

Resview Article

CRISPR/Cas9 Genome Editing in Livestock: Mechanisms, Delivery, Applications, and Regulatory Considerations

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Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein 9 (Cas9) system has emerged as the most transformative genome-editing technology in the history of animal biotechnology. Since the demonstration of its programmable nuclease activity in mammalian cells in early 2013, CRISPR/Cas9 has been rapidly adopted in livestock research, enabling modifications in cattle, pigs, sheep, goats, and poultry with a speed and precision that was inconceivable with earlier zinc-finger nuclease (ZFN) or transcription activator-like effector nuclease (TALEN) platforms. This review provides a comprehensive examination of the CRISPR/Cas9 mechanism, including guide RNA design principles, PAM sequence requirements, double-strand break repair pathways (NHEJ and HDR), and the growing family of Cas9 orthologs and variants. Delivery methods applicable to livestock zygotes and somatic cells — including microinjection, electroporation, lentiviral vectors, lipofection, and AAV — are systematically compared with respect to efficiency, off-target activity, and practical feasibility. A comprehensive survey of CRISPR/Cas9 applications in livestock covering disease resistance (PRRS, African swine fever), production trait improvement (myostatin knockout, cashmere enhancement, polledness), biomedical modelling, and xenotransplantation is provided. Methods for detecting and quantifying off-target editing events are discussed, along with the implications of off-target activity for food safety. The review concludes with a comparative analysis of regulatory frameworks across major jurisdictions, highlighting the spectrum from the permissive (Argentina) to the precautionary (European Union), and discussing the science-policy interface challenges that will determine the pace at which CRISPR-edited livestock products enter commercial production and consumer markets.

Keywords: CRISPR/Cas9, Livestock genome editing, Pigs, Cattle, NHEJ, HDR, Guide RNA, Disease resistance, Xenotransplantation, Regulatory framework

1. Introduction

The ability to modify specific sequences in the genome of living organisms with high precision has been a central goal of molecular biology since the discovery of restriction enzymes in the 1970s. Early approaches to targeted genome editing in livestock— including pronuclear microinjection of transgenes, homologous recombination in embryonic stem cells (not available in most livestock species), and ZFN-mediated editing— were either imprecise, technically demanding, or severely limited in efficiency. TALENs represented a significant advance in programmability and reduced off-target activity, but their design-requiring engineering of new protein–DNA binding domains for each target remained a major barrier to widespread adoption.

The discovery that the type II CRISPR/Cas9 system from *Streptococcus pyogenes* could be reprogrammed to cleave any genomic sequence adjacent to a protospacer adjacent motif (PAM) by simply changing a 20-nucleotide guide RNA sequence (Cong et al., 2013; Mali et al., 2013) triggered an explosion of genome-editing activity across the biological sciences. The simplicity, low cost, and versatility of CRISPR/Cas9 democratized genome editing, bringing it within reach of research groups without specialist protein engineering expertise. In livestock, the first CRISPR/Cas9 studies began appearing in late 2013 and accelerated rapidly through 2014 and 2015, generating results that demonstrated both the technical feasibility and the remarkable biological specificity achievable in pigs, cattle, sheep, and goats.

This review provides a structured overview of CRISPR/Cas9 as applied to livestock species as of mid-2015, synthesising the key mechanistic, technical, and regulatory dimensions of this rapidly evolving field. We

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focus particularly on the aspects that distinguish livestock CRISPR applications from those in laboratory rodents, including the larger genome size, the necessity of working primarily through embryo editing or somatic cell nuclear transfer (SCNT) rather than germline-competent embryonic stem cells, and the complex regulatory and social licence considerations that attend the prospect of commercially producing gene-edited food animals.

2. Mechanism of CRISPR/Cas9

2.1 Guide RNA Design and PAM Requirements

The CRISPR/Cas9 system requires two components for site-specific cleavage: the Cas9 endonuclease and a single guide RNA (sgRNA) that provides target specificity through Watson-Crick base pairing with the genomic sequence complementary to the 20-nucleotide spacer of the sgRNA. Target recognition also requires the presence of a PAM sequence (5'-NGG-3' for the canonical SpCas9) immediately 3' of the target site in the non-template strand. Cas9 undergoes conformational activation upon sgRNA-directed target recognition, positioning the RuvC and HNH nuclease domains to introduce a blunt-ended double-strand break (DSB) three nucleotides upstream of the PAM.

Guide RNA design is critical for achieving high on-target editing efficiency with minimal off-target activity. Numerous sgRNA design tools (CRISPOR, Benchling, E-CRISP, Cas-OFFinder) have been developed that predict on-target efficiency based on empirically derived scoring matrices and flag potential off-target sites across the reference genome. Key design rules include avoiding sequences with runs of four or more thymidines (which can terminate RNA Pol III transcription of the sgRNA), ensuring GC content of 40–70%, and minimising mismatches with off-target

genomic sequences, particularly in the seed region (positions 1–12 adjacent to the PAM).

2.2 Repair Pathway Choice: NHEJ vs HDR

Following Cas9-mediated DSB induction, cellular DNA repair proceeds via one of two primary pathways. Non-homologous end joining (NHEJ), which predominates in somatic and post-fertilisation embryonic cells, religates the broken ends in an error-prone fashion, frequently generating small insertions or deletions (indels) at the cut site. When the targeted sequence encodes a protein-coding region, indels often cause frameshift mutations leading to premature stop codons and functional gene knockout. In contrast, homology-directed repair (HDR) utilises a supplied donor DNA template to introduce precise sequence changes, insertions, or substitutions. HDR is inherently less efficient than NHEJ and is restricted to the S/G2 phases of the cell cycle, making it particularly challenging in the predominantly G1-phase early embryo. Strategies to improve HDR efficiency in livestock cells include synchronisation of donor cells at G2/M, use of small-molecule HDR enhancers (M3814, NU7441), and delivery of templates as single-stranded oligodeoxynucleotides (ssODN) rather than double-stranded donor plasmids.

3. Delivery Strategies in Livestock

The choice of delivery method for CRISPR/Cas9 components in livestock depends on whether editing is being performed directly in zygotes (producing founder animals without SCNT) or in somatic cells (that will serve as nuclear donors for SCNT). Table 2 summarises the major delivery approaches with their relative performance characteristics.

Table 2. Comparison of CRISPR/Cas9 delivery methods for livestock genome editing, with efficiency and off-target characteristics

Delivery Method	Efficiency	Off-target Risk	In Zygote?	Key Advantage
Microinjection (plasmid DNA)	Moderate	Moderate	Yes	Standard; widely validated
Microinjection (RNP complex)	High	Low	Yes	Transient expression; gold standard
Zygote electroporation	High	Low	Yes	High throughput; no injection skill
Lentiviral vector	High	High	Via SCNT donor	Stable integration; insertional risk
Lipofection (somatic cells)	Moderate	Moderate	Via SCNT donor	Simple; for somatic cell editing
AAV vector delivery	Moderate-High	Low	Via SCNT donor	Tissue-specific tropism; size limited

Direct zygote microinjection of Cas9 mRNA or protein and sgRNA has been the most widely validated approach, producing edited founder animals in cattle, pigs, and sheep. Cytoplasmic or pronuclear injection of ribonucleoprotein (RNP) complexes-Cas9 protein pre-assembled with sgRNA-is now considered best practice because the transient residence time of the RNP substantially reduces off-target editing relative to

plasmid delivery, which leads to prolonged Cas9 expression. Electroporation of zygotes with RNP, pioneered in mice and rats, has been successfully adapted to bovine and porcine zygotes, offering higher throughput than microinjection without requiring the expensive micromanipulation equipment and specialist skills that injection demands (Tanihara et al., 2016).

4. Applications in Livestock Species

Table 1 provides a comprehensive survey of CRISPR/Cas9 editing studies in major livestock species

reported through 2016, illustrating the breadth of biological targets and phenotypic outcomes achieved.

Table 1. Selected CRISPR/Cas9 genome editing studies in major livestock species and their phenotypic outcomes

Species	Target Gene	Edit Type	Resulting Phenotype	Reference
Pig	GGTA1	KO	Reduced xenoreactivity	Petersen et al., 2016
Pig	CD163	KO	Complete PRRS resistance	Whitworth et al., 2016
Pig	PERV (62 loci)	KO (multiplex)	PERV-free pig for xenotransplantation	Yang et al., 2015
Cattle	POLLED locus	KI	Hornless dairy cattle	Carlson et al., 2016
Cattle	MSTN	KO	Increased muscle mass	Lian et al., 2015
Sheep	MSTN	KO	Double-muscling phenotype	Wang et al., 2015
Goat	FGF5	KO	Increased cashmere fibre length	Wang et al., 2016
Chicken	DDX4/DAZL	KO	Germ cell disruption model	Oishi et al., 2016

KO = knockout (gene disruption by NHEJ-induced indels); KI = knock-in (precise sequence replacement by HDR); MSTN = myostatin; FGF5 = fibroblast growth factor 5; PERV = porcine endogenous retrovirus; PRRS = porcine reproductive and respiratory syndrome.

4.1 Disease Resistance

Disease resistance represents the most immediately commercially relevant application of CRISPR/Cas9 in livestock. Porcine reproductive and respiratory syndrome (PRRS) causes annual losses estimated at over \$660 million in the United States alone. PRRSV entry into macrophages requires CD163 as a receptor. Whitworth et al. (2016) demonstrated that CRISPR/Cas9 knockout of CD163 produced pigs completely resistant to both PRRSV Type 1 and Type 2 infection, with no adverse phenotypic effects, providing proof of concept for a revolutionary approach to PRRS control that could eliminate the need for repeated vaccination.

4.2 Production Trait Improvement

Myostatin (MSTN), a member of the TGF-beta superfamily, negatively regulates skeletal muscle mass. Natural loss-of-function mutations in MSTN are responsible for the double-muscling phenotype in Belgian Blue and Piedmontese cattle. CRISPR/Cas9-mediated MSTN disruption in sheep (Wang et al., 2015) and goats recapitulated this phenotype, producing animals with significantly increased muscle mass that could have commercial applications in lean meat production. In cattle, Carlson et al. (2016) demonstrated precise knock-in of the POLLED Celtic allele-which naturally prevents horn development-into the genome of Holstein bulls using CRISPR/Cas9,

offering a pathway to eliminating the welfare and labour cost associated with dehorning in the dairy industry.

5. Off-Target Effects and Safety Considerations

A critical safety requirement for CRISPR/Cas9-edited livestock intended for the food supply is comprehensive characterisation of off-target editing events. Off-target cleavage is driven by imperfect sgRNA-DNA complementarity, particularly when mismatches are located outside the seed region. Detection methods range from in silico prediction followed by PCR/Sanger sequencing of predicted off-target sites, to unbiased whole-genome approaches including GUIDE-seq, Digenome-seq, CIRCLE-seq, and whole-genome sequencing (WGS) at 30x or higher coverage.

Comprehensive off-target analyses published to date in livestock suggest that RNP delivery with well-designed sgRNAs produces very low frequencies of detectable off-target editing, often below the sensitivity threshold of standard detection methods. However, given the potential for heritable off-target mutations to propagate through breeding, regulatory agencies require exhaustive off-target characterisation data before approving genome-edited livestock. High-fidelity Cas9 variants including eSpCas9 (1.1), SpCas9-HF1, HypaCas9, and evoCas9 offer substantially reduced off-target activity with largely maintained on-target efficiency and represent best practice for production-intent livestock editing.

Table 3. Comparison of CRISPR/Cas9 nuclease variants and their applications in livestock genome editing

Nuclease System	PAM Sequence	DSB Type	Livestock Relevance
SpCas9 (<i>S. pyogenes</i>)	NGG	Blunt 3-bp upstream	Most widely used; large toolbox; well validated
SaCas9 (<i>S. aureus</i>)	NNGRRT	Blunt	Smaller size; better for AAV delivery in livestock
Cas9 nickase (D10A)	NGG	Nick only	Paired nickases reduce off-targets ~1,500-fold
dCas9 (dead)	NGG	None	CRISPRa/i gene regulation without editing
Cas12a/Cpf1 (<i>AsCpf1</i>)	TTTV	5-nt 5' overhang	T-rich PAM; staggered cuts; useful for AT-rich regions
Base editors (BE3/ABE)	NGG	None (nick only)	Precise C>T or A>G conversions without DSB

Step 1: Target site selection + sgRNA design (CRISPOR/Benchling) → Step 2: Cas9-sgRNA RNP assembly → Step 3: Delivery (microinjection/electroporation into zygotes, or lipofection into somatic cells for SCNT) → Step 4: Embryo culture/transfer → Step 5: Founder genotyping (PCR + Sanger/NGS) → Step 6: Off-target analysis (WGS/GUIDE-seq) → Step 7: Breeding to homozygosity → Step 8: Phenotypic characterisation

Figure 1. Workflow for CRISPR/Cas9-mediated genome editing in livestock, from target design through founder animal production and phenotypic validation.

6. Regulatory Landscape

The regulatory treatment of genome-edited livestock varies dramatically across jurisdictions, creating a complex global landscape for researchers and commercial developers. In the United States, the FDA has asserted jurisdiction over genome-edited animals as 'new animal drugs' under the Federal Food, Drug and Cosmetic Act, applying the same pre-market approval pathway to a small CRISPR indel as to a transgenic animal expressing a foreign gene—an approach widely criticised by the scientific community as disproportionate. Under this framework, even edits that could theoretically have arisen through natural mutation or conventional breeding require extensive safety data and regulatory review before commercialisation.

Argentina presented a contrasting model in 2015, issuing Resolution 763/2011 criteria that exempt genome-edited plants and animals from GMO regulations provided no foreign DNA from a different species is inserted. This approach, grounded in product characteristics rather than process, has been adopted or adapted by Brazil, Chile, and Australia. The European Union, under its 2001/18/EC GMO Directive and a 2018 European Court of Justice ruling, currently treats genome-edited organisms as GMOs regardless of edit type, imposing stringent pre-market safety assessments, labelling requirements, and extensive post-market monitoring. This regulatory divergence creates significant competitive asymmetry in agricultural biotechnology development.

7. Conclusions

CRISPR/Cas9 has established itself as the dominant platform for livestock genome editing, offering

capabilities—including simultaneous editing of multiple genes, precise base changes, and large structural modifications—that were practically unachievable with prior tools. The applications already demonstrated in pigs and cattle for disease resistance, production trait enhancement, and xenotransplantation illustrate the transformative potential of the technology for animal biotechnology. Realising this potential in commercial production systems will require continued improvement in editing efficiency and HDR frequency, comprehensive off-target characterisation, and most critically, the development of evidence-based, internationally harmonised regulatory frameworks that enable responsible innovation while maintaining appropriate safety oversight.

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