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Applications of CRISPR/Cas9 Guided Genome Editing in Livestock: An Update

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Abstract

The generation of genetically modified large animals has several advantages for different applications including meat, dairy and wool production and reproductive traits. However, the precise modification of the animal's genome is still challenging because traditional gene modifications by homologous recombination is only efficient in authentic embryonic stem cells. Recent advances in the development of novel, robust and efficient genome editing technologies based on programmable nucleases have substantially improved our ability to make precise changes in the genomes of eukaryotic cells. Targeted genome editing by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) system, has received unprecedented acceptance in the scientific community for development of novel approaches for augmenting productivity in livestock. It has currently emerged as novel approach to modify endogenous genes in various cell types and organisms including livestock such as pig, cattle, goat and sheep. In this article, we review the history of the discovery of CRISPR/Cas9 system, mechanism of action and the applications of CRISPR technology in livestock.

***Keywords**: Genome Editing; CRISPR/Cas9; Animal Welfare; Livestock*

Introduction

As global food demand continues to rise at an unprecedented pace, projections indicate a doubling by the year 2050, driven predominantly by an exponential surge in population. Concurrently, agricultural productivity faces significant challenges due to the diminishing availability of critical resources, particularly arable land. In this context, the livestock sector assumes a role of paramount importance, addressing the burgeoning food requirements and contributing significantly to nutritional security and employment generation. The livestock sector has emerged as a pivotal component of agricultural growth, especially in developing nations. It plays a crucial role in the socio-economic advancement and welfare of India's rural populace, providing not only essential food sources such as milk, meat, and eggs but also serving as a vital resource for draught power, manure, and hides. Recent advancements in genome editing techniques have facilitated the creation of gene-modified large animals that are used for biological, biomedical, and agricultural research. These innovations enhance the performance of livestock both qualitatively and quantitatively. Among the cutting-edge techniques, CRISPR/Cas9 technology has garnered significant attention as a novel method for precise genetic modifications in various cell types. Genome editing, involving the insertion,

deletion, or replacement of DNA at specific genomic loci using synthetic nucleases, enables meticulous and targeted alterations at the genome level. In the genetic era, the CRISPR/Cas9 system is revolutionizing genetic research across several animal models. Three primary methods dominate the landscape of genome editing in animal production. The first method, zinc finger nucleases (ZFNs), involves binding specific DNA domains that align with target DNA sequences. The second method, transcription activator-like effector nucleases (TALENs), employs nuclease domains to induce double-strand breaks (DSBs) in the genome. However, the most advanced and widely adopted technique is the CRISPR-associated protein 9 (CRISPR/Cas9), which allows for precise modification of gene functions within the animal genome, facilitating applications in drug delivery, gene targeting, transcriptional regulation and epigenetic modification.

Background of the CRISPR/Cas system

The genesis of CRISPR technology can be traced back to the fortuitous discovery by Japanese researcher [16]. While investigating the iap gene in *Escherichia coli*, which plays a role in converting alkaline phosphatase isozyme, they inadvertently cloned an unusual sequence characterized by repeated sequences interspersed with spacer sequences in an intergenic region upstream of the gene [16]. The purpose of these arrays remained enigmatic due to the limited DNA sequence information available at that time. Subsequent research revealed the presence of CRISPR arrays in various prokaryotic organisms, including bacteria and archaea, identifying 20 distinct types of microorganisms with these interrupted repeating sequences [25]. Francisco Mojica from University of Alicante in Spain played a pivotal role in elucidating the CRISPR system, identifying its presence in archaea in the 1990s [25] and in bacteria by 2005 [16].

The term "CRISPR" was first coined in 2002, following the structural and functional characterization of CRISPR-associated (Cas) proteins [17]. Alexander Bolotin's examination and sequencing of Streptococcus thermophilus further highlighted a unique CRISPR locus (Bolotin., *et al*. 2005). By 2005, multiple research teams had confirmed the existence of the CRISPR/Cas system in prokaryotes [25]. Bolotin., *et al*. 2005 [29], with [1] providing the first experimental evidence of its role as an adaptive defense mechanism against viral attacks. The CRISPR/Cas system, found in approximately 50% of bacterial and 90% of archaeal genomes, operates as a formidable shield against foreign genetic material introduced through viruses and plasmids [2,23]. Despite the possibility of multiple CRISPR loci, most bacterial genomes typically harbor only one CRISPR/Cas locus [2]. Stan J. J. Brouns and colleagues' research in 2008 revealed that spacer sequences derived from phages in *Escherichia coli* are transcribed into small RNAs, known as CRISPR RNAs (crRNAs), which guide Cas proteins to the target DNA. The CRISPR mechanism, akin to eukaryotic RNA interference (RNAi) silencing pathways, targets RNA molecules [22]. It identified an alternative CRISPR system that targets RNA [12], while [9] demonstrated that the CRISPR/Cas9 system induces double-strand breaks (DSBs) at specific DNA locations, with Cas9 being the sole protein required for cleavage. This feature is distinctive to Type II CRISPR systems, which utilize a single large protein (Cas9) in conjunction with crRNAs to facilitate interference. In 2013, Feng Zhang and colleagues pioneered the use of CRISPR/Cas9 for genome editing in eukaryotic cells, achieving targeted genome cleavage and homology-directed repair (HDR) in mouse and human cells [6,13]. Concurrently, a research team led by George Church at Harvard University published similar findings, underscoring the system's potential for precise genetic modifications [21]. CRISPR/Cas technology has since been recognized as superior to RNAi for gene silencing, offering more robust gene knockout capabilities. Notably, while bacteria have developed the CRISPR/Cas system as a defence mechanism against phages, phages have evolved anti-CRISPR strategies to evade destruction. Despite these countermeasures, the CRISPR/Cas system remains a dominant tool in genome editing. In a recent study, researchers in Germany used CRISPR/Cas9 nickases to develop a gene therapy approach to treat severe congenital neutropenia caused by ELANE mutations. The team showed that this CRISPR-based strategy offers an effective and safe way to restore neutrophil development by inhibiting ELANE expression via promoter editing rather than modifying its coding sequence [6,13].

CRISPR/Cas9 system and mechanism of action

The CRISPR/Cas9 system comprises three main components: the endonuclease Cas9, CRISPR RNA (crRNA), and trans-activating crRNA (tracrRNA). The Cas9 endonuclease, which is part of the Type II CRISPR/Cas system, has the capability to cut both strands of DNA at specific sites, producing double-strand breaks (DSBs). This precision is guided by a molecule known as guide RNA (gRNA), which

includes a short, approximately 20-base, pre-designed RNA sequence (crRNA) that binds to the target DNA, ensuring that Cas9 cuts the correct part of the genome [18]. The gRNA also contains a longer RNA component, tracrRNA (trans-activating crRNA), which forms a scaffold linking the crRNA to the Cas9 enzyme [18]. The guide RNA is designed to match and bind a specific DNA sequence due to its complementary bases. Cas9 locates and binds to a common sequence in the genome known as the Protospacer Adjacent Motif (PAM). Once Cas9 has identified the correct sequence, it cuts the DNA across both strands [18]. After the DNA is cut, the cell can repair the damage through two mechanisms: non-homologous end joining (NHEJ) or homologous-directed repair (HDR). NHEJ is a highly error-prone repair mechanism that introduces random mutations during the repair process, often resulting in deletions, insertions (indels), or substitutions in the genome. In contrast, HDR repairs the DSB through sequence homology, requiring a homologous template to guide the repair. HDR is primarily active during the S and G2 phases of the cell cycle and is generally less active compared to NHEJ [36]. This difference in activity levels is because HDR necessitates the presence of a homologous sequence to facilitate accurate repair, while NHEJ operates without such a template, leading to its higher error rate.

Application of CRISPR in livestock

Since the discovery of the CRISPR/Cas9 system, it has become a cornerstone of genome engineering in various animal species. The deployment of genome editing technologies in livestock holds the potential to significantly enhance animal welfare, productivity, and performance (Table 1 and 2), paving the way for a more sustainable livestock farming industry in the future. The applications of CRISPR technology have been discussed here.

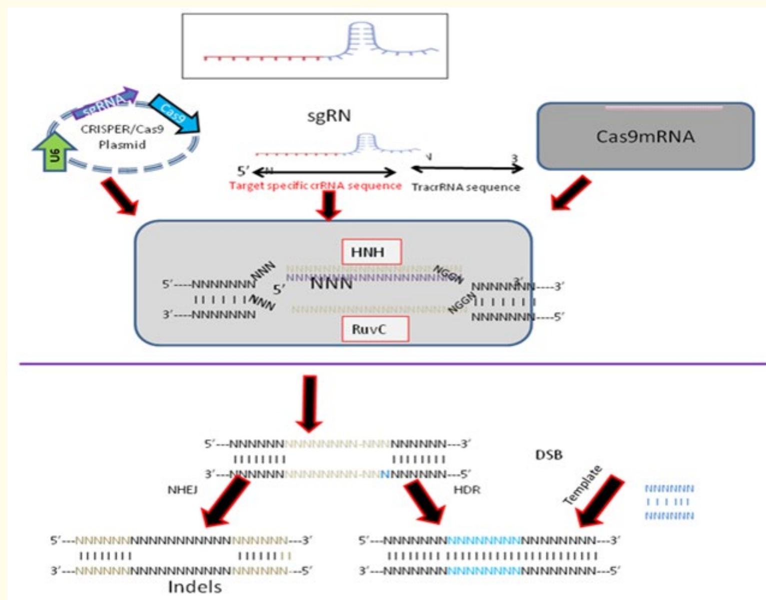

Figure: *Schematic illustration showing CRISPR/Cas9 mediated genome editing.*

Species	Genes	Effect/Goal	References
Cattle	*NANOS2*	Surrogate sire for genetic dissemination	[5]
	Insertion of TB-resistant gene *NRAMP-1* into bovine foetal cell	Suppression of tuberculosis	[8]
	GGTA and CMAH	Xeno transplantation	[28]
Goat	MSTN and FGF5	Muscle development and hair length	[33]
	MSTN	Muscle production	[11]
	BLG (Beta lactoglobulin)	Elimination of milk allergen	[38]
	EDAR	Cashmere yield	[12]
Pig	CD163	PRRSV Resistance	[32]
	MITF (Melanocyte Inducing Transcription Factor)	To generate knockout pigs	[33]
	NANOS2	Surrogate sire for genetic dissemination	[27]
	MSTN disruption	To promote growth and increase lean meat production	[38]
Sheep	Myostatin (MSTN)	To increase muscle growth	[7]
	MSTN, ASIP, and BCO2	Economically important traits	[33]

Table 1: *Applications of CRISPR/Cas9 technology in livestock.*

Gene	Application	Species	References
NMRAP1	It helps in increasing the resistance to tuberculosis	Cattle	[8]
CD163	It helps in protecting the pigs from porcine reproductive and respiratory syndrome virus (PRRSV).	Pig	[34]
CD163 SCRC5	It assists in making pigs fully resistant to both PRRSV genotypes, i.e., European genotype and North American genotype) while maintaining biological function	Pig	[4]
FGF, GDF8	To enhance the production of skeletal muscles	Goat	[33]
NANOS2	Pigs are predictable to serve as a perfect replacement for transplantation of donor spermatogonial stem cells to enhance the accessibility of gametes from genetically desirable sires	Pig	[27]
WWF	It increases bleeding competence for slaughtering procedures and blood collection	Pig	[12]
HBG1, CD34+	Hematopoietic stem and progenitor cell	Non-human primate	[14]

CFTR, B-cell Lymphoma 11A (BCL11A)	Once this gene is disabled using CRISPR/ Cas-9, the production of fetal hemoglobin containing γ globin in the red blood cells will increase, thereby alleviating the severity and manifestations of SCD	Human	[23]
DSB, HDR gene	High-throughput functional genomics screen in *in vitro* (cellular and organoid models), *vivo*, and *in vivo* systems, *In vitro* and *in vivo* disease modeling (knock-in and knockout models), Cancer diagnostic	Model animal	[30]
eIF4E gene	The findings of this research offer new target genes for the biological breeding of wheat yellow mosaic disease and show that wheat and barley share a similar recessive viral resistance mechanism	Wheat	[19]
HBB, BAF, TTR, ALL	Urinary tract infections; Thalassemia genetic diseases; Leukemia lymphocytic acute in relapse, acute lymphocytic leukemia (ALL) refractory lymphoma, B-cell	Human	[20]
LCA2 (LCA2, OMIM	Effectively treated with subretinal infusion of adenine base editors utilizing RNPs	Mice	[20]
HbF, HBG1	Gene abnormalities affecting the beta protein cause beta-hemoglobinopathy, which includes illnesses including sickle cell anemia and beta-thalassemia	Human	[35]

Table 2: *A selective list of gene-edited for different purposes.*

Improving productive trails

The genetic modification of livestock emerges as a paramount strategy for the enhancement of animal welfare encompassing the amelioration of animal populations and the mitigation of disease alongside the augmentation of animal health and the efficiency of food production, such as meat and milk. Genome engineering in bovines addresses pivotal concerns including the eradication of diseases such as tuberculosis, the elimination of allergens exemplified by the knock-out of beta-lactoglobulin, and the tailored production of commodities such as meat from male specimens and milk from females. Furthermore, it encompasses sex selection (animal sexing), the introduction of desirable traits such as stress tolerance and disease resistance, and the assurance of well-being, exemplified by the breeding of hornless animals. Comparative studies reveal that knockout lambs surpass Merino lambs by 25% in weight, while maintaining comparable wool quality characteristics [8], unpublished data). The advent of CRISPR technology has facilitated the germline transmission of MSTN mutations, evident in the gonads of founder sheep [33] and goats [33], as well as their progeny, culminating in animals that exhibit enhanced meat yield and superior wool quality. These genetically modified animals display a phenotype characterized by double muscling, with significantly increased muscle mass while retaining viability [10,26]. Moreover, the systemic administration of pharmacological agents that inhibit MSTN activity in adult mice has been demonstrated to substantially amplify muscle growth [3] underscoring the critical role of MSTN in regulating postnatal muscle homeostasis through the suppression of muscle growth.

Modification of milk composition

Beta-Lactoglobulin (BLG), a predominant whey protein in ruminant milk, is recognized as a significant allergen when it is devoid of iron, thereby contributing to cow milk allergy. Various methods such as heat treatment, fermentation, hydrolyzed protein desensitization, and glycation have been employed to mitigate the allergenicity of BLG, however, these approaches have proven suboptimal. In a pioneering effort, Ni., *et al*. (2014) successfully utilized CRISPR/Cas9 technology to edit the β-lactoglobulin gene in goats. Subsequently, BLG knock-

out (KO) goats and cows were produced through CRISPR/Cas9 mediated zygote microinjection and ZFNs gene editing followed by somatic cell nuclear transfer (SCNT), respectively [31,37]. Western blot analyses confirmed the abolition of the BLG protein in the milk of BLG KO goats. Comparative studies indicated that BLG KO goats exhibited reductions in milk fat, protein, lactose, and solids-not-fat by 5.49%, 7.68%, 7.97%, and 7.7%, respectively, when compared to wild-type (WT) goats. Furthermore, an innovative study employed the TALEN technique to insert the human lactoferrin (hLF) gene at the BLG locus in goats. These genetically edited goats produced human lactoferrin, a glycoprotein that facilitates iron absorption and enhances non-specific immune responses within the intestinal tract [31].

Improvement of meat production and reproduction performance

One prominent example in meat production is the deletion of the myostatin gene (MSTN), which encodes Myostatin, a protein that inhibits muscle development. A mutation in the MSTN gene results in double-muscling, a phenomenon first observed in cattle and subsequently in sheep, dogs, and humans [33]. The Belgian Blue and Piedmontese cattle, as well as Texel sheep, are renowned examples of naturally occurring MSTN gene mutations [33] harnessed CRISPR/Cas9 and somatic cell nuclear transfer to induce a myostatin mutation in Erhualian pigs, leading to mutant piglets with reduced levels of myostatin precursor protein. These myostatin knockout pigs exhibited a partially double-muscled phenotype, characterized by significant muscular protrusion and broader backs and hips compared to their wild-type counterparts. Achieving a double-muscling phenotype through genome editing holds significant promise for enhancing meat production in livestock. In addition to improving muscle mass, CRISPR technology has been utilized to enhance thermoregulation in animals. The UCP1 gene plays a crucial role in thermoregulation. Pigs lacking functional UCP1 protein are more susceptible to cold stress and fat deposition, which leads to higher neonatal mortality rates and reduced production efficiency. This advancement is particularly valuable for the pig industry, as it enhances animal welfare and reduces economic losses. Furthermore, CRISPR/Cas9 technology has been employed to deactivate the NANOS2 gene in domestic pigs, resulting in offspring with monoallelic and biallelic mutations NANOS2 knockout boars do not exhibit a germline phenotype but show normal testicular development. NANOS2-null boars have the potential to host germ cells from superior males, thereby increasing their genetic potential. Spermatogonial stem cell transplantation (SSCT) is a technique for transferring germ cells between male donors and recipients, which can be utilized for biomedical research, preserving endangered species, and transferring desirable traits in food animal populations. To fully realize these applications, recipient males must lack endogenous germlines but possess normal testicular structure and somatic cell function capable of allogeneic engraftment of donor stem cells, leading to the restoration of spermatogenesis. It demonstrated that CRISPR-Cas9 editing of the NANOS2 gene in cattle effectively removes the male germline [5]. These findings pave the way for surrogate sires to be used for germplasm transmission and regeneration across animal populations.

Disease resistance

Mycobacterium bovis, a causative agent of tuberculosis in livestock, presents a significant threat to both agriculture and public health, leading to considerable economic losses for farmers. The NRAMP-1 gene endows innate resistance to various intracellular infections, including those caused by *Mycobacterium*, *Leishmania, Salmonella*, and *Brucella*. Utilizing CRISPR technology, the NRAMP-1 gene has been successfully introduced into the cow genome, conferring resistance to tuberculosis [8]. Porcine reproductive and respiratory syndrome (PRRS) stands as the most economically detrimental disease affecting swine globally, caused by the Porcine reproductive and respiratory syndrome virus (PRRSV). Despite the development of vaccines, they have not proven effective in controlling the disease. The CD163 receptor mediates the entry of PRRSV into cells. Remarkably, the deletion of the CD163 protein-expressing gene in pigs via CRISPR resulted in pigs exhibiting no clinical signs of PRRS, demonstrating that a single gene deletion can create PRRSV-resistant pigs [34]. Additionally, the Niemann-Pick C1-Like 1 (NPC1L1) protein plays a crucial role in inhibiting cholesterol absorption, thereby reducing blood cholesterol levels in humans. This protein is extensively expressed in the human liver. To gain a deeper understanding of cardiovascular and metabolic disorders, inserted the NPC1L1 gene into pigs, creating a model to study these conditions more effectively [33].

Biomedical applications

Pigs are an optimal choice for human organ development due to their anatomical and physiological similarities to human organs and their cost-effectiveness as a breed. However, significant challenges such as immune rejection and potential cross-species infection need to be addressed. Scientists have made strides in overcoming these issues by introducing human organ development genes into the pig genome to facilitate the production of human organs in pigs. The availability and rejection of allografts remain critical concerns for patients awaiting organ transplantation. Xenotransplantation, which involves transplanting animal cells, tissues, or organs to replace damaged human tissues or organs, presents a viable solution. It was successfully inhibited pancreatogenesis in pig embryos through the zygotic co-delivery of Cas9 mRNA and dual sgRNAs targeting the PDX1 gene, qualifying pigs for the xeno-generation of human organs and tissues. However, the risk of retroviral transmission from pigs to humans poses another challenge in xenotransplantation. Porcine endogenous retroviruses (PERVs), integrated into the pig genome, persist in every organ and tissue and are hereditary. It demonstrated that using CRISPR/Cas9, all PERVs were eliminated in a pig kidney epithelial cell line (PK15), significantly reducing PERV transmission to human cells by over 1000-fold. To advance the development of human antibody repertoires in large animal models, researchers have focused on creating B cell-defective mutants. The IgM heavy chain gene, essential for B cell differentiation and maturation, was knocked out in pigs using CRISPR technology, resulting in pigs devoid of B cells. This work highlights the potential of CRISPR technology in pigs as an animal model for human disease research. Human serum albumin (HSA), the most prevalent plasma protein, plays a crucial role in maintaining homeostasis. Although various methods have been used to generate recombinant human serum albumin (rHSA), challenges in separation and purification have limited their effectiveness. To overcome these obstacles, produced rHSA in pigs by inserting human albumin cDNA into the swine albumin locus, thereby creating a bioreactor for human albumin synthesis. Neuronal ceroid lipofuscinoses (NCLs/CLN1) are hereditary, neurodegenerative, lysosomal storage disorders affecting children and young adults. The more severe infantile forms of this disease are caused by mutations in the palmitoyl-protein thioesterase 1 (PPT1) gene, resulting in a reduced lifespan of approximately nine years. Chen., *et al*. (2015) utilized CRISPR/Cas9 technology to introduce the disease-causing PPT1 (R151X) human mutation into the orthologous sheep locus, creating a sheep model for the study of CLN1 disease. This demonstrates the transformative potential of CRISPR technology in advancing medical research and therapeutic applications across species.

Conclusion

Since its introduction as a gene editing tool in mammalian cells ten years ago, CRISPR technology has significantly broadened the field of genetic study by allowing scientists to easily and precisely change DNA. In addition to helping us treat genetic diseases, this ground-breaking technology has created new opportunities for treating non-genetic diseases through epigenome editing. Furthermore, recent developments in base and prime editors have significantly enhanced our capacity to model and characterise mutations linked to disease, and these developments will be crucial in locating new treatment targets and comprehending resistance mechanisms. Without a doubt, these initiatives will speed up the creation of precision medicine-based strategies for treating various cancer forms. Future developments in CRISPR hold great potential since they will continue to spur fresh findings that expedite drug discovery. In the discipline of genetic engineering, CRISPR-Cas9 is a groundbreaking method. Due to its many uses in livestock (cattle, sheep, cows, goats, and pigs), it simplicity and potency. It offers unique traits in genetically altered species that are economically meaningful and advantageous for further study in other domains like genetics, biotechnology, and medicine. CRISPR, a direct genome editor application in the developing embryo. Breeding techniques for crops and cattle are advanced by the Cas-9 technique's simple, fast, and adaptable characteristics. Because of CRISPR's ability to target several locations at once and its specificity, it is conceivable to replicate rare human illnesses and generate xenotransplants safer. These two CRISPR uses have significantly advanced medical research, increasing understanding of the condition and paving the way for the development of novel treatments. As a result, CRISPR has significantly advanced genetic engineering and is currently regarded as the most popular genome editing method in molecular biology.

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