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Title of the doctoral dissertation: Transcriptome analysis of early stages of bovine embryos obtained in vivo from Polish Holstein-Friesian cows

Abstract

Background: The objective of the research was to investigate how maternal age and season affect the transcriptome of bovine embryos derived in vivo from Holstein-Friesian cows. The study aimed to analyze changes in gene expression and metabolic pathways related to embryo quality and developmental potential. Previous studies have documented the impact of maternal age on reproductive outcomes; however, the mechanisms underlying transcriptomic changes in early bovine embryos remain unclear. Therefore, by comparing embryos collected during warm and cold seasons and of younger and older cows, the dissertation study sought to identify molecular markers of embryo competence and environmental adaptation.

Methods: The RNA-Seq-based transcriptome profiling was conducted on 34 in vivo-derived embryos collected from seven Polish Holstein-Friesian dairy cows differing in age (2–7 years) and season (spring and summer). Embryos were obtained after superovulation, artificial insemination, and uterine flushing according to IETS guidelines, morphologically evaluated, and stored at -80 °C. RNA extraction, cDNA synthesis, amplification, and library preparation were performed according to the Takara Smart-seq2 protocol. Sequencing was carried out on the Illumina NovaSeq6000 platform (2×150 bp, 30 million reads per embryo). The raw reads were filtered for quality and mapped to the Bos taurus UMD3.0 reference genome (assembly accession GCA_002263795.3). Differential gene expression analysis was performed in DESeq2, comparing embryos by maternal age and seasons. The functional annotation of differentially expressed genes was conducted using PANTHER and STRING databases. The enrichment analysis of Gene Ontology Biological Processes and KEGG pathways was performed in Cytoscape (v3.9.1) with ClueGO (v2.5.9), applying Bonferroni step-down correction (adjusted $p<0.05$). Networks of enriched functional terms were generated separately for up- and downregulated gene sets to visualize molecular processes potentially affected by age and seasonal conditions. Additionally, deconvolution analysis employing the MuSiC and DeTREM algorithm was applied to estimate the contribution of specific embryonic developmental stages to the bulk transcriptomes, using reference single-cell RNA-seq datasets.

Results: Transcriptome analysis revealed that both advanced maternal age and seasonal conditions significantly influenced gene expression profiles in bovine embryos, affecting pathways essential for embryonic development, metabolism, implantation, immune regulation, and stress adaptation. In cotext to maternal age, the study identified the top 30 age-dependent upregulated differentially expressed gene (DEG) transcripts, including: *Inducible T cell costimulator (ICOS), Signal Transducer and Activator of Transcription 4 (STAT4), Matrix Gla Protein (MGP), Cancer/Testis Antigen Family 47 Member B1 (CT47B1), Family With*

Sequence Similarity 13 Member C (FAM13C), Cytochrome P450 Family 11 Subfamily A Member 1 (CYP11A1), Cytochrome P450 Family 4 Subfamily V Member 2 (CYP4V2), Hypoxanthine Phosphoribosyltransferase 1 (HPRT1), C-C Motif Chemokine Ligand 24 (CCL24), Phospholipase A2 Group VII (PLA2G7), SYT 4 (Synaptotagmin 4), Phosphodiesterase 10A (PDE10A), MAGE Family Member B2 (MAGE B2), INSC Spindle Orientation Adaptor Protein (INSC), Leucine rich repeats and calponin homology domain containing 2 (LRCH2), Zinc Finger Protein 514 (ZNF514), Molybdenum Cofactor Sulfurase (MOCOS), Homer Scaffold Protein 1 (HOMER1), RNA binding motif protein 46 (RBM46), Pyruvate Dehydrogenase Kinase 4 (PDK 4), Carbonic Anhydrase 2 (CA2), Transketolase Like 2 (TKTL2), Early growth response 2 (Egr 2), Zinc finger B-box domain (ZBBX), Chromosome 5 Open Reading Frame 75 (C5H12orf75) and 5 novel unannotated genes. The study identified the top 30 age-dependent downregulated differentially expressed gene (DEG) transcripts, including: *Neuropeptide S Receptor 1 (NPSR1), Synaptotagmin (SYT9), ADP Ribosylation Factor Like GTPase 13A (Arl13a), Prominin 1 (PROM1), RNA binding motif single-stranded interacting protein 3 (RBMS3), Fibronectin type III and SPRY domain containing 1 like (FSD1L), ATPase copper transporting alpha (ATP7A), Inosine monophosphate dehydrogenase 1 (IMPDH1), Serpin family B member 4 (SERPINB4), Calcyphosine-like (CAPSL), Fanconi anaemia, complementation group A (FANCA), CAMP Responsive Element Binding Protein 5 (CREB5), LY6/PLAUR Domain Containing 6B (LYPD6B), SANT and BTB domain regulator of CSR (SANBR), Lysozyme (LYZ), Ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), FEM-1-Like Death Receptor Binding Protein (FEM1B), Junction-Mediating And -Regulatory Protein (JMY), V-Akt Murine Thymoma Viral Oncogene Homolog 2 (AKT2)* along with eleven unannotated genes. In context to season, the study identified the top 30 season-dependent upregulated differentially expressed gene (DEG) transcripts, including: *HSPA6 (Heat shock protein family A (Hsp70) member 6), NR1I3 (Nuclear Receptor Subfamily 1 Group I Member 3), Cytochrome P450 Family 4 Subfamily V Member 2 (CYP4V2), C-X-C Motif Chemokine Receptor 4 (CXCR4), Immunoglobulin Heavy Constant Mu (IGHM), Hypoxanthine Phosphoribosyltransferase 1 (HPRT1), Follicle Stimulating Hormone Receptor (FSHR), MAGE Family Member B2 (MAGEB2), FosB Proto-Oncogene, AP-1 Transcription Factor Subunit (FOSB), Cytochrome P450 Family 11 Subfamily A Member 1 (CYP11A1), Zinc Finger B-Box Domain Containing (ZBBX), Forkhead Box A1 (FOXA1), Lysosomal Protein Transmembrane 5 (LAPTM5), MAGE Family Member D2 (MAGED2), Eva-1 Homolog C (EVA1C), SH3 Domain Binding Glutamate Rich Protein (SH3BGR), Carbonic Anhydrase 2 (CA2), Branched Chain Amino Acid Transaminase 2 (BCAT2), SH3 Domain Containing GRB2 Like 2, Endophilin A1 (SH3GL2), BMP Binding Endothelial Regulator (BMPER), Fibrinogen Beta Chain (FGB), Family With Sequence Similarity 13 Member C (FAM13C), Kallikrein Related Peptidase 8 (KLK8), Myosin Light Chain 7 (MYL7), CD8 Subunit Beta (CD8B)* and 4 novel unannotaed genes. However, the study identified the top 30 season-dependent downregulated differentially expressed gene (DEG) transcripts, including: *RNA binding motif single stranded interacting protein 3 (RBMS3), Ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), Proteasome 26S subunit, ATPase 6 (PSMC6), Dynein axonemal heavy chain 12 (DNAH12), ETS variant transcription factor 5 (ETV5), Synaptotagmin 9 (SYT9), Fibronectin type III and*

SPRY domain containing 1 like (FSD1L), F-box protein 48 (FBXO48), Dedicator of cytokinesis 8 (DOCK8), Adrenoceptor beta 2 (ADRB2), Zinc finger protein 845 (ZNF845), Fem-1 homolog B (FEM1B), Hepcidin antimicrobial peptide (HAMP), BEN domain containing 4 (BEND4), Aquaporin 7 (AQP7), Corneodesmosin (CDSN), and ATP binding cassette subfamily C member 3 (ABCC3), and 13 novel unannotated genes. In the context of deconvolution-based gene expression analysis, the study identified several overlapping marker genes across four successive stages of embryo development: the early-stage group comprising 2-cell and 4-cell embryos, the 8-cell stage, the morula stage, and the late blastocyst stage. Most of these marker genes exhibit stage specificity, with the majority being unique to the 8-cell stage (2832; 45.6%) and the early-stage group (1444; 23.3%), followed by morula (895; 14.4%) and late blastocyst (640; 10.3%). Only limited overlap was detected between clusters, indicating that each developmental stage is characterized by a distinct gene expression profile.

Conclusions: The findings demonstrate that maternal age and season jointly shape the bovine embryonic transcriptome, affecting key developmental, metabolic, and stress-adaptive processes. This knowledge may help improve embryo selection criteria, refine assisted reproduction protocols in cattle, and offer insights relevant to reproductive management in other mammalian species. In the context of maternal age, the study concludes that relative to embryos derived from younger cows, embryos obtained from older cows exhibited up-regulation of genes involved in the metabolic pathways of steroidogenesis, metabolic regulation, and immune signaling, including *CYP11A1, PDK4, HPRT1, STAT4, and EGR2,* suggesting compensatory activation of endocrine and metabolic pathways. In contrast, embryos from older cows showed down-regulation of key developmental and mitochondrial genes, such as *AKT2, UQCRC2, FANCA, JMY,* and *PROM1,* indicating impaired energy metabolism, cytoskeletal organization, genomic stability, and implantation-related processes. In context to season, the study concludes that when embryos collected during the warm season were compared with those obtained during the cold season, warm-season embryos displayed increased expression of stress-response, endocrine, and implantation-associated genes, including *HSPA6, FSHR, CXCR4, CYP11A1, BMPER, and FGB*, consistent with enhanced cellular stress adaptation and altered hormonal signaling. Conversely, reduced expression of genes involved in mitochondrial function, cytoskeletal dynamics, vesicle trafficking, and epigenetic regulation, such as *UQCRC2, DNAH12, SYT9, ETV5, and RBMS3*, was observed in warm-season embryos, suggesting a slower developmental tempo and altered metabolic homeostasis under elevated environmental temperatures. A deconvolution-inspired analytical framework was applied to bulk RNA-seq data from individual in vivo–derived bovine embryos to assess transcriptional similarity across stages of preimplantation development. This approach enabled the identification of shared gene expression signatures and stage-specific transcriptional programs. Clustering and dimensionality reduction analyses revealed a clear separation of developmental stages, with pronounced transcriptomic transitions between early cleavage stages and later stages. Comparative analyses using MuSiC and DeTREM indicated a predominance of late blastocyst–associated transcriptional signatures in whole-embryo transcriptomes, highlighting the dominance of later-stage gene expression programs in bulk RNA-seq data.

Keywords: Holstein-Friesian cattle, Bovine embryos, Maternal age, Transcriptome analysis, RNA-seq

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