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Decoding gene expression dynamics in bovine embryonic development: insights into key regulatory networks and implications for reproductive technologies

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Abstract

Objective

The expression patterns of development-related genes during bovine embryo development are highly regulated processes influenced by genetic and environmental factors. This study provides novel insights into these patterns, offering an enhanced insight into embryonic development and identifying promising candidates to improve reproductive technologies in bovine breeding.

Materials and methods

We examined gene expression profiles from bovine embryos (GEO accession: GSE18290) using GEO2R to detect genes showing differential expression across critical phases of development: oocyte to 2-cell, 4-cell to 16-cell, and morula to blastocyst. Following this, gene ontology (GO) and KEGG pathway enrichment analyses were conducted to reveal key biological processes linked to the discovered DEGs. Moreover, a protein-protein interaction (PPI) network was built to explore connections among central genes, together with identifying relevant microRNAs (miRNAs) and transcription factors.

Results

The analysis identified differential expression of 2,244 genes (1,026 upregulated, 1,218 downregulated) during the transition from oocyte to 2-cell, 2,379 genes (1,146 upregulated, 1,233 downregulated) across the progression from 4-cell to 16-cell, and 1,544 genes (821 upregulated, 723 downregulated) through the shift from morula to blastocyst periods. GO and KEGG analyses highlighted enriched biological processes such as mRNA catabolism, nonsense-mediated decay, mast cell activation, protein phosphorylation, nerve growth factor signaling, regulation of angiogenesis, MAPK and Rap1 signaling pathways, and protein processing in the endoplasmic reticulum. Key hub genes (*HSPA1A*, *PPARGC1A*, *AKT2*, *EGR1*, *HSDL2*) were pinpointed, along with related miRNAs (bta-miR-103a-3p, bta-miR-769-5p) and transcription factors (*HSF1*, *FOXA2*, *FOXO1*, *CREB*).

Conclusions

Our analysis revealed stage-specific gene expression changes and highlighted key biological pathways and regulatory networks involved in bovine embryonic development. Central hub genes, miRNAs, and transcription factors were identified as potential biomarkers and targets. These findings offer valuable molecular insights that can be applied to improve reproductive technologies and fertility in cattle breeding.

Keywords: bovine fetal development, gene expression patterns, hub genes, reproductive biology

Paper Type: Research Paper.

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Introduction

Bovine embryo research is central to meeting the challenges of sustainable food production and genetic advancement in livestock industries. The embryonic development of the bovine species involves an intricate and tightly controlled sequence of events that begins with fertilization and continues until birth. After fertilization occurs, the zygote experiences multiple rounds of cleavage divisions, resulting in the formation of a blastocyst. During these early stages, from fertilization to blastocyst formation, accurate control over gene expression is essential for proper embryonic growth and development. Understanding these gene expression dynamics is not only fundamental to developmental biology but also holds significant implications for advancing reproductive technologies and improving breeding strategies in livestock (Graf et al., 2014). Low fertility rates remain a major challenge in the dairy industry, with substantial embryonic losses occurring between days 8 and 16 post-mating (Hoelker et al., 2019). These losses are often attributed to disruptions in gene expression patterns and environmental factors. Identifying the molecular mechanisms underlying these disruptions can provide valuable insights into improving embryo survival rates. Maternal components, especially those encoded by maternal-effect genes, are crucial in the earliest stages of embryo formation. These genetic elements, which build up during the maturation of the oocyte, control the initial developmental processes before the embryonic genome becomes active (Svoboda et al., 2015). The shift from maternal RNA dependence to the initiation of the embryo's own gene expression, known as the maternal-to-zygotic transition, marks a crucial phase in early development. This phase involves substantial alterations in genetic activity, including the breakdown of inherited transcripts to avoid disruption of the embryo's development (Kropp et al., 2014). The patterns of gene activity set during the initial phases play a crucial role in determining the outcome of pregnancy and the proper development of the embryo. Identifying key gene networks and their regulatory mechanisms can lead to the discovery of predictive markers for embryo viability, which could revolutionize embryo transfer practices and improve animal health outcomes(Fujii, et al., 2024; Hernández-Vargas et al., 2020). For instance, studies in human embryogenesis have demonstrated that embryos exhibiting stage-specific gene expression patterns are more likely to be viable, suggesting that these patterns can serve as biomarkers for assessing embryo quality (Assou et al., 2011; Hasegawa et al., 2015). The identification of molecular markers capable of predicting the viability of bovine embryos can significantly improve the efficiency of breeding and reproductive programs. For example, using these markers during embryo selection allows farmers and IVF specialists to choose embryos with a higher likelihood of successful implantation and normal development for transfer to the uterus(Lonergan & Fair, 2014). This approach not only increases pregnancy rates and reduces the costs associated with unsuccessful transfers, but can also contribute to improved health and performance in subsequent generations. Integrating gene expression data with morphogenetic and metabolic parameters could further enhance the accuracy of embryo viability assessments, thereby improving the outcomes of assisted reproductive technologies (ART) (Mantikou 2013; Zanatta et al., 2021). To address the challenges in bovine embryonic development, this study employs a comprehensive bioinformatics approach to analyze the expression patterns of development-related genes from the oocyte to the blastocyst stage. Using bioinformatics resources such as GEO2R for analyzing differential gene expression, along with building protein-protein interaction networks to pinpoint key hub genes, this study seeks to gain a comprehensive, systems-level insight into gene regulatory mechanisms. A systems-level understanding refers to analyzing how multiple genes and their products interact as part of complex networks that govern embryonic development. In this study, we use systems biology approaches to move beyond isolated gene analysis and instead examine the collective behavior of gene networks throughout the stages from oocyte to blastocyst. Studies have indicated that GEO2R's results align well with other methods of differential expression analysis, suggesting that it is a reliable tool for identifying DEGs in various types of molecular datasets (Clough et al., 2024). Our primary objectives include identifying differentially expressed genes (DEGs) and analyzing their functional roles through Gene Ontology (GO) and pathway enrichment analyses. Additionally, we seek to construct interaction networks to determine regulatory relationships and explore the roles of miRNAs and transcription factors in modulating these genes. The epigenome, which comprises various mechanisms such as DNA methylation, chromatin remodeling, histone tail modifications, microRNAs, and long non-coding RNAs, interacts with environmental factors like nutrition, pathogens, and climate to influence gene expression profiles and the emergence of specific phenotypes (Shahsavari et al., 2023; Mohammadabadi et al., 2023). These interactions are complex and occur across multiple levels, involving dynamic crosstalk between the genome, epigenome, and environmental stimuli (Amiri Roudbar et al., 2020). Increasing evidence suggests that epigenomic variation plays a crucial role in determining health outcomes and production traits (Alavi et al., 2022; Safaei et al., 2022). The expression of eukaryotic genes is temporally and spatially regulated through multidimensional control mechanisms (Hajalizadeh et al., 2021). Only a limited subset of the entire genome is actively expressed in each tissue type, and gene expression is closely tied to developmental stages (Heidarpour et al., 2011; Khabiri et al., 2023). Consequently, gene expression patterns in eukaryotes are tissue-specific (Mohammadabadi et al., 2021; Mohammadabadi et al., 2024). Additionally, the levels of gene products synthesized within a given tissue, as well as those contributed by other tissues, collectively regulate gene expression (Shokri et al., 2023). Epigenetic modifications serve as a critical mechanism by which environmental factors leave a lasting imprint on the genome (Mohammadabadi et al., 2022a).

These modifications can be heritable, influencing successive generations without altering the DNA sequence itself (Mohammadabadi et al., 2025). For instance, maternal nutrition during pregnancy has been shown to induce epigenetic changes that affect offspring metabolism, growth, and disease susceptibility (Safaei et al., 2024). Similarly, environmental stressors such as exposure to toxins or pathogens can trigger epigenetic responses that modify gene expression and cellular function (Noori et al., 2017). Understanding epigenetic mechanisms can improve breeding strategies, enhance productivity, and promote disease resistance (Amiri Roudbar et al., 2015). Additionally, epigenetic markers are increasingly used to predict performance and health outcomes, providing a valuable tool for precision (Mohamadinejad et al., 2024). A fundamental aspect of genetic research involves the investigation of genes and proteins associated with specific traits, examined at both cellular and chromosomal levels (Bordbar et al., 2022). Advancing our understanding of these regulatory processes holds significant potential for improving biological insights and practical applications in health and production (Arabpour et al., 2021). Further research into epigenetic biomarkers and their role in gene regulation will contribute to more effective therapeutic strategies and enhanced understanding of complex biological systems (Mohammadabadi et al., 2022b). Thus, this research aimed to elucidate the molecular mechanisms governing bovine embryonic development, providing valuable insights for improving embryo viability predictions and optimizing assisted reproductive technologies (ART) in bovine breeding.

Materials and methods

This study aimed to investigate gene expression dynamics during the development of bovine embryos across several key stages, from oocyte to blastocyst. The approach used is depicted in Figure 1, which presents the step-by-step process followed in this study, including data acquisition, identification of genes with altered expression (DEGs), pathway and function analysis, and the development of protein interaction networks.

Data collection: The raw gene expression data for bovine embryos at successive developmental stages were downloaded from the Gene Expression Omnibus (GEO) database under the accession number GSE18290. The dataset included samples from the following stages: oocyte, two-cell, four-cell, sixteen-cell, morula, and blastocyst, with two biological replicates for each stage. The data were generated using Affymetrix microarrays. Total RNA was isolated from embryos at each developmental phase, then amplified and applied to Affymetrix microarrays to obtain gene expression data (Table 1) (Xie et al., 2010). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois.

Justification for sample size and data selection: Two biological replicates per developmental stage were included to maintain reproducibility and address biological differences.

This sample size is consistent with previous studies on bovine embryonic development (Xie et al., 2010). The dataset was chosen based on the availability of high-quality raw gene expression data from the GEO database. Only datasets generated using Affymetrix microarrays were included to ensure consistency in data generation and analysis. Samples were selected to represent key developmental stages (oocyte, two-cell, four-cell, sixteen-cell, morula, and blastocyst) to capture the dynamic changes in gene expression during early bovine embryogenesis.

Identification of differentially expressed genes (DEGs): The differentially expressed genes (DEGs) were identified using GEO2R, an online tool designed to compare sample groups within GEO datasets and detect genes with statistically significant expression changes under defined experimental conditions (Clough et al., 2024). For normalization of gene expression data, the quantile normalization method implemented in GEO2R was used, which ensures that the distribution of gene expression levels is the same across all samples and guarantees data comparability. GEO2R employs the Limma package (a linear modeling framework in R/Bioconductor) to perform differential expression analysis. The Limma package assumes a linear model for gene expression data. DEGs were filtered using thresholds of Fold Change > |2| and P-value ≤ 0.05 to account for biological relevance and statistical significance. A Venn diagram was then generated to visualize overlapping DEGs at different stages of bovine fetal development.

Functional enrichment analysis of DEGs: To uncover biologically relevant pathways and terms associated with co-expressed DEGs, overrepresentation enrichment analysis was performed through DAVID (version 2024). DAVID enabled the classification of GO enrichment terms into biological processes, molecular functions, and cellular components (Dennis et al., 2003), coupled with pathway analysis based on the KEGG database (Kanehisa et al., 2000). Enriched GO terms and KEGG pathways were filtered using a significance threshold of P-value < 0.05 to prioritize statistically robust associations. This method was intended to clarify the molecular mechanisms involved in important biological processes throughout bovine embryo development.

Construction and topological analysis of protein-protein interaction networks: Protein-protein interaction (PPI) networks involving the differentially expressed genes (DEGs) were constructed using Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING) (version 12.0) (Szklarczyk et al., 2023). Interactions were restricted to experimentally confirmed data (e.g., biochemical assays, crystallography) to ensure biological relevance. A confidence score threshold of > 0.15 was applied to filter interactions. The resulting networks were visualized and analyzed using Cytoscape software (Version 3.10.2) (Kohl et al., 2010). To evaluate the topological importance of nodes (proteins) within the PPI network, centrality metrics including degree centrality (number of connections), betweenness centrality (bridging role in pathways), and closeness centrality (proximity to other nodes) were calculated using the CytoNCA plugin. Statistical significance for enriched interactions or pathways was defined as P-value ≤ 0.05.

Analysis of miRNA and transcription factor associations: To investigate microRNAs (miRNAs) associated with hub genes, we utilized the miRWALK platform (http://mirwalk.umm.uni-heidelberg.de/). For the identification of transcription factors (TFs), the TF2DNA resource (https://www.fiserlab.org/tf2dna_db/index.html) was consulted. Additionally, we employed FFLtool, an online tool (http://bioinfo.life.hust.edu.cn/FFLtool#!/) specifically designed for analyzing Feed Forward Loop (FFL) regulatory motifs that involve interactions among transcription factors, miRNAs, and genes. FFLs were identified by constructing a regulatory network from gene expression and TF data, then detecting motifs where a TF regulates a target gene directly and indirectly (via a microRNA or another TF). To ensure relevance, FFLs were filtered based on statistical significance, consistent expression patterns, and, when available, epigenetic data. FFLs play a critical role in regulating various target genes, enabling spatio-temporal regulation and buffering noise, and are essential to many biological processes (Tang et al., 2015). The identified network connections were visualized using Cytoscape.

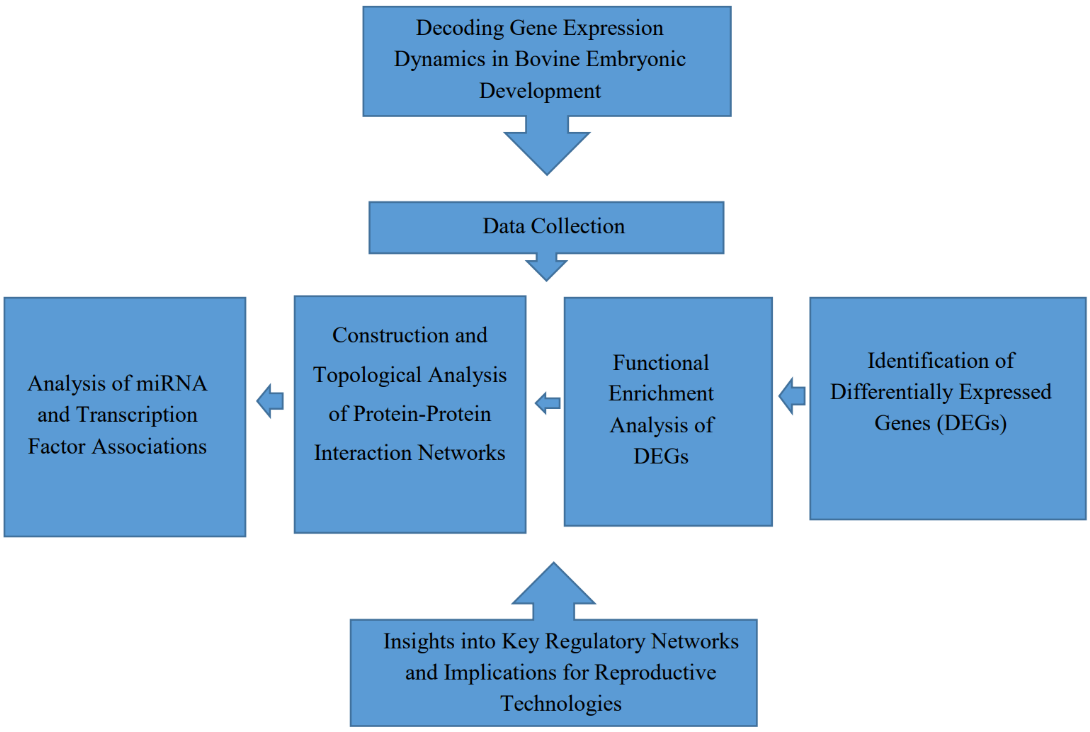

Figure 1. This flowchart outlines the key stages of the research process, from the initial planning phase through to the final reporting of results. Each step is presented in sequence, with a focus on the methodologies applied throughout the study

Table 1. Accession Numbers for bovine Embryonic Samples at different post-fertilization time points

Species	Stages of fetal development	Accession Numbers
Bovine	Oocyte	GSM456627
		GSM456628
	two-cell	GSM456631
		GSM456632
	four-cell	GSM456633
		GSM456634
	sixteen-cell	GSM456637
		GSM456638
	Morula	GSM456639
		GSM456640
	Blastocyst	GSM456641
		GSM456642

Results and discussion

Identification of differentially expressed genes (DEGs): To pinpoint genes with significant alterations in expression across important stages of bovine embryo development, differential gene expression analysis was carried out. The analysis revealed a total of 2,244 differentially expressed genes (DEGs) between the oocyte and 2-cell stages, with 1,026 genes upregulated and 1,218 genes downregulated. The comparison of the 4-cell and 16-cell stages revealed 2,379 DEGs, including 1,146 upregulated and 1,233 downregulated genes. Furthermore, the morula and blastocyst stages exhibited 1,544 significantly different genes, with 821 upregulated and 723 downregulated genes. To identify shared regulatory mechanisms across these developmental stages, a Venn diagram was constructed to visualize the overlap of DEGs (Figure 2). Notably, 90 genes were found to be common across all stages, including the oocyte, 2-cell, 4-cell, 16-cell, morula, and blastocyst stages, may play critical roles in fundamental biological processes essential for early embryonic development (Table 2). The Venn diagram (Figure 2) highlights the shared and unique DEGs across the developmental stages, providing insights into the dynamic regulation of gene expression during bovine embryogenesis.

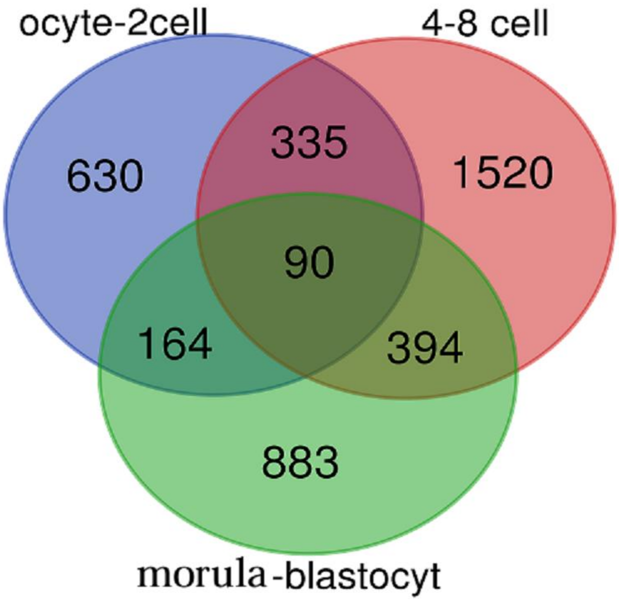

Figure 2. The Venn diagram displays the overlap of DEGs shared among various stages of bovine embryo development, highlighting the common genes present across these developmental phases.

Table 2. Common Genes During Different Stages of Embryonic Development (Oocyte, 2-cell, 4-cell, 16-cell, Morula, and Blastocyst Stages)

common genes	*ITPK1, NR4A2, PIR, SORL1, FAHD1, ITM2B, ACSL3, CDK20, EED, FABP5, NOP9, CACNB1, ATF1, SLC9A3, AHCYL1, PBX1, RBBP6, ECE1, PPARGC1A, TPT1, C18H19orf48, GRHL1, DMTF1, POFUT2, EEF2K, RNPC3, PLS3, YES1, LIX1L, PAM, PPP3CB, AKT2, NDRG1, CHD2, KLF5, FUCA1, HDAC11, HBB, TFPI, PAXBP1, PPIP5K2, PDGFRA, MIA3, MRPL23, RNF11, NINJ1, HSDL2, C18H16orf87, THNSL2, ESPL1, UGCG, MED20, RAP1A, MRI1, CRYM, ADGRL2, CHGA, SNX10, RGS19, EGR1, MAGT1, SYNGR2, SLC38A2, CD2AP, ADAM9, HSPA1A, PNRC1, SLC4A7, GDPD1, KCTD20, SEC23A, SCYL2, MAPKAPK5, GSTK1, MYO19, ZBTB9, FGF2, BAG2, SMG5, GPN1, G6PC, ANKRD12, UBA5, MEST, HNRNPLL, ITGB3, FAM120B, C25H16orf58, SCAF11, and RNF128*

Functional enrichment analysis of DEGs: DAVID software was utilized to carry out functional enrichment analysis aimed at uncovering the biological significance of DEGs present throughout the developmental stages of the bovine embryo. DAVID is a widely used tool for pathway analysis, enabling researchers to identify biological functions and pathways associated with DEGs. The GO analysis revealed significant enrichment of DEGs, identifying 11 biological processes with a P-value ≤ 0.05. Key biological processes related to DEGs at different developmental stages of the cow embryo are presented in Table 3. These processes include nuclear-transcribed mRNA catabolic process (nonsense-mediated decay), mast cell activation, protein phosphorylation, nerve growth factor signaling pathway, and positive regulation of angiogenesis, among others.

Table 3. Enriched Biological Processes Associated with Common DEGs in Bovine Embryo Development

Category	Term	Count	P-value
GOTERM_BP	GO:0000184~nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	4	3.2E-04
GOTERM_BP	GO:0045576~mast cell activation	2	7.8E-3
GOTERM_BP	GO:0006468~protein phosphorylation	6	2.7E-2
GOTERM_BP	GO:0038180~nerve growth factor signaling pathway	2	3.5E-2
GOTERM_BP	GO:0045766~positive regulation of angiogenesis	3	4.6E-2

Following the GO analysis, to pinpoint key pathways linked to the shared DEGs, KEGG pathway analysis was performed. The analysis revealed enriched pathways with a P-value ≤ 0.05. The most notable pathways included the MAPK signaling pathway, Rap1 signaling pathway, and protein processing in the endoplasmic reticulum, as shown in Table 4.

Table 4. Enriched KEGG Pathways Associated with Common DEGs in Bovine Embryo Development

Category	Term	Count	P-value
KEGG_PATHWAY	bta04010:MAPK signaling pathway	8	3.01E-04
KEGG_PATHWAY	bta04015:Rap1 signaling pathway	5	1.5E-2
KEGG_PATHWAY	bta04141:Protein processing in endoplasmic reticulum	4	4.1E-2

Construction and analysis of protein-protein interaction networks: To examine the functional interactions among the 90 shared differentially expressed genes, a PPI network was generated using the STRING database and subsequently visualized with Cytoscape software for further analysis. Proteins that lacked connections or were only partially connected were excluded from the network (see Figure 3). The network was analyzed using key centrality metrics degree centrality (reflects the number of direct interactions a protein has within the network), betweenness centrality (the extent to which a node lies on the shortest paths between other nodes, indicating its role as a communication bridge or bottleneck), and closeness centrality (quantifies how quickly a node can interact with all other nodes in the network, reflecting its potential for efficient communication) resulting in the identification of five critical hub genes: *HSPA1A*(Its central position suggests it may safeguard cellular homeostasis during the rapid cellular proliferation and differentiation characteristic of fetal development), *PPARGC1A* (Its high centrality indicates a fundamental role in meeting the energetic demands of developing fetal tissues, supporting processes such as cell growth and differentiation), *AKT2*(Its hub status highlights its involvement in signaling pathways essential for embryonic development and tissue morphogenesis), *EGR1*(to gene expression programs driving differentiation and development), and *HSDL2* (critical for membrane synthesis and energy storage during development). Interpreting their biological significance is essential to translate network topology into functional insights. Among these, *HSPA1A* emerged as the most significant hub gene, demonstrating the highest values across all three centrality measures, indicating its pivotal role in the network. Table 5 lists the top five hub genes, along with their degree, betweenness, and closeness centrality values. These hub genes are central to the regulatory network governing bovine fetal development, suggesting their potential importance in key biological processes during embryogenesis.

Identification of interactions among miRNAs, transcription factors, and hub genes: To further investigate the regulatory mechanisms underlying bovine fetal development, we analyzed the interactions between miRNAs, transcription factors (TFs), and hub genes. Our analysis identified several key TFs, including *HSF1*, *FOXA2*, *FOXO1*, and *CREB*, which play significant roles in modulating the expression of hub genes. Additionally, we uncovered a set of miRNAs, notably bta-miR-103a-3p and bta-miR-769-5p, that are strongly associated with the hub genes (see Figure 4). These interactions suggest a complex regulatory network involving TFs, miRNAs, and hub genes that may govern critical processes during bovine fetal development. Bovine embryo research is vital for improving beef and dairy production, conserving endangered cattle breeds, and enhancing genetic resilience against diseases and environmental stressors. It provides insights into molecular mechanisms of early embryonic development, aiding reproductive health and addressing developmental disorders (Ramos et al., 2013). Gene expression analysis reveals 2,244 DEGs (oocyte to 2-cell), 2,379 DEGs (4-cell to 16-cell), and 1,544 DEGs (morula to blastocyst), highlighting the shift from maternal control to embryonic genome activation, a key process for development (Goossens et al., 2007; Kropp et al., 2014).

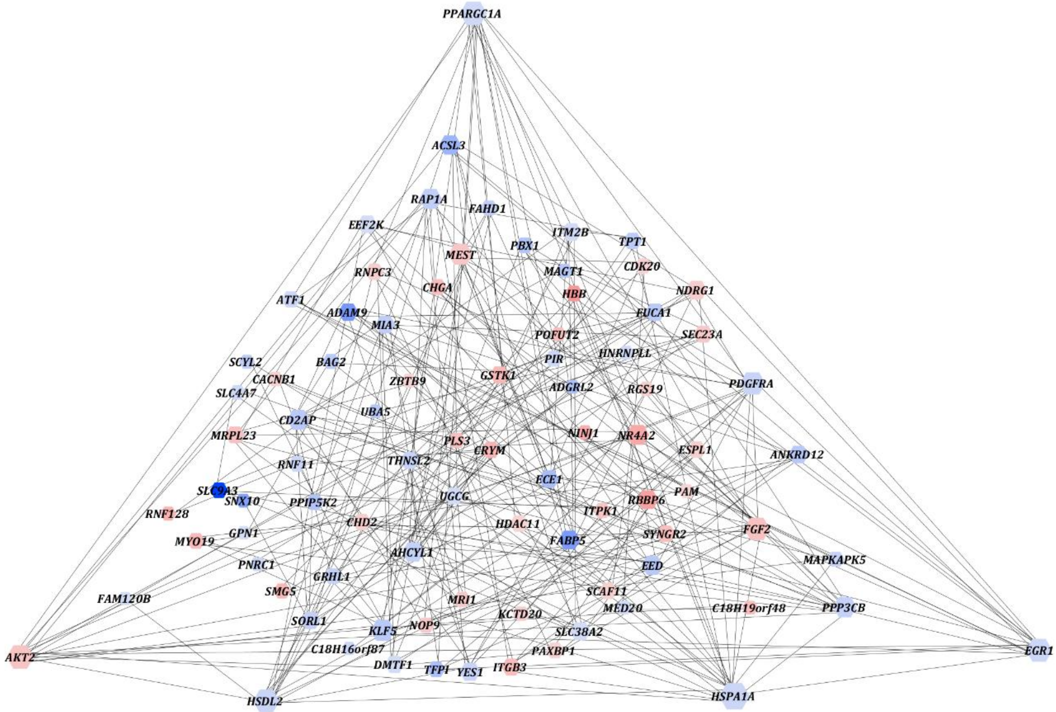

Figure 3. PPI Network Constructed from Common DEGs Involved in bovine fetal development. The PPI network illustrates the interactions among the 90 common differentially expressed genes (DEGs) identified across different stages of bovine fetal development. Node size is proportional to the degree of centrality, with larger nodes indicating greater involvement in the development process. Red nodes represent upregulated DEGs, while blue nodes indicate downregulated DEGs

Table 5. Hub Genes Identified in the PPI Network Based on Centrality Metrics

Gene	Degree	Betweenness	Closeness
HSPA1A	23.0	825.60706	0.5119048
PPARGC1A	18.0	473.67346	0.49142858
AKT2	18.0	626.075	0.48863637
EGR1	17.0	323.34247	0.46994534
HSDL2	17.0	606.733	0.4673913

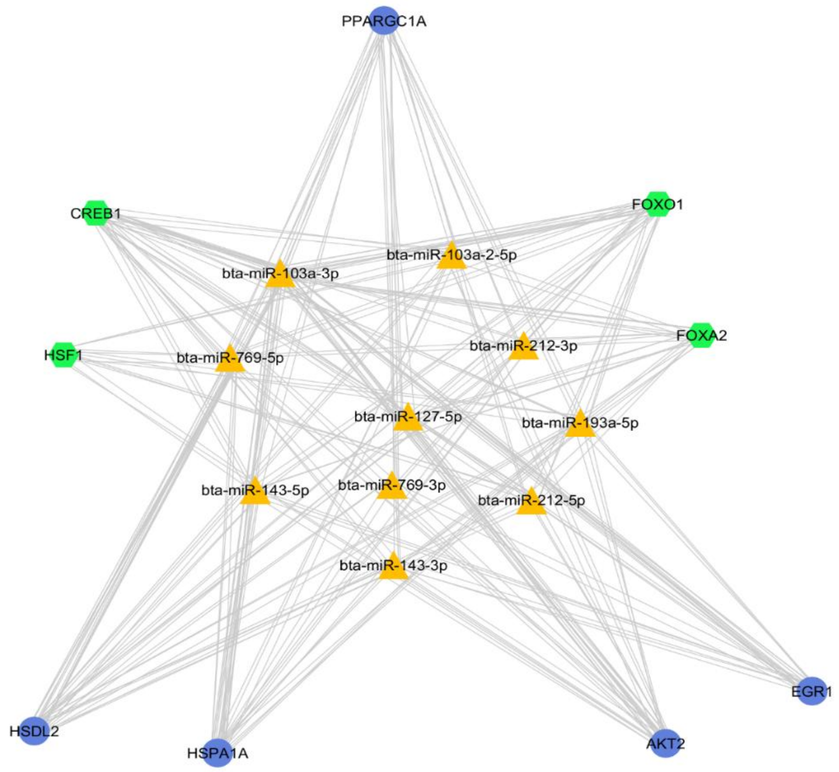

Figure 4. Regulatory Network of miRNAs, Transcription Factors, and Hub Genes: The network illustrates the interactions between miRNAs, transcription factors (TFs), and hub genes. Key TFs (e.g., *HSF1*, *FOXA2*, *FOXO1*, *CREB*) and miRNAs (e.g., bta-miR-103a-3p, bta-miR-769-5p) are shown to regulate hub genes, highlighting their potential roles in bovine fetal development

The identification of 90 common DEGs across all developmental stages suggests that these genes play fundamental roles in early embryonic development. These genes, including *PPARGC1A*, *HSPA1A*, *ITPK1*, and *NR4A2*, are involved in diverse processes such as signal transduction, metabolism, and stress response. Their consistent differential expression highlights their importance in maintaining cellular homeostasis and regulating developmental transitions. For instance, *PPARGC1A*, a key regulator of mitochondrial biogenesis and energy metabolism, may be crucial for meeting the energy demands of rapidly dividing embryonic cells (Fontecha-Barriuso et al., 2020). Similarly, *HSPA1A*, a heat shock protein, likely plays a role in protecting cells from stress during this period of rapid change (Fontecha-Barriuso et al., 2020). *ITPK1* is implicated in regulating calcium and phosphoinositide levels, impacting cellular signaling during crucial developmental phases (Ng et al., 2025). *NR4A2* is critical in various biological processes, including embryonic development, cell differentiation, and metabolism. It influences the expression of numerous genes involved in these processes (Ashraf et al., 2019). Functional Enrichment Reveals Key Biological Processes and Pathways: The role of DEGs in the outlined processes and pathways is likely fundamental to ensuring normal fetal development. Functional enrichment analysis of the common DEGs revealed significant enrichment of biological processes such as nuclear-transcribed mRNA catabolic process (nonsense-mediated decay), protein phosphorylation, and positive regulation of angiogenesis (Table 3). The relationship between fetal growth and development, particularly about nuclear-transcribed mRNA catabolic processes such as nonsense-mediated decay (NMD), is significant due to NMD's roles in cellular monitoring and gene regulation. During fetal development, proper expression levels of various genes are critical as they directly influence the growth, differentiation, and overall health of the developing organism. The proper functioning of NMD is essential to ensure that only correctly spliced and fully functional mRNAs are translated, thus maintaining the integrity of protein synthesis necessary for proper growth (Vicente-Crespo et al., 2011). Mast cells are essential cells in the immune system that contribute significantly to reproductive processes. They reside in the endometrial tissue and participate in several critical aspects of pregnancy, including implantation, placentation, and fetal growth. Specifically, mast cells release various mediators, such as histamine and cytokines, which promote trophoblast invasion and remodeling of uterine blood vessels necessary for a successful pregnancy (Chia et al., 2023). Mast cells are crucial for maintaining fetal growth and development through their roles in immune regulation, angiogenesis, and tissue remodeling. However, their dysregulation can lead to significant pregnancy complications, including IUGR and preterm births. Further research is necessary to understand better the specific mechanisms by which mast cell activation influences fetal development and how to manage conditions related to mast cell activation during pregnancy effectively (Chia et al., 2023). protein phosphorylation is vital for many aspects of fetal development, encompassing growth regulation, signaling transduction, and the orchestration of complex developmental processes (Ardito et al., 2017). The NGF signaling pathway is integral to fetal development, influencing neuronal growth, survival, and the overall health of the developing brain. Disruptions in this signaling pathway can have significant implications for fetal growth and long-term developmental outcomes (Sánchez-Infantes et al., 2018; Sofroniew et al., 2001). The formation of new blood vessels from preexisting ones, known as angiogenesis, is essential for maintaining proper blood flow and nutrient absorption. Especially important in the placenta, this mechanism supports the exchange of nutrients, gases, and metabolic waste products between maternal and fetal bloodstreams (Wang et al., 2022). By elucidating the roles of DEGs in these biological processes, our study provides valuable insights into the multifaceted mechanisms driving fetal development. Understanding how these processes interact and influence each other offers potential avenues for improving reproductive outcomes and addressing complications associated with fetal growth. Further research into these processes will be essential for enhancing our understanding and management of reproductive health in cattle. KEGG pathway analysis revealed significant enrichment in the MAPK signaling pathway, Rap1 signaling pathway, and protein processing within the endoplasmic reticulum (Table 4). These pathways were identified as common factors influencing fetal development, aligning with our overarching goals of understanding the molecular mechanisms underpinning this process. The mitogen-activated protein kinase (MAPK) signaling pathway plays a significant role in fetal development, particularly through its influence on placental growth and function. This pathway, alongside various growth factors, is critical for ensuring that the placenta can effectively supply nutrients and oxygen to the developing fetus (Perry et al., 2022). Excessive MAPK signaling, often due to mutations in upstream components like KRAS or BRAF, leads to uncontrolled cell proliferation. This disrupts lineage specification in the blastocyst, which can result in implantation failure or early embryonic lethality (Cheng et al., 2003). The Rap1 signaling pathway is vital in fetal development, influencing morphogenesis, cell adhesion, and migration. Its intricate interactions with other signaling pathways underscore its importance in ensuring normal embryonic development and potentially providing insight into developmental complications when dysregulated (Perez-Vale et al., 2023). Hyperactive Rap1 enhances cell adhesion excessively, impairing cell migration. This can disrupt germ layer formation, leading to malformed embryos or developmental arrest(Larsen et al., 2003). Protein synthesis, modification, and interaction in the endoplasmic reticulum are critical for early embryonic development in cattle. These processes ensure that the embryonic cells communicate effectively with the maternal environment, promote proper fetal development, and ultimately influence pregnancy outcomes(Banliat et al., 2020). Disruption in ER function impairs the synthesis of proteins essential for cell structure, signaling, and metabolism, further compromising embryo viability (Lin et al., 2019). By clarifying the involvement of these KEGG pathways in the development of bovine fetuses, this research contributes valuable knowledge about the molecular foundations of embryonic health. Protein-Protein Interaction Network Highlights Hub Genes: The construction of a protein-protein interaction (PPI) network revealed key hub genes among the common DEGs, indicating their central roles in regulating gene expression and cellular function. *HSPA1A*, exhibiting a degree of 23, was recognized as the primary hub gene in our analysis. Playing a key role, this gene contributes to embryonic development and stress regulation across beef cattle and additional livestock species. *HSPA1A*, a member of the heat shock protein 70 (*HSP70*) family, is activated in response to cellular stressors, such as elevated temperatures or oxidative stress. Its expression helps protect cells during stressful conditions. In bovine embryos, increased levels of *HSPA1A* have been observed following exposure to heat shock, indicating its role as a stress marker(Mori et al., 2015). The *HSPA1A* gene is pivotal for managing stress in bovine embryos, particularly during periods of high environmental temperature *HSPA1A*, as a major stress-responsive chaperone, underscores the critical importance of cellular defense mechanisms during early embryogenesis, particularly under environmental stressors such as heat. Its elevated expression suggests that embryos actively engage protective pathways to maintain protein homeostasis and viability, making *HSPA1A* a promising biomarker for embryo quality assessment. *PPARGC1A* was recognized as the second hub gene, characterized by a degree of 18 in this study. This gene is a crucial transcriptional coactivator extensively involved in liver metabolism, mitochondrial biogenesis, and muscle development, including its roles in the early stages of embryonic development in cattle. Studies have demonstrated that *PPARGC1A* is active throughout the early stages of bovine embryonic development, beginning at the oocyte and continuing through to the blastocyst phase. This expression is essential for proper early development and is linked to key processes such as mitochondrial function and energy metabolism (Suwik et al., 2020). *PPARGC1A* is implicated in both the early developmental stages of bovine embryos and the genetic traits that influence milk production. It regulates mitochondrial biogenesis, which is crucial for embryo viability and developmental competence, and its expression levels are linked to the overall quality of the developing embryo. This gene also potentially mediates metabolic pathways critical for the embryo and maternal health during pregnancy, further emphasizing its significance in bovine reproductive success (Suwik et al., 2020). *PPARGC1A* emerges as a pivotal regulator of mitochondrial biogenesis and energy metabolism throughout early development stages. Given the high energy demands of embryogenesis, *PPARGC1A*'s role in sustaining mitochondrial function likely underpins developmental competence and may influence traits extending beyond embryonic stages, such as lactation performance. Emphasizing *PPARGC1A*'s expression throughout all stages, from oocyte to blastocyst, aligns perfectly with our data and suggests its essential role in ensuring proper development. *AKT2* was recognized as the third hub gene, characterized by a degree of 18 in the study. This gene is vital for bovine embryonic development, impacting both metabolic regulation and early cleavage progression. Its phosphorylation status, influenced by factors such as follistatin and signaling pathways like TGF-β, plays a significant role in ensuring developmental competence(Ashry et al., 2018; Zhou et al., 2006). The involvement of *AKT2* in metabolic signaling and cleavage progression highlights the interplay between growth factor pathways and embryonic cell cycle regulation. Modulating *AKT2* activity could therefore represent a strategic target to enhance in vitro embryo production and improve reproductive technologies. *EGR1* was recognized as the fourth hub gene, characterized by a degree of 17 in the study. Known as a transcription factor, this gene plays a vital role in diverse biological functions during embryonic development and reproduction in cattle (Guo et al., 2014). *EGR1* is a multifunctional transcription factor vital for successful reproductive development in cattle. Its involvement spans from early ovarian development through to the complex signaling cascades leading to ovulation. *EGR1*, a transcription factor linked to ovarian development and ovulation, connects embryonic gene networks with maternal reproductive physiology. Understanding its regulatory mechanisms may offer novel avenues to address fertility challenges in cattle, with potential economic benefits. In our analysis, *HSDL2* emerged as a key hub gene characterized by a degree value of 17, plays a significant role in bovine embryo development, primarily through its involvement in metabolic pathways that influence fertility and embryonic health. The complex interplay between maternal metabolic status, hormone regulation, and embryo viability underscores the importance of further investigating the specific functions of *HSDL2*. Such research could provide critical insights for enhancing reproductive technologies and improving outcomes in cattle farming. By effectively monitoring and modulating factors that regulate *HSDL2* expression, it may be possible to optimize embryo transfer protocols and accelerate genetic progress in dairy cattle (Rhoads 2023). Overall, the association of *HSDL2* with metabolism and fertility highlights the profound impact of maternal metabolic health on embryo development, suggesting that targeted studies on this gene could pave the way for novel strategies to improve reproductive efficiency and productivity in the livestock industry. microRNAs that regulation hub genes: The modulation of key hub genes by microRNAs like bta-miR-103a-3p and bta-miR-769-5p plays an important role in bovine embryonic development, affecting processes including cell growth, specialization, and reaction to environmental stimuli. Since miR-103a-3p has been shown to promote cellular behaviors such as proliferation and invasion, it is conceivable that alterations in the levels of heat shock proteins, such as *HSPA1A*, may be part of the broader regulatory network involving bta-miR-103a-3p (Li et al., 2021). The regulatory role of bta-miR-103a-3p on the *PPARGC1A* gene involves its function as a microRNA that significantly influences various cellular processes, particularly in association with mitochondrial function and fat metabolism (Ropka-Molik et al., 2020). The *AKT* genes, including *AKT2*, are crucial for cellular growth and survival. miR-103a-3p might influence *AKT2* levels indirectly through its regulation of *PTEN* (Li et al., 2021). since both autophagy and apoptosis are crucial processes regulated by factors including *EGR1*, the modulation of miR-103a-3p can impact the network that governs *EGR1* expression. The balance of apoptosis and autophagy in cells could therefore affect *EGR1*'s role in cell survival or death (Zhang et al., 2019). The microRNA bta-miR-103a-3p plays a significant role in regulating the *HSDL2* gene, particularly through its interaction with various target genes essential for cell processes like proliferation and differentiation (Sol et al., 2015). MiR-769-5p may indirectly influence the expression of *HSPA1A* through its known interactions with other regulatory proteins that manage heat shock proteins' response, including stress-activated signaling pathways (Ni et al., 2020). The regulatory impact of bta-miR-769-5p on the *PPARGC1A* gene involves post-transcriptional mechanisms, where it may repress the expression of this gene. In turn, this can influence pathways related to lipid metabolism, and energy homeostasis (Ojo et al., 2023). *AKT2* is part of the *AKT* signaling pathway, which is crucial in mediating cell proliferation and survival—processes influenced by miRNA regulation. The implication arises from the fact that miRNAs often modulate multiple target genes, and given that *AKT* signaling is involved in oncogenesis, it stands to reason that bta-miR-769-5p could indirectly impact *AKT2* activity by modulating its associated pathways, such as survival and growth through effects on target proteins like *RYBP* (Xian et al., 2019). bta-miR-769-5p plays a significant role in the regulation of the *EGR1* gene, particularly in the context of embryonic growth and development. *EGR1*, or Early Growth Response 1, is a transcription factor that functions as both a tumor suppressor and a promoter in various biological processes, including cellular growth and differentiation (Krones-Herzig et al., 2005). The interaction between miR-769-5p and the *HSDL2* gene influences cellular proliferation and differentiation during embryonic development. It has been observed that modulation of miR-769-5p impacts cell cycle regulation (Ni et al., 2020). In summary, bta-miR-103a-3p is poised as a key regulator in bovine embryonic development, with bta-miR-769-5p further contributing alongside essential hub genes that mediate critical developmental processes. Transcription factors that control hub genes: The transcription factors *HSF1*, *FOXA2*, *FOXO1*, and *CREB* contribute significantly to managing the activity of different hub genes such as *HSPA1A, PPARGC1A*, *AKT2*, *EGR1*, and *HSDL2. HSF1* is crucial for the heat shock response, activating the expression of heat shock proteins to protect cells from stress. In bovine embryos, the expression of *HSPA1A*, which is driven by heat shock promoters, is regulated by *HSF1* during early developmental stages, particularly around the embryonic genome activation (EGA) phase. *HSPA1A* is modulated by *HSF1*, which responds to heat stress and cytotoxicity. *HSPA1A* is crucial for protein folding, assembly, and protection against stress, making it significant during the critical stages of embryo development (Barna et al., 2018). *FOXA2* acts as a transcriptional regulator, essential for the differentiation and maintenance of various endodermal tissues. It plays a role in glucose homeostasis by regulating genes involved in metabolic processes, which is critical for the developing embryo (Golson et al., 2016). For instance, *FOXA2*'s interaction with other factors is required for organ development and function, particularly in the pancreas (Golson et al., 2016). *PPARGC1A* is a master regulator of energy metabolism, influencing mitochondrial biogenesis and oxidative metabolism. This gene's expression is regulated by *FOXA2* and *FOXO1*, linking metabolic health to embryo viability (Golson et al., 2016). *FOXO1* functions as a key regulator of various biological activities, including apoptosis, the cell cycle, and metabolic processes. It functions as a master regulator for genes involved in insulin signaling and metabolism management (Yang et al., 2021). FOXO1's interaction with the hub gene *AKT2* underscores its importance in the insulin signaling pathway, promoting glucose uptake and glycogen synthesis while inhibiting gluconeogenesis. *CREB* is crucial for neural development and memory formation and acts as a transcriptional activator for numerous genes involved in cell survival and metabolism (Yang et al., 2021). Its regulatory influence extends to EGR1, a gene known to be involved in growth factor responses and various signaling pathways related to cell proliferation and differentiation. The interplay between *HSF1*, *FOXA2*, *FOXO1*, and *CREB* is pivotal in orchestrating the expression of hub genes like *HSPA1A*, *PPARGC1A*, *AKT2*, *EGR1*, and *HSDL2* during crucial stages of bovine embryo development. Understanding these regulators not only provides insights into bovine reproduction but also highlights pathways that might be targeted in metabolic therapies. Collectively, these hub genes form an integrated framework linking stress response, metabolism, developmental regulation, and fertility. Future studies should focus on functional validation of these genes and explore their interactions within the embryo-maternal interface to advance reproductive efficiency in cattle. Although microarray is a commonly used tool for gene expression analysis, it has limitations compared to RNA-Seq, including the inability to detect novel transcripts, isoform diversity, and non-coding RNAs. RNA-Seq offers higher sensitivity and a broader dynamic range, enabling a more comprehensive analysis of the transcriptome. Therefore, the results obtained from microarray should be interpreted with caution, and it is recommended that future studies utilize RNA-Seq for more in-depth transcriptomic profiling.

Conclusions: In this study, we identified several critical hub genes (*HSPA1A*, *PPARGC1A*, *AKT2*, *EGR1*, and *HSDL2*) that play pivotal roles in bovine fetal development. These genes are involved in essential biological pathways including cellular stress response (*HSPA1A*), mitochondrial biogenesis and energy metabolism (*PPARGC1A*), metabolic regulation and early embryonic cleavage (*AKT2*), transcriptional control of reproductive processes (*EGR1*), and metabolic modulation linked to fertility and embryo viability (*HSDL2*). Investigations into the expression patterns of genes involved in development during various stages of bovine fetal growth have uncovered important information about the molecular pathways governing fetal growth and differentiation. These findings emphasize the dynamic nature of gene expression and the essential roles these genes fulfill in orchestrating development. Understanding these patterns enriches our comprehension of cattle biology. It supports the development of strategies to improve reproductive performance and health, with implications for selective breeding advancements and optimized livestock management. Furthermore, the identification of key differentially expressed genes (DEGs), biological processes, and pathways has significant implications for enhancing reproductive technologies in bovine breeding, as these identified genes could serve as potential biomarkers for embryo viability, allowing for the selection of higher-quality embryos for transfer, while a deeper understanding of the regulatory mechanisms governing these genes could lead to novel strategies aimed at improving conception rates and reducing embryonic losses. To better understand the regulatory roles of hub genes, future research could use CRISPR-Cas9 gene editing, single-cell RNA sequencing, and ChIP-seq to study gene function and regulation. Proteomic analyses and in vitro embryo culture with gene manipulation would further clarify their impact on embryo development. These approaches will enhance insights into bovine fetal development and improve reproductive technologies.

Author contributions

Conceptualization, Z.R., F.M.; funding acquisition, Z.R.; methodology, Z.R., F.M.; formal analysis, Z.R., F.M., M.M., A.B.; resources, Z.R., investigation, Z.R., M.M., A.B.; writing—original draft preparation, F.M., Z.R., writing review and editing, Z.R., M.M., A.B.; project administration, Z.R. All authors read and approved the final manuscript.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article

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Ethical considerations

In this study, we analyzed data sets freely available in the public domain. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois.

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Conflict of interest

The authors declare no competing interests.

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رمزگشایی الگوی بیان ژن در تکامل جنینی گاو: بینش هایی در مورد شبکه های تنظیمی کلیدی و پیامدهای آن برای فناوری های تولیدمثل

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چکیده

هدف: الگوهای بیان ژن‌های مرتبط با رشد در طول رشد جنین گاو، فرآیندهای بسیار تنظیم‌شده‌ای هستند که تحت تأثیر عوامل ژنتیکی و محیطی قرار دارند. این مطالعه بینش‌های جدیدی در مورد این الگوها ارائه می‌دهد و بینش پیشرفته‌ای را در مورد رشد جنین و شناسایی کاندیداهای امیدوارکننده برای بهبود فناوری‌های تولید مثل در اصلاح نژاد گاو ارائه می‌دهد.

مواد و روش‌ها: در تحقیق حاضر، داده‌های بیان ژن مربوط به جنین‌های گاوی با شماره دسترسی (GEO: GSE18290) با استفاده از GEO2R تحلیل شدند تا ژن‌هایی با بیان متفاوت (DEGs) را در مراحل کلیدی رشد از اووسیت تا مرحله ۲ سلولی، مرحله ۴ سلولی تا ۱۶ سلولی، و مورولا تا بلاستوسیت شناسایی شوند. سپس، آنالیز غنی‌سازی ژن (GO) و مسیرهای KEGG به منظور شناسایی فرآیندهای زیستی مهم مرتبط با DEGهای شناسایی شده انجام شد. سپس، شبکه برهمکنش پروتئین-پروتئین

(PPI) برای بررسی ارتباطات بین ژن‌های هاب، همراه با شناسایی میکروRNAهای (miRNAها) و فاکتورهای رونویسی مربوطه ساخته شد.

نتایج: این تجزیه و تحلیل، بیان متفاوت ۲۲۴۴ ژن (۱۰۲۶ ژن بیان بالا، ۱۲۱۸ بیان پایین) را در طول گذر ازمرحله اووسیت به ۲ سلولی، ۲۳۷۹ ژن (۱۱۴۶ ژن افزایش بیان، ۱۲۳۳ ژن کاهش بیان) را در طول پیشرفت از ۴ سلولی به ۱۶ سلولی و ۱۵۴۴ ژن (۸۲۱ ژن افزایش بیان، ۷۲۳ ژن کاهش بیان) را در طول تغییر از دوره‌های مورولا به بلاستوسیست شناسایی کرد. آنالیزهای GO و KEGG فرآیندهای بیولوژیکی غنی‌شده‌ای مانند کاتابولیسم mRNA، تخریب واسطه کدهای بی‌معنی، فعال‌سازی ماست‌سل‌ها، فسفوریلاسیون پروتئین، سیگنال‌دهی فاکتور رشد عصبی، تنظیم رگ‌زایی، سیگنال‌دهی MAPK و Rap1 و پردازش پروتئین شبکه آندوپلاسمی را برجسته کردند. ژن‌های هاب کلیدی، از جمله *HSPA1A* ، *PPARGC1A*، *AKT2*، *EGR1* و *HSDL2*، به همراه miRNA های (bta-miR-103a-3p و bta-miR-769-5p) و فاکتورهای رونویسی (*HSF1*، *FOXA2*، *FOXO1* و *CREB*) مرتبط شناسایی شدند.

. **نتیجه‌گیری:** نتایج تجزیه و تحلیل مطالع تغییرات بیان ژن در هر مرحله را نشان داد و مسیرهای بیولوژیکی کلیدی و شبکه‌های تنظیمی دخیل در رشد جنین گاو را برجسته کرد. ژن‌های مرکزی، miRNAها و فاکتورهای رونویسی به عنوان نشانگرهای زیستی و اهداف بالقوه شناسایی شدند. این یافته‌ها بینش‌های مولکولی ارزشمندی را ارائه می‌دهند که می‌توانند برای بهبود فناوری‌های تولید مثلی و باروری در پرورش گاو به کار روند.

کلمات کلیدی: الگوهای بیان ژن، رشد و نمو جنین گاو، زیست شناسی تولید مثل، ژن هاب

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