

Transcriptomic profiling of the donkey endometrium reveals dynamic molecular transitions across the estrous cycle

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Abstract

The endometrium undergoes extensive cyclical remodeling essential for reproductive success; however, the molecular mechanisms governing these processes in donkeys (*Equus asinus*) remain poorly characterized. To address this gap, we performed high-throughput RNA sequencing on endometrial tissues collected from Dezhou donkeys (n = 16) at four post-ovulation stages: Day 0 (ovulation), Day 3 (early luteal), Day 7 (mid-luteal), and Day 18 (late luteal/pre-luteolysis). Differential gene expression analysis, Gene Ontology enrichment, and KEGG pathway mapping were conducted to characterize temporal transcriptomic dynamics. We identified 2,474, 6,548, and 1,288 differentially expressed genes in Day 0 versus Day 3, Day 0 versus Day 7, and Day 0 versus Day 18 comparisons, respectively. A biphasic transcriptional pattern emerged: transient suppression of immune and cytokine signaling pathways at Day 3 was followed by robust activation of TNF, NF- κ B, PI3K-Akt, and extracellular matrix–receptor interaction pathways by Day 7, consistent with progesterone-driven stromal proliferation and tissue remodeling. By Day 18, enrichment of cytochrome P450, steroid hormone biosynthesis, and amino acid metabolism pathways indicated metabolic reprogramming associated with luteal regression. This study provides the first comprehensive temporal transcriptomic atlas of the donkey endometrium, revealing conserved endocrine–immune–metabolic regulatory networks comparable to those in mares and cattle. These findings establish a molecular foundation for developing precision strategies to improve uterine health, fertility management, and assisted reproductive technologies in donkeys.

Keywords: endometrium, *Equus asinus*, estrous cycle, RNA-seq, transcriptomics

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Introduction

As a domesticated livestock species, the donkey (*Equus asinus*) holds substantial economic value and occupies an important place in human cultural history (Milevski and Horwitz, 2019; Huang *et al.*, 2023). Among the indigenous breeds of Shandong Province, China, the Dezhou donkey is regarded as one of the country's most important large-framed breeds, distinguished by its large body size, robust conformation, strong environmental adaptability, and superior meat and hide quality—attributes that have established it as a foundational genetic resource for the Chinese donkey industry and a preferred sire breed for improving local donkey populations through crossbreeding (Zhang *et al.*, 2022; Liu *et al.*, 2024). On the basis of coat pigmentation, the breed is conventionally classified into two distinct strains, namely Sanfen and Wutou (Zhang *et al.*, 2022; Liu *et al.*, 2024). These two strains are readily distinguishable by their coat pigmentation. In the Sanfen strain, depigmented (light-colored) markings appear in three characteristic regions—surrounding the eyes and muzzle and along the ventral abdomen—whereas the Wutou strain exhibits a uniformly black coat throughout (Zhang *et al.*, 2022; Liu *et al.*, 2024). Within China, the Dezhou donkey is valued chiefly for its contribution to the production of meat, milk, and Ejiao, the gelatin extracted from donkey hide (for which it serves as the principal source animal) (Khan *et al.*, 2024a, 2024b; Li *et al.*, 2024; Ullah *et al.*, 2025a). Over recent decades, however, the national donkey population has experienced a marked decline attributable to intensive slaughter practices, inherently low reproductive efficiency, and underutilization of genetic resources (Bennett and Pfuderer, 2019; Bennett and Pfuderer, 2020; Norris *et al.*, 2021). This demographic trajectory poses substantial risks for biodiversity conservation and threatens the long-term sustainability of donkey-derived industries. Accordingly, enhancing female reproductive performance has emerged as a critical priority for supporting population recovery and establishing scalable breeding programs (Miragaya *et al.*, 2018; Canisso *et al.*, 2019; Yang *et al.*, 2021; Ullah *et al.*, 2025b).

In jennies, the estrous cycle is generally longer than that of mares and typically ranges from approximately 23 to 27 days, although cycle characteristics may vary according to breed, season, age, nutritional status, and management conditions (Vandeplasseche *et al.*, 1981; Meira *et al.*, 1995; Taberner *et al.*, 2008; Díaz-Duran *et al.*, 2017). The cycle is broadly divided into two phases: the follicular phase (estrus) and the luteal phase (diestrus). Estrus usually lasts about 4–10 days and is characterized by sexual receptivity, dominant follicular growth, uterine edema, low progesterone concentrations, and increased estrogenic activity, with ovulation generally occurring near the end of estrus (Vandeplasseche *et al.*, 1981; Meira *et al.*, 1995; Taberner *et al.*, 2008; Díaz-Duran *et al.*, 2017). Following ovulation, corpus luteum formation marks the onset of the luteal phase, during which rising progesterone secretion promotes endometrial glandular activity, stromal remodeling, immune modulation, and

preparation of the uterus for possible embryo survival. These phase-dependent physiological changes provide the biological rationale for sampling the endometrium at defined post-ovulation stages in the present study.

The uterine endometrium is a highly dynamic, multifunctional tissue central to mammalian reproductive success (Critchley *et al.*, 2020; Hong, 2024). Successful pregnancy establishment requires precisely regulated endometrial remodeling throughout the estrous cycle to achieve optimal uterine receptivity (Yip *et al.*, 2013; Billhaq *et al.*, 2020). In equids, this cyclical transformation is orchestrated by ovarian steroid hormones and involves coordinated changes in cellular proliferation, differentiation, extracellular matrix composition, immune surveillance, and vascular dynamics—processes that collectively establish uterine receptivity for embryo implantation (Schöniger and Schoon, 2020; Amaral *et al.*, 2025; Radar-Chafirovitch *et al.*, 2025). Notably, endometritis represents a primary contributor to reproductive failure among domestic equids (Canisso *et al.*, 2020; Morris *et al.*, 2020; Ravaioli *et al.*, 2022), and in mares' chronic endometrial inflammation has been strongly implicated in persistent infertility and early embryonic loss (Canisso *et al.*, 2020; Morris *et al.*, 2020; Díaz-Bertrana *et al.*, 2021). Despite belonging to the Equidae family, donkeys exhibit suboptimal reproductive efficiency under managed breeding conditions, with both infectious and non-infectious disorders—including endometritis, metritis, placental retention, and hormonal dysregulation—substantially impairing fertility (Miragaya *et al.*, 2018; Van den Branden *et al.*, 2021; Wang *et al.*, 2024; Akhtar *et al.*, 2025). Given these physiological parallels, elucidating the molecular mechanisms underlying endometrial cyclical changes is essential for improving fertility outcomes and preventing uterine pathologies in this taxonomic group.

Conventional reproductive assessments—including estrus behavior monitoring, transrectal ultrasonography, circulating hormone quantification, and gross uterine evaluation—provide reliable information on follicular dynamics, ovulation timing, luteal presence, and overt uterine pathology in jennies. However, these approaches characterize the reproductive tract only at the anatomical, physiological, and endocrine levels; they cannot resolve the coordinated, genome-wide changes in gene expression that drive cyclic endometrial remodeling, nor can they identify the specific regulatory pathways, candidate genes, or molecular markers that determine uterine receptivity and endometrial health. Transcriptomic profiling by RNA sequencing overcomes these limitations by providing an unbiased, quantitative, genome-wide readout of endometrial gene activity at defined cycle stages, thereby enabling the discovery of stage-specific molecular signatures, pathway-level regulatory mechanisms, and candidate biomarkers that are inaccessible to conventional clinical examination (D'Agostino *et al.*, 2022; Jin *et al.*, 2023). These methodological approaches permit global profiling of transcriptomic dynamics, revealing regulatory pathways central to tissue homeostasis,

remodeling, immune regulation, and metabolic adaptation (Monteiro de Barros *et al.*, 2021; Weber *et al.*, 2021; Chen *et al.*, 2025). Consequently, the temporal gene expression landscape of the donkey endometrium throughout the estrous cycle remains poorly characterized, representing a significant knowledge gap that impedes mechanistic insight and applied reproductive improvement (Wang *et al.*, 2024; Wang *et al.*, 2025).

To address this knowledge gap, the present study employed RNA sequencing to characterize the temporal transcriptomic landscape of the donkey endometrium across four defined post-ovulation stages (Day 0, Day 3, Day 7, and Day 18). Differential gene expression analysis was integrated with Gene Ontology and KEGG pathway enrichment to identify stage-specific gene expression patterns and the biological pathways associated with cyclic endometrial remodeling. The findings are intended to provide a molecular basis for understanding endometrial physiology in the donkey and to support the future development of improved reproductive management and fertility-related strategies in this species.

Materials and Methods

Ethical statement: The study protocol was approved by the institutional Special Committee of Scientific Research Ethic of Liaocheng University (AP2026031403).

Study location and period: This study was conducted in Liaocheng City, Shandong Province, China, during April 2024.

Animal management: Sixteen clinically healthy female Dezhou donkeys (*Equus asinus*), aged 4–8 years and weighing 350–420 kg, were selected for this study. This age range was chosen because jennies reach puberty at approximately 2 years. In contrast, breeding is generally recommended after sufficient body development, around 2.5 years of age or after reaching 95% of adult body height and 70% of adult body weight (Miragaya *et al.*, 2018; Wang *et al.*, 2021, Wissdorf *et al.*, 2021). Therefore, the selected animals represented mature adult jennies suitable for minimizing age-related reproductive variation. Animals were selected from the available herd based on predefined inclusion criteria rather than formal randomization. Reproductive history was reviewed from farm records where available, and animals with a recent history of reproductive disease or treatment were excluded. Parity was not used as a grouping factor in the present study. All donkeys were maintained under comparable management conditions, including clean and well-ventilated housing, natural photoperiod, ad libitum access to clean water, a nutritionally balanced diet, and routine veterinary supervision throughout the study period.

Estrous cycle monitoring and experimental design: Estrous cycle monitoring was performed daily by transrectal ultrasonography using a linear-array transrectal probe. All examinations were performed by

the same experienced operator to reduce operator-related variation. Ovulation was confirmed by the disappearance or collapse of the dominant preovulatory follicle on ultrasonography, with subsequent corpus luteum formation used as supporting confirmation. Endometrial tissue samples were collected at four distinct stages; Day 0 (D0, ovulation), Day 3 (D3, early luteal phase), Day 7 (D7, mid-luteal phase), and Day 18 (D18, late luteal/pre-luteolysis phase) post-ovulation. Each experimental group comprised four biological replicates ($n = 4$ per group; $N = 16$ total).

Clinical evaluation of reproductive health: Clinical reproductive health was assessed before sample collection based on general physical examination, normal estrous cyclicity, absence of abnormal vaginal discharge, absence of uterine fluid or obvious uterine abnormalities on transrectal ultrasonography, and no history of reproductive tract disease. Histopathological examination was not performed; therefore, samples were described as endometrial tissues collected from clinically healthy jennies rather than histologically confirmed healthy endometrium.

Sample collection: Endometrial tissue samples were collected from each animal at the designated time points using a sterile transcervical biopsy approach, following established equine endometrial biopsy protocols (Merkl *et al.*, 2010). Briefly, animals were appropriately restrained, and the procedure was performed by an experienced veterinarian under routine animal welfare monitoring. No general anesthesia was used; however, animals were monitored during and after sampling for signs of discomfort or adverse reactions. Biopsy samples were collected using sterile instruments and immediately placed into RNase-free cryovials. No preservation solution was added because tissues were snap-frozen directly in liquid nitrogen after collection. Samples were transported under liquid nitrogen conditions and subsequently stored at -80°C until RNA extraction.

RNA extraction and quality assessment: Total RNA was isolated from endometrial samples using a commercial RNA extraction kit according to established protocols (Kurar *et al.*, 2010). RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), with only samples exhibiting RNA Integrity Number (RIN) values > 7.0 retained for library preparation.

Library construction and RNA sequencing: Transcriptome libraries were constructed following standard procedures. Briefly, poly-A messenger RNA was enriched from total RNA using Oligo(dT) magnetic beads, followed by fragmentation into short sequences serving as templates for first-strand cDNA synthesis using random hexamer primers and reverse transcriptase. Following RNA template degradation

with RNase H, second-strand cDNA synthesis was performed using DNA polymerase I and dNTPs. Double-stranded cDNA was purified, end-repaired, A-tailed, and ligated to sequencing adapters. Fragment size selection (170–420 bp) was performed using AMPure XP beads (Beckman Coulter, Brea, CA, USA), followed by PCR amplification and purification to obtain final sequencing libraries. Library quality was assessed using the Agilent 2100 Bioanalyzer, and concentration was measured using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Quantitative real-time PCR was used for precise quantification of effective library concentration, with libraries exceeding 2 nM proceeding to sequencing. Qualified libraries were sequenced on an Illumina platform (Illumina, San Diego, CA, USA) using paired-end 150 bp (PE150) configuration based on sequencing-by-synthesis technology (Meldrum *et al.*, 2011).

Data processing and bioinformatic analysis: Raw sequencing reads were processed to remove adapter sequences, poly-N regions, and low-quality reads. Clean reads were subjected to Q20, Q30, and GC content analysis to evaluate base quality and composition. Differential expression analysis was performed to identify differentially expressed genes (DEGs) between experimental groups. Functional annotation was performed using Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Results

Quality assessment and differential expression analysis: To comprehensively assess the quality of the transcriptome data and the overall expression patterns of the various samples, this study conducted RNA-seq sequencing on 16 endometrial samples from four different time periods, with 4 biological replicates for each period. The results of the sequencing quality control are summarized in Table 1. The number of raw reads per sample ranged from 42,849,878 to 47,958,586; after quality control (removal of low-quality reads, adapter sequences, and reads with a nitrogen proportion greater than 10%), each sample had 41,516,430 to 46,358,238 high-quality reads. The Q30 base quality ratio ranged from 93.95% to 94.6%, which is above 90%, while the GC content ranged from 50.44% to 53.94%. Pearson correlation analysis of the transcriptome expression profiles across the samples showed (Fig. 1A) that the correlation coefficients between biological replicates within the same period were high, indicating good reproducibility across these periods and thus reliable data. Principal component analysis (PCA) revealed that D3, D7, and D18 groups demonstrated robust intra-group clustering and favorable inter-group separation, while the D0 group exhibited relatively greater intra-group variability (Fig. 1A). Overall, sample quality was deemed acceptable for downstream analyses. Transcriptome sequencing identified a total of 28,325 expressed genes across all samples. Differential expression analysis revealed distinct transcriptomic patterns across the estrous cycle. The D0 vs. D3 comparison identified 2,474 DEGs,

comprising 1,727 upregulated and 747 downregulated genes (Fig. 1B). The D0 vs. D7 comparison revealed the most extensive transcriptomic remodeling, with 6,548 DEGs identified (3,610 upregulated; 2,938 downregulated) (Fig. 1C). The D0 vs. D18 comparison yielded 1,288 DEGs (520 upregulated; 768 downregulated) (Fig. 1D). Venn diagram analysis demonstrated overlapping DEG subsets: 1,898 genes were shared between D0 vs. D3 and D0 vs. D7 comparisons, 189 genes between D0 vs. D3 and D0 vs. D18, and 551 genes between D0 vs. D7 and D0 vs. D18 comparisons (Fig. 1E).

Gene ontology functional enrichment analysis: D0 vs. D3 comparison: GO enrichment analysis revealed that biological processes enriched among D3 DEGs included immune regulation, metabolic pathways, cell adhesion, G protein-coupled receptor signaling, and cell growth control. Cellular component differences were predominantly observed in extracellular regions and MHC protein complexes. Molecular function enrichment included regulatory mechanisms, cytokine activity, receptor binding, chemokine receptor binding, G protein-coupled receptor binding, and peptidase regulatory activity (Fig. 2A). Notably, the majority of DEGs at D3 were downregulated relative to D0 (Fig. 2B). D0 vs. D7 comparison: Enriched biological processes included intracellular signal transduction, small GTPase-mediated signaling, transmembrane transport, cell communication regulation, and stimulus response regulation. Cellular component enrichment was observed in extracellular regions, extracellular matrix, endoplasmic reticulum membrane, and endosomal membrane systems. Molecular function enrichment included molecular function regulation, cytokine activity, and chemokine receptor binding (Fig. 2C). By D7, differential gene expression demonstrated substantial upregulation (Fig. 2D). D0 vs. D18 comparison: Biological processes enriched at D18 included cell adhesion, intracellular signal transduction, and stimulus response regulation. Cellular components showed enrichment in extracellular regions and matrices, kinesin complexes, and microtubule cytoskeletal components. Molecular functions were primarily associated with calcium ion binding, microtubule motor activity, and nucleotide exchange factor activities (Fig. 2E). Following the suppression observed at D3, endometrial gene expression demonstrated significant upward recovery by D18 relative to D0 (Fig. 2F).

KEGG pathway enrichment analysis: D0 vs. D3 comparison: KEGG analysis revealed significant enrichment of cytokine-receptor interaction, TNF signaling, NF- κ B signaling, cell adhesion molecules, PI3K-Akt signaling, JAK-STAT signaling, and viral infection-associated pathways (Figs. 3A and 3B). D0 vs. D7 comparison: Significantly enriched pathways included TNF signaling, cytokine-receptor interactions, NOD-like receptor signaling, ECM-receptor interactions, PI3K-Akt signaling, NF- κ B signaling, branched-chain amino acid degradation (valine, leucine, isoleucine), cGMP-PKG signaling,

cancer-related pathways, and viral infection pathways (Figs. 3C and 3D). D0 vs. D18 comparison: Enriched pathways included chemical carcinogenesis, cytochrome P450-mediated xenobiotic metabolism, protein digestion and absorption, sphingolipid

biosynthesis, alanine-aspartate-glutamate metabolism, arachidonic acid metabolism, steroid hormone biosynthesis, linoleic acid metabolism, glycerophospholipid metabolism, pyrimidine metabolism, and mineral absorption (Figs. 3E and 3F).

Table 1 The summary table of sequencing data quality for each sample lists the values for raw_reads, clean_reads, clean_bases, error_rate, Q20 (%), Q30 (%) and GC_pct for the 16 endometrial samples: D0 represents the ovulation period, while D3, D7 and D18 correspond to the 3rd, 7th and 18th days after ovulation respectively; the final numbers indicate the biological replicate number. The number of clean reads per sample ranges from 41.5M to 46.3M, and the Q30 value lies between 93.95% and 94.60%, both of which are above 90%, indicating that the quality of the sequencing data is excellent and meets the requirements for further analysis.

Sample	library	raw_reads	clean_reads	clean_bases	error_rate	Q20	Q30	GC_pct
D0_1	FRAS202201684-1r	43394828	42278824	6.34G	0.03	97.83	94.05	51.64
D0_2	FRAS202201685-1r	42849878	41516430	6.23G	0.03	97.88	94.19	51.73
D0_3	FRAS202201686-1r	46930870	45010110	6.75G	0.03	97.89	94.23	51.38
D0_4	FRAS202201687-1r	46803722	45270642	6.79G	0.03	97.84	93.95	51.07
D3_1	FRAS202201688-1r	45560598	44059440	6.61G	0.02	98.07	94.47	51.21
D3_2	FRAS202201689-1r	45400358	43832504	6.57G	0.02	98	94.3	51.29
D3_3	FRAS202201690-1r	47007664	45529776	6.83G	0.02	97.99	94.32	50.92
D3_4	FRAS202201691-1r	43009524	41656166	6.25G	0.02	97.99	94.26	50.44
D7_1	FRAS202201692-1r	43610258	42257416	6.34G	0.02	98.02	94.32	51.06
D7_2	FRAS202201693-1r	42934924	41740520	6.26G	0.02	98	94.22	50.86
D7_3	FRAS202201694-1r	47400472	45975456	6.9G	0.02	98.14	94.6	51.06
D7_4	FRAS202201695-1r	42954630	41623392	6.24G	0.02	97.95	94.24	51.25
D18_1	FRAS202201696-1r	44139516	42691650	6.4G	0.02	98.09	94.55	52.75
D18_2	FRAS202201697-1r	47958586	46358238	6.95G	0.03	97.99	94.2	50.9
D18_3	FRAS202201698-1r	45407086	44063818	6.61G	0.03	97.85	93.97	51.07
D18_4	FRAS202201699-1r	46626776	43878626	6.58G	0.02	97.96	94.36	53.94

Discussion

This study presents the first temporal transcriptomic atlas of the donkey endometrium across four post-ovulation stages (D0, D3, D7, and D18), revealing dynamic molecular transitions underlying uterine remodeling and reproductive function. The D0 vs. D7 comparison yielded the highest number of DEGs (6,548), indicating that the most extensive transcriptomic reprogramming occurs during the mid-luteal phase, coinciding with corpus luteum establishment and peak progesterone secretion in jennies (Segabinazzi *et al.*, 2022). In D0 versus D7, the upregulated and downregulated genes together indicate two key adaptation strategies of the endometrium during this period: metabolic activation and signal remodeling. Metabolically, the upregulation of ENO2, PKM, and NAMPT meets the energy demands associated with secretory activities; as for signal remodeling, it is manifested by a reduction in chemokine/GPCR signals (CXCR4, CCR4/9) along with coordinated adjustments in the cytoskeleton/ECM (depression of cell adhesion molecules and reprogramming of Rho GTPase signals). Notably, there was a transient inflammatory-metabolic inhibition in the endometrium at D3, which was characterized by the simultaneous down-regulation of pro-inflammatory factors (TNF, IL1B, CXCL8) and key enzymes of glycolysis (ENO2, PKM), and these genes quickly recovered to the baseline level at D7. In

contrast, another group of genes, including the core components of calcium signaling pathway (RYR2, CACNB3), TGF- β signal transcription factor (SMAD3), connective tissue growth factor (CTGF) and matrix metalloproteinases (MMP7/11/13), increased continuously at D7 and D18 after a short downregulation at D3. This expression trend shows that with the development and maintenance of corpus luteum, calcium signal transduction and TGF- β -mediated tissue remodeling activities in endometrium are gradually enhanced. It is noteworthy that several transporter genes (SLC7A8, SLC7A11, SLC39A2/8) in this study also showed a continuous upward trend, indicating that the demand for amino acids and zinc ions in D18 endometrium increased, which may be related to the vigorous protein synthesis and cell metabolism during the secretory phase. In addition, ciliary movement-related genes (DNAH6/9/12, KIF5C/6) and ion channel genes (KCNMA1, SCNN1A) were temporarily up-regulated in D7 but were inhibited again in D18. This suggests that endometrial genes exhibit a biphasic transcriptional pattern: an early suppressive phase reflecting the transition from follicular to luteal dominance, and a subsequent activation phase associated with progesterone-driven endometrial remodeling. This temporal pattern is consistent with studies in mares and other mammals demonstrating that maximal endometrial transcriptional activity coincides with mid-luteal progesterone peaks and preparation for potential

embryo arrival (Bauersachs *et al.*, 2005, Bauersachs and Wolf, 2012).

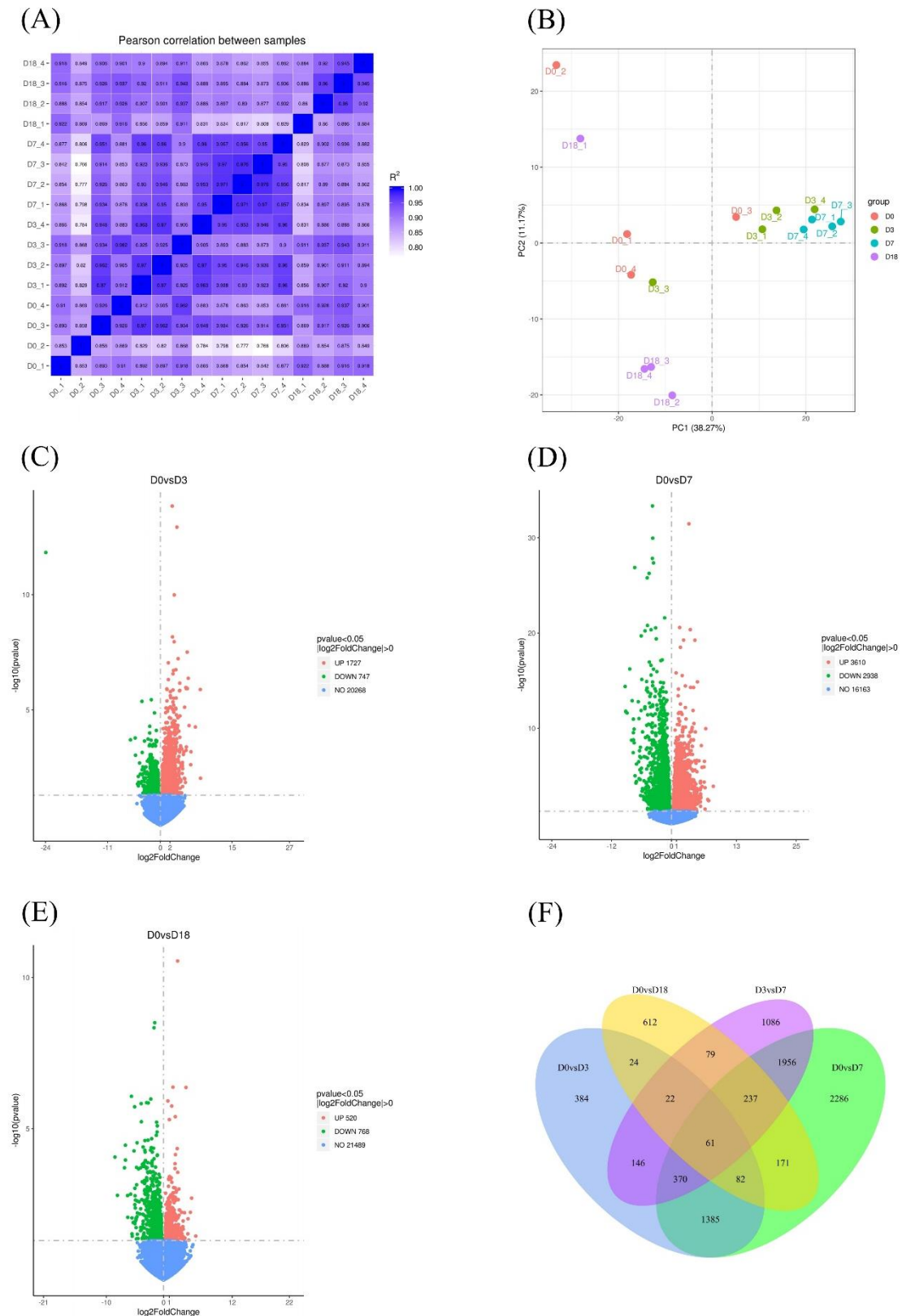
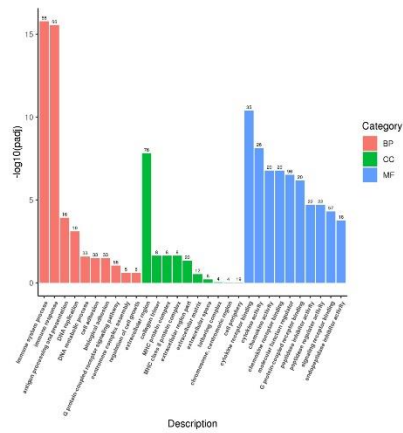


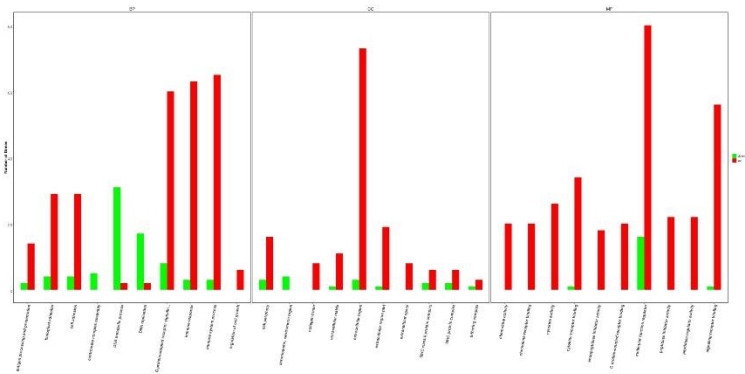
Figure 1 (A) The values shown in the graph represent the Pearson correlation coefficient (R^2) of the FPKM values for all genes between the two samples; the color range, from purple to blue, indicates an increase in the correlation coefficient from low to high. The correlation coefficients between samples taken at the same time period are generally higher than those between samples taken at different times, which suggests good biological reproducibility. (B) Principal Component Analysis (PCA) plot. It is constructed based on the gene expression profiles of all samples; the horizontal axis represents

the first principal component (PC1, with a contribution rate of 38.27%), while the vertical axis represents the second principal component (PC2, with a contribution rate of 11.17%). Different colors denote different time periods: D0 (ovulation period), D3 (3 days after ovulation), D7 (7 days after ovulation), and D18 (18 days after ovulation). The samples from D0, D3, and D7 cluster together in the principal component space, whereas the sample from D18 is distinctly separated, indicating that the transcriptome of the endometrium at stage D18 differs the most from those at other stages. (C-E) In these plots, the horizontal axis shows the log₂FoldChange in gene expression between the treatment and control groups, while the vertical axis indicates the significance level of the expression difference (-log₁₀padj or -log₁₀pvalue). For D0 vs D3: red dots represent genes with significantly increased expression (UP: 1727 genes), blue dots represent genes with significantly decreased expression (DOWN: 747 genes), and gray dots represent genes with no significant difference (NO: 20,268 genes). For D0 vs D7: red dots represent genes with significantly increased expression (UP: 3,610 genes), blue dots represent genes with significantly decreased expression (DOWN: 2,938 genes), and gray dots represent genes with no significant difference (NO: 16,163 genes). For D0 vs D18: red dots represent genes with significantly increased expression (UP: 520 genes), blue dots represent genes with significantly decreased expression (DOWN: 768 genes), and gray dots represent genes with no significant difference (NO: 21,489 genes). Compared to D0 vs D3, the number of genes showing significant differences in D0 vs D18 is significantly reduced, suggesting that the transcriptome of the endometrium at stage D18 is closer to that at stage D0. (F) Venn diagram of differentially expressed genes, showing the number of genes that are common to all comparison groups as well as those that are unique to each group.

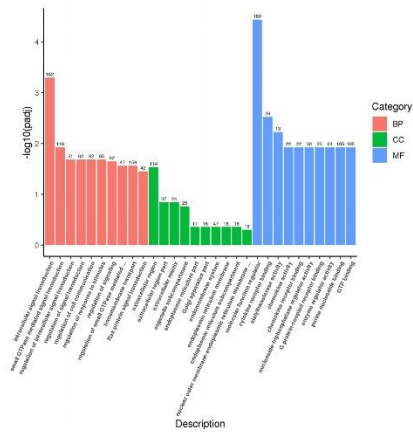
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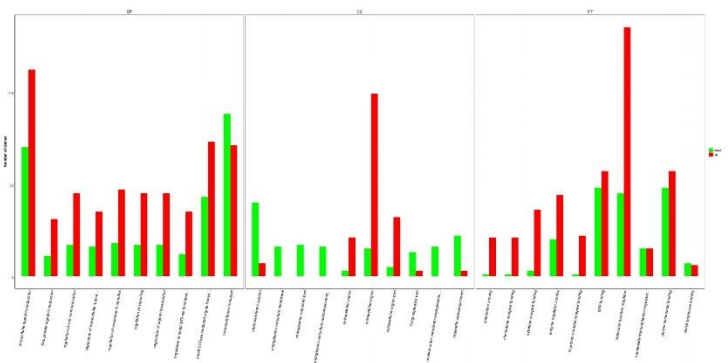
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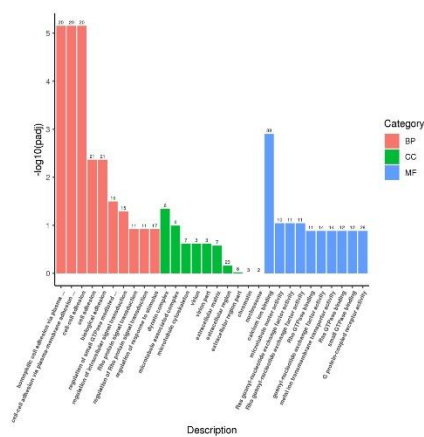
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(D)



(E)



(F)

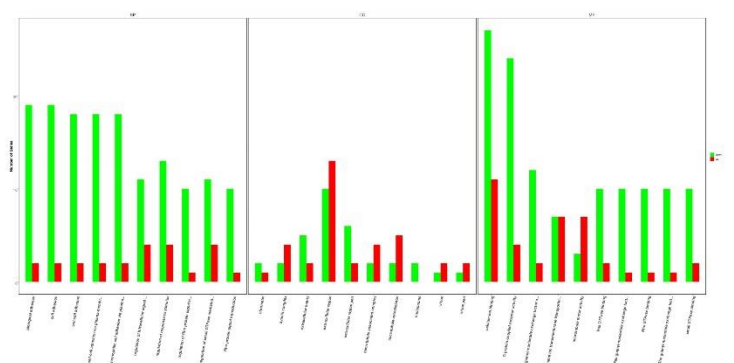
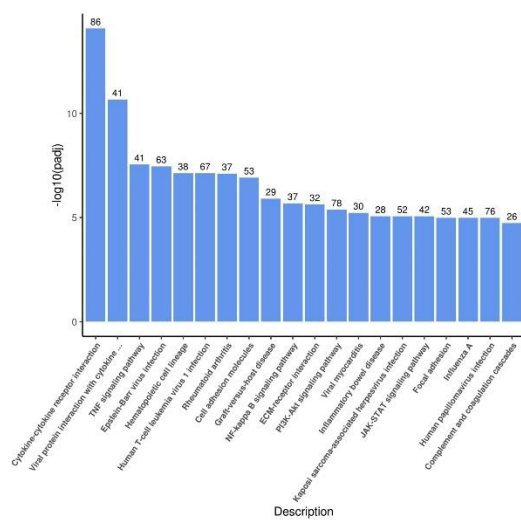
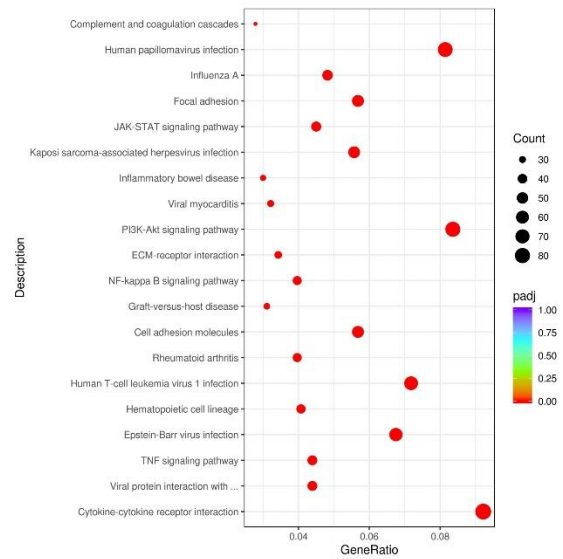


Figure 2 (A) From the results of GO enrichment analysis, the 30 most significant terms are selected to be presented in the form of bar charts; if there are fewer than 30 terms, then all terms are shown. In these charts, the horizontal axis represents the GO terms, while the vertical axis indicates the significance level of GO term enrichment, expressed as $-\log_{10}(\text{padj})$. A higher value corresponds to greater significance. Different colors are used to represent the three GO subcategories: BP, CC, and MF. (B) Based on the GO enrichment analysis results, the 30 most significant terms are used to create bar charts; if there are less than 30 terms, all terms are included. These bar charts are organized into three categories: biological processes, cellular components, and molecular functions, as well as according to whether the genes show upregulation or downregulation. In these charts, the horizontal axis represents the GO terms, and the vertical axis shows the number of genes associated with each GO term. (C) From the results of GO enrichment analysis, the 30 most significant terms are selected to be presented in the form of bar charts; if there are fewer than 30 terms, then all terms are shown. In these charts, the horizontal axis represents the GO terms, while the vertical axis indicates the significance level of GO term enrichment, expressed as $-\log_{10}(\text{padj})$. A higher value corresponds to greater significance. Different colors are used to represent the three GO subcategories: BP, CC, and MF. (D) Based on the GO enrichment analysis results, the 30 most significant terms are used to create bar charts; if there are less than 30 terms, all terms are included. These bar charts are organized into three categories: biological processes, cellular components, and molecular functions, as well as according to whether the genes show upregulation or downregulation. In these charts, the horizontal axis represents the GO terms, and the vertical axis shows the number of genes associated with each GO term. (E) From the results of GO enrichment analysis, the 30 most significant terms are selected to be presented in the form of bar charts; if there are fewer than 30 terms, then all terms are shown. In these charts, the horizontal axis represents the GO terms, while the vertical axis indicates the significance level of GO term enrichment, expressed as $-\log_{10}(\text{padj})$. A higher value corresponds to greater significance. Different colors are used to represent the three GO subcategories: BP, CC, and MF. (F) Based on the GO enrichment analysis results, the 30 most significant terms are used to create bar charts; if there are less than 30 terms, all terms are included. These bar charts are organized into three categories: biological processes, cellular components, and molecular functions, as well as according to whether the genes show upregulation or downregulation. In these charts, the horizontal axis represents the GO terms, and the vertical axis shows the number of genes associated with each GO term.

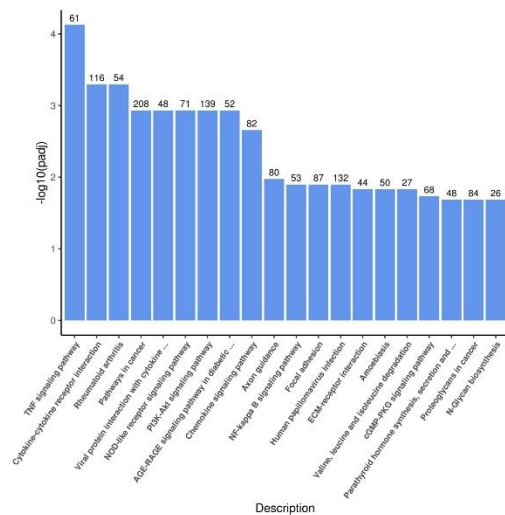
(A)



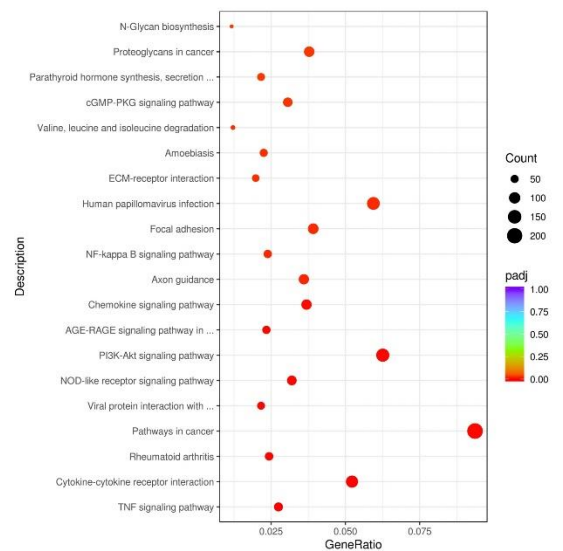
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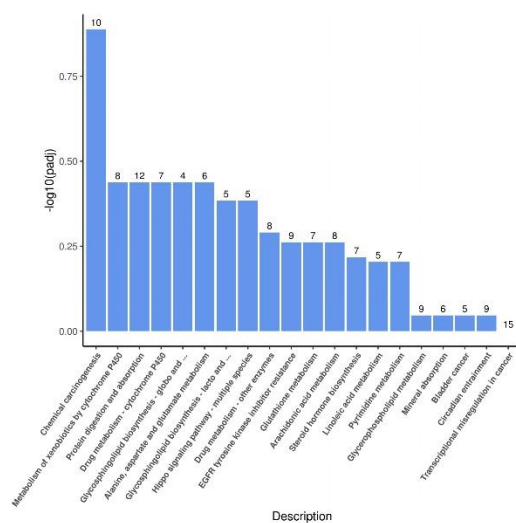
(C)



(D)



(E)



(F)

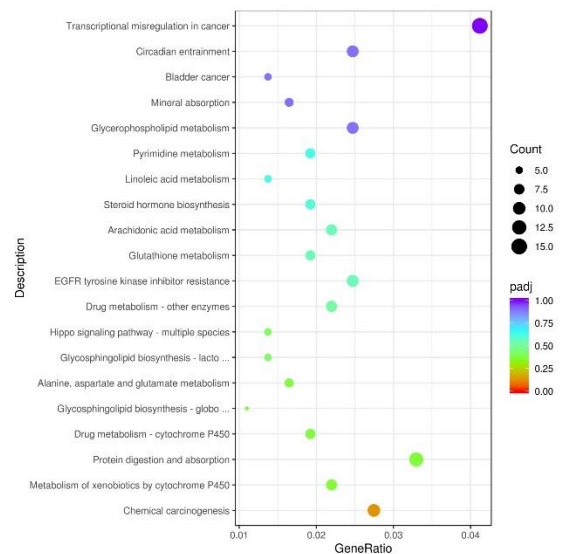


Figure 3 (A) From the KEGG enrichment results, the 20 most significant KEGG pathways are selected and presented in the form of bar charts; if there are fewer than 20 pathways, then all of them are shown. In these charts, the x-axis represents the KEGG pathways, while the y-axis indicates the level of significance of each pathway – higher values denote greater significance. (B) From the KEGG enrichment results, the 20 most significant KEGG pathways are chosen and displayed as scatter plots. If there are less than 20 pathways, then all of them are included in the plot. As shown in the diagram, the x-axis represents the ratio of the number of different genes associated with a particular KEGG pathway to the total number of such genes; the y-axis still represents the KEGG pathway itself. The size of the dots corresponds to the number of genes associated with that pathway, and the color of the dots ranges from red to purple, with red indicating lower significance and purple indicating higher significance. (C) From the KEGG enrichment results, the 20 most significant KEGG pathways are selected and presented in the form of bar charts; if there are fewer than 20 pathways, then all of them are shown. In these charts, the x-axis represents the KEGG pathways, while the y-axis indicates the level of significance of each pathway – higher values denote greater significance. (D) From the KEGG enrichment results, the 20 most significant KEGG pathways are chosen and displayed as scatter plots. If there are less than 20 pathways, then all of them are included in the plot. As shown in the diagram, the x-axis represents the ratio of the number of different genes associated with a particular KEGG pathway to the total number of such genes; the y-axis still represents the KEGG pathway itself. The size of the dots corresponds to the number of genes associated with that pathway, and the color of the dots ranges from red to purple, with red indicating lower significance and purple indicating higher significance. (E) From the KEGG enrichment results, the 20 most significant KEGG pathways are selected and presented in the form of bar charts; if there are fewer than 20 pathways, then all of them are shown. In these charts, the x-axis represents the KEGG pathways, while the y-axis indicates the level of significance of each pathway – higher values denote greater significance. (F) From the KEGG enrichment results, the 20 most significant KEGG pathways are chosen and displayed as scatter plots. If there are less than 20 pathways, then all of them are included in the plot. As shown in the diagram, the x-axis represents the ratio of the number of different genes associated with a particular KEGG pathway to the total number of such genes; the y-axis still represents the KEGG pathway itself. The size of the dots corresponds to the number of genes associated with that pathway, and the color of the dots ranges from red to purple, with red indicating lower significance and purple indicating higher significance.

GO and KEGG enrichment analyses revealed dynamic regulation of immune-related pathways across the cycle. During stage D3, the endometrium downregulates in a synchronized manner genes related to immune activation, cytokine signaling, and cell adhesion, such as pro-inflammatory factors (IL1B, TNF, IL6), chemokines (CXCL8, CXCL10, CCL5), and innate immune receptors (TMEM173/STING), suggesting transient suppression of inflammatory pathways during the early luteal phase—consistent with observations in mares and cattle where post-ovulatory immune modulation creates an anti-inflammatory environment promoting embryo tolerance—consistent with observations in mares and cattle where post-ovulatory immune modulation creates an anti-inflammatory environment promoting embryo tolerance (Bauersachs and Wolf, 2012, Forde and Lonergan, 2012, Mazzoni *et al.*, 2020). By D7, robust activation of TNF, NF- κ B, PI3K-Akt, and cytokine-receptor interaction pathways indicate controlled re-engagement of immune regulatory networks supporting tissue remodeling while maintaining immunological balance. The D7 transcriptome was additionally characterized by significant enrichment of ECM-receptor interaction pathways, critical for stromal proliferation, angiogenesis, and glandular differentiation—hallmarks of progesterone-induced uterine transformation (Sharom, 2014; Crisóstomo, 2018; Chi *et al.*, 2020; Muderspach *et al.*, 2025). In mares and other species, genes encoding collagens, integrins, and matrix metalloproteinases are highly expressed during early diestrus to enable structural reorganization (Boakari *et al.*, 2019; Zhang *et al.*, 2024), and the activation of these pathways in donkeys indicates conserved mechanisms of progesterone-mediated uterine remodeling across equids.

At D18, KEGG enrichment revealed a shift toward metabolic and catabolic pathways, including

cytochrome P450-mediated metabolism, steroid hormone biosynthesis, and amino acid metabolism, likely reflecting preparation for luteal regression and transition to a new ovulatory cycle. The expression of cytochrome P450 enzymes and lipid-metabolizing genes suggests local production and degradation of steroid intermediates and prostaglandins—key mediators of luteolysis and endometrial renewal (Arosh *et al.*, 2002; Waclawik, 2011). Similar upregulation of metabolic genes during late luteal regression has been reported in mares and cattle (Sánchez *et al.*, 2019; Sponchiado, 2019).

The transcriptomic timeline observed in donkeys parallels patterns documented in mares (Klein and Troedsson, 2011; Bauersachs and Wolf, 2012), cattle (Forde and Lonergan, 2012; Sandoval, 2016), and pigs (Hale, 2017; Gutierrez, 2019), confirming conserved hormonal control of endometrial gene expression across mammals. However, the inter-ovulatory interval in donkeys (22–24 days) is slightly shorter than in mares (Segabinazzi *et al.*, 2022), potentially compressing the window of maximal transcriptomic remodeling—an aspect relevant for optimizing artificial insemination and embryo transfer protocols. Several limitations should be acknowledged, including the modest sample size per time point ($n = 4$) and the absence of concurrent hormonal measurements. Future studies integrating hormonal assays, histological evaluation, and single-cell transcriptomics will enable finer dissection of cell-type-specific regulatory networks and establish causal relationships between gene expression patterns and endometrial physiology.

In conclusion, this study provides the first comprehensive transcriptomic profiling of the donkey endometrium across the estrous cycle, revealing a biphasic expression pattern characterized by early transcriptional suppression (D3), robust mid-luteal activation (D7), and late-cycle metabolic normalization

(D18). The coordinated regulation of immune tolerance, extracellular matrix remodeling, and steroid metabolism underscores the intricate hormonal control of endometrial physiology. Upregulation of TNF, NF- κ B, PI3K-Akt, and ECM-related pathways during mid-luteal stages highlights progesterone's pivotal role in establishing uterine receptivity, while late-cycle enrichment of oxidative and detoxification pathways reflects preparation for endometrial renewal. These findings demonstrate that donkeys share fundamental reproductive regulatory mechanisms with other domestic equids while potentially exhibiting species-specific temporal adaptations. This molecular atlas establishes a foundation for developing precision breeding strategies, diagnostic biomarkers for uterine health assessment, and improved reproductive technologies in this economically significant species.

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References

- Akhtar MF, Ali S, Hassan F and Changfa W 2025. Molecular pathways affecting reproductive efficiency in seasonal breeders: prospects and implications for improving fertility in donkeys. *Front Vet Sci*. 12: 1633945.
- Amaral A, Sadowska A, Cerveira-Pinto M, Kordowitzki P, Skarzynski D, Ferreira-Dias G and Szóstek-Mioduchowska A 2025. Ovarian steroids modulate mRNA expression of ecm associated genes and collagen deposition induced by tgf β 1 in equine endometrium in vitro. *Sci Rep*. 15(1): 538.
- Arosh JA, Parent J, Chapdelaine P, Sirois J and Fortier MA 2002. Expression of cyclooxygenases 1 and 2 and prostaglandin e synthase in bovine endometrial tissue during the estrous cycle. *Biol Reprod*. 67(1): 161–169.
- Bauersachs S, Ulbrich SE, Gross K, Schmidt S, Meyer H, Einspanier R, Wenigerkind H, Vermehren M, Blum H and Sinowatz F 2005. Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. *J Mol Endocrinol*. 34(3): 889–908.
- Bauersachs S and Wolf E 2012. Transcriptome analyses of bovine, porcine, and equine endometrium during the pre-implantation phase. *Anim Reprod Sci*. 134(1–2): 84–94.
- Bennett R and Pfuderer S 2019. Demand for donkey hides and implications for global donkey populations. In: 93rd Annual Conference of the Agricultural Economics Society. Warwick: University of Warwick.
- Bennett R and Pfuderer S 2020. The potential for new donkey farming systems to supply the growing demand for hides. *Animals* 10(4): 718.
- Billhaq DH, Lee SH and Lee S 2020. The potential function of endometrial-secreted factors for endometrium remodeling during the estrous cycle. *Anim Sci J*. 91(1): e13333.
- Boakari YL, El-Sheikh Ali H, Dini P, Loux S, Fernandes CB, Scoggin K, Esteller-Vico A, Lawrence L and Ball B 2019. A high protein model alters the endometrial transcriptome of mares. *Genes* 10(8): 576.
- Canisso IF, Panzani D, Miró J and Ellerbrock RE 2019. Key aspects of donkey and mule reproduction. *Vet Clin Equine Pract*. 35(3): 607–642.
- Canisso IF, Segabinazzi LG and Fedorka CE 2020. Persistent breeding-induced endometritis in mares—a multifaceted challenge: from clinical aspects to immunopathogenesis and pathobiology. *Int J Mol Sci*. 21(4): 1432.
- Chen Y, Zhang S, Sun Y, Zou J, Qiu X, Xi H, Xu Y, Li Y, Chen B and Fan J 2025. Bisphenol A impairs oocyte maturation by dysfunction of cumulus cells. *Theriogenology* 233: 139–146.
- Chi R-pA, Wang T, Adams N, Wu S-p, Young SL, Spencer TE and DeMayo F 2020. Human endometrial transcriptome and progesterone receptor cistrome reveal important pathways and epithelial regulators. *J Clin Endocrinol Metab*. 105(4): e1419–e1439.
- Crisóstomo MRRCS 2018. Mare endometrium: physiological and pathological involvement of hormones and neutrophil extracellular traps. Lisbon: Universidade de Lisboa.
- Critchley HO, Maybin JA, Armstrong GM and Williams AR 2020. Physiology of the endometrium and regulation of menstruation. *Physiol Rev*. 100(3): 1149–1179.
- D'Agostino N, Li W and Wang D 2022. High-throughput transcriptomics. *Sci Rep*. 12(1): 20313.

- Díaz-Bertrana ML, Deleuze S, Pitti Rios L, Yeste M, Morales Fariña I and Rivera del Alamo MM 2021. Microbial prevalence and antimicrobial sensitivity in equine endometritis in field conditions. *Animals* 11(5): 1476.
- Forde N and Lonergan P 2012. Transcriptomic analysis of the bovine endometrium: what is required to establish uterine receptivity to implantation in cattle? *J Reprod Dev.* 58(2): 189–195.
- Gutierrez K 2019. Refinement of CRISPR/Cas9 gene editing efficiency and its application to the study of porcine early embryo development. Montreal, QC: McGill University.
- Hale BJ 2017. Two components of maintaining developmental competence: microRNA-21 in the maturing oocyte and induction of autophagy in the follicular-stage ovary. Ames, IA: Iowa State University.
- Hong I-S 2024. Endometrial stem cells: orchestrating dynamic regeneration of endometrium and their implications in diverse endometrial disorders. *Int J Biol Sci.* 20(3): 864.
- Jin B, Yang L, Ye Q and Pan J 2023. Ferroptosis induced by DCPS depletion diminishes hepatic metastasis in uveal melanoma. *Biochem Pharmacol.* 213: 115625.
- Khan MZ, Chen W, Li M, Ren W, Huang B, Kou X, Ullah Q, Wei L, Wang T and Khan A 2024a. Is there sufficient evidence to support the health benefits of including donkey milk in the diet? *Front Nutr.* 11: 1404998.
- Khan MZ, Chen W, Wang X, Liang H, Wei L, Huang B, Kou X, Liu X, Zhang Z, Chai W and Khan A 2024b. A review of genetic resources and trends of omics applications in donkey research: Focus on China. *Front Vet Sci.* 11: 1366128.
- Klein C and Troedsson MH 2011. Transcriptional profiling of equine conceptuses reveals new aspects of embryo-maternal communication in the horse. *Biol Reprod.* 84(5): 872–885.
- Kurar E, Atli M, Guzeloglu A, Ozsensoy Y and Semacan A 2010. Comparison of five different RNA isolation methods from equine endometrium for gene transcription analysis. *Kafkas Univ Vet Fak Derg.* 16: 851–855.
- Li M, Sun L, Du X, Zhao Y, Ren W, Man L, Zhu M, Liu G, Khan MZ and Wang C 2024. Characterization and discrimination of donkey milk lipids and volatiles across lactation stages. *Food Chem X.* 23: 101740.
- Li T, Qu H, Wang L, Ma Q, Wang C, Khan MZ and Chai W 2026. Transcriptomic analysis provides molecular insights into skin development in Dezhou donkey foals. *Vet Sci.* 13(1): 107.
- Liu S, Su J, Yang Q, Sun M, Wang Z, Yu J, Jafari H, Lei C, Sun Y and Dang R 2024. Genome-wide analyses based on a novel donkey 40K liquid chip reveal the gene responsible for coat color diversity in Chinese Dezhou donkey. *Anim Genet.* 55(1): 140–146.
- Mazzoni G, Pedersen HS, Rabaglino MB, Hyttel P, Callesen H and Kadarmideen HN 2020. Characterization of the endometrial transcriptome in early diestrus influencing pregnancy status in dairy cattle after transfer of in vitro-produced embryos. *Physiol Genomics.* 52: 269–279.
- Miragaya MH, Neild DM and Alonso AE 2018. A review of reproductive biology and biotechnologies in donkeys. *J Equine Vet Sci.* 65: 55–61.
- Monteiro de Barros MR, Davies-Morel MC, Mur LA, Creevey CJ, Alison RH and Nash DM 2021. Characterization of an ex vivo equine endometrial tissue culture model using next-generation RNA-sequencing technology. *Animals* 11(7): 1995.
- Morris LH, McCue P and Aurich C 2020. Equine endometritis: A review of challenges and new approaches. *Reproduction* 160(5): R95–R110.
- Muderspach ND, Daucke R, Nielsen MW, Scoggin KE, Fedorka CE, Miller LM, May MG, Troedsson MH, Ferreira-Dias G, Agerholm JS, Schoof EM and Christoffersen M 2025. Proteomic profiling of endometrial tissue and plasma from mares with endometrial fibrosis. *Biol Reprod.* 114: 158–173.
- Norris SL, Little HA, Ryding J and Raw Z 2021. Global donkey and mule populations: Figures and trends. *PLoS One* 16(2): e0247830.
- Radar-Chafirovitch A, Quaresma M, Yáñez-Ortiz I, Leiva B, Ferreira-Dias G, Payan-Carreira R, Miro J and dos Anjos Pires M 2025. Donkey endometrium: characterization of resident immune cells. *Res Vet Sci.* 184: 105516.
- Ravaioli V, Raffini E, Tamburini M, Galletti G and Frasnelli M 2022. Infectious endometritis in mares: microbiological findings in field samples. *J Equine Vet Sci.* 112: 103913.
- Sánchez JM, Simintiras CA and Lonergan P 2019. Aspects of embryo-maternal communication in establishment of pregnancy in cattle. *Anim Reprod.* 16(3): 376–385.
- Sandoval GMB 2016. Utero-ovarian relationships during establishment and maintenance of pregnancy in lactating dairy cows. Madison, WI: University of Wisconsin-Madison.
- Schöniger S and Schoon H-A 2020. The healthy and diseased equine endometrium: a review of morphological features and molecular analyses. *Animals* 10(4): 625.
- Segabinazzi LG, Gilbert RO, Ambrosia RL, Bergfelt DR, Samper JC, Peterson EW and French HM 2022. Structural and functional dynamics of the ovary and uterus during the estrous cycle in donkeys in the Eastern Caribbean. *Animals* 13(1): 74.
- Sharom FJ 2014. Complex interplay between the P-glycoprotein multidrug efflux pump and the membrane: its role in modulating protein function. *Front Oncol.* 4: 41.
- Sponchiado M 2019. Deciphering the embryo-maternal interactome: Embryo-dependent programming of endometrial function during early pregnancy in cattle. Antwerp: University of Antwerp.
- Ullah A, Chen W, Shi L, Wang M, Geng M, Na J, Akhtar MF, Khan MZ and Wang C 2025a. Challenges and enhancing strategies of equine semen preservation: nutritional and genetic perspectives. *Vet Sci.* 12(9): 807.

- Ullah A, Khan MZ and Wang C 2025b. Overview of donkey welfare and husbandry practices in Asia. *Animals* 15(23): 3464.
- Weber KS, Wagener K, Blanco M, Bauersachs S and Bollwein H 2021. A comparative analysis of the intrauterine transcriptome in fertile and subfertile mares using cytobrush sampling. *BMC Genomics*. 22(1): 377.
- Yang F, Li N, Liu B, Yu J, Wu S, Zhang R, Yang W, Ji C, Sun Q and Ma J 2021. Practical protocols for timed artificial insemination of jennies using cooled or frozen donkey semen. *Equine Vet J*. 53(6): 1218–1226.
- Yip KS, Suvorov A, Connerney J, Lodato NJ and Waxman DJ 2013. Changes in mouse uterine transcriptome in estrus and proestrus. *Biol Reprod*. 89(1): 11–13.
- Zhang M, Wu X, Guo T, Xia Y, Wang Z, Shi Z, Hu K, Zhu X, Zhu R and Yue Y 2024. Involvement of METTL3-mediated m6A methylation in the early development of porcine cloned embryos. *Theriogenology* 226: 378–386.
- Zhang Z, Gao X, Faheem M, Wang Y, Wang T, Shi X, Huang B, Zhu M and Wang C 2022. Comparative analysis of growth and development characteristics of two Dezhou donkey strains. *Livest Sci*. 263: 105024.