A Comparative Transcriptome and Proteomics Study of Postpartum Ovarian Cycle Arrest in Yaks

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Abstract

Background: Postpartum ovarian cycle arrest is the main factor affecting yak reproductive efficiency. There are few reports regarding the molecular regulatory mechanism of postnatal estrus on transcriptome and proteome in yaks. Our previous studies focused on the ovaries of yaks with postnatal ovarian cycle arrest and postnatal estrus yaks. In this study, RNA sequencing transcriptomic was combined with quantitative proteomic analyses to identify postpartum ovarian cycle-related genes and proteins.

Results: Consequently 1,149 genes and 24 proteins were found to be up- or downregulated during postnatal estrus. The analysis of differentially regulated genes identified three gene or protein pairs that were synchronously upregulated and no gene or protein pairs that were synchronously downregulated, suggesting that these upregulated genes may regulate the postpartum ovarian cycle. The functional classification of these differentially expressed genes and proteins indicated their connection with oocyte meiosis, the estrogen signaling pathway, progesterone-mediated oocyte maturation and the gonadotrophin releasing hormone (GnRH) signaling pathway.

Conclusions: In this study, a total of six genes and two proteins involved in the oocyte meiosis, the estrogen signaling pathway, progesterone-mediated oocyte maturation and the GnRH signaling pathway were identified. This is the first report of a comprehensive transcriptomic and proteomic analysis of postnatal estrus on Yaks.

1. Introduction

The adverse environment of high altitude and extreme cold on the Tibetan Plateau is a challenge to the survival of yaks and they have evolved multiple strategies to adapt to environmental conditions. Postpartum ovarian cycle arrest is an adaptive strategy occurring postpartum, which assists yaks to survive unfavorable environments. Postpartum ovarian cycle arrest is regulated through nutrition, hormones and genetic factors [1–3]. However, due to the severe living conditions, yak reproductive characteristics include late reproductive onset after five years of age, seasonal estrus starting every July and a long generation interval resulting in one fetus in two years or two fetuses in three years [4–7]. Yak ovaries show periodic activity, with the estrus cycle lasting 19 to 21 days and the gestation period lasting 250 days.

The reproduction processes of yaks are regulated by hormones that involve the hypothalamus-pituitary-gonadal axis. Gonadotrophin releasing hormone (GnRH) is produced by the hypothalamus. The level of gonadotrophin releasing hormone receptor (GnRHR) mRNA in the hypothalamus is higher than that in the pineal gland, pituitary gland, and ovary during estrus of yaks [3]. This GnRH leads to accessory corpus luteum formation, but neither the plasma progesterone concentration or the pregnancy rate is affected by having an accessory corpus luteum [8–11]. The follicle-stimulating hormone receptor (*FSHR*) mRNA expression level is higher in the pituitary gland tissue compared with luteinizing hormone receptor (*LHR*)

during estrus of yaks, whereas there is low expression of *FSHR* and *LHR* mRNA in the pineal gland and hypothalamus. The *FSHR* mRNA expression is higher than that of *LHR* in the ovary, whereas *LHR* mRNA expression is higher than that of *FSHR* in the uterus during the estrus of yaks [12]. Luteinizing hormone (LH) support is important during the final part of follicle maturation through its influence on cumulus cells [13, 14]. Dominant follicles experience a reduction in FSH dependence, but acquire increased LH dependence as they grow during the low FSH levels of follicular waves [15, 16]. The estradiol receptor (*ER*) mRNA is greater in the pituitary gland tissue than in other glands during estrus of yaks [17] and this estradiol (E_2) peak is smaller than that of the follicular phase [18, 19].

2. Materials And Methods

2.1. Sample Collection and Preservation

In September 2019, 16 female yaks with calves were selected in Haiyan County, Qinghai Province and observed twice daily for estrous under natural grazing conditions. Until October 2019, after two consecutive rectal examinations, the surface of ovary was smooth without large follicles or corpus luteum. If the concurrent content of progesterone in plasma was below 0.5 μg / L, they were classified as being in postpartum ovarian cycle arrest. Three yak ovaries in each of postnatal ovarian cycle arrest and postnatal estrus was obtained immediately at the time of slaughter and stored immediately in liquid nitrogen until further use.

2.2 RNA extraction, Library Construction and RNA-Seq

The total RNA of the mixed sample of each group ovaries were extracted according to the instruction manual of TRIzol Reagent (Life Technologies, CA, USA). RNA integrity and concentration were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). The mRNA was isolated by the NEBNext Poly(A)mRNA Magnetic Isolation Module (NEB, E7490). The cDNA library was constructed following the manufacturer's instructions for the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, E7530) and NEBNext Multiplex Oligos for Illumina (NEB, E7500). The enriched mRNA was fragmented into approximately 200 nt RNA inserts, which were used to synthesize the first-strand cDNA and the second cDNA. The double-stranded cDNA underwent end-repair/dA-tail and adaptor ligation. 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 Illumina HiSeq™ sequencing platform.

2.3 Transcriptome Analysis Using Reference Genome-based Reads Mapping

Low quality reads, such as only adaptor, unknown nucleotides over 5%, or Q20 less than 20% where the percentage of sequences with sequencing error rates was less than 1%, were removed by perl script. The clean reads that were filtered from the raw reads were mapped to the yaks genome using Tophat2 software [20]. The aligned records from the aligners in BAM/SAM format were further examined to

remove potential duplicate molecules. Gene expression levels were estimated using fragments per kilobase of exon per million fragments mapped (FPKM) values by the Cufflinks software [21].

2.4 Identification of Differential Gene Expression (DEGs)

The DESeq and Q-value were employed to evaluate differential gene expression between postnatal ovarian cycle arrest and postnatal estrus of yaks. Gene abundance differences between those samples were then calculated based on the ratio of the FPKM values. The false discovery rate (FDR) control method was used to identify the threshold of the P-value in multiple tests to compute the significance of the differences. In this study, only genes with an absolute value of $\log_2$ ratio greater than or equal to two and an FDR significance score less than 0.01 were used for subsequent analysis.

2.5 Sequence Annotation

Genes were compared against various protein databases by BLASTX, including the National Center for Biotechnology Information (NCBI) non-redundant protein (Nr) database and the Swiss-Prot database with a cut-off E-value of 10^{-5}. Genes were also searched against the NCBI non-redundant nucleotide sequence (Nt) database using BLASTn by a cut-off E-value of 10^{-5}. Genes were retrieved according to the highest score based on the best BLAST hit along with their protein functional annotation.

To annotate the gene with gene ontology (GO) terms, the Nr BLAST results were imported into the Blast2 GO program. This analysis mapped all the annotated genes to GO terms in the database and counted the number of genes associated with each term. Perl script was then used to plot GO functional classification for the unigenes with a GO term hit to view the distribution of gene functions. The obtained annotation was enriched and refined using TopGo. The gene sequences were also aligned to the Clusters of Orthologous Group (COG) database to predict and classify functions. KEGG pathways were assigned to the assembled sequences by perl script.

2.6 TMT Quantitative Proteomic Analysis.

Mixed samples from postnatal ovarian cycle arrest and postnatal estrus yak ovaries were ground into powder in liquid nitrogen, homogenized in lysis buffer (8 M urea, 1% Triton X-100, 65 mM DTT and 0.1% Protease Inhibitor Cocktail III) 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 rpm at 4 °C for 10 min, the remaining precipitate was washed with cold acetone three times, then the protein was dissolved in a buffer of 8 M urea and 100 mM TEAB at a pH of 8 and the protein concentration was determined using a 2-D Quant kit (GE Healthcare, USA).

Total protein (100 μg) solution was reduced with 10 mM 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 at 1:50 trypsin-to-protein mass ratio for the first digestion overnight, and at 1:100 trypsin-to-protein mass ratio for a second four hour digestion. After trypsin digestion, peptides were desalted using a Strata X C18 SPE column

(Phenomenex) and 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 μm 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 strategy51 against Uniprot_Neohemiptera.fasta database. 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.

2.7 Gene Expression Assay Using PCR

Total tissue RNA was extracted with RNAiso Plus (Takara, Dalian, China) and the quality was checked with A260/280 (1.9 to 2.0). Reverse transcription was performed using a RNA PCR kit (AMV) (TaKaRa, Dalian, China) in a total volume of 10 μL of the reaction mixture, which contained 0.5 μg RNA, 5 mM MgCl2, 1 × RT buffer, one μL dNTP mixture, 10 U RNase inhibitor, 2.5 U AMV-RT, 1.25 pmol oligo(dT) primer and 3.75 μL RNase free dH_2O, at 37°C for 15 min and 85°C for five seconds. The real-time PCR assay was developed based on SXBR Premix Ex TaqTM II (Perfect Real Time) (Takara). The housekeeping gene *18 s* was used to correct potential variations in the RNA loading. Amplification reactions were performed in a total volume of 20 μL with 12.5 μL SXBR Premix Ex TaqTM II (2×), 2 μL template cDNA, 1 μL of each primer (10 μmol/L) and 4.5 μL deionized H_2O. The amplification program was carried out as an initial denaturation step at 95 °C for two min, followed by 40 cycles of denaturation for 10 s at 95 °C and annealing and extension at 60 °C for 30 s. Fluorescence was measured at the end of each annealing and extension step. The cycle threshold (Ct) was returned to baseline during each reaction and whether a specific product or a primer dimer was present in the PCR during each reaction was determined based on the melting curve.

3. Results

3.1 Expression Profile of Postnatal Ovarian Cycle Arrest and Normal Ovaries

To clarify the mechanism of postnatal ovarian cycle arrest, the transcriptome and proteome of estrus and anestrus ovaries were surveyed using RNA-Seq and iTRAQ techniques. A total of 18,036 genes were identified and quantified in the transcripts of ovary (Table 1). After filtering with FDR, 1,149 genes were found to be differentially expressed between estrus and anestrus ovaries, with 626 upregulated differentially expressed genes (DEGs) and 523 downregulated ones detected. A total of 56,530 transcripts were identified between estrus and anestrus ovaries with fold-changes greater than or equal to two and an FDR less than 0.01, where 2,294 differentially expressed transcripts (DETs) had 1,210 upregulated and 1,084 downregulated genes. A protein library was established by transcriptome results and differential protein analysis was carried out, where 4,457 proteins were identified. A total of 24 differentially

expressed proteins (DEPs), of which 14 were upregulated and 10 were downregulated, were identified between estrus and anestrus ovaries with at least a 1.2-fold difference, Thirty-three transcriptome/protein pairs were synchronously upregulated. (Table S2).

Table 1
Overall features of the oestrus and anestrus ovaries expression profile.

Group name	Type	Number of genes	Number of proteins	Number of correlations
Anestrus VS Oestru	Identification	18036	4934	708
Anestrus VS Oestru	Quantitation	18036	3889	487
Anestrus VS Oestru	Differential expression	2294	24	3

3.2 Function Classification of DEGs and DEPs by COG

The COG database, an international standardized gene functional classification system was used to classify orthologous gene products. The upregulated and downregulated DEGs between postnatal estrus and anestrus ovaries were distributed in 21 and 17 COG categories, respectively. In Fig. 1, general function, the largest category in transcriptome, had 55 DEGs, 44 of which was upregulated. The upregulated DEPs between postnatal estrus and anestrus were distributed in translation, ribosomal structure and biogenesis, signal transduction mechanisms, posttranslational modification, protein turnover, chaperones and nucleotide transport and metabolism, all of which had one DEP. The downregulated DEPs were distributed in transcription and post-translational modification, protein turnover and chaperones, which had one DEP and two DEPs. The categories of cell cycle control, cell division, chromosome partitioning and cell wall, membrane and envelope biogenesis were directly associated with ovary growth.

3.3. Functional Classification of Enriched DEGs and DEPs by GO

The GO database was again used to classify the enriched DEGs and DEPs between postnatal estrus and anestrus ovaries. Based on sequence homology, they were categorized into three main categories as molecular function, biological process, and cellular component. A gene product might be associated with or located in one or more cellular components, so that it is active in one or more biological process, performing one or more molecular function. The DEGs were distributed in up to 7,495 GO terms and DEPs by contrast were classified into only 188 GO terms. The GO terms related to yak reproduction, environmental effect and rhythmic were selected. In the biological process category, the upregulated DEGs (Fig. 2A) and DEPs (Fig. 3A) were distributed in 51 and 26 GO categories, respectively. The terms of DEPs were all included in DEGs. In the category of biological process (Fig. 2A, Fig. 3A), the largest terms

in transcripts and proteomics were cellular processes including 354 DEGs and cellular process with five DEPs. In respect to reproduction, a total of 75 DEGs were involved in reproduction and reproduction process. Contrastingly, no DEP was associated with the reproduction and reproduction process term. In respect of environmental effect, a total of 507 DEGs were associated with the terms of biological regulation and response to stimulus. Regarding proteomics, 30% of DEPs were associated with the terms of biological regulation and response to stimulus. Four DEGs were associated with the term of rhythmic process and no DEPs were associated. In biological process category (Fig. 2D, Fig. 3D), the downregulated DEGs and DEPs were distributed in 47 and 36 GO categories, respectively. In the category of biological process (Fig. 2D, Fig. 3D), the largest terms in transcripts and proteomics were cellular process with 187 DEGs and cellular process with seven DEPs. In respect to reproduction, a total of 43 DEGs and two DEPs were involved in the two terms of reproduction and reproduction process. In the aspect of environmental effect, a total of 284 DEGs were associated with the terms of biological regulation and response to stimulus. Regarding proteomics, 15.22% of DEPs were associated with the terms of biological regulation and response to stimulus. Three DEGs were associated with the term of rhythmic process and no DEPs were associated. In the cellular component category of upregulation (Fig. 2B, Fig. 3B), the term plasma membrane had the largest DEG number, occupying 14.56% of total annotated DEGs. Unlike DEGs, 25.64% of DEPs were distributed in the terms of cell and cell part which included 10 DEGs. Notably, most GO terms of proteomics were identical to those of transcripts. In the cellular component category of downregulation (Fig. 2E, Fig. 3E), the DEG number in the terms of cell part and cell were the largest, occupying 34.89% of total annotated DEGs. Like DEG, 25.64% of DEPs distributed in the terms of cell and cell part which included 14 DEG and most GO terms of proteomics were identical to those of transcripts. In the category of upregulated molecular function (Fig. 2C, Fig. 3C), most GO terms were associated with enzyme activity and binding. A total 247 DEGs fell into six GO terms having enzyme activities. In proteomics, two terms, including three DEPs, were related to enzyme activity. Thirty-five DEGs and three DGPs were involved the term of molecular function regulator. There were seven and six DEGs in the terms of electron carrier activity and antioxidant activity, respectively, but neither contained DEPs. Binding was the term that contained the largest DEG number at 305, which represented 50.33% of all DEGs annotated in GO terms. In the category of downregulated molecular function (Fig. 2F, Fig. 3F), most GO terms were also associated with enzyme activity and binding. A total of 158 DEGs fell into six GO terms having enzyme activities. In proteomics, four terms, including eight DEPs, were related to enzyme activity. Eleven DEGs and three DGPs were involved the term of molecular function regulator. There were four and two DEGs in the terms of electron carrier activity and antioxidant activity respectively, but neither contained DEP. Binding contained the largest DEG number at 165, which represented 47.97% of all DEGs annotated in GO terms.

3.4. Function Annotation of Enriched DEGs and DEPs by KEGG

The KEGG data base is a knowledge base for systematic analysis of gene functions that link genomic information with higher order functional information. Genes within the same pathway usually cooperate

with each other to exercise their biological function, indicating that pathway-based analysis is a useful instrument for further understanding of the biological functions of genes. A KEGG analysis of DEGs and DEPs between postnatal estrus and anestrus ovaries was carried out. 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 in Fig. 4. The upregulated DEGs related to cellular processes had eight pathways and 15 DEGs (Fig. 4A), especially the pathways involved in reproductive processes, except p53 signaling and peroxisome. The DEPs were involved in reproductive processes except for focal adhesion (Fig. 4C). Six downregulated DEGs had two pathways associated with reproductive processes in endocytosis and phagosome, but no DEPs associated with reproduction (Fig. 4B-D). One upregulated DEG and two DEPs were in the cAMP signaling pathway and calcium signaling pathway associated with the reproduction of environmental information processing. In the category of upregulated DEGs organismal systems, progesterone oocyte maturation and the estrogen signaling pathway were involved in reproduction (Fig. 4A). Phototransduction and the GnRH signaling pathway were associated with the reproduction of DGPs. In the downregulated DEGs in organismal systems, two DEGs related to the estrogen signaling pathway were associated with reproduction, but not with DEPs associated with reproduction (Fig. 4B-D).

3.5 Validation of Selected DEGs and DEPs by PCR Analysis

To evaluate the gene expression profile, 25 genes including 22 upregulated genes and three downregulated genes based on the results of GO analysis belonged to response to stimulus, reproductive process, reproduction and rhythmic process were selected to undergo PCR (Table 2). Some of the selected genes were involved in reproduction directly, where ten genes in the two categories were all upregulated. In addition, some genes related to environmental effect were also chosen as response to stimulus related genes and they were also all upregulated. Rhythmic related genes were also examined where four genes were upregulated, and three genes were downregulated.

Table.2. Validation of selected DEGs by qRT-PCR analysis.

Gene ID	Gene product	qPCR		ONT	
		log_2^{FC}	regulated	log_2^{FC}	regulated
rhythmic process					
gene16171	Lutropin-choriogonadotropic hormone receptor, partial [Bos mutus]	3.89	Up	3.95	Up
gene9978	PREDICTED: bone morphogenetic protein 15 [Bos mutus]	5.14	Up	5.11	Up
gene8885	Forkhead box protein R1	3.45	Up	3.43	Up
gene7394	Casein kinase I isoform alpha, partial [Bos mutus]	1.33	Up	1.23	Up
gene1350	Slit-like protein 3 protein, partial [Bos mutus]	-1.10	Down	-1.06	Down
gene533	hypothetical protein M91_01346 [Bos mutus]	-1.24	Down	-1.26	Down
gene1328	Inhibin beta A chain [Bos mutus]	-1.43	Down	-1.41	Down
reproductive process					
gene21158	PREDICTED: placenta-specific protein 1 [Bos mutus]	2.36	Up	2.33	Up
gene17252	PREDICTED: cyclin-dependent kinases regulatory subunit 2 [Balaenoptera acutorostrata scammoni]	2.21	Up	2.15	Up
gene9481	PREDICTED: insulin-like 3 [Bos mutus]	2.25	Up	2.23	Up
gene9237	Zona pellucida sperm-binding protein 2 [Bos mutus]	4.67	Up	4.70	Up
gene7773	Calmegin, partial [Bos mutus]	1.77	Up	1.78	Up
reproduction					
gene5675	Gap junction beta-5 protein [Bos mutus]	1.74	Up	1.75	Up
gene21158	PREDICTED: placenta-specific protein 1 [Bos mutus]	2.31	Up	2.33	Up
gene9210	PREDICTED: ubiquitin-conjugating enzyme E2 C isoform X1 [Bos mutus]	1.67	Up	1.62	Up
gene4775	PREDICTED: 15-hydroxyprostaglandin dehydrogenase [NAD(+)] isoform X1 [Bos mutus]	1.89	Up	1.95	Up
gene13254	oocyte-expressed protein homolog [Bos taurus]	3.22	Up	3.25	Up

response to stimulus					
gene9034	Zinc finger protein 300, partial [Bos mutus]	2.33	Up	2.31	Up
gene14959	PREDICTED: placenta-specific gene 8 protein-like [Bos mutus]	1.44	Up	1.48	Up
gene14082	cell division control protein 2 homolog [Ovis aries]	1.65	Up	1.66	Up
gene9161	PREDICTED: securin isoform X1 [Bos mutus]	2.16	Up	2.17	Up
ONT.2585	Cleavage stimulation factor subunit 2 [Bos mutus]	1.67	Up	1.65	Up
gene6739	PCNA-associated factor, partial [Bos mutus]	1.89	Up	1.80	Up
gene4364	Macrophage-capping protein, partial [Bos mutus]	1.35	Up	1.31	Up
gene15253	Scavenger receptor class B member 1 [Bos mutus]	1.24	Up	1.21	Up
gene4783	Cyclin-A2, partial [Bos mutus]	1.92	Up	1.96	Up
gene3262	PREDICTED: growth/differentiation factor 9 [Bos mutus]	3.09	Up	3.01	Up

To sum up, the above results showed that the expression patterns of all selected genes as determined by PCR were in line with the results from the RNA-seq analysis, despite some differences in the expression levels. The results using samples from independent experiments at different reproductive stage indicated that the responses of ovary to hormone were reproducible. Therefore, the PCR results confirmed the reliability of results from RNA-seq based transcriptome analysis for identifying DEGs.

4. Discussion

4.1 Photoperiod and Postpartum Anestrus of Yaks

In this study, GO analysis of combined proteomics and transcriptomic data showed that rhythm process included *CSNK1A1*, *M91_09723, M91_11326, M91_21439* upregulated DEGs and *M91_01346, M91_08632*, *M91_03244* downregulated DEGs. At the same time, KEGG pathway classification analysis showed that phototransduction which had only one DEP Calmodulin and this was upregulated in the postpartum anestrus ovary. Illumination is a rhythmic process and the change of light rhythm has an important effect on the reproduction process of animals [22–25]. The yak is an important mammal on the Qinghai Tibet Plateau, begins its breeding season in the autumn and is considered to be a short day breeder [26–28]. It is generally postulated that the pineal hormone melatonin synchronizes the ovine

reproductive cycle with the environmental photoperiod through neuroendocrine changes in the hypothalamus-pituitary-gonadal axis[29]. The yaks breeding season begins not because the days are short and the nights are long, but because the days are becoming longer and the days are getting shorter in the summer solstice, so the photoperiod induces endogenous hormones that play a role in regulating the reproductive cycle of yaks [29]. In mammals, photoperiod had triggered the seasonal breeding [30] and therefore animals are either short- or long-day breeders. Therefore, it is postulated that most yaks will upregulate the *CSNK1A1*, M91_0*9723, M91_11326, M91_21439* and calmodulin between summer solstice to winter solstice for rhythmic light cycle. The mechanism of yak postpartum anoestrus caused by these biological factors through hypothalamus-pituitary-gonadal axis needs to be studied next.

4.2 Hormone and Postpartum Anestrus of Yaks

Yaks are seasonal breeders with mating and conception restricted to the warm season. Puberty generally occurs during the second to the fourth warm season following birth, between 13 and 36 months of age. The yaks usually have a long post-partum anestrous period and only a small proportion of yaks return to estrus during the first breeding season after calving, with most coming into estrus during the second and third years [31]. In biological processes, 60 DEGs including 38 upregulated were associated with reproductive process and 58 DEGs including 37 upregulated were associated with reproduction. In KEGG, *M91_03062* was upregulated in oocyte meiosis, *HSPCA* was upregulated in the estrogen signaling pathway and progesterone mediated oocyte maturation. At the same time, *SHC2* and *ATF6B* were downregulated in the estrogen signaling pathway contact with reproduction. There was no gene downregulated in the oocyte meiosis and progesterone-mediated oocyte maturation. Calmodulin was upregulated associated with oocyte meiosis and the GnRH signaling pathway. The expressions of *FST*, *IL10RB*, *ATP6AP2*, *GNAI2* and *PIK3R2* were significantly downregulated and relative expressions of *PIK3R1* and *CYP1A1* were significantly upregulated in the ovarian tissues of non-breeding yaks [32].Before fertilization, the mature oocyte seems to absorb nutrients through receptor-mediated endocytosis, indicating active energy use or storage [33]. Estradiol benzoate treatment can induce a new synchronized follicle wave emergence and increase the pregnancy rate of resynchronized non-pregnant heifers [34]. Reproductive seasonality, driven by either photoperiod or nutritional resource availability, has a significant influence on the success of assisted reproductive technologies on wild cattle [35]. Progesterone was directly linked to undersized deviation but the progesterone effect on decreasing future largest subordinate follicle diameter occurs independently of the progesterone effects on follicle stimulating hormone and luteinizing hormone concentrations [36]. The results also showed that the *M91_03062, HSPCA* and Calmodulin were upregulated in the ovary of the anoestrus yak. In other words, calving caused *M91_03062, HSPCA* and calmodulin to be highly expressed in the ovaries of most but not all yaks, which led to the occurrence of anestrus in yaks.

5. Conclusion

In conclusion a combination of multi-omics data analysis was used to identify the putative effectors regulating postpartum anestrus in the yak. The integrated quantitation and comparison of mRNA and

protein abundances revealed extensive translational and post-translational regulation of postpartum anestrus. This study also confirmed that *CSNK1A1*, M91_0*9723, M91_11326, M91_21439, M91_19073, SHC2, Atf6b*, *M91_03062, HSPCA* and calmodulin could regulate estrus, respectively, in the postpartum so as to control the anestrus status. There are still many issues to be studied in the future, such as which factors regulate postpartum estrus. The present results offer new insights into postpartum anestrus and reproduction of the yak.

Abbreviations

GnRH: Gonadotrophin Releasing Hormone; GnRHR:Gonadotrophin Releasing Hormone Report; FSHR:Follicle-Stimulating Hormone Receptor; LHR:Luteinizing Hormone Receptor; LH:Luteinizing Hormone; ER:Estradiol Receptor; E_2:Estradiol; FPKM:Fragments Per Kilobase of exon per million fragments Mapped; FDR:False Discovery Rate; KEGG:Kyoto Encyclopedia of Genes and Genomes; GO:Gene ontology; COG:Clusters of Orthologous Groups; DEGs:Differentially Expressed Genes; DEPs:Differentially Expressed Proteins; DETs:Differentially Expressed Transcripts; CSNK1A1:Casein Kinase Alpha; HSPCA:Heat Shock Protein HSP 90-alpha; SHC2:SHC adaptor protein 2; ATF6B:Activating Transcription Factor 6 beta; FST:Follistatin; IL10RB:Interleukin 10 receptor subunit beta; GNAI2:G protein subunit alpha i2; PIK3R2:Phosphatidylinositol 3-kinase regulatory subunit beta; *CYP1A1*:cytochrome P450, family 1, subfamily A, polypeptide 1;

Declarations

Acknowledgments

Not applicable.

Authors' contributions

The contributions of the authors were as follows: SH. H. was the principle investigator. He designed the experiment, oversaw the development of the study and wrote the last version of the manuscript. Y. Y. conducted the animal work and most of the laboratory work and wrote the manuscript. Y.ZH., ZH. C., SH. L., Y. ZH. and D. ZH. helped to conduct animal trial and part of the laboratory work and helped to revise the manuscript. R. L. provided ETEC inoculum and helped to revise the manuscript. The authors declare no conflicts of interest. The author(s) read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All animal management and experimental procedures followed the animal care protocols approved by the Northwest Minzu University Animal Care and Use Ethics Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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whether circulating FSH or LH are decreased or elevated. Theriogenology. 2020;143:148–56.

Figures

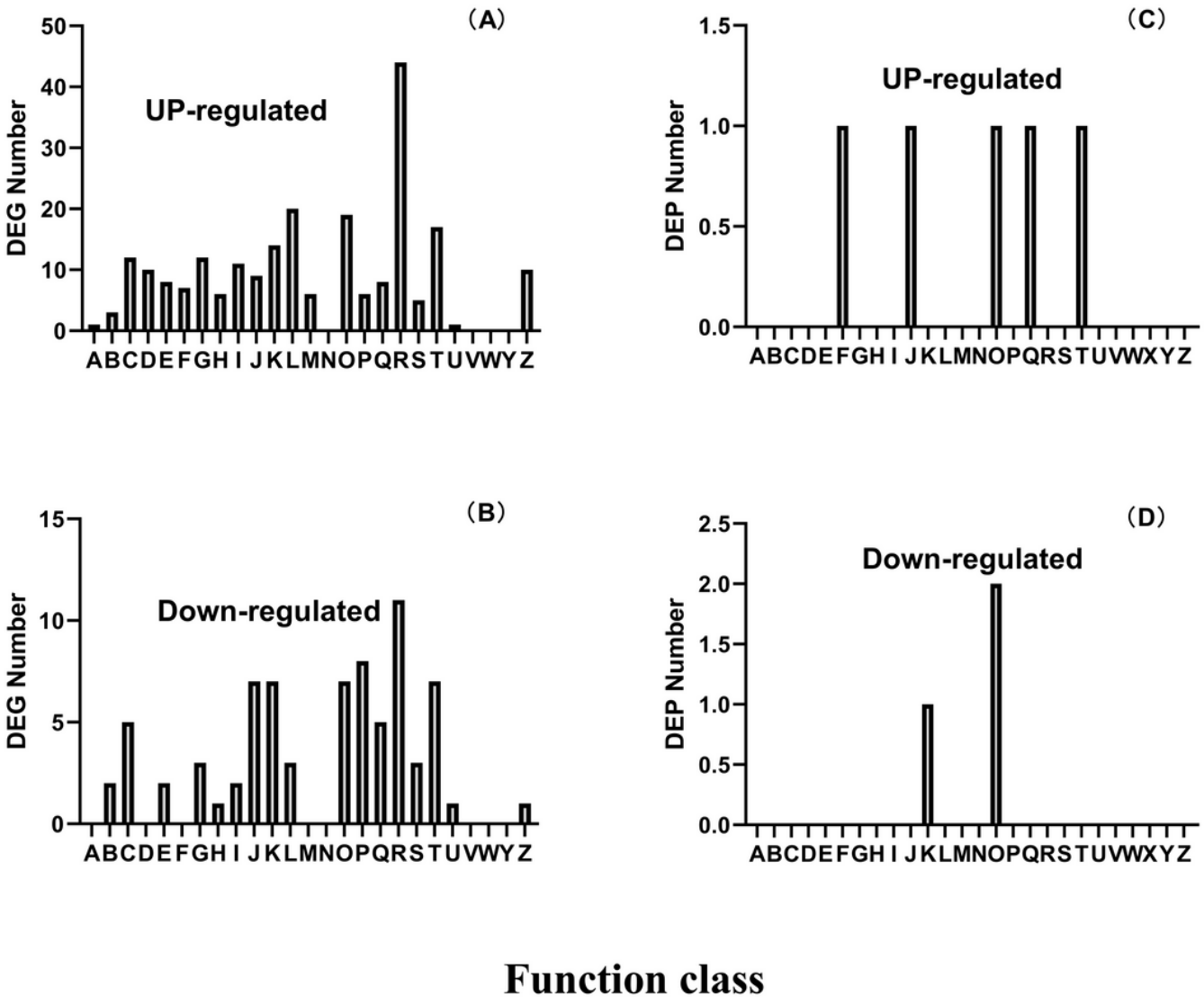
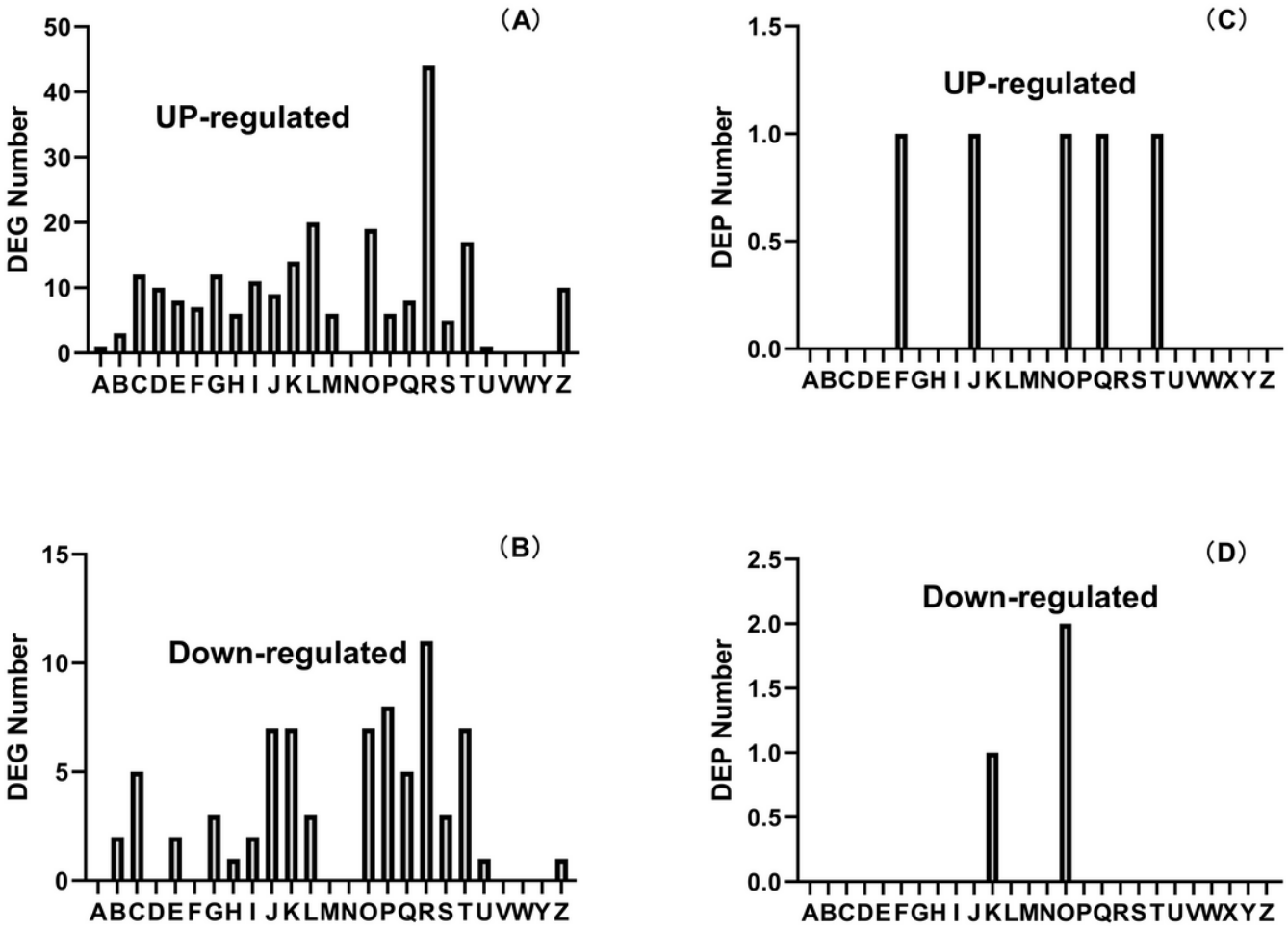


Figure 1

COG function classification of DEGs and DEPs. 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 metabolites biosynthesis, transport and catabolism, R: General function prediction only, S:

Function unknown, T: Signal transduction mechanisms, U: Intracellular trafficking, secretion, and vesicular transport, V: Defense mechanisms, W: Extracellular structures, Y: Nuclear structure, Z: Cytoskeleton. The horizontal/vertical axis indicates the numbers of DEGs and DEPs up and downregulated.



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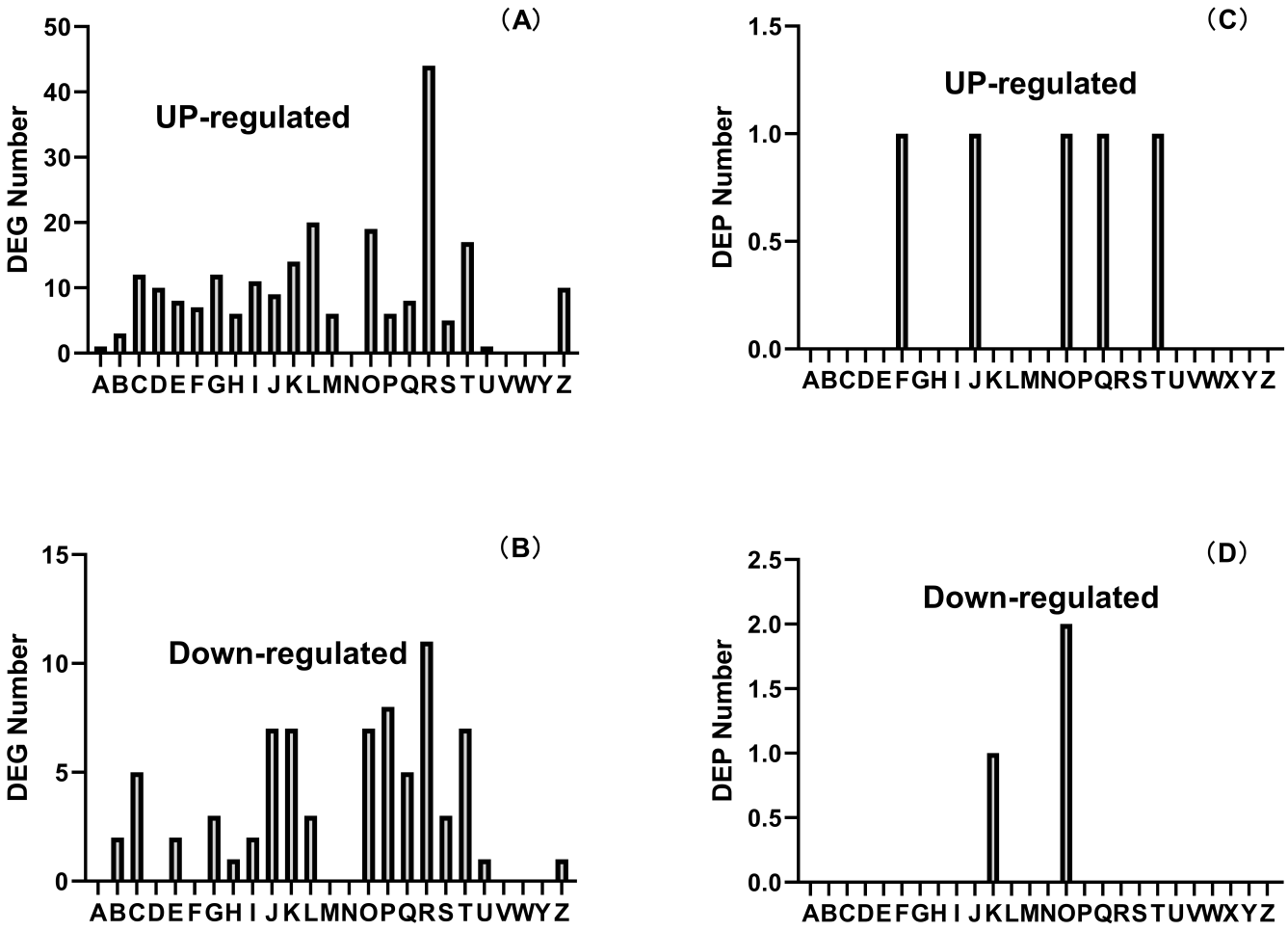

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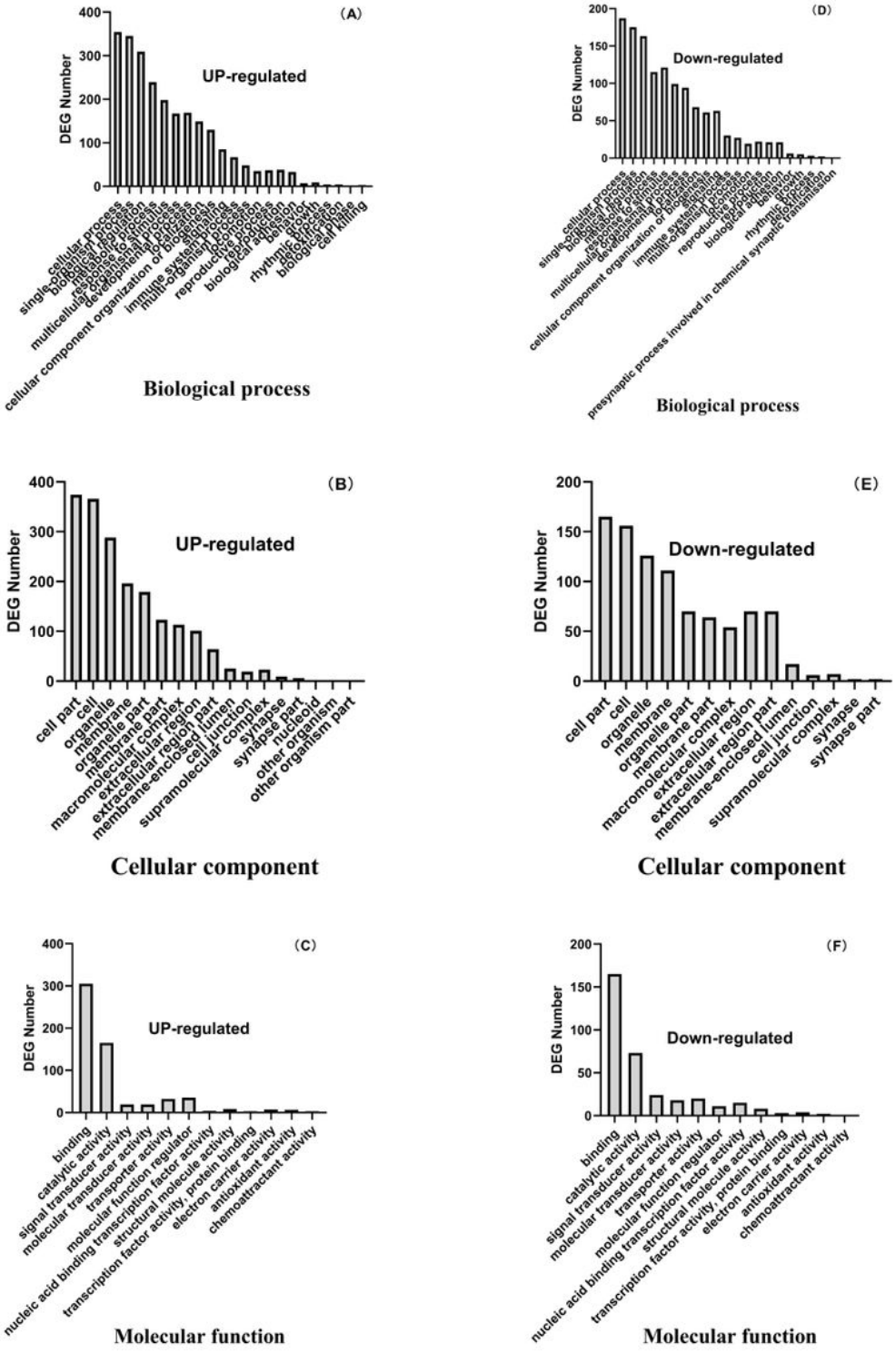
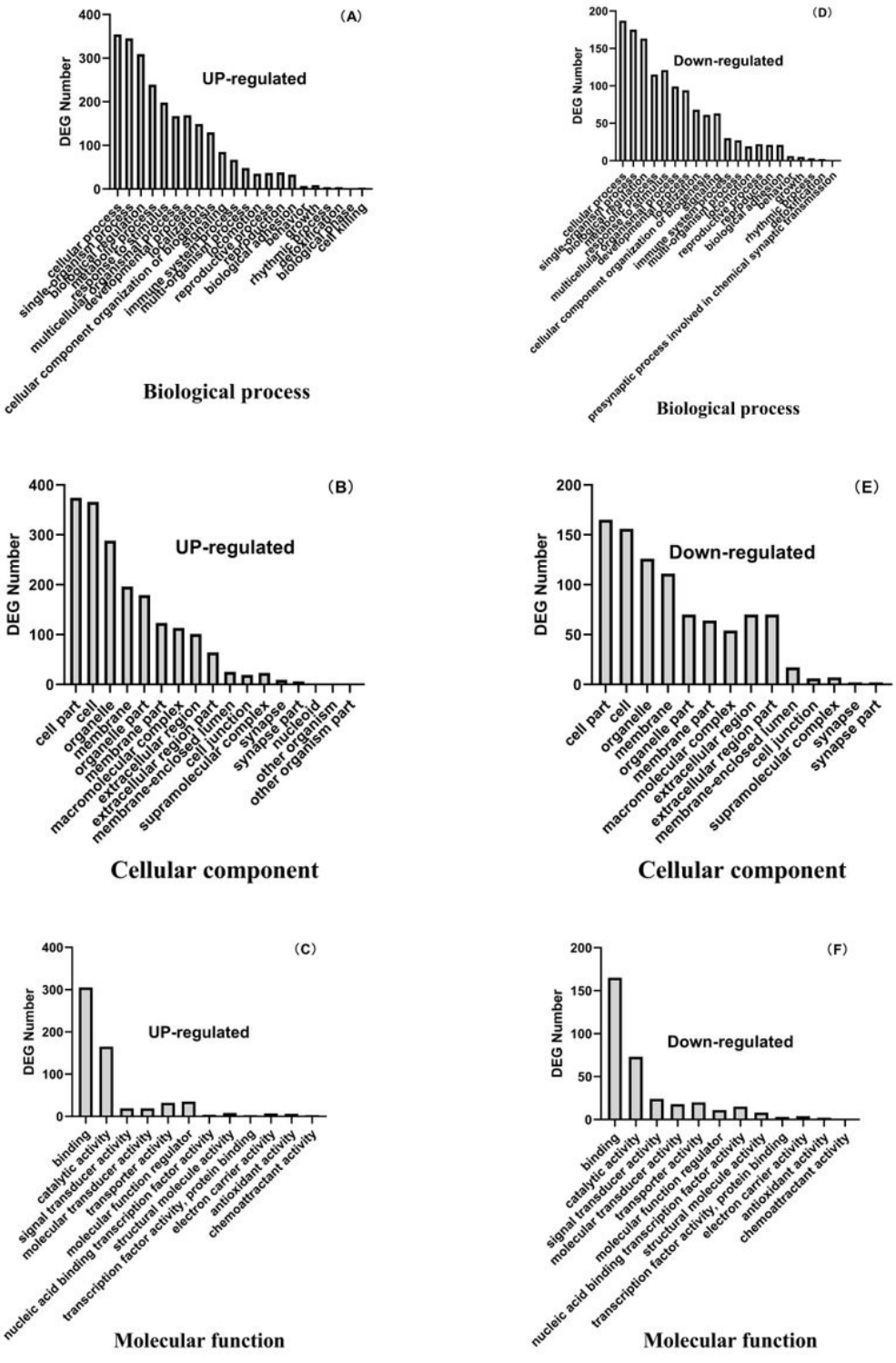


Figure 2

GO enrichment analysis of DEGs. The DEGs were categorized based on biological process (A and D), cellular component (B and E), and molecular function (C and F). The vertical axis indicates the numbers of DEGs.



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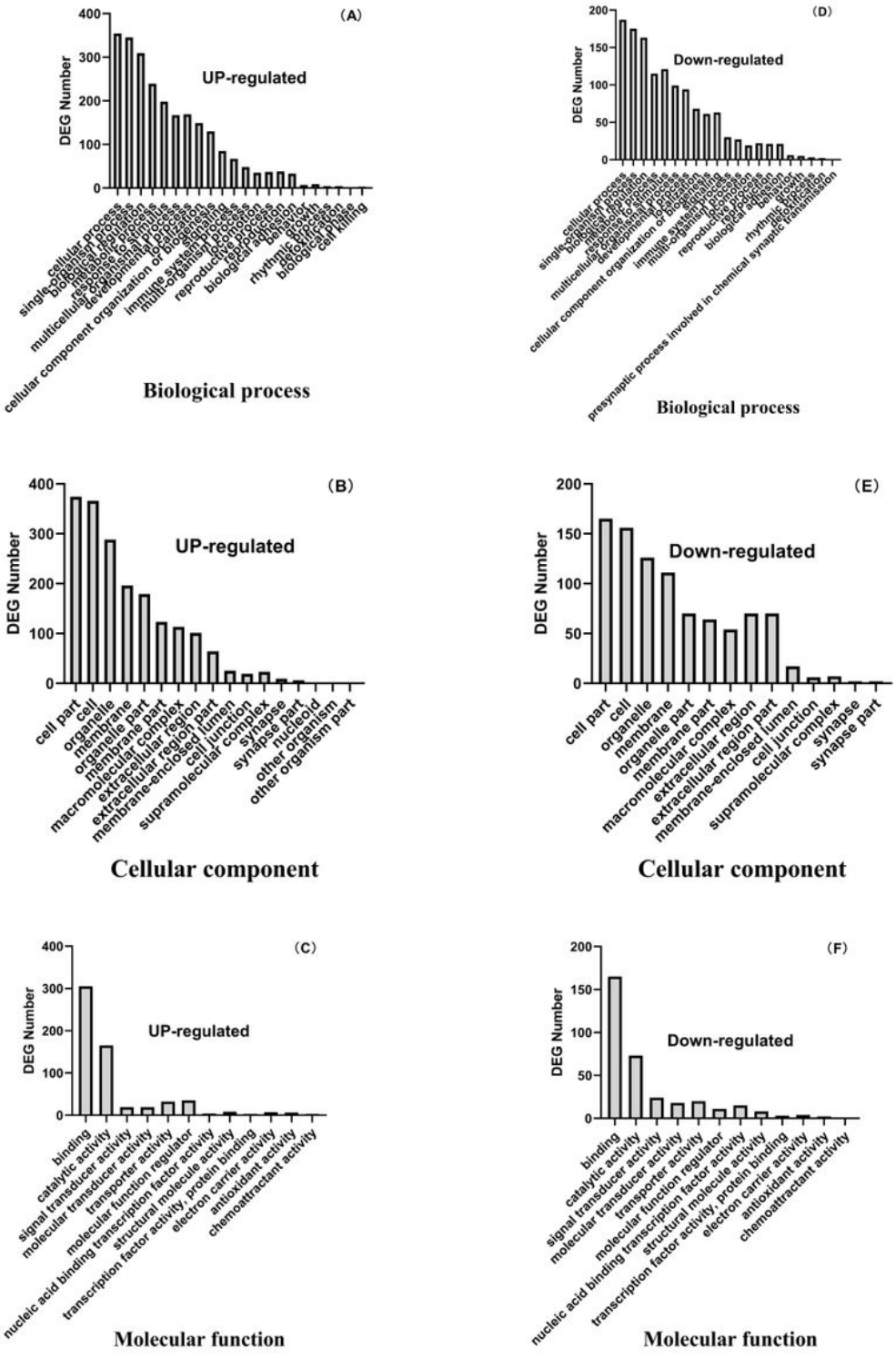

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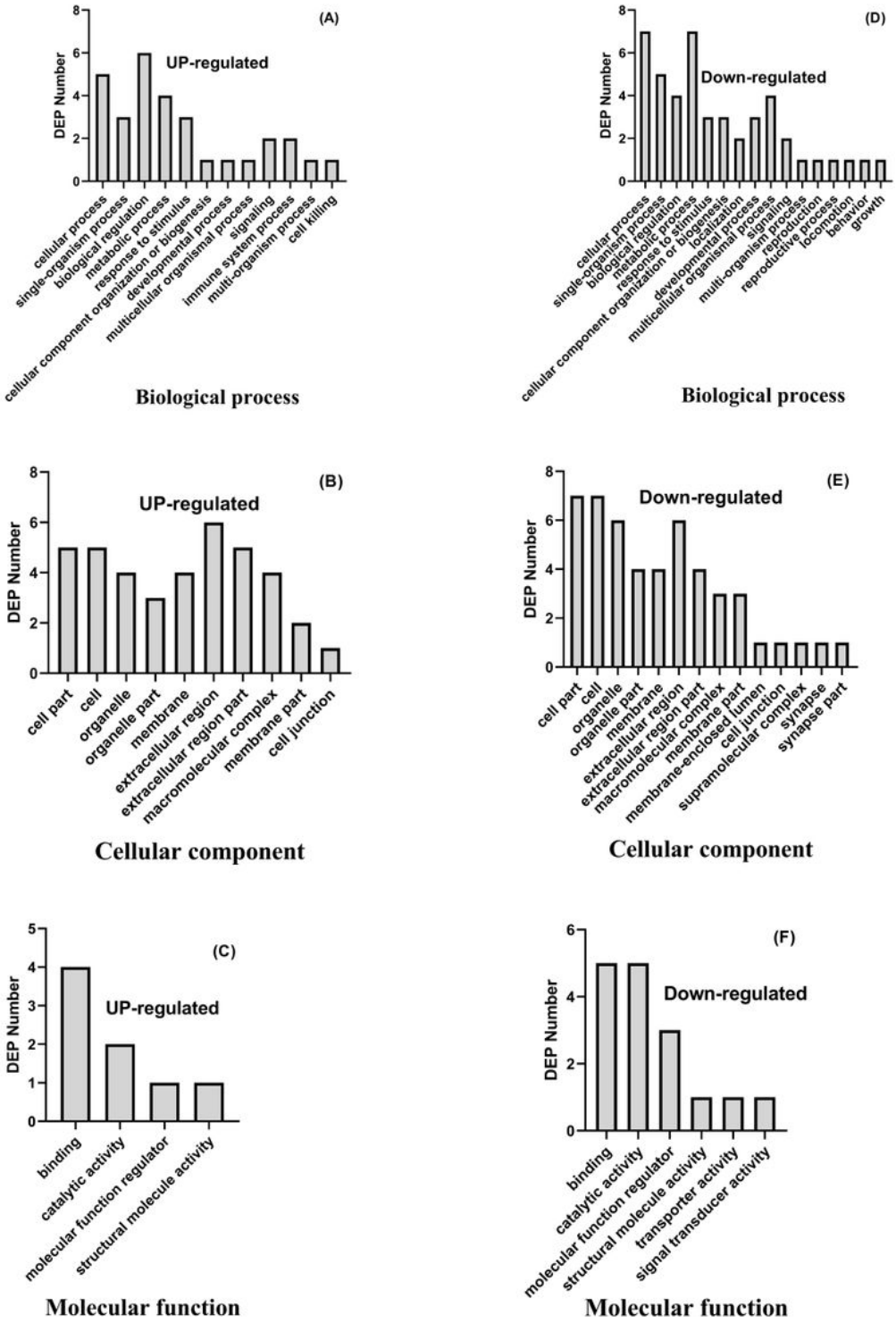

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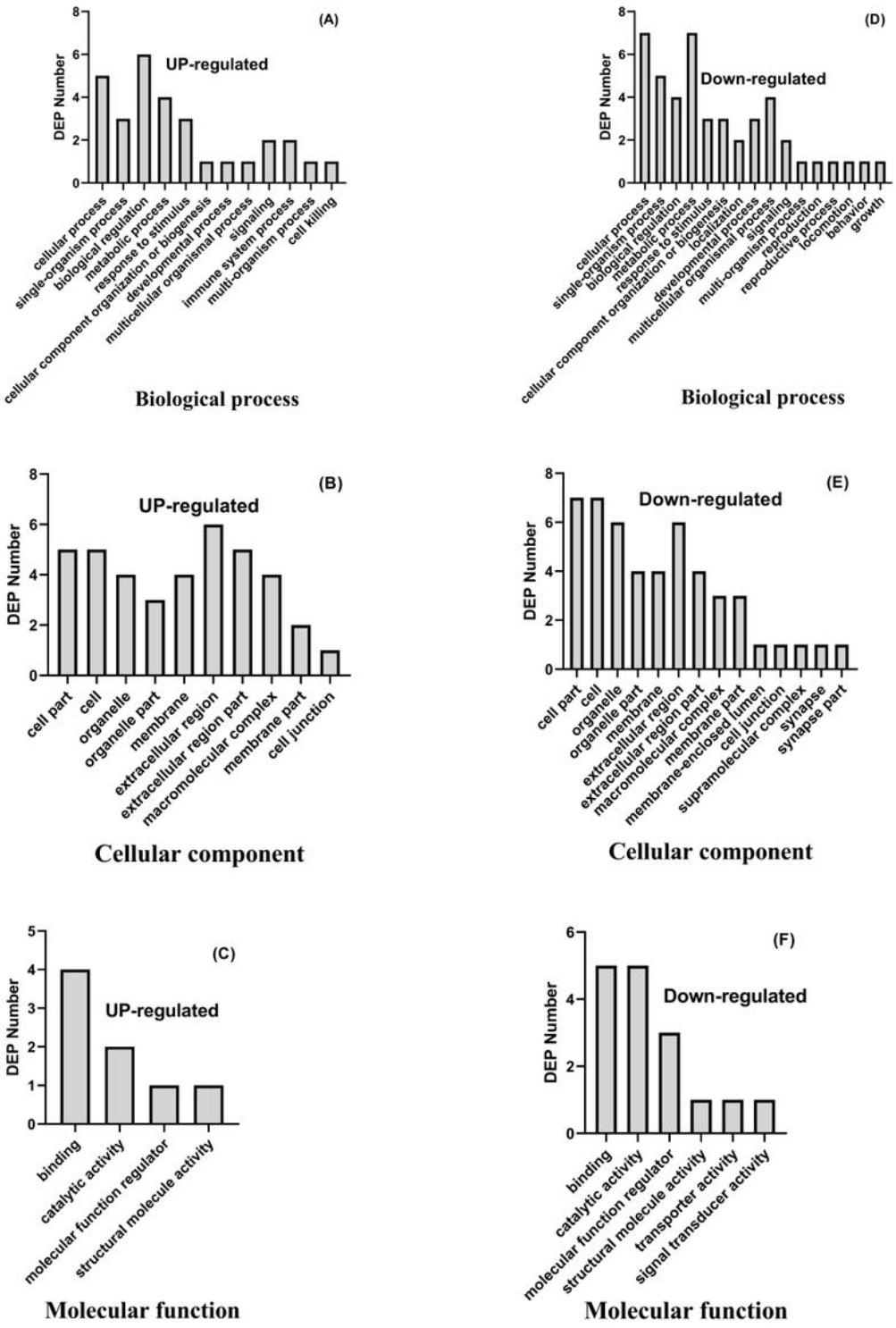

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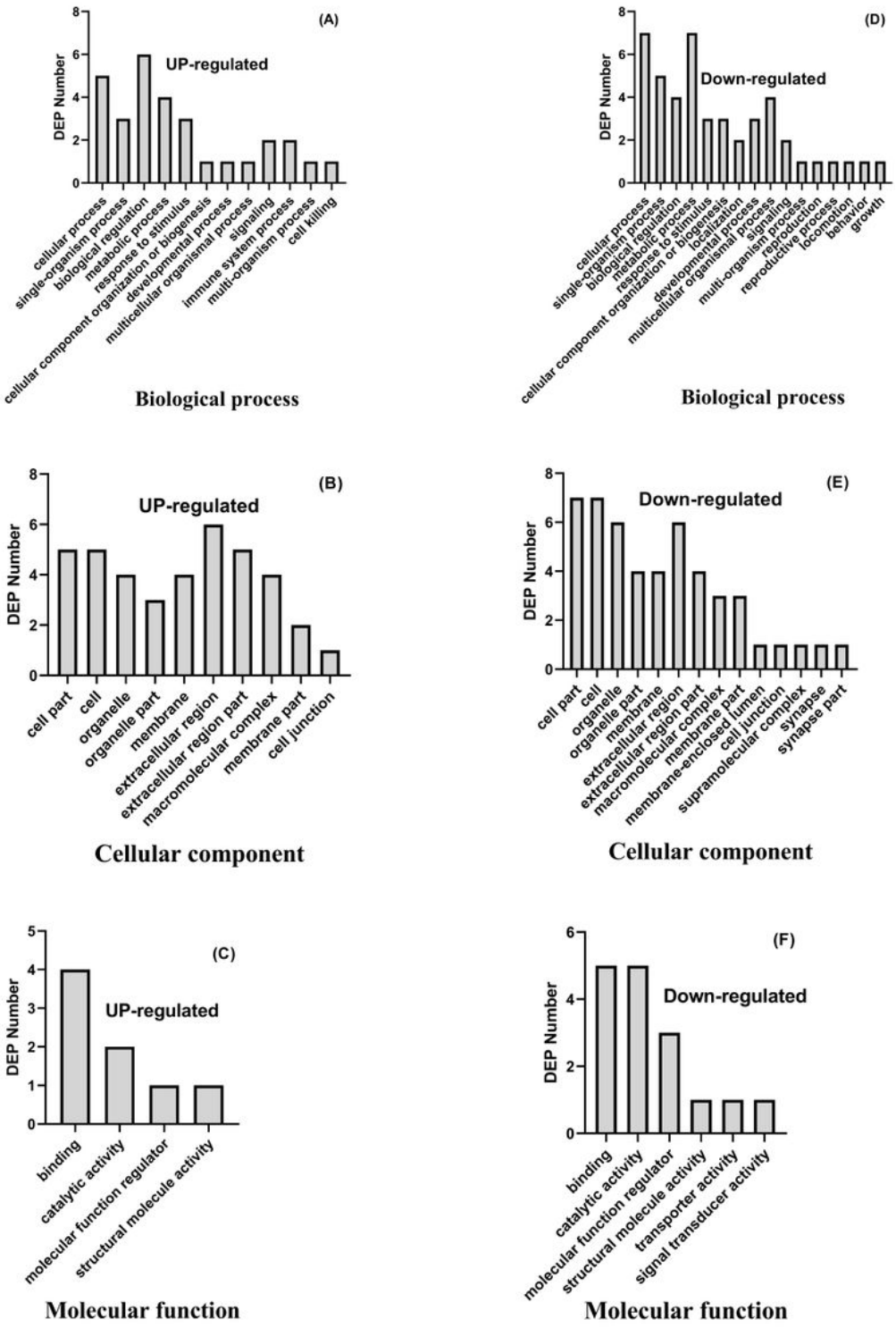

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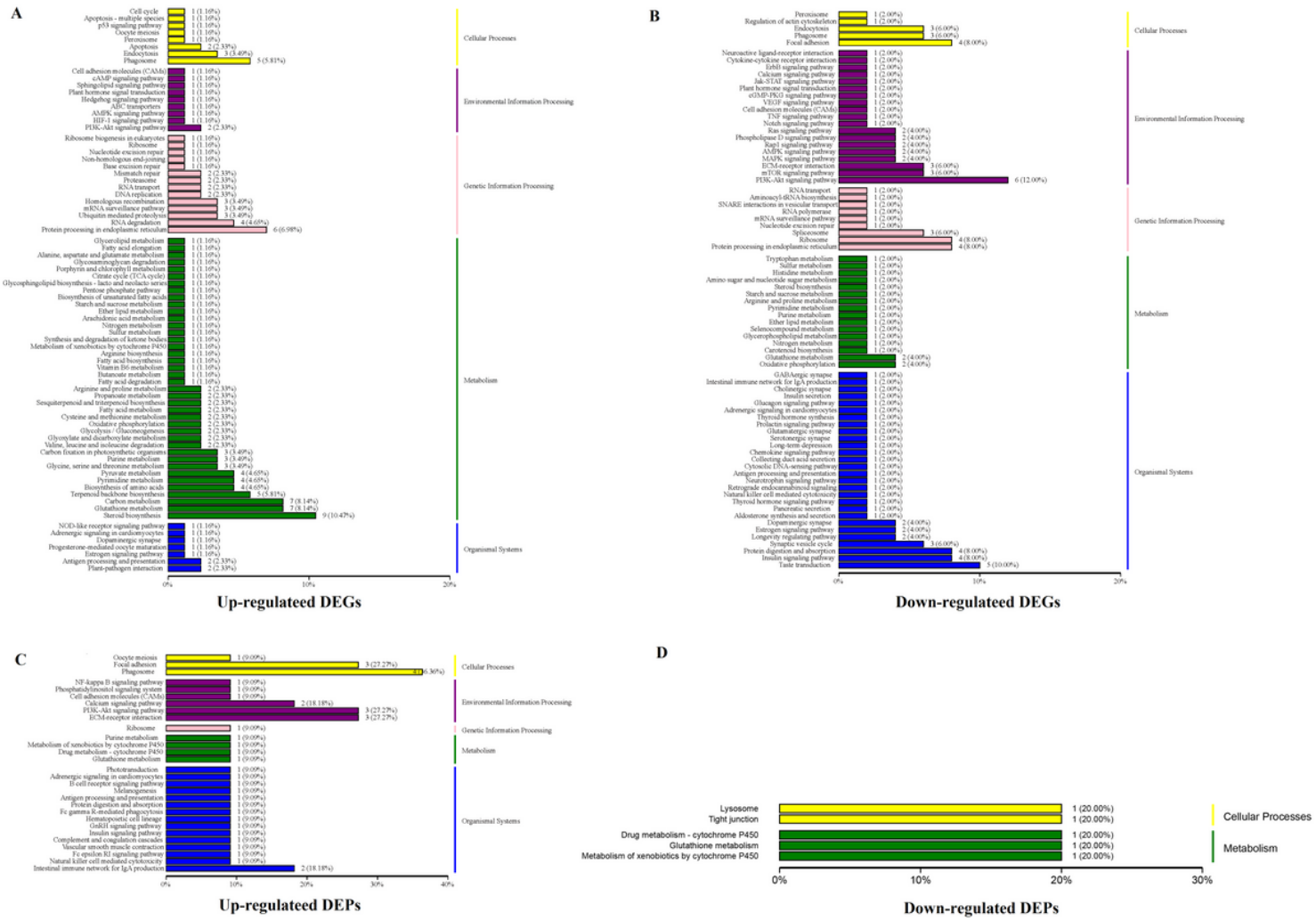
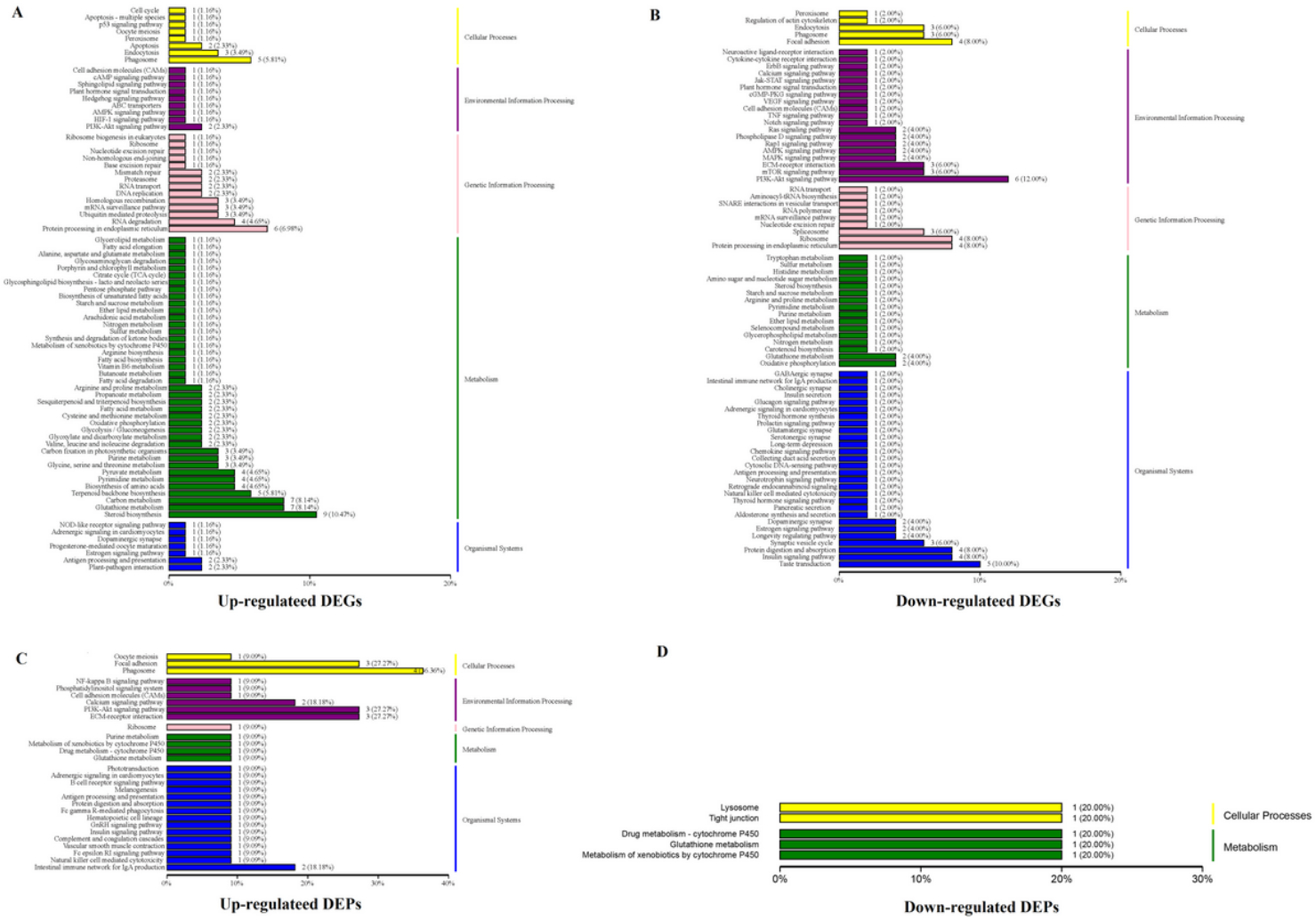


Figure 4

KEGG pathway enrichment analysis of DEGs and DEPs. The presented chart shows the distribution of DEGs (A and B) and DEPs (C and D) indicated in enriched KEGG pathway.



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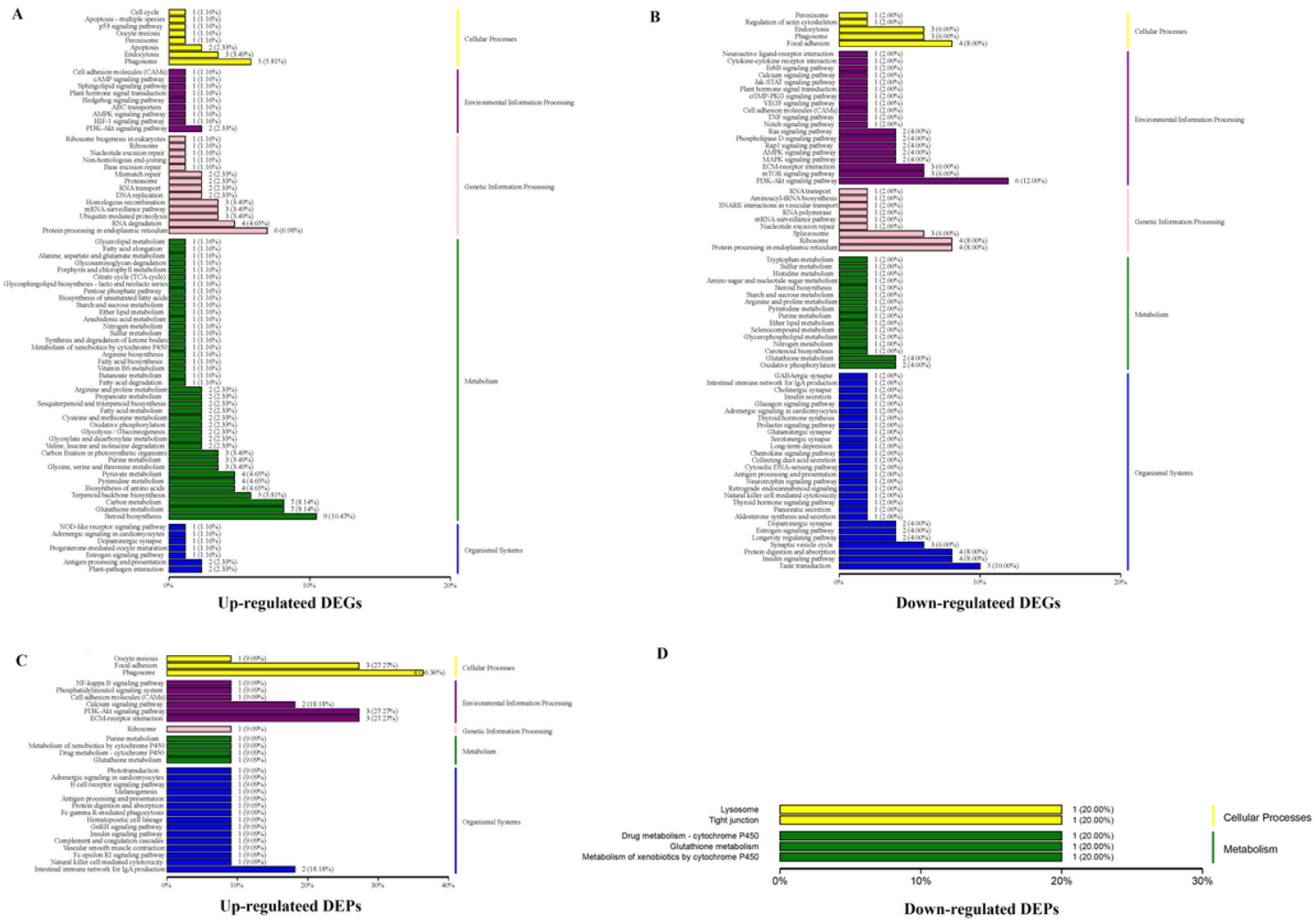

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Supplementary Files

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