Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
28 (3): 327-335, 2022
DOI: 10.9775/kvfd.2022.27004

RESEARCH ARTICLE

Differential Expression of Proteins in Tibetan Sheep Ovary and Relation to Litter Size Traits at Different Follicular Development Stages

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Article ID: KVFD-2022-27004 Received: 23.12.2021 Accepted: 27.05.2022 Published Online: 29.05.2022

Abstract: Reproductive performance of Tibetan sheep is the most important factor affecting the sheep industry's production efficiency in Qinghai-Tibet Plateau. To identify differences in lambing traits of Tibetan ewes and the ovarian proteome at different stages of follicular development during the reproductive cycle, the ovarian proteome of Tibetan sheep with clear lambing records and different litter sizes were screened and analyzed using label-free mass spectrometry. The results showed that of 2508 proteins detected, there were 57 differentially expressed proteins during the anestrus season, and compared with single ewes, in twin lambing ewes expression was up-regulated in 43 proteins and down-regulated in 14 proteins; 2664 proteins, with 69 differentially expressed proteins, were detected on the 1st day of the ewe estrus period. Expression of 39 proteins in twin ewes was up-regulated and down-regulated in 30 proteins when compared with single ewes; 2704 proteins and 96 differentially expressed proteins were detected in 11th day of the ewe estrus period. Expression of 16 proteins in twin ewes was up-regulated and expression of 80 proteins was down-regulated compared with single ewes. The pathways in twin sheep that were significantly up-regulated were oxidative phosphorylation (phagosome and endocytosis), oocyte meiosis, extracellular matrix receptor interaction, and cell adhesion. The amino acids (lysine, valine, leucine, isoleucine) and fatty acid degradation, TGF-β signal and GnRH signal pathways were significantly down-regulated in twin sheep. GDF9, BMPR-1B, MTHFR and Cu/Zn-SOD were relatively active in different stages of follicular development during the reproductive cycle. This study contributes to the research of twin production in Tibetan sheep, the in-depth study of Tibetan sheep fertility, and promotes the development of animal husbandry in the Qinghai-Tibet Plateau.

Keywords: *Tibetan sheep, Twin trait, Reproductive cycles, Follicular development, Label-free Mass Spectrometry*

Tibet Koyun Ovaryumlarında Proteinlerin Diferansiyel Ekspresyonu ve Farklı Foliküler Gelişim Aşamalarında Batın Büyüklüğü Özellikleriyle İlişkisi

Öz: Tibet koyunlarının üreme performansı, Qinghai-Tibet Platosu'ndaki koyun endüstrisinin üretim verimliliğini etkileyen en önemli faktördür. Üreme döngüsü sırasında foliküler gelişimin farklı aşamalarında bulunan Tibet koyunlarının kuzulama özelliklerindeki ve yumurtalık proteomundaki farklılıkları belirlemek için net kuzulama kayıtları ve farklı batın büyüklükleri olan yumurtalık proteomları, etiketsiz kütle spektrometrisi kullanılarak tarandı ve analiz edildi. Sonuçlar, tespit edilen 2508 proteinden, anöstrus sezonu boyunca 57'sinin farklı şekilde eksprese edildiğini ve tek kuzu doğuran koyunlarla karşılaştırıldığında, ikiz kuzulayanlarda 43 proteinin daha fazla ve 14 proteinin daha az eksprese edildiğini gösterdi. Koyunlarda östrus periyodunun 1. gününde farklı şekilde eksprese edilmiş 69 protein dahil 2664 protein tespit edildi. Tek kuzu doğuran koyunlarla karşılaştırıldığında ikiz kuzulayan koyunlarda 39 protein daha fazla eksprese edilirken, 30 protein daha az eksprese edildi ve östrusun 11. gününde 96'sı farklı şekilde eksprese edilmiş 2704 protein tespit edildi. Tek kuzu doğuran koyunlarla karşılaştırıldığında, ikiz kuzulayan koyunlarda 16 proteinin ekspresyonu daha fazla iken, 80 proteinin ekspresyonu ise daha azdı. İkiz kuzulayan koyunlarda önemli ölçüde fazla eksprese edilen yolaklar, oksidatif fosforilasyon (fagozom ve endositoz), oosit mayozu, hücre dışı matris reseptör etkileşimi ve hücre adezyonuydu. Amino asitler (lizin, valin, lösin, izolösin) ve yağ asidi yıkımı, TGF-β sinyali ve GnRH sinyal yolaklarının ikiz kuzulayan koyunlarda önemli ölçüde ekspresyonu azalmıştı. GDF9, BMPR-1B, MTHFR ve Cu/Zn-SOD, üreme döngüsü sırasında foliküler gelişimin farklı aşamalarında nispeten aktifti. Bu çalışma, Tibet koyunlarında ikizlik ve doğurganlığının derinlemesine incelenmesine katkıda bulunmakta ve Qinghai-Tibet Platosu'nda hayvancılığın gelişimini teşvik etmektedir.

Anahtar sözcükler: *Tibet koyunu, İkizlik, Üreme döngüleri, Foliküler gelişim, Etiketsiz Kütle Spektrometrisi*

How to cite this article?

Cui Y, Zhang L, Li J, Chen Q, Ren L, Jia J: Differential expression of proteins in Tibetan sheep ovary and relation to litter size traits at different follicular development stages. *Kafkas Univ Vet Fak Derg,* 28 (3): 327-335, 2022.
DOI: 10.9775/kvfd.2022.27004

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Introduction

Tibetan sheep is the most common breed in Qinghai province where sheep cultivation is an important industry [1]. With the development of modern animal husbandry, improving lambing rate and reducing stocking density, while increasing economic benefits is occurring in Qinghai province's sheep cultivation industry. The ideal lambing performance of ewes is to produce twins; however, Tibetan sheep have a low reproductive rate with a twin rate of approximately 5% [2]. Sheep reproductive traits are usually regulated by many related genes in complex mechanisms. Improving lambing rate and reducing feed and management costs are an important area of research in Tibetan sheep production.

In recent years, the inherent reproductive performance of Tibetan sheep can no longer meet the current production needs as the sheep farming industry in Qinghai Province, the main production area in China, has grown rapidly. How to improve sheep reproductive performance has been the main puzzle of sheep industry in Qinghai and even the whole country for a long time. Over the past decade, many studies have shown that polymorphisms of fecundity genes, BMPR-1B, BMP15, and GDF9, would affect amino acids of the translated protein, increase ovulation rate, and ultimately affect the lambing characteristics of specific breeds [3-5]. Some studies have indicated that BMPR-1B, one of the oocyte-derived members of transforming growth factor-β family, has played an imperative role in follicular growth and ovulation [6-8]. Mutation at locus 746 in the coding region of the BMPR-1B gene (FecB mutation) increases the signal strength to downstream receptors during BMPR-1B signaling, promotes steroid production, alters SMAD expression and phosphorylation status, accelerates follicle maturation, and causes an additive effect on ovulation number in sheep [9]. During follicle development, the BMP system increased average ovulation in Booroola Merino sheep, Garole sheep, and Hu sheep [10]. The reproductive performance of sheep is not only controlled by small effects of multiple genes but is also affected and regulated by one or more quantitative trait loci (QTL). It has a non-additive dominant gene action mode, and its heritability (H^2) is very low. The heritability of sheep double lambs is only 0.126 [11].

Mammalian follicle development and ovulation are regulated by the downstream action of GnRH in the hypothalamus and FSH and LH in pituitary gonadotropins on follicular granulosa cells and/or membrane cells, as well as intraovarian effects of growth factors and steroids [12]. Research confirmed that various intraovarian factors could control the release of these gonadotropin hormones (FSH and LH) and extraovarian feedback effects on hypothalamic and/or pituitary gonadotropins. Genes affecting the development and control of the reproductive axis are potential candidate genes for twin production [13]. From long-term studies and observations of animal twins, it is clear that twins are mainly controlled by genetic factors. There are great differences in the rate of twin lambs among different sheep breeds, and the traits of twin lambs are greatly affected by their parents. In addition, they are also affected by parity, season and nutrition [14,15]. Therefore, the most effective way to improve the rate of sheep production is to further our understanding of the genetic basis of twin production in Tibetan sheep, find the main genes and molecular markers of twin production, and carry out molecular breeding. Here, we identified and analyzed different proteins in ovarian tissue of Tibetan ewes, with a history of producing single and twin ewes, at different stages of follicular development using label-free mass spectrometry and identified signal pathways and molecular mechanisms that have an impact on twin production in Tibetan sheep.

Material and Methods

Approval was granted by the Animal Care Committee of Qilu Normal University, China, for all the animal related protocols (QLNU-2019-10-0014). Furthermore, for slaughtering the animals, approval was granted by the National Administration of Mutton Sheep Slaughtering and Quarantine (Qinghai, China, 20200407).

Study Location

This work was carried out in the Hainan Tibetan Autonomous Prefecture, Qinghai Province, China, located at an altitude of more than 3200 meters from sea level in southeastern Qinghai-Tibetan Plateau, which has a desiccated cool climate.

Animals and Sample Collection

Twenty-four healthy Tibetan ewes (3-4 years old, 2 lambing records) from Wayu town, Qinghai province, China, were chosen. Twelve were sheep that produced twins (T group) and 12 produced singletons (S group). All experimental sheep were healthy, free of reproductive diseases, and without hormone treatment. All ewes were not pregnant. The experimental sheep were slaughtered during estrus (October 2020, Day1 and Day 11 of estrus cycle) and anestrus (April 2020), respectively. Three ewes each of different lambing types slaughtered at each stage. The reproductive state of the ewes (estrus) was identified based on the combination of ram test and vulvar observation. The ewes were deemed in estrus when they accepted mounting by the rams aged 2 to 3 years with high serving capacity as teasers with canvas apron conducted by leash. Experimental ewes were killed by bloodletting via the carotid artery at 7 a.m. before feeding and watering. The ovaries of Tibetan ewes were collected in accordance with

approved guidelines [16], and ovary samples were peeled and washed to remove all surface fat and ligaments. The ovaries were then transferred to sterile plastic tubes and stored at -80°C.

Ovary Samples Proteomics Collection and Measurement

The surface contaminants from the Tibetan sheep ovary were washed away with the help of phosphate buffer saline solution before their transfer to 1.5 mL centrifugal tubes. Prior to proteomics analysis, samples were kept at -80°C. Proteomics analysis was carried out by SMBPT Co., Ltd (China, Shanghai). Each group had 3 replicates. First, the lysis buffer method was used for protein extraction [17]. Next, 250 μg of protein was digested by complying with the described FASP procedure. Q Exactive TM mass spectrometer and Easy nLC system (Thermo Fisher Scientific, MA, USA) was used to perform label-free mass spectrometry (MS). For the analysis of MS data, we used Max Quant software while the UniProt database *(Ovis aries https://www.uniprot.org/uniprot/?query=Ovis+aries&sort=score)* was accessed for reference. Relative quantitative real-time PCR (qPCR) was performed to determine the copy number of target genes. RT-PCR system was 20 μL: 2×PerfectStart@Green qPCR SuperMix 10 μL, 1 μL of primers (0.5 μL each for F primer and R primer), 1.5 μL of 50 mg/μL template cDNA, and RNase-free ddH2O 8 μL. Reaction conditions: pre-denaturation 94°C for 30 s; denaturation 94°C for 5 s, annealing 60°C for 30 s, 72°C for 40 cycles. The primers were synthesized at Shanghai Biological Engineering Ltd., China *(Table 1)*.

Statistical Analysis

Differential proteomics analysis was conducted by Applied Protein Technology (Shanghai, China). 5800 MALDI-TOF/TOF (AB Sciex, USA) was used for mass spectrometer (MS) data analyses. For quantitative analysis, a protein must have at minimum of one unique peptide match with the MS ratios. A ≥1.5 or ≤1.5-fold cutoff value was used to identify up-regulated and down-regulated proteins with a P-value <0.05. We passed all the identified proteins through the Blast2GO program for functional annotation as well as classification against the UniProt database which contains cell components along with the biological process and molecular function. Furthermore, we applied the search pathway tool from the KEGG Mapper platform. Fisher's exact test was used to test for significant pathway enrichment. Pathways that had an adjusted P value of <0.05 were deemed significant. DPEs were used for cluster analysis. GeneMANIA was used to predict both physical, as well as functional, interactions among genes/proteins. Finally, the PPIs were analyzed.

Table 1. *Primer sequence of target genes*

Gene Name	Primer Sequences (5'-3')
GDF9	F: ACTGAATGAATAGGGTGTTG R: ATCTGTACCATATCTAAGTCC
BMPR1B	F: CCGCTCGAGAACATGCTTTTGCGAAGTTCAG R: CGCGCGATCCCAGAGCTTAATGTCCGGGACT
MTHFR-P1	F: AAGCTGCGTGATGATGAAATCG R: CTCCCGCAGACACCTTCTCC
MTHFR-P2	F: AACGAAGACTTCAAAGACACTT R: CTTCACTGGTCAGCTCCTCCCC
Cu/Zn-SOD	F: CTCTGCGGCATTATCACA R: GGAAAAGCCATAGAAGGT

Results

Changes in Proteome Profiles During Anestrus and Estrus Season

Single and twins producing Tibetan ewes of similar age exhibit differences in protein composition of ovarian tissue during different stages of follicular development. 2508 proteins and 57 differential proteins were detected during the anestrus season. In twin ewes expression of 43 proteins were up-regulated and 14 proteins were down-regulated compared with single ewes; 2664 proteins, with 69 differentially expressed proteins, were detected in the 1st day of ewe estrus period. In twin ewes 39 proteins were up-regulated and 30 proteins were down-regulated compared with single ewes; 2704 proteins and 96 differentially expressed proteins were detected during the 11th day of ewe estrus *(Fig. 1)*. The expression of 16 proteins in twin ewes were up-regulated and 80 proteins were down-regulated compared with single ewes. On the 11th day of the ewe estrus period, there were significantly more differentially expressed protein compared to during the ewe anestrus season and the 1st day of ewe estrus period, indicating that on the 11th day of estrus *(Fig. 2)*. There was an increase in ovarian activity. The protein expression of GDF9, BMPR-1B and MTHFR in twin producing ewes was lower than that of single lamb producing ewes during estrus period, but the expression trend of these genes during estrus period was opposite to that of the anestrus season.; The expression trend of Cu/Zn-SOD was opposite on the 1st day and 11th day of the ewe's estrus period.

Gene Ontology (GO) Analyses

To explore biological functions associated with differentially expressed proteins, enrichment analysis in the Gene Ontology (GO) was used *(Fig. 3)*. Using GO, we identified cellular components, molecular function and biological process. There were 21 terms in cellular components, which were mainly related to the following terms: enriched in cytoplasm, cytoplasmic matrix, nucleus and calcium complex. A total of 31 molecular function terms were enriched. These included ATP binding, signal transduction, calcium binding, oxygen transport activity, growth factor activity, DNA binding and carbohydrate binding. There

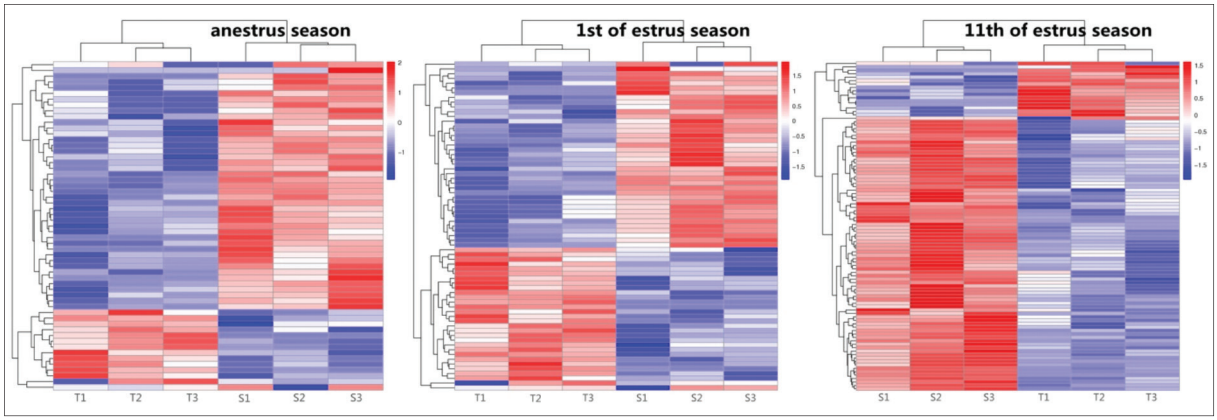

Fig 1. Differentially expressed proteins in three follicular development stage of Tibetan ewes. Three replicates for the double lambing group (T) and three replicates for the single lambing group (S). The image presents the relative abundance of proteins using different colors, where deeper red represents higher intensity and blue represents lower intensity

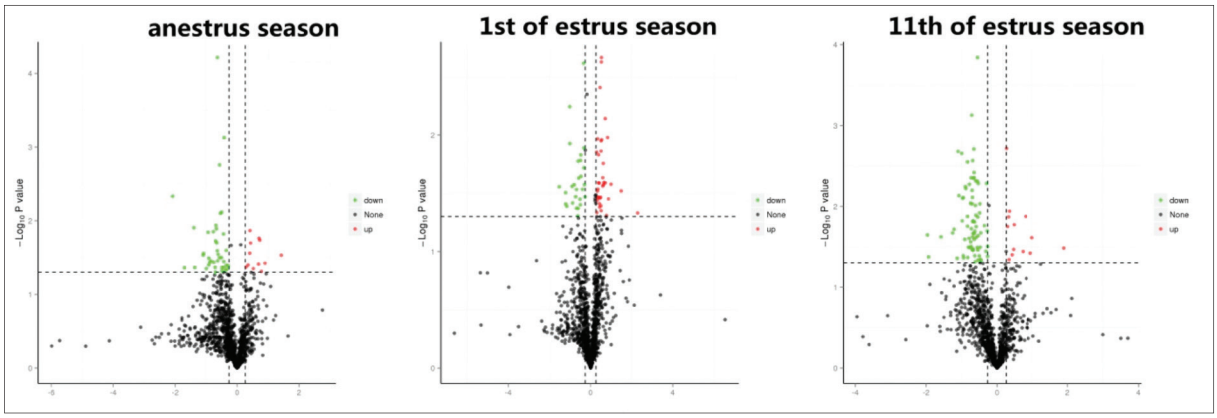

Fig 2. Differentially Expressed Proteins (DEPs) Cluster Evaluation in three follicular development stage of Tibetan ewes. Down was the DEPs of twins lambing group (T) were less than single lambing group (S); Up was the DEPs of twins lambing group (T) were more than single lambing group (S); None was the DEPs of twins lambing group (T) were no significant differences than single lambing group (S)

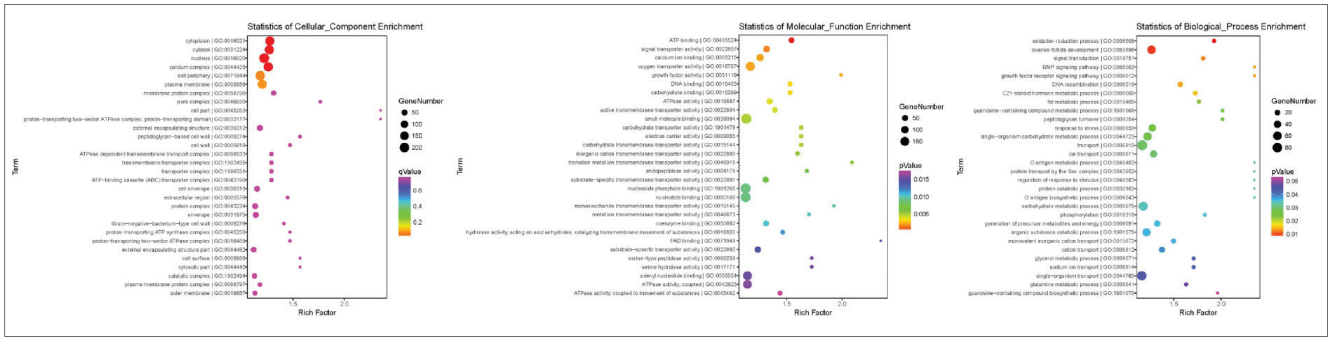

Fig 3. Differentially Expressed Proteins (DEPs) GO Evaluation in Tibetan ewes

were 35 enriched terms related to biological processes. These include oxidative phosphorylation, oocyte meiosis, signal transduction, BMP signal pathway, growth factor receptor signal pathway, C21 steroid hormone metabolism and fatty acids β oxidation process. Enriched biological processes in twin producing sheep are ovarian specific proteins including oxidative phosphorylation, oocyte meiosis, signal transduction and C21 steroid hormone metabolism which were all were up-regulated. These accounted for about 54% of the total number of up-regulated proteins in the ovary. Some proteins related to transcription regulation, protein translation and protein modification. These proteins were also up-regulated (accounting for 7% of the total up-regulated proteins); proteins related to intracellular pyrimidine synthesis accounted for about 4% of the up-regulated proteins. The

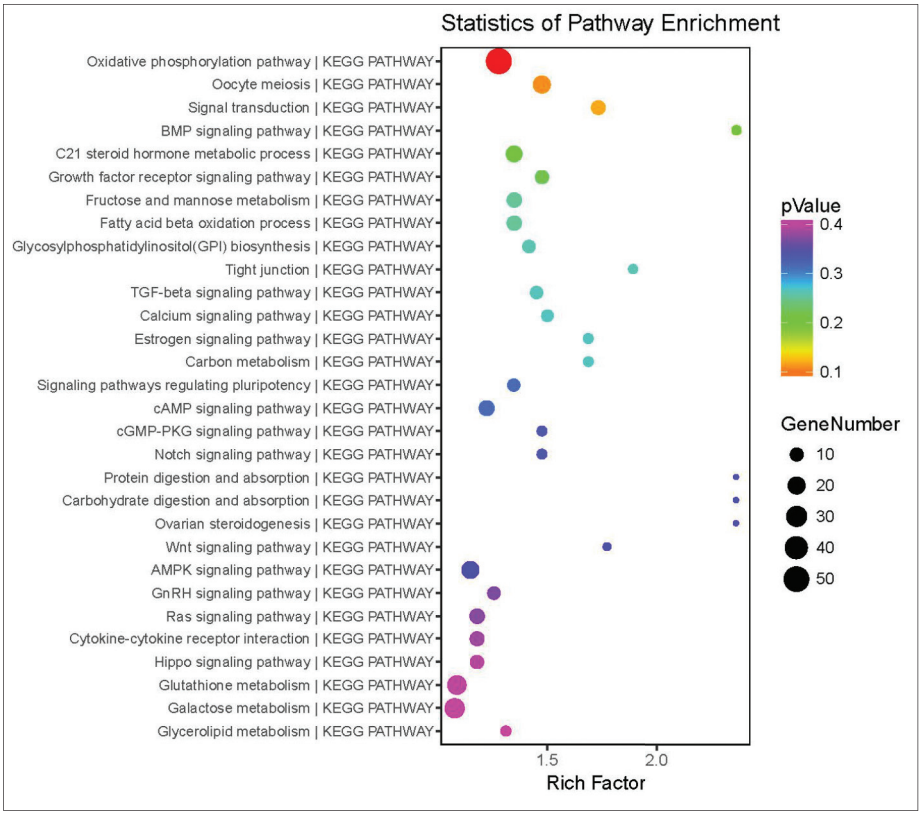

Fig 4. Differentially Expressed Proteins (DEPs) KEGG Evaluation in Tibetan ewes

synthesis, processing and transportation rate of protein in the ovaries of twin producing sheep were all accelerated, intracellular and extracellular signal transduction were enhanced, and the level of anaerobic respiration and metabolism was improved. BMP signaling and growth factor receptor signaling pathways were down-regulated, accounting for 26% of the total down-regulated proteins in the ovary. Fatty acids, β Oxidation, lipid, and small molecule transport were also partially down-regulated (accounting for 13% of the down-regulated proteins), indicating that the rate of transport and metabolism of small organic molecules such as amino acids and biotin decreased in the cells of twin producing sheep, and the aerobic respiration in the ovarian cells decreased.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Analyses

The major pathways associated with differentially expressed proteins were identified using KEGG pathway analysis *(Fig. 4)*. A total of 99 differentially expressed proteins across 63 pathways were significantly enriched ($P<0.05$). Of these, 34.6% of the up-regulated protein and 48.3% of the down-regulated protein were enriched. The significantly up-regulated pathways in twin producing sheep were oxidative phosphorylation (phagosome and endocytosis), oocyte meiosis, extracellular matrix receptor interaction, and cell adhesion, indicating that the activity of ovarian cells in twin producing Tibetan sheep was increased and the material exchange between cells was more frequent. Amino acid (lysine, valine, leucine, isoleucine) and fatty acid degradation, TGF-β signalling pathway and GnRH signalling pathway were significantly down-regulated in twin producing sheep. The degradation of related amino acids synthesizing Smads family proteins was significantly inhibited in the ovarian cells of twin producing sheep. Intracellular metabolic efficiency decreased, and the degradation process of amino acids and fatty acids appeared.

Protein-Protein Interaction Network (PPI Network) Analyses

Protein networks among the 99 proteins were analyzed via STRING *https://string-db.org/cgi/input.pl (Fig. 5)*. Among the screened up-regulated differential proteins, ribosomal proteins interacted with glycolysis related proteins. Among the down-regulated differential proteins, lipid metabolism related proteins interacted. These differentially expressed proteins created a complex network of interactions. Interestingly, GDF9, BMPR-1B, MTHFR and Cu/Zn-SOD were at the intersection of this interaction network, indicating that they were relatively active during different stages of follicular development. Protein expression of GDF9, BMPR-1B and MTHFR in twin producing sheep was higher than that of single lamb producing sheep during anestrus season, but the expression trend is opposite during estrous season. Expression trends of Cu/Zn-SOD were opposite during the 1st day of anestrus, estrus and during the 11th day of estrus.

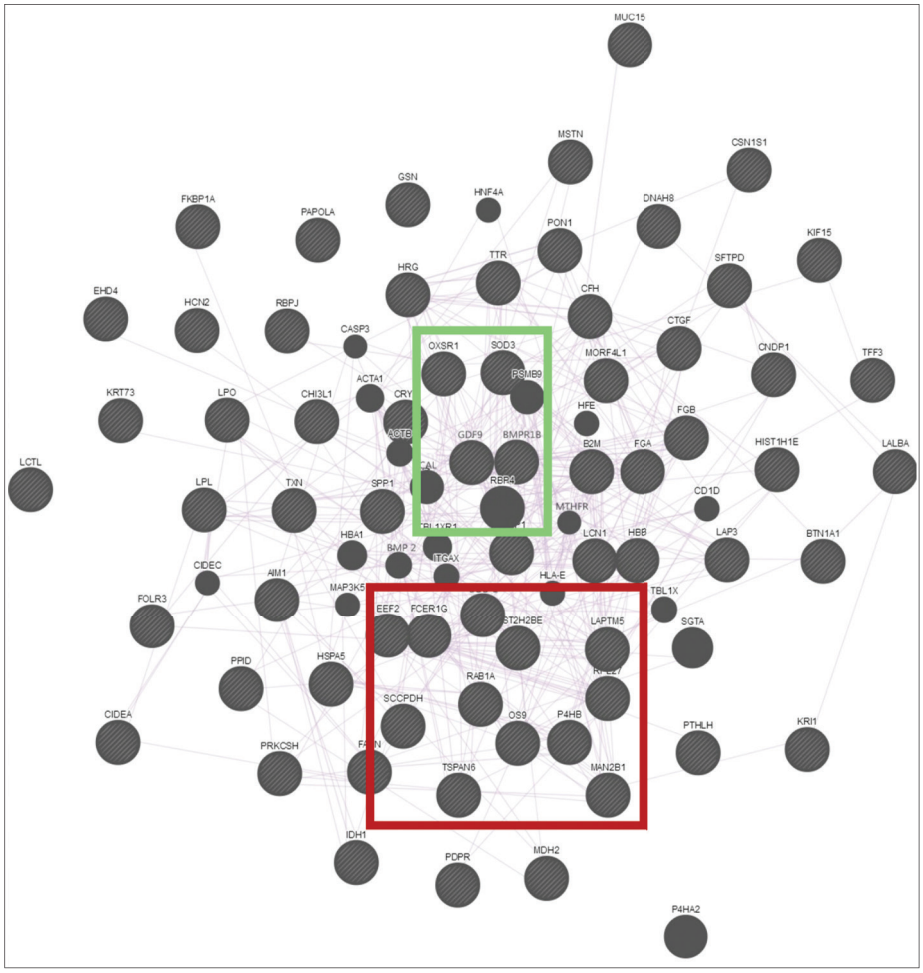

Fig 5. Differentially Expressed Proteins (DEPs) PPIs Evaluation in two follicular development stage of Tibetan ewes. The green zone was the differentially expressed proteins in twins lambing sheep higher than that in single lambing sheep; The red zone was the differentially expressed proteins in twins lambing sheep lower than that in single lambing sheep

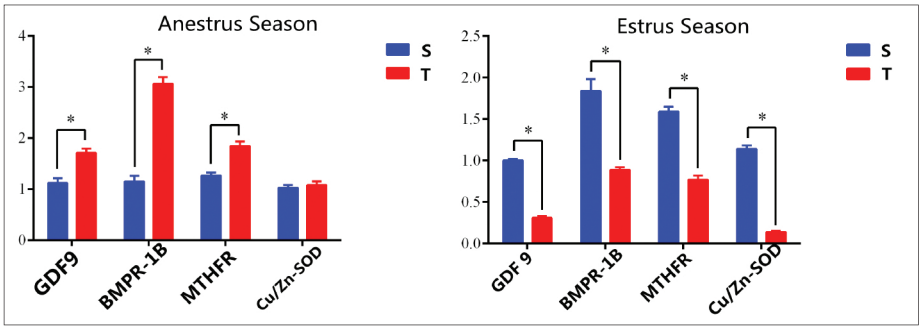

Fig 6. Effects of different litter size on the differentially expressed proteins coding genes in Tibetan sheep ovary in aneustrus and estrus seasons

Validation of Differentially Expressed Proteins Coding Genes by qPCR

The gene expression of GDF9, BMPR-1B and MTHFR in twins trait sheep was higher than that in single trait sheep at anestrus season, but the expression trend is opposite at estrous season ($P<0.05$) via RT-PCR, however, the gene expression of Cu/Zn-SOD were opposite at the 1st of anestrus, estrus and at 11th of estrus ($P<0.05$). In summary, the results of selected differentially expressed proteins coding genes by RT-PCR were the same expression tendency of the label-free analysis *(Fig. 6)*. The RT-PCR assay illustrated that the label-free results were reliable for further analyses.

Discussion

Tibetan sheep production has been the main revenue source of Tibet plateau residents. Sheep reproductive performance is economically important for the sheep industry and plays a key role in the development of the sheep industry [18]. Sheep fertility has complex molecular regulatory mechanisms and the regulatory mechanism controlling sheep reproductive performance is an exciting research area. At present, research on lambing traits of sheep at home and abroad mainly focuses on gene polymorphism [19,20]. There are few reports on sheep proteins from the perspective of proteomics. Studies show that molecular regulation of tissues and cells depends on many

cytokines and catalytic enzymes. Most cytokines and catalytic enzymes are essentially proteins [21]. Therefore, proteins drive cellular activities. Twin production in sheep is related to many factors including the development of ovarian follicles and ovulation frequency. Seasonal estrus, which results from changes in both ovarian function and hormonal secretions during the different seasons, is a critical factor limiting sheep fecundity and productivity [22]. Expression regulation in sheep ovarian tissue is important for understanding follicular development and ovulation regulation. The ovulation number of sheep controls lambing traits, and the ovary is the main site of follicular development and ovulation in animals. In the ovary, the morphology and function of the follicles change continuously with the different stages of development, during which the cells need to synthesize sufficient amounts of biomolecules to ensure normal cell proliferation and differentiation [23,24]. This may be an important physiological mechanism, explaining differences of the ovarian proteome between twin- and singleton-producing Tibetan ewes during different stages of follicular development.

Follicular development depends on the mutual regulation of oocytes, surrounding granulosa cells, and theca cells. The initiation of primordial follicle development is related to various factors secreted inside and outside the follicle, and neurotransmitters or signal transmission between oocyte granulosa cells [25]. Basic fibroblast growth factor, stem cell factor, leukemia inhibitory factor, nerve growth factor, bone morphogenetic protein, insulin and keratinocyte growth factor can induce the initial recruitment of primordial follicles and develop into mature follicles [26]. The ovarian proteome of Tibetan sheep with recorded differences in twin production were screened and analyzed by label-free Mass Spectrometry at different stages of follicular development. Results showed that of 2706 proteins 99 were differentially expressed. Of 2508 proteins, 57 were differentially expressed during ewe anestrus. Expression of 43 proteins in twin-producing ewes was up-regulated and expression of 14 proteins was down-regulated compared with single ewes; 2664 proteins and 69 differentially expressed proteins were detected in the 1st day of ewe estrus period. Expression of 39 proteins was up-regulated and expression of 30 proteins was down-regulated in twin-producing ewes compared with single ewes. On the 11th day of ewe estrus, 2704 proteins and 96 differentially expressed proteins were detected. The expression of 16 proteins in twin-producing ewes was up-regulated while expression of 80 proteins was down-regulated compared with single ewes. Differentially expressed proteins in twin-producing sheep were related to oxidative phosphorylation, follicular growth and development, signal transduction, growth factor receptor signal pathway, fatty acid degradation, tricarboxylic acid cycle, lipid metabolism, BMP signal pathway, TGF-β signaling pathway, metabolism of C21 steroids, and fatty acids β. Expression decreased significantly during oxidation. GDF9, BMPR-1B, MTHFR and Cu/Zn-SOD are relatively active during different stages of follicular development in ewe reproductive cycle. Our study showed that the protein expression of GDF9, BMPR-1B and MTHFR in twin-producing sheep was higher than in singleton-producing sheep during anestrus season. The expression trend is opposite during estrous season. Expression trends of Cu/Zn-SOD were opposite at the 1st of anestrus, estrus and at 11th of estrus. BMPR-1B, a member of BMPs, is a major gene affecting ovine ovulation rate, and plays a pivotal role in follicle development and litter size [27]. During anestrus, ovarian physiological activity is reduced with the follicular stage not developing. During the selection of the dominant follicle, the expression of BMPR-1B protein in the follicular granule cells declined followed by an increase in follicle size. Granular cells in atretic cells continued to demonstrate high expression of BMPR-1B mRNA [28]. Studies of the FecB gene have shown that the signaling pathway in FecB mutant ewes leads to increased intensity of signaling to downstream receptors during follicular development, promotes steroid production, and alters SMAD expression and phosphorylation status, leading to reduced follicular granulosa cell apoptosis, early follicular maturation, and increased ovulation numbers [29]. This is the same result as the enrichment of the KEGG pathway. cAMP, as the second messenger of hormones, activates protein kinase, enhances the activity of metabolic enzymes, strengthens the synthesis of proteins *in vivo*, and induces hormones (such as growth hormone, follicle stimulating hormone, etc.) or enzyme synthesis to promote the body's anabolism. In the ovary, follicle stimulating hormone increases the synthesis of estradiol and progesterone through the cAMP pathway [30,31]. Growth differentiation factor 9 (GDF9) belongs to the growth differentiation factor-β superfamily, which is only expressed in ovaries or oocytes, and has an important impact on follicular growth and development, and reproductive function [32]. GDF9 is an oocyte derived growth factor that affects ovine ovulation in a dose-dependent manner and is expressed at all stages of ovine follicular development. GDF9 can affect the distribution of organelles in oocytes, the integrity of the zona pellucida, stimulate granulosa cell proliferation, inhibit the differentiation of granulosa cells induced by FSH, regulate the expansion of cumulus, stimulate the formation of follicular membrane, and affect the synthesis of hormones in ovary [33]. A mutation in the methylenetetrahydrofolate reductase (MTHFR) gene will lead to a decrease in folate metabolism, DNA methylation, and increase concentration of homocysteine in plasma, resulting in disorders of biological processes such as cell

cycle regulation, DNA replication, and DNA and protein modification [34]. Superoxide dismutase (SOD) is an important biological antioxidant enzyme and plays a very important role in the immune system of organisms. Cu/Zn-SOD can inhibit the mitosis and meiotic maturation of oocytes [35]. Transgelin in the ovary will prevent the formation of ara54 dimers, thus blocking the combination of androgen receptors and ara54, resulting in the retention of androgen receptors in the cytoplasm, changing the transmission pathway of downstream signals, and then affecting the reproductive performance of animals [36]. The regulation and expression of GDF9, BMPR-1B, MTHFR and Cu/Zn-SOD were significantly different in ovarian tissues of Tibetan sheep of different lambing types. The regulation of these differentially expressed proteins and their protein pathways can significantly affect follicle development and ovulation, and a more in-depth study of this as a candidate target protein for Tibetan sheep double lambing can effectively elucidate the intrinsic molecular mechanisms of Tibetan sheep double lambing traits and provide a basis for subsequent studies of Tibetan sheep double lambing production. This study is not only beneficial to the research and utilization work on the double lamb trait of Tibetan sheep, but also to the in-depth research on the breeding power of Tibetan sheep and to promote the development of animal husbandry in the Tibetan Plateau region.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (J. Jia).

Acknowledgments

The authors are grateful to all the participants who took part in this study.

Funding Support

This work was supported by Special Projects of Agricultural Industry (No. 201303062-5) and National Natural Science Foundation of Qinghai Province (2020-ZJ-735).

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

YC and LZ: the hypothesis of this study; YC and JL: work management, article writing; YC, QC and LR: experimental procedure follow-up, statistical analysis; YC, JL, QC and LR: literature review, review of results; JJ: final decision, funding support.

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