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# Transcriptome and proteome analysis of pregnancy and postpartum anoestrus ovaries in yak

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## ABSTRACT

**Background:** Domestic yaks are the most important livestock species on the Qinghai-Tibetan Plateau. Adult female yaks normally breed in the warm season (July to September) and enter anestrus in the cold season (November to April). Nevertheless, it is unclear how ovarian activity is regulated at the molecular level.

**Objectives:** The peculiarities of yak reproduction were assessed to explore the molecular mechanism of postpartum anestrus ovaries in yaks after pregnancy and parturition.

**Methods:** Sixty female yaks with calves were observed under natural grazing in Haiyan County, Qinghai Province. Three yak ovaries in pregnancy and postpartum anestrus were collected. RNA sequencing and quantitative proteomics were employed to analyze the pregnancy and postpartum ovaries after hypothermia to identify the genes and proteins related to the postpartum ovarian cycle.

**Results:** The results revealed 841 differentially expressed genes during the postpartum hypoestrus cycle; 347 were up-regulated and 494 genes were down-regulated. Fifty-seven differential proteins were screened: 38 were up-regulated and 19 were down-regulated. The differential genes and proteins were related to the yak reproduction process, rhythm process, progesterone-mediated oocyte maturation, PI3K/AKT signaling pathway, and MAPK signaling pathway categories.

**Conclusions:** Transcriptome and proteomic sequencing approaches were used to investigate postpartum anestrus and pregnancy ovaries in yaks. The results confirmed that BHLHE40, SF11X1, FBPX1, HSPCA, LHCGR, BMP15, and ET-1R could affect postpartum hypoestrus and control the state of estrus.

**Keywords:** Postpartum anestrus; pregnancy; transcriptomics; proteomics; yak

## INTRODUCTION

The yak is a dominant livestock species and a valuable resource in the Qinghai-Tibet Plateau. Yaks provide milk, meat, wool, service power, fuel, and other daily necessities for local herders and is an essential species in the plateau area [1,2]. Female yaks only reproduce when they are more than 2 years old, and they have a higher rate of lethargy and a lower pregnancy rate than ordinary cattle [3]. The average reproductive rate of adult yaks is only 48.61%, and more than half have one fetus in 2 years or 2 fetuses in 3 years [4]. In addition, female yaks

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#### Conflict of Interest

The authors declare no conflicts of interest.

#### Author Contributions

Data curation: Ma J; Formal analysis: Wang J; Investigation: Li S; Project administration: Yang Y; Supervision: Zhaxi Y, Zhao Y, Zhang D; Writing - original draft: Chen Z; Writing - review & editing: Chen Z, Huo S.

have a low rate of estrus, and more than 90% of postpartum female yaks do not reenter estrus in the same year [5].

The ovaries of yaks during pregnancy are generally irregular, with a corpus luteum, an important endocrine gland that secretes progesterone and estrogen. These hormones regulate the estrus cycle and maintain pregnancy [6,7]. The proportion of corpus luteum ovaries decreases from 3–6 months in the second trimester, but the tissue structure is still relatively tight [8]. Natural progesterone secreted by the corpus luteum regulates the hypothalamic-pituitary-ovary axis, together with estrogen [9]. The low pulse frequency of luteinizing hormone (LH) and a series of follicle waves is a characteristic of the postpartum period, until the LH pulse frequency increased sufficiently to induce sufficient oestradiol concentrations, thus caused luteinizing hormone surge and ovulation [9]. The development and functional maintenance of the reproductive system are regulated by the hypothalamus-pituitary-gonad axis [10]. After calving, in the mechanism of postpartum fatigue caused by lactation, stimulation of lactation behavior on the nipple excites the nipple receptors, which sends impulses to the hypothalamus via the afferent nerves, and afferent neuron terminals, then secrete neurotransmitters to stimulate the hypothalamus. Neurons are sensitive to the negative feedback effect of estrogen, thereby reducing the function of the pulse generator [11,12]. The secretion of gonadotropin-releasing hormone (GnRH) is inhibited, resulting in low levels of follicle-stimulating hormone (FSH) and LH. Hence, the development of follicles is blocked, causing apathy in yaks [13-15]. Yak lactation can cause prolonged periods of postpartum anestrus. In addition, lactation can increase the concentration of corticosteroids in plasma, thereby inhibiting the secretion of LH and reducing the sensitivity of the pituitary to GnRH [16,17].

Studying the yak ovary transcriptome could reveal the molecular mechanisms and specificity of yak reproduction. RNA sequencing (RNA-seq) was used to compare the transcriptome data between yak and common cattle ovaries. RNA-seq and Tandem Mass Tags (TMT) quantitative proteomics approaches were employed to gain insights into the genomic and proteomic deviations in the expression patterns between ovaries in these species. These results provide a basis for examining the general molecular mechanisms of yak ovaries and illuminating the specificity of yak reproduction.

## MATERIALS AND METHODS

### Sample collection and preservation

Healthy female yaks aged 6 years and raised in Haiyan County of Qinghai Province were selected to analyze anestrus and pregnancy. Three yak ovaries each in pregnancy and postpartum anestrus were obtained immediately at the time of slaughter, washed with normal saline, labeled, and stored in liquid nitrogen until further use.

### RNA extraction, library construction, and RNA-seq

The total RNA from the mixed samples of each group of ovaries was extracted according to the instruction manual supplied with TRIzol Reagent (Life Technologies, USA). The RNA integrity and concentration were checked using a Nano Drop One/One<sup>c</sup> instrument (Thermo Fisher Scientific, USA). One microgram total RNA was prepared for cDNA libraries using protocol provided by Oxford Nanopore Technologies (ONT). Briefly, SuperScript IV First-Strand Synthesis System (Invitrogen) was used for full length mRNA reverse transcription and following cDNA PCR with LongAmp Tag (NEB). The PCR products were then subjected

to FFPE DNA repair and end-repair (NEB) steps and following adaptor ligation using T4 DNA ligase (NEB). The suitable fragments were isolated by Agencourt AMPure XP beads (Beckman Coulter, Inc.) and enriched by PCR amplification. The constructed cDNA libraries of the Yaks were then sequenced on a flow cell using an Nanopore sequencing platform.

The gene expression levels were calculated using the fragments per kilobase of exon per million fragments mapped (FPKM) method with Cufflinks software [18]. Differentially expressed genes (DEGs) between postpartum anestrus and pregnancy ovaries were identified using DESeq [18]. The differences in gene abundance between samples were calculated based on the ratio of FPKM values. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with a FDR < 0.01 and foldchange  $\geq 2$  found by DESeq were assigned as differentially expressed. The DEGs were searched against the Gene Ontology (GO) database, the Clusters of Orthologous Groups (COG) database, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. GO enrichment analysis of the differentially expressed genes (DEGs) was implemented using the Goseq R packages based Wallenius non-central hyper-geometric distribution, which can adjust for gene length bias in DEGs [19]. The gene sequences were also aligned to the Clusters of Orthologous Group (COG) database to predict and classify functions. Furthermore, we used KOBAS software (KOBAS (pku.edu.cn)) to test the statistical enrichment of differential expression genes in KEGG pathways [20].

### TMT quantitative proteomic analysis

Mixed samples from the pregnancy and postpartum anestrus yak ovaries were ground to a powder in liquid nitrogen and homogenized in a lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail) and then centrifuged at 12,000 rpm at 4°C. The supernatant was precipitated with cold 15% trichloroacetic acid/acetone for 2 hours at -20°C. After centrifugation at 12,000 g at 4°C for 10 min, the supernatant was collected, and the protein concentration was determined using a 2-D Quant 166 kit (GE Healthcare, USA) [21].

Total protein (100  $\mu$ g) solution was reduced with 10mM DTT for one hour at 37°C and alkylated with 20 mM IAA for 45 min at room temperature in the dark. Proteins were then diluted by adding 100 mM TEAB to reach a urea concentration lower than 2 M. Finally, trypsin was added of 1:50 trypsin-to-protein mass ratio for the first digestion overnight, and at 1:100 for a second four hours' digestion. After trypsin digestion, peptides were desalted using a Strata X C18 SPE column (Phenomenex) and further dried under vacuum. Peptides were reconstituted in 0.5 M TEAB and labeled with TMT reagents (ThermoFisher Scientific, USA). The samples were fractionated by high pH reverse-phase HPLC using an Agilent 300Extend C18 column as five  $\mu$ m particles, 4.6 mm ID and 250 mm length. All the MS/MS data were processed using Mascot search engine (v.2.3.0) with the target-decoy database searching strategy<sup>51</sup> against Uniprot\_Neohemiptera.fasta database [22]. Trypsin/P was specified as cleavage enzyme allowing up to two missing cleavages. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. The FDR was adjusted to less than 0.01 and peptide ion score was set to greater than 20. Proteomic difference analysis was performed by T test between the two groups. The screening conditions were the  $p < 0.05$  and the two group sample ration ratio FC > 1.50 were considered differentially expressed proteins (DEPs).

### Gene expression analysis by quantitative real-time PCR (qRT-PCR)

The total RNA from the yak ovary tissue was extracted using the TRIzol method. The concentration and purity of the RNA were measured using a NanoDrop One/One<sup>c</sup>

spectrophotometer. RNA with an absorbance of 1.8–2.0, and primeScript RT master mix (perfect real-time) was used for reverse transcription. The total volume of the reaction mixture was 20  $\mu\text{L}$  and contained 1  $\mu\text{g}$  RNA, 4  $\mu\text{L}$  5 $\times$  PrimeScript RT Master Mix (Perfect Real Time), and RNase-free  $\text{dH}_2\text{O}$  to 20  $\mu\text{L}$ . The reactions were incubated at 37°C for 15 min, then at 85°C for 5 sec. Subsequently, qRT-PCR was performed using a TB Green Premix Ex Taq II kit (Tli RNaseH Plus, Takara, China) and a Bio-Rad cfx96 Touch Real-time PCR System (Bio-Rad, USA). The amplification reactions contained 12.5  $\mu\text{L}$  TB Green Premix Ex Taq II (2 $\times$ ), 2  $\mu\text{L}$  template cDNA, 1  $\mu\text{L}$  of upstream and downstream primers (10  $\mu\text{mol/L}$ ), and 8.5  $\mu\text{L}$  of deionized water in a total volume of 25  $\mu\text{L}$ . Thermal cycling included an initial denaturation step at 95°C for 30 sec, followed by 40 denaturation cycles at 95°C for 5 sec, and annealing and extension at 60°C for 30 sec. The fluorescence was measured at the end of each annealing and extension step. During each reaction, the cycle threshold (Ct) was restored to the baseline, and a melting curve was used to determine if there was a specific product or primer dimer for each reaction. The final melting curve was analyzed after the reaction,  $\beta$ -actin was used as an internal reference gene, and the relative expression of each gene was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method [23].

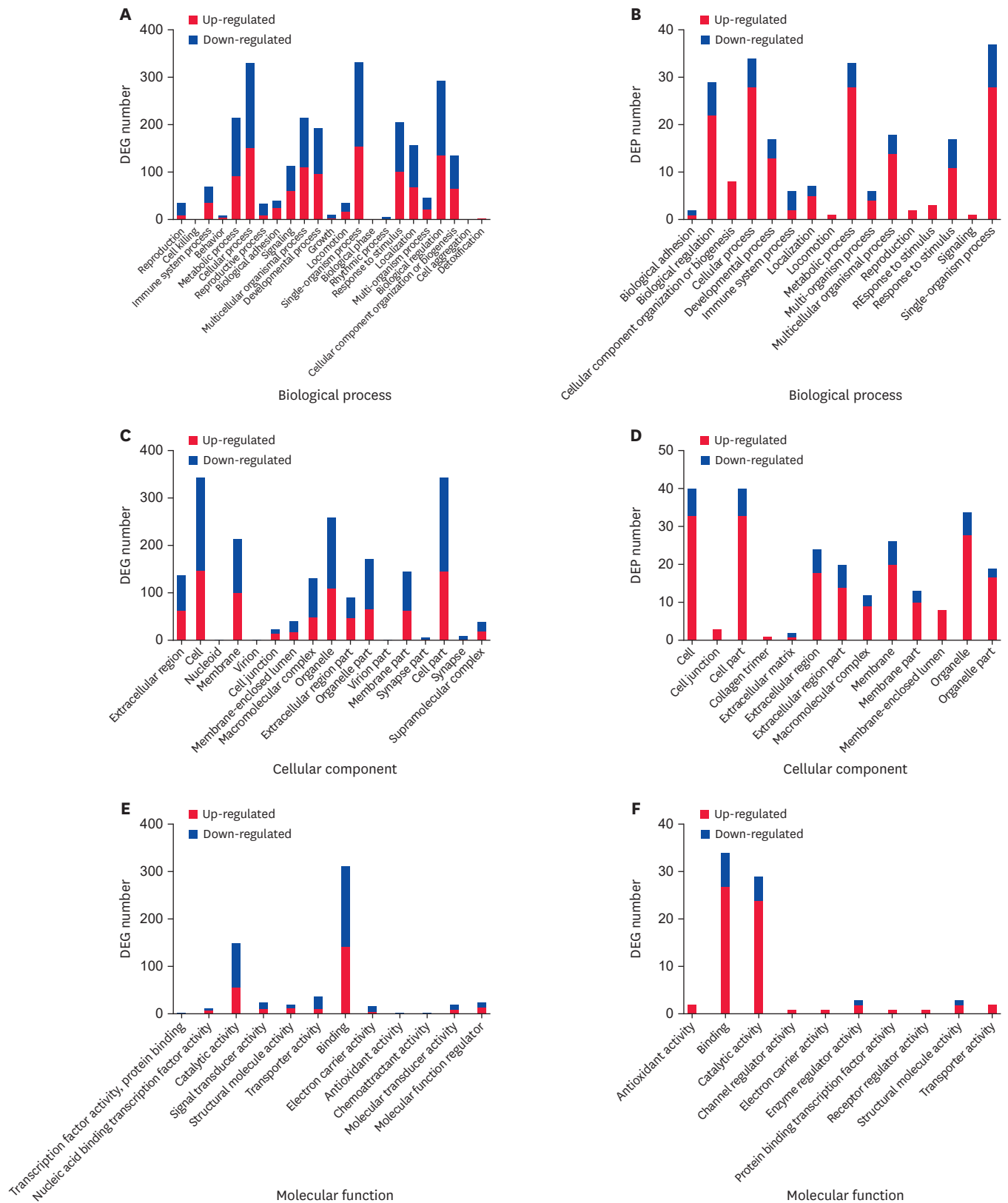
## RESULTS

### Expression profiles of postnatal ovarian cycle arrest and normal ovaries

After FDR screening, 841 DEGs were identified from the postpartum anestrus and pregnancy ovaries; 347 were up-regulated, and 494 were down-regulated. Fifty-seven differential proteins were detected; 38 were up-regulated and 19 were down-regulated.

### GO functional classification of enriched DEGs and DEPs

The GO database was again used to classify the enriched DEGs and DEPs between postpartum anestrus and pregnancy ovaries. They were categorized into 3 main categories based on the sequence homology: molecular function, biological process, and cellular component. A gene product might be associated with or located in one or more cellular components, such that it is active in one or more biological processes, performing one or more molecular functions. The DEGs were distributed in up to 437 GO terms, whereas the DEPs were classified into only 52 GO terms. The GO terms related to the growth and development of yak follicles were selected. In the biological process category (**Fig. 1A and B**), most of the annotated genes were involved in a ‘single-organism process’ (332 DEGs), followed in order by a ‘cellular process’ (330 DEGs) and ‘metabolic process’ (215 DEGs). In terms of proteomics, the largest term in proteomics was ‘cellular process’ (35 DEPs), followed in order by a ‘single-organism process’ (32 DEPs) and a ‘metabolic process’ (31 DEPs). In addition, 269 genes were associated with development and reproduction, i.e., ‘developmental process,’ ‘reproduction,’ and ‘reproductive process.’ In terms of yak reproduction, 269 DEGs were involved in 4 aspects: ‘developmental process,’ ‘reproduction,’ ‘reproductive process,’ and ‘rhythmic process.’ The ‘developmental process’ has 195 DEGs. Ninety-eight of these were up-regulated ‘reproduction’ and had 35 DEGs. Twenty-five were down-regulated ‘reproductive process’ and had 34 DEGs. Ten were up-regulated ‘rhythmic process’ and had 5 DEGs. Four were down-regulated. In addition, ‘cellular process’ contained 330 DEGs, and ‘biological regulation’ had 295 DEGs. Two hundred and twenty-six DEGs were related to the ‘metabolic process’ and ‘growth’. In terms of proteomics, 29 DEPs were annotated as ‘biological regulation.’ ‘Developmental process,’ ‘reproduction’ and ‘reproductive process’ and had 23 DEPs. These results are consistent with the biological characteristics and function of the ovary. No DEP was associated with the ‘rhythmic process.’



**Fig. 1.** GO enrichment analysis of the DEGs and DEPs. The vertical axis indicates the number of DEGs and DEPs up- and down-regulated. GO, Gene Ontology; DEG, differentially expressed gene; DEP, differentially expressed protein.

In the cellular component category (**Fig. 1C and D**), most of the genes were involved in 'cell,' followed by 'cell part' and 'organelle.' The DEG number in the terms, 'cell part' and 'cell' (343 DEGs), was the largest. The largest terms in proteomics were 'cell' and 'cell part' with 40 DEPs. In addition, 'organelle' had 260 DEGs, and 'membrane' had 215 DEGs. One hundred and seventy-two DEGs and 34 DEPs were annotated as the 'organelle part'.

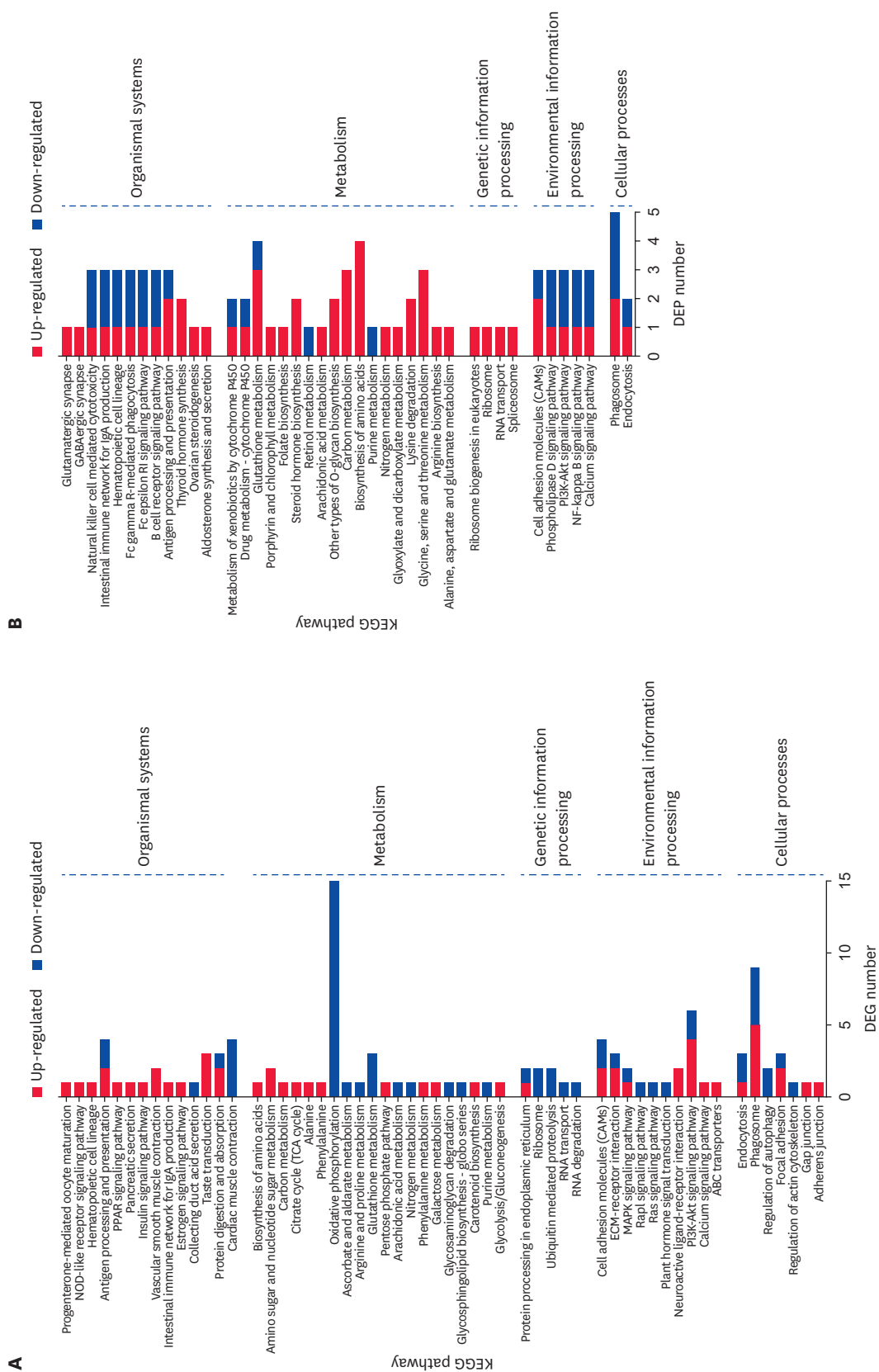
In the molecular function category (**Fig. 1E and F**), most of the genes were involved in 'binding,' followed by 'catalytic activity.' The largest DEG number (312 DEGs) was in the term 'binding,' among which 141 were up-regulated and 171 were down-regulated. 'Catalytic activity' terms had 148 DEGs; 56 were up-regulated and 92 were down-regulated. In proteomics, 32 DEPs were annotated to 'binding,' and 27 of them were up-regulated; 'catalytic activity' had 29 DEPs, and 25 were up-regulated. Previous microarray studies showed that the RNA-binding molecular function category accounted for a large proportion of the genes expressed in bovine oocytes, thereby confirming that 'binding' plays a vital role in the normal physiological activities of the yak ovary.

### KEGG functional annotation of enriched DEGs and DEPs

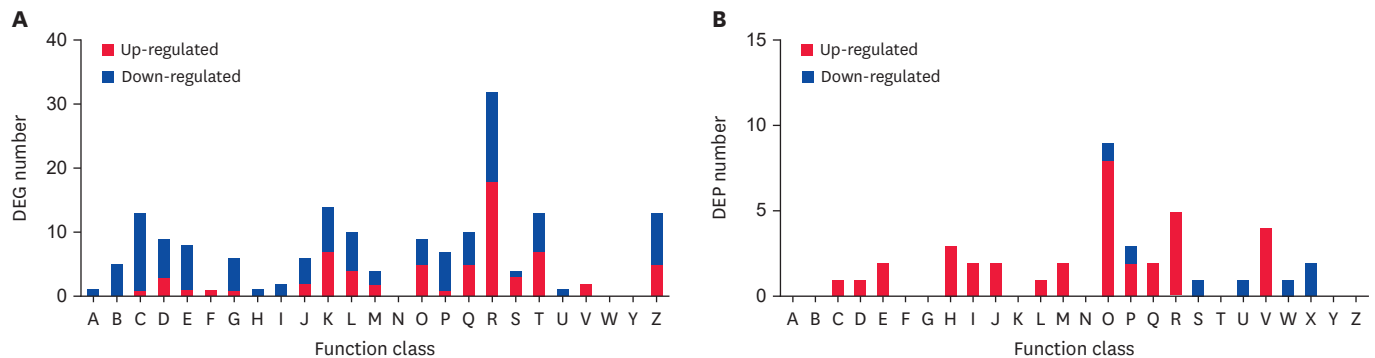
The KEGG database is a knowledge base for the systematic analysis of gene functions that link genomic information with higher-order functional information. Genes and proteins involved in biochemical metabolism and signal transduction can be detected with pathway analysis and KEGG ways possessing DEGs and DEPs were evaluated. KEGG analysis of DEGs/DEPs between postpartum anestrus and pregnancy ovaries was carried out. The genes and proteins involved in biochemical metabolism and signal transduction can be identified by pathway analysis. One hundred and fifty-six DEGs were identified in the KEGG database. In postpartum anestrus and pregnancy ovaries, 63 KEGG pathways were significantly enriched with DEGs, mostly related to cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems (**Fig. 2A**). The category with the most DEGs was 'metabolism' (23 sub-categories and 43 DEGs), followed by 'organismal systems' (16 sub-categories and 27 DEGs). By contrast, fewer DEGs were associated with 'genetic information processing' (12 DEGs). The 'cellular processes' category included 7 sub-categories and 20 DEGs. In addition, 26 DEGs were associated with the 'environmental information process' pathway. With respect to proteomics, 41 pathways were enriched with DEPs (**Fig. 2B**), and the total number of DEPs was smaller than for DEGs. The largest category was 'metabolism,' with 18 sub-categories and 33 DEPs. The second-largest category was 'organizational systems' (12 sub-categories and 27 DEPs). By contrast, fewer DEPs were associated with 'cellular processes' (7 DEPs), and the 'environmental information process' category included 5 sub-categories and 15 DEGs.

### COG function classification of DEGs and DEPs

The COG database is used to classify orthologous gene products. DEGs and DEPs between postpartum anestrus and pregnancy ovaries were distributed in 22 and 17 COG categories, respectively (**Fig. 3**). As shown in **Fig. 3A**, the 'general function' was the largest category for transcriptome data (32 DEGs), 18 of which were up-regulated. For proteins, the largest category was 'posttranslational modification, protein turnover, chaperones' (9 DEPs), 8 of which were up-regulated (**Fig. 3B**). The 'RNA processing and modification,' 'cell cycle control, cell division, chromosome partitioning,' and 'signal transduction mechanisms' categories were associated directly with follicular growth and development of yak ovaries. 'Cell cycle control, cell division, chromosome partitioning' was enriched with both DEGs and DEPs, most of which were up-regulated. 'RNA processing and modification' and 'signal



**Fig. 2.** KEGG pathway enrichment analysis of the DEGs and DEPs. The chart shows the distribution of DEGs (A) and DEPs (B) indicated in the enriched KEGG pathways. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed gene; DEP, differentially expressed protein.



**Fig. 3.** COG function classification of DEGs and DEPs. Abscissa is the content of COG classification, and ordinate is the number of genes. Grey and dark bars represent up-regulation and down-regulation, respectively.

A: RNA processing and modification; B: chromatin structure and dynamics; C: energy production and conversion; D: cell cycle control, cell division, chromosome partitioning; E: amino acid transport and metabolism; F: nucleotide transport and metabolism; G: carbohydrate transport and metabolism; H: coenzyme transport and metabolism; I: lipid transport and metabolism; J: translation, ribosomal structure, and biogenesis; K: transcription; L: replication, recombination and repair; M: cell wall/membrane/envelope biogenesis; N: cell motility; O: posttranslational modification, protein turnover, chaperones; P: inorganic ion transport and metabolism; Q: secondary metabolite biosynthesis, transport, and catabolism; R: general function prediction only; S: function unknown; T: signal transduction mechanisms; U: intracellular trafficking, secretion, and vesicular transport; V: defence mechanisms; W: extracellular structures; Y: nuclear structure; Z: cytoskeleton.

COG, Clusters of Orthologous Groups; DEG, differentially expressed gene; DEP, differentially expressed protein.

transduction mechanisms' were only enriched with DEGs (one and 13 DEGs, respectively). Among the categories associated with primary metabolic functions, the 'amino acid transport and metabolism' cluster was the largest group with 8 DEGs, 7 of which were down-regulated. This category was followed by 'carbohydrate transport and metabolism,' 'nucleotide transport and metabolism,' and 'coenzyme transport and metabolism' (6 DEGs, 1 DEG, and 1 DEG, respectively). Regarding proteins, 'coenzyme transport and metabolism' and 'lipid transport and metabolism' were the largest groups, each with 3 DEPs. This category was followed by 'amino acid transport and metabolism' and 'nucleotide transport and metabolism' (2 DEPs and 1 DEP, respectively). The 'second metabolites biosynthesis, transport, and catabolism' category included 5 DEGs and 3 DEPs, respectively. In addition, 7 DEGs were assigned to 'inorganic ion transport and metabolism,' whereas only 2 DEPs were associated with this category. Although the total number of DEGs annotated in the COG database was larger than that for DEPs, there were more DEPs in the 'defense mechanisms' and 'coenzyme transport and metabolism' categories. There were more up-regulated DEGs than down-regulated DEGs in 6/22 categories. By contrast, 13 categories for the proteomics data included more up-regulated DEPs. This suggests that in postpartum anestrus and pregnancy ovaries, the expression patterns of proteins were different from those of the genes. Thus, numerous unknown changes may occur during transcription and translation.

### Validation of selected DEGs/DEPs by qRT-PCR

The genes with different expression levels were screened out from the reproductive endocrine system process and rhythm process and verified by qRT-PCR. The expression levels of the BHCHE40, TFRI-2, sFRP-4, C4BX2, and PPP2A2B genes were significantly up-regulated in the anestrus yak ovaries. On the other hand, the expressions of BMP15, INSL3, HMGB3, SPINK2, PLAC1, and UBE2X1 genes were significantly down-regulated in anestrus yak ovaries. These results are consistent with the data measured by RNA-seq (**Table 1**).



**Table 1.** Validation of selected DEGs by qRT-PCR analysis

Gene ID	Gene product	qRT-PCR		ONT	
		log <sub>2</sub> <sup>FC</sup>	Regulated	log <sub>2</sub> <sup>FC</sup>	Regulated
<b>Reproductive process</b>					
Gene19652	Tissue factor pathway inhibitor 2 precursor (Bos taurus)	1.34	Up	1.77	Up
Gene33	Secreted frizzled-related protein 4, partial (Bos mutus)	2.15	Up	1.72	Up
Gene9481	PREDICTED: insulin-like 3 (Bos mutus)	-2.02	Down	-1.82	Down
Gene4332	PREDICTED: C4b-binding protein alpha chain-like isoform X2 (Bos mutus)	5.98	Up	5.39	Up
Gene2827	High mobility group protein B3 (Bos mutus)	-1.06	Down	-1.23	Down
Gene16923	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform, partial (Bos mutus)	1.05	Up	1.32	Up
Gene10424	Serine protease inhibitor Kazal-type 2 precursor (Bos taurus)	-2.46	Down	-2.36	Down
Gene21158	PREDICTED: placenta-specific protein 1 (Bos mutus)	-3.31	Down	-2.58	Down
<b>Rhythmic process</b>					
Gene9978	PREDICTED: bone morphogenetic protein 15 (Bos mutus)	-3.21	Down	-3.42	Down
Gene8079	Class E basic helix-loop-helix protein 40 (Bos taurus)	1.47	Up	1.42	Up
<b>Reproduction</b>					
Gene9210	PREDICTED: ubiquitin-conjugating enzyme E2 C isoform X1 (Bos mutus)	-0.47	Down	-1.22	Down

DEG, differentially expressed gene; qRT-PCR, quantitative real-time polymerase chain reaction.

## DISCUSSION

The hypothalamic-pituitary-gonadal axis is the main reproductive endocrine axis in animals. The pituitary secretes FSH and LH, both of which bind to specific receptors on the ovary to promote growth, development, maturation, ovulation, and luteinizing formation of ovarian follicles, and thereby regulating estrus, fertilization, pregnancy, parturition, and other processes [15,24]. The hypothalamus and pituitary gland functions are low after delivery when the function of the neuroregulatory system has not been restored. During this period, GnRH is released at a lower frequency; the pituitary gland responsiveness to GnRH decreases; LH is released in small amounts, and its biological activity is low [25,26].

In the present study, GO analysis combined with transcriptomics and proteomics data showed that the rhythm process involves DEGs up-regulated by class E basic helix-loop-helix protein 40 (BHLHE40) and down-regulated by steroidogenic factor 1 isoform X1 (SF1X1), forkhead box protein R1 (FBPR1), bone morphogenetic protein 15 (BMP15) and lutropin- hormone/ choriogonadotropic receptor (LHCGR). Changes in the light rhythm have an important influence on the reproduction process of yaks [27]. The estrus cycle of light-illuminated rats was prolonged, the light time was prolonged, and the anestrus cycle appeared disordered [28]. In mammals, the photoperiod triggers seasonal breeding, hence animals are either short- or long-day breeders [29]. LHCGR has a certain regulatory effect on estrus and follicular development. The combination of LHCGR and LH can activate the cAMP signal pathway and promote the development and maturation of ewe follicles [30]. Pakarainen et al. [31] reported that high doses of FSH could not induce follicular development and ovulation in mice in the absence of LHCGR expression. Shao et al. [32] reported that sheep LHCGR protein mainly interacts with some proteins related to the sheep reproductive performance and helps regulate sheep high fecundity. In this study, the LHCGR gene was down-regulated during the rhythm process. As a result, LHCGR could not induce estrus and follicular development of animals.

The yak is a seasonal breeding animal. Only a few yaks (< 10%) return to estrus during the first breeding season after calving [27]. In the GO biological process category, 34 DEGs, including 10 up-regulated DEGs, were related to reproduction, and 35 DEGs, including 10 up-regulated DEGs, were related to reproduction. The presence of BMP15 in the pituitary gland

enables this tissue to secrete and express FSH, thereby promoting follicular development. In this study, the BMP15 gene was down-regulated during reproduction, and the function of FSH was inhibited and its activity was low, causing the follicles to lock, affecting estrus. In KEGG analysis, the DEGs related to the oxidative phosphorylation and glutathione metabolism were down-regulated, resulting in a negative energy balance, which blocked the development of follicles. In one study, a negative energy balance was found to be an important risk factor for the lowered reproductive performance and increased rate of apathy in dairy cows during early lactation [33]. Glycoprotein  $\alpha$ -1,2-mannosidase was down-regulated in the Mitogen-Activated Protein Kinase (MAPK) pathway and the phosphatidylinositol 3 kinase-Akt (PI3K/Akt) signaling pathway. The endothelin-1 receptor (ET-1R) is up-regulated in the calcium-signaling pathway and the cyclic adenosine monophosphate-PKG (cAMP-PKG) signal transduction system, and ET-1R damages cells. The MAPK signal pathway components are phosphorylated and activated during oocyte maturation, which plays an important role in MII phase arrest in oocyte maturation [34,35]. Components of the PI3K/Akt signaling pathway and some downstream effector molecules are present in follicles, and changes occur during formation, growth, ovulation, and luteinization [36,37]. High cAMP concentrations can inhibit the occurrence of oocyte vesicle rupture, and maintain oocyte meiosis arrest [38], and cAMP, which is required to maintain meiosis arrest, is produced by oocytes [39]. Heat shock protein CA (HSPCA) is up-regulated in progesterone-mediated oocyte maturation. When the secretion of progesterone reaches a threshold value, it causes negative feedback, acting on the hypothalamus and pituitary to inhibit the secretion of adeno-pituitary FSH, and inhibiting ovarian follicles. The development of the brain inhibits the sex center in the central nervous system of the brain and stops the estrus performance [40].

In conclusion, transcriptome and proteomic sequencing approaches were used to investigate postpartum anestrus and pregnancy ovaries in yak. The results confirmed that BHLHE40, SF11X1, FBPX1, HSPCA, LCHGR, BMP15, and ET-1R could affect postpartum hypoestrus, thereby control the state of estrus. Bioinformatics was used to explore differences between yak postpartum fatigue and pregnancy ovaries. The findings provide a reference for revealing the functional characteristics of yak ovaries from a molecular perspective, and provide a novel approach for the in-depth and early resolution of reproductive problems that plague yak development.

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