Review

The Prospect and Application Research Progress of Precision Gene Editing Technology Based on CRISPR/Cas9 in Livestock Genetics and Breeding

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Review

The Prospect and Application Research Progress of Precision Gene Editing Technology Based on CRISPR-Cas9 in Livestock Genetics and Breeding

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Abstract: With the continuous update of gene editing technology, great progress has been made in livestock breeding, researchers have produced gene edited pigs, cattle, sheep and other improved livestock. Gene editing technology is a genetic means to knock in, knock out, delete, inhibit, activate or replace specific bases of DNA or RNA sequences at the genome level to achieve accurate modification, and can edit genes at a fixed point without the need for DNA templates. In recent years, although regular clusters of interspaced short palindromic repeats (CRISPR)/Cas9 system mediated gene editing technology has been widely used in animal genetic breeding research, but so far, the system's precise insertion efficiency of foreign genes is not high enough, and there are certain off-target effects, and it is not enough to be used in genome editing of large livestock such as cashmere goats. In this paper, the development status, challenges, application prospects and look into the distance of CRISPR/ Cas9-mediated precisiongene editing technology in livestock breeding were reviewed, in order to provide theoretical reference for livestock gene function analysis, genetic improvement and livestock breeding with local economic characteristics.

Keywords: precision gene editing; CRISPR/Cas9; livestock genetic breeding; cashmere goat; editing efficiency; fixed point integration

1. Introduction

In the 1990s, genome editing technology began to rise, and with the rapidupdate and iteration of technology, gene editing methods that accurately targeted modification genomes emerged [1,2], such as transcription activator like effector nuclease (TALEN), zinc-finger endonuclease (ZFN), clustered regularly interspaced short palidromic repeats (CRISPR) appeared in the late 1990s [3]. Due to the advantages of CRISPR, such as low cost, simple operation, accurate editing, high efficiency, fast development rate, stable structure of Cas9 nuclease, easy design, system maturity, and carrier ability to simultaneously target multiple sites of genes [4–6], CRISPR exceeds any other technology. It has been widely used in livestock and poultry gene function analysis, molecular breeding, genetic improvement, disease resistance breeding, molecular biology and molecular cytology and other genome editing fields [7–9]. CRISPR-Cas9, a genetic tool, is a miracle in the 21st century [10–13]. Table 1 summarizes the advantages and disadvantages of the three gene editing techniques [13,14]. In addition, the epigenetic traits that affect livestock genetics and breedingare essentially regulated by genes. In order to breed better varieties and improve animal husbandry productivity, it is necessary to accurately edit the genome through specificity and multiple loci simultaneously. In this paper, the

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principle of CRISPR/Cas9 technology and its application in livestock and poultry breeding were reviewed, with a view to providing a reference for CRISPR/Cas9 technology to further study new breeding methods such as genome transcriptional regulation and epigenetics in large livestock.

Table 1. Comparison of advantages and disadvantages of CRISPR/Cas9, TALEN and ZFN gene editing technologies.

Characteristic	CRISPR-Cas9	TALEN	ZFN
Price	Low	High	Low
Precision	Pinpoint	Moderate	Low
Combination mode	RNA-DNA	Protein-DNA	Protein-DNA
Design and construction	Easy	Difficulty	Moderate
Target fragment size	20-50bp	30-40bp	18-36bp
Application	Wide	Small	Small
Off-target effect	High	Low	Low

2. CRISPR/Cas9 Gene Editing Technology

2.1. Overview and Principle of CRISPR/Cas9

CRISPR is an adaptive immune system discovered in the genomes of bacteria and archaea. It is composed of Cas9 protein, functional genes, short regularly clustered repeatsequences, and similarly long spacer sequences arranged from the 5 'to 3' [15,16],which can effectively defend against the invasion of exogenous viral DNA [17] and protect one's own genetic information from destruction [18]. The proteins encoded by CRISPR is called Cas (CRISPR association proteins), which is a nucleic acid endonucleasecomposed of a Nuclease domain (RuvC-like) and a Nuclease functional region (McrA-like HNH), and it mainly encodes functional proteins that bind to nucleic acids [19]. According to the different mechanisms of action of Cas proteins, the CRISPR/Cas9 system is divided into two classes and six types [20]. The class 1 includes type I, type III and type IV, and the class 2 includes type II, type V and type VI. Among them, the CRISPR/Cas9 system of type II is widely used as the most advanced gene editing technology in improving genetic breeding, reproductive performance, and nutrient intake levels of livestock due to its simplest structure, strongest specificity and highestefficiency [21]. The mechanism of action of the Type II CRISPR/Cas9 system is based on the principle of base complementary pairing, that the Cas9 protein is guided by single guide RNA (sgRNA) to carry out targeted editing of the target DNA, causing double strand break (DSB), thereby stimulating the repair mechanism of DNA [22].

The Cas9 protein needs to undergo cleavage through the combined action of specific CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) by base pairing binding [23]. Replacing crRNA and tracrRNA with sgRNA [24] simplifies the operation of theCRISPR/Cas9 system. sgRNA is adjacent to protospacer adjacent motif (PAM) [25], so it canensure that Cas9 protein accurately recognizes exogenous DNA without destroying its own Genetic material. However, not all sequences are efficient and specific for induction cutting. Currently, different PAM (5'-NGG-3', N as A/T/C/G) forms of CRISPR/Cas9 systems can be used depending on the sequence characteristics of editing sites [26]. The CRISPR/Cas system immunoregulates invading elements through three stages: acquisition, transcription, and interference [27]. It is divided into two different subsystems, the highly conserved "information processing" subsystem (acquisition) and the "implementation" subsystem (transcription and interference). The first stage is to obtain recognition and insert the ingested PAM into the dominant or adjacent area [6]; In the second stage of transcription, the CRISPR sequence is transcribed together with PAM to form a long transcript called pre-CRISPR RNA, and mature small crRNAs are processed by a unique Cas protein and combinedwith tracrRNA to form complementary chains [28];

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In the third stage, interference, the crRNA-tracrRNA complex accurately induces the Cas9 protein targeted cleavage of exogenous DNA.

The CRISPR/Cas9 system generally repairs genetic material by nonhomologous end joining (NHEJ) and homologous recombination (HR) [29]. NHEJ is mainlyused for the study of gene knockout such as insertion, deletion or chromosomerearrangement of small fragments, which is the first successful method for theCRISPR-Cas system has prevented the function of endogenous genes in domestic animals such as goats [30], sheep [31], cattle [32] and pigs [33]. When thehomologous template of heterologous mutated DNA serves as the repair template of DSB, HDR can insert exogenous DNA fragments into specific locations of the genome to achieve accurate editing of target sites [34]. Following NHEJ,HDR achieved gene knockout in large animal for the first time. Although HDR can be accurately repaired, it also has certain drawbacks. Randomly introduced insertions or deletions may lead to gene knockout, new functions may be generated, and even activate DNA damage regulatory mechanisms to induce cellapoptosis [35].

Based on the continuous research on the powerful Cas9 protein [36], moreand more new CRISPR/Cas9 tools [37] have emerged, such as Streptococcus pyogenesCs9 (SpCas9) is the main tool for studying animal gene editing [38], the "BE" that makes C/T replace each other [39,40], and the "PrimeEditor" [41] that can accurately target editing without the template of donor genetic material, etc. These new tools not only have a low probability of missing the target, but also there will be no new functional changes [42].

2.2. Comparison between CRISPR/Cas9 and Other Gene Editing Techniques

At present, the mainstream gene editing technology is the CRISPR/Cas9 system mediated by a shorter RNA. Previously, research on gene editing techniques using Zinc-fingernuclease (ZFN) and transcript activator-like effector nucleases (TALEN) was more common. ZFN and TALEN both use the Fok I familyrestrictive endonuclease to form dimers to specifically recognize and non-specific cut the targeted DNA [43], but ZFN recognizes triple bases, while TALEN can only recognize single nucleotides. Because of this feature of TALEN, it will not be affected by external factors when designing, and TALEN can target longer sequences [44], which has a low probability of miss the advantages of short preparation cycle and low experimental cost are more favored than ZFN. Like CRISPR/Cas9, they also repair genetic material through HR and NHEJ mechanisms. Compared with traditional gene editing techniques, ZFN and TALEN achieve gene expression regulation through targeted insertion, base deletion and replacement, transcriptional inhibition or activation, etc., and improve gene targeting efficiency [45]. However, the development of these two technologies islimited due to their complex operation and high cost.

However, compared with the above two technologies, CRISPR/Cas9 has incomparable advantages in livestock genetics and breeding. Table 1 summarizes the main differences among the three genome editing technologies. Firstly, the construction of the CRISPR/Cas9 system is extremely simple, requiring no significant human or material resources to design, process, and screen proteins thatcan recognize DNA. Simply designing and synthesizing a gRNA that complements DNA can achieve twice the result with half the effort [46], which is the main reason why this technology is widely used in the entire scientific research field; Secondly, the CRISPR/Cas9 system can simultaneously edit multiple independent target gene sites based on multiple gRNAs at different sites without the need for multiple domain connections [47], with high efficiency being its significant advantage. Despite its high efficiency and specificity, the limitationsof the CRISPR-Cas9 system structure also result in off target effects. Therefore,researchers have calculatedand designed the most suitable editing sites to improve the specificity of sgRNA [45], controlled the number of sgRNAs [48], and continuously constructed new Cas9 protein mutants, such as EvoCas9 [49], Sniper Cas9 [50], xCas9 [51], and AsCpf [52], These gene editing tools with simpler structures and higher editing efficiency [43] can be flexibly used for any nucleic acid sequence. Through continuous innovation and improvement, CRISPR/Cas9's editing ability will be stronger.

Overall, although several gene editing technologies have their own advantages, the CRISPR/Cas9 system has led a technological revolution in different fields due to its simple design

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and operation, low cost, ability to edit multiple genes simultaneously, and high mutation efficiency [8,53].

3. Application of CRISPR/Cas9 System in Livestock and Poultry

3.1. Application of CRISPR/Cas9 Technology in Pig Genetic Breeding

As an important livestock for meat, the scale of pig farming is gradually expanding, and intensive breeding has led to the emergence of different diseases. It is urgent to cultivate genetically modified pigs with disease resistance. Pigsarethe first large animals to be edited by CRISPR/Cas9 [54], and CRISPR/Cas9 gene editing technology provides new ideas in genetic improvement and disease resistance breeding.

GUANGHAI et al. [55] used the CRISPR/Cas9 system to edit the non-coding region of insulin-like growth factor 2 (IGF2) in Bama pigs, and found thatit could improve meat production in Bama pigs. Mitochondrial uncoupling protein 1 (UCP1) can regulate body temperature in a cold environment, thereby reducing the body fat production rate of newborn piglets and improving the leanmeat rate and birth rate [56]. Using CRISPR/Cas9 technology to knock UCP1 into the chromosomes of pigs to construct healthy lean pigs with cold resistance and low fat, thereby reducing economic losses in agriculture and animal husbandry [57].

Classical swine fever virus (CSFV) and Porcine reproductive and respiratory syndrome virus (PRRSV) are both envelope single-stranded sense RNA viruses [58], and the replication process does not produce intermediate products. Transgenic pigs resistant to CSFV were constructed using the CRISPR/Cas9 system, and it was found that growing pigs, pregnant sows, and their fetuses are all resistant to PRRSV infection [59]. CD163 knockout pigs were also constructed, and found that the replication of CSFV was restricted, and disease resistance could be stably transmitted to offspring. African swine fever virus (ASFV) has a higher mortality rate than ordinary swine fever, reaching 100%. HUBNERet al. utilized CRISPR/Cas9 targeting ASFV phosphoprotein p30 to reduce the number of African swine fever viruses [60]. Xu et al. [61] constructed CD163and porcine aminopeptidase (pAPN) Double-gene Knockout (DKO) pigs, which inactivated the virus receptor CD163 and pAPN proteins. This not only prevented both PRRSV and TGEV virus infections, but also did not affect reproductive performance and meat quality [62]. The construction of these disease-resistantgene editing pigs can reduce economic losses caused by diseases and promote a healthier and faster development of the pig industry.

3.2. Application of CRISPR/Cas9 Technology in Chicken Genetic Breeding

Due to the unique structure of chicken fertilized eggs, the development ofCRISPR/Cas9 technology in poultry breeding lags behind that of mammals [63].Therefore, CRISPR/Cas9 gene editing technology needs to accelerate the pace in genetic aspects such aspoultry reproductive development and disease resistance breeding [64].

Primordial germ cells (PGCs) in chickens can carry genetic information to the next generation, but both the donor PGC and the recipient PGC develop simultaneously in the recipient testis, leading to rejection of the donor PGC and reduced sperm count. Researchers used gene editing technology to construct sterile hosts to eliminate the role of endogenous receptor PGC. Ballantyne et al. [65] obtained DAZL gene edited chickens using CRISPR/Cas9 technology, theiCaspase9 gene was fixedly point inserted into the chicken PGC and drugs were added to inhibit the growth of PGC in the gonads, and the effectiveness of infertile hosts was verified by carrying exogenous PGC with feather color. Later, researchers used the CRISPR/Cas9 system to knockout the key gene DMRT1 that determines gonadal differentiation and gender development in male chicken PGC [66]. The edited chicken PGC was injected into the aforementionedsterile host chicken, and the obtained embryos and chicks' gonads eventually developed into ovaries, proving that the DMRT1 gene is particularly important in testicular development [67]. Zhang et al. [68] used CRISPR/Cas9 technology to knockout the important gene Stra8 (Stimulated by recurrent acid gene8) that affects reproductive development in chicken embryonic stem cells,and found that ECS cannot be transformed into male germ cells.

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MSTN (Myostatin) negativelyregulates the proliferation and differentiation of skeletal muscle cells [69]. Xu et al. [70] injected the MSTN-knockout sgRNAs adenovirus vector into theleg muscles of chicks, and the expression of MSTN was significantly downregulated, but gene editing offspring could not be obtained. It can be seen that the study of combining PGC with CRISPR/Cas9 for poultry gene editing is more reasonable.

Poultry is highly susceptible to viruses, such as the MDV (Mark's diseasevirus), the highly pathogenic ALV-J (Avian leukosis virus J) [71], and the AIV (Avian influenza virus) [72], with fast transmission speed and high mortalityrate. Koslova et al. successfully cultivated chNHE1-KO homozygous mutant chickens resistant to ALV infection using CRISPR/Cas9 technology [73]. Use CRISPR/Cas9 to modify the residues of the chANP32A gene to reduce AIV replication [74]. Hellrich et al. [75] used the CRISPR/Cas9 system to knockout Trp38 encoded by the chNHE1 (Chicken Na+/H+ exchange type 1) of the ALV-J receptor in chicken PGC, obtaining anti ALV-J chickens. Liu et al. [72] successfully constructed a CRISPR/Cas9 vector that blocks the expression of ALV-J in vivo using coinfected virus MDV for the first time. In summary, CRISPR/Cas9 gene editing technology has achieved significant results in poultry disease prevention and breeding.

3.3. Application of CRISPR/Cas9 Technology in Cattle Genetic Breeding

Cattle are premium species that produce meat, milk, and derivative leather.At present, research on gene editing in cattle mainly includes disease resistant breeding, increasing meat production, and eliminating allergens. By using Cas9 technology to precisely insert beneficial genes into animals, thus promoting thehealthy and efficient development of the industry, reducing economic losses, and ensuring human food safety.

Bovine tuberculosis is a zoonosis induced by Mycobacterium bovis (M.bovis) infection. Gao et al. [76] for the first time used Cas9 nickase (Cas9 n) to prepare naturally resistant associated macrophage protein-1 (NRAMP1) gene edited cows, enhancing their resistance to Mycobacterium infection and reducing the risk of human infection caused by contact with diseased cows and their by-products. Subsequently, Yuan et al. [77] used CRISPR/Cas9 system to integrate NRAMP1 gene into FSCN1-ACTB (F-A) site and bovine homologous mouse Rosa26 site, enhancing the resistance of cows to tuberculosis. SZILLAT et al. [78] constructed a CD46 gene knockout cell line dependent on the invasion of Bovine Viral Diarrhea Virus (BVDV) using CRISPR/Cas9 technology, laying the foundation for elucidating the mechanism of CD46's role in the replication cycle of plague viruses.

Prevention and treatment of mastitis is a major problem in dairy industry.Bovine paratuberculosis caused by Mycobacterium bovis paratuberculosis (MAP)has a huge impact on dairy industry. Using CRISPR/Cas9 technology to knockout the Interleukin-10 Receptor Alpha (IL-10RA)in bovine mammary epithelial cells that can immunoregulate MAP can inhibit or promote the expression of certain pro-inflammatory cytokines, providing a cellular model for further research on the anti-inflammatory mechanisms of IL-10RA [79].

Cattle horns are prone to accidental injuries during play and struggle, which can lead to miscarriages in severe cases. Therefore, the use of gene editing technology to cultivate hornless cattle is of great significance. Gu Mingjuan et al. [80] used CRISPR/Cas9 technology to insert the 202bp repetitive Pc (polling of cellular origin) site of chromosome 1, a gene that controls hornless traits, into the genome of fibroblasts from horned Mongolian cattle, providing a basic material for cultivating hornless cattle.

The OCT4 gene plays an important role in maintaining the pluripotency ofearly embryonic stem cells in mammals. SIMMET et al. [81] used CRISPR/Cas9 technology to knockout the OCT4 gene in bovine embryos and found that its blastocyst development process was similar to that of human blastocysts lacking OCT4. Bradford et al. [82] targeted the injection of the OCT4 gene intobovine fertilized eggs and found that on day 5, embryonic development stagnated and blastocyst formation was not possible. Based on this, CAMARGO et al. [83] further utilized optimized electroporation technology to transfect the CRISPR/Cas9 system targeting the sgRNA-Cas9 protein complex of OCT4 into bovine fertilized eggs, greatly reducing operational difficulties and making it easier to obtain transgenic animals. These results not only provide a model animal for treating early human

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embryonic developmental defects, but also lay a scientific foundation for promoting cattle reproduction.

4. Application of CRISPR/Cas9 Technology in Goat and Sheep Genetic Breeding

Due to the superiority of cashmere, meat, milk, and other by-products, sheep or goat are also one of the most important livestock in agricultural production and play an important role in animal husbandry. In the genetic breeding processof goats and sheep, gene editing is used to improve growth status, increase cashmere yield and fertility, and obtain individuals with multiple excellent traits. Scientists use gene editing technology to modify their traits according to different needs, in order to accelerate the breeding process of sheep and goat.

4.1. Promote Hair Follicle Growth and Development

Cashmere goats are the main local economic livestock in Inner Mongolia, and cashmere is an important natural raw material for the textile industry. As an important economic value structure for cashmere goats, it is also an important source of income for farmers and herdsmen. Using CRISPR/Cas9 gene editing technology to study the functions of genes related to hair follicle development is of great significance for improving the cashmere production performanceof cashmere producing goat varieties.

Studies have shown that CRISPR/cas9 inhibition of FGF5 expression can improve cashmere growth in sheep [84,85] or goats [86], increasing hair follicledensity, length, and improving quality. Fibroblast growth factor 5 (FGF5) is the main inhibitor that controls fiber length and growth. Wang et al. [86] targeted the knockout of the FGF5 gene in Shaanbei white cashmere goats using CRISPR/Cas9 technology, and found that the number and density of secondary hair follicles in edited cashmere goats significantly increased, and cashmere lengthsignificantly extended; They further performed HE staining on the skin tissues of aborted and normal goats, and showed that the number of secondary hair follicles in the skin of FGF5 mutant goats was more than that of wild-type goats, the diameters of primary and secondary hair follicles were longer than wild-type goats, and the SHF/PHF ratio was significantly higher than that of normalgoats. Zhang et al. [87] also confirmed that knocking out FGF5 resulted in a significant increase in fiber length and growth rate. Li et al. [88] used CRISPR/Cas9 to insert nonsense codons into the FGF5 gene, which improved cashmereyield. Hao et al. [89] revealed the inhibition of hair follicle growth and development in goats by producing SCNT mediated extracellular receptor abnormalities (EDAR) knockout through CRISPR/Cas9. In addition, Li [90] added exogenous thymosin β 4 (Tβ4) targeted integration into cashmere goats and discoveryof Tβ4 can induce hair follicle development,accelerate the differentiation of hair follicle stem cells, and play an important role in improving hair production. Hao [89] combined the CRISPR-Cas9 systemand somatic cell nuclear transfer technology to construct EDAR gene targeted cashmere goats, which exhibited baldness. Hu [85] utilized the CRISPR/Cas9 system to target Vascular Endothelial Growth Factor (VEGF) in Inner Mongoliawhite cashmere goats with FGF5 and CCR5 genes, which played a crucial rolein cashmere breeding by simultaneously knocking in hair follicle development promoting genes and knocking out hair follicle development inhibiting genes. Wool color is also a key production trait for cashmere. In order to change thecolor of cashmere, Zhang et al. [91] applied CRISPR/Cas9 technology to targetand knock out the ASIP gene. Compared with wild-type individuals with whitehair, the ASIP edited sheep wool color produced diversity, indicating that ASIPaffects fur color formation and serves as a marker gene. CRISPR/Cas9 currently has a prominent ability to disrupt genes that inhibit hair follicle development into an ideal phenotype, providing a scientific basis for further cultivating high-quality and high-yield cashmere goat breeds, and also providing insights for the treatment of androgenic alopecia.

4.2. Improve Muscle Growth and Development

Researchers [92–94] used Cas9/gRNA to block genes that inhibit muscleproduction in meat sheep [95], improving meat production and quality to meetpeople's livelihood needs for mutton.

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Myostatin (MSTN) can regulate skeletal muscle growth and promote muscle proliferation. Therefore, inhibiting the expression of MSTN can induce the appearance of "dual muscle" traits in developedmuscle areas such as the buttocks, shoulders, and legs, providing application value for improving meat quality and increasing lean meat percentage in livestock. Albertio and Wolf [96] boldly hypothesize the use of DNA nucleases to apply this phenotype to the growth of cattle, sheep, and pigs, promoting meat production performance in livestock and poultry.

He et al. [97] injected targeted MSTN into the cytoplasm of prokaryotic embryos to obtain gene edited goats with dual muscle traits. Wang et al. [92] demonstrated that the muscle development level of MSTN-edited sheep was significantly faster than that of wild-type sheep. Niu et al. [98] found that sheep modified with BCO2 biallelic genes exhibited yellow fat, indicating that the BCO2 gene may determine the formation of fat color. Wan et al. [99] used CRISPR/Cas9 system to knock out the conserved site of the goatCLPG1 gene for the first time in order to study the "beautiful buttocks" of sheep. In addition, Zhang et al. [93] demonstrated that the loss of MSTN function in sheep skeletalmuscle satellite cells (sSMSCs) promotes the differentiation and growth of sSMSCs. Zhou et al. [100] introduced single nucleotide point mutations in the Recombinant SuppressorsOf Cytokine Signaling 2 (SOCS2) using the CRISPR/cas9system, resulting in an increase in sheep weight and milk production; in 2022, they knocked out the MSTN bialleles with a high proportion of mutations and found that meat quality was almost unaffected [101]. On the basis of MSTN mutant sheep, not only were changes in transcriptome gene expression analyzedin MSTN knockout goats [102], but also confirmed how knockout alleles are inherited to offspring [92]. Mei obtained one double allele and one single allele MSTN gene mutated goat through microinjection of fertilized eggs. Both edited sheep fetuses had "double muscle" and significantly increased weight and length. Further sectioning revealed a significant increase in muscle cross-section and muscle fiber density [103]. CRISPR/Cas9 significantly highlights the function of genes related to muscle growthand development in sheep, and has potential roles in the cultivation of new breeds of meat sheep.

4.3. Improving Reproductive Capacity

Improving reproductive performance is a key goal of breeding. The Fec familygenes are associated with the fecundity of sheep. Zhou et al. [104] used CRISPR/Cas9 technology to inject single-strand FecB DNA oligonucleotides into thezygote of sheep, obtaining FecB homozygous mutant sheep, proving that FecB homozygous ewes not only have higher lambing rates but also improve reproductive rates. The CRISPR/Cas9 system simultaneously edits HYAL2 and PrP genes to improve lambing rate [8]. Niu et al. [28] introduced point mutations in goat growth differentiation factor 9 (GDF9) and found that it also affects ovulation rate and litter size. Zhang et al. [105] used the CRISPR/Cas9 systemto induce functional deletion mutations in the bone morphogenetic protein receptor type IB (BMPR-IB) gene in sheep, leading to increased ovulation rate and litter size. Based on ssODNs, Zhou et al. [104] further introduced point mutations to generate BMPR-1B gene edited sheep. Tian et al. [106] microinjected the AANAT gene into frozen and unfrozen sheep embryos, and the results showed that there was no significant difference in the reproductive ability of transgenic offspring between the two environments, but AANAT transgenic individuals had good reproductive ability. It can be seen that CRISPR/Cas9 technology is an effective editing tool for developing ideal traits of farm animals, promoting the development of animal husbandry.

4.4. Improving Milk Composition

At present, researchers have enriched the composition of sheep milk by genetically modifying essential nutrients and knocking out non essential proteins in sheep milk. Goat milk is rich in fat and protein, and its composition is similar to human milk, but β-Lactoglobulin (BLG) is prone to sensitization. Zhou et al. [107] prepared using CRISPR/Cas9 technology knock out of β-lactoglobulin in goats reduces the protein concentration of BLG, laying the foundation for improving the composition of goat milk. Wei et al. [108] constructed BLG gene knock out cows from fertilized eggs to broaden the audience for milk and its by-products. Another study used the CRISPR/Cas9 system to knock out factors that affect milk traits, such as Stearoyl CoA Desaturase 1 (SCD1) [109] and Acetyl

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CoA acyltransferase 2 (ACAA2) [110]. In breast cells, Ma et al. [111] used CRISPR/Cas9 technology to inject Arylkylamine N-acetyltransferase (AANAT) and Acetylserotonin O-methyltransferase (ASMT), which mediate melatonin expression, into the cytoplasm of sheep fertilized eggs, and constructed an AANAT/ASMT breast bioreactor, resulting in edited sheep with high-yield of melatonin milk. CRISPR/Cas9 technology plays an irreplaceable role in the development of non artificial natural low-fat orspecific functional dairy products, promoting the development of the dairy industry rich in specific nutrients or pharmaceutical ingredients.

4.5. Establishment of Animal Disease Resistance Breeding and Human Disease Models

The economic losses caused by livestock and poultry diseases not only limit the development of animal husbandry, but also pose a threat to human safety in the event of an outbreak of zoonotic diseases. The CRISPR/Cas9 systemhas been used in disease resistance breeding for several diseases, providing a good therapeutic model for studying the pathogenesis and improving animal health. In addition, the body size and anatomical structure of sheep are similar to humans, and CRISPR/Cas9 has been used in sheep to provide an effective modelfor studying human diseases.

Fan et al. [112] targeted knock out PRP in goat fibroblast donor cells to produce SCNT mediated anti-PRP goats, and using CRISPR/Cas9 to generate NUP155 gene knockout donor cells in goat fibroblasts, a NUP155 gene knockout goat model for studying heart disease was obtained through SCNT program. Menchaca et al. [113] knocked out hyaluronidase 2 (HYAL2) in lambs, leading to lung adenocarcinoma syndrome, and confirmed the possibility of the CRISPR/Cas9 system producing antiviral animals, they also discovered for the first time that the deletion of the sheep otoferlin (OOF) gene is an effective model for treating deafness [114]. Li [115] inserted Tβ4 gene into the CCR5 site in goat, laying the foundation for establishing a goat knock inmodel. Fan et al. [116] used CRISPR/Cas9 combined with SCNT technology to cultivate IFNA gene knock out sheep, providing a large-scale animal model for fetalresistance to Zika virus (ZIKV) infection. Subsequently, they pioneered the human cystic fibrosis (CF) sheep model with CRISPR/Cas9 mediated cystic fibrosis transmembrane transmission regulator (CFTR) deficiency using the same technology [59]. The liver and gallbladder disease phenotype of newborn CFTR−/− sheep was consistent with that of humans. Williams et al. [117] applied CRISPR/Cas9 to point mutate the non-specific alkaline phosphatase (TNSALP) gene alkaline phosphatase (ALPL) , resulting in edited lambs exhibiting human hypophosphatasia (HPP), providing an effective animal model for studying this rare metabolic bone disease. Vilarino et al. [118] constructed Pancreas/duodenumhomeobox protein 1 (PDX1) fetuses, laying the foundation for gene edited sheep as host organs for xenotransplantation. In addition, the interspecies blastocystcomplementary technology combining embryonic gene editing and pluripotent stem cells (PSCs) has achieved xenotransplantation of human organs in large animals as hosts. The production of these disease-related gene knock out sheep demonstrates the possibility and effectiveness of the CRISPR/Cas9 system in breeding antiviral livestock, as well as its enormous potential for the treatment of human diseases.

4.6. Current Problems of Gene Editing Sheep

Although CRISPR/Cas9 technology has broad prospects in the developmentof animal husbandry, the low targeting efficiency, poor chimerism, and off-target effects of the CRISPR/Cas9 system [119] seriously restrict the sheep breeding process. Scientists are constantly optimizing the CRISPR/Cas9 system to be more efficient, accurate, and safe. The CRISPR/Cas9 system has developmentaldefects and low survival rates when editing cells in vitro to obtain gene editedhomozygotes. Direct injection into the fertilized egg results in the formation ofchimeras by simultaneously editing multiple cells due to division. Therefore, electroporation of Cas9 protein and sgRNA into embryos to construct gene edited sheep not only obtained homozygous positive individuals but also improved editing efficiency. Improving the targeting efficiency of gene edited sheep is related to various factors, such as increasing the embryo injection concentration of the CRISPR/Cas9 system or improving the conception rate [120].

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Off-target effects cause unpredictable consequences such as base mutations,deletions, rearrangements, and immune responses [121]. Therefore, reducing theoff-target rate is of utmost importance in improving the CRISPR/Cas9 system. At present, strategies such as optimizing sgRNA length, modifying Cas9 nuclease specificity, and using other Cas variants are mainly used to reduce off target effects [122]. Wienert et al. [123] developed a method called DISCOVER-Seq for identifying Cas off-target. Due to its ability to recruit DNA repair factors in cells and individuals, this method is applicable to various sgRNAs and Cas proteins. Donohue et al. [124] proposed a technique called CRISPR hybrid RNA DNA (chRDNA) to reduce off-target rates while maintaining targeted editing ability and improving Cas9 specificity. Although the efficiency of using viral vectors to deliver CRISPR systems is high [125], the Cas9 protein is relatively large and needs to be packaged separately before co-injection. MOUT et al. [126] developed an engineered DNA-Free Virus Like Particles (eVLPs) that can be efficiently edited in various cells and organs, minimizing off-target rates and the risk of DNA integration. Perez et al. [127] developed an algorithm called CRISPR Specific Correction (CSC) system for correcting specific gRNAs targeting mismatches in non coding and repetitive regions. The SpCas9 mutant and other gene editing techniques have overcome the drawbacks of off target efficiency in the CRISPR/Cas9 system [128], but they have also changed the species gene pool and ecosystem to a certain extent. Therefore, it is necessary to upgrade and improve the development of more accurate and easily controllableCRISPR/Cas9 systems [129].

5. Conclusion and Prospect

Since the emergence of the CRISPR/Cas9 system, it has made a qualitative leap in the progress of modern breeding in agriculture and animal husbandrywith its characteristics of precision, efficiency, simplicity, and economy. As a popular method for studying target genes, the development of CRISPR has solved some new challenges in genetic breeding, production performance, animal health, environmental protection, and human health in modern animal husbandry. With the development of technology, molecular breeding and gene editing breeding that achieve gene recombination through targeted gene transfer have replaced traditional breeding methods such as artificial selection and multi generationhybridization. CRISPR/Cas9 technology can accurately integrate, delete, and replace genes across species, insert excellent trait genes according to needs, break reproductive isolation, and achieve mutual acquisition of disease resistance genes from different species [130], reducing the time and cost of long-term breeding, accelerating the breeding process, providing new research ideas for cultivating high-quality and high-yield disease resistant livestock varieties, and creating new production models for gene edited animals. In addition, constructing multiple sgRNA vectors while editing multiple genes of the same individual genomeis currently the main research direction of resistance breeding [61]. Despite the many benefits of gene editing breeding, there are still some issues and risks:how to ensure the safety of gene edited species and their by-products? How tofind effective genes that affect the disease resistance of livestock? Have national regulations introduced policies for the use and promotion of genetically modified animals? Can humans accept the consumption of genetically modified agricultural products? Once these issues are resolved, the application of CRISPR gene editing technology in animal husbandry breeding will be even more fruitful.

At present, the regulatory standards for gene edited animals in most countries are not clear. Although regulatory agencies attempt to restrict the production of genome edited livestock and poultry, scientists believe that as long as standardized biosafety testing is passed, it can be applied to commerce. Government departments have established policies such as monitoring the breeding and consumption mechanisms of gene edited agricultural and livestock products, gradually allowing genetically modified animals to enter the market [131]. With thecontinuous improvement of supervision mechanisms and scientific technology, the public's recognition of genetically modified livestock and poultry products will also continue to increase. The emergence of CRISPR/Cas9 whole genome scanning technology not only saves time and cost, but also avoids the risk of disease transmission, making it an effective tool for accurately screening resistance genes. However, for disease resistant traits that are not suitable for cell death and virus replication screening, it is currently

insufficient to explore the function of their target genes [61]. Although the existing CRISPR/Cas9 technologycan achieve the level of simultaneous editing of multiple genes, it cannot achieve over 20 or even hundreds of genes. It can be seen that in the future, continuous exploration and innovation are needed to optimize the CRISPR screeninglibrary and screening conditions, and build a more solid and efficient multi gene editing technology, in order to achieve simultaneous regulation of multiple important economic traits of livestock and poultry micro effect genes [132], thereby obtaining the most ideal genetic improvement species.

In summary, CRISPR/Cas9 technology has achieved fruitful results in genetic breeding and improvement of livestock, promoting the sustainable development of animal husbandry. With the continuous improvement of gene editing technology and the continuous exploration of the structure and function of Cas9, further enhancing the safety and superiority of gene editing, CRISPR technology will undoubtedly have a more profound impact. On the basis of innovation and protection, we will promote the progress of gene editing technology. We believe that gene edited animal products will eventually become market-oriented, and the development of human society will achieve a more inclusive and sustainable future.

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