Identification of Prolificacy-Related Differentially Expressed Proteins from Sheep (*Ovis aries*) Hypothalamus by Comparative Proteomics

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Reproduction, as a physiologically complex process, can significantly affect the development of the sheep industry. However, a lack of overall understanding to sheep fecundity has long blocked the progress in sheep breeding and husbandry. In the present study, the aim is to identify differentially expressed proteins (DEPs) from hypothalamus in sheep without FecB mutation in two comparison groups: polytocous (PF) versus monotocous (MF) sheep at follicular phase and polytocous (PL) versus monotocous (ML) sheep at luteal phase. Totally 5058 proteins are identified in sheep hypothalamus, where 22 in PF versus MF, and 39 proteins in PL versus ML are differentially expressed, respectively. A functional analysis is then conducted including Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis to reveal the potential roles of these DEPs. The proteins ENSOARP00000020097, ENSOARP00000006714, growth hormone (GH), histone deacetylase 4 (HDAC4), and 5′-3′ exoribonuclease 2 (XRN2) in PF versus MF, and bcl-2-associated athanogene 4 (BAG4), insulin-like growth factor-1 receptor (IGF1R), hydroxysteroid 11-beta dehydrogenase 1 (HSD11B1), and transthyretin (TTR) in PL versus ML appear to modulate reproduction, presumably by influencing the activities of gonadotropin-releasing hormone (GnRH). This study provides an alternative method to identify DEPs associated with sheep prolificacy from the hypothalamus. The mass spectrometry data are available via ProteomeXchange with identifier PXD013822.

1. Introduction

The achievement of reproduction is an essential but complex process involving the central nervous system and endocrine activities, especially those involving hormones released from the hypothalamic-pituitary-gonadal axis. The hypothalamus, as a critical organ in this axis, initiates the reproductive process through a gonadotropin-releasing hormone (GnRH) signal, which then travels to the pituitary, where follicle-stimulating hormone and luteinizing hormone are induced to function in both the ovary and testis, enabling the synthesis and release of ovarian estradiol and testicular hormone, facilitating folliculogenesis and spermatogenesis, respectively, and maintaining the functions of corpus luteum. Numerous studies involving GnRH and several neuroendocrine factors, such as kisspeptins and neurokinin B, known to be related to GnRH release have been widely studied. Furthermore, some metabolic
factors including leptin, insulin, and ghrelin have also been found to regulate GnRH release by mediating kiss-1 neuronal activities. The hypothalamus, therefore, plays a crucial role in mammalian reproduction.

Sheep is a species of significant importance to modern agriculture because of the production of meat, wool, and milk necessary for our daily life. Different sheep species are distributed in different countries and latitudes. Overall, sheep can be divided into two types based on their oestrous cycle: perennial oestrous species and seasonal oestrous species. The Dorset sheep, a characteristic seasonal oestrous breed is known for its high growth rate. In contrast, the Small Tail Han (STH) sheep is an excellent perennial oestrous breed mainly bred in northern China, and it has attracted much attention for its prolificacy. As a prominent indigenous species, the STH sheep has been well studied, especially for its reproductive traits, for many years. The FecB mutation, which was the first identified major gene significantly influencing sheep reproduction, was a point mutation (A to G) at base 746 in bone morphogenetic receptor type 1B (BMPR1B), resulting in an amino acid change from glutamine to arginine. Ewes with one or two copies of the FecB mutation produce 1 or 1.5 extra lambs at each birth. However, as studies go on, other candidate genes such as BMPR1B, growth differentiation factor 9, and bone morphogenetic protein 15 have also been shown to influence sheep reproduction. Although in-depth explorations of reproduction have been conducted, it has yet to be discovered how sheep prolificacy was established. Furthermore, STH ewes without the FecB mutation can also produce more than one lamb and the mechanism underlying this intriguing finding has yet to be elucidated.

In recent years, advanced approaches such as RNA-sequencing (RNA-seq) and bioinformatics analysis have greatly broadened our knowledge and understanding of the functions of the hypothalamus. Amar et al. identified many microRNAs, including let-7 family and miR-138, which may influence the hypothalamic functions in rats, Gao et al. found several key long non-coding (lnc) RNAs and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with goat puberty via RNA-seq, and it is important to note that all these miRNAs and lncRNAs function mainly through targeting mRNAs. Proteins, as the main manifestation of gene functions, participate in almost every physiological process. Recently, therefore, proteomics analysis has been conducted to resolve gene and genome issues. Tandem mass tag (TMT)-based quantitative proteomics analysis uses an isotope reagent to label the terminal amino group of a polypeptide or amino group of a lysine side chain and then analyzes protein expression levels of up to ten samples simultaneously by high-resolution mass spectrometry. This technique has been used to identify biomarkers in plasma to find candidate proteins that modulate muscular aging and to modify amino acids. Therefore, applying TMT-based quantitative proteomics analysis to reveal reproductive functions in the sheep hypothalamus at the protein level may provide a new insight into the hyper fecundity of sheep that lack the FecB mutation.

2. Experimental Section

2.1. Ethics Statement and Animal Processing

All experiments involving animals were authorized by the Science Research Department (in charge of animal welfare issue) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS; Beijing, China). In addition, ethical approval of animal survival was given by the animal ethics committee of IAS-CAAS (No. IASCAAS-AE-03, 12 December 2016). STH sheep with FecB ++ (without the effects of FecB mutation) and aged from 2 to 3 years were selected via TaqMan MGB probe technology. For the purpose of estrus synchronization, controlled internal drug releasing (CIDRs) (InterAg Co., Ltd., New Zealand) (progesterone 300 mg) were inserted into the vaginas of those sheep for 12 days, ovulation rate (the number of corpus luteum) of those sheep was determined using the laparoscopy procedure on day six after CIDR removal. According to littering records and ovulation rate, all STH ewes were categorized as being polytocous sheep (n = 6) and monotocous sheep (n = 6). All sheep had free access to water and food and were fed and housed at the Tianjin Institute of Animal Sciences, Tianjin.

2.2. Tissue Acquisition and Sample Preparation

Six sheep containing three polytocous sheep and three monoto- cous sheep were slaughtered within 45–48 h since CIDR removal (folllicular phase), the remaining six sheep containing three polytocous sheep and three monotocous sheep were slaughtered on day nine after CIDR removal (luteal phase). Totally, all STH ewes were categorized as being polytocous sheep at luteal phase (PF, n = 3), polytocous sheep at follicular phase (PL, n = 3), monotocous sheep at follicular phase (MF, n = 3), or monoto- cous sheep at luteal phase (ML, n = 3).

Twelve hypothalamic samples were then obtained after slaughter and immediately stored at −80 °C until they are used. First, the hypothalamic samples were processed with the method of
SDT²⁸ (constituted of 4% w/v SDS, 150 mM Tris/HCl (pH8), 100 mM DTT), an ultrasonic process (80W, 10 s on, 15 s off for ten cycles), and the samples with tubes were placed in a boiling water bath for 15 min. The homogenized tissue was centrifuged at 14 000 × g for 40 min and the supernatant was removed by filtering it through a 0.22 µm filter. Finally, the filtrate was collected and protein quantification was performed using BCA Protein Assay Kit (Bio-Rad, USA), and sodium dodecyl sulfate-PAGE was also applied to determine their purity. Finally, the processed samples were stored at −80 °C.

2.3. FASP Enzymatic Hydrolysis and TMT Labeling

Thirty microliters of high-quality protein solution per sample was treated with filter-aided sample preparation (FASP) enzyme, and the filtrates was obtained, and then peptide quantification was carried out (measured by optical density at 280 nm; OD280). For each sample, 100 µg of peptide was labeled with TMT using the TMT Mass Tagging kits and reagents (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions.

2.4. Reversed-Phase Rating

A high pH reversed-phase (RP) spin column was then performed to grade mixed peptide after balanced mix of labeled peptide using the pierce high PH reversed-phase fractionation kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The peptide label after grading was mixed and lyophilized. Subsequently, 100 µg of processed peptide was diluted with 300 µL of 0.1% trifluoroacetic acid and transferred to a high pH RP spin column. The flow component was collected by centrifugation of 12 000 × g, 5 min. A step gradient elution was begun after addition of 300 µL of pure water to collect the wash fraction by centrifugation of 12 000 × g, 5 min. The samples were then lyophilized and reconstituted with 12 µL of 0.1% formic acid (FA). The peptide concentration was determined by measuring absorbance (OD280).

2.5. Mass Spectrometry

2.5.1. High Performance Liquid Chromatography

Each sample was separated using a high-performance liquid chromatography (HPLC) liquid phase system Easy nLC (Thermo Fisher Scientific) with a nanoliter flow rate. The buffer solutions A and B were 0.1% formic acid aqueous solution and 0.1% formic acid acetonitrile aqueous solution (84% acetonitrile) respectively. Buffer solution A (95%) was used to balance the chromatographic column and sample was loaded from the autosampler to the loading column (Thermo Scientific Acclaim PepMap100, 100 µm × 2 cm, nanoViper C18). The peptides were separated by analytical column (Easy column, 10 cm, internal diameter 75 µm, 3 µm, C18-A2; Thermo Scientific) at a flow rate of 300 nL min⁻¹.

2.5.2. Mass Spectrometer Identifications

Mass spectrometry (MS) of separated samples was performed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific). The analytic time was around 60 min, positive ions were detected, and the scanned area of parent ion scan ranged from 300 to 1800 mass to charge ratio (m/z); the primary MS resolution, automatic gain control (AGC) target, maximum inject time, number of scan ranges, and dynamic exclusion were 70 000 at 200 m/z, 366, 10 ms, 1, and 40.0 s, respectively. The m/z of polypeptide and polypeptide fragments were obtained as followed: ten fragment maps were collected after each full scan, and MS2 activation type, isolation window was HCD and 2 m/z respectively. In addition, secondary MS resolution was 17 500 at 200 m/z (TMT6plex); microscans, secondary maximum inject time, normalized collision energy, and underfill were 1.60 ms, 30 eV, and 0.1%, respectively.

2.6. Data Analysis and Identification of DEPs

The raw data of MS were output as RAM files, and Mascot 2.2 (Matrix Science, Boston, Mass) was used to map the raw data to Proteome Discoverer 1.4 (Thermo Fisher Scientific) to identify and quantify proteins. Proteins with a fold change > 1.2 or < 0.83 and p-value < 0.05 in two comparable groups (PF versus MF or PL versus ML) were considered DEPs.

2.7. GO and KEGG Analysis

Functional annotation of Gene Ontology (GO) was performed using Blast2GO software.²⁹ The main steps were searching using the BLAST tool (https://blast.ncbi.nlm.nih.gov/) protein sequence database, mapping, annotation, and annotation augmentation. For functional annotation of KEGG, the KAAS (KEGG Automatic Annotation Server)²⁵ was used to map DEPs to the KEGG genes database, and then the mapped proteins were categorized based on KEGG orthology. Finally, the pathways involving those classified proteins were obtained automatically. A GO term or KEGG pathway with p < 0.05 after fisher’s exact test was considered significantly enriched.

2.8. Quantitative Analysis of Selected Proteins Using Parallel Reaction Monitoring Method

2.8.1. Sample Preparation

In order to validate the accuracy of the authors’ results, the parallel reaction monitoring (PRM) method was applied, a technique based on MS analysis, to verify protein abundance. After enzymatic hydrolysis, the peptides were desalted, lyophilized, and then redissolved with 0.1% FA, and the concentration of the peptides was determined by OD280.

2.8.2. LC-PRM /MS Analysis

According to pre-experimental results, peptide information suitable for PRM analysis was imported into Xcalibur software (Thermo Fisher Scientific) for PRM setting. Each sample shared 1 µg of peptide and 200 fmol of standard peptide was added (PRTC: GILFVGSGVSGGEEGAR) for chromatographic separation using HPLC system. A Q-Exactive HF MS (Thermo
Scientific) was used for PRM MS. Time for PRM MS analysis was 60 min and positive ions were detected. Then, 20 PRM scans were obtained according to inclusion list after each first-order full mass spectrum scanning. The raw data after LC-PRM/MS analysis was analyzed by Skyline 3.5.0.[31]

3. Results

3.1. Data Quality Control

In the current study, we applied TMT-based quantitative proteomic analysis using a Q-Exactive mass spectrometer with high accuracy and high resolution to identify DEPs in PF versus MF and PL versus ML after hypothalamus sampling (Figure 1A), the overview of TMT-based studies in polytocous sheep at follicular phase (PF), polytocous sheep at luteal phase (PL), monotocous sheep at follicular phase (MF), and monotocous sheep at luteal phase (ML) was displayed in Figure 1B. The results showed that more than 77.6% of the peptides had a score greater than 20, which means the data we obtained are relatively high-quality, and the median score was around 26.51 (Figure S1A, Supporting Information). Most peptides were 7 to 9 amino acids long, followed by peptides of 5 to 7 and 7 to 11 amino acids (Figure 2A). The counts of identified peptides showed a decreasing trend from 0–1 to >20 (Figure 2B). In terms of labeling quantification peptides, the abundance ratio of most proteins was nearly 1 (Figure 2C,D), and the isoelectric point of numerous proteins was around 7 (Figure S1B, Supporting Information). The molecular weights are also shown in Table S1, Supporting Information. These results suggested that the data we obtained was highly reliable.

3.2. Analysis of DEPs

We identified 5058 proteins by the multiple TMT labeling technique, where the intersection included 71.68% of detected proteins (Figure 3A and Table S2, Supporting Information). We obtained 22 DEPs (5 upregulated and 17 downregulated) in PF versus MF, and 39 DEPs (31 upregulated and 8 downregulated) in PL versus ML (Figure 3B,C and Table S3, Supporting Information). Detailed information of those DEPs was listed in Table S4, Supporting Information. We also conducted DEPs clustering using hierarchical cluster analysis and generated heatmaps in which the two comparable groups can be easily recognized (Figure 3D,E), highlighting the notable differences between PF and MF and between PL and ML, suggesting the rationality of identified DEPs.

3.3. Functional Enrichment Analysis Based on DEPs

To explore the potential roles of DEPs in PF versus MF and PL versus ML, we conducted GO terms and KEGG pathway analysis, with the top 20 enriched GO terms shown in Figure 3. The most enriched GO term in PF versus MF was osteoblast development (Figure 4A), whereas the most enriched GO term in PL versus ML was positive regulation of synaptic plasticity (Figure 4B). The top 20 enriched KEGG pathways were shown in Figure 4. The most enriched pathway in PF versus MF was histidine metabolism (Figure 5A) and that in PL versus ML.

![Figure 1. Quantitative proteome studies of sheep hypothalamus with FecB ++ genotype. A) Sample processing, where PS and MS represent polytocous and monotocous sheep respectively; FP and LP represent follicular phase and luteal phase, respectively. B) Overview of TMT-based studies in polytocous sheep at follicular phase (PF), polytocous sheep at luteal phase (PL), monotocous sheep at follicular phase (MF) and monotocous sheep at luteal phase (ML).]
was steroid hormone biosynthesis (Figure 5B). Several pathways related to metabolism were also in the top 20 enriched pathways, including glutathione metabolism and glycerophospholipid metabolism in PF versus MF, and histidine metabolism, butanoate metabolism, and glutathione metabolism in PL versus ML (for detailed GO, KEGG enrichment results, refer to the data which has been displayed in Tables S5 and S6, Supporting Information).

3.4. Data Validation

The PRM technique was performed to verify the accuracy of the proteomics data, and the validation results (Figure 6 and Table S7, Supporting Information) suggested that the abundance of nine selected proteins (NEBL, LGALS3, H1F0, TTR, ASPA, TPM1, LAP3, ENSORP00000004680, and ENSORP00000006714) obtained by the TMT method were similar to data from the PRM technique, indicating our results obtained from TMT technique were reliable.

4. Discussion

Recent advances in TMT-based proteomics and forward knowledge in protein analysis, compared with 2D electrophoresis which was first reported in 1975 and is still used to isolate proteins,[32] enable the detection of proteins at low abundance with high sensitivity and good repeatability in a short time and using a simple process. The hypothalamus can be divided into several differently functional regions according to the nerve cell types,[33] and the functions of different regions varies. Many studies have focused on the structure and functions of these different regions. Such as KNDy neurons, which play critical roles in GnRH secretion, project to around 60% GnRH cell bodies in the mediobasal hypothalamus (MBH) GnRH neurons and to 45% of the preoptic area,[34] and a study reported that the decrease of kisspeptin in MBH rather than in GnRH neurons in ewes may be responsible for the seasonal difference,[35] however, less researches have put efforts on the functions of the whole hypothalamus.

Therefore, for the purpose of investigating the hypothalamic protein abundance from a whole level, we applied a TMT-based proteomic strategy using the whole hypothalamus of STH ewes that lack the FecB mutation to identify DEPs associated with fecundity. In contrast to early studies on the hypothalamus[36–38] using 2D gel electrophoresis, our proteomics analysis based on TMT labels reported a relatively larger set of proteins (5058) in sheep hypothalamus. Of these, 22 were DEP, including 18 proteins (PRKCI, PDZD11, ACHE, LIMK1, GH, NEBL, GST, NCBP1, GIGYF2, WIPF, LGALS3, SDHC, HNMT, AGFG1, SEC11, H1F0, HDAC4, XRN2) that were known and well characterized (annotated by GO terms or KEGG pathways) in PL versus ML. Whereas, there were 39 DEPs, including 33 proteins (BAG4, IGF1R, HRG, ASPA, KLK6, E1.1.1.30, LY6E, BTBD17, S100A1, RP-L30, PRRC2A, LAP3, HSD11B1, CPLX1, RNF219, HPCA, NEXN, KIAA0556, S100A10, CPLX2, TPM1, SLC41A3, CALY, RASA2, PISD, RENBP, TTR, SEC61A, CNR1, H3, MAST1, ABCD3, WIPF) that were known and well characterized in PF versus MF (Table S7, Supporting Information).
Figure 3. A) Identification of proteins by different tandem mass tag labeling, where A, B, and C represent three TMT markers; Volcano plots of differentially expressed proteins (DEPs) in B) polytocous (PF) versus monotocous (MF) sheep at follicular phase and in C) polytocous (PL) versus monotocous (ML) sheep at luteal phase, where the red circles on the positive side represent upregulated proteins and those on the negative side represent downregulated proteins; Heatmaps of DEPs at D) PL versus ML and E) PF versus MF where the red and blue shades represent significantly up- and downregulated proteins, respectively, and grey areas indicate no quantitative information of proteins.

4.1. DEPs Analysis in PF versus MF

Our quantitative analysis of proteins showed that two undefined proteins, ENSOARP00000020097 enriched in GO terms including gene expression, binding, tRNA metabolic process, and ENSOARP00000006714 with little known about its function, were the top two differentially abundant proteins in PF versus MF, indicating that they may play important roles in the hypothalamus. Therefore, an additional validation experiments are necessary to explore their detailed functions in reproduction, especially the GnRH activities.

Growth hormone (GH) is a member of the somatotropin prolactin family and is enriched in many GO terms, including growth hormone receptor binding, hormone activity, cellular response to insulin stimulus, insulin secretion, and pathways including PI3K-Akt and Jak-STAT signaling pathways. The PI3K-Akt signaling pathway has been reported to function in neuronal survival, prolactin secretion, and insulin regulation. The Jak-STAT signaling pathway participates in leptin-mediated functions such as follicle maturation and apoptosis. Furthermore, one of the functions of leptin is to modulate insulin activities, and both insulin and leptin also have effects on...
Figure 4. The top 20 enriched GO terms concluding biological process (BP), molecular function (MF), and cellular component (CC) in A) polytocous (PF) versus monotocous (MF) sheep at follicular phase and in B) polytocous (PL) versus monotocous (ML) sheep at luteal phase.

Figure 5. The top 20 enriched KEGG pathways in A) polytocous (PF) versus monotocous (MF) sheep at follicular phase and in B) polytocous (PL) versus monotocous (ML) sheep at luteal phase.
Figure 6. Validation of abundance of nine selected proteins using parallel reaction monitoring and tandem mass tag methods in A) polytocous (PF) versus monotocous (MF) sheep at follicular phase and in B) polytocous (PL) versus monotocous (ML) sheep at luteal phase.

GnRH release. Chen et al. suggested that overexpression of GH can also disrupt the state of reproduction, mainly through the activity of leptin. Therefore, we speculate that both Jak-STAT signaling pathway and Jak-STAT signaling pathway enriched by GH could be involved in modulation of GnRH secretion.

It is well known that acetylation and deacetylation play crucial roles in gene transcription. Histone deacetylase 4 (HDAC4), as a critical deacetylase, can act as a repressor to modulate gene expression, mainly by influencing chromatin condensation and structure (summarized by Wang et al.). The amino-terminal fragment of HDAC4 cleaved by caspase can lead to nuclear localization, resulting in gene repression and neuronal cell death. HDAC4 was found to suppress peroxisome proliferator-activated receptor (PPAR)γ prosurvival activity, suppressing PPARγ transcription in cultured cortical cells, which further decreases metabolic activities such as insulin activity. Hence, we concluded that HDAC4 may inhibit expression of genes related to GnRH release by binding to the gene promoter region and further attenuating the GnRH activities.

In addition, 5′-3′ exoribonuclease 2 (XRN2) is enriched in GO terms such as RNA catabolic process, 5′-3′ exoribonuclease activity, rRNA processing, transcription termination site sequence-specific DNA binding, pathways including ribosome biogenesis in eukaryotes, and RNA degradation. The GO and KEGG analyses suggested that XRN2 was mainly involved in diverse RNA processes. Indeed, XRN2 mainly functions as a cleaner to degrade the fragment of target RNA generated by antisense oligonucleotides. Davidson et al. noted that XRN2 can perform a protective function by degrading aberrant pre-RNA, and some studies have reported its function in modulating the maturation and decay of mammalian pre-rRNAs, terminating premature transcription with the cooperation of other factors, and gene silencing at 3′ ends through interaction with other factors. One study reported that XRN2 can regulate the process of epithelial–mesenchymal transition and metastasis by modulating the maturation of miR-10a, raising the possibility that XRN2 operates as a regulator in the hypothalamus to modulate activities of RNAs such as miRNAs related to reproduction, likely influencing gene expression and silencing, additionally, the degraded RNA fragments that cannot be eliminated by XRN2 on time may also influence gene expression or others. Overall, some DEPs, such as GH, HDAC4, and XRN2, whose abundances differed between PF and MF, may be responsible for, but may not those DEPs itself, different hormone release associated with the success of reproduction.

4.2. DEPs Analysis in PL versus ML

In our comparison of PL versus ML, the top two differentially abundant proteins were bcl-2-associated athanogene 4 (BAG4) and insulin-like growth factor-1 receptor (IGF1R). BAG4, also called silencer of death domains (SODD), has been shown to have crucial roles in inhibiting apoptosis, which further decreases metabolic activities such as insulin activity. Hence, we concluded that HDAC4 may inhibit expression of genes related to GnRH release by binding to the gene promoter region and further attenuating the GnRH activities.

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4.2. DEPs Analysis in PL versus ML

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a change of function of IGF1. Furthermore, the activation effect of IGF1 on kiss1 gene expression\cite{3} was observed in female rats during follicular phase, the presumably reverse effects of IGF1 on GnRH release may occur during luteal phase, as the physiological situation-specific or cooperating with other proteins such as BAG4 or TTR by IGF1R. All in all, considering the negative feedback control of estradiol and progesterone on GnRH secretion, and the key roles of insulin on GnRH secretion,\cite{66–69} demonstrating its crucial roles in GnRH release.

Histone H3 (HH3), as an important component of chromatin, was enriched in GO terms such as gene silencing by RNA, histone binding, positive regulation of gene expression, regulation of gene silencing, and pathways including transcriptional misregulation in cancers. Most discoveries about HH3 were mainly focused on methylation\cite{70,71} and acetylation\cite{72,73} raising the possibility that the hypothalamus in STH sheep with hyper fecundity at the luteal phase may need lesser methylation and acetylation activities to maintain the luteal functions.

In conclusion, in the current study, we applied TMT-based quantitative proteomics for the first time to identify DEPs from sheep hypothalamus in PF versus MF and PL versus ML. In total, we obtained 22 and 39 DEPs in the two comparison groups. Furthermore, several DEPs such as GH, HDAC4, IGF1R, HSD11B1, and TTR appear to directly or indirectly regulate GnRH activities by mediating metabolic factors such as insulin, leptin, and other protein activities. These results may add to our knowledge of sheep fecundity based on the hypothalamus at the protein level, and deepen our understanding of sheep prolificacy.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

GnRH, hypothalamus, reproduction, sheep, TMT-based quantitative proteomics

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