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Integrated analysis of differential expression profile of miRNA in the uterus of seasonal estrus sheep

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Abstract

Background Photoperiod is one of the important factors affecting seasonal estrus of sheep. The importance of the uterus to reproduction is self-evident. However, the uterine molecular mechanisms involved in photoperiodic regulation of seasonal estrus events in sheep remain poorly understood. In recent years, the role of uterine microRNA (miRNA) in mammalian reproduction has been continuously revealed, and it is necessary to analyze and discuss their participation in the seasonal estrus of sheep.

Results We compared the miRNA expression profile in uterine tissues of Sunit sheep at 3 different photoperiods, short photoperiod (SP), short transfer to long photoperiod (SLP), and long photoperiod (LP). The results showed that 31, 29 and 21 differentially expressed miRNA (DEM) were identified between SP and LP, SP and SLP, LP and SLP, respectively. Subsequently, we constructed co-expression networks of DEM and target genes in different periods. Among all DEM, nove_320, nove_338 and nove_339 target the largest number of mRNAs. Functional annotation analysis showed that these DEM target genes can be enriched in multiple GO and KEGG signaling pathways, such as cell proliferation, apoptosis, reproductive process and biological adhesion, which are related to animal reproduction and uterine receptivity. Finally, the expression level of DEM was verified by RT-qPCR, and the binding regulation relationship between DEM and their target genes in the co-expression network was confirmed by double luciferase reporter gene experiment.

Conclusions This study revealed the changes of miRNA expression in sheep uterus under different photoperiod, providing valuable resources for understanding the molecular mechanism of sheep seasonal estrus.

Keywords MicroRNA, Photoperiod, Uterus, Seasonal estrus, Sheep

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Background

Reproductive performance has a crucial impact on the economic benefits of the sheep breeding industry, and seasonal estrus is one of the important factors limiting the reproductive efficiency of sheep. Seasonal estrus can be divided into long-day (LD) estrus and short-day (SD) estrus [1], in which sheep is SD estrus, and a shorter duration of sunlight will promote the onset of seasonal estrus activities [2]. Seasonal estrus is tightly controlled by seasonal variations in relative day-night length [3]. Among sheep, some sheep breeds such as Small-tailed Han sheep is year-round estrous. Interestingly, in contrast, some breeds such as Sunit sheep develop gonads and show seasonal estrous behavior only at certain times of the year [4]. The uterus plays an important role in reproduction in sheep while participating in the regulation of the estrous cycle, but the molecular mechanisms linking the uterus to seasonal estrous remain largely unknown [5, 6]. Therefore, an in-depth understanding of the molecular mechanisms involved in uterine-related functions is important for studying seasonal estrous in sheep.

Recently, microRNA (miRNA) has been recognized as key regulator due to their critical roles in gene expression and transcriptional regulation at the post-transcriptional level [7]. The mechanism of miRNA action in most mammals is to pair with the base complementary to the 3' untranslated region (3'UTR) of mRNA, thereby inhibiting the translation process of mRNA [8]. More and more studies have shown that miRNA play important roles in animal reproduction. In studies related to human male reproduction, a consistent association between aberrant miRNA expression and infertility has been reported, and each tissue/cell contains a well-defined and stable population of miRNA associated with spermatogenesis and reproductive development [9]. In livestock studies, miRNA have also been shown to be part of the reproductive physiology of animals and can serve as important candidate biomarkers [10]. Furthermore, it was demonstrated that miR-484 regulates granulosa cell function and reduces human ovarian reserve function through Yes-associated protein 1 (YAP1)-mediated mitochondrial function and apoptosis [11]. Transfection of miR-155 into the cumulus-oocyte complexes (COC) of B6D2F1 female mice for in vitro culture was found to inhibit oocyte expansion and maturation [12]. RNA sequencing (RNA-seq) analysis of ovarian tissues from single- and multi-lambing dairy goats identified several miRNA involved in reproductive processes, among which miR-101-3p was found to regulate the growth process of goat ovaries by targeting the Stanniocalcin-1 (STC1) gene [13]. Subsequently, miR-89 was found to have potential binding sites to the 3'UTR of aralkylamine N-acetyltransferase (AANAT) mRNA in the pineal gland of sheep, which may be involved in the physiological process of pineal gland regulating sheep seasonal estrous [14]. These studies have revealed the important role of miRNA in reproductive physiology. In utero, miRNA is also considered to play an important role in a variety of physiological activities, including immune response, embryo attachment, decidualization, and embryo-endometrial interaction [15]. For example, early studies have shown that miR-451, miR-155, miR-335-5p and miR-365 are regulated by 17β-estradiol (E_2) in the uterus of mice, and the target genes of these miRNA are involved in the regulation of cell growth [16]. Similarly, miRNA in extracellular vesicles in the uterus of sheep have also been found to be regulated by progesterone, promoting the establishment of pregnancy in sheep [17]. In addition, mouse experiments have shown that miR-183 obstructs embryo implantation by regulating heparin-bound epidermal growth factor like growth factor (Hbegf) and laminin gamma-1 (Lamc1) in mouse uterus, which also indicates the important role of miRNA in uterus [18]. A study in cattle suggests that miRNA contained in extracellular vesicles present in uterine fluid may regulate early embryonic development in cattle by participating in lipid metabolism [19]. Spatial transcriptomic studies from pigs have shown that miR-9 interacts with CXCR4 and CXCL11 to provide a microenvironment for endometrial implantation [20]. In sheep, it has also been found that miR-200a regulates postpartum recovery of maternal uterus morphology and function through Hippo signaling pathway by targeting two target genes, ZEB1 and YAP1 [21]. Interestingly, a study from chickens showed that miR-449c-5p, controlled by clock genes, is involved in regulating uterine Ca^{2+} transport in hens and plays a role in egg shell calcification [22], further demonstrating the important role and extensive involvement of uterine miRNA in reproductive physiology. However, it is important to emphasize that despite these advances, our understanding of the role of uterine miRNA in reproductive physiology is still very limited. So far, more studies on miRNA in utero have focused on the correlation analysis between abnormal expression of miRNA and diseases in human pathological conditions [23–25]. However, the role of miRNA in regulating uterine development and function under normal physiological conditions has not attracted enough attention, especially the role of miRNA in the uterus of seasonal estrus sheep.

In conclusion, uterine miRNA may play an important role in the seasonal estrus activity of sheep. In this study, in order to better understand the role of uterine miRNA in seasonal breeding of sheep, we took Sunit sheep as the research object and used transcriptome sequencing technology to study the expression profile of miRNA in the uterus of Sunit sheep under different photoperiod. On the basis of the previous research, the miRNA-mRNA

co-expression network was constructed and the GO and KEGG pathways were analyzed. And the relevant experimental verification was carried out to explore the relevant biomarkers. This study revealed the important role of uterine miRNA in the seasonal estrus events of sheep, and provided a new theoretical basis for the study of seasonal estrus regulation of sheep.

Methods

Experimental animals

9 healthy 3-year-old Sunit ewes with a body weight of 37 Kg ± 0.78 were selected from Wulat Middle Banner, Bayannur City, Inner Mongolia Autonomous Region, China. All ewes had at least three lambing records. The sheep were raised on a farm at the Tianjin Institute of Animal Science in China and had free access to water and food.

All sheep were transferred to a controlled photoperiod chamber after simultaneous estrus treatment [4]. Room setting: Use the special lighting for breeding. When the light is on, the light intensity of the plane where the sheep's eyes are is about 200 lx, and when the light is off, the light intensity is about 5 lx. The humidity is controlled at 50-70%, the temperature is controlled at 14–22 ℃, and the environmental monitoring equipment is used to monitor the environmental parameters in real time and adjust them in time.

All sheep were divided into 3 groups. Among them, 3 sheep were treated with short photoperiod for 42 days (SP42, SP = 8 h light, 16 h dark). 3 sheep were treated with long photoperiods for 42 days (LP42, LP = 16 h light, 8 h dark). 3 sheep were moved to LP42 processing only after SP42 processing (SLP42). Then, all the animals were euthanized under anesthesia by injection of pentobarbital at a dose of 60–100 mg/kg to obtain uterine tissue for subsequent experiments.

RNA extraction and library construction

The RNA sample quality assessment and library construction in this study were completed by Annoroad Gene Technology (Beijing) Co., LTD. In simple terms, after the sample test is qualified, 3 μg of total RNA is taken from each sample as the starting amount. cDNA is synthesized by adding splices to both ends of the small RNA. After PCR amplification, electrophoresis was used to isolate the target DNA fragments and complete the library construction. Finally, the constructed library was sequenced by Illumina. For raw data quality control, Phred quality scoring threshold: single base quality ≥ Q20(error rate ≤ 1%); Sliding window filtering: window size 4 bp, average quality ≥ Q15; Retention length ≥ 18nt. Cutadapt 5.0 is used for joint removal, allowing a maximum mismatch of 1–2 and a minimum overlap length of 6 bp. For more detailed steps, please refer to the Annoroad website: https://www.annoroad.com/.

Table 1 Information of RT-qPCR primers

Primer name	Primer sequence (5'-3')
novel_320-F	TATAAAGGTGGTGGGCTGCG
novel_338-F	AAAGGATTCTGCTGTCGGTCCCA
novel_339-F	GCAAACAAACATGGTGCACTTCTT
oar-miR-493-3p-F	CCTGGGGAAAGCGAGTAGGGAC
oar-miR-154a-5p-F	GGCGGTGGGAAAGACAAACTCAGAGT
oar-miR-543-5p-F	CGGCTTGTGCTTGATCTAACCATGT
U6-F	GGGCCATGCTAATCTTCTCTGTATCG

Identification of miRNA

High-throughput sequencing was also performed by Annoroad. In simple terms, after sequencing obtains raw data in fastq format, quality control, data filtering, and pruning results in clean data that can be used for subsequent analysis. After classification annotation, the expression of miRNA of species was analyzed by comparing with the reference genome, and novel miRNA sequences were found. For more detailed steps, please refer to the Annoroad website: https://www.annoroad.com/.

Differential expression analysis of miRNA

The difference analysis of miRNA was performed by DEGseq software. The screening criteria for differential miRNA were: pval ≤ 0.05, padj ≤ 0.05,|log2(Fold_change)| ≥ 1.

Target gene prediction of miRNA

Based on the previous research of our team, the differential expression gene (DEG) profiles of seasonal estrus sheep were obtained [4]. Then, the miRanda and TargetScan were used to predict the target genes of miRNA obtained by sequencing in this study, and the intersection of the two target prediction results was taken as the target gene of miRNA.

GO and KEGG analysis

To predict the miRNA target genes, by using the online enrichment of microscopic letter (https://www.bioinformatics.com.cn/) to complete the GO and KEGG analysis. The screening conditions for significant enrichment were: $P < 0.05$.

Gene expression validation by RT-qPCR

We selected 6 miRNA to validate the accuracy of RNA-sequencing by RT-qPCR. The U6 was used as an internal reference to normalize miRNA expression. Each RT-qPCR experiment was performed 3 times, and the relative RNA expression value was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study are shown in Table 1.

Construction of miRNA-mRNA co-expression network

miRNA and predicted target genes are presented in the form of miRNA-mRNA co-expression network. The visualization software is Cytoscape (V3.9.0) software.

Construction of double luciferase vector

PCR primers were designed according to the sequence matching novel_339 in the protein tyrosine phosphatase non-receptor type 13 (PTPN13) – 3'UTR sequence in the NCBI database, the enzyme cut site CTCGAG of *Xho I* was added upstream of the PCR primers, and the enzyme cut site GCGGCCGC of *Not I* was added downstream of the PCR primers. The PCR product was linked to the double-cleaved psiCHECK2 vector and transformed into *E. coli*. Twelve colonies of *E. coli* grown overnight were picked separately for PCR identification to finally obtain the PTPN13 – 3'UTR wild-type vector (PTPN13 – 3'UTR-WT). The PTPN13 – 3'UTR wild-type vector was mutated using the site-directed mutation method, the novel_339 seed sequence target UAAAGGCCACA was mutated to AUUUCCGG UGU, *E. coli* was transformed using the same method as above, and the PTPN13 – 3'UTR mutant vector (PTPN13 – 3'UTR-MuT) was finally obtained. The tripartite motif containing 71 (TRIM71) – 3'UTR wild-type vector (TRIM71 – 3'UTR-WT), TRIM71 – 3'UTR mutant vector (TRIM71 – 3'UTR-MuT), PR domain zinc finger protein 1 (PRDM1) – 3'UTR wild-type vector (PRDM1 – 3'UTR-WT) and PRDM1 – 3'UTR mutant vector (PRDM1 – 3'UTR-MuT) were constructed by the same method. All plasmids were constructed by GenePharma (Shanghai, China) and confirmed by sequencing.

Validation of miRNA–mRNA targeting relationship

According to the manufacturer's instructions, the constructed double luciferase plasmid and miRNA mimics were transfected into HEK293T cells and the cell contents were collected 48 h later. The chemiluminescence intensity was then measured on a photometer using the dual luciferase reporting system (Vazyme, Nanjing, China) to detect firefly and Renilla luciferase activity. Relative luciferase activity = activity of sea kidney luciferase reporter gene/activity of firefly luciferase reporter gene.

Table 2 Summary of mapping data of uterine tissue

Sample	Total reads	Mapped reads	Mapping rate (%)
	miRNA		
LP42-1	22,922,460	10,149,429	44.28
LP42-2	28,505,246	13,299,111	46.65
LP42-3	27,185,883	13,723,311	50.48
SP42-1	29,220,022	12,972,757	44.4
SP42-2	22,159,737	11,470,604	51.76
SP42-3	20,706,982	9,358,581	45.2
SLP42-1	24,559,976	11,267,645	45.88
SLP42-2	26,206,405	13,204,815	50.39
SLP42-3	25,572,228	11,762,308	46
Total	227,038,939	107,208,561	

Note: LP42 and SP42 represented a long photoperiod every day for 42 days and a short photoperiod every day for 42 days. SLP42 represented a short photoperiod every day for 42 days, followed by a long photoperiod for 42 days

Statistical analysis

The relative expression of miRNA was analyzed using an independent samples *t*-test. $P < 0.05$ indicates a significant difference. Statistical analysis and plots were performed using GraphPad Prism 9.

Results

Overview of uterine tissue sequencing data

The overall miRNA expression profile of Sunit ewe uterus under different photoperiod was obtained. The data for subsequent analysis, the clean reading, is based on the filtered raw offline data, the reading without adapter contamination, the base error rate < 0.1%. Overall, we obtained clean readings of 227 million miRNA. To obtain more accurate sequences and the accuracy of subsequent analyses, the clean reads were mapped to the reference genome using bioinformatics analysis software, and the results were statistically compared. We found that the average comparison rate of miRNA was about 50%. In addition, perfectly matched reads for 107 million miRNA were obtained (Table 2).

Identification of miRNA in uterine tissues

RNA-seq yielded about 227 million clean reads after filtering and mapping. We classified and annotated all clean reads, including known miRNA, non-coding RNA (ncRNA) (rRNA, tRNA, snRNA, snoRNA and other rfam), repetitive sequences, as well as sRNAs and novel miRNA annotated by perfect matching to mRNA exons and introns. The proportion of clean reads matching known miRNA maturity in each sample was less than 2%, followed by more than 15% of clean reads matching other ncRNAs in the rfam database (Table 3).

In addition, we mainly focused on the identification of known miRNA and novel miRNA. Overall, we identified miRNA ($n = 449$), including known miRNA ($n = 25$) and novel miRNA ($n = 424$). The total number of clean reads identified as known miRNA and novel miRNA was 80,066,077 and 66,644, respectively, and the number of unique clean reads identified as known miRNA and novel miRNA in the 3 experimental groups was 16,791 and 597, respectively. Figure 1 showed an example of the miRDeep2 output. The top of Fig. 1 showed the scores and total counts assigned to each part of the miRNA. Different colors were used to indicate different parts of the predicted hairpin secondary structure. All readings related to miRNA are also shown at the bottom of the figure. Therefore, about the detailed miRDeep2 output information

Table 3 Summary of the classification statistics of sRNA total reads mapped to reference genome

Types	LP42	Proportion	SP42	Proportion	SLP42	Proportion
Total	37,171,851	76.25%	33,801,942	74.05%	36,234,768	73.66%
Known_miRNA	28,343,600	1.57%	25,031,627	1.55%	26,690,850	1.68%
rRNA	583,687	1.24%	524,753	3.88%	608,321	2.39%
tRNA	461,665	0.03%	1,311,045	0.03%	864,343	0.03%
snRNA	9788	4.22%	8544	6.06%	9483	6.04%
snoRNA	1,568,194	14.73%	2,049,038	12.86%	2,187,205	14.32%
Other_rfam	5,476,628	0.15%	4,345,641	0.12%	5,187,079	0.16%
Repeat	57,442	0.30%	40,710	0.26%	56,203	0.32%
Exon:+	112,192	0.04%	87,420	0.03%	114,593	0.04%
Exon:-	14,111	0.65%	10,995	0.50%	14,085	0.62%
Intron:+	242,579	0.09%	168,504	0.10%	223,700	0.11%
Intron:-	33,927	0.08%	33,749	0.05%	41,029	0.06%
Novel_miRNA	28,994	0.64%	17,179	0.51%	20,471	0.60%
Other	239,044	76.25%	172,737	74.05%	217,406	73.66%

Note: The data of LP42, SP42 and SLP42 were the total number of 3 biological repetitive samples, respectively. All the data and proportion calculations are based on mapped reads

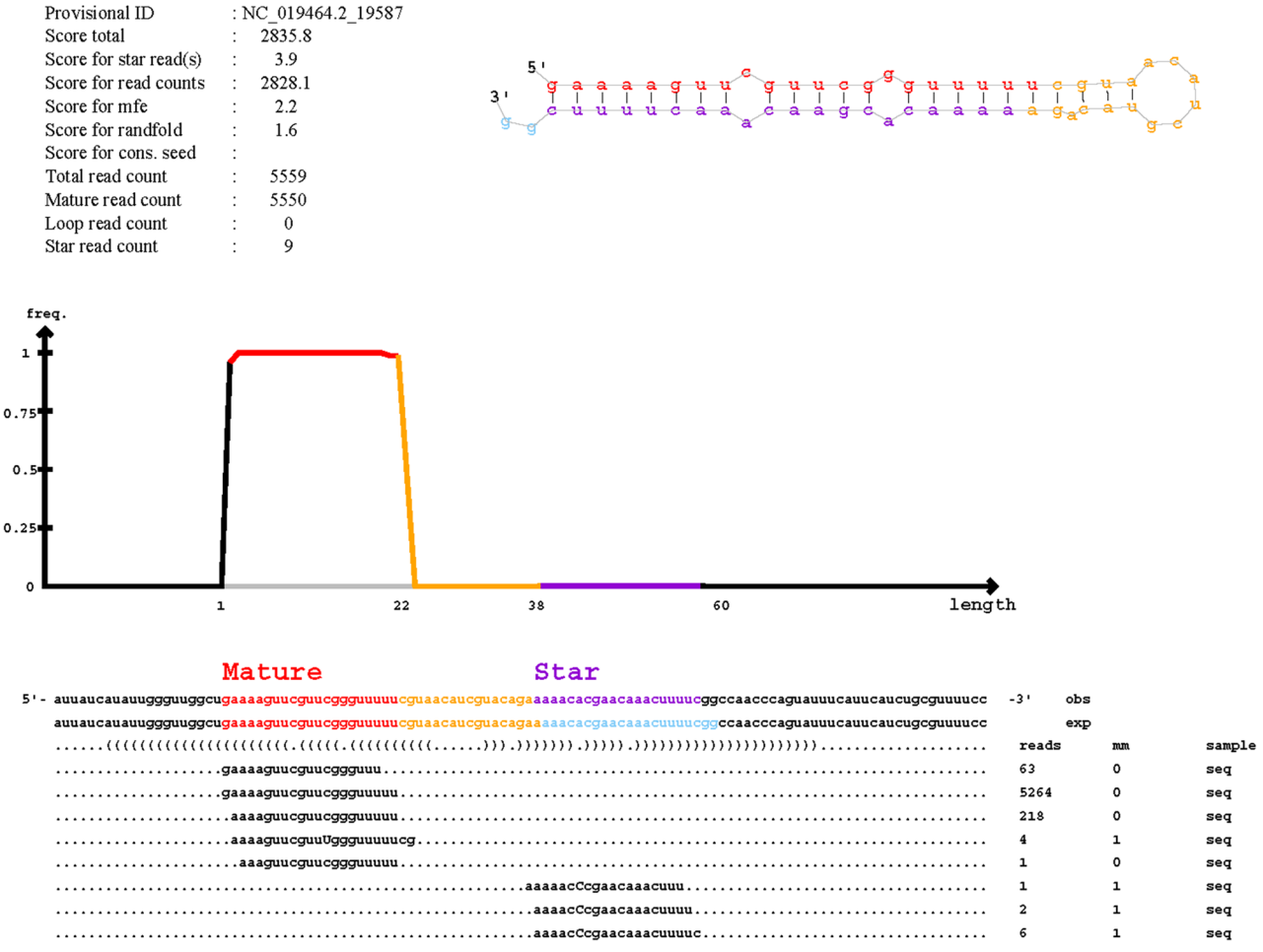

Fig. 1 An example of the miRDeep2 output. The figure illustrated the output for the miRNA of novel_555. The upper part of Fig. 1 showed the read count and the total count of each part. The predicted secondary structure of the hairpin is also depicted, with mature (red), star (purple) and circular (yellow) sequences highlighted in different colors. The bottom of the map shows the results of miRNA alignment with predicted precursor sequences on the genome (OBS line) and experimental sequences reported in miRBase (EXP line). For each sequence, the frequency and mismatch with the genome sequence (mm sequence) are given. Mismatches are also highlighted in uppercase letters

of novel miRNA, we described in detail the 3 biological repetitive samples of the 3 processing groups (LP42-1, 64; LP42-2, 93; LP42-3, 80; SP42-1, 45; SP42-2, 52; SP42-3, 42; SLP42-1, 67; SLP42-2, 64; SLP42-3, 90), including the precursor secondary structure, count number and read sequence shown in the picture. Known miRNA was also described in detail (LP42-1, 105; LP42-2, 105; LP42-3, 104; SP42-1, 105; SP42-2, 105; SP42-3, 102; SLP42-1, 103; SLP42-2, 105; SLP42-3, 104).

Differential expression analysis of miRNA

There 21 DEM (10 upregulated and 11 downregulated) were identified in the LP42 vs. SLP42 group (Fig. 2A). There were 31 DEM (11 upregulated and 20 downregulated) in the SP42 vs. LP42 (Fig. 2B). There were 29 DEM (11 upregulated, 18 downregulated) in SP42 vs. SLP42 group (Fig. 2C). The heat map showed the number and expression pattern of DEM, and all DEM were statistically significant (Fig. 2D) ($P < 0.05$).

Analysis of miRNA–mRNA co-expression network

To better understand the role of differentially DEM in Sunit sheep uterus, after obtaining differentially expressed genes by the aforementioned methods, the target genes of DEM in the 3 comparison groups were predicted and a miRNA-mRNA co-expression network was constructed (Fig. 3). There were 9 DEM targeted a total of 49 DEG in the LP42 vs. SLP42 group (Fig. 3A). In the SP42 vs. LP42 group, 14 DEM targeted a total of 67 DEG (Fig. 3B). 12 DEM targeted a total of 54 DEG in the

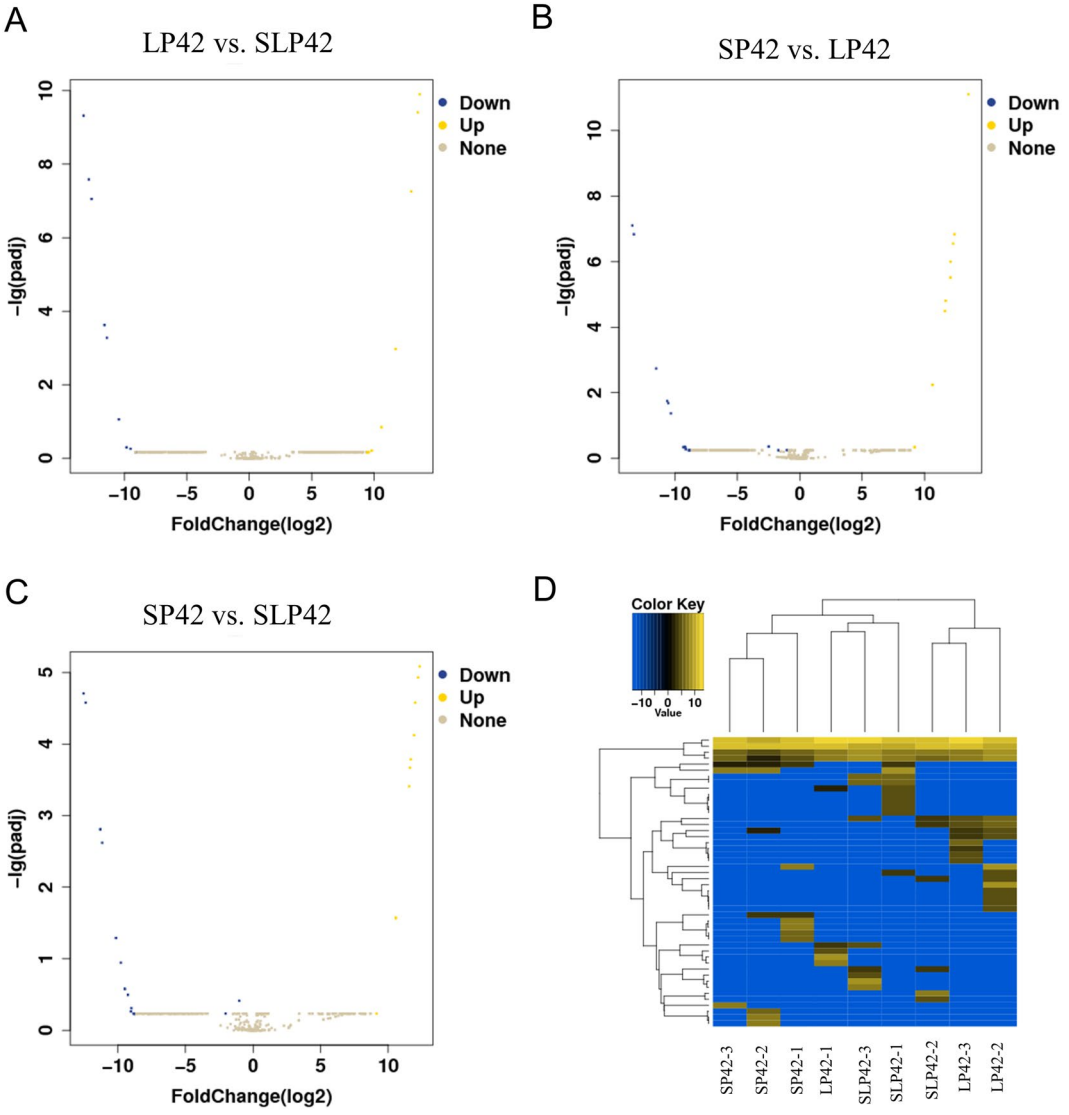

Fig. 2 Analysis of DEM among 3 groups. Volcano plots show the up – and downregulation distribution of DEM in (**A**) LP42 vs. SLP42, (**B**) SP42 vs. LP42 and (**C**) SP42 vs. SLP42, where yellow and blue represent up- or down-regulation, respectively. Heatmap (**D**) shows the expression patterns of the 3 groups of DEM

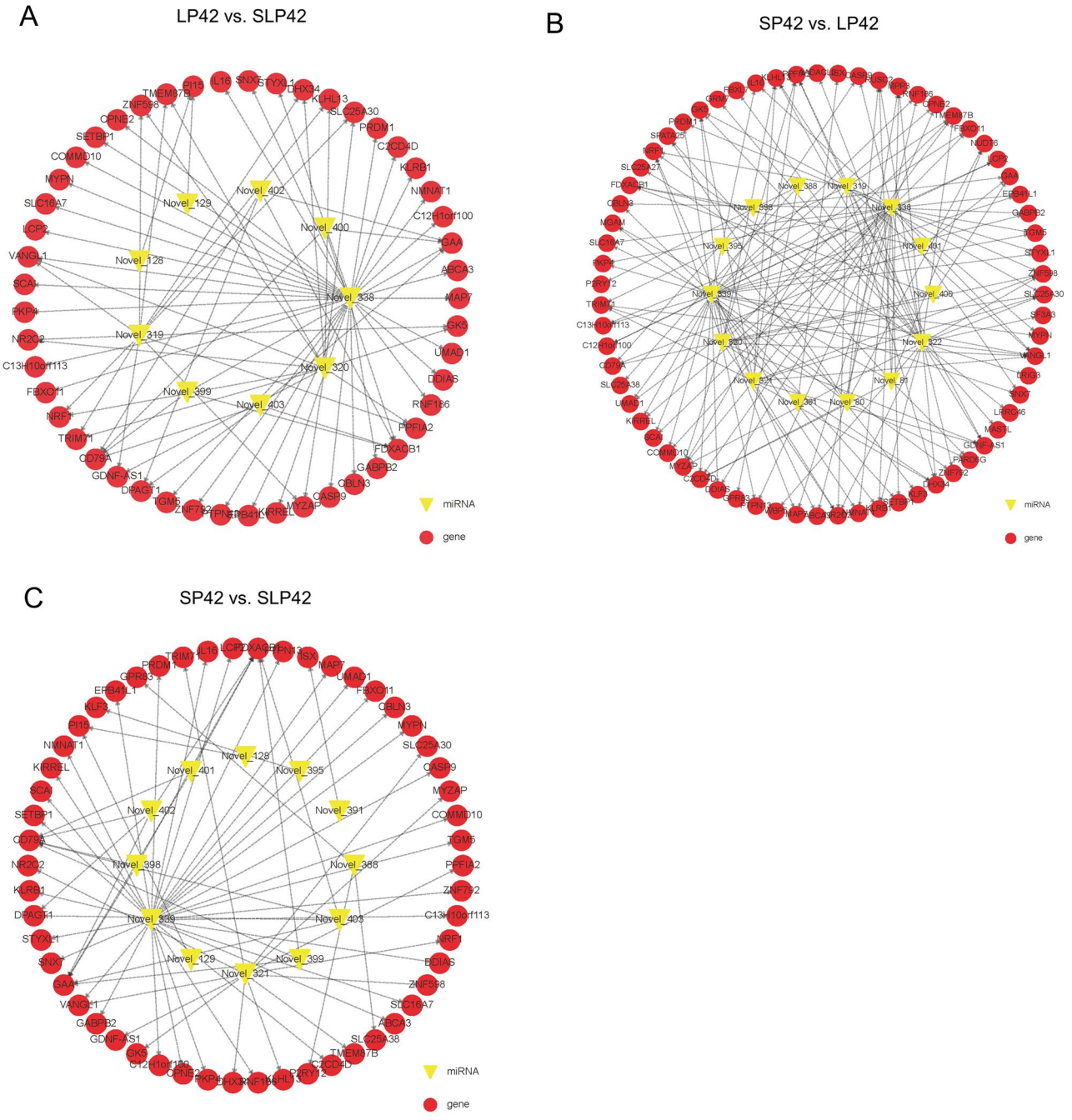

Fig. 3 DEM – DEG network interaction analysis. The DEM–DEG network between (**A**) LP42 vs. SLP42, (**B**) SP42 vs. LP42, (**C**) SP42 vs. SLP42. Note: Nodes represented DEM or DEG, and edges represented the interaction between DEM and DEG. Red and yellow represented DEG and DEM, respectively

SP42 vs. SLP42 group (Fig. 3C). Among them, novel_320, novel_338 and novel_339 targeted the most regulated mRNAs, implying that they play an important role in the regulation of seasonal estrus in Sunit sheep.

Functional enrichment analysis of miRNA target genes

In the GO and KEGG bioinformatics analysis of the identified DEM, GO enrichment revealed that among the 3 comparison groups LP42 vs. SLP42 (Fig. 4A, Supplementary Tables 1–3), SP42 vs. LP42 (Fig. 4B, Supplementary Tables 4–6), and SP42 vs. SLP42 (Fig. 4C, Supplementary Tables 7–9), the most significantly enriched GO entry in the biological process (BP) category was cellular process; the cellular component (CC) category cell part was the most enriched; the binding was the most enriched term in the molecular function (MF) process. KEGG enrichment analysis revealed that the most significantly enriched signaling pathways in the 3

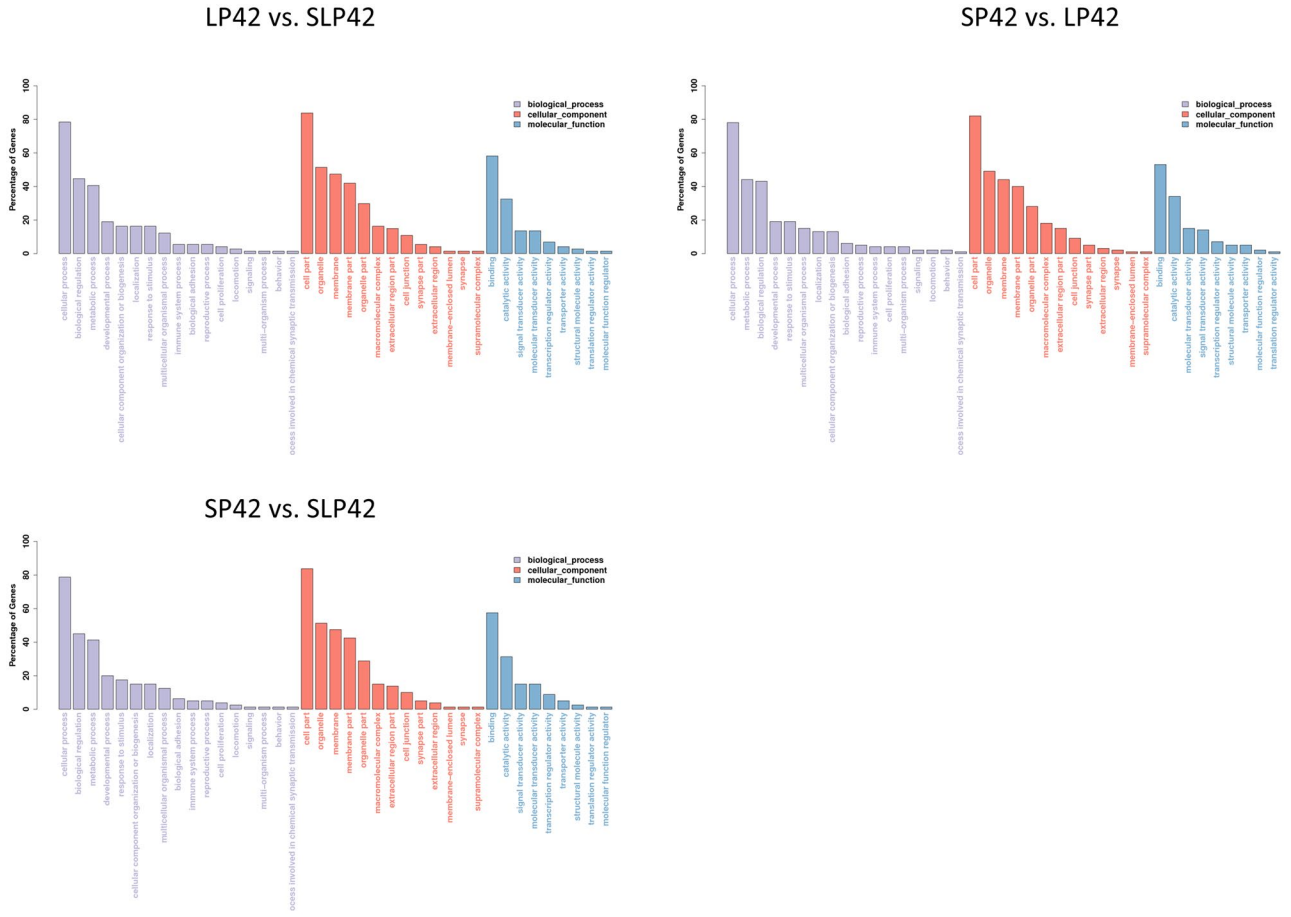

Fig. 4 Enriched GO terms of genes targeted by DEM in 3 groups. Enriched GO terms of genes targeted by DEM in (**A**) LP42 vs. SLP42 (**B**) SP42 vs. LP42 (**C**) SP42 vs. SLP42. The horizontal and vertical coordinates represent the GO terms and −lg (p−Value) of the enriched genes, respectively

groups of DEM target genes were Apoptosis and Platelet activation signaling pathway (Fig. 5, Supplementary Tables 10–12).

Validation of sequencing data

To verify the accuracy of the sequencing data, we randomly selected six miRNA (novel_320, novel_338, novel_339, oar-miR-493-3p, oar-miR-154a-5p and oar-miR-543-5p) for RT-qPCR. The results showed that the trends of RT-qPCR were consistent with the RNA-seq expression trends, indicating that the sequencing results were reliable (Fig. 6).

Dual-luciferase activity assay

In the 3 comparison groups of LP42 vs. SLP42, SP42 vs. LP42, and SP42 vs. SLP42, 3 DEG-DEM pairs PRDM1-novel_338, TRIM71-novel_320 and PTPN13-novel_339 were randomly selected for dual luciferase activity assay, respectively. In LP42 vs. SLP42 group, novel_338 could bind to PRDM1-WT plasmids to reduce the dual-luciferase activity. Co-transfection of the plasmids carrying PRDM1-MuT with novel_338 mimics did not significantly change the dual-luciferase activity, indicating that PRDM1 was the target gene of novel_338 (Fig. 7A). In the SP42 vs. LP42 group, novel_320 and TRIM71 had two binding sites predicted by the website, so the mutant sequences MuT-1 and MuT-2 were designed for the two sites, respectively. Dual luciferase assay showed that novel_320 could bind to TRIM71-WT to reduce the dual luciferase activity. Mut-1 site had no significant change in dual luciferase activity, indicating that mut-1 site was a binding site, and mut-2 site had reduced dual luciferase activity, indicating that mut-2 site was not a binding site. Therefore, novel_320 can target TRIM71, and the binding site was the sequence corresponding to MuT-1 (Fig. 7B). In addition, a binding relationship between PTPN13 and novel_339 was demonstrated in the SP42 vs. SLP42 group (Fig. 7C).

Discussion

As an adaptive outcome of animals to adapt to environmental changes, seasonal estrus characteristics are crucial for the survival of offspring individuals and the continuation of species [26]. However, the molecular

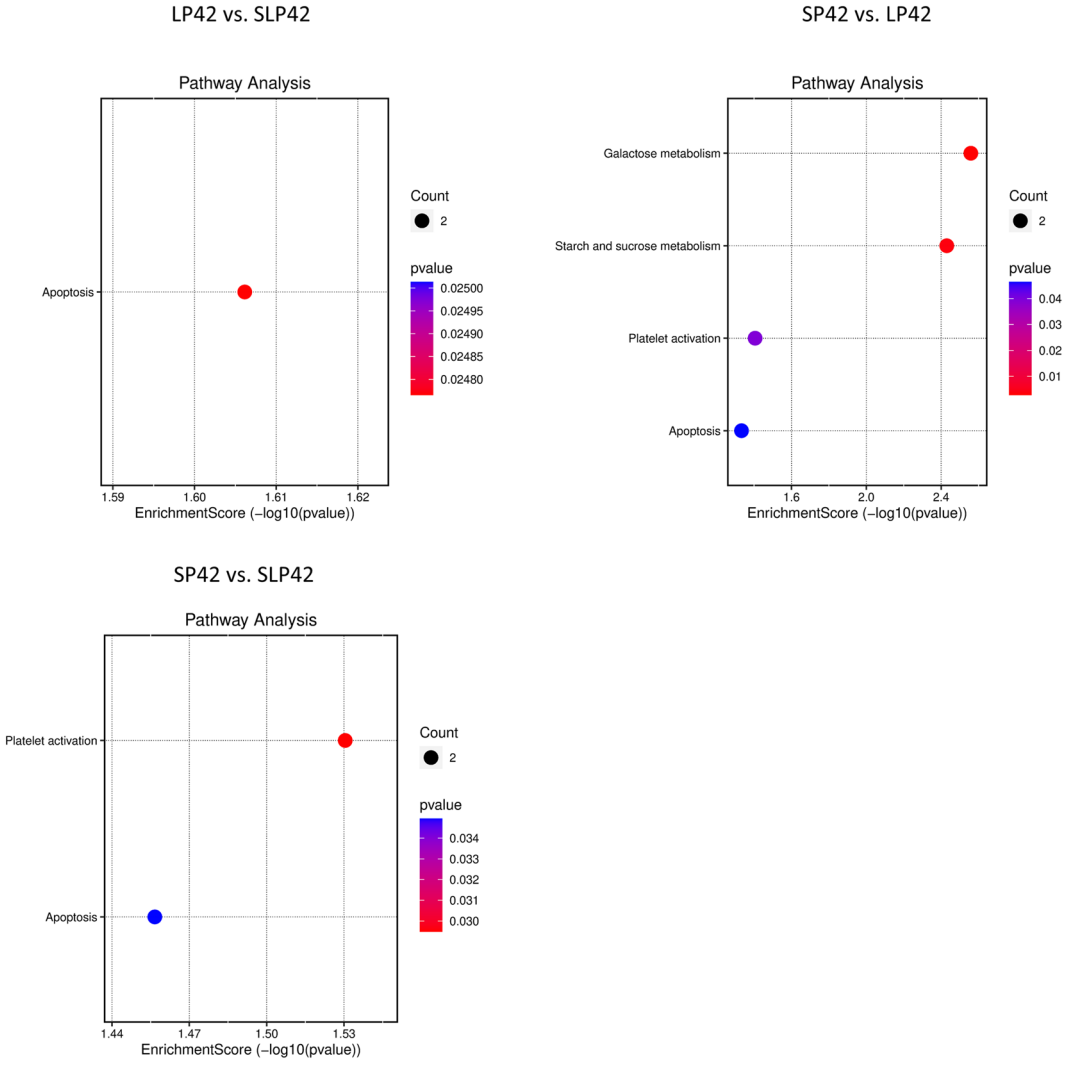

Fig. 5 Enriched KEGG of genes targeted by DEM in 3 groups. Enriched KEGG of genes targeted by DEM in (**A**) LP42 vs. SLP42 (**B**) SP42 vs. LP42 (**C**) SP42 vs. SLP42. The horizontal and vertical coordinates represent the – lg (p – Value) and KEGG pathway of the enriched genes, respectively

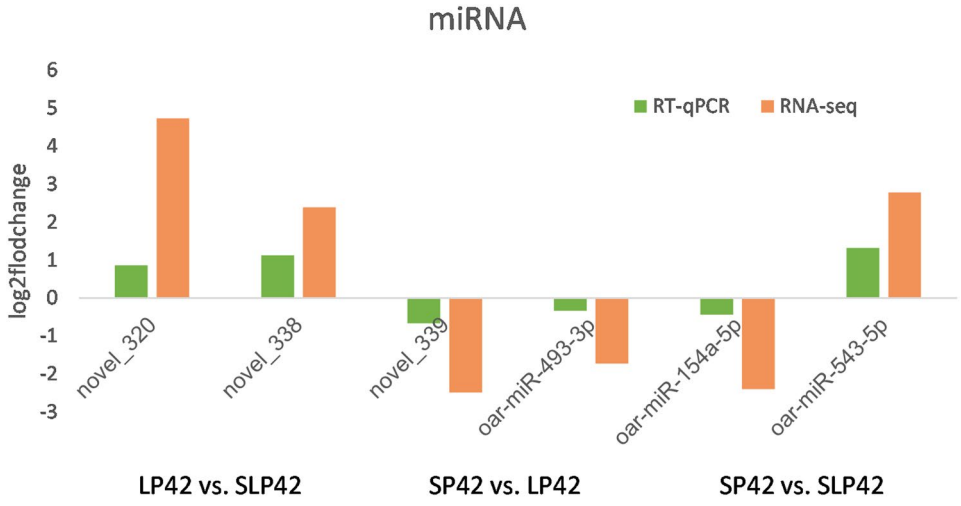

Fig. 6 Validation of RNA-seq data using RT-qPCR. RNA-Seq and RT-qPCR results of two selected differentially expressed miRNA in the uterus of Sunit sheep at different photoperiods

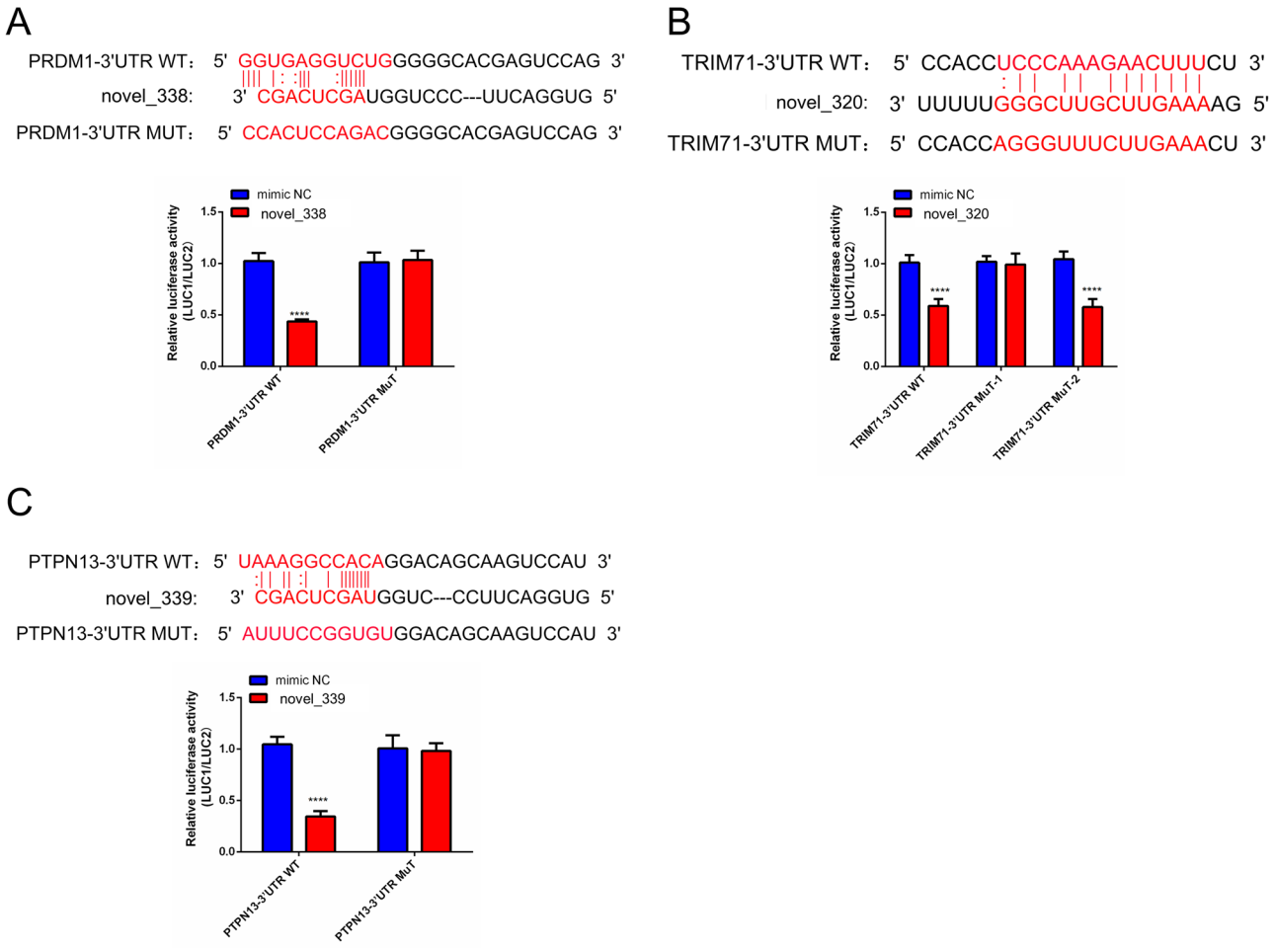

Fig. 7 The results of dual-luciferase activity assay. (**A**) Schematic illustration of PRDM1-WT and PRDM1-MuT luciferase reporter vectors (up); the relative luciferase activities were detected in HEK-293T cells after co-transfection with PRDM1-WT or PRDM1-MUT and novel_339 mimics respectively (down). (**B**) Schematic illustration of TRIM71-WT and TRIM71-MuT luciferase reporter vectors (up); the relative luciferase activities were detected in HEK293T cells after co-transfection with TRIM71-WT or TRIM71-MUT and novel_320 mimics respectively (down). (**C**) Schematic illustration of PTPN13-WT and PTPN13-MuT luciferase reporter vectors (up); the relative luciferase activities were detected in HEK293T cells after co-transfection with PTPN13-WT or PTPN13-MUT and novel_339 mimics respectively (down)

mechanisms underlying seasonal estrus in mammals have not been elucidated in detail. As the place where pregnancy occurs, the importance of the uterus for reproduction is self-evident. So far, it has been recognized that the health of the uterus is crucial for the reproductive process, and in the evaluation of fertility of patients with uterine malformations, it has been found that uterine malformations can cause severe reproductive dysfunction [27]. Circadian clock gene oscillation has been observed in the mouse uterus, and a study by Ono et al. found that muscle Arnt-like protein-1 (Bmal1), an important protein that regulates transcription of core clock genes, that the presence of depleted decidual cells protects against trophoblast invasion. Highlighting the importance of the endometrial clock throughout pregnancy, experiments in mice demonstrated that animals with uterus-specific Bmal1 deletion also had poor placental development, and these mice also had intrauterine stillbirth, suggesting the importance of the uterus in reproductive physiology outside pregnancy [28]. In addition, in more branched fields, the interaction between the uterus as part of the maternal system and the embryo is also valued and considered to be the basis of the process leading to a successful pregnancy [29]. Notably, the non-pregnant uterus also plays an important role in the regulation of reproduction during physiological processes in the organism. Previous studies have shown that the non-pregnant uterus exhibits a pattern of undulating activity throughout the menstrual cycle, which is associated with successful reproduction in spontaneous cycles and assisted reproduction, and is innervated by steroid hormones [30]. Little is known about the involvement of the uterus in photoperiod-induced seasonal reproduction, and in our previous study, the differential expression profiles of mRNA, long

non-coding RNA (lncRNA), and circular RNA (circRNA) in the uterus of seasonal breeding sheep were outlined and discussed [4], while the role of uterine miRNA in the context of seasonal estrus in sheep has not been reported. Therefore, the main purpose of this study is to screen miRNA markers related to seasonal reproduction in sheep uterus, and to conduct a preliminary study on their potential target gene regulatory network, so as to provide a reference for research in related directions.

In this study, a total of 529 miRNA were identified in the 3 control groups, of which, the newly discovered miRNA accounted for about 80.15% of the total miRNA, and the known miRNA accounted for only 19.85%. According to miRBase database (http://mirbase.org/index.shtml), the current 153 mature known miRNA, we only had 105 stem ring sequence. When the 3 biological replicate samples in each treatment group were identified, 104, 102, 103 and 104 stem-loop structures were identified for LP42-3, SP42-3, SLP42-1 and SLP42-3, respectively, and 105 stem-loop sequences were identified for the remaining individuals. It is worth noting that miR-1193 was not detected in the stem-loop structure. It has been found to be associated with the occurrence and development of various diseases [31–33], including cervical cancer, etc [34]. However, its function under normal physiological conditions is poorly understood. Unfortunately, in our study, especially in the SLP42 group, miR-1193 was detected only in SLP42-2 individuals, so the relationship between miR-1193 and sheep photoperiod and uterus could not be determined, and further research is needed to explore.

It is well known that the mode of action in which miRNA exerts their primary function is to influence phenotypic changes by regulating the expression of target genes [35]. In this study, the DEM-DEG co-expression network was constructed, and the KEGG and GO enrichment of DEM targets of the 3 comparisons were similar. The most significant signaling pathways were apoptosis and platelet activation signaling pathways, and it is hypothesized that DEM may affect seasonal estrus in sheep by inhibiting target genes through these two signaling pathways. Studies have shown that pregnancy loss in mice may be associated with apoptotic processes, suggesting an important role of apoptotic processes in maintaining uterine homeostasis [36]. Other studies have demonstrated that pineal excision and continuous light exposure can inhibit uterine implantation in rodents and increase the level of apoptosis in the uterus of rats with continuous light exposure [37], which to some extent reveals the regulatory relationship of photoperiod on uterine reproductive physiology.

In our study, three new miRNA, novel_320, novel_338 and novel_339, were identified, targeting the largest number of target genes. In view of the widespread existence and expression of miRNA in sheep seasonal estrus events, this suggests that miRNA may be a biomarker of seasonal estrus. novel_338-PRDM1, novel_320-TRIM71 and novel_339-PTPN13 are differentially expressed miRNA-gene pairs of LP42 vs. SLP42, SP42 vs. LP42 and SP42 vs. SLP42, respectively. Among them, previous studies have shown that members of the positive regulatory domain-containing (PRDM) family encode histone methyltransferases that are essential for reproduction [38], including primordial germ cell specification and differentiation [39–41], embryo development [38], and adult germ cell meiotic recombination [42]. In our study, *PRDM1* expression was found for the first time to appear to be affected by photoperiod changes, suggesting its potential role in seasonal estrous in sheep. Considering that the protein encoded by *PRDM1* is a transcription factor [43], it can be considered whether it directly combines the promoter region of photoperiod related genes (such as *Eya3* and *TSHβ*) to regulate their transcriptional activity, thus affecting the start or termination of seasonal reproduction in subsequent studies. *TRIM71* is an RNA-binding protein with ubiquitin ligase activity. In mammals, *TRIM71* plays a role in a variety of physiological processes, including cell cycle regulation, embryonic stem cell (ESC) self-renewal and pluripotent stem cell reprogramming [44]. TRIM71 deletion has been shown to cause germ cell loss during mouse embryogenesis and is associated with male infertility [45]. Although the function of TRIM71 in the uterus is still unknown, our study has identified a potential role of TRIM71 in photoperiod control. Given its important role in reproduction and RNA-binding properties, TRIM71 may play a specific role in photoperiod regulation in the uterus. PTPN13 is a class I non-receptor protein tyrosine phosphatase discovered in 1994 [46]. At present, the research focus is mainly on the physiology of cancer and other diseases [46, 47]. Studies have shown that PTPN13 acts as a tumor suppressor in a variety of cancers [48, 49]. In our study, the expression level of PTPN13 was found to be increased under long light condition. Considering that Sunit sheep are short-day breeding animals, PTPN13 may play a potential role in physiological control during the non-breeding season. Finally, this study used dual luciferase reporter gene technology to preliminarily identify possible targeting relationships between novel_338 and PRDM1, novel_320 and TRIM71, and novel_339 and PTPN13, providing a basis for further understanding the role of the uterus in seasonal reproduction in sheep.

To our knowledge, this is the first attempt to link the miRNA of the sheep uterus to the photoperiod. It should be emphasized that in addition to the genes already mentioned above, other genes are also worthy of attention. For example, novel_322 also had multiple target genes in the SP42 vs. LP42 comparison group. Among them, RUSC2

genes may be involved in endometrial proliferation and decidualization by regulating chromatin accessibility or epigenetic modification. Studies have shown that chromatin remodeling factors (such as CHD family proteins) can maintain reproductive cell self-renewal by enhancing chromatin openness and mRNA stability, suggesting that RUSC2 may affect uterine function through a similar mechanism [50]. In addition, studies have shown that VANGL1 plays an important role in embryonic development, cell adhesion, migration, and polarity. The results of animal experiments showed that silencing the expression of VANGL1 in uterus would inhibit mouse embryo implantation, which proved that VANGL1 existed as a necessary condition for mouse embryo implantation in uterus [51]. In our study, linking it to photoperiodic mediated miRNA (novel_322) further illustrates the important potential role of this gene in uterine-related physiological processes. In addition, AADACL3 has also recently been reported to exist as a candidate gene for important economic traits in sheep, and may be related to animal production and reproductive performance [52]. In this study, the association between AADACL3 and reproductive performance is located in the uterus and is related to photoperiod mediated seasonal estrus, which provides the direction and basis for further study of the function of this gene. Of course, there are some limitations to the current study. First, although we have identified some miRNA and their target genes that may be associated with seasonal estrus in sheep, especially in the womb, direct experimental evidence from individual sheep is lacking. Second, the downstream mechanism of uterine reproductive physiology involved in PRDM1, TRIM71 and PTPN13 still needs to be further explored. Finally, our sample size is not large enough and group representation may be limited.

Conclusion

This study provided the expression profiles of miRNA in sheep utero under different photoperiods, and combined with previous studies, identified several miRNA-gene pairs that may be involved in sheep seasonal estrus, such as novel_338-PRDM1, novel_320-TRIM71 and novel_339-PTPN13. These results provide a valuable reference for understanding the molecular mechanism of seasonal estrus in sheep.

Abbreviations

AANAT	Aralkylamine N-acetyltransferase
AADACL3	Aryacetamide deacetylase-like 3
circRNA	Circular RNA
COC	Cumulus-oocyte complexes
DEM	Differentially expressed miRNA
DEG	Differential expression gene
Hbegf	Heparin binding epidermal growth factor like growth factor
LP	Long photoperiod
LD	Long-day
Lamc1	Laminin gamma 1
lncRNA	Long non-coding RNA
miRNA	microRNA
ncRNA	Non-coding RNA
PRDM1	PR domain zinc finger protein 1
PTPN13	Protein tyrosine phosphatase non-receptor type 13
RUSC2	Homo sapiens RUN and SH3 domain containing 2
SP	Short photoperiod
SLP	Short transfer to long photoperiod
sRNA	Small RNA
SD	Short-day
STC1	Stanniocalcin-1
TRIM71	Tripartite motif containing 71
VANGL1	Vang-like protein 1
YAP1	Yes-associated protein 1
3'UTR	3' untranslated region

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions

F. Y. and J. L. conceived the study and wrote and prepared the manuscript. Y. L., R. D., X. H., and P. W. analyzed the data and provided suggestions. Y. L. and M. C. Z. directed the study and conducted the review. All authors read and approved the final manuscript.

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Data availability

The differentially expressed genes analyzed during the current study are from previous work and the datasets are available in the SRA repository (accession number: SRP241010). miRNA datasets are available in the GSA repository (accession number: CRA020735).

Declarations

Ethics approval and consent to participate

All experiments were performed following the relevant guidelines and regulations set by the Ministry of Agriculture of the People's Republic of China. Ethical approval on animal survival was given by the animal ethics committee of CAAS-IAS (No. IAS2018-3). The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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