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Review Article

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Traditional Strategies and Cutting-Edge Technologies Used for Animal Breeding: A Comprehensive Review

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Abstract
Food security continues to remain a critical challenge confronting humanity. Worldwide, the demand for food products is extensively increasing. Today, the research of generating transgenic organisms is one of the fastest-developing biotechnology fields, as it promises sustainability of agriculture and aquaculture. Many of the animals observed today, including top-quality agricultural breeds such as Holstein cows and Landrace swine, have undergone significant changes and are entirely distinct from their wild ancestors. The reason behind this is that they went through decades of selective breeding (SB) techniques, particularly crossbreeding and mutation breeding. Genome or gene editing (GE) refers to the process of targeted restructuring of a gene at a specific site through genetic engineering (GEN) technology. It ultimately leads to the construction of 'custom-made animals' with better hereditary features and amendments. It effectively imitates the natural methods that form the origin of SB programs. However, farmers' attempts to provide stable productivity and sustainability using traditional breeding proved difficult, inefficient, and time-consuming. So, there is a pressing need for novel strategies for efficient animal breeding today. Compared to conventional breeding techniques, GE is receiving considerable attention, since it can efficiently adjust the target gene to get a GEN animal with desired traits. Developments in animal breeding methods are ongoing, and as each innovation is made, it potentially offers a range of opportunities. The prospects vary between providing targeted production traits such as yield and nutritional profile, improving disease resistance, and expanding animal welfare. This article gives an overview of GE techniques used in breeding livestock models, birds, and fishes. It concludes that applications of GE to improve the traits are the present and future of innovation in animal production. The limitations and future of the breeding process and the development of GE technology in farm animal breeding are also addressed.

Keywords:
animal breeding; farm animals; gene editing; genetic engineering; genomics; livestock

1. Introduction

The United Nations predicted that the world population will grow to 9.7 billion by 2050 and reach 11.2 billion in 2100 [1]. According to the Food and Agriculture Organization (FAO) of the United Nations [2], the need for more or better animal proteins such as (eggs, meat, milk, etc.) is expected to rise to 1,800 million tons while the population increases [3]. Significant threats to animal production practices are added by the destructive impacts of climate change, increased urbanization, and political conflicts [4,5]. The cumulative effect of these developments in agriculture and food production is anticipated to be between 800 billion and 1.2 trillion over the next 20 years, accounting for about 36 percent of the total potential impact [6,7]. Given all this, the global demand for food security will substantially grow as well. One potential solution to this problem is the development of sustainable aquaculture and agricultural resources. In addition, there is an increasing focus on creating substitute, such as lab-grown

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meat, to relieve environmental pressures from traditional livestock and seafood production [7].

Aquaculture produced from fisheries for human use is the quickest-growing food-making sector and promptly becomes a principal element for international seafood security because of the elevated animal protein in human nutrition [2,8]. In contrast to land-based agriculture, which competes with habitable land areas, aquaculture has the advantage of having enormous, accessible ocean areas. Another benefit of fish farms is that they have a higher adequate feed conversion level than other food production methods [2].

Traditionally, scientists have used two significant strategies i.e., directed selection breeding and crossbreeding, to develop key traits of fish, poultry, and livestock [9]. However, these approaches could not provide an effective solution, mainly due to the inherent drawbacks, such as slow genetic progress, animals' long cycle times, and prediction complexity [10] (Table 1). The classical methods have been incapable of introducing or improving high-quality genes rapidly and precisely without introducing undesirable genes. Some bad genes include those that would result in trait deficiencies in animals, such as low growth, infertility, susceptibility, etc.) [11]. For example, selective breeding for over 60 years has produced high-quality meat. However, it has also caused the buildup of recessive mutations that brought about genetic diseases [12]. Moreover, traditional strategies were inadequate for breeding certain traits, such as disease resistance. Resistance to numerous diseases is a complex trait controlled by more than one gene, making conventional genetic selection for disease-resistant breeding much more expensive, time-consuming, and inefficient [13].

To get out of the bottleneck and the associated technical problems, traditional breeding methods such as artificial selection and multi-generation hybridization have been replaced by more precise genome editing/gene editing (GE) breeding techniques. Genetic engineering covers a broad range of techniques that enable targeted modifications in an organism's DNA (gene recombination). Using GE approaches, targeted mutations can be achieved in particular genes, and new lines of animals with important phenotypes can be produced. Gene editing technology has been deployed to create more innovative solutions for expanding the genetic improvement of fish and livestock, thereby promoting disease resistance in bred animals.

Thanks to gene editing methods such as zinc-finger endonuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR), significant advancements have been made in genetic modification. Later, other GE methodologies, such as base editor (BE) and prime editor (PE), were introduced into the field, but they are not widely used and are being tested and evaluated.

Research continues on the GE tools to enhance the effectiveness of targeted animal modifications, increasing the number of available GEN animal models. Figure 1 presents some of the milestones in animal breeding. The recent development of GE, along with the animal production technologies, gives the potential for speeding up successful animal genetic development. This development includes modification of production traits, disease resistance breeding, and improvement of animal health [14]. Despite the many profits of GE breeding, several concerns remain regarding the bioethics and biosafety of transgenic foods that carry foreign genes [15,16].

Embryonic stem cells (ESCs) can be grown in vitro for many passages and transformed with transgene constructs, causing genome modifications. The constructs used allow the selection of effectively transformed cells and permit gene targeting to be achieved. Consequently, genes can be precisely introduced, replaced, or deleted (so-called knock-ins (KIs) and knock-outs (KOs), respectively [17]. The injection of genetically modified embryonic stem cells (ESCs) into blastocysts, primarily through gene targeting, results in various genetic modifications. Many laboratories globally have tried to construct ESC from farm animals for several years. However, no robust and reproducible procedure has been published in this regard. Even in mice, the assembly of ESC is an expensive and labor-intensive technology [18]. Among farmed animals, the ability to engineer the genome has been restricted in livestock species. This restriction is due, in part, to the shortage of stable ESC that can give the germline [19]. As the actual embryonic stem cells (ESCs) capable of donating the germline have not been fully characterized in livestock species, their use remains limited. The expertise in targeted genetic modification of somatic cell nuclear transfer (SCNT) [20] permitted the generation of genetically engineered livestock. However, although this was possible, the efficacy of producing GEN animals using this route was poor; thus, only selective GEN models have been constructed [21].

Genetic engineering in agriculture refers to actively producing valuable plants or animals. Agricultural production implies cultivating crops and breeding animals for food or other resources, which are then distributed to markets for public use. Like plants and animal modification, attempts to breed plants and animals started hundreds of years before people recognized what DNA was. Genome editing technologies offer scientists a rapid and precise method to modify definite features of plants and animals, letting agriculture remain sustainable and productive in the face of a fluctuating climate and increasing world pop-

2025, Vol. 1, Article ID. 2025.0002
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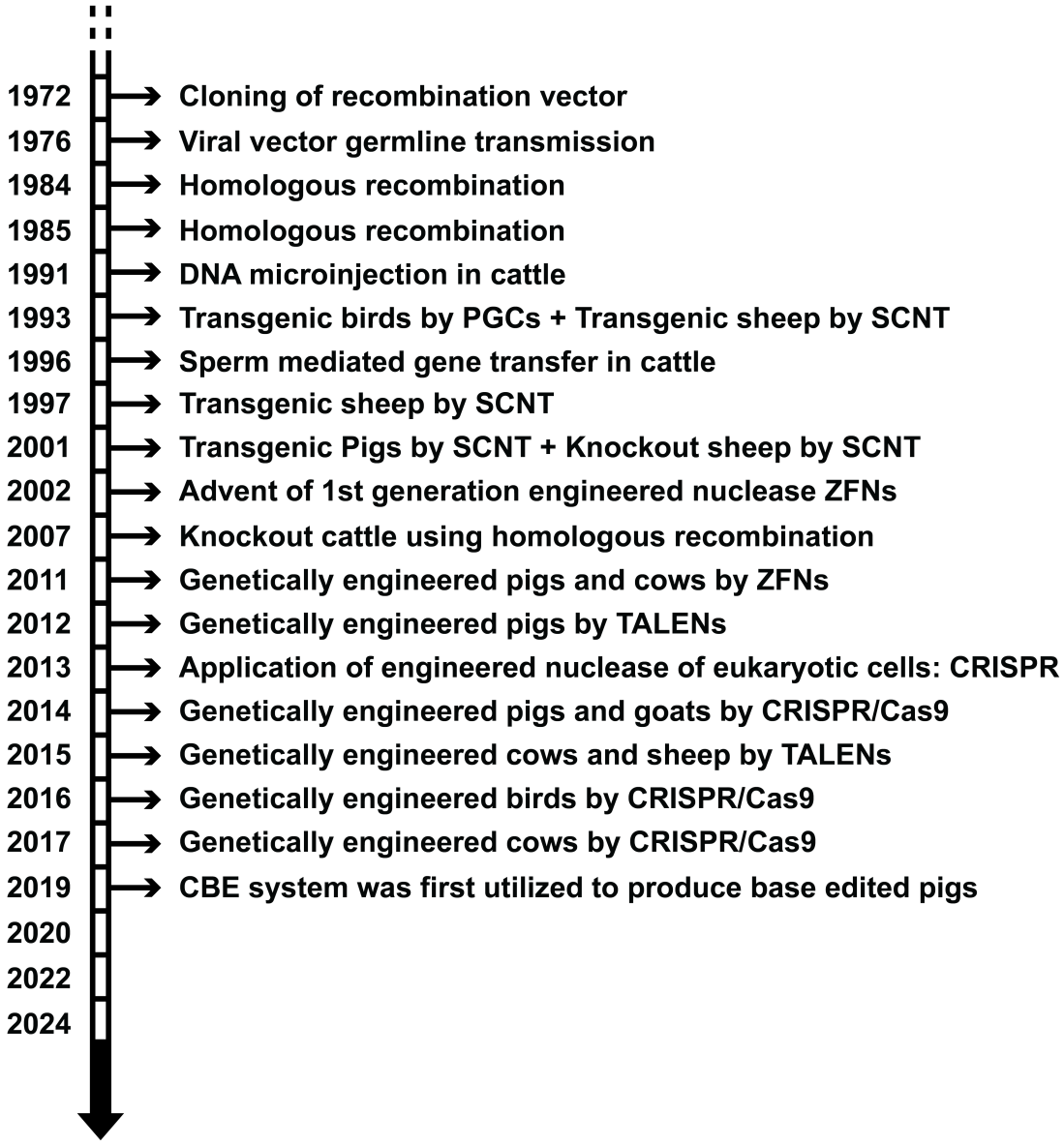

Figure 1: Time scale-based evolutionary landmarks in constructing genetically engineered animals and the significant roles of advancement of genome editing systems. CRISPR: Clustered regularly interspaced short palindromic repeats; CBE: Cytosine base editor; PGCs: Primordial germ cells; SCNT: Somatic cell nuclear transfer; TALENs: Transcription activator-like effector nuclease; ZFNs: Zinc-finger endonucleases. Source: [3,19,22–24].

ulation pressure [25]. These technologies significantly impact agricultural farming since they can breed plant and animal lines with valuable traits that will display new settings, boost yields, generate higher-quality food, and deliver items for new markets. The applications of GE strategies, particularly TALENs, in several plant species, have been discussed in a previous chapter [26]. A brief comparison of genome editing (GE) approaches in plants and animals reveals that the methodology for genome editing in farm animals differs significantly from that in plants [27]. This difference is partly due to biological variations between the two kingdoms, like the mode of reproduction (for instance, plants can self-pollinate) and the number of offspring per reproduction cycle. Regarding GE traits, one transgenic plant transformation can re-develop from somatic cells, while animals can only grow from germline cells. Another significant difference between plant and animal breeding is that plant breeders focus on developing a distinguishable plant variety, often secured by plant breeders' rights. In contrast, animal breeders focus choice

on elite animals in the breeding scheme, with millions of genetically discrete individuals produced primarily by the multiplication of elite genetics via outcrossing [23].

The applications of GE technologies for livestock production offer huge promises. They are very diverse, varying between providing targeted production traits such as yield and nutritional profile, improving disease resistance, and expanding animal welfare. However, it is difficult to construct specific gene-targeted livestock because of technical difficulties and the long generation time of large animals. It also raises serious safety concerns related to the expansion and use of genetically modified products in contrast to conventional breeding, which is envisioned as safe [28,29].

There is an ongoing debate on the potential of GE technologies and their association with other methods in agriculture for developing food security. A Genetically Modified Organism (GMO) is one in which the genetic material has been changed in a way that does not happen naturally by mating and/or recombination. In contrast, a "Living Modified Organism" (LMO) does not contain foreign genes and is created through natural mutations or conventional breeding programs without human interference. The established international regulatory courses typically exclude genome-edited plants and animals, using novel biotechnological techniques, from GMO/LMO classification [29,30]. Accordingly, organisms generated by the GE techniques are excluded from the GMOs. Concerns have also been voiced over transparency for consumers. So far, consumers have expressed very little interest in genetically engineered products. For instance, in Canada, the demand for transgenic salmon, which is expected to grow faster and be commercially viable, was so limited that the economic viability of AquaBounty (the company behind it) is at risk [23]. Several reasons for the slow progress in this formerly promising field include, the lack of public research financial support, regulatory hurdles, and concern about public opinion [23]. Added to the safety concerns are the raised ethical worries and welfare issues focused on animal breeding, especially in vertebrates, because hundreds of genetic transformation experiments are conducted in living embryos, causing the loss of embryos during impregnation and pregnancy [31]. In this context, it is worth mentioning that this type of research is costly. It is also true that both public sector and private funding is scanty, as compared with applied and basic biomedical research.

Several review articles about the obstacles to the widespread adoption of GE food animals have appeared in the published literature. The present review does not reiterate these matters but focuses on some of the new applications of GE. Also, it does not address genetically engineered farm animals developed for biomedical research applications. A new animal drug or nonfood species is reared and utilized in contained and controlled conditions, such as GE laboratory animals employed in research institutions.

2. Genome/Gene Editing Technologies

2.1. Past Technologies

Gene engineering techniques refer to processes that involve overexpressing or inhibiting a unique gene. It was reported that recombinant gene cloning was successfully achieved in 1972 (Figure 1) [3]. The first attempts at generating transgenic mice were carried out during the 1970s by exogenous gene transfer and germline transmission into a fertilized embryo (Figure 1) [32]. However, gene modification through viruses had disadvantages, including experimental safety concerns, undesired incorporation into the host genome, and possible oncogenic effects. The other methods of DNA delivery are predominantly microinjection and electroporation (Figure 2) [33–35].

To improve GEN technologies, HR was used in the year 1984, for more precise gene insertion. With HR, nucleotide sequences are switched between similar or identical DNA molecules. The SCNT [36] or microinjection (MI) (Figure 2) techniques is used to initiate the genome modifications for the construction of genetically engineered livestock. In an HR method, exogenous genes have to be transferred into somatic cells or in vivo fertilized embryos. Homologous recombination occurs when cells divide, and a cycle of checking processes is required over a long time for the selection of gene-engineered cell or embryo [37]. The technically complicated process of constructing GM animals involves three critical steps at early embryonic stages: isolation of zygotes from pregnant females, transferring nuclei into the separated zygotes, and subsequently implanting the modified embryos into pseudo-pregnant female recipients to obtain viable progeny.

Traditional breeding methods are based on phenotypic information. Unfortunately, long-term selection led to cell senescence and death in embryos and somatic cells [38]. Therefore, it wasn't easy to efficiently generate genetically engineered livestock animals in the 1990s [39]. Research on animal production with the required trait through SCNT concentrates on animal production by constantly applying GE tools to SCNT, since it can decrease the aging of somatic cells [3]. The following paragraphs give descriptive examples from each corresponding field.

2025, Vol. 1, Article ID. 2025.0002
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Table 1: A brief comparison between the significant available conventional and existing GE strategies used in animal breeding.

Aspect	Conventional Breeding Strategies	Gene editing Existing Strategies
Genetic change	Selects desirable traits that have occurred by natural mutation	Deliberate development of characteristics that are difficult to achieve by traditional breeding methods.
Major strategies in breeding	Directed selection breeding and crossbreeding	Gene editing: Knock-in/ knock out particular gene(s)
Insertion of the desired gene in the genome	Random at multiple sites	Site-specific and can target single and multiple sites
Genetic consequences	The process is slow and may result in the loss of many other existing desirable traits.	Fast and specific for removing undesirable genes
Off-target modifications	high	Less
Risk of introducing bad genetic traits like mosaicism and disease resistance	High	Less
Long-term risk of random insertion of undesired exogenous genes	High	Less risky, but not fully known
Possible threats to animal welfare	unlikely	higher
Efficiency at modifying the DNA of cells	Low and Poor	Much Higher
Time required to choose and stabilize genetic traits in a population	Long: Slow and requires more than one generation	Short: Fast and requires only one generation
Incidence of stillborn female	High	Not existing
Cost of breeding	Expensive	Less cost
Safety concerns about transgenic foods	Safe	Questions remain

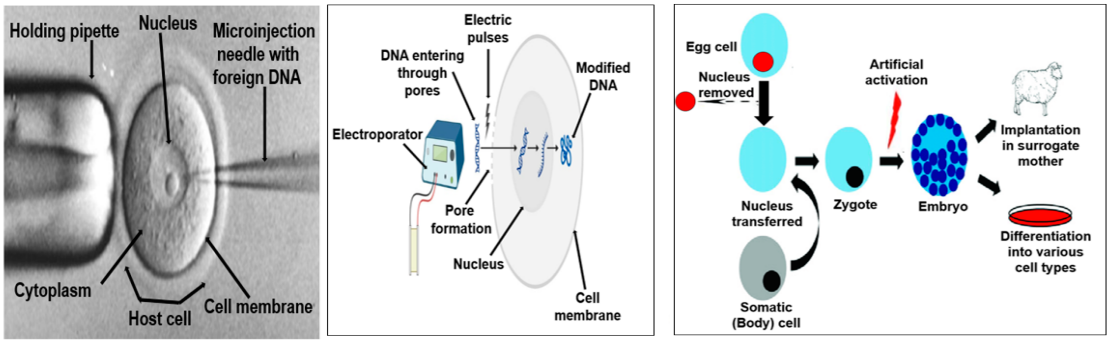

Figure 2: The main cytological techniques used to deliver nucleases for livestock genome modification are: (1) cytoplasmic microinjection, (2) electroporation, and (3) somatic cell nuclear transfer, regardless of the genome editing technology applied.

Before the advancement of SCNT in livestock in 1997, most genetically engineered animals were generated mainly by MI of DNA into the cytoplasm of the pro-nuclei of embryos (zygotes) (Figure 1). The first transgenic large animals were produced in 1985 by MI to improve the growth of agricultural livestock, and this allowed the successful production of genetically modified animals, including rabbits, goats, sheep, pigs, and cattle [40]. The initial stages of GE are followed by embryo transfer into a recipient. Direct DNA MI was the first practice that led to regular and relatively easy success in mammals [41]. The first GEN farm animals were constructed by MI of the genes of interest into the pronucleus of zygotes [42]. Microinjection was the first practical method for the generation of trans-

genic animals through the injection of an extraneous DNA construct into a fertilized ovum [43]. The construct is randomly incorporated into the recipient oocyte's genome. Following this, the zygote resumes embryonic development and the embryo is translocated to a foster mother and ultimately grows into a transgenic animal [44].

The transgenic recombinant DNA (rDNA) integration levels were low, 10.4% and 1.3% in pigs and sheep, respectively, an issue that has continued to be problematic for livestock. Also, less than 1% of embryos are injected, and about 12% of farm animals are transgenic [39]. In addition, MI was unsuitable for use on farm animals, due to long gestation periods, small litter sizes, and reduced transgene integration rates. Furthermore, multiple transgene copies are integrated at random, since genes can only be added, not replaced or deleted. These conditions hinder the correct control of gene expression and interfere with endogenous gene function. Thus, large amounts of oocytes are needed to be injected because the overall efficacy of the process is very low [45].

In invertebrates [46] and lower vertebrates [46,47], pronuclei are not observable genes. Therefore, MI must be executed in the cytoplasm, using more significant quantities of DNA. For unclear reasons, the success rate of this method is quite variable depending on the species. Microinjection into a fertilized egg is the preferred method for GE in fish [46,47]. However, GE can also be made on fish cell lines. Genome editing utilizing an electric pulse to transfer the protein tools that execute the GE into cell lines from some fish species, including Atlantic salmon (*Salmo salary*), rainbow trout (*Oncorhynchus mykiss*), and Chinook salmon (*Oncorhynchus tshawytscha*) has been indicated to be highly successful [48,49]. Further, microinjection remains ineffective in the laboratory fish medaka, *Xenopus*, and chicken. As previously reported [50], the extraneous DNA usually does not incorporate into the genome of these species.

Initially, transgenesis via pronuclear MI suffered substantial limitations since targeted disruption was not promising using these approaches [20,51]. With MI, mosaicism is possible [52], which occurs when an organism has two or more genetically different cell populations, due to a genetic mutation that did not influence all the cells [53]. Thus, the desired genetic change is not found in all cells of GEN animals produced using zygote MI. Not every copy of the genome in every cell is modified; rather, only certain copies of the genome are altered. Therefore, animals resulting from modified embryos don't generally have 100% altered cells. Some cells have changed DNA, and others have unaltered DNA. Mosaic animals may express the desired trait; however, whether or not they can pass it to their progeny depends on whether their sperm or egg cells possess the DNA changes. Mosaicism is particularly problematic in large livestock species having long reproductive cycles, as it can take years to generate non-mosaic homozygous progeny through crossbreeding [35]. Creating the desired phenotype coupled with germline transmission could require generating some transgenic founder lines, which will ensue by breeding. Mosaicism can prevent the organism from developing distinct observable traits and present challenges for breeding the wanted genetic alteration [19]. Furthermore, pronuclear injection could not regulate the integration site or the number of copies of the integrated genes.

The most profound restriction was that DNA can only be added but neither deleted nor modified in situ. In addition, the incorporation of exogenic DNA was random, resulting in erratic transgene expression due to the integration site effect. Moreover, random integration into the genome poses a danger of disrupting critical endogenous DNA sequences or stimulating cellular oncogenes, which could potentially harm the animal's health [35].

Additionally, despite the widespread use of the MI method in the generation of edited animal models, it requires expensive micromanipulation equipment and expert personnel. This requirement introduces operator-dependent experimental differences as a confounding factor in editing experiments [54]. Moreover, it is time-consuming, which limits the number of microinjections that can be performed in each attempt [55].

To increase the efficacy of MI, lentivirus or retrovirus was inserted into a zygote, revealing higher efficiency than DNA injection. GEN has become considerably more efficient and precise since the emergence of GE tools such as ZFNs, TALENs, and CRISPR/Cas9. Furthermore, MI's efficiency in constructing genetically engineered animals has significantly improved as well [56,57]. With the introduction of the ribonucleoprotein (RNP) complex in the CRISPR/Cas9 structure, electroporation, one of the most usual techniques for RNP delivery, has become feasible [5,58].

To deliver DNA sequences more significant than four kilobases, viruses carrying the DNA may be inserted in the electroporation technique, which utilizes an electric pulse to make pores in the fertilized egg [59], across which the DNA carrying the GE tools can pass [60–62]. The 'foreign' viral DNA integrates into the genome at minor frequencies (~0.1%), though its relative incidence within the developing egg cell drops with each round of cell division [63,64]. The electroporation is appropriate for inducing indel mutations, large deletions, and small insertions [65]. Consequently, different kinds of gene-engineered animals have been generated by GE tools, leading to improved productivity, disease resistance, and

2025, Vol. 1, Article ID. 2025.0002
https://doi.org/10.69709/SustainFoodConn.2025.106617

biomedicine improvement. Electroporation is an efficient method that has permitted the generation of gene-engineered animals, with an easier and faster approach than the previously laborious and expensive MI technique [5,54,65]. It can be concurrently applied to many zygotes and only requires a stereomicroscope, electroporator, and fusion chamber [54]. This approach allows for a more significant number of oocytes or zygotes per female receiver at a time and, finally, to achieve live offspring with targeted gene modifications [54,66]. Combining the mass production of In vitro fertilization (IVF) embryos with the GE by electroporation of gRNA/Cas9 RNPs, Cas9 mRNA, and gRNA strategy compensates for poor IVF outcomes with high editing frequency [54].

To overcome mosaicism and low efficacy associated with MI, SCNT was introduced and successfully used to generate cloned livestock. Before the advance of GE tools (ZFNs, TALENs, and CRISPR/Cas9), SCNT was favored over MI [5]. Since the 2000s, SCNT or cloning (Figure 2) has been the most widely used strategy for breeding genetically engineered or genome-edited farm animals like pigs [67], cows [67,68], and goats [69]. In addition, SCNT has been employed in generating genetically engineered animals with genetically engineered somatic cells [3,70]. This method is a protocol in which the nucleus of a somatic cell with a desired mutation is moved to the cytoplasm of an enucleated metaphase II stage oocyte, resulting in a new animal genetically identical to the somatic cell donor [71].

The production of GEN animals using site-specific modifications is possible at present. The SCNT technology was further found for other vital livestock species (Figure 1): cattle [72], goats [73], pigs [74,75], and sheep [17,76], offering the first cell-mediated stage for livestock GEN. The strategy is performed by injecting an exogenous gene and the targeted interruption of an endogenous gene [77], known to cause hyperacute refusal after organ transplantation. Many other GEN livestock models have been described [67,77].

Manipulating the preferred gene in somatic cells (usually fetal fibroblasts) is easier and more accurate than in embryos. To verify that the wanted genetic changes are present in the cells, single-cell-derived colonies are separated and checked for the presence of these changes. Next, the cells are donated for SCNT [67,76,78]. The genetic modifications hosted in the genome of somatic cells should be performed over to offspring by SCNT involving targeted modifications. Compared with the zygote manipulation method for GEN animal production, SCNT has several advantages, including a simple selection of donor cells with the wanted gene or mutation. Furthermore, since the whole animal originated from a single GEN donor nucleus, the risk of mosaicism is eliminated [75]. This cell-mediated GE strategy enables researchers to verify that the gene-edited cells contain the desired genetic modification before implantation in recipient mothers, ensuring successful animal production. GEN animals created by SCNT often need fewer recipient animals than the number of animals required for the zygote micromanipulations [76]. This approach reduces the occurrence of genetic mosaicism, reduces the time frame for generating the sought genotype, and decreases the overall cost of animal production [12]. Still, this approach is more technically demanding and typically has little incidence of term development. Due to low efficiency, only a few GEN animal models are registered. Since targeted modifications in somatic cells depend on the incidence of endogenic HR, their efficacy in these cells is extremely low [67,77]. The reduced effectiveness causes modifications of only one out of two alleles at a time. Since only heterozygous mutations can be established, breeding steps are necessary to produce genetically engineered (GEN) animals with homozygous modifications. The prolonged gestation period of some livestock species, particularly the large animals, adds days needed for finalizing breeding steps. The low rate of HR obligated the use of a selectable indicator, for instance, Neomycin, to select somatic cells holding the correct genetic modification [21,67,77].

In addition, potential cloning-associated epigenetic changes might add to the GEN animal phenotype. Thus, GEN animals generated through this procedure often carry a foreign gene that can improve antibiotic resistance in the animals. Despite these limitations, SCNT remains the primary method for constructing the KI gene-edited livestock [78]. Moreover, about 50% of the published KO farm animals were generated by SCNT.

Currently, SCNT allows gene KO and gene KI generation in farm animals [35]. The SCNT technology is being used to develop the poor fertility of buffalo, which are principal livestock in many countries. Although SCNT cloning of GE cells solved some of the problems associated with zygote micromanipulation, the low efficiency of generating live and healthy cloned progeny has limited the usefulness of this approach to produce GE livestock [79].

Moreover, the productivity of SCNT is poor [80], and animals born through SCNT often experience a high incidence of health problems such as abortions, mortality, and both pre- and post-birth abnormalities, raising animal welfare concerns [80–82]. The SCNT procedure is inefficient due to two main problems. Firstly, effective implantation has a set of logistical challenges. Specifically, foster mothers have to be biologically prepared for implantation [83]. Nevertheless, the success rate for implanting a fertilized egg or embryo is significantly lower than that

of natural fertilization through mating. Second, the minimal levels of DNA methylation and elevated expression of the *Xist* gene in transgenic cloned calves. These conditions increase the incidence of stillborn female cloned buffalo relative to naturally breeding female buffaloes [84]. It has been reported that siRNA-mediated knock-down of the *Xist* gene successfully inhibits gene expression and stimulates the development of SCNT-cloned female buffalo embryos [45]. The possibility of GM animals being generated by SCNT was one of the powerful forces behind its development. The CD163 and CD1D KO transgenic pigs were produced using both SCNT and cytoplasmic injection into zygotes in vitro [85]. The targeting efficiency in both cases was 100%. Nevertheless, several technical hurdles limit the application of this method. First, the efficacy of cloning is very low in almost all kinds of farm animals (0.5–1.0%). Second, SCNT is accompanied by side effects such as aberrant reprogramming during embryogenesis and other developmental abnormalities in extraembryonic tissues of the produced animals, such as the placenta [86]. These abnormalities result in a low pregnancy frequency and higher instances of sudden death following delivery. In addition, anomalies such as obesity, immunodeficiency, and defects in the respiratory system are also seen in cloned animals after birth [87,88]. Therefore, the application of SCNT in agriculture has lagged primarily because of concerns about the time-consuming production process and the potential public criticism against the consumption of livestock, containing transgenes in the food industry. Several attempts have been made to improve SCNT efficiency, including addressing epigenetic factors to control reprogramming challenges. Two studies were devoted to injecting epigenetic factors throughout embryo development [89]. Yet, the aforementioned matters have not been fully resolved to date, and SCNT still has a reduced implantation rate compared to IVF, as reported lately [3].

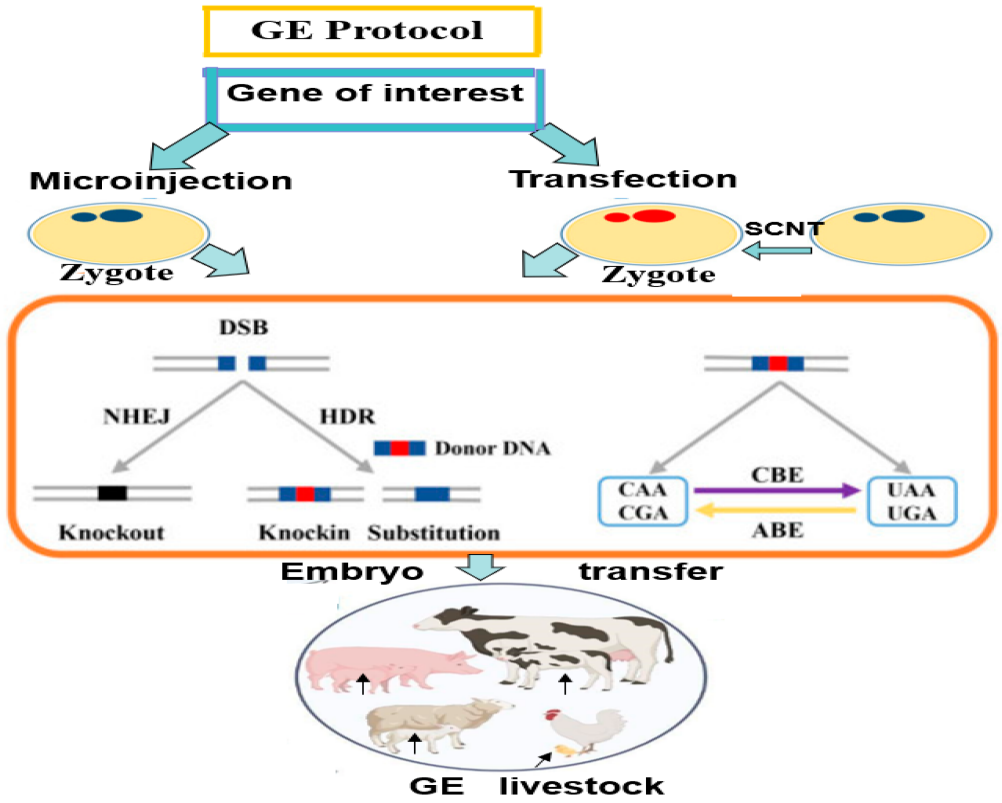

Figure 3: Schematic summary of combining genome editing and either zygote microinjection or "cloning" ('somatic cell nuclear transfer') or for production of livestock animals for different applications protocols including ZFNs, TALENs, CRISPR, and BE. ABE: adenine base editors; CBE: cytosine base editors; CRISPR: Clustered regularly interspaced short palindromic repeats; DSB: double-stranded break; GE: gene editing; HDR: homologous directed repair; NHEJ: non-homologous end junctions; SCNT: somatic cell nuclear transfer; TALENs: Transcription activator-like effector nuclease; ZFNs: Zinc-finger endonucleases. Adopted from: [35,90].

2025, Vol. 1, Article ID. 2025.0002
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Figure 3 outlines a protocol combining access at the embryo stage with genetic tools for producing genetically engineered livestock. SCNT necessitates the fusion of an enucleated egg cell acquired from the egg cell donor and a somatic cell resulting from the nucleus donor. Before this step, an exogenous gene is delivered to the somatic cell stage using methods such as virus, MI, or electroporation to produce a gene-engineered cell using one of the available techniques. After that, the developed engineered embryos are transferred to a foster mother to generate gene-engineered livestock.

2.2. Present Technologies

Over the past hundreds of years, traditional methods for selecting desired traits have proved to be slow, inaccurate, and insufficient to meet the rapidly growing global population demands. Without established ESC lines, SCNT had to be utilized to generate numerous genetically engineered livestock models. With the GEN by SCNT, genetically engineered livestock models could be produced by bringing site-specific modifications.

Although successful, only a few genetically modified livestock models have been developed due to low productivity and associated congenital disabilities. Owing to their precision, efficiency, and versatility, the modern GE systems ZFN, TALEN, CRISPR-Cas9, BE, and PE have emerged as cutting-edge biotechnological approaches [91] in animal research and sustainable food security. Compared to conventional breeding techniques (Table 1), notably crossbreeding and mutation breeding, these tools markedly decrease the time and effort required to establish a GEN animal. The emerging technological innovations in GE systems are "ideal" approaches that have improved efficiency in creating GE livestock models, altering a genomic sequence, and presenting higher DNA sequence precision with low or no off-target effects. Another advantage of using GE techniques is the application of direct inoculation into developing embryos to cause targeted mutations, consequently excluding side effects associated with SCNT.

Conventional genetic modification methods put genes at multiple and random locations in the genome, demanding researchers to decide which insertion is the most beneficial [92]. The application of conventional technologies to livestock breeding depends on the positive and negative selection of the favorite traits, and it usually needs generations to choose and stabilize genetic characteristics in a population. In contrast, the relatively modern technologies of GE offer a one-step generation of an animal with predetermined genetic and phenotypic characteristics based on the knowledge of gene functions [35,64]. The past GE techniques were developed by randomly inserting the desired gene into the host genome. Technically, random insertion has several possible disadvantages [93]. First, the targeted host gene integration site can only be identified after examining the transgenic animals by polymerase chain reaction (PCR) and DNA sequencing. Second, gene insertion can change the expression of endogenous genes. Third, several gene copies can be incorporated into the host genome. The random modification of the genome was ineffective and costly for animal breeding. Moreover, there were safety concerns about malfunctioning original genes or influences on the regulation of gene expression [93].

Due to their ability to precisely edit target loci, modern breeding techniques result in faster and more efficient livestock improvements than traditional breeding methods. However, the long-term consequences of GE are not fully known, and there are concerns that it may threaten animal welfare despite being less risky than classical GEN methods [5].

Present GE tools enable quick investigation of first-class traits, such as productivity and resilience to diseases and climate changes, without minor issues. The following sections discuss the basic principles of these present GE strategies and the major insights into their recent applications and achievements.

Basic Principles

The early gene targeting experiments relied on the spontaneous integration of exogenous DNA into the genome. However, the rate of successful genetic modification using this procedure is very low (1 in 1000,000 sites and 1 in 10,000 cells). As a new GEN technology, GE can precisely identify and adjust a definite sequence of a specific gene in an organism's genome. The precision of these systems originates from the ability to identify particular DNA sequences on the genome and induce site-specific DSBs [94]. The DNA breaks lead to activation of the intrinsic DNA repair process to fix the DSBs since the DNA breaks may cause cell death if not corrected. The insertion of site-specific genomic modification is simplified by two major DNA repair pathways: NHEJ or HDR (Figure 4).

The error-prone nature of the NHEJ mechanism causes random base insertions or deletions (indels) and deletions of nucleotides at the sites of DSBs. This mechanism leads to gene KO as it often produces a frameshift of the amino acid codons and the development of a premature stop codon. On the other hand, if a donor DNA with homologous sequences to the target genome site is present, definite modifications at the nucleotide level can be intro-

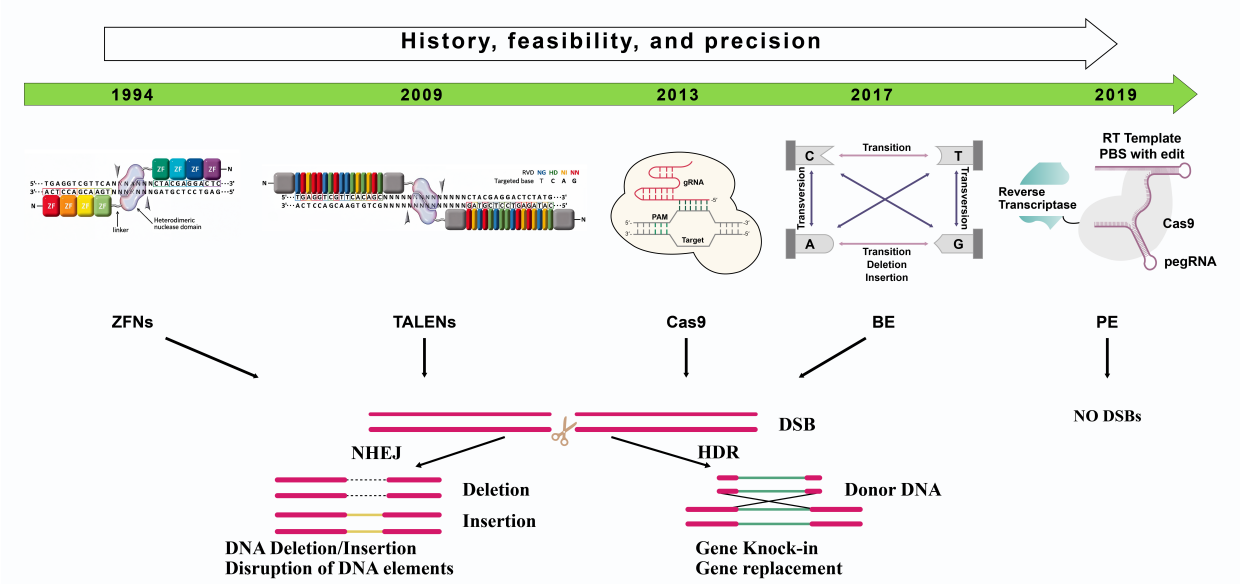

Figure 4: Schematic illustration of the mechanism of the present-day genome editing techniques: ZFN: Zinc finger nuclease; TALENs: Transcription activator-like effector nucleases; CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/ CRISPR associated protein system 9; BE: Base editing; PE: Prime editing. The first four strategies edit target DNA sites by a similar mechanism. The nuclease component of these systems induces DSBs, which are recognized by the cellular intrinsic DNA repair pathways. Two principal repair mechanisms are possible: Homologous recombination (HR) and nonhomologous end joining (NHEJ). The latter frequently creates small insertions and/or deletions (indels) that result in frameshift mutations and disturb the function of genes. Prime editors comprise a Cas9 nickase domain fused to a reverse transcriptase domain. Compared to Cas nucleases or BEs, PEs have unique advantages: they do not need DSBs or donor templates of DNA for repair. Therefore, PEs offer high editing purity and target specificity. Adapted from: [24,95,96].

duced throughout the HDR pathway [97], In this process, the cell utilizes the sister chromatid of the injured DNA as a template for reparation. By integrating a designed donor template, particular modifications can be carried out at the DSB site through the HDR pathway [98]. In general, most cell types have seen a lower incidence of HDR than NHEJ. Whether the cells follow the NHEJ or HDR mechanism depends on the cell cycle, the make-up of the ends, and end resection [99].

A new BE platform, a C-to-G base editor (CGBE1), has been introduced [100]. The new system can distinguish specific sequences on the genome and initiate site-specific DNA breaks. This platform is the first CRISPR C-to-G base editor to present a transversion mutation (C→G) without a DSB. The latest GE tool is the PE, one of the most precise approaches for point mutation [101]. This novel protocol of PE has been designed to introduce point mutations with no donor DNA template for the HDR track or even the presence of a DSB in the target sequence (Table 2 and Figure 4).

Table 2 compares and summarizes the advantages and disadvantages of the GE techniques available for application in livestock research. The CRISPR/Cas9 system has reached unprecedented levels of efficacy for gene inactivation in almost all species. Notably, it enables the concurrent inactivation of multiple genes by utilizing more than a single target-specific guide RNA.

Through sequential nuclear transfer, one allele of gene A can be inactivated, followed by somatic cell nuclear transfer (SCNT) and re-isolation of somatic cells.

Table 2: Differences between advantages and disadvantages of ZFN, TALEN, CRISPR–Cas9, BE and PE gene-editing technologies.

Characteristic	ZFN	TALEN	CRISPR–Cas9	BE	PE
Endonuclease	Fok I	Fok I	Cas9	dCas	pegRNA
DNA binding system	Protein–DNA	Protein–DNA	RNA–DNA	R NA-DNA-Protein	R NA-DNA-Protein
DNA binding determinant	Zinc finger Protein	TALE protein	CrRNA/sgRNA	dCas/nCas	nCas9/pegRNA

2025, Vol. 1, Article ID. 2025.0002
https://doi.org/10.69709/SustainFoodConn.2025.106617

Design and construction	Difficult	Moderate	Easy	Easy	Easy
Target fragment size/monomer	9–18 bp	14–20 bp	20–22 bp	4–8 bp	8–15 bp
Target recognition efficiency	High	High	High	Variable	Variable
Average mutation rate	Medium	Low	Low	Very high	Very high
Precision	Low	Moderate	Precise	More precise	Most precise
In vivo delivery	Easy	Difficult	Moderate	Difficult	Difficult
Multiplexing ability	No	No	Yes	*	*
Off-target effect	High	Lower	Variable	Low	Very low
Cytotoxicity	High	Lower	Low	*	*
Cost	Very high	High	Low	Very high	Very high
Cytotoxicity	High	Intermediate	Low	*	*
Time needed	Time-consuming	Time-consuming	Less time needed	Time-saving	Time-saving
Application	Not widely used	Widely used	Most widely used	*	*
Mechanism of action	DSBs with either NHEJ or HDR	DSBs with either NHEJ or HDR	DSBs with either NHEJ or HDR	DSBs with either NHEJ or HDR	No DSBs. Instead of replacing a base, it modifies the base chemical structure into another one

Abbreviations: BE: Base editor; bp: Base pair; CRISPR: Clustered regularly interspaced short palindromic repeats; Cas9: CRISPR associated protein system 9; CrRNA: CRISPR RNA; dCas: Catalytically inactive (dead) Cas; nCas: Nickase Cas; pegRNA: Prime editing guide RNA; PE: Prime editor; sgRNA: single-guide RNA; TALEN: transcription activator-like effector nucleases; ZFN: zinc finger nuclease. * Not evaluated. Source: [5,10,102–104].

Subsequently, the second allele of gene A can be targeted, followed by another round of SCNT and the isolation of cells to focus on gene B. If the genome-edited pig is produced by SCNT, followed by a simple selection of the edited cells by magnetic beads, it can score efficiencies of >90% for multi-gene inactivation [105]. CRISPR system was first applied to the mammalian genome in 2013 [106], and after that, it extended to a broad collection of cell lines and fish, bird, and mammalian groups, including livestock. This new GE system revolutionized the construction of GM animals. It enables researchers to modify genetic makeup that leads to improvements in livestock traits and construct more improved large animal models of human diseases. The pig recorded the maximum number of concurrently inactivated gene copies: 25 copies of porcine endogenic retroviruses [107].

Progresses in CRISPR/Cas9 GE have appreciably improved the ability to accurately disrupt genes and/or produce particular mutations by direct zygote handling (pronuclear or cytoplasmic injection or electroporation) [108,109]. Electroporation has been reported to be highly productive in creating indel mutations in bovine and porcine zygotes [110]. This method significantly simplifies the production of genetically engineered livestock, as it does not need micromanipulation expertise. Yet, genetic mosaicism remains a major problem using the zygote manipulation method [111].

As in the past GE techniques, which resulted in mosaicism [112], mosaicism occurs when DNA replication occurs before CRISPR-mediated genome editing, significantly reducing the possibilities for direct KO generation. The influence of mosaicism could be even more overwhelming if both somatic and germline mosaicism were found in the offspring. One of the strategies suggested for reducing genetic mosaicism is a transfer of CRISPR/Cas9 into either metaphase II (MII) oocyte or a very early zygote stage [113].

Nevertheless, the injection of CRISPR/Cas9 into MII oocytes did not decrease mosaicism compared to the zygote injection in sheep [114] and cattle [115]. The inability of CRISPR to identify its target site be-

fore some level of chromatin decondensation took place could cause these somewhat surprising results [12]. Another possible tactic for lowering mosaicism is shortening the longevity of Cas9 by speeding up its degradation [8]. This strategy can be achieved by marking Cas9 with ubiquitin-proteasomal degeneration signals mediating Cas9 degradation. Otherwise, the nuclear transfer technique using GEN cells could eradicate the threat of mosaicism. Recent studies showed that the microinjection of CRISPR/Cas9 constituents into swinish germinal vesicle oocytes efficiently reduces the risk of mosaicism [116]. About 83% of the mutated embryos were non-mosaic [116].

CRISPR/Cas9 system-mediated GE strategy has been used widely in studies involving the genetic breeding of animals. However, the system's efficacy at inserting foreign genes is insufficient, mainly due to specific off-target effects [104]. CRISPR)/Cas9 technology is unsuitable for use in the GE of large livestock. New target GE systems, BE systems, have been developed using CRISPR/Cas systems [90]. The first BEs were created in 2017 [117], and the BE technique was the first innovation in the GE field after CRISPR/Cas9, because of the ability to complete precise point-mutation without a DSB. Unlike traditional CRISPR/Cas systems, the new system does not depend on cellular repair, employing the NHEJ or HDR mechanism. In contrast to DSB-mediated mutation, BE-mediated mutation is more precisely controllable. The advent of the BE system offers a powerful tool for accurate GE.

The principle of the BE systems relies on combining a nucleotide deaminase and unnaturally modified nCas9 protein (D10A nickase) to replace bases at the target location by creating a single cut in the double-strand. The latter is a single-strand break (SSB) instead of a DSB, as the D10A mutation in the RuvC domain alters Cas9 into a DNA nickase [118]. Generating an SSB instead of a DSB escapes the formation of indels [100]. According to different base modification enzymes, the BE systems consist of cytosine base editors (CBE) and adenine base editors (ABE). These changes can achieve C–G to T–A and A–T to G–C edits, respectively [119].

The application of ABEs overcomes the constrictions of CBEs, which can only edit C or G bases, and gives a wider range of base transformation choices. Unlike CBEs, ABEs do not need to stop the activity of alkyl adenine DNA glycosylase (AAG) [120,121]. The optimized CBE system was first efficiently utilized to produce base-edited pigs to obtain larger animal models [122]. Subsequently, many research groups have deployed CBE and ABE to KO various mammalian genes, for example, GGTA, MSTN, CD163, GHR, and others, for the improvement of rabbit and pig embryos [123,124], and human cells [122,125,126]. These results also suggest that the BE systems can improve livestock traits like reproduction, milk, and wool production.

In 2019, PE was introduced as a tool that can achieve targeted editing, including all 12 possible combinations of base-to-base conversions, insertions, and deletions, without the need for the template of the donor genetic material [101,117]. While PE represents meaningful promising progress in GE, this technology is still in its early stages, and further research and investigations are needed to unlock its competencies and potential. Prime editing is a catalytically inactivated nCas9 (H840A) that is fused with a reverse transcriptase (RT-nCas9) with the capacity to be transfected along with a prime editing guide RNA (pegRNA). The molecular mechanism of PE involves identifying and attaching the primer-binding site (PBS) of the target to the 5′ region of the primer-editing guided RNA (PegRNA), thus exposing non-complementary strands. Cas9 nickase (nCas9) cuts the ssDNA complementary to the template RNA via the RuvC nuclease domain, attaching primers for RT enzymes to nCas9. The PegRNA serving as the gRNA identifies the target DNA to achieve base-to-base editing [127,128]. These new procedures have a low chance of missing their target, and no new functional alterations will occur. Furthermore, base editing (BE)-mediated gene editing is not reliant on double-strand breaks (DSBs) DSBs-induced NHEJ repair pathway is random and may cause unnecessary indels production. In addition, excessive DSBs cause cytotoxicity. On the other hand, researchers also are not required to screen for very active sgRNA and Cas nucleases. Also, the BE system does not need donor DNA, a crucial part of the HDR repair pathway. Designing practical donor DNA for livestock and delivering donor DNA effectively into livestock cells are practical challenges [129].

The PE has been practiced in mouse cells [mouse neuro-2a (N2a) cells], of which the prime editor 3 (PE3) facilitated base transversion at three target locations of Hoxd13 and androgen receptor genes with an effectiveness rate between 8% and 40% [124]. Additionally, zygote MI of pegRNAs, targeting the same Hoxd13 gene, resulted in successful conversion mutations. The conversions G-to-C and G-to-T were obtained in 44% and 75% of blastocysts, respectively, with mutation incidences varying from 1.1 to 18.5% in each embryo. Although PEs are notably versatile tools for precise GE and have distinct advantages, they show lower efficiency than other GE strategies [130].

2025, Vol. 1, Article ID. 2025.0002
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3. Applications of Genome Editing in Animals

In agriculture, GE is used to improve the characteristics of crops and livestock, such as enhancing their resistance to pests and diseases or improving their nutrient content. Genetic development of economically important traits is a key topic of livestock research. The continued development and refinement of GE technologies are anticipated to have a profound impact on various industries, with the potential to drive significant breakthroughs across multiple fields in agriculture. The following studies have been carried out to increase production based on farm animal species (Figure 5).

3.1. Gene Editing in Farm Animals

Breeding farm animals has become a primary focus in the field of animal breeding. In the last 10 years, GE technology has been used to breed various farm animals, including fish, chickens, rabbits, pigs, cattle, sheep, goats, and other species [10,90]. Genome editing technologies hold meaningful promise for reforming agriculture by allowing precise and effective genetic modifications in livestock. Using Cas9 to insert favorable genes into animals accurately, scientists can support the industry's healthy and effective development, cut economic losses, and guarantee high food safety standards. Recent investigations suggest that the GE systems can improve effectively animal traits like growth and reproduction as well as production of egg, milk, and wool [56,131,132].

In aquaculture species, the genes associated with main traits, such as growth, disease resistance, and robustness, are still very inadequate, which impedes the CRISPR/ Cas applications in this field [9]. CRISPR/Cas assembly needs to be transported into fertilized eggs at the one-cell stage in fish [47,133]. The time required for the progress of the zygote to first cell division differs from one fish species to another; thus, it is essential to know this timing for successful GE. Microinjection of many eggs quickly is labor intensive and requires an expensive specific MI platform. Additionally, access to newly fertilized embryos is difficult for species such as certain shrimp. Consequently, the substitute methods of CRISPR/Cas9 delivery have to be advanced and examined for breeding purposes, for instance, electroporation, the MI of unfertilized ova, sperm-mediated transfer, or editing PGCs [9].

3.1.1. Breeding of Improved Characteristics

Table 3 shows examples of GE applications for fish, poultry, and livestock production trait perfections. The main interest fields covered under this umbrella include reproductive performance, egg and meat production, and improvements in milk quality. However, it should be remembered that the full KO of a gene that negatively controls growth may lead to welfare worries. These concerns involve miscarriage, difficulties during birth, developmental abnormalities, or even offspring mortality, as observed in pigs [134], as well as in cattle [135], goats [136], and rabbits [136]. By contrast, spontaneous changes in this gene that have happened naturally are not substantially associated with such extreme alterations in muscle mass. Avoiding this using GE will likely need more accurate mutations in this gene [137].

Breeding in Fish

The aquaculture industry provides potential promises to meet the demand for high-quality food as it offers nutritionally rich, affordable supplies [138]. The first fish species to be exposed to an organized family-based SB program began in 1975. These programs have generated genetically discrete lines of fast-growing salmon. However, since the 1970s, millions of these fertile farmed salmon have leaked into the wild [139]. These sea cage escapees are a significant threat to the fish farm business. For example, mating between wild salmon and domestic salmon escapees endangers the genetic integrity of the wild salmon [138]. Using the CRISPR/Cas9 system, researchers KO the dead-end (dnd) gene in novel sterility models in Atlantic salmon (*Salmo salar*), resulting in a loss-of-function mutation that prevents germ cell differentiation [50,140] (Table 3). The resulting mutation prevents sexual maturity, leading to a sterile phenotype and consequently protecting the integrity of the wild-type population. Subsequently, dnd KO individuals have increased growth, higher flesh quality, and lesser susceptibility to infectious diseases [49,140]. Germ-cell-free Atlantic salmon stayed immature and did not experience puberty [141]. In another investigation, sterile channel catfish (*Ictalurus punctatus*) were developed by editing the luteinizing hormone (*LH*) gene utilizing a modified ZNF technology with electroporation [142]. In a similar study, scientists at Kyushu University used platinum TALEN to KO the *LH* gene in Japanese anchovy (*Engraulis japonicus*) [8].

Myostatin (MSTN; previously called GDF-8) gene is extraordinarily well conserved in many different species and is expressed almost exclusively in skeletal muscles [10,12]. The gene assembles the transforming growth factor-beta superfamily, which harms skeletal muscle growth. Inhibition of *MSTN* benefits aquaculture as it creates fish with improved development and

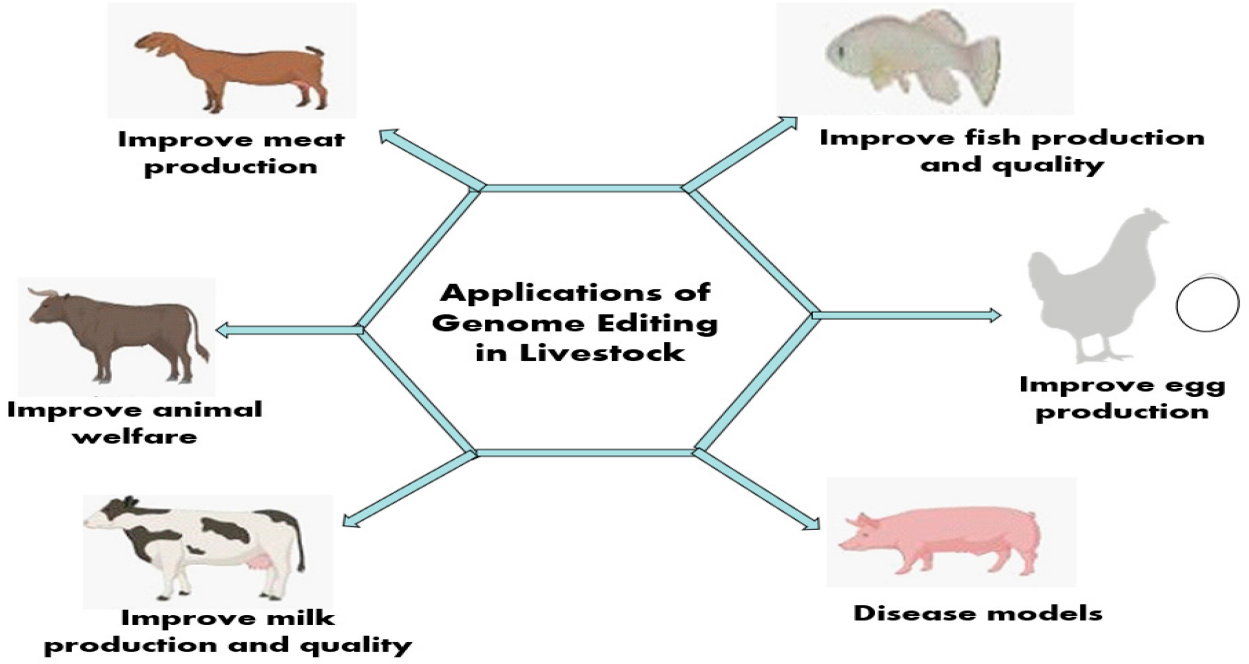

Figure 5: Creating genome-edited animals: Fishes, chickens, pigs, cows, sheep, and goats. Potential applications of genome editing include making animals better in the production of eggs, meat, and milk, as well as improved welfare and disease resistance.

increased muscle mass [143]. As shown in Table 3, CRISPR tools have been utilized for growth improvement by deactivating the *MSTN* gene in many fishes such as the common carp (*Cyprinus carpio*) [144], channel catfish (*Ictalurus punctatus*) [145], Red Sea bream (*Pagrus major*) and tiger puffer (*Takifugu rubripes*) [146], as well as olive founder (*Paralichthys olivaceus*) [121], Pacific oyster (*Crassostrea gigas*) [147], blunt snout bream (*Megalobrama amblycephala*) [148], and Nile tilapia (*Oreochromis niloticus*) [28]. The *MSTN* KO Red Sea bream was reported to significantly enlarge its muscle mass (16% increase of skeletal muscle), decrease body length, and better feed efficacy, leading to better overall growth, which was not seen in other fishes [146,149]. Furthermore, the CRISPR/Cas9 strategy was utilized for *MSTN*-targeted interference to obtain the first growing mud loach (*Misgurnus anguillicaudatus*) [150]. Aside from the MSTN gene, CRISPR was also used to improve growth-associated characteristics in the tiger puffer (*T. rub ripe*) by disturbing the leptin receptor gene that regulates appetite, making fish eat more [146,151].

Breeding in Poultry

Chicken is a key animal species globally; thus, genome-tailored chickens will spread avian biology and be valuable for agricultural and industrial applications. Genome editing technology can develop avian models for different applications. However, the generation of gene-edited poultry using various approaches, including CRISPR–Cas9, has been less fruitful than for mammals. It is more challenging due to the complex structure of the fertilized bird egg [10,152]. The application of genetic techniques established in mammals to bird species is impeded by the difficulty of getting into the fertilized oocyte nucleus and handling avian oocytes [153]. Since poultry has unique reproductive features, the lack of ESC continues in poultry species, and the traditional SCNT technology utilized in cloning mammals cannot be used in poultry. In chickens, genetic information to offspring is carried by the PGCs, and both donor and recipient PGCs grow concurrently in the recipient testes, resulting in the rejection of the donor PGC and a low sperm count [154]. Although PGC is an excellent tool for precise genome adjustments, only chicken PGC can be reliably cultured in vitro [155]. Using germline-competent chicken PGCs, which can be preserved in culture with no loss of germ cell properties, it is possible to produce particular gene-tailored chickens without genomic integration of foreign transgenes [155]. A primordial germ cell-mediated germline transfer system has been established to produce gene-edited chickens [153,155]. Despite being possible, the procedure's success largely relies on the quality of PGCs, which could be inconsistent. Due to its low efficiency, the

2025, Vol. 1, Article ID. 2025.0002
https://doi.org/10.69709/SustainFoodConn.2025.106617

first knockout (KO) chicken was not produced until 2013 [156].

Together with in vitro culture and manipulation of chicken PGCs, ovalbumin-KO chickens were produced using TALEN [157] (Table 3). In the chicken egg white, total proteins account for approximately 11% of the egg's weight. Ovalbumin (OVA) constitutes about 54% of whole egg white proteins [157]. Due to its richness in protein content, the chicken egg is the best system for constructing bioactive materials. However, too much OVA obstructs the purification of biofunctional proteins. Consequently, adjusting the egg-white composition would allow for agricultural and industrial uses [157]. Using a CRISPR/Cas9-mediated KI system, bioreactor chickens for human interferon β (hIFN-β) were produced [158]. They inserted the *hIFN-β* gene into a precise locus of the chicken *OVA* gene. That study proved the possibility of a CRISPR/Cas9-mediated KI transgenic system at the chicken *OVA* locus leading to elevated-level production of bioactive biomaterials. However, producing KO birds employing the CRISPR-Cas9 system is technically challenging due to avian and mammalian species' developmental and physiological differences [157]. The *G0/G1* changeover gene 2 (*G0S2*)-edited chickens was constructed using the CRISPR-Cas9 system [158]. The G0S2 protein is a repressor of adipose triglyceride lipase (ATGL, also called patatin-like phospholipase domain-having protein 2), activating the initial lipolysis reaction [158]. It can be inferred that the consistent reactivity of the ATGL enzyme, resulting from the removal of G0S2 suppression, would lead to the continuous hydrolysis of triacylglycerols in adipose tissue, thereby reducing lipid content and fat accumulation.

Additionally, researchers discovered a phenotype of decreased fat deposition in the abdomen of the G0S2 KO chickens, which may be a possible target gene for trait development [159]. Notably, the mutated animals have been found to survive and reproduce normally [10]. Too much body fat in broiler chickens frequently causes leg weakness. Other problems, such as diminished reproductive performance, have been reported [160].

An *MSTN* chicken model using D10ACas9 has been effectively constructed [155] (Table 3). The skeletal muscles of the chest and the leg of *MSTN* mutant chickens were extensively expanded due to myofiber hyperplasia and hypertrophy [155]. Furthermore, compared to the corresponding wild types, these *MSTN* mutant chickens had significantly less fat deposition in their abdomens [155]. CRISPR recombinant adenovirus was injected into the EGK stage XI Japanese quail (Coturnix japonica) blastoderm [161]. *MSTN* mutant quails with improved feed significantly in quail with targeted *MSTN* gene were obtained. Further examination showed a higher feed conversion rate in the *MSTN* mutant quails [161].

Investigating the combination of PGC using CRISPR–Cas9 for poultry GE has more potential for a scientific breakthrough. Improved meat production performance was achieved in livestock by injecting MSTN–KO sgRNAs' adenovirus vectors into chicks' leg muscles [162]. Although MSTN expression was significantly downregulated, gene-edited offspring were still not bred.

Breeding in Pigs

The ZFN technique was fruitfully used to KO the exogenous enhanced Green Fluorescent Protein (eGFP) gene in porcine somatic cells, proving that the ZFN-KO system might be applied to domestic animals [163]. The first ZFN-mediated KO pig of an enhanced GFP transgene was bred in 2011 [164]. Mutations in the MSTN gene were targeted by ZFNs to generate a double-muscled phenotype in Meishan pigs (*Sus scrofa domesticus*) [165] (Table 3).

Based on the advantages of TALEN, many researchers have started to apply TALEN procedures to modify and improve livestock genomes, proving its excellent potential for application. It was reported that 64% of TALENs had high action in primary porcine cells [166]. Furthermore, direct injection of TALEN mRNA into zygotes of livestock resulted in the KO of target genes in about 75% of embryos (29% in swine). Also, TALENs successfully resulted in MSTN-KO in porcine somatic cells and the generation of KO swine with significant changes in muscle phenotypes (Table 3).

Pigs were the first large farmed animals edited using CRISPR-Cas9. Researchers [167–171] have used CRISPR and other gene editing procedures (Table 3). They successfully edited the MSTN gene in both local Chinese pig breeds and commercial Large White pigs, creating MSTN knockout (KO) pigs with an increased lean meat percentage. In addition to having comparatively high muscle mass, all pigs with MSTN mutation also displayed improved productivity. However, editing the third exon of MSTN in commercial Large White pigs led to a reduced survival rate and limb abnormality of piglets after birth [134,170], though targeting the first exon could efficiently avoid the deformities reported with the third exon GE. Another research group [165,167] found that homozygous MSTN KO Meishan pigs and Erhualian pigs did not have congenital disabilities.

Another likely candidate gene for refining meat assembly in livestock and developing curative medication for muscle disorders is the *FBXO40* protein-coding gene,

whose expression is confined to muscle. *FBXO40* KO pigs generated by CRISPR/Cas9 have been made recently but showed only marginal amplification in muscle mass (4%) compared to controls [172]. Interestingly, the KO pigs grew usually, with no pathological signs found in major organs. In addition, CRISPR-Cas9 was used to knock the non-coding region of insulin-like growth factor 2 *(IGF2*) into Bama pigs [173]. They reported that the production of excellent meat quality did not change the health of the pigs. The CRISPR–Cas9 was used to knock mitochondrial uncoupling protein 1 (UCP1), which regulates and maintains the body temperature into piglet chromosomes to farm cold-resistant pigs with healthy lean with low fat [174].

Researchers [175] have reported a G to A mutation at position 3072 in the third intron of the porcine *IGF2* gene, which affects skeletal muscle growth, fat deposition, cell proliferation, and differentiation. This mutation prevents the binding of transcription repressor ZBED6, improving the expression of the *IGF2* gene and thus increasing the growth rate [175]. Such preferred mutations are commonly found in foreign marketable breeds but rarely in Chinese pig breeds. The binding sequence of *ZBED6* in the *IGF2* gene locus was deleted in 2018 [173]. This work was accomplished by MI of Cas9 nickase mRNA and a pair of sgRNAs into swinish zygotes. As a result, the expression level of the *IGF2* gene was improved, and, therefore, the meat production of Bama pigs significantly increased. Using the CRISPR system, the ZBED6 binding sequence was disrupted in porcine fetal fibroblasts [176]. After that, they combined the SCNT technology and obtained miniature spotted pigs in Liang Guang with high lean meat levels. The gene *FBXO40* is another possible candidate gene for the development of meat production in livestock, which is specifically expressed in muscles. CRISPR was used successfully to generate *FBXO40* gene KO pigs, and the muscle weight of the genetically engineered pigs increased by about 4% compared to the wild-type control [172].

Finally, a one-step method that joined the HA3A-BE3-Y130F BE with the porcine zygote injection produced concurrent gene mutations in *CD163, MSTN*, and *IGF2* [126]. The analyses indicated that the expression degree of *CD163* and *MSTN* in the triple gene-edited pigs declined while the expression level of IGF2 improved, as designed. Interestingly, the growth performance and disease resistance were positively developed.

The analyses indicated that the expression degree of *CD163* and *MSTN* in the triple gene-edited pigs declined while the expression level of *IGF2* improved, as designed. Interestingly, the growth performance as well as the disease resistance were positively developed.

Breeding in Cattle

Cattle are acknowledged as the most incredible animals for producing significant amounts of meat and/or milk, making them an important source of protein [22]. However, cattle have low fecundity and a long generation interval compared to other livestock species, which slows genetic progress [137]. Yet, the dairy industry was well-positioned for the fast adoption of gene deletion. Beta-lactoglobulin (BLG) is a key milk allergen found in goat and cow milk but not found in human milk. Neither fermentation nor heat treatment can remove BLG from milk [177]. ZFNs GE and SCNT technology solved this long-standing problem by constructing a cow that lacked the protein BLG [178] (Table 3). Subsequently, cow milk was "humanized" using ZFNs and SCNT to insert human milk protein, Lactoferrin or Lactalbumin, into the endogenic *BLG* gene region [178]. The *BLG* gene KO cows were structured from fertilized eggs to expand the market for milk and its derivatives [179]. This gene can be KO through the CRISPR/Cas9 technique to generate hypoallergenic cow milk [180].

MSTN biallelic mutations in cattle were efficiently generated using ZFNs to improve meat production [181]. Direct injection of TALEN mRNA into zygotes of livestock resulted in the KO of target genes in about 75% of embryos (43–75% in bovines). In 2015, the *MSTN* gene was edited in cattle using TALENs and SCNT to produce high-quality meat [135] (Table 3). These experiments resulted in the successful birth of cattle with better muscle mass. Generation of *MSTN*-mutated cattle without exogenous gene insertion using CRISPR-Cas9 in vitro fertilized embryos has been published [5]. This research team transmitted *MSTN KO* germline into cattle via CRISPR-Cas9. They successfully produced beef cattle with higher muscle mass. These GE outcomes in cattle proved that the *MSTN* mutation is passed on to the next generation via the germline, laying the basis for the mass construction of *MSTN* mutation cattle.

CRISPR-Cas9 was used to KO the *OCT4* gene, which plays a vital role in maintaining the pluripotency of mammals' early embryonic stem cells [182]. Improved electroporation technology was used to deliver CRISPR-Cas9 targeting the sgRNA-Cas9 protein complex of OCT4 into fertilized bovine eggs, decreasing operational difficulties and producing breeding transgenic animals easier [183]. These data offer an animal model for handling early human embryonic growth-related defects and assist further research into enhancing cattle reproduction.

Another important field in modern livestock for the application of GE technology is the cultivation of hornless cattle to reduce the dangers of possessing horns for the an-

2025, Vol. 1, Article ID. 2025.0002
https://doi.org/10.69709/SustainFoodConn.2025.106617

imals and the farmers or animal caretakers [103,160,184]. Therefore, dehorning of cattle is a demanding action to avoid accidental injury. Traditional means for physically removing animal horns are costly and painful, and this action raises animal welfare concerns. Using natural selection and breeding in naturally polled cattle breeds to produce polled livestock can take several generations to show effect. Using a TALEN-based GE approach, the genome makeup of the horn-free Holstein cow was changed by the polled (hornless) locus. After that, polled-edited cattle were generated following a nuclear transfer of gene-edited somatic cells. Later on, CRISPR–Cas9 was used to transfer a gene that controls dehorned traits into the genomes of fibroblasts derived into the genomes of fibroblasts originated from horned Mongolian cattle [185]. Furthermore, the Polled gene was isolated and integrated into the genome of fibroblasts extracted from a horned bull using the CRISPR/Cas12a system, followed by somatic cell nuclear transfer (SCNT) [186] (Table 3).

The black-coated Holstein dairy cows are prone to high-heat stress due to absorbing much light. Heat stress substantially negatively impacts growth, reproduction, and milk production [112]. In this context, a critical GE application was the induction of a mutation in the Pre-melanosome protein (*PMEL*), a gene that controls the black color in the coat; using CRISPR-Cas9, gray-coated mutated calves were successfully born [187,188]. Another method to mitigate heat stress in Holstein cattle is by editing the Slick hair coat (*SLICK*) locus located in the Prolactin hormone receptor (*PRLR*) gene, which determines hair length [189]. Animals with SLICK haplotypes possess short, fine hair and can handle heat stress. The *PRLR* gene's exon 11 (*SLICK2)* region, which is well adjusted to hot climatic situations, has a mutation (C > T) in Limonero and Carora cattle [190]. Consequently, inserting this (*SLICK*) mutation into the *PRLR* gene of heat-sensitive breeds reduces heat stress in animals subjected to hot and humid weather conditions [188]. Interestingly, the cows with SLICK gene mutations have very low health risks.

Breeding in Sheep and Goats

Sheep and goats are essential livestock in farming production and play a vital role in animal gardening. For goats and sheep, scientists use GE technology to modify different species' traits: growth status, fertility, milk production, and cashmere yield [104].

It is known that the fecundity (*Fec*) family of genes is linked with the fecundity of sheep. CRISPR-Cas9 proved to be an effective editing strategy for developing superior traits in farm animals, boosting the progress of animal husbandry [191]. The Booroola fecundity (*FecB*) homozygous mutant sheep were bred using CRISPR–Cas9 by transferring ssFecB DNA oligonucleotides into the zygote of sheep (Table 3). The resulting ewes had superior lambing rates and better reproductive rates. The lambing rate was also improved by a simultaneous edition of the Hyaluronidase 2 (*HYAL2)* and Ovine prion Protein (*OPrP*) genes using CRISPR-Cas9 [192]. In this context, efficiently generated goats were obtained by introducing defined point mutation (*I397V*) in goat growth differentiation factor 9 (*GDF9*) through CRISPR/Cas9 [193]. It was found that this technique can enhance the ovulation rate and increase the litter size. Further, the Aralkylamine N-acetyltransferase (*AANAT*) gene was microinjected into unfrozen and frozen sheep embryos [194]. The results indicated no significant difference in the reproductive capabilities of transgenic offspring between the two environments, however, *AANAT* transgenic animals had good reproductive potential.

The use of SCNT and TALENs in sheep was found to have increased muscle production phenotype [195]. Previous reports [196] revealed that TALEN pairs could effectively induce KO of target genes in numerous livestock, with a 20–60% knockout efficacy. Amazingly, TALEN-mediated gene KI effectiveness is greater than 30% at some loci [196].

Also, in sheep, studies used CRISPR/Cas9 technology to generate sheep with the *MSTN* KO gene [56,129,196–198]. The same strategy was efficiently employed to increase meat production in goats [136]. Targeted MSTN was inserted into the cytoplasm of prokaryotic embryos to produce gene-edited goats with dual muscle features [196]. Moreover, it was confirmed that the loss of MSTN function in sheep skeletal muscle satellite cells (sSMSCs) enhances the differentiation and growth of sSMSCs [199]. Single-nucleotide point mutations in the Recombinant Suppressors of Cytokine Signaling 2 (*SOCS2*) were induced using CRISPR–Cas9, increasing sheep weight and milk production. More recently, the MSTN bialleles KO had many mutations without affecting meat quality [200]. A double-allele and a single-allele MSTN gene-mutated goat were bred through the microinjection of fertilized eggs [201]. Fetuses of both edited sheep had "double muscle" and substantially increased weight and length.

As for milk, researchers have improved the constitution of sheep milk by genetically amending essential nutrients and knocking out non-essential proteins. For example, goat milk is rich in protein and fat, and its composition is comparable to human milk. However, β- BLG is not found in human milk. The β- BLG is a key nonhuman milk allergen prone to sensitization [104]. Interestingly, the KO BLG gene was followed by human lacto-

ferrin gene KI using TALENs in goats [202]. This elegant work indicated that GE, through TALEN-mediated HDR, could make genetically engineered livestock. Also, it can be utilized as mammary gland bioreactors to generate specific products efficiently. The advent of CRISPR–Cas9 was crucial to producing non-artificial milk or dairy byproducts, with low-fat or rich in particular nutrients or pharmaceutical ingredients [203].

It was discovered that using CRISPR–Cas9 KO *BLG* in goats cuts the protein amounts in BLG, laying the basis for improving the makeup of goat milk [204] (Table 3). The latter study recorded declined levels of fat, protein, lactose, and solid not fat in the milk by 5.5, 7.7, 8.0, and 7.7%, respectively. To improve the nutritional value of milk, CRISPR–Cas9 was utilized to KO components that affect milk characteristics, such as Stearoyl CoA Desaturase 1 (SCD1) [205] and Acetyl CoA acyltransferase 2 (ACAA2) [206]. CRISPR-Cas9 was used to transfer the AANAT and Acetylserotonin O-methyltransferase (ASMT) genes, which regulate melatonin expression, into the cytoplasm of fertilized sheep eggs, resulting in the creation of an AANAT/ASMT breast bioreactor [203]. The resulting genetically edited sheep had melatonin-rich milk. Also, the *BLG* gene has been KO by employing the CRISPR/Cas9 tool to construct hypoallergenic goat milk [207].

Cashmere goats are the principal local economic crucial livestock raised in Inner Mongolia. Cashmere is a significant natural raw material for the textile industry and, thus, an important source of income for farmers and herders. Researchers used CRISPR–Cas9 to stop Fibroblast Growth Factor 5 (FGF5), a secreted signaling protein that stops hair growth by inhibiting dermal papilla cell activation. This protein is the causative underlying the angora phenotype (long hair coat) [12]. Expression of *FGF5* improved cashmere density and length in cashmere-producing goats [208] and sheep [131].

The Gene-edited Cashmere goats resulting from the KO of the *FGF5* gene in Shaanbei white were followed in Cashmere goats using CRISPR–Cas9 [208] (Table 3). Compared with wild-type goats, significant increases in the number, density, and diameters of the secondary hair follicles in the skin of FGF5 mutant goats, as did cashmere length, were recorded [208]. Further studies confirmed the reported improvement of cashmere yield in sheep [131]. Cashmere goats carrying a mutant Ectodysplasin receptor-A (EDAR) gene, which inhibits hair follicle growth and development, were generated using CRISPR-Cas9-mediated somatic cell nuclear transfer (SCNT) to induce extracellular receptor abnormalities [209]. The SCNT technology and CRISPR–Cas9 were joined to produce *EDAR* gene-targeted Cashmere goats, which showed baldness [209]. Using CRISPR–Cas9 [208]. Compared with wild-type goats, significant increases in the number, density, and diameters of the secondary hair follicles in the skin of *FGF5* mutant goats, as did Cashmere length, were recorded [208]. Further studies confirmed the reported improvement of cashmere yield in sheep [131]. Cashmere goats carrying an Ectodysplasin receptor-A (*EDAR*) gene mutant with inhibited hair follicle growth and development were generated using CRISPR–Cas9-SCNT-mediated GE extracellular receptor abnormalities [209]. using CRISPR–Cas9 [208].

Also, adding the exogenic thymosin β 4 (*Tβ4*) targeted incorporation into Cashmere goats stimulated hair follicle development, quickened the differentiation of hair follicle stem cells, and improved hair assembly [210]. CRISPR–Cas9 can disrupt genes that prevent hair follicle development into a perfect phenotype. This new knowledge would offer a scientific foundation for additional breeding of high-quality and high-yield Cashmere goat strains [104]. It would also provide insights into the therapy of androgenic alopecia.

In several investigations, two or three genes were targeted concurrently, resulting in double or triple gene KOs [208]). For example, to increase goat meat production and Cashmere yield harvest, Cas9 mRNA and sgRNAs targeting *MSTN* and *FGF5* KO genes were injected into goat zygotes [211]. The KO efficacy of *MSTN* and *FGF5* in 98 experimented animals was 15 and 21%, respectively. There was 10% of the goats had double-gene KO. Three GE sheep were obtained by targeting *MSTN*, *ASIP*, and *BCO2* genes utilizing CRISPR/Cas9 and zygote microinjection protocols [211].

3.1.2. Breeding of Disease-Resistance Animals

One potential application of GE in animals is to produce animals with increased disease resistance [212]. A disease-resistant animal model formed through GE, which is raised in a limited space and susceptible to the spread of disease, can decrease time and economic loss by decreasing dependence on antibiotics. It also can reduce the pain caused by disease and improve animal welfare.

Previously, the two major strategies for disease control were vaccination and antibiotic feeding [213]. However, prolonged use of antibiotics pollutes the environment and expands resistance to harmful microorganisms [213]. Moreover, in traditional breeding methods, it is not easy to choose complex traits that are hard to detect and measure, such as disease-resistance traits. Getting traits such as robust health, elevated muscle mass, and high fecundity through genetics, rather than using antibiotics, growth hormones, or other drugs, is usually favored

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Table 3: Examples of transgenic farmed animals produced using gene editing strategies for improved characteristics.

Animal	Breed/Common name	Scientific Name	Editing Tool	Edited Gene Abbreviation *	Trait Targeted	Reference
Fish	Atlantic Salmon	*Salmo salar*	CRISPR	*dnd*	Sexual maturity	[50,140]
	Channel Catfish	*Ictalurus punctatus*	ZNF+ electroporation	*LH*	Fertility	[142]
		Ictalurus punctatus	CRISPR	*MSTN*	Muscle growth	[145]
	Japanese Anchovy	*Engraulis japonicus*	TALEN	*LH*	Fertility	[8]
	Common Carp	*Cyprinus carpio*	CRISPR	*MSTN*	Skeletal muscle growth	[144]
	Red Sea Bream	*Pagrus major*	CRISPR	*MSTN*	Skeletal muscle growth	[146]
	Tiger Puffer	*Takifugu rubripes*	CRISPR	*MSTN*	Skeletal muscle growth	[146]
		Takifugu rubripes	CRISPR	*LPR*	Appetite regulation	[146,151]
	Olive Founder	*Paralichthys olivaceus*	CRISPR	*MSTN*	Skeletal muscle growth	[121]
	Pacific Oyster	*Crassostrea gigas*	CRISPR	*MSTN*	Skeletal muscle growth	[147]
	Blunt Snout Bream	*Megalobrama amblycephala*	CRISPR	*MSTN*	Skeletal muscle e growth	[148]
	Nile Tilapia	*Oreochromis niloticus*	CRISPR	*MSTN*	Skeletal muscle growth	[28]
	Mud Loach	*Misgurnus anguillicaudatus*	CRISPR	*MSTN*	Skeletal muscle growth	[150]
Poultry	White Leghorn chicken	*Gallus gallus domesticus*	TALEN	*OVA*	Egg white proteins	[157]
		Gallus gallus domesticus	D10A-Cas9 nickase (Cas9n)	*MSTN*	Skeletal muscle growth	[159]
	Broiler chicken	*Gallus gallus domesticus*	CRISPR	*G0/G1 (G0S2)*	Lipid content/Adipose tissue	[158]
	Japanese quail	*Coturnixjaponica*	CRISPR	*MSTN*	Skeletal muscle growth	[161]
Pig	Meishan pig	*Sus scrofa domesticus*	ZFN	*MSTN*	Skeletal muscle growth	[163–165]
	Large White pig	*Sus scrofa domesticus*	TALEN	*MSTN*	Skeletal muscle growth	[170]
	Chinese Erhualian pig	*Sus scrofa domesticus*	CRISPR	*MSTN*	Skeletal muscle growth	[167]
	-	-	TALEN- SCNT	*MSTN*	Skeletal muscle growth	[166]
	Chinese experimental mini-pigs	*Sus scrofa domesticus*	CRISPR- SCNT	*Fbxo40*	Skeletal muscle growth	[172]
Cattle	Beef cattle		ZFN	*MSTN*	Skeletal muscle growth	[181]
	Beef cattle	*Bos aurus/indicus*	TALENs- SCNT	*MSTN*	Skeletal muscle growth	[135]
	Dairy cattle	*Bos taurus*	ZFNs- SCNT	*BLG*	Milk allergen	[178,179]
	Dairy cattle	*Bos taurus*	CRISPR	*BLG*	Milk allergen	[180]
	Holstein dairy cattle	*Bos taurus*	CRISPR	*PMEL*	Coat color	[187,188]

Sheep	Booroola sheep	*Ovis aries*	CRISPR	*FecB*	Reproduction rate	[191]
	Domestic sheep	*Ovis aries*	CRISPR	*HYAL2,O PrP*	Reproduction rate	[192]
	Domestic sheep	*Ovis aries*	CRISPR-Microinjection	*MSTN*	Skeletal muscle growth	[56,197]
	Merino wool sheep	*Ovis aeries*	CRISPR	*FGF5*	Hair growth	[131]
Goat	Arbas cashmere goat	*Capra hircus*	CRISPR	*FAT-1/MSTN*	Skeletal muscle growth	[129]
	Dairy goat	*Capra aegagrus hircus*	TALEN	*BLG*	Milk allergen	[202]
	Inner Mongolia cashmere goat	*Capra* spp.	CRISPR	*FGF5*	Hair growth and development	[208]
	Shaanbei white Cashmere goats	*Capra* spp.	CRISPR–SCNT	*EDAR*	Hair growth and development	[208,209]

* Abbreviations: *FecB:* Booroola fecundity gene; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; *dnd*: dead-end gene; *EDAR*: Ectodysplasin receptor-A; *eGFP*: Enhanced Green Fluorescent Protein gene; *FAT-1*: FAT atypical cadherin 1; *FGF5*: Fibroblast growth factor 5 gene; *G0/G1* (*G0S2*): Changeover gene 2; *HYAL2*: Hyaluronidase 2 gene; *LH*: Luteinizing hormone gene; *LPR*: Leptin receptor gene; *OVA:* Ovalbumin gene; *OPrP:* Ovine prion Protein; *PMEL:* Pre-melanosome gene; SCNT: Somatic-cell nuclear transfer; TALEN: Transcription Activator-Like Effector Nuclease; ZFN: Zinc-Finger Endonuclease.

by both breeders and consumers. For now, the high disease resistance practiced by large-scale animal breeding may cause some production traits [214].

Due to this, researchers have directed their efforts towards modifying animal genomes to improve their disease resistance and general health, assist animal welfare and reproductive efficacy, and develop novel biomedical models to better understand the etiology of diseases [215].

The following sections summarize some of the GE research that has been done to increase resistance to diseases according to species in livestock (Table 4).

Breeding in Fish

One major challenge facing the aquaculture industry is the high mortality rate among fish populations in fish farms [216]. Infectious diseases were the principal cause of mortality, with the most common viral diseases being pancreas disease, gill disease, and heart and skeletal muscle inflammation [216]. A potential strategy for solving problems is to improve fish robustness by adjusting gene control mechanisms implicated in the immune system function [138].

A targeted disturbance of the Toll-like receptor (*TLR22*) gene was completed in farmed Indian major carp Rohu (*Labeo rohita*) that can be employed as a model system to study the role of *TLR22* against parasites such as fish lice [217] as well as bacteria and pathogenic dsRNA viruses. As an efficient strategy to control the hemorrhagic disease caused by grass carp reovirus (GCRV), CRISPR/Cas9 was used to KO the grass carp (*Ctenopharyngodon idellus*) Junctional adhesion molecule-A (*JAM-A*) gene [218]. Recently, the CRISPR-Cas technology has been applied to mutate miRNA binding sites in the three prime untranslated region (3′ UTR) of immune-related genes [138]. Consequently, this prevents miRNA binding and blocks miRNA-induced mRNA breakdown or translational inhibition. Eventually, the mRNA half-life was increased, leading to a notable increase in protein levels that strengthened the immune response.

Breeding in Poultry

Poultry is incredibly susceptible to viruses, such as Mark's disease virus (MDV), the highly pathogenic avian influenza virus (AIV) [13,124], and the Avian leukosis virus subgroup J (ALV-J) [91,219]. The Chicken Na + /H + exchanger type I (*chNHE1)*-KO homozygous mutant chicken was successfully engineered using PGCs and CRISPR/Cas9 technologies [219]). This transgenic chicken, which was created by knocking out the retrovirus receptor Tva gene using CRISPR/Cas9, has shown resistance to ALV subgroups A and K, enabling it to combat ALV infection [219].

CRISPR/Cas9 system was utilized to change the recognized residues within the chANP32A gene to diminish AIV replication of the AIV [220]. Also, the CRISPR–Cas9 technique was used to remove Trp38 encoded by *chNHE1 W38* of the ALV-J receptor, acquiring the bred chicken resistance against the retroviral infection [219]. In this regard, for the first time, developing a CRISPR–Cas9 vector that stopped the expression of ALV-J in vivo was successful using coinfected MDV. Following this, breeding with GE quickly grows new livestock forms with disease-resistance traits by deleting disease predisposition genes and pathogens receptor genes or introducing disease-resistance genes [13]. Additionally, scientists can test new livestock genetic traits that affect disease resistance and do not exist from natural genetic resources.

2025, Vol. 1, Article ID. 2025.0002
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Recently, chickens resistant to ALV-J were produced by deleting one amino acid, W38, inside the ALV-J receptor NHE1 using CRISPR/Cas9 GE. This resistance phenotype was confirmed in vitro and in vivo [219].

Breeding in Pigs

Swine are one of the primary livestock resources and are considered an ideal animal model for biomedical research and xenotransplantation due to their physiological and genetic similarities to humans [221]. The Porcine Reproductive and Respiratory Syndrome (PRRS) infection is estimated to cost pig farmers about six million dollars daily in North America and Europe [222]. Due to the genetic diversity of the virus, the vaccine has limited efficiency on PRRS [223], and therefore, attention was directed to the cellular receptors involved in the PRRSV entrance into the target cell. Cluster of Differentiation (CD163) is a transmembrane protein on the surface of macrophages acting as a receptor for PRRSV [224]. CRISPR/CAS9 was used to produce a KO mutation in the exon 7 of the *CD163* gene [225].

Mutant pigs showed strong resistance to the PRRS virus. Following this, several research groups have successfully produced various types of CD163-KO pigs using CRISPR/Cas9 tools [222,226–229]. Confrontation with either the NVSL 97-7895 PRRSV virulent virus isolates [225] or the highly pathogenic PRRSV (HP-PRRSV) strain [229] revealed that CD163-KO pigs are resistant to viral disease shown by the nonexistence of viremia, antibody response, high fever or any other PRRS-associated clinical symptoms. Furthermore, neither PRRSV nor its antibodies were found in pig serum. For the first time, gene-edited breeding for livestock disease resistance has solved an obstacle neither selection programs nor vaccines can solve entirely.

One GE design in the specific deletion of the Scavenger Receptor Cysteine-Rich domain 5 (SRCR5) of the *CD163* gene while keeping other CD163 domains unharmed. Macrophages from these pigs resisted both *PRRSV* genotypes [226,227] but not Porcine Epidemic Diarrhea Virus (PEDV) infection after virus challenge tests [227,228]. Furthermore, CRISPR/Cas9 and SCNT were utilized to produce pigs with the concurrent KO of CD163 and Porcine Aminopeptidase N (*APN*) genes [207]. Further analysis revealed that the double-KO (DKO) pigs were utterly resistant to genotype 2 PRRSV and transmissible gastroenteritis virus (TGEV) infection simultaneously. These findings confirmed that DKO *CD163* and *pAPN* pigs were produced [230]. This study reported that the pigs with DKO mutation had no difference in meat production and reproductive performance relative to wild-type pigs. Lung fibroblast-like cells originating from these animals maintained a prominent level of Porcine Delta Coronavirus (PDCoV) infection, suggesting that *APN* is a dispensable receptor for PDCoV [231]. These *CD163* null mutations resist several PRRSV isolates [222,229].

Additionally, many GM pigs created using CRISPR/Cas9 are appropriate donors for disease models and xenotransplantation [107,229,232]. Transgenic pigs resistant to the classical swine fever virus (CSFV) were bred using CRISPR–Cas9 [233]. These researchers observed that growing pigs, pregnant sows, and fetuses were resistant to PRRSV infection. CD163 KO pigs were also bred, and the replication of CSFV was confined while disease resistance was stably transferred to offspring. The African swine fever virus (ASFV) has a higher mortality rate than ordinary swine fever, reaching 100% in some cases. CRISPR–Cas9 was used to target ASFV phosphoprotein p30 to decrease the number of viruses by inhibiting viral replication [234]. In 2020, the CD163 and pAPN DKO pigs were assembled [207], turning off the virus receptor CD163 and pAPN proteins and confirming more excellent disease resistance. Other infection challenge trials have indicated that these DKO pigs showed reduced susceptibility to PDCoV, providing in vivo evidence that pAPN could serve as one of the receptors for PDCoV. This procedure blocked PRRSV and TGEV infections and conserved reproductive capacity and meat quality [235].

Finally, it should be mentioned that knocking out of another PRRSV receptor gene, CD169, was attempted, but no significant differences in the viral challenge were reported between edited and wild-type pigs [222]. This negative finding suggests that this gene may not necessarily affect the occurrence of PRRS. Commentators have stressed that, at present, GE is most useful for traits affected by one major gene (monogenic) [236], for instance, resistance to PRRS. Monogenic resistance to infections in pigs is rare [237], and conventional breeding is likely not to be used for traits affected by multiple genes in livestock [137]. The Royal Society for the Protection of Cruelty to Animals debated that genome-edited disease resistance can be short-lived if diseases develop to overcome changes in single genes [238].

Breeding in Cattle

Mycobacterium bovis (*Mbv*) infection can cause large debts to the livestock business and poses zoonotic dangers because humans mainly acquire *Mby* infection through infected food or drinks, unpasteurized dairy foods, or exposure to infected animals [103].

In cattle, bovine *CD18* is a gene that codes for the leukotoxin receptor found on the leucocyte surface. The use of ZFNs to insert a single amino acid substitution (glutamine [Q] to glycine [G]) at amino acid position five in both alleles resulted in the production of transgenic cattle resistant to the cytotoxicity of leukotoxin secreted by Mannheimia (Pasteurella) haemolytica, the bacterium responsible for the respiratory disease Pasteurellosis [239] (Table 4). This study confirmed the feasibility of developing gene-modified cattle resistant to *M. haemolytica*-caused pneumonia.

The TALE nickase-mediated *SP110* gene KI was utilized to produce Tuberculosis (TB)-resistant cattle, and the gene-edited cows were capable of inhibiting the growth and reproduction of *Mby* [232]. Also in bovine, Gao et al. [240] were the first researchers to use Cas9 nickase (Cas9n)-mediated homologous recombination to integrate naturally resistant-associated macrophage protein-1 (*NRAMP1*) into the bovine genome, promoting their resistance to *Mbv* infection. Combined with SCNT, gene-edited cattle with significantly enhanced TB resistance were produced. Gene-edited TB-resistance cattle were generated using the CRISPR/Cas 9 with NRAMP gene inclusion [241] (Table 4).

The CRISPR–Cas9 strategy was used to develop a *CD46* gene KO cell line reliant on the highly contagious bovine viral diarrhea virus (BVDV) attack [242]. This virus can cause severe intestinal and respiratory diseases in cattle. The virus also causes abortion in pregnant cows, and certain infected calves that acquire it at birth can transmit the infection for the rest of their lives. These experiments enabled researchers to clarify the mechanism underlying *CD46*'s role in plague viruses' replication cycles. The virus binds to a cellular receptor (CD46), causing infection in cows. In vivo, CRISPR-mediated homology-directed repair and SCNT were used to generate a mutation in the BVDV binding domain of bovine CD46 [243]. The outcome was a GE CD46 receptor in the virus unable to bind and a live calf with drastically reduced susceptibility to the infection. The first CD46 gene-edited calf was produced.

In dairy cows, Mastitis is a severe disease caused by Bovine paratuberculosis, resulting in considerable economic losses to the dairy industry. Using ZFNs-mediated gene recombination approach, the human lysozyme gene and lysostaphin gene were precisely inserted into the endogenous Casein beta (*CSN2*) gene locus of the cow [244] (Table 4). Then, transgenic cows were produced by SCNT. The gene-edited cows were capable of secreting lysostaphin or human lysozyme, and consequently killing *Staphylococcus aureus* in their milk [244]. Furthermore, the ZFN GE method was used to direct the human lysozyme gene to the beta-casein locus, creating a transgenic mastitis resistance cow [183].

Using TALEN, the mouse *SP110* gene was introduced into the Methionine Adenosyltransferase 1a (*MAT1A*) and Pulmonary surfactant protein A1 (*SFTPA1*) sites of the Holstein-Friesian dairy cow genome [232]. Integration of the *NRAMP1* gene into the Fascin actin-bundling protein 1- Beta-actin (*FSCN1-ACTB)* locus and the bovine homology of the mouse Rosa26 locus using the CRISPR/Cas9 system was a significant step. This genetic modification conferred high resistance in the *NRAMP1* transgenic cows against tuberculosis [232], underscoring the potential of genetic research in enhancing disease resistance in livestock. The transgenic cows showed elevated resistance to *Mbv* infection. In vitro, CRISPR/Cas9-mediated NO of the *CD46* gene in Madin–Darby bovine kidney (MDBK) cells led to a significant decline in BVDV susceptibility [242].

Breeding in Sheep and Goats

As sheep's body size and functional structure are comparable to those of humans, CRISPR–Cas9 tools have been used in sheep to offer an effective model for studying disease resistance breeding for several diseases. Therefore, the CRISPR–Cas9 system provides a promising therapeutic model for investigating pathogenesis and improving animal health. The thymosin β4 (*Tβ4*) gene was implanted into the chemokine receptor type 5 (CCR5) site in goats, enabling the establishment of a goat knock-in model [195].

African researchers are now working to use the CRISPR/Cas9 system to KI the Apolipoprotein L1 (*ApoL 1*) gene, which confers resistance to trypanosomiasis in primates [245], into an Indigenous goat breed [246]. If successful, this GE scheme could also be used to oppose the destructive disease of trypanosomiasis in Indigenous cattle breeds. The phenotypes of the liver and gallbladder diseases of newborn Cystic Fibrosis Transmembrane conductance regulator (CFTR)-/- sheep agreed with those of humans. In sheep, CRISPR–Cas9 was applied to induce one point mutation in the tissue non-specific alkaline phosphatase (*TNSALP*) gene (ALPL) (1077 C > G) [247]. The resulting engineered lambs exhibited a rare human bone disease, hypophosphatasia, thus, providing an efficient animal model for investigating this rare metabolic bone disease. Also, in lambs, the possibility of using CRISPR–Cas9 for engineering antiviral animals has been established [248]. The hyaluronidase 2 (*HYAL2*) gene involved in lung adenocarcinoma syndrome was KO. It was for the first time that removing the sheep otoferlin (*OOF*) gene is an efficient model for treating deafness [248]. An-

2025, Vol. 1, Article ID. 2025.0002
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Table 4: Examples of transgenic farmed animals produced using gene editing strategies for disease-resistance.

Animal	Common Name	Scientific Name	Editing Tool	Edited Gene Abbreviation *	Trait Targeted	Reference
Fish	Grass carp	*Ctenopharyngodon idellus*	RISPR	*JAM-A*	GCRV hemorrhagic disease infection	[218]
Poultry	Broiler chicken	*Gallus gallus domesticus*	CRISPR-MIPGCs	*chNHE1*	ALV infection	[219]
Pig	-	*Sus domesticus*	CRISPR	*CD163*	Genotype 1 PRRS infection	[225]
	Chinese pig	*Sus domesticus*	CRISPR-SCNT	*CD163*	Genotype 1 PRRS infection	[227]
	Wild pig	*Sus scrofa*	CRISPR- SCNT	*CD163/APN*	Genotype 2 PRRSV/TGEV infections	[207,230]
Cattle	Cow	*Bos taurus*	ZFN	*CD18*	Pasteurellosis resistance	[239]
	Holstein–Friesian dairy cow	*Bos taurus*	TALE	*SP110*	Tuberculosis resistance	[232]
	Holstein–Friesian dairy cow	*Bos taurus*	Single Cas9n-SCNT	*NRAMP*	Tuberculosis resistance	[240,241]
	Mardin-Darby cow	*Bos taurus*	CRISPR- SCNT	*CD46*	BVDV resistance	[242]
	Chinese dairy cow	*Bos taurus*	ZFN-SCNT	*CSN2*	Mastitis resistance	[244]
	Holstein–Friesian dairy cow	*Bos taurus*	TALEN	*SP110/MAT1A/ SFTPA1*	Tuberculosis resistance	[245]
	Holstein–Friesian diary cow	*Bos taurus*	CRISPR	*NRAMP1 /FSCN1-ACTB (F-A)*	Tuberculosis resistance	[245]
Sheep	Domestic sheep	*Ovis aries*	CRISPR–SCNT	*IFNA*	ZIKV resistance	[233]

* Abbreviations: ACTB: Beta-actin gene; ALV: Avian leukosis virus; APN: Aminopeptidase N gene; APOL1: Apolipoprotein L1 gene; BVDV: Bovine viral diarrhea virus; Cas9n: Cas9 nickase; CSN2: Casein beta gene; CD: Cluster of Differentiation gene; CSFV: Classical swine fever virus; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; FSCN1: Fascin actin-bundling protein 1 gene; GCRV: Grass carp reovirus; chNHE1: Chicken Na + /H + exchanger type I gene; IFNA: Interferon Alpha gene; JAM-A: Junctional adhesion molecule-A gene; MAT1A: Methionine Adenosyl transferase 1a gene; MI: Microinjection; NRAMP1: Naturally resistant-associated macrophage protein-1; (PRRS: Porcine Reproductive and Respiratory Syndrome; PGCs: Primordial Germ Cells; SFTPA1: Pulmonary surfactant protein A1; SCNT: Somatic-cell nuclear transfer; SP110: SP110 nuclear body protein gene; TGEV: Transmissible gastroenteritis virus; ZIKV: Zika virus.

other report pointed out that the CRISPR/Cas9 system adjusts *HYAL2* and *PrP* genes to produce disease-resistant lambs [192].

The CRISPR–Cas9 and SCNT technologies were combined to breed Interferon Alpha (*IFNA*) gene KO sheep [233]. This engineered sheep offered a large-scale animal model for fetal resistance to Zika virus (ZIKV) infection (Table 4). Thereafter, using the same technology, the human cystic fibrosis (CF) sheep model with CRISPR–Cas9–mediated CFTR deficiency was constructed [249]. The SCNT program targeted KO *PRP* in goat fibroblast donor cells to generate SCNT-mediated anti-PRP goats. The CRISPR–Cas9 produced the *NUP155* gene KO donor cells in goat fibroblasts [249]. These experiments developed a *NUP155* gene KO goat model for investigating heart disease. Pancreas/duodenum homeobox protein 1 (*PDX1*) fetuses were composed [250], permitting gene-edited sheep to serve as host organs for xenotransplantation. Furthermore, interspecies blastocyst-paired technology, combining embryonic gene editing and pluripotent stem cells (PSCs), has enabled xenotransplantation of human organs by using large animals as hosts [104].

These studies confirm that the CRISPR/Cas9 system can significantly improve modern livestock breeding systems. The DNA BE can produce new varieties without limitation to individuals or species. Single nucleotide variants (SNVs) are the critical genetic basis for trait alteration in livestock and the top cause of 2/3 of human diseases [251]. Therefore, developing a new BE system in GE technology based on the CRISPR/Cas system is very important and urgent [91].

4. Conclusions and Future Perspectives

With the increase in the world population and the necessity for social development, the genetic improvement of farm animals has been and will continue to be a key focus, driving sustainability in both animal agriculture and aquaculture. Gene editing technology currently under development has massive potential to increase animal husbandry productivity, profitability, and sustainability appreciably. Compared to old techniques, hybridized GE technologies can considerably speed up and improve efficiency and accuracy in animal breeding. In the near future, the design and production of large animals may revolutionize agricultural and biomedical fields.

With the advancement of efficient GE technology, the off-target effect has become a significant concern for researchers, and a challenging problem that severely restricts animal breeding. Consequently, researchers have combined SCNT and whole genome sequencing technology to advance a commonly applicable off-target activity assay (NT-SEQ) for various GE tools, including BE tools [252]. The finding of this off-target activity recognition method guides the continuous optimization of GE tools and increases their future application. In the future, the constant optimization of the efficiency and precision of GE technology may significantly enhance the broad application of gene-edited large animal models in molecular breeding, and offer technical support for solving global food safety and ensuring food supply.

Currently, there are many significant safety concerns surrounding GMOs, especially farm animals, as they are sentient. These concerns include technical defects, ethical considerations, and public acceptance of GM animals and their products. It is still uncertain if GE has to be controlled similarly to bioengineered animals; nonetheless, studies show that it is unnecessary to regulate GE. Unlike genetic modifications, where new foreign genes are inserted into the original DNA, GE does not certainly introduce any extraneous or new DNA into the genome of the edited species. Instead, a slight controlled alteration in the original DNA develops the targeted trait by deleting the inferior alleles [47]. Modifications produced are not distinct from naturally found gene alleles. For instance, the GE of a particular gene is made using a template DNA dictated by the sequence of a usually present allele from the same species. Still, other activists believe that GE can cause genomic abnormalities in the resultant GMOs, even if genes are not introduced or inserted only transiently [31]. Studies should focus on identifying and minimizing the possibility of introducing off-target sequence variants during GE, as this will be critical to avoid any possible adverse health effects due to GM livestock products.

Application for genetic developments in aquaculture is still in its early stages. Up to now, the primary research works in aquaculture are based on conventional genetic improvement plans such as growth, production, and reproduction. The continuous progress of the CRISPR/Cas9 tool and its appliance in aquaculture as a novel breeding strategy will revolutionize the area by increasing production and quality. Furthermore, various solutions in aquaculture are anticipated shortly, including disease resistance and sterile breeding, which could not have been achieved before by conventional means.

Another logistical challenge still unresolved and under continuous research is that many of the economically related traits for animal production are controlled by more than one gene, and each gene has minor effects (polygenic traits) [253]. For such traits, editing numerous loci would be needed to make significant improvements, which adds a lot of complications for the existing technologies. Every practitioner should be conscious of the significance of early screening. Due to diverse laws and regulations, gene-edited animals are only commercially available in a few countries. The primary challenge in applying and promoting GM agricultural food animals lies not in the technology itself but in government regulations and public acceptance. In 2009, The U.S. Food and Drug Administration (FDA) accepted a recombinant anti-thrombin medication derived from the milk of genetically edited goats [254], a drug expressing genetically edited chicken in 2015 [255]. In 2020, GalSafe pigs α-galactose free were permitted for food and medical purposes [10]. This development indicates that more transgenic animals will be acknowledged, and the market will be enhanced.

Abbreviations

ABE	Adenine Base Editor
AIV	Avian Influenza Virus
ALV	Avian Leukosis Virus
ASFV	African Swine Fever Virus
BE	Base Editor
BLG	Beta-lactoglobulin
Cas9	CRISPR Associated Protein System 9
CBE	Cytosine Base Editor
CSFV	Classical Swine Fever Virus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
dCas9	Dead Cas9
DSB	Double Strand Breaks
ESC	Embryonic Stem Cells
eGFP	Enhanced Green Fluorescent Protein
FAO	Food and Agriculture Organization
GE	Genome/gene editing

GEN	Genetic Engineering
GMO	Genetically Modified Organism
HDR	Homologous Directed Repair
HR	Homologous Recombination
Indel	Insertion or deletion
KI	Knock In
KO	Knock Out
LH	Luteinizing Hormone
LMO	Living Modified Organism
Mbv	*Mycobacterium bovis*
MI	Microinjection
MSTN	Myostatin
NHEJ	Nonhomologous End Joining
OVA	Ovalbumin
PAM	Protospacer Adjacent Motif
PCR	Polymerase Chain Reaction
PE	Prime Editor
pegRNA	Prime-editing guide RNA
PGCs	Primordial Germ Cells
PRRS	Porcine Reproductive and Respiratory Syndrome
RT	Reverse Transcriptase
RNP	Ribonucleoprotein
SB	Selective Breeding
SCNT	Somatic Cell Nuclear Transfer
sgRNA	Single guide RNA
SNVs	Single Nucleotide Variants
SSB	Single Strand Break
TALEN	Transcription Activator-Like Effector Nuclease
ZFN	Zinc-Finger Endonuclease

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The author confirms that he was solely responsible for the conception, design, analysis, interpretation, drafting, and final approval of the article.

Conflicts of Interest

The author declares no conflicts of interest.

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