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Identification and analysis of microRNAs-mRNAs pairs associated with nutritional status in seasonal sheep

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ABSTRACT

Given the important role of nutritional status for reproductive performance, we aimed to explore the potential microRNA (miRNA)-mRNA pairs and their regulatory roles associated with nutritional status in seasonal reproducing sheep. Individual ewes were treated with and without 0.3 kg/day concentrates, and the body condition score, estrus rate, and related miRNAs and target genes were compared. A total of 261 differentially expressed miRNAs were identified, including 148 hypothalamus-expressed miRNAs and 113 ovary-expressed miRNAs, and 349 target genes were predicted to be associated with nutritional status and seasonal reproduction in sheep. Ultimately, the miR-200b-*GNAQ* pair was screened and validated as differentially expressed, and a dual luciferase reporter assay showed that miR-200b could bind to the 3′-untranslated region of *GNAQ* to mediate the hypothalamic-pituitary-ovarian axis. Thus, miR-200b and its target gene *GNAQ* likely represent an important negative feedback loop, providing a link between nutritional status and seasonal reproduction in sheep toward enhancing reproductive performance and productivity.

1. Introduction

Nutrition is very important in many aspects of reproductive performance in sheep, such as age at puberty in both sexes, fertility, ovulation rate, embryo survival, and the parturition to re-breeding interval [1–5]. Previous studies [6–8] indicated that sustained low levels of nutrition could lead to an extended period of anestrus and delay the onset of the next breeding season. By contrast, increased dietary energy and protein levels modify ewe fertility either through their influence during pregnancy or on the duration of the breeding season [9–11], suggesting a potential correlation between estrous activity and current nutrition levels and body weight.

Over the past few years, several microRNAs (miRNAs) have been shown to be expressed and/or enriched in the hypothalamus and ovary [12]. Moreover, recent research has suggested that miRNAs play a role in hypothalamic control of the energy balance. For example, administration of a pegylated leptin antagonist was shown to predispose high fat-fed rats to obesity through leptin damage in early life, which promoted insulin/leptin resistance and modified hypothalamic miRNA expression during adulthood [13–17]. However, there is little knowledge on the links between miRNA profiles, and their effects on the relationship between nutritional status and reproductive performance in sheep. Identification of such targets could provide novel opportunities for improving productivity in ewes and inform livestock management. Toward this end, we investigated whether potential miRNA-mRNA pairs might regulate the seasonal reproductive pattern and play a role in the altered expression of key genes that mediate nutrition factors and the hypothalamus-pituitary-ovary (HPO) axis in sheep.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted in strict accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health

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(eighth edition 2011). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Medical College of Shihezi University, Xinjiang, China.

2.2. Animals and experimental design

A total of 40 healthy Kazakh ewes were provided by the Animal Laboratory of Shihezi University, which were maintained at the Animal Experimental Station of Shihezi University, China. The ewes were randomly chosen and divided into two groups (20 per group). The control group (CG) was raised using a normal feeding method with *ad libitum* access to alfalfa (1.5 kg/sheep per day) and water, including 16.05% crude protein, 58.3% crude fiber, 9.11% crude ash, 2.73% crude fat, 1.43% calcium, and 0.15% phosphorus. The experimental group (EG) was induced toward an enhanced nutritional status by feeding a concentrate supplement (0.3 kg/sheep per day) including 18.0% crude protein, 11.0% crude fiber, 11.0% crude ash, 2% crude fat, 0.8–1.5% calcium, and 0.4% phosphorus, with *ad libitum* access to alfalfa (1.5 kg/sheep per day) and water throughout the anestrous season (February to June in Xinjiang, China).

2.3. Estrus identification and tissue collection

During the anestrous season, the date when a ewe first accepted mounting by the "teaser" ram along with detection of vaginal bleeding and pro-genital swelling was recorded, which were considered characteristics of estrous onset, representing the first day of the estrous cycle. Moreover, to eliminate the ram effect on inducing the estrus cycle in ewes, the teasing in our study was conducted according to the method described by Zarazaga et al. [18]. The estrus stage of the ewe was also identified through a blood serum hormone test by collecting blood from the jugular vein every morning (9:00–10:00 a.m.). In the EG, three ewes with standing estrous behavior and ovulatory follicles in the ovaries reaching 5.5 ± 0.2 mm in diameter were selected and humanely killed for obtaining hypothalamus and ovary samples. These tissues were then used to construct miRNA libraries designated HEN and OEN, respectively. Similarly, in the CG group, three other ewes in a confirmed state of anestrus during the entire non-breeding season and without active follicles in the ovaries (no follicles greater than 2.0 mm in diameter or ovulation marks) were humanely killed for obtaining hypothalamus and ovary samples, designated HAN and OAN, respectively. All samples were immediately frozen in liquid nitrogen until total RNA extraction.

2.4. RNA extraction

Total RNAs extracted from the hypothalamus and ovary of the three estrus and three anestrous Kazak sheep were pooled, respectively. Subsequently, low-molecular-weight RNAs were separated by 15% polyacrylamide gel electrophoresis, and RNA molecules in the range of 18–30 nt were enriched.

2.5. MiRNA sequencing and bioinformatics analysis

Four libraries (HEN vs. HAN, OEN vs. OAN) for the different estrus and nutrition conditions were compared to the standard using an Agilent 2100 Bioanalyzer and ABI StepOnePlus RT-PCR System, along with sequencing using Illumina HiSeq2500 technology (Beijing Genomics Institute, China). An overview of the experimental procedure is shown in Supplementary Fig. S1.

2.6. Target prediction, gene ontology and pathway analysis

The TargetScan (http://www.targetscan.org/) and RNAhybird (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/) were used for target gene prediction. And then GO Term (http://www.geneontology.org/) and KEGG pathway analyses of these were performed on. Genes with FDR $\leq$0.05 were considered as significantly enriched as target gene candidates.

2.7. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) validation

Twelve miRNAs, with six miRNAs in the hypothalamus and ovary libraries each, were randomly selected to verify the reliability of the sequencing results. miRNAs and mRNAs were reverse-transcribed to cDNA with the miRcute miRNA First-strand cDNA Kit (TIANGEN, Beijing, China). qRT-PCR was then performed using the MX3000p qRT-PCR System (Stratagene, US) and miRcute miRNA Premix SYBR (TIANGEN), following the manufacturer's instructions. All reactions were performed in triplicate. U6 RNA was chosen as an endogenous internal control, and the relative expression levels were calculated based on the $2^{-\Delta\Delta Ct}$ method. The miRNA-specific primers are listed in Supplementary Table S1.

2.8. Dual-luciferase reporter system validation

Based on the selected candidate target genes from the analyses described above, primer pairs with the addition of different restriction sites were designed to amplify pri-miR-200b [containing 3′-untranslated region (UTR)-binding sites], sent to BGI Genomics for sequencing, and then the sequence was subjected to follow-up experiments. The sequences of all primers and β-actin as a reference gene are shown in Supplementary Table S2. After successfully constructing the vector with the 3′-UTRs (containing miRNA-binding sites) of the chosen target genes, it was recombined to the psi-CHECK^{TM}-2 dual luciferase miRNA target expression vector (Promega, USA) according to the manufacturer's introductions. After verification by restriction digestion and sequencing, the recombined vectors were co-transfected into isolated HeLa cells (BGI Genomics, China), including the transfection groups with the psi-CHECK^{TM}-2 plasmid, target gene-3′UTR-psi-CHECK^{TM}-2, target gene-3′UTR-psi-CHECK^{TM}-2 and pcDNA3.1 (+) co-transfection, and target gene-3′UTR-psi-CHECK^{TM}-2 and pri-miR-200b-pcDNA3.1 (+) co-transfection. After 48 h of HeLa cell culture, the luciferase activities were detected with Dual-Luciferase Reporter Assay System (Promega, USA) on a multi-plate reader (Biotek, USA) according to the manufacturer's protocols. All experiments were repeated three times with three replicates for each sample.

2.9. Verification of interactions of miRNA target genes and estrus-related genes in target cells

The recombined interference vectors, including the miR-200b-pcDNA3.1 (+) group and empty vector group, were respectively co-transfected into isolated hypothalamus and ovarian granulosa cells. After 48 h of culture, total RNA was extracted from the hypothalamus and ovarian granulosa cells as described above, and the downstream genes of the target gene and the HPO axis-related genes were selected for detection by qRT-PCR, respectively. All experiments were repeated three times with three replicates for each sample.

2.10. Data analysis

All statistical analyses were performed with SPSS 19.0 statistical

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