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RESEARCH ARTICLE

Identification of LncRNA Expression in the Estrous Cycle of Qira Black Sheep and Its Combination with miRNA Analysis

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Abstract

In order to investigate the expression of lncRNAs in the ovaries of Qira black sheep at different stages of the estrous cycle, Qira black sheep were used as experimental materials in this experiment, and after estrus synchronization, ovarian tissues at four different stages of the estrous cycle were collected to extract total RNA.The samples at different stages were first subjected to genome-wide analysis using RNA-seq technology; target genes of lncRNAs were predicted using co-expression methods; and then GO and KEGG analysis of target genes was performed. Genes related to the estrous cycle of Qira black sheep were also selected to study their transcriptional differences. Finally, lncRNA-miRNA and mRNA-lncRNA-miRNA interaction networks were established to further analyze the effect of lncRNA alignment on Qira black sheep reproduction. The results showed that in the differentially expressed part, 14 lncRNAs were differentially expressed in Estrous VS Diestrus; the differential expression levels of lncRNAs in the two comparison groups of Estrous VS Metestrus and Estrus VS Proestrus were 18 and 24, respectively. The results of GO and KEGG functional enrichment analysis showed that differentially expressed lncRNAs and their target genes were mainly involved in reproduction-related pathways such as retinol metabolism, ovarian sterol production and endosterol biosynthesis. In the combined analysis of lncRNA-miRNA and mRNA-lncRNA-miRNA, genes related to reproduction, such as LNC011583, LNC003443 and bta-miR-202, were found, thus it can be seen that lncRNAs have some effect in the reproduction of Qira black sheep.

Keywords: *Estrous, Ovary, LncRNA, Qira black sheep*

Qira Kara Koyunlarında Östrus Siklusunda LncRNA Ekspresyonunun Belirlenmesi ve miRNA Analizi İle Kombinasyonu

Öz

Bu çalışmada, Qira kara koyunlarının ovaryumlarında lncRNA'ların ekspresyonunun araştırılması için materyal olarak östrus siklusunun farklı aşamalarında olan Qira kara koyunları kullanıldı. Total RNA eldesi amacıyla, östrus senkronizasyonunu takiben siklusun dört farklı aşamasından ovaryum dokuları toplandı. Farklı aşamalardaki örnekler, önce RNA-seq teknolojisi kullanılarak genom çapında analize tabi tutuldu, lncRNA'ların hedef genleri birlikte ifade edilme yöntemleri kullanılarak tahmin edildi ve takiben hedef genlerin analizi GO ve KEGG ile gerçekleştirildi. Qira kara koyunlarında östrus siklusu ile ilgili genler, transkripsiyonel farklılıklarının araştırılması amacıyla seçildi. Son olarak, lncRNA hizalamasının Qira kara koyunlarında üreme üzerine etkisini daha fazla analiz etmek için lncRNA-miRNA ve mRNA-lncRNA-miRNA etkileşim ağları kuruldu. Sonuçlar, çeşitli ekspresyon bölgelerinde, 14 lncRNA'nın diöstrusa göre östrusta farklı şekillerde eksprese edildiğini gösterdi ve metöstrusa karşı östrus ve proöstrusa karşı östrus gruplarında lncRNA'ların diferansiyel ekspresyon seviyeleri sırasıyla 18 ve 24 saptandı. GO ve KEGG fonksiyonel zenginleştirme analizleri, farklı şekilde eksprese edilen lncRNA'ların ve bunların hedef genlerinin, esas olarak retinol metabolizması, yumurtalık sterol üretimi ve endosterol biyosentezi gibi üreme ile ilgili fonksiyonlarda yer aldığını gösterdi. lncRNA-miRNA ve mRNA-lncRNA-miRNA'nın kombine analizi sonucu, LNC011583, LNC003443 ve bta-miR-202 gibi üreme ile ilgili genler saptandı, dolayısıyla lncRNA'ların Qira kara koyunlarının üremesinde bir miktar etkisinin olduğu görülebilir.

Anahtar sözcükler: *Östrus, Ovaryum, LncRNA, Qira kara koyunu*

INTRODUCTION

In general, the boundary of mammalian singleton and multiple birth traits is relatively obvious, but there is diversity in this trait in sheep, and it is of great scientific value to study the mechanism of multiple birth in sheep [1].

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Qira black sheep is a multiparous fine landrace mainly producing lamb skin and is a characteristic sheep in Xinjiang. Due to its special ecological environment, it is characterized by resistance to coarse feeding, early sexual maturity, perennial estrus, and high fertility [2-4]. Among them, annual estrus and high reproductive rate are prominent breed characteristics of Qira black sheep and these characteristics are rare among sheep breeds in Xinjiang. Therefore, it is important to fully study and develop the excellent performance and multiparity of Qira black sheep for the long-term development of Xinjiang sheep.

Long non-coding RNAs (lncRNAs) are non-coding RNAs greater than 200 nucleotides in length. Studies have shown that lncRNAs play an important role in regulating cellular processes, including transcriptional regulation, intracellular substance transport and chromosome remodeling [5]. Their low expression, poor conservation between species and cellular expression specificity of lncRNAs, make them hot spots [6,7]. LncRNAs are involved in the regulation of various processes in the nucleus and cytoplasm, involving biological development processes such as immunology, neurobiology, cancer and stress [8], so the majority of studies on lncRNAs are still the related regulation and effects of lncRNAs on human cancers.

In recent years, more and more studies have shown that lncRNAs play an important role in sheep reproduction [9]. During the estrous cycle, follicles undergo multiple processes such as development, maturation, ovulation, and luteinization, and the development of follicles is regulated by many regulatory factors [10]. Zheng et al.[11] demonstrated the role of lncRNAs in the initial stages of follicular development. But the expression of lncRNAs in various stages of follicles is unknown. The aim of this study was to analyze and compare the expression of lncRNAs in the ovaries of Qira black sheep at different stages of the estrous cycle. To understand the regulatory mechanism of lncRNAs on follicles at different stages of their growth and development. And the effect of lncRNA countermeasures on the fecundity of Qira black sheep. It provides some reference for future studies on the regulation of follicular growth and development and the effect of lncRNAs on fertility.

Material and Methods

Ethics Statement

All experimental procedures were approved (A2016-085) by the Ethics Committee of Animal Experimental of the First Affiliated Hospital, Shihezi University School of Medicine. All methods were carried out in accordance with relevant guidelines and regulations. During the experiment, every effort was made to minimize suffering by the animals.

Sample Collection and Preparation

In this experiment, Xinjiang Qira Black Sheep, which is multiparous and productive, was selected as the experimental animal. Twelve healthy Qira black sheep (about 3-4 years old and weighing about 35-40 kg and all of them are multiparous) were selected from sheep farms in Qira County, Xinjiang Province, China. All sheep were kept under the same conditions with free access to food and treated with a vaginal sponge (injected with human synthetic progesterone) to synchronize the estrous cycle. Estrus was monitored twice daily by oestrus rams and vaginal examination. After determination of the second estrus, ovarian tissues were collected separately according to the following criteria according to groups: day 0 is estrus; 9-10 days before estrus is diestrus; 1-2 days before estrus is proestrus; and 2-3 days after estrus is diestrus. Then follow the test needs, follicular development rules and reproductive endocrine changes, the ovaries of the four periods were divided into four groups with three replicates in each group: these were diestrus (QD1, QD2, QD3), proestrus (QP1, QP2, QP3) estrus (QE1, QE2, QE3), and metestrus (QM1, QM2, QM3) *(Table 1)*. After the test sheep were slaughtered, ovarian tissue with follicles was obtained.

RNA Library Construction and Sequencing

Total RNA was extracted from each sheep ovarian tissue according to the manufacturer's recommendations, and the extracted RNA was then subjected to quality testing and integrity assessment (Agilent Technologies, CA, USA). A total of 3 μg RNA was used for each sample as input material for RNA sample preparation, for a total of 12 samples. First, ribosomal RNA was removed by the Epicenter-Ribo-zero rRNA Removal Kit (Epicentre, USA), and residues without rRNA were cleared by ethanol precipitation. Subsequently, sequencing libraries were generated using rRNA depleted RNA using the NEBNext Ultra Directed RNA Library Preparation Kit for Illumina (NEB, USA) according to the manufacturer's recommendations. Finally, sequencing libraries constructed using appropriate PCR cycles were sequenced by Novogene (Beijing, China) on an Illumina genome analyzer. Samples were averaged for subsequent assays.

Table 1. *Experimental grouping*

Grouping	Diestrus (QD)			Proestrus (QP)			Estrus (QE)			Metestrus (QM)		
Reoeat	QD1	QD2	QD3	QP1	QP2	QP3	QE1	QE2	QE3	QM1	QM2	QM3
Quantity	1	1	1	1	1	1	1	1	1	1	1	1

Data Analysis

Quality Control and Screening of lncRNAs: Raw data in fastq format (raw reads) were first processed through an in-house perl script to obtain clean data, then bowtie2 (v2.2.8) *(http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml)* was used to build the index of the reference genome, and finally HISAT2(v2.0.4) [12] *(http://daehwankimlab.github.io/hisat2/)* was used to align the clean reads at the paired ends to the reference genome (reference genome and gene model annotation files were downloaded directly from the genome website). StringTie (v1.3.1) [13] *(http://ccb.jhu.edu/software/stringtie/#contact)* was used, the mapped reads for each sample were assembled. Finally, we predicted target genes by co-expression.

Differential Expression and Functional Enrichment: First, by observing the distribution of differential transcripts on the chromosome, and the linked expression of their surrounding transcripts, we can pick out the target genes related to the study. Then, hierarchical cluster analysis was performed by FPKM expression levels of differential transcripts under different experimental conditions. Finally, through the volcano plot, the overall distribution of differential transcripts or genes can be visually seen. In this experiment, we selected candidate lncRNAs for subsequent analysis based on the transcriptional assembly results of lncRNAs, combined with the functional characteristics of lncRNA non-coding proteins as well as their structural characteristics. Differential expression in gene expression data was determined using Cuffdiff (v2.1.1) *(http://cole-trapnell-lab.github.io/cufflinks/manual/)* software and the Ballgown suite, where transcripts with $P<0.05$ were designated as differentially expressed.

Gene Ontology (GO) enrichment analysis of differentially expressed genes or lncRNA target genes was implemented by the GOseq R software package. We used KOBAS software to test the statistical enrichment of differential expression genes or lncRNA target genes in KEGG pathways [14]. KEGG is a database resource for understanding high-level functions and utilities of the biological system [15] *(http://www.genome.jp/kegg/)*.

lncRNA-miRNA-mRNA Association Analysis: LncRNAs, as a type of ceRNAs (competing endogenous RNAs) [16,17], can competitively bind miRNAs with genes, so we searched for lncRNA-miRNA-gene pairs that also possess miRNA binding sites for combined analysis.

qRT-PCR: QRT-PCR was used in this experiment to validate gene expression levels. The amplification procedure was performed using a two-step method: 95°C 30 s; 95°C 5s, 60°C 30 s, for 45 cycles in total. Each sample was repeated three times for a total of 12 samples. Information about primers for qRT-PCR using GAPDH as the reference gene is shown in *Table 2*. Finally, the statistical analysis of the test data was performed using the $2^{-\triangle\triangle ct}$ method for relative quantitative analysis, and the statistical software SPSS 17.0 was used for significance test.

Result

Overview of Sequencing Data

In total, there were 12 libraries in this study, as shown in *Table 1*, resulting in 1,694,019,460 raw reads. In order to ensure the quality of the data and remove the adapter-bearing, low-quality reads inside, 1,647,629,538 clean reads were obtained after screening. Among them, clean reads accounted for 99.99% of raw reads.91.73% ~ 93.99% of clean reads in all libraries were successfully mapped to the reference genome *(Table 3)*. To further validate the data, we selected 2 differentially expressed lncRNAs targeting reproduction-related genes and determined their expression levels by qRT-PCR, and the results were verified to be consistent with those obtained by sequencing *(Fig. 1)*.

Screening of lncRNAs

A total of 11981 lncRNAs, 13,831 TUCPs and 22,824 mRNAs were identified in all samples for further analysis *(Fig. 2-A)*. We obtained Pearson correlation coefficients between 0.875 and 0.949 among the samples, of which the highest correlation with QD1 is QM3 (0.949) *(Fig. 2-B)*. The results showed that the expression levels of ovaries were almost consistent during the four stages of the estrous cycle *(Fig. 3-A)*, while the expression levels of lncRNAs and TUCP were much lower than those of mRNAs *(Fig. 3-B)*.

Differential Expression Analysis

In this experiment, we identified 11981 lncRNAs and 22,824 mRNAs from the ovaries of Qira Black sheep for further

Table 2. *Lnc-010801, lnc-008562 and internal reference amplification primers*

Primer	Gene Sequence	Fragment Size/bp
GAPDH-F	CCTGCCAAGTATGATGAGAT	119
GAPDH-R	TGAGTGTCGCTGTTGAAGT	
LNC-010801-F	GCGGGAAGTCCTGTCCTCT	124
LNC-010801-R	CGAAAAGTCCGAAACACCAG	
LNC_008562-F	CGCCAAATCGGAGTAAACA	100
LNC_008562-R	AATTCATCCAGGCAGGGTC	

Table 3. *The summary of sample data quality*

Sample Name	Raw Reads	Clean Reads	Clean Bases	Error Rate(%)	Q20 (%)	Q30 (%)	GC Content (%)
He1	90840870	88180586	13.23G	0.01	97.79	94.2	49.09
He2	120787312	117362696	17.6G	0.01	97.7	93.94	48.18
He3	114504810	111147030	16.67G	0.01	97.88	94.37	48.6
QD1	121671830	118548894	17.78G	0.01	97.73	94.03	48.26
QD2	111730482	108979944	16.35G	0.01	97.8	94.18	47.82
QD3	123522800	121471712	18.22G	0.01	97.15	93.23	48.47
QE1	109926982	106702328	16.01G	0.01	97.66	93.95	48.42
QE2	115505728	112006036	16.8G	0.01	97.75	94.15	48.63
QE3	121606340	118252002	17.74G	0.01	97.72	94.08	47.17
QM1	113856982	110990072	16.65G	0.01	97.78	94.15	49.28
QM2	121209526	117616398	17.64G	0.01	97.62	93.8	47.37
QM3	116530402	113689300	17.05G	0.01	97.75	94.1	47.42
QP1	110697120	107454358	16.12G	0.01	97.85	94.35	50.25
QP2	103318274	100061160	15.01G	0.01	97.82	94.27	49.01
QP3	98310002	95167022	14.28G	0.01	97.44	93.39	47.96

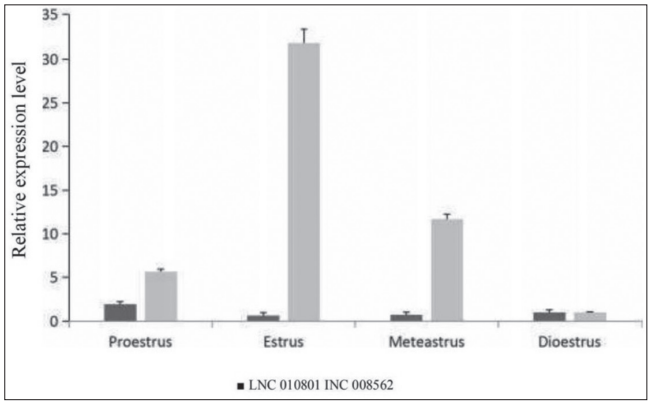

Fig 1. QRT-PCR validation of differentially expressed genes

was the most differentially expressed, with 15 up-regulated and 9 down-regulated. The remaining two comparison groups also had up- and down-regulated genes, respectively. From the lncRNA differential expression clustering results plot *(Fig. 4-D)*, we can see that the three proestrus groups (QP1/2/3) and the three estrus groups (QE1/2/3) were clustered, indicating that the differences between these two groups were quite different.

GO and KEGG Enrichment Analysis

In this experiment, target genes were predicted by means of co-expression. A total of 13,0152 potential target genes were identified by co-expression, and GO and KEGG analyses were performed on these potential target genes.

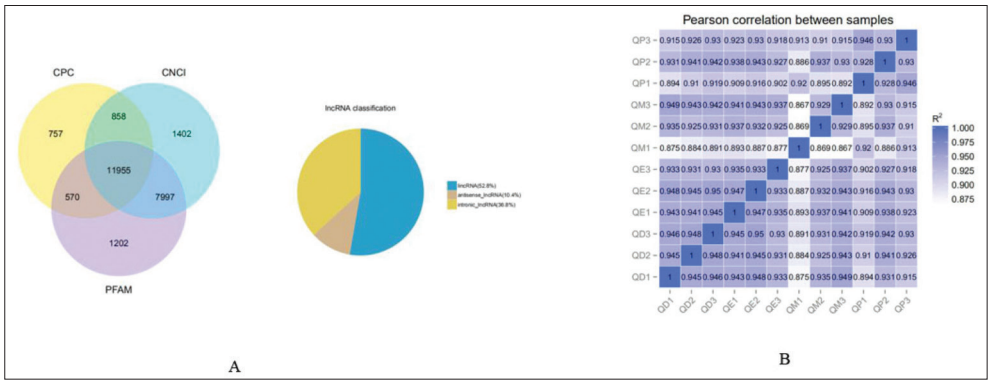

Fig 2. Screening results **(A)** and correlation expression **(B)**

analysis. Differential expression analysis was performed using Ballgown. In this experiment, we will elaborate the overall differential expression of lncRNAs from three comparison groups: QE_VS_QD, QE_VS_QM and QE_VS_QP.

We obtained differential expression results for the three comparison groups *(Fig. 4-A,B,C)*. Among them, QE_VS_QP

After obtaining the 56 differentially expressed lncRNAs, the target genes of these lncRNAs were subjected to GO enrichment analysis and their functions were described. GO analysis showed that all were divided into BP (biological process), CC (cellular component) and MF (molecular function). From the GO enrichment histogram *(Fig. 5-A)*, the GO terms of these differentially expressed lncRNA

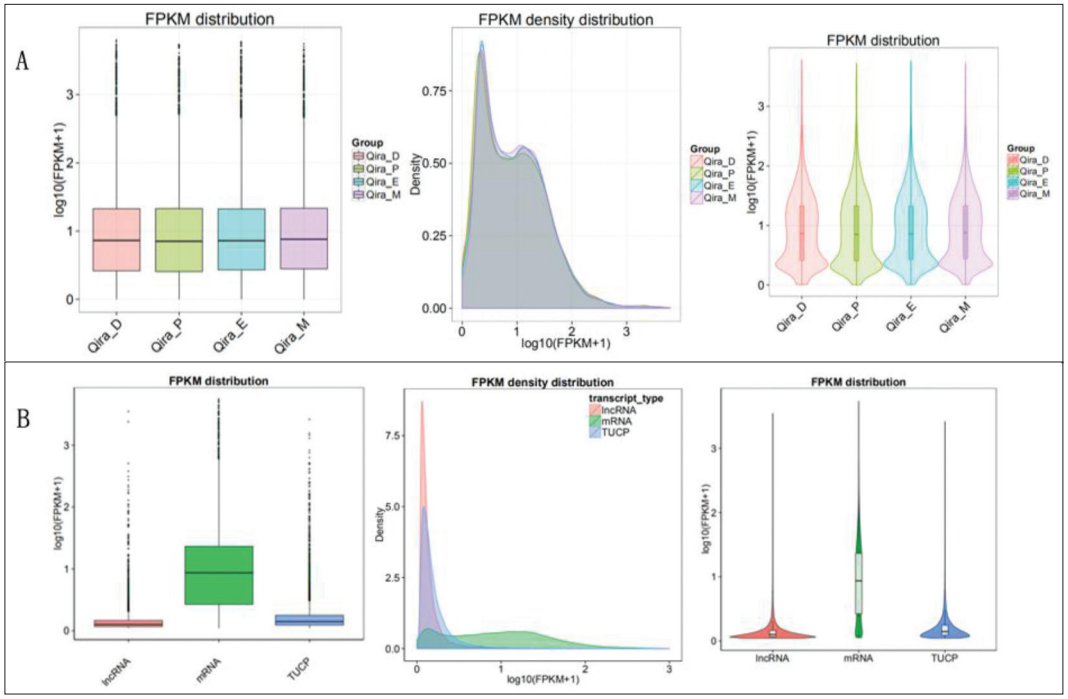

Fig 3. Comparison of expression levels of each group **(A)** and each gene **(B)**

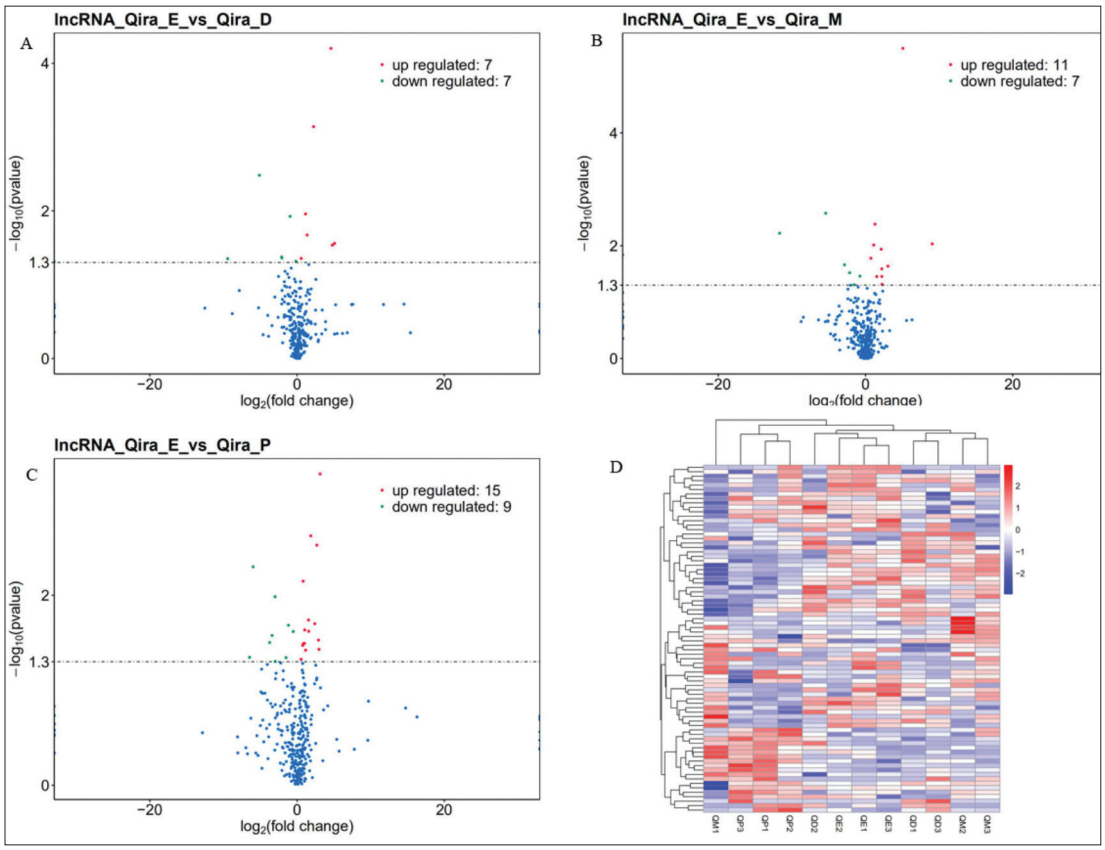

Fig 4. Differential expression in each comparison group **(A,B,C)** and differential expression clustering results **(D)**

target genes are enriched for several processes, such as: transcription, DNA-dependent, RNA biosynthetic process, neutral lipid catabolic process, etc.

The scatter plot is a graphical presentation of the KEGG enrichment analysis results, and we picked the 20 pathway entries with the most significant enrichment for

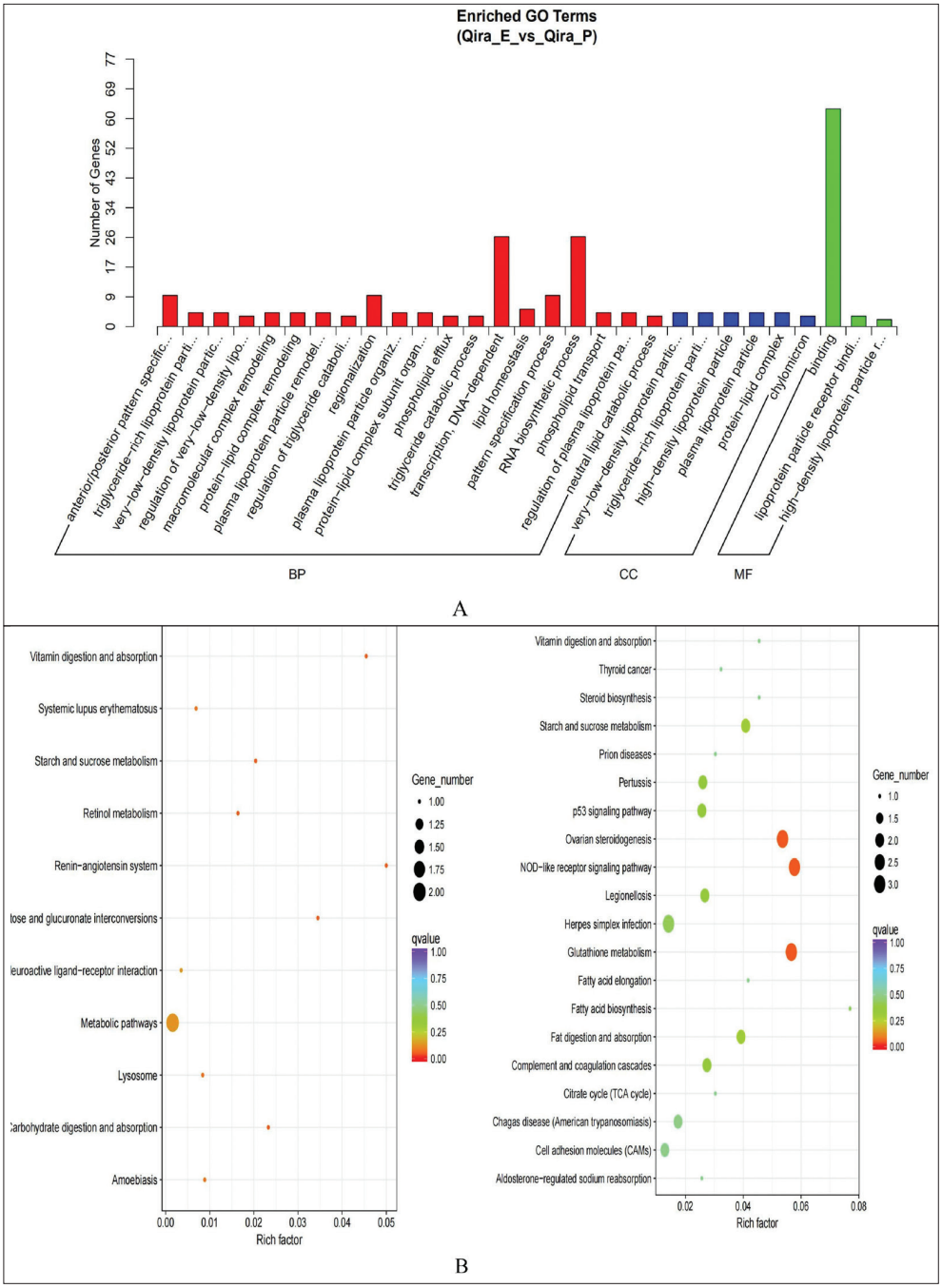

Fig 5. GO **(A)** and KEGG **(B)** analysis results of differentially expressed lncRNA target genes

presentation in this plot. KEGG analysis of heterologously expressed co-expressed lncRNA target genes showed that they were rich in pathways related to reproduction: Jak-STAT signaling pathway, retinol metabolism, Ovarian steroidogenesis, etc. *(Fig. 5-B)*.

LncRNA-miRNA-mRNA Association Analysis

By constructing an interactive network diagram of miRNA-lncRNAs (miRNAs have been proposed in other literature), we can better understand the involvement and function of lncRNAs in the estrous cycle of Qira Black Sheep. We constructed the interaction network of miRNA-lncRNAs separately for the three comparison groups *(Fig. 6-A,B,C)*. We obtained 248 miRNA-lncRNA interaction combinations with potential relationship compared by QE_VS_QP, of which LNC001917, LNC011056, LNC003367, LNC009395, LNC003443, etc. could interact with multiple different miRNAs, so they were in the middle position, and it can be speculated that these genes play a role in the regulation of estrous cycle in Qira black sheep. However, in the QE_VS_QD and QE_VS_QM comparison groups, we also obtained 175 and 22 interaction network graphs with potential relationships, respectively.

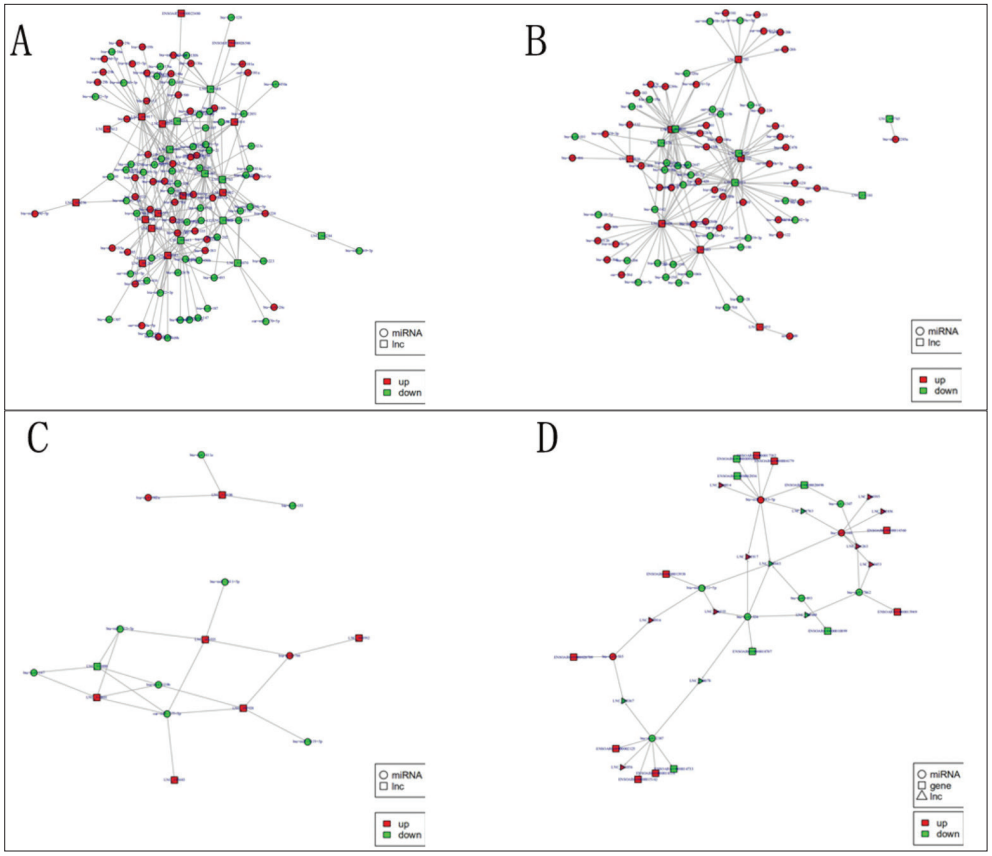

Fig 6. Target lncRNA-miRNA interaction net **(A,B,C)** and Target miRNA-lncRNA-mRNA interaction net **(D)**

We also constructed an interaction network map of miRNA-lncRNA-mRNA *(Fig. 6-D)*. We obtained that in QE_VS_QP, bta-miR-1343-5p, bta-miR-2411-5p, bta-miR-2387, etc. are at the comparative core position because they are able to interact with the most genes. Among them, LNC001917, LNC003443 and LNC001763 can interact with multiple different miRNAs, so we speculated that these genes may play a role in the regulation of the estrous cycle in Qira Black sheep.

Discussion

From the results of GO and KEGG enrichment analysis in this experiment, it can be seen that the most significantly different GO term is the term of the cofactor. However, it has been shown that [18], the cofactors may catalyze the synthesis of steroid hormones by increasing enzyme activity. In the results of KEGG enrichment analysis of lncRNAs, there is also a pathway of cytochrome P450 enzymes, which are expressed in both ovarian testes and play an important role in the synthesis of anabolic steroids and maintenance of sex hormones [19]. In KEGG enrichment analysis of lncRNA target genes, we obtained pathways related to fecundity as: retinol metabolism, ovarian endosterol production and endosterol biosynthesis. Studies have found that retinol and its derivatives play an important role in ovarian steroidogenesis, oocyte maturation and early embryonic development, are abundant in cumulus granulosa cells, and are involved in signaling related to ovarian development [20-22].

In the combined analysis of lncRNA-miRNAs, we obtained that bta-miR-26b was present in the gene interacting with LNC011583 in the QE_VS_QD comparison, which was pointed out by [23] that it belongs to the miR-26 family and plays a key role in estrogen stimulation [24]. So we speculated that LNC011583 gene, which interacts with bta-miR-26b, may play a role in estrogen stimulation, thereby affecting the content of estrogen. We also obtained oar-miR-432 and bta-miR-202 genes interacting with LNC003443 gene in QE_VS_QP comparison. Among them, the oar-miR-432 gene is likely to function by directly or indirectly affecting gonadotropin-releasing hormone (GnRH) activity associated with reproductive hormone release [25]. It has been proposed [26] that the bta-miR-202 gene is expressed only in the gonads. On the one hand, we can speculate that when LNC003443 gene interacts with oar-miR-432, it may have an effect on the reproduction of Qira Black sheep by affecting the activity of gonadotropin-releasing hormone (GnRH); on the other hand, when LNC003443 gene interacts with bta-miR-202 gene, it may have an effect on the ovary and thus affect the reproduction of Qira Black sheep.

In this experiment, the target genes of lncRNA-controlled

reproduction-related pathways were obtained by GO and KEGG enrichment analysis. The results showed that the target genes of lncRNAs had an effect on reproductive hormone synthesis such as steroids and retinol, thus we speculated that lncRNAs had some effect on Qira black sheep reproduction. Finally, we also performed a combined analysis combining mRNA and miRNA, speculating on genes that may be associated with reproduction, such as LNC011583 and LNC003443. It further illustrates the expression of lncRNAs during the estrous cycle in Qira black sheep. This experiment provides some reference for the study of fertility in Qira black sheep.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

XC Zeng designed the study, conducted the experiments, analysed the data, and drafted the manuscript. HY Chen and X Chen designed the study and drafted the manuscript. S Jiang, H Shen conducted parts of the experiments and collected samples.

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