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Integrative Transcriptomic and Proteomic Analysis of Ovaries at Different Physiological Periods in Dolang Sheep

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ABSTRACT

Background: The Dolang sheep is a well-known indigenous breed from Xinjiang, China. Two of its main advantages are year-round estrus and high prolificacy. Although the molecular regulatory mechanisms of reproductive processes have been studied in other animals and humans, relevant information on its year-round estrus and high prolificacy remains limited, particularly for Dolang sheep breeds from Xinjiang.

Methods: To obtain differentially expressed genes (DEGs) and proteins that might be responsible for the year-round estrus and high fecundity, ovaries from Dolang sheep were studied at different periods using an integrative strategy of transcriptomics and proteomics via high-throughput sequencing and iTRAQ technologies.

Result: RNA-seq yielded 28,717 unigenes, 987 candidate genes and 308 DEGs in the ovaries (at estrus) of Dolang sheep compared with the dioestrus and gestation period (about 45 days of pregnancy). GO and KEGG analysis revealed that the gene products were mainly involved in metabolism, hormone secretion and regulation, ovulation, estrus and regulation of reproduction. At the protein level, a total of 4847 proteins and 470 differentially expressed proteins were identified. The latter were potentially associated with metabolism, endocytosis, the glycolytic pathway and skeletal muscle growth. In conclusion, the current study provides a basis and prospective understanding of the molecular regulatory mechanism underlying the high prolificacy and year-round estrus of Dolang sheep.

Key words: Dolang sheep, iTRAQ, Next-generation RNA sequencing, Ovary.

INTRODUCTION

Sheep are important livestock within modern animal husbandry, providing meat, milk and wool for human use, fur and fiber for the textile industry, as well as being frequently used as model organism for studying comparative genomics, functional genomics and other omics (Chang *et al*., 2021). The most famous sheep breeds are seasonal breeders, that is, the ewes can only go into estrus and mate during a certain several-months period annually. Thus, seasonal breeding is a major limitation for increasing sheep fertility (Getachew *et al*., 2017; Mei and Liu, 2021; Berean *et al*., 2021). However, some sheep breeds are in year-round estrus or have a long estrus period. For example, Dolang sheep have perennial estrus and are famous for their high prolificacy (Xing *et al*., 2019). Currently, the regulation mechanism of perennial estrus in sheep remains unclear, especially with regard to the main genes involved in estrus regulation in Dolang sheep. For the last 10 years, transcriptomics have widely been applied to analyze DEGs, identify novel candidate genes, describe metabolic pathways and predict relationships between genes and target organs (Marguerat 2010; Ramayo *et al*., 2012). Furthermore, next-generation sequencing technology has provided a new and fast method to quantify and analyze gene expression on a global genomic scale. A typical research method employed within proteomics is iTRAQ, which is used in quantitative proteomics to determine the amount of proteins from different tissues or organs in a single experiment through tandem mass spectrometry (Zieske 2006). iTRAQ has the advantages of being rapid, accurate and convenient, which has been widely used to study protein expression and function in animals, including small-tailed Han sheep (Miao *et al.* 2016), swine (Hu *et al*., 2019) and humans (Lizandra *et al*., 2019).

Combining transcriptomics and proteomics could be used to analyze changes from mRNA expression to protein abundance and this approach has been increasingly used to study gene function. Furthermore, post-transcriptional regulation is very important for RNA splicing, mRNA stability, transfer, translation initiation and protein stability. Chalmel and Rolland (2015) reviewed the correlation between transcription and translation during spermatogenesis by comparing the testicular transcriptome and proteome, which provided a basis for addressing scientific questions by combining RNA-seq and MS-based proteomics. Sun *et al.* (2018) investigated protein and mRNA expression levels in

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fast and slow muscles of scallops, which revealed the regulatory mechanisms essential for maintaining muscle structure and function at the transcriptional and posttranscriptional levels. Similar methods have been employed in the investigation of indigenous Chinese Shaziling pigs (Yang *et al*., 2016) and bacterial denitrification (Zheng *et al*., 2018). Thus, combining transcriptomic and proteomic analysis provides deep insights into the underlying mechanisms of biological processes (Conrad *et al*., 2018). Dolang sheep are widely bred in the south Xinjiang, China and are famous for their high prolificacy. However, the regulation mechanisms of perennial estrus, especially in Dolang sheep, remain unclear, as there has been no adequate research into associated genes. Thus, it is necessary to combine transcriptomic and proteomic methods to analyze the high prolificacy and year-round estrus of Xinjiang Dolang sheep.

MATERIALS AND METHODS

Materials and treatment

In this study, estruses of eight healthy 3-year-old Dolang sheep were synchronized using hormonal treatment. The sheep received a CIDR vaginal plug for 13 days, followed by an injection of 400 IU Pregnant Mare Serum Gonadotropin and 0.1 g prostaglandin at the time of vaginal plug removal. Estrus occurred 48 h after vaginal plug removal. Two ewes with synchronized estruses were slaughtered on the day of their estrus, whereas two other ewes were slaughtered on the 10th day after estrus during dioestrus. The four remaining ewes were mated on the day of their estrus, followed by copulation again on the next day. Two pregnant ewes were slaughtered at the next estrus cycle after B ultrasonic monitoring around the 45th day. Thus, three groups of ewes were available for analysis: synchronized estrus, synchronized luteal phase, pregnancy, with each group comprising two ewes. Subsequently, ovaries and other reproductive organs were collected after slaughter and immediately frozen in liquid nitrogen. The experiment was completed in Xichang University from 2020-2021.

Isolation of total RNA, cDNA library construction and transcriptome sequencing

Library construction, transcriptome sequencing and analysis of sequence data were as same as Chang *et al.* (2021).

Protein preparation

Protein was extracted from the Dolang sheep ovarian tissue, according to methodology described in previous studies (Chalmel and Rolland 2015). Briefly, the ovarian tissue (0.25 g) was ground in mortar by liquid nitrogen and homogenized using Buffer A (700 mM sucrose, 50 mM Tris-HCl pH 8.0, 100 mM KCl and 2 mM EDTA). The sample was then mixed with equal volume of Buffer B (Tris-HCl pH 7.5 saturated phenols), homogenized for 3 min on ice and centrifuged at 15,000 rpm for 10 min using a high-speed low-temperature refrigerator. Protein was extracted from the upper organic phase with Buffer A and then precipitated at -20°C overnight using ice-cold Buffer C (saturated ammonium acetate in methanol, 4× volume). Protein was pelleted by centrifugation, washed three times with ice-cold Buffer C and then washed twice in ice-cold acetone. Next, solubilizing buffer (40 mM Tris-HCl, pH 8.5,7 M Urea, 4% CHAPS, 2 M Thiourea, 2 mM EDTA, 1 mM PMSF) was used to suspend the samples, followed by ultrasonic crushing in ice (pulse-on 2 s, pulse-off 3 s, power 180 W). After centrifugation at 20,000 rpm for 30 min using the high-speed low-temperature refrigerator, protein was reduced in 10 mM dithiothreitol (DTT) at 56°C for 1 h, alkylated by IAM (55 mM) in darkness for 1 h and precipitated in chilled acetone (4 × volume) at -20°C overnight, followed by centrifugation at 20,000 rpm for 30 min at 4°C again using high-speed low-temperature refrigerator. The pellet was dissolved in 400 µL of 0.5 M TEAB and sonicated on ice for 3 min. After centrifugation at 20,000 rpm for 30 min at 4°C using high-speed low-temperature refrigerator, the supernatant was collected and protein concentration was determined using the Bradford method (Deutsch *et al*., 2014).

iTRAQ labeling, SCX fraction and LC-ESI-MS/MS analysis

One hundred micrograms of each protein sample were digested using Trypsin Gold at 37°C for 16 h (protein: trypsin = 50:1). Protein peptides were then dried by vacuum centrifugation, reconstituted in 0.5 M TEAB and processed with 8-plex iTRAQ labeling reagent according to the manufacturer's instructions. Each sample was labeled with the iTRAQ tags as follows: Ovary-O1 (115), Ovary-O2 (116), Ovary-A1 (117), Ovary-A2 (118), Ovary-P1 (119) and Ovary-P2 (121). Protein peptides were then labeled using isobaric tags, incubated at room temperature for 2 h and then pooled and dried by vacuum centrifugation. SCX chromatography was performed with a LC-20ABPump system and tandem mass spectrometry (MS/MS) was carried out by Q EXACTIVE, following a previously described operational process (Yang *et al*., 2016). iTRAQ proteomics analyses were performed twice for each sample.

Proteome data analysis

The Proteome Discoverer 1.2 software was used to convert the raw data files acquired from the Orbitrap to mascot generic format (MGF) files. The MGF files were used to search against Dolang sheep proteins (https://www.ncbi.nlm.nih.gov/genome/gdv/?org=ovis-aries) by Mascot v2.3.02. To identify and quantify the proteins in Dolang sheep ovary tissue, the following parameters were used for quantification: iTRAQ 8-plex; enzyme: trypsin/P; variable modifications: dioxidation (M), oxidation (M), fixed modification: carbamidomethyl (C); iTRAQ 8-plex (N-term), iTRAQ 8-plex (K) and iTRAQ 8-plex (Y), mass values: monoisotopic; peptide mass tolerance: ± 20 ppm; fragment mass tolerance: ± 20 mmu; max missed cleavages: 1; charge states of peptides: + 2 and + 3. In order to reduce the probability of false peptide discovery, peptides with

significant scores (≥20) at 99% confidence interval from Mascot probability analysis above the identity threshold were counted as identified. Besides, each confident protein identification involved at least one unique peptide. The iTRAQ proteomics analysis was performed twice and differentially expressed proteins (DEPs) were identified if the ratio was > 1.5 in both experiments and the *p*-value <0.05, as previously described (Yang *et al*., 2016).

Functional analysis

To analyze the potential functions of identified genes and proteins, we performed functional enrichment analysis of Dolang sheep ovarian genes. Briefly, Dolang sheep ovarian genes and proteins were mapped to multiple public databases, such as Swiss-Prot/UniProt, NCBI non-redundant (NR), GO and KEGG databases. With all identified genes/proteins as background, we used the number of DEGs/DEPs to calculate the *p*-value (<0.05) and *q*-value (<0.05), which represent the significance of enriched GO/KEGG pathways of genes and proteins and FDR, respectively. The *p*-values and *q*-values were calculated by Fisher's exact test and the "*q* value" package in R (Dabney and Storey 2010), respectively.

Weighted gene co-expression network analysis

To identify co-expressed reproduction-related genes and proteins, weighted gene co-expression network analysis (WGCNA) was applied for both genes and proteins, following a protocol from a previous study (Langfelder and Horvath 2008). First, lowly expressed genes (<5 RPKM) were excluded. There was no restriction for protein data. We then transformed gene/protein expression quantities into a log2 (RPKM +1) format and calculated the correlation between samples and Hierarchical clustering analysis was performed. We performed visualization using R and the Cytoscape software. The following criteria were used to filter modules: Pearson p > 0.8 and p-value < 0.05.

One-way ANOVA was used to determine the significance of differences in expression of both genes and proteins during different physiological stages of Dolang sheep ovaries.

RESULTS AND DISCUSSION

Overview of Dolang sheep ovary transcriptome

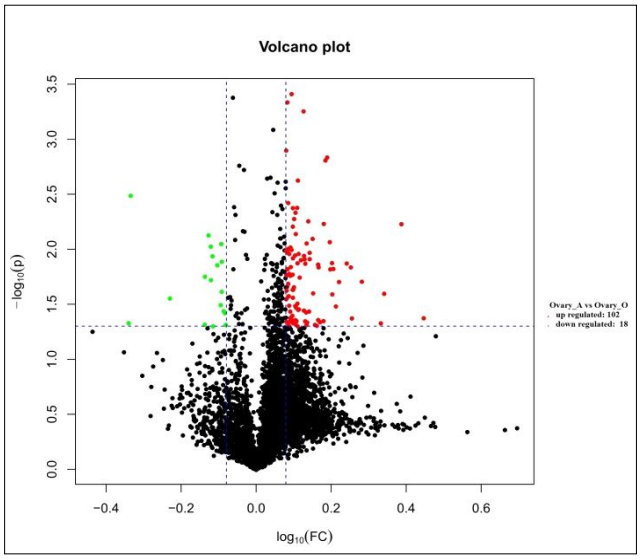
The overview of Dolang sheep ovary transcriptome was same as described by Chang *et al.* (2021).

Identification of DEPs in Dolang sheep ovaries using iTRAQ

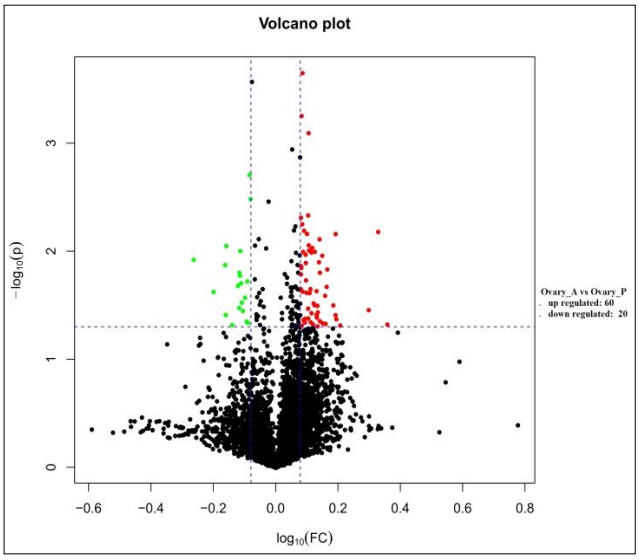
Proteomics is a recently developed technology that can be used for large-scale study of protein structure and function in complex biological samples (David *et al*., 2017). In this study, we employed iTRAQ technology, which is a relative proteomics quantitation method, to study differential protein expression changes in Dolang sheep ovaries during different physiological stages. Initially, we obtained 80,938 total peptides and 42,703 total proteins. In all, 4,845 ovarian proteins (out of 42,703) were identified, of which 470 were differentially expressed during estrus, dioestrus and pregnancy period. We first identified 102 up-regulated proteins and 18 down-regulated proteins in dioestrus compared to estrus ovaries. 60 proteins were up-regulated and 20 proteins were down-regulated in the luteal phase compared to pregnancy period ovaries. 24 proteins were up-regulated and 50 proteins were down-regulated during estrus compared to pregnancy period (Fig 1). Up-regulated proteins included follistatin-related protein 1 isoform X2, cleavage stimulation factor subunit 1 isoform X1, integrin alpha-M precursor, cyclin-Y-like protein 1 isoform X1 and others. Down-regulated proteins included histone H2B type 2-F, cyclin-Y isoform X1 and heat shock protein beta-6 isoform X1.

Through annotation of 3442 DEPs in the GO database, we found that 1924 DEPs were classified into biological processes category, 960 were classified into molecular function and 558 were classified into cellular components. Further analysis of DEPs clustered into biological processes revealed that 71 were related to the biological process, cellular process (52), metabolic process (52) and single-organism process (42), followed by organic substance metabolic process (41), primary metabolic process (41) and cellular metabolic process (37) (Fig 2A). In the molecular function categories, DEPs were primarily associated with molecular function and binding, indicating that proteins relating to molecular and binding functions experienced significant changes between physiological periods (Fig 2B). Cellular component ontology annotation revealed that a quarter of the DEPs belonged to cell parts and cell, followed by those belonging to the intracellular category (Fig 2C). Lizandra *et al.* (2019) confirmed that a major function of small extracellular vesicles (sEVs) is to promote cellular adhesion by using iTRAQ technology and western blot. iTRAQ has also been used in studies of other animals, such as rats (Zou *et al*., 2019) and canines (Wu *et al*., 2018). Thus, iTRAQ technology is quite relevant in the field of proteomics research

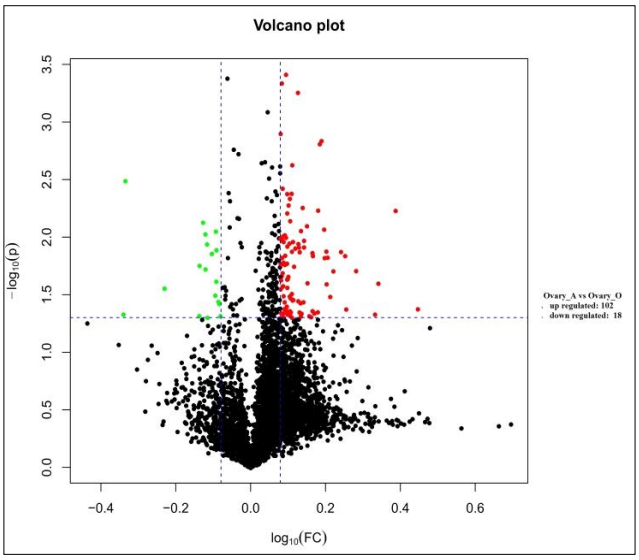
The KEGG pathway database is a powerful tool for analysis of gene and protein function within regulatory networks (Kanehisa *et al*., 2012). In the current study, in order to identify the biological pathways associated with changes in protein expression between different-stage ovaries, all functional proteins were submitted for KEGG pathway analysis. A total of 9731 proteins were assigned to 298 KEGG pathways (Fig 3), falling into 30 main categories, including Metabolic pathways (575), Endocytosis (139), Pathways in cancer (136), the PI3K-Akt signaling pathway (120), Huntington's disease (104), Focal adhesion (104), Spliceosome (104) and RNA transport (101). Among these 30 main categories, the metabolic pathways category included the largest number of proteins (575). These metabolic pathways included energy metabolism,



A: Ovary_A vs Ovary_O;



B: Ovary_A vs Ovary_P;



C: Ovary_O vs Ovary_P

Fig 1: Volcano plot of proteins expression

Each point in the figure represents a protein. The red points represent up regulated proteins (FC≥1.2); The green points represent down regulated (FC≤0.83); the black points represent no significant difference (0.83<FC<1.2). Abscissa represents the log10 logarithmic of quantitative ratio of two group samples (FC=fold change), Ordinate represents logarithm - log10 significance test *P* value. A: Ovary_O vs Ovary_A; B: Ovary_P vs Ovary_A; C: Ovary_O vs Ovary_P; Ovary-O: oestrum ovary; Ovary-A: dioestrus ovary; Ovary-p: pregnancy ovary.

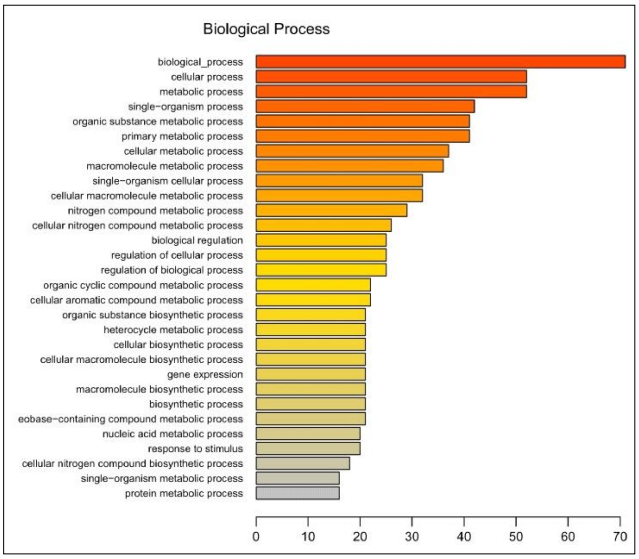
carbohydrate metabolism, lipid metabolism, intracellular respiratory metabolism and amino acid metabolism, providing valuable basis for the investigation of processes underlying high prolificacy and year-round estrus. The above results were consistent with transcriptomic analysis results.

Integration of transcriptome and proteome analysis

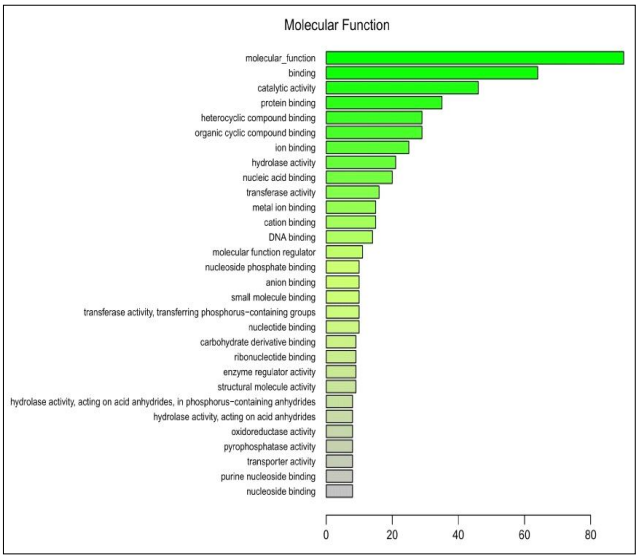
To identify pathways associated with high prolificacy and year-round estrus in Dolang sheep for both datasets, we integrated transcriptomic and proteomic data to find the corresponding genes and proteins. We believed that there is an association when a certain identified protein is also differentially expressed at the transcriptomic level. However, we identified no intersection between transcription and protein levels based on Wayne intersection analysis of the three groups in this study. Thus, the following conjoint analysis of protein levels was not possible. In the current study, transcriptomic and proteomic data seldom overlapped, which was similar to previous reports. These differences were probably caused by differential regulation of translation,

alternative splicing and database annotation errors (Hornshøj *et al.*, 2009). Yang *et al.* (2016) analyzed the growth and development of skeletal muscle in Shaziling and Yorkshire pigs using transcriptome and proteome technology, but only three genes overlapped between transcriptomic and proteomic data.

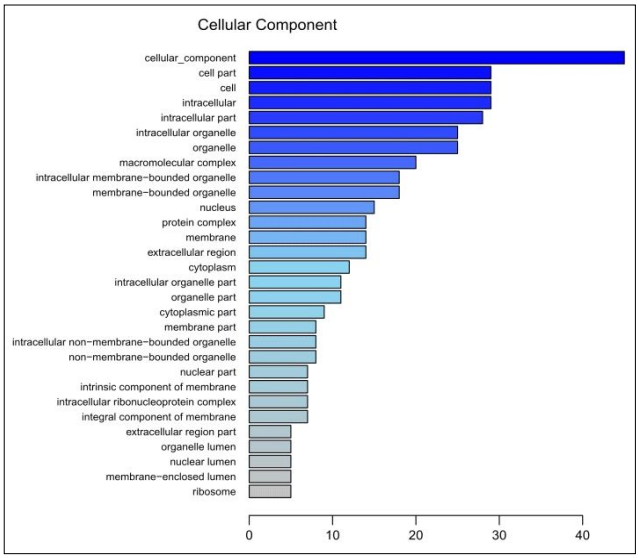
Although transcriptomics and proteomics data had no overlap, interaction GO and KEGG pathway analyses both indicated shared important biological significance (Kim *et al.*, 2010). Considering this, the differentially abundant DEGs and DEPs converging in the same metabolic pathways, especially the regulation of reproductive process, were quite relevant. We found that some processes and pathways jointly play an important role in transcription and translation, as revealed by GO and KEGG pathway correlation analysis. As revealed by GO, 239 (29.7%) genes (proteins) had important roles in the biological processes of transcription and translation, corresponding to 557 (69.3%) and 8 (1%), respectively (Fig 4A). Among the 10 main entries, the biological process (GO: 0008150) included the largest number of genes and proteins (142 up-regulated, 33 down-regulated), while cellular process (GO: 0009987) was second (98 up-regulated, 22 down-regulated), followed by single-organism process (GO: 0044699, 94 up-regulated, 19 down-regulated). Processes associated with reproduction included aromatic compound biosynthetic process (GO: 0019438, 29 up-regulated, 7 down-regulated), reproductive process (GO: 0022414, 5 up-regulated, 2 down-regulated), reproduction (GO: 0000003, 4 up-regulated, 2 down-regulated), asexual reproduction (GO: 0019954, 1 up-



A: Biological process



B: Molecular function



C: Cellular component

Fig 2: GO Function analysis.

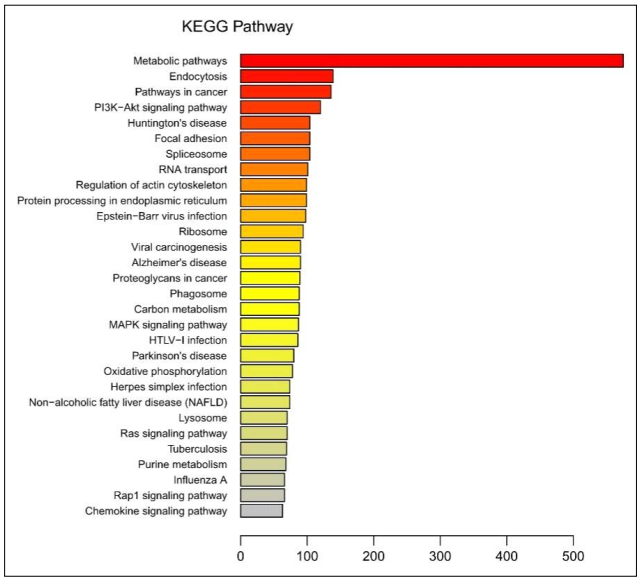
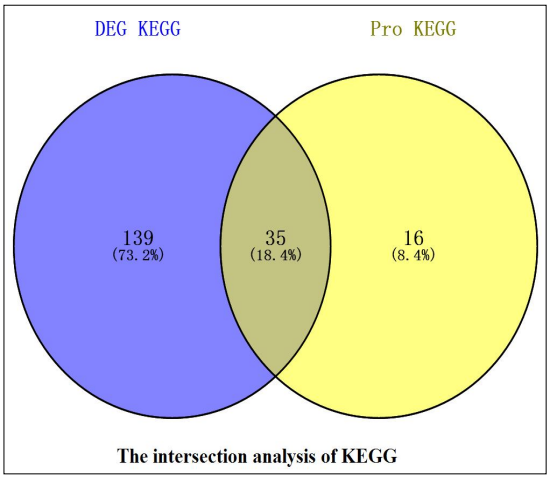
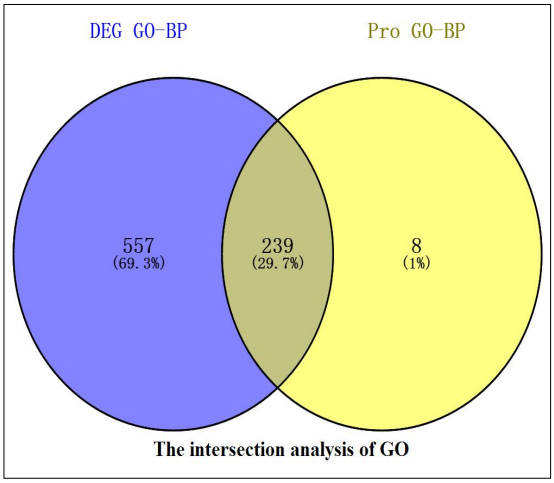


Fig 3: The KEGG pathway analysis of all functional proteins.



A: The intersection analysis of GO



B: The intersection analysis of KEGG

Fig 4: The intersection analysis of GO and KEGG pathway between DEGs and DEPs from transcriptome and proteome.

regulated, 0 down-regulated), reproduction of a single-celled organism (GO: 0032505, 1 up-regulated, 0 down-regulated) and other pathways, which were regulated at the transcriptomic and proteomic level. KEGG pathway analysis revealed 35 (18.4%) genes (proteins) that were commonly affected, as well as 139 (73.2%) genes and 16 (8.4%) proteins with important roles in pathways at different levels (Fig 4B). Among the 35 entries, Metabolic pathways (KO 01100) included the largest number of genes (25) and proteins (20), Phagosome (KO 04145, 7 genes and 8 proteins), Glutathione metabolism (KO 00480, 5 genes and 4 proteins), Calcium signaling pathway (KO 04020, 4 genes and 2 proteins), Thyroid hormone synthesis (KO 04918, 3 genes and 2 proteins), Endocrine and other factors-regulating calcium reabsorption (KO 04961, 2 genes and 2 proteins) and other pathways, which were regulated at both transcriptomic and proteomic level. In summary, various trends in DEP abundance were consistent with the DEG data, which revealed consistency between transcriptome sequencing results and iTRAQ proteomics analysis, suggesting that there exist important correlation between different omics data.

CONCLUSION

The combined use of proteomic and transcriptomic analyses was effective in identifying DEGs and DEPs. Based on the putative results of GO and KEGG pathway analyses, we found that many of the differentially abundant proteins and genes were related to reproduction or reproductive processes (ovulation, estrus and regulation of reproduction), metabolic pathways, as well as endocrine and other factor-regulated calcium reabsorption. The results of our study will provide valuable information that could contribute to a deeper understanding of the molecular mechanisms contributing to the high prolificacy and year-round estrus of Dolang sheep.

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Conflict of interest: None.

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