is the availability and rejection of the allograft. Xenotransplantation is one of the solution in which the transplantation of animal cells, tissues or organs could replace an injured tissue or whole organ in humans. Pigs are the best choice for human organ development because anatomical and physiological similarities to the human organs and are cost-effective in breeding but the major problems are the immune rejection and potential cross species infection. So to overcome these problems scientists have been introduce human organ development gene into the pig genome to produce human organs into the pig. Wu successfully inactivated the pancreatogenesis in pig embryos *via* zygotic co-delivery of Cas9 mRNA and dual sgRNAs targeting the PDX1 gene, which makes the pigs suitable for the xeno-generation of human tissues and organs.

Generating B cell deficient mutant is the first step to produce human antibody repertoires in large animal models. IgM heavy chain gene is crucial for B cell development and

differentiation. This IgM heavy chain gene has been knock-out in pig through CRISPR technology, resulted in a B cell-deficient pig. This study highlights the potential of CRISPR technology in pig, which can used as animal model for study of human diseases. Human Serum Albumin (HSA) is the most abundant plasma protein that plays important role in homeostatic functions in human physiolog. Several approaches have been used to produce recombinant Human Serum Albumin (rHSA), however not so effective because of separation and purification of the rHSA are problematic. To overcome these problems Peng knocking human albumin cDNA into swine albumin locus to produce rHSA in pigs, which can used as bioreactor for the production of human albumin.

The Neuronal Ceroid Lipofuscinoses (NCLs/CLN1) are a group of inherited, neurodegenerative, lysosomal storage disorders that affect children and young adults. The more critical infantile forms of this disease are due to mutations in the Palmitoylprotein Thioesterase 1 (PPT1) gene, which reduces the child's lifespan to approximately 9 years of age. Eaton produced disease-causing PPT1 (R151X) human mutation into the orthologous sheep locus through CRISPR/Cas9 technology and generated a sheep model for the study of CLN1 disease.

Application in aquaculture: The fish farming contributes billions of dollars to the world economy. The genome editing technology has also been demonstrated in area. Escaping of farmed salmon fish into wild stocks is a major threat to the genetic integrity of wild populations. To overcome this problem dnd-gene (required for germ cell survival in vertebrates) has been knock-out by using CRISPR-Cas9 system. Similarly genome edited ridgetail white prawn has been produced by microinjection of Cas9 mRNA.

Application in animal welfare: The horned animal in the farm caused many problem such as they injure the animal, require more space for feeding, are dangerous to handle and transport than hornless animals. To over these problem farmers generally destruct the horn-producing cells before they grow and attach to the skull (disbudding) to prevent horn growth. But this practice is very painful to the animal. In 2016 Carlson et al., has been produced the hornless dairy cattle by Transcription Activator-Like Effector Nucleases (TALENs) approach but no such type of animal has been produce through CRISPR technology which is more efficient and easy technique compare to other genome editing technology.

CONCLUSION

Genome editing by CRISPR/Cas9 system has become a prevailing tool in biological research. It have the ability to generate site-specific indels to the genome, which allows help in the identification of gene function, allelic variants between breeds or species, or to create novel phenotypes. Compare to other genome editing techniques (ZFN and TALEN), CRISPR/Cas9 system is more efficient and easy to handle. The CRISPR technology has been successfully used for generation of many valuable animals for human disease models, xeno-transplantation and the agricultural economy such as improves the milk composition, meat production and disease resistant livestock animals. Mostly this tool used on the pig because of anatomical and physiological similarities to human, however very less effort has been made on cattle. Buffalo contribute more than 50% milk production in India and like cow milk; buffalo milk also contains beta-lactoglobulin which act as allergen in many children and adults. So to overcome this problem, CRISPR technology could be used for the production of beta-lactoglobulin knock out buffalo in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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