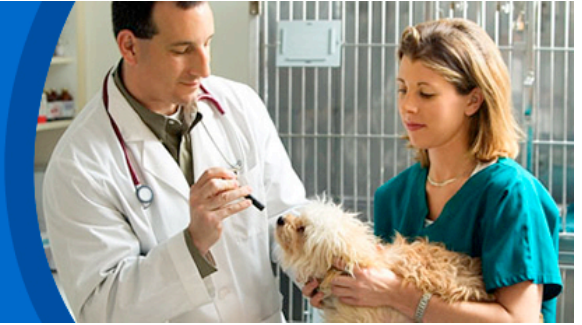




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Recent advances and future prospects of gene editing technology for enhancing livestock health and productivity

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ABSTRACT

This review aims to explore recent advances and future prospects of gene editing technology for enhancing livestock health and productivity. The authors examine various engineered nucleases, including Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and the CRISPR/Cas9 system, alongside emerging tools like base editing and prime editing. The discussion covers delivery techniques such as microinjection, electroporation, hydrodynamic delivery, and viral and non-viral vectors, as well as production methods like zygote manipulation and somatic cell nuclear transfer. Results highlight successful applications in improving production traits through MSTN and BLG knockouts, enhancing health via resistance to PRRSV and TGEV, and developing biomedical models for xenotransplantation. The authors conclude that while genome editing offers enormous potential for animal welfare and sustainable production, challenges regarding off-target effects, ethical concerns, and the preservation of genetic diversity must be addressed.

Keywords: CRISPR/Cas9, Genome editing, Livestock productivity, Animal health, Somatic cell nuclear transfer

Introduction

Traditionally, the genetic improvement of livestock has relied on selective breeding, a process that spans generations and consumes significant time and resources. However, genome editing offers a more rapid and precise approach to achieve genetic enhancements in livestock. This technology has the potential to revolutionize the livestock industry, addressing critical challenges such as disease outbreaks, animal welfare concerns, and the need for sustainable production practices. Precise genome editing hinges on the capacity of engineered nucleases, including Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system, to make targeted cuts in the genome. These cuts result in double-stranded breaks (DSBs), which stimulate the cell's repair mechanisms, leading to either non-homologous end-joining (NHEJ) or homology-directed repair (HDR). This repair process introduces specific mutations into precise genomic locations. While ZFNs and TALENs require the assembly of engineered proteins for each target, the CRISPR/Cas9 system stands out as a simpler and more versatile method. Its efficiency has advanced to the point where it can facilitate multiple edits simultaneously. Consequently, CRISPR-based genome editing has become the preferred choice for introducing targeted genomic modifications in livestock species. The landscape of CRISPR/Cas9-based genome editing tools is continually expanding. In this discussion, we will explore various gene editing tools, including emerging technologies like base editing and prime editing. We will also delve into the diverse delivery techniques employed to introduce gene editing tools into cells. Furthermore, we will explore how genome editing in livestock can enhance both health and production in this industry.

The rise of genome editing

Genome editing stands as a potent and exacting technology empowering scientists to manipulate an organism's DNA, spanning humans, animals, and plants alike. This technology revolves around the precise modification of particular genes, encompassing the addition, removal, or substitution of DNA sequences, all with the intent of modifying an organism's traits or rectifying genetic anomalies. The progression of gene editing constitutes a captivating voyage characterized by the continual refinement and enhanced accessibility of techniques over the years.

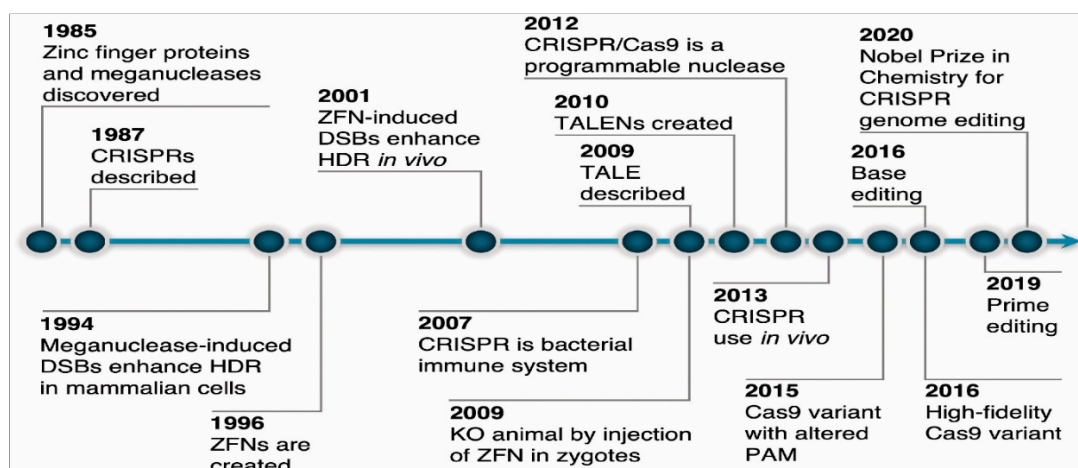
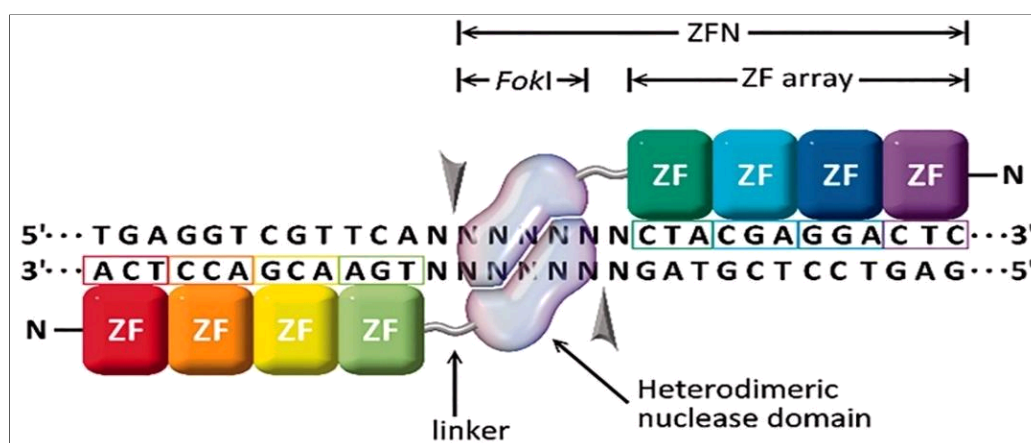


Figure. History of genome editing: From meganucleases to CRISPR (Troder *et al.*, 2021). DSBs, double-strand breaks; HDR, homology-directed repair; KO, knock-out; PAM, protospacer adjacent motif; TALENs, transcription activator-like effector nucleases; ZFN, zinc finger nucleases.

Zinc finger nucleases



Reference: (Lino *et al.*, 2018) [1]

Zinc fingers (ZFs) represent common DNA-binding motifs found in numerous transcription factors (Kim *et al.*, 1996). The ZF domain is composed of 20–30 amino acid residues, forming two opposing β sheets along with an α -helix. Typically, ZFs bind to DNA through the interaction of the α -helix region with the major groove of the DNA. ZFNs are created by combining a non-sequence-specific cleavage domain with a site-specific DNA-binding domain attached to the zinc finger. Each zinc-finger unit selectively recognizes three base pairs (bp) of DNA. The FokI type II restriction endonuclease acts as the domain responsible for DNA cleavage. To cleave DNA, FokI nuclease needs to dimerize, necessitating the involvement of two ZFN molecules that bind to the target site in the correct orientation (Smith *et al.*, 2000).

Transcription activator-like effector nucleases

TALENs consist of a non-specific DNA cleavage domain combined with a customizable, sequence-specific DNA-binding domain, resulting in a double-strand break. This DNA-binding domain utilizes a highly conserved repeat sequence derived from transcription activator-like effectors (TALE), initially identified in phytopathogenic *Xanthomonas* bacteria, which naturally modulate gene transcription in host plant cells (Bogdanove *et al.*, 2010). TALE binds to DNA through a central region containing an array of 33- to 35-amino-acid sequence motifs. Each repeat's amino acid sequence is structurally similar, except for two highly variable amino acids known as the repeat variable di-residues (RVDs) at positions 12 and 13. DNA-binding specificity is determined by RVDs: HD specifically binds to C nu-

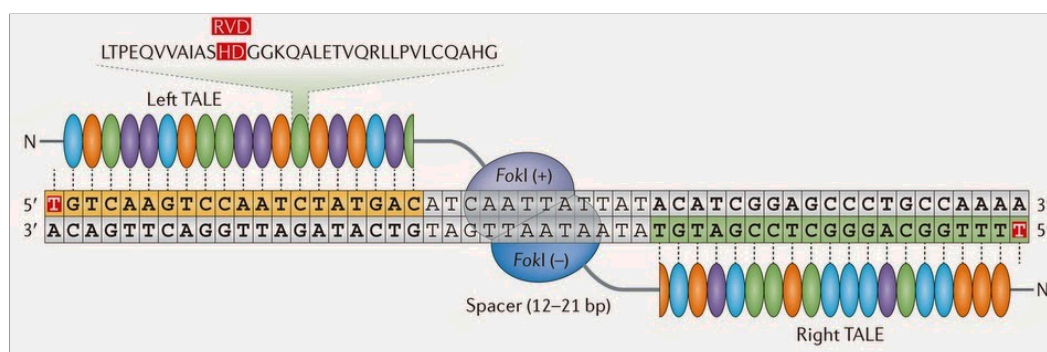
cleotides, NI to A nucleotides, NN to A or G nucleotides, NH to G nucleotides, and NS to all nucleotides (Becker *et al.*, 2021) [2]. The cleavage domain in TALENs is provided by FokI nuclease. Therefore, like zinc fingers, TALEN modules are designed in pairs to target opposing DNA loci, with appropriate spacing (12–30 bp) between the two binding sites.

Reference: (Kim & Kim, 2014)

CRISPR/Cas system

Clustered regularly interspersed short palindromic repeats (CRISPRs) were first identified in *E. coli* and later in many bacteria and archaea, where they function as an adaptive immune system against foreign genetic elements. When a bacteriophage infects a bacterium, short fragments of viral DNA are captured by Cas enzymes and integrated into CRISPR loci as spacers between repeat sequences. These spacers serve as a genetic memory of prior infections. Upon reinfection, the CRISPR array is transcribed and processed into mature CRISPR RNAs (crRNAs), which guide Cas nucleases to complementary target sequences (protospacers), leading to sequence-specific cleavage of invading DNA (Marraffini, 2015).

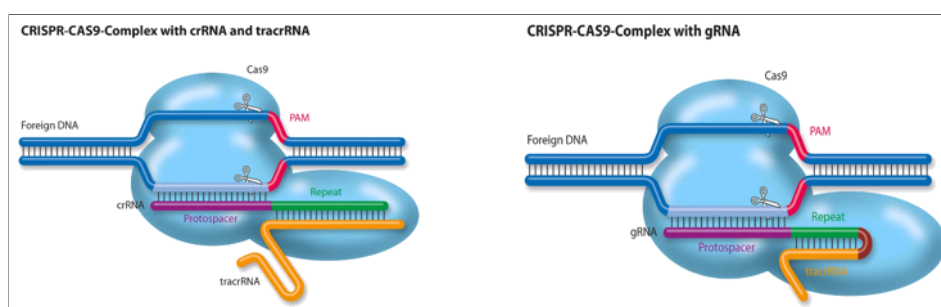
Mature crRNAs associate with Cas proteins to form effector complexes that recognize and destroy foreign nucleic acids. Target recognition typically requires a short adjacent motif known as the protospacer adjacent motif (PAM). CRISPR–Cas systems are classified into two major classes: Class 1 systems use multi-protein effector complexes, whereas Class 2 systems rely on a single effector protein. Among them, the Type II CRISPR/Cas9 system, derived from *Streptococcus pyogenes*, is the most widely used for genome editing.



The CRISPR/Cas9 system consists of a Cas9 endonuclease and a guide RNA (sgRNA). In its natural form, a crRNA pairs with a *trans*-activating crRNA (tracrRNA) to guide Cas9 to a complementary 20-nt DNA target adjacent to a PAM sequence (Jinek *et al.*, 2012). In engineered systems, these RNAs are fused into a single sgRNA for simplicity. Cas9 introduces a site-specific Double-Strand Break (DSB) 3 bp upstream of the PAM using its HNH domain (target strand cleavage) and RuvC domain (non-target strand cleavage). Unlike ZFNs and TALENs, which require protein redesign for each target, CRISPR/Cas9 can be easily reprogrammed by altering only the guide RNA sequence, making it a versatile and efficient genome-editing tool.

Figure. (Max Planck Society) CRISPR-Cas9 consists of three components: With its spacer region from a previous virus attack, the CRISPR-RNA (crRNA) recognises a matching region in a foreign DNA strand. Together with the tracrRNA it forms highly stable hair-pin-shaped structures. The Cas9 enzyme can cleave the two DNA strands; however, another short sequence known as the protospacer adjacent motif (PAM) must follow immediately after the targeted DNA strand.

Gene disruption using CRISPR/Cas9 is achieved by inducing a site-specific Double-Strand Break (DSB) in the target gene. Following cleavage, the cell repairs the DSB through two major pathways: Non-Homologous End Joining (NHEJ) or homology-directed repair (HDR) (Riordan *et al.*, 2015).



Non-Homologous End Joining (NHEJ) is the predominant DSB repair pathway. It directly ligates broken DNA ends through the action of the Ku70/80 complex and DNA Ligase IV (Pannunzio *et al.*, 2018). NHEJ is error-prone and frequently introduces small insertions or deletions (indels) at the cleavage site. These indels often cause frameshift mutations, disrupting the coding sequence and generating premature stop codons (Dow, 2015). This strategy is widely used to create gene knockouts (KO). CRISPR/Cas9-mediated KO has been successfully demonstrated in multiple species, including humans, sheep, goats, cattle, pigs, and mice, with efficiencies ranging from 10% to over 90% (Mei *et al.*, 2016; DeWitt *et al.*, 2017; Xie *et al.*, 2017; Seki and Rutz, 2018; Jin *et al.*, 2019). Delivery of CRISPR/Cas9 as ribonucleoprotein (RNP) complexes enhances knockout efficiency (Perisse *et al.*, 2020), enables rapid DSB induction, and reduces off-target effects because the RNP is degraded within approximately 24 hours.

Homology-Directed Repair (HDR) is a high-fidelity repair pathway that enables precise genome modification using a homologous DNA template. HDR occurs less frequently than NHEJ in eukaryotic cells and is mainly active during the S and G2 phases of the cell cycle (Zhao *et al.*, 2017). Upon DSB formation, cyclin-dependent kinases (CDK1/2) activate CtIP endonuclease, which cooperates with the MRN complex (Mre11–Rad50–Nbs1) to initiate DNA end resection (Yeh *et al.*, 2019). This process generates 3' single-stranded DNA (ssDNA) overhangs that are initially coated by replication protein A (RPA). Rad51 subsequently replaces RPA, facilitating homologous sequence search and strand invasion. After alignment with the donor

template, DNA synthesis occurs, Rad51 dissociates, and the DNA strands are ligated to complete precise repair (Pawelczak *et al.*, 2018). Thus, while NHEJ is primarily used for gene knockouts due to its efficiency, HDR enables accurate gene insertion or correction but occurs at lower frequencies.

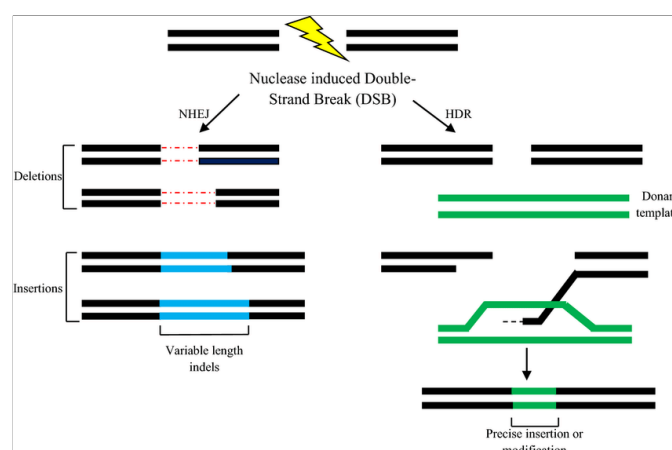


Figure. Pathways of DSB (double-strand break) repair (Barman *et al.*, 2019). DSBs generated during genome editing can be repaired by either of the two pathways, i.e., non-homologous end-joining (NHEJ) and homology-directed repair (HDR). At DSB sites, NHEJ includes short insertions and/or deletions (termed as indels) while HDR includes accurate insertions or mutation using donor template.

Improvements of CRISPR/Cas9

Cas9 nickase (nCas9)

A modified Cas9 endonuclease has been developed to enhance the efficiency of introducing single point mutations. This enzyme has been engineered to induce a single-strand break using either the RuvC or HNH domain. Referred to as "nCas9" or nickase, it combines one inactive domain with another functional domain capable of creating a single-strand DNA break. When two different guide RNAs (gRNAs) targeting nearby locations are used in conjunction with nCas9, a technique called "double nicking" is employed. This approach enhances gene editing specificity and reduces the risk of off-target mutations without compromising on-target effectiveness (Adli, 2018). When employing double nicking, it's estimated that off-target activity in cell lines can be reduced by 50- to 1,500-fold (Harrison and Hart, 2018).

Dead Cas9 (dCas9)

The dCas9, also known as nuclease-null or "dead Cas9," is specifically designed to avoid creating double or single-strand DNA breaks. By rendering the RuvC and HNH nuclease domains inactive, the CRISPR/dCas9 complex can precisely locate the target DNA sequence and induce direct transcriptional changes in the gene without causing any damage to the DNA itself. This dCas9 can be combined with proteins to either inhibit gene expression (CRISPRi) or activate it (CRISPRa). For example, when Cas9 is fused with Kruppel-associated box (KRAB), it promotes gene repression, while fusing it with VP₁₆ or VP₆₄ leads to the activation of gene expression.

Base editing

Following CRISPR/Cas9, base editing emerged as a major advancement, enabling precise point mutations without generating double-strand breaks (DSBs). The first cytidine base editor (BE1) was created by fusing catalytically inactive Cas9 (dCas9) with rat APOBEC1 cytidine deaminase, allowing direct conversion of cytidine (C) to uridine (U), resulting in C→T (or G→A) substitutions (Komor *et al.*, 2016). BE1 targeted bases within a 4–8 bp editing window but showed limited efficiency due to cellular repair of the intermediate U:G mismatch. To improve editing efficiency, BE2 incorporated a uracil DNA glycosylase inhibitor (UGI), preventing base excision repair and increasing C→T conversion rates approximately threefold (Rees and Liu, 2018). Further refinements led to more efficient variants such as BE3, BE4max, and YFE-BE4max, which enhanced editing precision and activity.

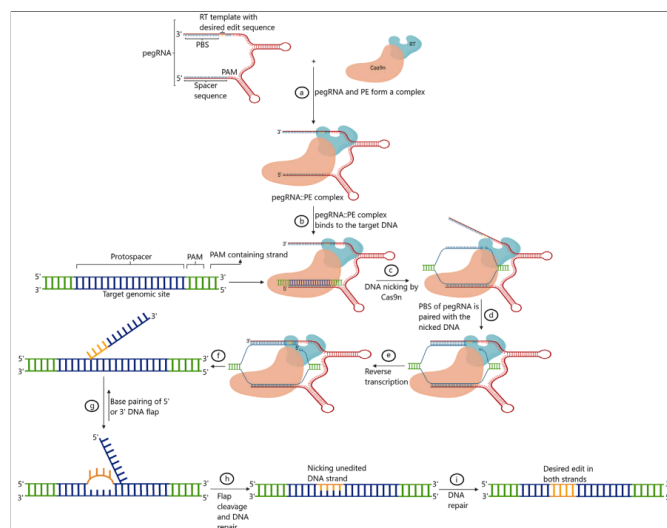
The development of Adenine Base Editors (ABEs) expanded base editing capabilities by enabling A:T to G:C conversions. ABEs utilize engineered TadA tRNA adenosine deaminases fused to dCas9 to convert adenine to inosine, which is interpreted as guanine during DNA replication (Anzalone *et al.*, 2020). Although ABEs primarily mediate A→G transitions, occasional C→G edits were observed (Kim *et al.*, 2019), leading to the development of C-to-G Base Editors (CGBE1) (Kurt *et al.*, 2020). CGBE1, derived from BE4max, combines a Cas9 nickase, rat APOBEC1 cytidine deaminase, and *Escherichia coli* uracil DNA N-glycosylase (eUNG). Notably, it enables C→G transversion mutations without inducing DSBs, representing the first base editor capable of this modification. Collectively, these advancements significantly enhance the precision and safety of genome editing, offering promising applications for correcting inherited genetic mutations and enabling targeted genomic modifications.

Prime editor

Reference: (Hassan *et al.*, 2020)

The gene editing tool known as "PE" stands for Prime Editing. It utilizes a catalytically impaired nCas9 (H840A) fused with a reverse transcriptase (RT-nCas9) and can be transfected alongside a prime editing guide RNA (pegRNA). Prime editing operates through a specific molecular mechanism. It starts by recognizing the DNA target with 20 nucleotides located at the 5' end of the pegRNA. The pegRNA also features a long 3' end that extends to interact with the

complementary strand of the target sequence. The RT-nCas9, with its RuvC nuclease domain, breaks the single-strand DNA. Subsequently, the tip of the 3' end of pegRNA, which includes a Primer Binding Site (PBS), aligns with the broken DNA strand. Here's where the magic happens: the RT-nCas9 utilizes the pegRNA template, containing the modification site upstream of the PBS, to synthesize a new DNA sequence (Anzalone *et al.*, 2019). Dr. David Liu's laboratory has effectively demonstrated how prime editing can introduce targeted insertions and deletions without the need for creating a double-strand break (DSB) in cells. Prime editing holds immense promise for gene editing, but it's important to note that this technology is still in its early stages, and further research is required to fully unlock its potential (Yan *et al.*, 2020).



Delivery techniques

CRISPR-Cas delivery methods can be categorized into two main groups: delivery modes and delivery carriers. There are three distinct CRISPR-Cas delivery modes, which include using the Cas protein as a ribonucleoprotein complex (RNP) along with the guide RNA, delivering the Cas protein and guide RNA through a plasmid encoding both, and using mRNA to facilitate Cas protein translation alongside a separate guide RNA.

Physical delivery methods

Microinjection

Microinjection is regarded as the "gold standard" for introducing CRISPR components into cells due to its high efficiency (Hori *et al.*, 2014). In this technique, individual cells can be directly injected with either plasmid DNA containing both the Cas9 protein and the sgRNA, mRNA containing Cas9 and sgRNA, or Cas9 protein paired with sgRNA. Using a microscope and a needle with a diameter ranging from 0.5 to 5.0 μm, the cell membrane is punctured, allowing the delivery of cargo directly to a specific location within the cell. This approach overcomes challenges associated with traversing extracellular matrices, cell membranes, and intracellular components. Moreover, microinjection isn't constrained by cargo molecular weight, which is a notable limitation in viral vector delivery systems. Additionally, this method permits precise control over the quantity of cargo delivered, thereby enhancing control over potential off-target effects.

Electroporation

This method employs pulsed high-voltage electrical currents to temporarily create tiny openings in the cellular membrane of cells that are suspended in a buffer. These openings, which are nanometer-sized pores, enable the entry of various components into the cell. Nucleofection is a technique based on electroporation, and it utilizes a specific combination of specialized solutions and precise electrical parameters to achieve the direct delivery of plasmid DNA into the nucleus of the cell. This process leads to an increase in gene expression.

Hydrodynamic delivery

Hydrodynamic delivery is an *in vivo* technique wherein a substantial volume of solution, equivalent to 8-10% of the body weight, containing the gene editing cargo is rapidly infused into an animal's bloodstream. This infusion generates a significant surge in hydrodynamic pressure, temporarily enhancing the permeability of endothelial and parenchymal cells. This allows cargo, which wouldn't typically traverse cellular membranes, to enter the cells. It's important to note that hydrodynamic delivery is primarily employed for *in vivo* applications because it relies on temporarily elevating pressure in a closed system to force cargo through otherwise impermeable barriers.

However, it's worth mentioning that the process of hydrodynamic delivery can be quite disruptive and may lead to potential physiological complications, such as cardiac dysfunction, increased blood pressure, and liver enlargement (Bonamassa *et al.*, 2011). This method also carries a risk of accidental mortality. Furthermore, the transfection rates achieved through hydrodynamic delivery tend to be very low, and it's effective only for certain types of cells.

Viral vector delivery methods

Adeno-associated virus (AAV)

Adeno-associated virus (AAV), a single-stranded DNA virus belonging to the *Dependovirus* genus of the *Parvoviridae* family, is widely used in gene therapy due to its high transduction efficiency and low immunogenicity and toxicity. In CRISPR/Cas9 applications, AAV serves as an efficient delivery vehicle through multiple strategies: (i) packaging SpCas9 and sgRNA in a single vector; (ii) delivering them separately using dual AAV co-infection; or (iii) employing a split-Cas9 system, where N⁻ and C-terminal fragments are delivered in separate AAV vectors.

Because of AAV's limited packaging capacity (~4.7 kb), smaller Cas9 variants are often preferred. *Staphylococcus aureus* Cas9 (SaCas9), approximately 70% the size of *Streptococcus pyogenes* Cas9 (SpCas9), retains comparable nuclease activity and enables single-vector delivery while leaving ~1 kb space for additional elements such as tags or multiple sgRNAs. Other compact Cas9 variants from *Streptococcus thermophilus* (Cong *et al.*, 2013) and *Neisseria meningitidis* (Esvelt *et al.*, 2013) are also promising for AAV-based delivery.

Alternative viral vectors such as lentivirus (LV) and adenovirus (AdV) can also deliver CRISPR components. Compared to AAV (~20 nm), LV and AdV particles are larger (80–100 nm) and offer greater packaging capacity, making them advantageous for delivering larger Cas9 constructs or multiple sgRNAs for multiplex genome editing.

Non-viral vector delivery vehicles

Lipid nanoparticles

Nucleic acids are inherently unstable when outside of cells, and due to their strong negative charge, they encounter difficulty passing through cell membranes. Nonetheless, one effective approach involves enclosing nucleic acids within highly positively charged liposomes, which facilitates their delivery into cells. It's important to note that lipid nanoparticles lack viral components, which is advantageous in mitigating safety concerns and potential immune reactions.

In a related development, Lee *et al.* (2017) also explored the use of gold nanoparticles (AuNPs) for delivering Cas9-sgRNA ribonucleoprotein complexes (RNPs). AuNPs are chemically inert and do not induce an immune response against the nanoparticles themselves, further contributing to their suitability for this purpose.

Production of gene edited farm animals

Zygote manipulation

Advancements in CRISPR/Cas9 genome editing have significantly enhanced our ability to precisely disrupt genes or introduce specific mutations through direct manipulation of zygotes. However, a signifi-

cant challenge that persists with this zygote manipulation approach is the issue of genetic mosaicism (Mehrvavar *et al.*, 2019). Mosaicism arises when DNA replication occurs before CRISPR-mediated genome editing, greatly reducing the likelihood of achieving a direct knockout (KO). The impact of mosaicism can be particularly problematic if it involves both somatic and germline mosaicism in the offspring. One proposed strategy to mitigate genetic mosaicism involves introducing CRISPR/Cas9 into either metaphase II (MII) oocytes or very early-stage zygotes. For instance, Hashimoto *et al.* in 2016 demonstrated the elimination of mosaic mutants through electroporation of Cas9 ribonucleoprotein complexes (RNPs) into early-stage zygotes.

Another potential tactic to reduce mosaicism is to shorten the lifespan of Cas9 by accelerating its degradation. This can be achieved by tagging Cas9 with signals that target it for ubiquitin-proteasomal degradation, thereby promoting its rapid breakdown. Alternatively, to completely eliminate the risk of mosaicism, one can consider the nuclear transfer approach, which involves using genome-edited cells for transferring the nucleus into an enucleated egg cell.

Somatic cell nuclear transfer

To achieve precise genetic modifications, the process typically begins with the introduction of targeted genetic changes into somatic cells, often fetal fibroblasts. After this, single-cell-derived colonies are isolated, and thorough screening is conducted to verify the presence of the desired genetic alterations within these cells. Subsequently, these modified cells serve as donor cells for somatic cell nuclear transfer (SCNT). This method offers a significant advantage compared to the zygote manipulation approach in the production of genetically engineered (GE) animals. This advantage arises from the fact that the entire animal is generated from a single GE donor nucleus, completely eliminating the risk of mosaicism (Polejaeva and Campbell in 2000).

Improving livestock production traits

This area of interest encompasses several key domains, including the enhancement of meat and fiber production, improvements in milk quality, reproductive performance, disease resistance, and animal welfare. Among these, myostatin (MSTN), a protein known to negatively regulate skeletal muscle mass (as demonstrated by McPherron *et al.* in 1997), has been a prominent target of gene editing. In livestock production, knocking out the MSTN gene offers a strategy for promoting muscle growth in animals. Myostatin knockout mice (McPherron *et al.*, 1997), exhibit roughly twice the skeletal muscle mass throughout their bodies, resulting from a combination of muscle fiber hyperplasia and hypertrophy. Successful MSTN gene disruption via gene editing has been reported in sheep, goats, and pigs, leading to enhanced animal growth performance (Deng *et al.*, 2014; Ni *et al.*, 2014; Wang K. *et al.*, 2015). Another notable target is whey protein β -lactoglobulin (BLG), a major milk allergen which is absent in human milk. CRISPR/Cas9 and zygote microinjection, as well as ZFNs gene editing and somatic cell nuclear transfer (SCNT), have been employed to create BLG knockout goats and cows (Zhou *et al.*, 2017; Sun *et al.*, 2018). BLG knockout goats, when compared to their wild-type counterparts, have demonstrated reduced levels of fat, protein, lactose, and solid not fat in their milk by 5.49%, 7.68%, 7.97%, and 7.7%, respectively.

In some instances, multiple genes have been targeted simultaneously, resulting in double or triple gene knockouts. For example, MSTN and FGF5 knockout goats were created to enhance both meat production and cashmere yield (Wang X. *et al.*, 2015a). Fibroblast growth factor 5 (FGF5), a protein that inhibits hair growth and is linked to the angora phenotype (long hair coat), was one of the targets. Recently, the concept of "surrogate sires" was validated for pigs, goats, and cattle (Ciccarelli *et al.*, 2020). This approach involves generating males with knockout of the NANOS2 gene using CRISPR/Cas9 editing, leading to germline ablation in the testes while retaining normal structural development. Subsequent spermatogonial stem cell transplanta-

tion (SSCT) using allogeneic donor stem cells resulted in sustained donor-derived spermatogenesis.

Improving health and welfare

Porcine reproductive and respiratory syndrome virus (PRRSV) is responsible for causing significant economic losses in swine production. (Yang *et al.*, 2018) demonstrated that pigs with a knockout (KO) of the CD163 gene are entirely resistant to viral infection. This resistance is evident through the absence of viremia, antibody response, high fever, or any other clinical signs associated with PRRS.

Similarly, research by Whitworth *et al.* in 2019 showed that pigs deficient in amino peptidase N (APN) are fully resistant to the transmissible gastroenteritis virus (TGEV). Combining these findings, double-gene-knockout (DKO) pigs, which have knockouts for both CD163 and pAPN receptor proteins, were found to be completely resistant to genotype 2 PRRSV and TGEV (Xu *et al.*, 2020).

Prion diseases, such as scrapie in goats or sheep and bovine spongiform encephalopathy (BSE) in cattle, are fatal infectious neurodegenerative disorders affecting the central nervous system (CNS). These diseases are associated with the misfolding of the normal cellular prion protein (PrP) (Prusiner, 1998). Accumulation of misfolded PrP disrupts cell function, leading to cell death. Studies involving the disruption of PrP expression in cattle have shown that these animals appear clinically, histopathologically, immunologically, and physiologically normal. Brain tissue homogenates from PrP knockout cattle are also resistant to prion propagation in vitro.

In the context of livestock management, the practice of dehorning horned cattle is associated with risks of injury to both the animals and farmers. Naturally occurring structural variants that result in polledness (lack of horns) are known in most beef cattle. Schuster *et al.*, 2020 isolated the Polled Celtic variant from the genome of an Angus cow and integrated it into the genome of fibroblasts obtained from a horned bull using the CRISPR/Cas12a system, followed by somatic cell nuclear transfer (SCNT).

Biomedical applications

The development of a wide range of genome editing tools has greatly expanded our ability to create genetically engineered (GE) livestock models. Pigs, in particular, have gained prominence in biomedical research due to their physiological and anatomical similarities to humans, which enable them to accurately mimic human disease symptoms. These GE pig models have found applications in various fields, with a particular focus on xenotransplantation. One approach involved the use of Zinc Finger Nucleases (ZFNs) to generate GE pigs lacking both alleles of the GGTA1 gene. This modification is significant for xenotransplantation, as it reduces the risk of immune rejection when pig organs are transplanted into humans.

GE pigs have been developed for biomedical purposes as immunodeficient models. These animals, lacking a functional immune system, are invaluable for studying disease mechanisms, immune responses to infections, vaccine development, and cell transplantation. Through the use of Transcription Activator-Like Effector Nucleases (TALENs), GE pigs lacking functional RAG2 were created (Lee *et al.*, 2014). These pigs were capable of supporting the proliferation and differentiation of transplanted induced pluripotent stem (iPS) cells. Beyond pigs, genome editing tools have also been applied to small ruminants in biomedicine. For example, the CRISPR/Cas9 system was utilized to disrupt the PDX1 gene in sheep, resulting in an apancreatic phenotype. This model has potential applications in growing human pancreas tissue through chimeric approaches.

Additionally, cystic fibrosis (CF) sheep models were generated using the CRISPR/Cas9 system. These models effectively recapitulated many of the symptoms seen in human CF patients (Fan *et al.*, 2018). These GE livestock models play a crucial role in advancing our understanding of various diseases and in developing potential therapies and treatments.

Conclusion

Genome editing in cattle has enormous potential to raise animal welfare, production, and health. However, current challenges include off-target effects, ethical concerns, public perception and acceptance of genome edited livestock products, editing complex traits (polygenic traits) and economic viability. Overusing genome editing techniques in livestock can reduce genetic diversity within populations. Reduced genetic diversity can make animals more vulnerable to diseases and environmental changes. Monitoring and assessing the health and well-being of edited animals over their lifetimes is essential to ensure that unintended consequences do not arise. Continued research and development in genome editing technologies, such as base editing and prime editing, may further enhance precision and reduce off-target effects. The adoption of genome editing in livestock may increase as the technology matures and as the need for sustainable and efficient food production continues to grow.

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