REVIEW

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Special Topic: Gene Editing towards Translation

Molecular breeding of farm animals through gene editing

Fei Gao[1,3,#], Naipeng Hou[2,3,#], Xuguang Du[1,3], Yu Wang[4], Jianguo Zhao[4] & Sen Wu[1,3,*]

[1]*State Key Laboratory of Animal Biotech Breeding, College of Biological Sciences, National Engineering Laboratory for Animal Breeding, Frontiers Science Center for Molecular Design Breeding, China Agricultural University, Beijing 100193, China;*
[2]*College of Animal Science and Technology, China Agricultural University, Beijing 100193, China;*
[3]*Sanya Institute of China Agricultural University, Sanya 572025, China;*
[4]*Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China*

#Contributed equally to this work.
*Corresponding author (email: swu@cau.edu.cn)

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Abstract: The rapid development of biotechnology has facilitated our understanding of the biological functions of candidate genes for important economic traits in farm animals. Molecular breeding by gene editing has greatly revolutionized the breeding of farm animals. Through gene editing and embryo manipulation, breeds with designed economic or disease-resistant traits can be readily generated. Along with this fast progress, the safety assessment of gene-edited farm animals has attracted public and regulatory attention. This review summarizes the research progress of gene editing in farm animals, focusing on performance improvement, disease resistance, bioreactors, animal welfare, and environmental friendliness. The limitations and future development of gene editing technology in farm animal breeding are also discussed.

Keywords: molecular breeding, farm animals, gene editing

Introduction

Farm animals, including pigs, cattle, sheep, goats, and chickens, provide animal protein and fat necessary for human daily life worldwide. Driven by a combination of population growth, urbanization, and rising incomes, the global demand for animal products is growing substantially. Despite the great progress in agriculture and animal husbandry, there are still about 1 billion people in the world with chronic malnutrition [1]. Global climate change will aggravate the lack of animal protein production [2], and the current efforts to meet the global food demand have also worsened the already overburdened environment [3,4]. According to the prediction of the United Nations, the world population will reach 10 billion by the middle of this century [5]. Breeding farm animals with the advantages of fast growth, strong disease resistance, good meat quality, low feed consumption, and high feed conversion rate has become the goal of the breeding field.

In the past few decades, researchers have used traditional crossbreeding strategies to improve important traits of livestock and poultry, which has a long time cycle, slow genetic progress, and cannot provide an effective solution for breeding traits such as disease resistance. In recent years, with the rapid development of

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gene editing technology, precision molecular breeding has provided innovative solutions for accelerating the genetic improvement of livestock and poultry for better production performance, enhancing disease resistance, reducing the threat of zoonosis transmission, and improving animal welfare [6–8].

At present, the public still has concerns about the safety of transgene. Different from transgenic technology, gene editing can achieve precise modification of target genomic sites without introducing foreign DNA sequences. Gene editing can quickly obtain specific genotypes that occur naturally at low frequencies and have the same effect as natural mutations [9–11]. Up to now, several gene-edited animals have been approved to enter the market in the world [12–14], which reflects the great value of molecular breeding through gene editing.

This review will introduce the development history of gene-edited farm animals, and systematically outline the progress and application prospects of gene-edited farm animals with aspects of production performance improvement, disease resistance, bioreactor, animal welfare, and environmental friendliness. Additionally, we provide new insights regarding the safety and supervision of gene-edited farm animals.

Gene editing technology and the development of farm animals

With the growth of the world population and the need for social development, the genetic improvement of farm animals has been a hot topic in agricultural research. Traditional breeding methods, including directed selection breeding, have become insufficient to meet the needs of sustainable agricultural development in recent years. Researchers are focusing on faster and more efficient genetic improvement strategies. Gene editing is a kind of genetic engineering technology that targets a specific site in the genome of an organism and achieves targeted modifications, such as insertions, deletions, or mutations, through non-homologous end joining or homologous recombination.

Zinc finger nucleases (ZFNs), transcriptional activator-like effect nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR), and other gene editing tools were developed and applied successively, and then played a pivotal role in the field of life science and medicine, marking the arrival of the era of gene editing. Table 1 briefly summarizes the recent progress of gene editing tools. From the gene editor ZFNs [15] to TALENs, which uses TALE to specifically recognize and bind DNA sequences [16], to the widely used CRISPR/Cas9 [17], farm animals have been genetically modified through gene editing tools, such as *myostatin* (*MSTN*) deficient double-muscled pigs generated by ZFNs [18], hornless cattle generated by TALENs [19], and avian leukemia-resistant chickens edited by CRISPR/Cas9 [20]. Varieties of gene editing tools have been widely used in livestock [21–23] and poultry [24], greatly promoting molecular breeding of farm animals.

Generally, the production of gene-edited livestock species includes the modification of the target gene in cultured cells followed by the production of animals through embryonic technologies such as somatic cell nuclear transfer (SCNT) technology and handmade cloning (HMC). Also, gene-edited farm animals are produced through more simplified methods such as electroporation after *in vitro* fertilization (gene editing via electroporation, GEEP) and microinjection (MI) (Figure 1).

Because poultry has unique reproductive characteristics, the production of gene-edited poultry utilizes different methods than those used for mammals. In recent years, with the development of the *in vitro* culture

Table 1 A comparison of different gene editing tools

Gene editing tools	ZFNs	TALENs	CRISPR/Cas9	Base editors
Target DNA sequence recognition elements	Zinc-finger protein	TALE protein	Single guide RNA	Single guide RNA
The length of recognition elements	9–18 bp/ZFN	14–20 bp/TALE	20 bp gRNA + PAM sequence	20 bp gRNA + PAM sequence
DNA double-strand break element	*Fok* I endonuclease	*Fok* I endonuclease	Cas9 protein	–
Advantages	Efficient, small size and easy delivery	High efficiency, low off-target rate, low toxicity, and easy to design	Efficient, easy to construct, and edit multiple sites simultaneously	Safe, DSB-free, high efficiency in dividing cells and non-dividing cells
Disadvantages	Requires large-scale screening and engineering of large numbers of proteins, time-consuming and costly, off-target effects and toxicity	It is time-consuming and expensive, requires sequencing, and molecular cloning is complex and difficult to perform	May induce large DNA deletion, reversion, translocation, and complex rearrangements; the efficiency of editing can be affected by the sequence context of the targeted locus; may induce a p53-mediated DNA damage response	Undesired bystander mutations, gRNA independent DNA and RNA off target, too large for efficient *in vivo* delivery by single AAV vectors, the efficiency of editing can be affected by the sequence context of the targeted locus

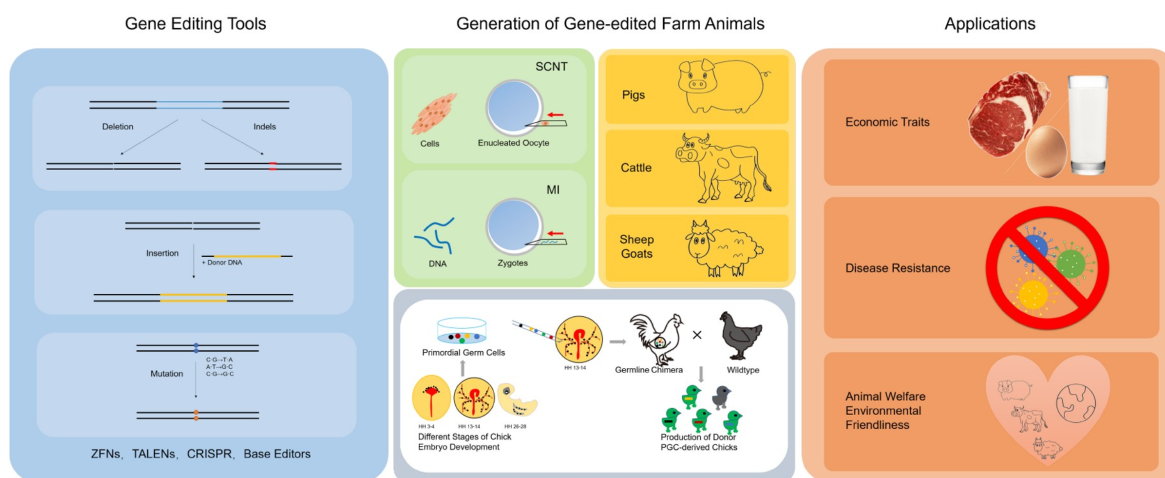

Figure 1 The production and application of gene-edited farm animals. Left: gene editing tools, including ZFNs, TALENs, CRISPR, and Base editors. Middle: simplified diagram of the methods for generating gene-edited farm animals (livestock and poultry). Right: applications of gene-edited farm animals.

of chicken primordial germ cells (PGCs) [25], the chimera preparation technology based on PGCs, and more efficient gene editing technology, precise modification of target genes in chickens has become efficient and reliable. To date, homologous recombination, TALENs, and CRISPR have all been successfully applied in chickens [26–28]. Although PGC is a powerful tool for accurate genome modification, only chicken PGC can be reliably cultured *in vitro*, other poultry species have yet to develop their PGC culture methods. Besides the PGC approach, researchers have also developed adenovirus-mediated blastoderm injection [29], vascular injection of plasmids [30,31], and sperm transfection assisted gene editing (STAGE) [32] methods for the preparation of gene-edited birds. Yet, these methods need to be further optimized, and new methods also need to be developed for more simple, efficient, and versatile gene editing in poultry. It is anticipated that

molecular breeding of poultry will have a bright future in performance improvement, disease resistance breeding, and egg bioreactor (Figure 1).

In the past decade, the supervision, evaluation, and regulation of gene-edited animals have also moved quickly, and several farm animal products have been approved to enter the market. The U.S. Food and Drug Administration (FDA) approved a recombinant anti-thrombin drug derived from the milk of transgenic goats in 2009 [12], a drug expressing transgenic chicken in 2015 [13], the AquAdvantage transgenic salmon in 2015 as the first edible gene-edited animal [14], and GalSafe pigs without α-galactose for both food and medical applications in 2020 (https://www.fda.gov/news-events/press-announcements/fda-approves-first-its-kind-intentional-genomic-alteration-line-domestic-pigs-both-human-food), indicating more gene-edited animals will be recognized, and the market will be enriched.

Application of gene editing in farm animals

Improvement of production performance

Over the past decades, researchers have identified numerous single nucleotide polymorphisms (SNPs) sites associated with economic traits of livestock and poultry, offering potential genetic targets for precise molecular breeding [33]. One of the important breeding goals in animal husbandry is to improve the meat yield and lean meat percentage of farm animals. *MSTN*, a member of the TGF-β superfamily, plays a negative regulatory role in the development of mammalian skeletal muscle. Inhibiting the expression of *MSTN* can promote the proliferation and differentiation of skeletal muscle cells, increase the number of muscle fibers, accelerate muscle growth, and increase lean meat percentage [34]. *MSTN* is conserved in various species. Natural mutations of *MSTN* have been reported in cattle, sheep, and dogs, and these mutant animals all have the double-muscle phenotype with significantly increased muscle mass and can survive and reproduce normally [9–11]. Therefore, the *MSTN* is often used as the preferred target gene for molecular breeding of livestock and poultry to promote muscle growth and meat yield. As early as the late 20th century, researchers have shown that *MSTN* knock-out mice approximately double their total body skeletal muscle weight due to myofiber hyperplasia and hypertrophy [35]. In 2015, Qian *et al*. [18] generated *MSTN* gene-edited Meishan pigs using ZFNs and observed an obvious double-muscle phenotype. At present, researchers have used ZFNs, TALENs, CRISPR, and other types of gene editing tools to successively edit the *MSTN* gene of various Chinese local pig breeds and commercial pigs, and generated *MSTN* knock-out pigs with high lean meat rate [36–39]. Among them, editing of the third exon of *MSTN* in commercial Large White pigs resulted in a low survival rate and limb deformity of piglets after birth, while targeting the first exon could effectively avoid the abnormalities associated with the third exon gene editing [39–41]. Another group reported that homozygous *MSTN* knock-out Meishan pigs and Erhualian pigs did not have birth defects [18,42]. Subsequently, different groups also generated *MSTN* mutant sheep [43], rabbits [44], goats [44,45], and cattle [46] by gene editing, which greatly improved the meat production performance in livestock.

Researchers have produced *MSTN* mutant chickens and quails [47,48]. Kim *et al*. [47] successfully generated an *MSTN* chicken model using D10ACas9 and found that the chest and leg skeletal muscles of *MSTN* mutant chickens were significantly enlarged, and the abdominal fat deposition of *MSTN* mutant chickens was significantly lower than that of wild types. Lee *et al*. [48] injected CRISPR recombinant

adenovirus into the EGK stage XI quail blastoderm and finally obtained *MSTN* mutant quails. Further analysis showed that *MSTN* mutant quails had a higher feed conversion rate [49]. In addition, researchers also found a phenotype of reduced abdominal fat deposition in the *G0S2* knock-out chickens, which may be a potential target gene for trait improvement [50].

Insulin-like growth factor 2 (*IGF2*) is a regulatory factor, which affects cell proliferation and differentiation, as well as skeletal muscle growth and fat deposition [51]. Some researchers have reported that there is a G to A mutation at position 3072 in the third intron of the porcine *IGF2* gene, which can effectively avoid the binding of transcription repressor ZBED6, thus improving the expression of *IGF2* gene and increasing the growth rate [52,53]. Such favorable mutations are widely found in foreign commercial breeds, but rarely in Chinese pig breeds. In 2018, Xiang *et al*. [54] deleted the binding sequence of *ZBED6* in the *IGF2* gene locus by microinjection of Cas9 nickase mRNA and a pair of sgRNAs into porcine zygotes, thereby improving the expression level of the *IGF2* gene and preparing Bama pigs with significantly increased meat production. Liu *et al*. [55] used CRISPR to disrupt the *ZBED6* binding site in porcine fetal fibroblasts, and further combined the SCNT technology to obtain Liang Guang small spotted pigs with increased lean meat rate. Another potential candidate gene for improving meat production in livestock is *FBXO40*, a member of the F-box family of proteins, which is a muscle-specific expression gene [56]. Mice with nonsense mutations in *FBXO40* exhibit muscle hypertrophy phenotype [57]. Zou *et al*. [58] successfully generated *FBXO40* gene knock-out pigs, and compared with the wild-type control, the muscle weight of knock-out pigs increased by about 4%. Gene-edited farm animals mentioned above are summarized in Table 2.

Improvement of disease resistance

Farm animal breeding is mainly focused on the improvement of production and economic traits. Besides these, various diseases are also important factors restricting the development of animal husbandry because they can cause huge economic losses. Yet, it is difficult to eliminate the disease just by relying on vaccines and drug treatments. In recent years, researchers have also reported promising results in disease-resistance breeding in livestock and poultry using gene editing (Table 3).

Table 2 Gene-edited farm animals with improved production performance

Species	Gene	Modification	Approach	Reference
Pig	*MSTN*	Knock-out	ZFNs	[18]
Pig	*MSTN*	Knock-out	CRISPR/Cas9	[36]
Pig	*MSTN*	Knock-out	CRISPR/Cas9 and *Cre*/*lox*P	[37]
Pig	*MSTN*	Knock-out	CRISPR/Cpf1	[38]
Pig	*MSTN*	Knock-out	TALENs and CRISPR/Cas9	[39]
Pig	*IGF2*	Knock-out	CRISPR/Cas9	[54,55]
Pig	*FBXO40*	Knock-out	CRISPR/Cas9	[58]
Sheep	*MSTN*	Knock-out	CRISPR/Cas9	[43]
Rabbit	*MSTN*	Knock-out	CRISPR/Cas9	[44]
Goat	*MSTN*	Knock-out	CRISPR/Cas9	[44,45]
Cattle	*MSTN*	Knock-out	ZFNs	[46]
Chicken	*MSTN*	Knock-out	D10ACas9	[47]
Chicken	*G0S2*	Knock-out	CRISPR/Cas9	[50]
Quail	*MSTN*	Knock-out	CRISPR recombinant adenovirus	[48,49]

Table 3 Gene-edited animal models of disease resistance

Species	Gene	Disease/Traits	Modification	Reference
Pig	*CD163*	Porcine reproductive and respiratory syndrome (PRRS)	Mutant	[66,67]
Pig	*CD163*	Porcine reproductive and respiratory syndrome (PRRS)	Knock-out	[68–71]
Pig	*CD163*	Porcine reproductive and respiratory syndrome (PRRS)	Homologous replacement	[72,73]
Pig	*Sn/SIGLEC1/CD169*	Porcine reproductive and respiratory syndrome (PRRS)	Knock-out	[75]
Pig	*ANPEP*	Transmissible gastroenteritis virus (TGEV)/ porcine epidemic diarrhea virus (PEDV)	Knock-out	[79–81]
Pig	*ANPEP*	Porcine delta coronavirus (PDCoV)	Knock-out	[82]
Pig	*CMAH*	Porcine epidemic diarrhea virus (PEDV)	Knock-out	[84]
Pig	*shRNA*	Classical swine fever virus (CSFV)	Knock-down	[86,87]
Pig	*pRSAD2*	Classical swine fever virus (CSFV)/pseudo rabies virus (PRV)	Knock-in	[88]
Pig	*shRNA*	Pig foot-and-mouth disease virus	Knock-down	[89]
Sheep	*shRNA-VP1*	Sheep foot-and-mouth disease virus	Knock-down	[90]
Bovine	*shRNA-VP1*	Bovine foot-and-mouth disease virus	Knock-down	[91]
Bovine	*NRAMP1*	Bovine tuberculosis	Knock-out	[93]
Bovine	*SP110*	Bovine tuberculosis	Knock-out	[92]
Bovine	*CD18*	Bovine pneumonia	Mutant	[94]
Bovine	*PRNP*	Bovine spongiform encephalopathy	Knock-out	[100,101]
Bovine	*PRNP*	Bovine spongiform encephalopathy	Knock-in	[101]
Chicken	*ANP32*	Avian influenza virus (aivs)	Knock-out	[102]
Chicken	*3D8 scFv*	Avian influenza virus (aivs)	Knock-in	[103]
Chicken	*Tva*	Avian leukemia virus (ALV)-A/K	Knock-out	[104]
Chicken	*NHE1*	Avian leukemia virus (ALV)-J	Mutant	[20,105]
cow	*HSPA1L*	Heat resistant cow	Mutant	[107]
Pig	*ANTXR1*	Porcine vesicular disease (Seneca virus A)	Knock-out	[106]
Sheep	*TLR4*	Improve sheep disease resistance	Transgene	[108]

Porcine reproductive and respiratory syndrome (PRRS)

PRRS, commonly known as “blue ear disease”, seriously affects the economic interests of producers. PRRS virus (PRRSV), a class of RNA viruses with highly contagious and quickly mutating features, infects porcine alveolar macrophages and causes PRRS [59,60]. Since 2006, highly pathogenic (HP) PRRSV has become the main epidemic strain in China, further exacerbating the economic impact on China’s pig industry [61].

Due to the genetic diversity of the virus, the vaccine has limited efficacy on PRRS [62]. As a result, more attention is paid to the cellular receptors that directly determine whether PRRSV can enter the target cell. To date, several important PRRSV receptors have been identified, such as heparin sulfate (HS), sialoadhesin (*Sn/CD169*), *CD163*, *CD151*, and vimentin [63]. *CD163*, a member of the scavenger receptor cysteine-rich (SRCR) family, is a transmembrane protein on the surface of macrophages serving as a receptor for PRRSV [64]. The fifth SRCR domain (SRCR5) is considered necessary for PRRSV infection [65]. Whitworth *et al.* [66,67] in 2014 produced pigs with a mutation in the exon 7 of *CD163* gene using CRISPR/Cas9, resulting in CD163 protein inactivation. Following the live virus challenge test, mutant pigs did not show viremia, antibody response, high fever, or any other PRRS clinical symptoms, demonstrating strong resistance to PRRSV infection. This is the first study using gene editing technology to produce pigs resistant to porcine viral infectious diseases.

Later, researchers successively generated different types of *CD163* gene mutant pigs [68–73]. Among

them, one gene editing design is the specific deletion of the SRCR5 domain of the *CD163* gene while keeping other *CD163* domains intact. Macrophages from these pigs are fully resistant to both PRRSV genotypes [69–71]. This study shows that gene editing can successfully delete the virus-resistance-related region yet still maintain the important role of other regions of *CD163* in inflammation and immune response. In addition, by homologous substitution of the porcine SRCR5 domain with corresponding human *CD163* domain, the gene-edited pigs were also resistant to PRRSV [72,73]. Interestingly, maybe because of herd immunity [74], feeding *CD163* knock-out and wild-type pigs together can reduce the mortality rate of pigs [70]. It is of great significance for improving the health of pig populations. In addition to *CD163*, other important receptor genes for PRRSV, such as *CD169*, were also knocked out with gene editing in pigs, but no significant differences in viral resistance were found between edited and wild-type pigs, so *CD169* may not be a key gene influencing the occurrence of PRRS [75]. Also, gene-edited animals may be combined with mass vaccination for disease elimination [76]. In summary, disease-resistance breeding for the *CD163* gene is a great and successful example of gene editing in animal breeding.

Coronaviruses

Piglet diarrhea is an important factor affecting pig survival. Several coronaviruses, including porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and porcine deltacoronavirus (PDCoV), can cause intestinal infection and necrosis in piglets, which can further lead to malabsorption, diarrhea, and death.

Porcine aminopeptidase N (*APN*), considered a receptor for TGEV and PEDV, is a metallopeptidase highly expressed in the intestinal epithelial cell membrane. It is involved in the removal of N-terminal amino acids from protein substrates and affects protease activity rather than functions as a receptor for virus infection [77]. Although studies have shown that APN is not necessary for infection [78], it still promotes infection. *APN* knock-out pigs were found to be effective against TGEV but not PEDV infection after virus challenge tests [79–81]. In addition, alveolar macrophages of *APN* knock-out pigs are resistant to PDCoV infection, while pulmonary fibroblast-like cells can be infected by high-titer PDCoV, suggesting that APN is the receptor for PDCoV in porcine alveolar macrophages, but is not necessary for pulmonary fibroblasts infection by PDCoV [82].

PEDV infection approach is mainly through villi by the S protein, which is exposed to sialic acids (NA) in the host intestine and binds to APNs on epithelial cells. An important PEDV receptor is *N*-glycolyl NA (NGNA) in the villi of the small intestine. NGNA is formed by inserting oxygen into the acetyl group of *N*-acetyl NA (NANA) by CMP-*N*-glycolylneuraminic acid hydroxylase (CMAH) [83]. In CMAH knock-out pigs, NGNA is not expressed. Although PEDV infection is still present in these CMAH knock-out piglets, the symptoms and mortality caused by PEDV infection are alleviated [84]. At present, there is no effective coronavirus-resistant pigs generated, these gene-edited pigs still provide a valuable reference for solving coronavirus related diseases in the future.

Classical swine fever virus (CSFV) and foot-and-mouth disease virus (FMDV)

CSFV, a member of the genus Pestivirus within the family Flaviviridae, is a small, enveloped, positive-strand

RNA virus that has been in the spotlight for a long time. RNAi strategies can be used to interfere with CSFV *in vitro* [85]. Xie *et al*. [86] used shRNA to knock down CSFV and obtained anti-CSFV pigs that slowed down the clinical phenotype of CSF. Lu *et al*. [87] also integrated anti-CSFV shRNA into porcine endogenous miR-17–92 clusters, effectively inhibiting CSFV replication. In a broader research perspective, RNAi can provide a valuable reference, although it does not belong to the category of gene editing technology.

It has been reported that the *pRSAD2* gene overexpression can resist a variety of viruses by inhibiting virus replication [88]. In fibroblasts isolated from the *pRSAD2* knock-in pigs, virus challenge experiments found obvious resistance to both CSFV and pseudorabies virus (PRV) [88], but *in vivo* virus challenge experiments are still lacking.

Researchers have regarded the RNAi technique just mentioned as an important method to effectively inhibit viral infection. Except for the anti-CSFV pigs, RNAi combined with gene editing technology is also used in the preparation of anti-FMDV models. Pigs [89], sheep [90], cattle [91], and other different transgenic animals showed obvious resistance to FMDV. Although a small number of individuals also have symptoms such as ulcers, they all appear much later than wild-type individuals. The spread of FMDV can be prevented through vaccines, but gene-edited animal models offer an alternative approach to FMDV prevention.

Tuberculosis (TB) and pneumonia

Bovine TB is a chronic infectious disease that affects a wide range of mammalian hosts, and not only seriously affects the agricultural economy, but also threatens human health as a zoonotic disease. Mycobacterium is an important intracellular pathogen that causes bovine TB. In 2015, Wu *et al*. [92] used TALE nickase-mediated SP110 gene knock-in to generate TB-resistant cattle, and the gene-edited cattle were able to inhibit the growth and reproduction of *Mycobacterium bovis*. In 2017, Gao *et al*. [93] used Cas9 nickase-mediated homologous recombination to integrate natural resistance-associated macrophage protein-1 (NRAMP1) into the bovine genome. In combination with SCNT, they obtained gene-edited cattle with greatly enhanced TB resistance.

Similarly, pneumonia caused by *Mannheimia haemolytica* made huge economic losses in the cattle industry. In 2016, Shanthalingam *et al*. [94] used ZFNs to induce a Q(–5)G substitution in both alleles of *CD18* in bovine fetal fibroblast cells. By SCNT, they produced a bovine fetus homozygous for the Q(–5)G substitution. Leukocytes were resistant to leukotoxin-induced cytolysis. This study demonstrated the feasibility of developing gene-edited cattle resistant to *M. haemolytica*-caused pneumonia.

Prion diseases

Prion diseases are a class of transmissible neurodegenerative diseases that can be fatal, including bovine spongiform encephalopathy, scrapie in goats and sheep, and Creutzfeldt's disease in humans [95]. Prions are the pathogens that cause such degenerative lesions of the central nervous system. They are not viruses in the conventional sense because no nucleic acids are contained. Prions can be understood as infectious protein particles that can support their accumulation and spread in host cells. There is considerable evidence that

some prion proteins (PrP) are misfolded within the host cell, resulting in a change in physicochemical properties, becoming pathogenic PrP that can polymerize and accumulate continuously, resulting in disruption of cell function and cell death [96]. At present, there is no effective treatment for prion disease, so in the rearing of ruminants such as cattle and sheep, active precautions such as prohibiting the addition of animal-derived feed and culling and eliminating known infected livestock are the only practical measures. Interestingly, although PrP in cells is necessary for the pathogenesis of prion disease, it is dispensable for normal growth and development in animals [97,98]. In normal goat populations, there are non-sense mutations in the natural PrP-encoded gene, which have the potential to resist prion disease and are of great value for researching and producing prion-free products [99]. In addition, PrP-deficient cattle are normal clinically, physiologically, histopathologically, immunologically, and reproductively [100]. And their brain tissue homogenates do not support *in vitro* proliferation of prions [100]. These PrP-deficient cattle were produced by the conventional gene targeting method, which is time-consuming and costly. To produce more PrP-deficient farm animals, gene editing technology is now the obvious choice to facilitate this process [101]. Although there are no successful cases of gene editing techniques against prion diseases, we believe such PrP gene-edited livestock will be reliable models for prion research that may improve food safety.

Avian influenza virus (AIV) and avian leukemia virus (ALV)

The outbreak and transmission of the AIV can cause serious economic losses to the poultry industry. Some strains of the AIV can also infect humans, posing a great threat to human life and public health. The current vaccines or antiviral drugs cannot prevent and cure the infection of all strains of the virus, and an in-depth study of the host factors that interact with AIV is key to the development of new avian influenza prevention and control strategies. The completion of the life cycle of influenza viruses depends on the specificity of the host, and AIV can replicate well in birds rather than in mammals. Acidic ribophosphate 32 family member A (*ANP32A*) was found to play an important role in the pathogenesis of avian influenza and targeted *chANP32A* knock-out significantly inhibited AIV virus polymerase activity [102], suggesting that *ANP32A* is a good candidate gene for anti-influenza molecular breeding.

In addition to hindering the replication of the virus, another intervention is the degradation of the viral genome. The 3D8 single-stranded variable fragments (*3D8 scFv*) exhibit hydrolytic activity against RNA and DNA viruses. The *3D8 scFv* transgenic chickens produced by recombinant lentiviral vector systems have good resistance to contact transmission of the H9N2 virus [103]. Although the expression of *3D8 scFv* does not inhibit direct viral infection, it can prevent contact transmission from the external environment, which is very useful for the prevention and control of the disease.

Another disease that seriously affects the development of the poultry industry is the ALV, a retrovirus that causes bird tumors and contains seven ALV subtypes (A to E, J, and K). It is currently believed that ALV virus infection of host cells requires four receptor proteins, including the Tva protein associated with low-density lipoprotein receptors (LDLR), the Tvb protein of the tumor necrosis factor receptors family, the Tvc protein similar to the mammalian butylophilic class, and the Tvj protein as a Na^+/H^+ exchanger 1. *Tva* gene knock-out chickens obtained by CRISPR/Cas9 showed complete resistance to ALV subsets A and K [104]. By removing tryptophan residues of chicken Na^+/H^+ exchanger 1 (*chNHE1*) W38, complete resistance to ALV-J infection was achieved [20,105]. These gene-edited chickens have effectively resisted different

subtypes of the ALV virus. Research on other subtypes and key receptor proteins needs to be further explored by gene editing.

Outlook of disease resistance breeding

Researchers have produced many other disease resistance models, the model of porcine vesicular disease caused by Seneca virus A (SVA) by knocking out anthrax toxin receptor 1 (*ANTXR1*) [106], cow models that resist heat stress by the deletion of *HSPA1L* [107], and broad spectrum disease-resistant sheep models by overexpressing *TLR4* [108]. In short, the current research and development of disease resistance models mainly target key receptors of viruses to interfere their replication. The existing disease resistance animals have made up for the shortcomings of vaccines, but due to the lack of large-scale trials, it is unknown whether gene-edited disease-resistant animals will maintain resistance to the virus over time. Even if gene-edited animals cannot all enter the market, they are still valuable for the study of the pathogenesis of key diseases affecting the production of animal husbandry.

Multi-gene editing in farm animals

Traditional breeding livestock with specific and desirable traits is a slow process due to the long breeding cycles and generation intervals, which often take years in large animals. New gene-editing techniques enable the efficient and precise editing of multiple genes related to disease resistance and economic traits in livestock, which is expected to meet the urgent demand of breeders to assemble several desirable traits rapidly (Table 4). Xu *et al*. [109] used CRISPR/Cas9 and SCNT to generate pigs with the simultaneous knock-out of *CD163* and *APN* genes. Further analysis showed that the double-knockout pigs were able to resist PRRSV and TGEV infection at the same time, but had no difference in meat production and reproductive performance compared with wild-type pigs. Song *et al*. [110] used a one-step method that combined the HA3A-BE3-Y130F base editor with the porcine zygote injection to generate simultaneous gene mutations in three genes *CD163*, *MSTN*, and *IGF2*. The analyses showed that the expression level of *CD163* and *MSTN* in the triple gene-edited pigs was decreased and the expression level of *IGF2* was increased, as designed. The growth performance and disease resistance were significantly improved.

To improve the goat meat production and cashmere yield simultaneously, Wang *et al*. [111] injected Cas9 mRNA and sgRNAs targeting *MSTN* and *FGF5* genes into goat zygotes and obtained 98 goat progenies. The knock-out efficiencies of *MSTN* and *FGF5* genes were 15% and 21%, respectively. There was ten percent of the goats with double gene knock-out. Wang *et al*. [112] successfully obtained three-gene editing sheep using CRISPR/Cas9 and zygote microinjection technology, simultaneously targeting *MSTN*, *ASIP*, and *BCO2* genes.

Table 4 Multi-gene editing in farm animals

Species	Gene	Modification	Approach	Reference
Pig	*CD163* and *APN*	Knock-out	CRISPR/Cas9	[109]
Pig	*CD163*, *MSTN*, and *IGF2*	Mutation	Cytodine base editor	[110]
Goat	*MSTN* and *FGF5*	Knock-out	CRISPR/Cas9	[111]
Sheep	*MSTN*, *ASIP*, and *BCO2*	Knock-out	CRISPR/Cas9	[112]

These studies have theoretical and practical significance for the realization of molecular breeding in livestock. In recent years, with the development of efficient gene editing technology, the off-target effect has become a major concern of researchers and a challenging problem. Therefore, researchers have combined SCNT and whole genome sequencing technology to develop a widely applicable off-target activity assay (NT-SEQ) for various gene editing tools, including base editing tools [113]. The establishment of this off-target activity detection method will guide the continuous optimization of gene editing tools and increase the further application of gene editing tools. In the future, the continuous optimization of the efficiency and accuracy of gene editing technology may greatly promote the wide application of gene-edited large animal models in molecular breeding, and provide technical support for solving global food security and ensuring food supply.

Applications of bioreactors

The major studies of classical farm animal breeding would be generation of animal with special trait for the improvement of production performance and disease resistance. In order to provide a more comprehensive understanding of genome manipulation in farm animals, we have discussed the applications of bioreactors, as summarized in Table 5. At present, medicinal proteins are produced mainly using *Escherichia coli*, yeast, or mammalian cells cultured *in vitro*. However, humanized proteins in *E. coli* or yeast are often misfolding or lacking appropriate glycosylation modifications, which can affect the effectiveness of these proteins. Therefore, humanized animal models are excellent carriers of bioreactors. Mammary gland and egg bioreactors play an important role in the efficient expression and easy purification of target proteins. The successful marketing of recombinant human antithrombin III (ATryn) produced from transgenic goats marks the arrival of the era of animal bioreactors [12]. More recently, Liu *et al*. [114] used the ZFNs-mediated gene recombination strategy to precisely insert the lysostaphin gene into the endogenous CSN2 gene locus of cattle. Lysostaphin can be secreted into the milk of transgenic cows, which can effectively resist the infection of *Staphylococcus aureus* in the mammary gland. Ma *et al*. [115] generated *AANAT*/*ASMTZ* transgenic sheep using the CRISPR/Cas9 technology, which can be used as a bioreactor to produce sheep milk rich in melatonin.

Table 5 Gene-edited animal models as efficient bioreactor

Species	Products/Genes	Modification	Reference
Cow	Lysostaphin	Knock-in	[114]
Sheep	Melatonin	Transgene	[115]
Goat	BLG	Knock-out	[118]
Cow	BLG	Knock-out	[119]
Chicken	Human monoclonal antibodies	Transgene	[120]
Chicken	miR24 and IFN-β-1A	Transgene	[121]
Chicken	hEGF	Transgene	[122]
Chicken	hEPO	Transgene	[123]
Chicken	Interferon α2a and CSF1	Transgene	[124]
Chicken	CD20	Transgene	[125]
Chicken	HIFN-β	Knock-in	[126]
Chicken	Ovomucoid	Knock-out	[127]

Cow and goat milk are important protein sources with high nutritional value, but contain whey protein β-lactoglobulin (BLG) which is not found in human milk. BLG can cause intestinal allergic reactions, especially infant diarrhea, and seriously affect the absorption and utilization of nutrients in dairy products. However, neither heat treatment nor fermentation can remove BLG protein allergens from cow and goat milk [116,117]. In recent years, researchers have successfully generated BLG gene knock-out goats [118] and cows [119] using the CRISPR/Cas9 combined with zygote microinjection technology or ZFNs combined with SCNT technology, respectively. Whereafter, the protein components of milk produced by knock-out cows were detected by SDS-PAGE and Western blotting, the results showed that BLG protein was completely absent. Thus, these studies provide a new way to effectively change the composition of cow and goat milk and make it more suitable for human health [118,119].

In addition, with the development of avian gene editing technology, the poultry egg bioreactor can also be used as an effective platform for the production of protein drugs. A hen can lay 300 eggs per year and the protein composition of egg white is relatively simple, making it easier to purify the target proteins from the egg. In 2005, Zhu *et al*. [120] used chicken embryonic stem cells to prepare chimeric transgenic chicken and expressed human monoclonal antibodies in chimeric eggs. The maximum content of antibodies with good antigen recognition ability could reach 3 mg/egg. In 2007, Lillico *et al*. [121] successfully used lentiviral vectors to prepare transgenic chickens, which express the humanized SCFV-FC mini antibody, known as miR24 and IFN-β-1A. Subsequently, researchers have produced transgenic chicken models to express various humanized proteins, such as hEGF [122], hEPO [123], human cytokine interferon α2a, and two species-specific Fc fusions of the cytokine CSF1 [124], and CD-20 [125]. In addition, with the development of precision gene editing technology, researchers successfully inserted the target protein precisely into the ovalbumin locus and realized the efficient expression of HIFN-β [126]. Ovomucoid (OVM) is a kind of major protein in egg white and is considered the main allergen in eggs. The researchers generated an *OVM* knock-out chicken model and found that *OVM* knock-out chicken could almost eliminate ovomucoid from eggs without destroying fertility. Therefore, the hypoallergenic eggs developed in this study can be used as a food source for most patients with egg allergy [127].

Animal welfare and environmental friendliness

With the large-scale development of animal husbandry, people have gradually realized that animal welfare and environmental friendliness are also important parts of animal husbandry production (Table 6).

In the daily management of modern animal husbandry, physical dehorning of cattle to protect animals and producers from accidental injuries is not only costly but also painful for animals, raising concerns about animal welfare. In 2016, Carlson *et al*. [19] used TALENs and SCNT to integrate the *POLLED* alleles associated with cow hornless into the cow genome, and successfully bred two gene-edited hornless cattle, proving the feasibility of gene editing to solve the problem of dehorning. In 2020, Young *et al*. [128] further

Table 6 Gene-edited animal models to improve animal welfare and environmental friendliness

Gene	Target	Modification	Reference
polled	Hornless cattle	Knock-in	[19,128]
UCP1	Cold resistant pig	Knock-in	[130]
bgl7A, *eg1314*, *xynB*, *eappA*	Low-fecal nutrient emission pig	Transgene	[131]

performed phenotyping on the offsprings of gene-edited hornless cattle, and did not observe obvious health problems.

Newborn piglets are sensitive to cold, and insulation measures are often needed in animal production to ensure the normal survival and growth of newborn piglets. Uncoupling protein 1 (*UCP1*) plays an important role in body fat regulation and thermogenic cold resistance [129]. Since the evolution of domestic pigs, the *UCP1* gene has been partially missing and inactivated, which may be one of the reasons for the poor cold tolerance of piglets. Zheng *et al*. [130] used the CRISPR/Cas9-mediated non-homologous recombination to insert mouse adiponectin promoter and mouse *UCP1* gene into the *UCP1* locus of pigs that had been inactivated. By combining with SCNT, they generated *UCP1* knock-in pigs and realized the specific expression of *UCP1* in mature white fat cells in pigs. Compared with wild-type pigs, *UCP1* knock-in pigs have stronger thermoregulation ability under acute cold stimulation conditions, which not only improves animal welfare but also greatly saves the economic loss of pig farming. The pigs also exhibit excellent productivity with a significant reduction in dorsal thickness and fat percentage, as well as a significant increase in lean meat rate.

In addition to the concern for animals themselves and economic benefits, environmental globalization has also led people to pay attention to environmental problems in livestock production. In pig production, their excrement contains high levels of nitrogen and phosphorus, and other chemicals, which are hazardous to the environment. If pigs can digest food more efficiently, the nitrogen and phosphorus content in the excrement will be reduced. By *piggyBac* transposon system, researchers introduced a range of microbial genes *bg17A*, *eg1314*, *xynB*, and *eappA* to pigs, the digestion capacity of pigs was enhanced, resulting in a healthy, fast-growth, and low-fecal nutrient emission transgenic pig [131]. This is a very valuable solution to the issue of environmental emissions from animal husbandry, but of course, it also needs to be based on the health of the animals themselves.

Safety and regulation of gene-edited agricultural animals

The rapid development of gene editing technology in recent years has greatly promoted the research of trait improvement and provided new opportunities for the molecular breeding of farm animals. A good example is the PRRS-resistant pigs generated by gene editing of the *CD163* gene, which could not be readily obtained by traditional breeding that relies on natural variation within the population. From production traits to disease resistance traits, gene editing technology has undoubtedly had a huge impact on the development of animal husbandry. As an emerging technology, of course, there are still too many unknowns, which also creates concerns about these gene-edited animals.

The public concerns about gene-edited animals are mainly focused on off-target gene editing, the welfare of gene-edited animals themselves, and the safety of gene-edited animal products. Off-target gene editing has attracted more attention from researchers aiming at reducing the off-target rate. A low off-target rate means a lower level of introduced unintended mutations in the animal which may be of great significance for improving the public recognition of gene-edited animals. The evolution and development of more precise and accurate gene editing technology in recent years have been solving this issue. With better target site selection, whole genome sequencing, and whole genome off-target detection, gene-edited animals in the

future should be safe with few unwanted mutations.

As for the welfare of animals themselves, current studies generally focus on the ability of animals to exhibit their natural behavior and development. Researchers should compare and analyze the factors affecting the development of animals according to the target gene selection, to minimize the negative effect on the animal's physiology and behavior. On the other hand, researchers are also focused on generating gene-edited animals with improved animal welfare, such as hornless cattle.

With the rapid advancement of gene-editing technology, an increasing variety of gene-edited animals are being developed, presenting a wider range of applications. As far as the safety of gene-edited animal products is concerned, researchers have paid considerable attention to it and have carried out studies on off-targeting and rigorous phenotypic testing. Additionally, the Codex Alimentarius has established international guidelines for the safety of genetically modified (GM) animals, with stringent restrictions on the circulation of GM animals in the market. There may be an imbalance between policies and products, but overall, the current policies are relatively conservative which can ensure food safety to the greatest extent.

Chemical mutagenesis was used to alter plant DNA in the 1940s. Since the early 1990s, genetically engineered foods have been integrated into our life. Genetically engineered plants with the same safety standards as those of conventional plants are now being developed and sold (https://www.fda.gov/food/agricultural-biotechnology/science-and-history-gmos-and-other-food-modification-processes). In 2003, The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations developed international guidelines and standards for genetically engineered foods. With the development of genetic engineering technology, the term "bioengineered" began to be used for some foods. Similarly, with the approval of genetically modified salmon as the first genetically engineered animal in 2015, to the approval of GalSafe pig in 2020, the regulations governimg genetic engineering animals have also been continually improved. In the coming decades, we can expect to see an increasing number of genetically modified animals entering the market [132,133].

Although gene-edited farm animals show promise and have the potential to be immensely valuable in the future, from the perspective of promoting the sustainable development of world agriculture, there is an urgent need to address the current discordance between gene-edited farm animals and regulatory policies. The four basic principles of the evaluation of gene-edited farm animals (environment, society, human and animal evaluation, and practicality) should be strictly followed. For the regulation of gene-edited farm animals, there is still no unified international standard. Some countries regulate gene-edited animals and transgenic animals according to the same standard. Some others only identify animals with exogenous DNA insertions as transgenic animals. And some still maintain a more cautious attitude [134].

For a wide variety of gene-edited animals, more detailed and internationally unified regulatory policies are of great significance for both agricultural development and public recognition. Some scholars have proposed a more comprehensive classification method that divides gene-edited animals into seven categories which include mimicking beneficial natural mutations of the same breed (GE1), genetically dysfunctional breeds (GE2), mimicking beneficial mutations or genotypes across species (GE3), creating new artificial mutations (GE4), specific integration of foreign genes or regulatory elements into the genome of domestic animals (GE5), spatiotemporal expression of some endogenous genes through endogenous genes and endogenous primers, genetic modification by recombination of offspring (GE6), and gene-edited livestock using drugs and other means (GE7). More targeted management of different classes of gene-edited animals may be a

better regulatory approach. It is also worth noting that ethical considerations are an ongoing concern in the development of genetically modified animals [134,135].

Conclusions

The development of animal husbandry has always focused on exploring the improvement of economic traits, animal disease resistance, and animal welfare. Gene editing technology has brought precision breeding to a higher level. Whether for increasing the speed and gain of genetic improvement or for boosting population disease resistance, gene-edited animals have shown great advantages. With the improvement of regulatory policies, several gene-edited animals have successfully made their way to the food table. While more research is constantly evolving, from more in-depth research such as muscle gaining with *MSTN* mutation and disease resistance with *CD163* knock-out, to new research such as gender control of chickens [136], gene-edited farm animals have great potential. Animal models of human diseases and bioreactors also demonstrate that farm animals have broad prospects for applications in human medical research.

However, many current studies and models are still not recognized by the general public. On the one hand, it is necessary to further refine the regulatory system to strictly ensure food safety and animal welfare. On the other hand, there are still many technical problems to be solved, which have already promoted further development of whole genome sequencing technology and gene editing technology. Also, the development of efficient multi-gene editing technology is needed to accelerate the breeding process.

According to a mathematical model, the addition of gene editing techniques may greatly promote the development of animal husbandry [137]. It is believed that with further development and refinement of research and policies, the genetic improvement of farm animals will make greater progress, and more gene-edited animals will come to the table in the future.

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Author contributions

F.G., N.H., X.D., Y.W., J.Z. and S.W. conceived, performed and designed the topics. F.G. and N.H. wrote the first draft of the manuscript. X.D., Y.W., J.Z. and S.W. corrected and validated the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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