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Integration Stability and Phenotypic Regulation in Genetically Engineered Livestock from a Molecular Ecology Perspective

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Abstract This study systematically analyzes the genetic stability and phenotypic evaluation framework of transgenic livestock, providing a comprehensive overview of key technologies for phenotypic data collection and analysis. It focuses on the integration patterns of exogenous genes within host genomes, the effects of copy number variation, and the stability of gene expression across generations. Through case studies on transgenic cattle and pigs, the research reveals a strong correlation between genetic stability and phenotypic consistency. The results indicate that combining non-viral vectors with gene-editing technologies not only facilitates long-term stable gene expression but also effectively ensures physiological health and production reliability in livestock. This study offers a solid theoretical foundation and methodological support for the safety assessment and industrial application of transgenic livestock.

Keywords Transgenic livestock; Genetic stability; Phenotypic evaluation; Gene editing; Multi-omics integration

1 Introduction

With the rapid advancement of molecular biology and genetic engineering technologies, transgenic technology has become an essential tool in modern livestock breeding. It offers unprecedented opportunities to improve production performance, disease resistance, and the quality of animal products (Wheeler and Walters, 2001; Laible et al., 2015; Hryhorowicz et al., 2020). By introducing exogenous genes into animal genomes, scientists can endow livestock with new economic traits, such as accelerated growth rate, improved feed conversion efficiency, enhanced disease resistance, or superior product quality (Laible et al., 2015; Shaukat, 2021). For instance, transgenic cows can produce milk enriched with specific nutrients, while transgenic pigs are widely used in human disease modeling and xenotransplantation studies (Hryhorowicz et al., 2020; Park, 2023). These groundbreaking achievements have not only transformed livestock production systems but also provided new insights into biomedicine, food safety, and ecological sustainability. However, the research and application of transgenic livestock face critical scientific challenges related to genetic stability and phenotypic consistency, which pose obstacles at the technical, ethical, and regulatory levels (Laible et al., 2015; Eriksson et al., 2018).

Genetic stability serves as the foundation of transgenic livestock research, determining the accuracy and long-term controllability of exogenous gene transmission across individuals and generations (Van Cott et al., 1997; Yum et al., 2018). If the insertion site of an exogenous gene in the chromosome is unstable or if copy number variations and epigenetic modifications occur, this may lead to gene silencing, expression deviation, or undesirable phenotypes, thereby compromising the reliability of research results and the predictability of animal performance (Pursel et al., 1989; Evangelou et al., 2018). For example, studies have shown that certain transgenic pigs initially exhibit the desired disease resistance, but this trait diminishes in subsequent generations, suggesting that the exogenous gene may be influenced by epigenetic regulation or genomic rearrangement (Yum et al., 2018). Similar phenomena have been observed in transgenic sheep and goats, where expression levels of milk protein genes vary among individuals (Van Cott et al., 1997; Evangelou et al., 2018). Therefore, systematic evaluation of genetic stability in transgenic livestock not only helps elucidate the integration behavior of exogenous genes within host genomes but also provides theoretical guidance for improving gene-editing strategies and transformation efficiency (Laible et al., 2015; Wang et al., 2022).

Phenotypic consistency is a crucial indicator for assessing the practical value of transgenic livestock. Even if the insertion and expression of an exogenous gene are relatively stable, inconsistent phenotypic manifestations across different environments, sexes, or genetic backgrounds can undermine both the scientific significance and application potential of the transgenic line (Van Cott et al., 1997; Evangelou et al., 2018). Phenotypic consistency encompasses multiple aspects, including physiological metabolism, reproductive capacity, immune response, and behavioral characteristics. Through systematic phenotypic assessment, researchers can determine whether the introduced gene functions as intended and whether it exerts unintended effects on animal health or growth. For instance, although certain transgenic cows maintain stable gene expression, their milk composition fluctuates abnormally, suggesting that environmental factors and gene network regulation play critical roles in phenotype formation (Yum et al., 2018; Yum et al., 2024). Establishing a standardized phenotypic evaluation system is therefore essential for promoting the transition of transgenic livestock from laboratory research to industrial application (Hryhorowicz et al., 2020; Park, 2023).

This study aims to systematically investigate the methods for evaluating genetic stability and phenotypic performance in transgenic livestock lines, elucidating their intrinsic relationship and implications for breeding practices. By summarizing mainstream detection technologies, analyzing stability variations under different transgenic strategies, and integrating genetic and phenotypic data from representative cases (such as transgenic cattle and pigs), this study seeks to construct a scientific and reproducible evaluation framework. Through this systematic analysis, the study aspires to advance transgenic livestock research toward greater scientific rigor and application sustainability, thereby providing robust support for the modernization of animal husbandry and innovation in life sciences.

2 Transgenic Livestock Technologies

2.1 Common transgenic methods

The construction of transgenic livestock relies on various efficient and stable technologies for introducing and expressing foreign genes. Among them, pronuclear microinjection was the first method to be widely used. This technique involves directly injecting target DNA into the pronucleus of a fertilized egg, allowing the exogenous gene to integrate randomly into the host genome and thereby producing transgenic individuals (Robl et al., 2007).

This approach is relatively simple to perform and applicable to a wide range of species, laying the foundation for the creation of early transgenic animal models such as pigs, cattle, and sheep (Niemann and Kues, 2003). However, due to its uncontrolled integration sites, high mosaicism rates, and significant variability in gene expression, its transgenic efficiency remains low—only about 1%-2%—which limits its suitability for modern commercial breeding applications (Robl et al., 2007).

To overcome these limitations, researchers developed viral vector–mediated transduction. This method employs vectors such as lentiviruses or retroviruses to deliver target genes into the host genome. Viral vectors exhibit a high integration rate and broad host range, allowing for tissue-specific expression of transgenes.

In the past decade, the advent of genome editing technologies has brought revolutionary progress in transgenic livestock production. Among these, the CRISPR/Cas9 system has emerged as the mainstream tool due to its simplicity, low cost, and ability to perform multi-site genome editing. This technique uses an RNA-guided nuclease to induce double-strand breaks at target sites, enabling precise insertion of exogenous genes or knockout of endogenous genes through homology-directed repair (HDR) (Table 1).

Additionally, somatic cell nuclear transfer (SCNT) is often used in combination with the CRISPR system to clone embryos that have been successfully edited (Robl et al., 2007). This integration of techniques significantly improves transgenic efficiency and provides a more reliable model for studying phenotypic stability and genetic consistency in transgenic livestock.

Table 1 Comparison of different transgenic vector methods

Method	Efficiency	Control of Integration Site	Safety Risk	Typical Applications
Microinjection	Low	Random	Risk of uncontrolled insertion sites	Early models of mice, pigs, and cattle
Viral Vector	Medium	Random but stable integration	Risk of insertional mutagenesis	Transgenic large animals
Gene Editing (CRISPR)	High	Site-specific	Controllable off-target risk	Disease-resistant pigs, transgenic cattle, chickens

2.2 Major application areas

Transgenic livestock technology demonstrates broad and profound application potential in both agriculture and biomedicine. In growth performance improvement, the introduction of genes regulating growth hormone (GH) or insulin-like growth factor (IGF) can significantly enhance animal growth rate and feed conversion efficiency (Niemann and Kues, 2003). In disease resistance enhancement, researchers have successfully developed livestock resistant to specific pathogens by introducing antiviral or immune-related genes. For instance, transgenic cattle carrying the NRAMP1 gene exhibit remarkable resistance to tuberculosis, while pigs expressing interferon show improved defense against viral infections compared with conventional breeds.

In terms of product quality optimization, transgenic techniques can regulate metabolic pathways involving milk proteins, fatty acids, and amino acids, enabling the production of dairy and meat products with higher nutritional value and enhanced functionality (Wheeler and Walters, 2001).

Moreover, transgenic livestock are widely used as animal bioreactors for the efficient production of pharmaceutical proteins. Cows and goats capable of expressing recombinant human antithrombin III, insulin, or human serum albumin in their milk have become essential platforms for the continuous production of high-value biopharmaceuticals (Bertolini et al., 2016).

2.3 Ethical and regulatory challenges arising from technological development

Despite the significant scientific and economic value brought by transgenic livestock technology, its development continues to raise complex ethical debates and regulatory challenges.

On the ethical level, public concerns focus on issues such as animal welfare, the naturalness of life, and potential health risks (Eriksson et al., 2018). Early studies revealed that some transgenic animals exhibited reduced fertility and immune system disorders during experiments, sparking ethical reflections on the boundaries of biotechnological intervention in living organisms.

On the regulatory level, policy orientations differ significantly across countries. The United States and Europe typically adopt risk-based regulatory frameworks, emphasizing the properties and safety of the final product; whereas Asian regions tend to prioritize process-based supervision and ethical review (Bertolini et al., 2016). In China, research and commercialization of transgenic livestock are approached with prudence, following the guiding principle of “scientific research first, regulation in parallel”, which emphasizes both technological innovation and strict oversight.

3 Theoretical Basis of Genetic Stability

3.1 Definition and importance of genetic stability

Genetic stability refers to the ability of an exogenous gene to maintain structural integrity, a relatively constant level of expression, and stable transmission to offspring according to genetic laws across generations within the host genome. Its connotation encompasses three aspects: structural stability (absence of unintended mutations, rearrangements, or deletions), expression stability (absence of sustained silencing or abnormal fluctuations), and transmission stability (following Mendelian segregation and predictable inheritance in populations) (Yum et al., 2018; Yum et al., 2024).

For transgenic livestock, genetic stability not only determines the reproducibility and controllability of experimental outcomes but also directly affects the feasibility and safety of breeding and industrial application (Yum et al., 2018; Yum et al., 2024). Long-term population tracking has indicated that livestock produced via non-viral integration strategies such as transposon systems exhibit no significant accumulation of somatic mutations, abnormal copy number variations, or telomere anomalies after years of breeding and multigenerational propagation. This suggests that such methods maintain high levels of genomic integrity and physiological health (Yum et al., 2018; Yum et al., 2024).

3.2 Effects of insertion site and copy number on stability

The insertion site and copy number are critical determinants of genetic stability and predictable gene expression (Table 2). Random integration methods—such as pronuclear microinjection or certain viral vectors—are prone to position effects. When exogenous genes are inserted into heterochromatic regions, repetitive sequences, or areas near active transposable elements, they often experience epigenetic silencing, expression drift, or integration rearrangements. In contrast, integration into genomic safe harbors can substantially reduce insertional mutagenesis risks and enhance expression stability.

Regarding copy number, while high copy numbers may initially increase expression levels, they also elevate the likelihood of homologous recombination or tandem repeat-induced instability and silencing. Conversely, low copy numbers, particularly single-copy targeted insertions, favor long-term stable expression and predictable inheritance. Studies on transgenic goats and cattle have demonstrated that expression levels are not linearly correlated with copy number, indicating that local chromatin environment, promoter selection, and epigenetic modifications are equally crucial in maintaining genetic stability (Yum et al., 2018; Yum et al., 2024).

Table 2 Comparison of the effects of insertion site and copy number on genetic stability and expression

Factor	Favorable Conditions	Unfavorable Conditions	Main Risks	Countermeasures and Recommendations
Insertion Site	Euchromatin regions, genomic safe harbors, distant from key regulatory areas	Heterochromatin, repetitive sequences, regions near active transposons	Position effect, gene silencing, structural variation	Select genomic safe harbors; perform breakpoint sequencing; combine with homologous recombination repair
Copy Number	Single-copy or low-copy targeted integration	High-copy tandem insertions	Recombination, silencing, expression drift	Use single-copy knock-in (KI); perform ddPCR/qPCR quantification; optimize expression control
Regulatory Elements	Species-matched promoters, use of insulators/barriers	Heterologous strong promoters without protective elements	Epigenetic silencing, abnormal histone modifications	Introduce insulators; apply site-specific enhancer strategies

3.3 Molecular mechanisms of exogenous gene inheritance and potential variation risks

The inheritance of exogenous genes in livestock populations follows Mendelian laws, and their stability is influenced by multiple factors such as integration mechanisms, epigenetic regulation, and the host genomic environment. Transposon systems represented by Sleeping Beauty and PiggyBac integrate through a “cut-and-paste” mechanism, tending to insert into non-coding or low-risk regions, thereby reducing the potential hazards of insertional mutagenesis and ensuring the stable transmission of exogenous genes in the germline. Whole-genome sequencing and copy number variation analyses have shown that this type of integration has minimal impact on global genomic stability indicators such as SNP profiles, CNV, and telomere length (Yum et al., 2018).

The potential risks are mainly reflected in three aspects. In terms of structural variation, if the integration site is close to coding regions or key regulatory elements, it may lead to large fragment deletions, rearrangements, or functional disturbances. In terms of epigenetic silencing, changes in promoter methylation or histone modifications may cause intergenerational decreases in expression levels or even gene inactivation. Regarding

positional effect variation, changes in local chromatin plasticity may lead to expression differences among individuals or generations.

To address these issues, researchers have proposed a variety of optimization strategies, including using safe harbor targeting and breakpoint sequencing to ensure integration site safety, adopting single-copy targeted knock-in techniques to reduce structural interference risks, introducing insulator or barrier sequences to prevent epigenetic silencing, selecting promoters that match the host species, and conducting long-term population tracking to verify genetic stability. Long-term follow-up results show that livestock lines established following these design principles generally achieve stable inheritance and reproducible expression of exogenous genes, and no significant negative effects on animal health or genomic integrity have been observed (Yum et al., 2018; Yum et al., 2024).

4 Theoretical Framework and Indicator System for Phenotypic Evaluation

4.1 Significance of phenotypic evaluation in transgenic research

Phenotypic evaluation is the core link connecting genotype, expression, and functional traits, and it is a key method for verifying the biological effects and application value of exogenous genes. The goal of transgenesis is not only to achieve stable integration and expression of exogenous genes but also to continuously and reproducibly achieve the expected trait improvements (such as growth performance, disease resistance, and product quality). Therefore, it is necessary to conduct systematic, quantitative, and long-term phenotypic tracking of individuals and populations to identify expected effects and possible unintended effects (off-target/pleiotropy), and to provide evidence for safety and ethical assessments.

With the development of high-throughput and digital technologies, phenotypic evaluation is evolving from traditional single-trait observation toward multimodal integration (imaging phenomics, metabolomics/proteomics, wearable/environmental sensing, and behavioral monitoring), and combined modeling with genomic information, thereby improving the accuracy of breeding selection and enhancing the depiction of environmental adaptability and animal welfare.

4.2 Common phenotypic indicators: growth rate, physiological metabolism, reproductive performance, disease resistance

The phenotypic indicators of transgenic livestock vary according to target traits and application needs, typically covering five aspects: growth, metabolism, reproduction, health and disease resistance, and welfare and environmental adaptability (König and May, 2019).

For growth rate and body conformation, common indicators include body weight, average daily gain (ADG), feed conversion ratio (FCR), skeletal development, and body composition. For genetic modifications related to the GH/IGF axis and muscle development, the focus should be on verifying energy utilization efficiency and tissue development characteristics (Pinkert, 2014). The introduction of imaging measurement and automatic weighing technologies makes dynamic monitoring more accurate and objective, effectively reducing human error.

In terms of physiological metabolism and endocrinology, energy metabolism indicators such as blood glucose, insulin, cholesterol, and lipid profiles can be monitored, as well as endocrine levels including GH, IGF, and the thyroid axis. Combined with physiological parameters of organs such as liver enzymes and kidney function, and metabolomic and proteomic analyses, these help assess the remodeling of metabolic pathways and potential health risks caused by transgenic manipulation (Pinkert, 2014).

For reproductive performance and offspring traits, indicators such as age at first mating, estrous cycle, semen quality, conception rate, embryo survival and implantation rate, litter size, and lactation ability are examined to assess the impact of exogenous genes on the reproductive axis and their intergenerational inheritance effects.

In terms of disease resistance and immune response, for transgenic modifications aimed at improving disease resistance (such as antiviral, immune regulation, or pathogen recognition receptors), immunological tests

(antibody titer, cytokine profile), pathogen challenge experiments, and transcriptomic analyses can be combined to comprehensively assess infection rate, recovery time, and survival rate (König and May, 2019).

In animal welfare and adaptability, behavioral performance, stress physiological indicators (such as cortisol level), heat stress scores, feeding patterns, and activity rhythms are monitored to prevent excessive pursuit of productivity at the expense of animal welfare, while meeting the ethical concerns of regulators and the public (König and May, 2019).

4.3 Environmental influences on phenotypic expression and their control

The environment is a key external variable determining the reproducibility and generalizability of phenotypic traits. Genotype × Environment interactions (G×E) can cause the same genetic modification to exhibit heterogeneous performance under different ecological and management systems (Figure 1).

Common influencing pathways include: heat and humidity stress altering energy allocation and feed intake; diet composition regulating lipid metabolism and immunity; and density and light exposure affecting reproductive hormones and behavior. To ensure scientific validity and comparability, randomized block or stratified designs should be adopted during the design stage, and continuous records of temperature, humidity, feed composition, pathogen exposure, and stress levels should be maintained. During statistical analysis, covariate correction and stratified analysis should be introduced to ensure scientific rigor and comparability.

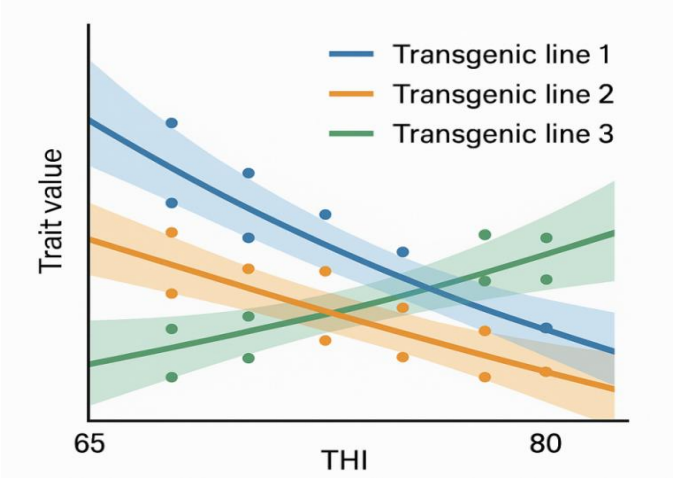

Figure 1 Reaction norms of key traits across THI gradients with random regression fits

5 Phenotypic Evaluation Methods for Transgenic Livestock

5.1 Principles of experimental design: control group setup and sample size

For the setup of control groups, non-transgenic individuals of the same breed, age, and management conditions should be selected as negative controls to minimize interference from genetic background and environmental factors. When studies involve different transgenic constructs, multiple treatment groups should be established, including a vector control, to identify potential nonspecific effects caused by regulatory elements. To further reduce environmental variation, it is recommended to use randomized grouping and block design, maintaining consistency in feeding management, housing density, lighting conditions, and disease prevention measures.

In determining sample size, a balance should be achieved between statistical power and experimental feasibility. Too small a sample size may lead to insufficient statistical power and high variability in results, while excessively large sample sizes may waste resources. For transgenic livestock, especially in large animal experiments, power analysis should be conducted based on the expected effect size, significance level (α), and statistical power (1-β) to determine a reasonable sample size. For high-dimensional studies such as metabolomics, simulation-based sample optimization methods can be used, combined with hybrid sampling strategies that integrate random sampling and extreme phenotype sampling, thereby improving parameter estimation and model prediction accuracy.

5.2 Phenotypic data collection techniques: imaging measurement, metabolomics, and behavioral analysis

Phenotypic data collection is rapidly evolving from traditional manual measurement to high-throughput, automated, non-invasive, and multimodal integration approaches. This transformation has greatly enhanced data accuracy, efficiency, and reproducibility, providing a solid foundation for the systematic phenotypic characterization of transgenic livestock.

In imaging measurement, RGB and depth cameras, laser scanning, structured light, and 3D reconstruction technologies are widely used for body size measurement, weight estimation, and surface or muscle thickness analysis. CT and MRI can analyze tissue distribution and fat deposition patterns. When combined with computer vision algorithms such as convolutional neural networks (CNNs), these technologies enable automatic landmark recognition and phenotypic feature quantification, reducing human error and improving assessment efficiency.

In metabolomics and biochemical detection, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) can be used to analyze dynamic changes in energy metabolism, lipid synthesis, and amino acid utilization in samples such as blood, urine, and milk. Combined with transcriptomic and proteomic data, these methods can construct metabolic pathway networks, revealing potential systemic metabolic effects induced by genetic modifications.

In behavioral and physiological monitoring, video tracking systems and wearable sensors enable continuous recording of feeding, rumination, activity rhythms, social behavior, and stress responses. Monitoring heart rate, body temperature, and respiration rate provides quantifiable indicators of livestock adaptability and recovery speed under environmental changes or pathogen exposure, offering multidimensional data support for health and welfare assessment.

5.3 Data analysis and statistical models: multivariate regression, genetic and environmental interaction analysis

The analysis of multidimensional high-throughput data requires an integrated statistical framework to effectively extract genetic signals and reveal the patterns of gene–environment interaction (G×E).

In multivariate analysis, when multiple correlated phenotypes are studied, multivariate regression, canonical discriminant analysis, or stepwise discriminant analysis can be applied for joint modeling. These methods enhance the detection power of quantitative trait loci (QTLs) and gene effects by accounting for trait correlations. For datasets with nonlinear relationships or strong collinearity, partial least squares regression (PLSR) or principal component regression (PCR) can be used for dimensionality reduction before further analysis to reduce noise and improve model stability.

In gene–environment interaction studies, linear mixed models (LMMs) and random regression models (RRMs) are widely used to analyze longitudinal data and multi-environment experimental results. These models describe reaction norms of gene effects across environmental gradients or time, thus revealing genotype performance differences and adaptive traits under varying environments.

In multilevel and machine learning modeling, for complex high-dimensional data such as imaging, metabolomics, and behavioral datasets, algorithms such as random forest (RF), support vector machine (SVM), and deep learning can be applied to capture nonlinear patterns and higher-order interactions. These models enable individual-level trait prediction and cluster analysis. To prevent overfitting, cross-validation and independent external validation sets should be incorporated to evaluate model performance and ensure robustness.

6 Correlation Analysis Between Genetic Stability and Phenotypic Consistency

6.1 Relationship models between stability and phenotypic variation

Genetic stability — the structural integrity and consistent expression of exogenous genes across generations — is the foundation for achieving phenotypic consistency. When copy number and integration sites remain stable over generations and expression variance is low, the intergroup dispersion of target traits decreases significantly; conversely, rearrangements, copy number drift, or epigenetic silencing can amplify phenotypic fluctuations (Van Cott et al., 1997).

At the statistical level, multivariate regression, structural equation modeling (SEM), and the animal model can be used to decompose phenotypic variance into genetic and non-genetic components, jointly assessing indicators of genetic stability (such as integration site consistency, single-copy targeting, and expression variance) and phenotypic uniformity. Cross-species evidence also suggests a significant correlation between the stability of gene expression in founder generations and the conservation of phenotypes in progeny — lineages with more stable expression tend to exhibit lower intergenerational phenotypic diversity.

In animal populations, this relationship often manifests as a negative correlation between phenotypic variance and genotypic consistency. For example, in transgenic pig lines, if the copy number and expression level of an exogenous growth hormone gene vary greatly among individuals, the coefficients of variation (CV) for weight gain rate and fat ratio increase significantly. Conversely, when integration sites are fixed and expression is stable, the phenotypic distribution becomes more concentrated. Furthermore, environmental factors (E) can be incorporated into a three-dimensional relationship model:

$$P = G + E + G \times E$$

where G represents genetic stability factors and G × E represents gene-environment interaction effects.

By analyzing variance components across generations, the contribution of genetic instability to phenotypic inconsistency can be quantified, allowing the prediction of trait performance trends of exogenous genes in breeding populations.

6.2 Effects of different transgenic integration sites on phenotypic consistency

The chromatin environment and local regulatory network of the integration site determine expression robustness and inter-individual consistency. A single, well-defined integration site with single-copy expression usually corresponds to more stable recombinant protein yields and lower variability among individuals (Van Cott et al., 1997). Conversely, random integration often causes position effects. If located near coding regions or key regulatory elements, it may cause insertional mutations or expression drift, thereby increasing phenotypic uncertainty.

By inserting exogenous genes into genomic safe harbors and using insulator sequences, position effects can be mitigated, neighboring chromatin influence reduced, and phenotypic consistency improved.

6.3 Molecular links between gene expression regulation and phenotypic stability

At the transcriptional regulation level, selecting appropriate tissue-specific or host-compatible promoters and optimizing the arrangement of regulatory elements can reduce spatiotemporal fluctuations in gene expression, improving the predictability and stability of transgene expression.

At the epigenetic level, DNA methylation status and histone modification patterns determine how gene expression is maintained over time. During generational transmission, accumulation of methylation may cause gene silencing, while the inclusion of insulator or barrier sequences during construct design can effectively block the influence of unfavorable chromatin environments, thereby delaying or preventing silencing.

At the post-transcriptional and translational regulation level, interactions between miRNAs and mRNAs, as well as RNA-binding protein (RBP)-mediated regulation, affect mRNA stability and protein yield. By optimizing sequences and adjusting codon usage, the risk of transgene sequences being targeted by endogenous miRNAs can be reduced, minimizing expression fluctuations and ensuring stable product output.

Genomic structural variations (CNVs and SVs) also play a key role in phenotypic consistency. These variations alter gene dosage and regulatory network structures, directly influencing key economic traits such as growth rate, muscle development, and reproductive performance. Within regulatory networks, redundant elements such as shadow enhancers can maintain target gene expression when disturbances occur, enhancing overall phenotypic robustness.

By integrating genomics, transcriptomics, and epigenomics data and combining them with eQTL analysis, researchers can systematically identify cis- and trans-regulatory loci contributing to expression stability and phenotypic consistency. This multi-omics approach provides molecular-level scientific evidence for screening genetically stable lineages and evaluating the long-term reliability of transgenic livestock.

7 Case Analysis: Genetic Stability and Phenotypic Studies in Transgenic Cattle and Pigs

7.1 Case background: representative experiments and commercial transgenic livestock projects

Cattle and pigs hold significant value in agriculture and biomedicine and are among the most representative species in transgenic livestock research. Transgenic cattle research focuses on optimizing milk composition and expressing exogenous pharmaceutical proteins, while transgenic pig research covers disease-resistant breeding, pharmaceutical protein bioreactors, and xenotransplantation donor construction (Van Cott et al., 1997; Yum et al., 2018; Yum et al., 2024). Over the past decade, transposon-mediated nonviral transfer and targeted gene editing/integration have provided feasible routes to achieve long-term genetic stability and predictable phenotypes (Yum et al., 2018; Yum et al., 2024).

7.2 Genetic stability testing and result analysis

7.2.1 Transgenic cattle (example: transposon-mediated integration and mammary gland expression)

Long-term follow-up has shown that over more than 10 years, physiological indicators and nutritional composition in these cattle show no significant difference compared with control groups. Whole-genome resequencing also revealed no increase in somatic mutation rate, copy number variation (CNV), or structural variation (SV), indicating good maintenance of genomic integrity. The exogenous gene was stably transmitted through the germline, and expression levels remained consistent across generations (Yum et al., 2018; Yum et al., 2024).

7.2.2 Transgenic pigs (two categories: pharmaceutical protein expression and disease resistance gene editing)

Lactating transgenic pigs expressing recombinant human protein C (rhPC) showed a strong correlation between a single integration site plus stable copy number and stable rhPC production in milk. The Western blot isoform profiles were highly consistent within the same lineage, suggesting that post-translational modifications contribute to inter-lineage differences (Van Cott et al., 1997).

For xenotransplantation applications, multi-gene–modified Yucatan miniature pigs achieved knockout of immunogenic loci and expression of human regulatory proteins, showing reduced immunogenicity when co-cultured with human immune cells.

Compared with random integration, site-specific editing (e.g., PRRSV-resistant pigs with targeted CD163 modification) showed no evidence of “reversion mutations” or “structural rearrangements” during generational tracking, and transcriptional balance in nearby regions remained intact, demonstrating that editing specificity and stability are superior to random integration (based on the key points of the second dataset).

Table 3 Evidence matrix of genetic stability

Species/Line	Transgenic Strategy	Integration/Edit Characteristics	Intergenerational Transmission	Whole-Genome Integrity	Key Conclusions
Cattle	Transposon-mediated (non-viral)	Single/few copies, noncoding region integration	expression across F1–F(_n) generations	Mutation rate/CNV/SV ≈ control	Long-term safety and stability (Yum 2018/2024)
Pig (rhPC)	Random integration (early stage)	Single defined site preferable	Stable across multiple litters and generations	Consistent isoform profiles within line	Single-site stability → predictable yield (Van Cott 1997)
Xenotransplantation pigs	Multigene modification	Immunogenic loci knockout + human gene insertion	Validated across generations	In vitro immune compatibility ↑	Significantly reduced immunogenicity

7.3 Phenotypic evaluation and comparison of production performance

7.3.1 Cattle (mammary expression of pharmaceutical proteins / functional improvement of dairy products)

In long-term breeding, growth, reproduction, and health indicators were comparable to controls; no significant differences were found in the nutritional composition of milk and meat, indicating agricultural application potential and food safety (Yum et al., 2024). The target proteins (such as lactoferrin or recombinant human proteins) were highly and stably expressed in milk, and metabolomic/proteomic and functional assays showed enhanced antibacterial and bioactive properties, consistent with the evidence chain presented in Section 7.2.

7.3.2 Pigs (rhPC bioreactor / disease resistance / xenotransplantation)

The milk expression level of rhPC in transgenic pigs ranged from 100-1800 μg/ml among different constructs or lines and remained stable within the same line. Reproductive and lactation performance were normal, making them suitable for large-scale biopharmaceutical production (Van Cott et al., 1997). For xenotransplantation purposes, multi-gene-modified miniature pigs exhibited stable health and physiological performance while successfully expressing multiple human complement-regulating and anticoagulant proteins, significantly improving organ compatibility indicators. In addition, the breed of recipient sows and embryo transfer conditions significantly affected pregnancy and delivery rates, providing a basis for optimizing breeding procedures.

7.4 Research outcomes and risk assessment conclusions

The genetic stability of exogenous genes is the core prerequisite for ensuring phenotypic consistency and production predictability. Defined insertion sites, single-copy integration, and stable expression patterns constitute the foundation of a reliable genetic system. The use of nonviral-mediated gene transfer and targeted integration technologies can significantly reduce the risks of structural variations and epigenetic silencing, thereby enhancing genetic safety and long-term expression consistency (Van Cott et al., 1997; Yum et al., 2024).

In terms of risks and countermeasures, early random integration strategies were often associated with potential issues such as repetitive sequence insertions and expression drift, leading to genetic instability or functional loss. To prevent such risks, comprehensive molecular characterization analyses should be performed, including breakpoint sequencing or whole-genome sequencing (WGS), copy number quantification, and methylation profiling, to accurately assess integration features. Meanwhile, combining insulators and safe harbor site strategies can effectively isolate adverse chromatin effects and reduce the probability of transgenerational silencing. Long-term population tracking can further verify the stable transmission of both genetic and phenotypic traits. Additionally, optimizing recipient breed selection and embryo manipulation procedures in breeding and transplantation stages helps improve reproductive success rates and maintain trait consistency.

At the application and translational level, research and industrial practice in cattle and pigs have fully demonstrated that transgenic livestock hold sustainable potential for agricultural production and biomedicine. The establishment of standardized phenotypic evaluation and continuous safety monitoring systems not only ensures product safety and functional reliability but also provides scientific and transparent evidence for regulatory review and public communication, thereby promoting the social acceptance and regulated development of transgenic technology (Yum et al., 2024).

8 Challenges and Future Directions

8.1 Technical aspects: precision of gene editing and controllability of insertion sites

Although transgenic livestock technologies have made remarkable progress, many challenges remain at the technical level. The precision of gene editing is a primary concern. Current mainstream systems such as CRISPR/Cas9, TALEN, and ZFN possess high editing efficiency but still may cause off-target effects, leading to unintended gene mutations or chromosomal rearrangements. These molecular events may disrupt key genes or regulatory elements, resulting in physiological abnormalities or phenotypic drift, which could affect the reliability of research conclusions and industrial safety. For instance, in some pig-editing experiments, off-target mutations disrupted immune gene expression, leading to reduced disease resistance. Therefore, improving editing system specificity and developing controllable gene repair mechanisms will be central to future technical optimization.

Meanwhile, the controllability of insertion sites directly determines the stability and consistency of exogenous gene expression. The "position effect" caused by traditional random integration remains a major source of phenotypic variation. Although the discovery of "safe harbor" loci (e.g., Rosa26, H11) has greatly improved this issue, safe sites in different species have yet to be systematically identified, and their tissue specificity and transcriptional activity still require further validation. In the future, site-specific targeting systems based on genome editing and precise recombination are expected to become a research focus. By combining high-fidelity Cas variants (such as Cas12, Cas13) with recombinase systems, precise chromosomal integration of exogenous genes at predetermined locations can be achieved, fundamentally eliminating random insertion risks. Furthermore, the use of long-read sequencing and whole-genome validation technologies will enable real-time verification and risk screening of editing results, establishing a safer and more traceable construction process for transgenic livestock.

8.2 Management and ethics: animal welfare and public acceptance

Beyond technical issues, transgenic livestock development faces complex management and ethical challenges. Animal welfare remains a central social concern. While gene editing improves traits, it may also cause physiological burdens and health risks such as metabolic overload, reproductive disorders, or shortened lifespan. For example, certain pigs with high growth hormone expression exhibited myocardial hypertrophy and endocrine imbalance, prompting ethical reflections within the scientific community on the moral boundaries of human intervention in nature. Therefore, in research and breeding practices, animal welfare assessment standards should be established, and the physiological conditions of edited animals should be monitored long-term to ensure that improved traits are not achieved at the cost of animal health.

Public acceptance and regulatory frameworks directly influence the future of transgenic livestock applications. Due to differing public perceptions of "genetically modified animal products", some regions maintain a cautious or even resistant stance toward commercialization. Western countries have implemented limited market access through comprehensive approval and labeling systems, while Asian nations remain in the exploratory phase of regulatory development. In recent years, China has strengthened legal regulation and ethical review of transgenic technology, though improvements in research transparency, public science communication, and risk dialogue are still needed. Enhancing public understanding of genetic science and promoting openness and consistency in regulatory systems are key to fostering rational social consensus and effective policy implementation.

From a global governance perspective, ethical issues surrounding transgenic livestock involve not only animal rights but also biodiversity conservation and food safety. In the future, an international framework for ethical evaluation and information sharing should be established to promote unified standards among nations in technology assessment, data transparency, and ecological risk evaluation, balancing scientific innovation with ethical responsibility.

8.3 Future research trends: multi-omics integration, digital phenotyping, and artificial intelligence evaluation

Future research on transgenic livestock will enter a data-driven and intelligent decision-making era. Multi-omics integration will become the core approach for evaluating genetic stability and phenotypic consistency. By systematically integrating data from genomics, transcriptomics, methylomics, proteomics, and metabolomics, researchers can uncover the dynamic molecular behavior of exogenous genes and their regulatory networks. For instance, combined analysis of DNA methylation and mRNA expression profiles can identify epigenetic regulation of gene silencing; integrating metabolomic and proteomic data can trace the physiological origins of phenotypic variation. This cross-level data fusion will shift research from "gene presence" to "functional realization."

The development of digital phenotyping provides new technological pathways for phenotypic evaluation. Using high-resolution imaging sensors, infrared scanning, behavior recognition, and automated monitoring systems,

researchers can continuously record livestock growth, health, and behavioral patterns. Through cloud databases and data-mining algorithms, precise dynamic phenotypic models can be built to enable individual tracking and group performance prediction. This digital monitoring approach not only improves the objectivity and timeliness of data collection but also supports large-scale breeding and health management with intelligent tools.

Artificial intelligence (AI) and machine learning algorithms will play an increasingly important role in genetic and phenotypic evaluation. AI can automatically identify key factors affecting genetic stability and trait expression from large-scale omics and phenotypic datasets, constructing predictive models. For example, deep neural networks can perform pattern recognition on phenotypic outcomes associated with different editing sites, assisting in selecting optimal insertion sites and gene constructs. In the future, AI will be deeply integrated with bioinformatics platforms to form a Smart Breeding System, enabling integrated decision support for gene design, phenotypic evaluation, environmental monitoring, and risk prediction.

Overall, future research on transgenic livestock will move toward precision, safety, intelligence, and sustainability. Through the coordinated advancement of technological innovation, ethical governance, and intelligent evaluation, transgenic livestock are expected to make forward-looking contributions to global food security, medical health, and agricultural modernization, while ensuring animal welfare and ecological safety.

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