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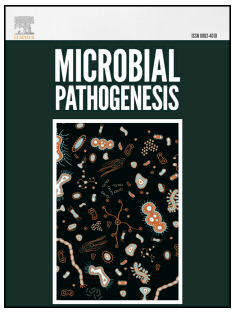

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Integrated analysis of miRNA and mRNA expression profiling in bovine endometrial cells in response to lipopolysaccharide-stimulation

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Abstract

MicroRNAs (miRNA) play an important role in regulating gene expression, making them important resources for exploring molecular mechanisms. Molecular mechanisms involved in the inflammatory responses of bovine endometrial cells induced by lipopolysaccharide (LPS) have not been widely studied. In the present study, miRNA and mRNA expression profiling of bovine endometrial cells treated with 1 μg/mL LPS for 24 h were evaluated by RNA-Seq (RNA-sequencing). The results showed that LPS induced 20 (11 up- and 9 down-regulated) differentially-expressed miRNAs and 108 (90 up- and 18 down-regulated) differentially-expressed mRNAs of bovine endometrial cells. The results for 5 mRNAs and 4 miRNAs were evaluated by quantitative real-time PCR (qRT-PCR) to validate the reliability of the RNA-seq data. Integrating analysis of the miRNA and mRNA expression profiles revealed 116 miRNA-target gene pairs. GO and KEGG pathway analysis of differentially expressed miRNAs and target genes predicted the likely roles of differentially expressed miRNAs in inflammatory responses in bovine endometrial cells induced by LPS. The reliability of the integrating analysis of the miRNA and mRNA data were validated by measuring the expression of three miRNA-target gene pairs by qRT-PCR. Our results improve the understanding of the role of miRNA involvement in inflammatory response of bovine endometrial cells induced by LPS.

Keywords: endometrial cells; lipopolysaccharide; RNA-Seq; miRNA

1. Introduction

Micro RNAs (miRNAs) are small non-coding RNAs that regulate gene expression by targeting specific mRNAs [1]. They regulate gene expression by blocking translation or degrading target mRNA [2]. The role of miRNA has been widely evaluated in different cells [3]. Specific miRNAs have been associated with infectious diseases, inflammation, and immune responses through regulation of the immune system[4, 5]. The ability of miRNAs to regulate inflammatory responses and specific diseases makes them important pathogenic factors for potential therapeutic targets. Inflammatory disorders caused by bacterial infection are a constant problem in the

dairy industry, particularly in the uterus of different animals.

The uterus is the reproductive tract in mammals and is prone to bacterial infections, particularly in the dairy industry [6, 7]. Infections caused by bacterial contamination are a major problem in reproductive management [8-10]. Bacterial infections in the uterus cause uterine inflammatory diseases and have a tremendously negative economic impact on the livestock industry [11]. Uterus infection has also been associated with lower conception rates in cows, which also results in economic loss [12]. Numerous studies have examined the uterus bacterial community, endometrial epithelial cells, and inflammation in the female genital tract [13-15]. Sheldon et al. [15] reported that bacterial infections in the uterus disrupt uterine and ovarian function, leading to the secretion of cytokines and chemokines by lipopolysaccharide (LPS) which is detected by Toll-like receptors (TLRs) on endometrial cells.

LPS from gram-negative bacteria is among the most important stimulus for mammary cell inflammation [16]. However, few studies have investigated the miRNA response to LPS stimulation of cells to understand the mechanism involved. Chi and Wu [17] reported that LPS stimulation differentially expressed 431 miRNAs and 430 target genes in bone marrow-derived macrophages. It has also been reported that several hundreds of genes undergo alternative promoter and 3′ untranslated region usage following LPS treatment from monocyte-derived macrophages [18].

In the current study, RNA-Seq was used for miRNA and mRNA expression profiling and to examine the enrichment of differentially expressed miRNAs and target genes and interactions of miRNAs and target genes in LPS-stimulated bovine epithelial cells.

2. Material and methods

2.1 Cell culture system

Uteri of non-pregnant cattle without evidence of genital disease were collected at a local abattoir after slaughter and kept on ice until further processing in the laboratory. The physiological stage of the reproductive cycle for each genital tract was determined by observation of the ovarian morphology [19]. Genital tracts at an ovarian stage I corpus luteum were selected for endometrial tissue culture, and only

the horn ipsilateral to the corpus luteum was used. For tissue explants, the endometrium was cut into strips and placed into serum-free DMEM/F12 (Invitrogen，Carlsbad, CA, USA) supplemented with 50 IU/mL penicillin (Sangon Biotech, Shanghai, China) and 50 μg/mL streptomycin (Sangon Biotech) under sterile conditions. The strips were then cut into 1 mm^3 pieces using a mechanical tissue chopper and, after three washes with serum-free DMEM/F12, placed into fresh media DMEM/F12 containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 50 IU/mL penicillin, and 50 μg/mL streptomycin. Tissue explants were incubated at 37℃ and 5% CO_2 in a humidified incubator overnight, and then the supernatants were removed and replaced with fresh media. Media was changed once every 48 h until the cells were spread across the bottom of the culture flask. The cells were digested by trypsin (0.25%) supplemented with 0.1% EDTA-2Na (Gibco). Stromal and epithelial cell populations were used for later experiments after 3 passages.

2.2 Experimental design

When bovine endometrium stromal and epithelial cell populations had grown to 80% confluence in the bottom of the culture flask, the cells were stimulated with or without 1.0 μg/mL LPS from *Escherichia coli* 055:B5 (Sigma–Aldrich, St. Louis, MO, USA) for 24 h, according to our previous study. Supernatants harvested after treatment with LPS for 24 h were used to determine the concentration of IL-6 and IL-8 using commercial ELISA kit (Lengton bio, China) according to the manufacturer's protocol. When concentration of IL-6 and IL-8 in LPS treatment group was significantly higher than that in control group, the samples were subjected to RNA-Seq.

2.3 RNA extraction

Total RNA was extracted from endometrial cells directly from the cell culture plate by using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA quantity and quality were measured using a NanoDrop ND-2000 spectrophotometer (Wilmington, DE, USA). Acceptable OD (A_{260}/A_{280}) ratios were between 1.8 and 2.0, and OD (A_{260}/A_{230}) ratios were greater than 1.8. RNA degradation and contamination was detected on a 1% agarose gel. RNA purity was evaluated using the NanoPhotometer® spectrophotometer (Implen, Inc., Westlake Village, CA, USA).

RNA concentration was measured using a Qubit® RNA Assay Kit in a Qubit® 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit with the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). RNA was subjected to high-throughput sequencing.

2.4 Library preparation for small RNA (miRNA) sequencing

A total of 3 μg total RNA per sample was used as input material for the small RNA library. Sequencing libraries were generated using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (New England Biolabs, Ipswich, MA, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, the NEB 3′ SR Adaptor was specifically ligated to the 3′ end of miRNA, siRNA, and piRNA. After the 3′ ligation reaction, the SR RT Primer was hybridized to excess 3″ SR Adaptor (which remained free after the 3 ligation reaction) and the single-stranded DNA adaptor was used to produce a double-stranded DNA molecule. This step is important for preventing adaptor-dimer formation, and double-stranded DNAs are not substrates for ligation mediated by T4 RNA Ligase 1 and therefore do not ligate to the 5′ SR Adaptor in the subsequent ligation step. The 5′ end adapter was ligated to the 5′ ends of miRNAs, siRNA, and piRNA. First-strand cDNA was synthesized using M-MuLV Reverse Transcriptase (RNase H–). PCR amplification was performed using LongAmp Taq 2X Master Mix, SR Primer for Illumina, and index (X) primer. PCR products were purified on an 8% polyacrylamide gel (100 V, 80 min). DNA fragments corresponding to 140–160 base pairs (bp; length of small non-coding RNA plus the 3′ and 5′ adaptors) were recovered and dissolved in 8 μL elution buffer. Finally, library quality was assessed on the Agilent Bioanalyzer 2100 system using DNA High Sensitivity Chips. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq SR Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2500/2000 platform and 50-bp single-end reads were generated. Mapped small RNA tags were used to search for known miRNA. miRbase

20.0 was used as a reference, and modified software mirdeep2 and srna-tools-cli were used to obtain the potential miRNA and draw secondary structures. Custom scripts were used to obtain miRNA counts and base bias for the first position of the identified miRNA with a certain length and at each position of all identified miRNAs, respectively. The software miREvo and mirdeep2 were used to predict novel miRNAs by exploring the secondary structures. miRNA expression levels were estimated from transcript per million (TPM) values (TPM = (miRNA total reads/total clean reads) × 10^6). Differential expression analysis of two samples was performed using the DEGseq (2010) R package. The P-value was adjusted using the q value. A q value < 0.01 and $|\log_2$ (fold-change)$|>1$ was set as the threshold for significantly differential expression.

2.5 Library preparation for mRNA sequencing

A total of 3 μg RNA per sample was used as input material for RNA sample preparations. Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs) following the manufacturer's recommendations and index codes were added to attribute the sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations at an elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, the NEBNext Adaptor with a hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments 200 bp in length, the library fragments were purified by the AMPure XP system (Beckman Coulter, Brea, CA, USA). Next, 3 μL USER Enzyme (New England Biolabs) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. PCR was performed using Phusion High-Fidelity DNA polymerase, Universal PCR primers and index (X) primer. PCR products were purified (AMPure

XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated. Reference genome and gene model annotation files were downloaded from the genome website. The index of the reference genome was built using Bowtie v2.2.3 and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12. We selected TopHat as the mapping tool for generating a database of splice junctions based on the gene model annotation file which provides a better mapping result than other non-splice mapping tools. mRNA expression levels were estimated as the expected number of fragments per kilobase of transcript sequence per million base pairs sequenced values (TPM = total exon fragments/(mapped reads × exon length)). Differential expression analysis under two conditions was performed using the DEGseq R package (1.20.0). P values were adjusted using the Benjamini & Hochberg method. A corrected P-value of < 0.01 and $|\log_2(\text{fold-change})| > 1$ were set as thresholds for significantly differential expression.

2.6 Target gene prediction and miRNA-mRNA regulatory network

The target genes of miRNAs were predicted using psRobot_tar in psRobot for plants or miRanda for animals. miRanda uses a dynamic programming algorithm to search for complementary regions between an miRNA and the 3′-untranslated region of the mRNA, and the scores are based on sequence complementary and minimum free energy of RNA duplexes, which are calculated using the Vienna RNA package. Thus, a hub miRNA was defined as having a connectivity ≥10. A hub miRNA and its regulated genes were then classified to a hub-miRNA module as follows: opposite modes of regulation of miRNAs and mRNAs were identified based on their expression profiles and used to determine each miRNA-mRNA and miRNA-target intersection. High reliability for the miRNA-target intersection was used to establish the hub miRNA module and predict target-gene function in the hub miRNA module. Subsequently, an overall network was established based on verified protein–protein

interactions using the STRING database and miRNA targets. Visual representations of interaction webs were designed and constructed using Cytoscape software and the igraph data package on an R platform. Finally, a closed connected network module was identified using the Markov cluster algorithm, and a module of ≥10 nodes was selected.

2.7 GO and KEGG enrichment analysis of differentially expressed target gene of differentially expressed miRNAs

The gene ontology (GO) database (http://geneontology.org/) includes the following three functional categories: biological process, cellular component, and molecular function. Genes can be further organized by directed acyclic graph according to their scope. In GO clustering, genes are considered significantly enriched based on the ratio of the observed GO term for all genes/GO term for a single gene set. GO enrichment analysis of differentially expressed miRNAs and target gene candidates were evaluated using the GOseq R package, in which gene length bias was corrected. GO terms with corrected P values ≤ 0.05 were considered significantly enriched by differentially expressed miRNAs and target genes.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html) was used to systematically analyze gene function and genomic information from biological pathways and further group biological pathways according to metabolism, enzyme, biochemical reaction, gene regulation, and protein–protein interaction. We used KOBAS software to test the statistical enrichment of differential expression target genes in the KEGG pathways

2.8 Reverse transcription and qRT-PCR

First, 1 μg RNA was reverse-transcribed into complementary DNA (cDNA) in 20-μL reaction mixtures using an RT system kit (Takara, Shiga, Japan). The relative mRNA and miRNA quantities were determined by qRT-PCR in 20-μL reaction volumes using the SYBR green Plus reagent kit (Takara) in the Mx3000P qRT-PCR detection system. Primers and Tm values used for amplification of relative mRNA and miRNA by qRT-PCR are shown in Table 1 and Table 2. U6 and β-actin were used as internal controls for miRNA and mRNA, respectively. The 20-μL qRT-PCR mixture contained

the following: 10 μL of 2 ×SYBR Premix Ex Taq II; 0.4 μL of 50 × ROX Reference Dye II; 0.8 μL of forward primer (20 μM); 0.8 μL of reverse primer (20 μM); 6 μL of double-distilled water, and 2 μL of cDNA. PCR was performed using the following program: 95℃ for 30 s; 40 cycles of 95℃ for 5 min, different Tm values for 34 s; 95℃ for 15 s, 60℃ for 60 s; and 95℃ for 15 s.

3. Results

3.1 Sequencing overview of miRNA

A total of 7,236,182 and 7,132,755 clean reads were obtained from the control and LPS groups, respectively (Table 3). Read length distributions are shown in Fig. 1. Additionally, 21–24 nucleotide (nt) miRNAs accounted for at least 75% of the population in the control and LPS groups. Other reads of longer than 25 nt, may mostly represent Piwi-interacting RNA (piRNA), a newly identified class of small regulatory RNAs reported to be abundantly generated in the mature testes of animals [20].

3.2 Sequencing overview of mRNA

A total of 41,263,672 and 35,251,970 clean reads were generated in the control and LPS groups, respectively. The error rate and GC content of each group were calculated to control for sequence quality (Table 4). The quality of the process used for library construction for sequencing was also assessed based on transcript homogeneity (Fig. 2).

3.3 Differentially expressed mRNA and miRNA

After normalization of the raw data, significantly differentially expressed genes including 20 miRNAs (Table 5) and 108 mRNAs (Table 6) were identified in the LPS group compared to in the control group; the 20 miRNAs consisted of 11 up-regulated and 9 down-regulated miRNAs; the 108 mRNA consisted of 90 up-regulated and 18 down-regulated mRNAs. bta-miR-183, bta-miR-744, and bta-miR-375 were the most significantly up-regulated miRNAs, while bta-miR-365-3p, bta-miR-20a, bta-miR-197, bta-miR-19b, and bta-miR-135a were the most significantly down-regulated miRNAs in the LPS group compared to in the control group. These results were verified by qRT-PCR (Fig. 4B). TLR4, CXCL3, C3, NUR77, YEAST2,

BET1, and CYB5R1 were the most significantly up-regulated mRNAs, while TSPAN7 and LYPLA2 were the most significantly down-regulated mRNAs in the LPS group compared to in the control group. The results were verified by qRT-PCR (Fig. 3B). The heat map (Fig. 3A), scatter plot (Fig. 3C), and volcano plot (Fig. 3D) also revealed variable expression patterns of the differentially expressed miRNAs and mRNA in the control and LPS groups.

3.4 qRT-PCR validation of differentially expressed miRNA and mRNA

To validate the RNA-seq results, we conducted qRT-PCR to investigate the relative expression levels by randomly selecting 6 mRNAs (TLR4, CXCL3, C3, TSPAN7, NUR77) (Fig. 3B) and 4 miRNAs (bta-miR-375, bta-miR-20a, bta-miR-183, bta-miR-365-3P) (Fig. 4B) that showed significant differences in expression. The RNA-seq data and qRT-PCR data were identical. In general, the results of qRT-PCR validated the RNA-seq results and added more confidence to the credibility of the differentially expressed miRNAs and mRNAs.

3.5 GO and KEGG enrichment of differentially expressed miRNAs and target genes

To determine the functional roles of miRNAs and target genes in biological pathways, we performed pathway enrichment analysis using KEGG pathways and gene ontology (GO) for biological functions. In GO enrichment analysis, a total of 118 GO terms were significantly enriched, with the 34 GO terms mainly related to molecular function, such as lipase activity, phospholipase activity, CoA-transferase activity, and carboxylic ester hydrolase activity. The 79 GO terms were mainly related to biological processes, such as lipid catabolic process, response to bacterium, defense response to bacterium, and regulation of gene expression. The 5 GO terms were related to the cellular component, such as outer membrane, septin ring, and septin cytoskeleton. Thirty significantly enriched GO terms are shown in Fig. 5A. Pathways that were enhanced under LPS stimulation were analyzed by KEGG analysis. Approximately 20 significant pathways were detected and are shown in Fig. 5B. Notably, most genes were associated with the valine, leucine, and isoleucine degradation pathways followed by adherens junction; Amoebiasis, cell adhesion molecules, and the AMPK

signaling pathways.

3.6 Interaction network of miRNAs and target genes

To understand how miRNAs affect mRNA expression, miRNA-seq and mRNA-seq data were correlated following the criterion of anti-regulation of an miRNA and corresponding mRNA. Based on the miRNA-target gene interaction pairs identified by reverse association, we compared the interaction network between miRNAs and target genes in the control and LPS groups. We used 116 miRNA-target gene pairs for reverse correlation, including 11 pairs of up-regulated miRNAs and 9 pairs of down-regulated miRNAs to construct the miRNA-target gene interaction network. In addition, one target (YEAST2) of bta-miR-19b and bta-miR-135a, three targets (YEAST2, BET1, and CYB5R1) of bta-miR-197, and one target (LYPLA2) of bta-miR-744 were validated by qRT-PCR. These results showed that this method is reliable for predicting target genes of miRNAs by integrating analysis of miRNA-seq and mRNA-seq data (Fig. 6).

4. Discussion

miRNAs act at the post-transcriptional level and inhibit gene expression by binding to a complementary sequence of an mRNA molecule [21, 22]. The identification of miRNAs and their target mRNAs in bovine endometrial cell responses to LPS is important for understanding their biological mechanisms and functions. Our results showed that LPS induced 20 differentially-expressed miRNAs and 108 differentially-expressed mRNAs in bovine endometrial cells, as well as enriched 118 GO terms and 66 KEGG pathways.

LPS was previously reported to induce pro-inflammatory gene expression to different degrees and increase cell counts[23]. Similar results were observed in the current study, with several genes such as regulation of cell proliferation genes, inflammatory response genes, and response to cytokine (inflammatory cytokines and chemokines related) genes were significantly enriched under LPS treatment. Mishra et al. [24] also found that several cytokines associated with inflammation were increased under LPS treatment in mice. Yu et al. [25] reported that LPS caused a substantial increase in the mRNA levels of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β,

IL-6, and IL-8, which are all associated with cell inflammation in bovine mammary epithelial cells.

The importance and expression of different miRNAs in relation to LPS treatment has been documented in different cells [26, 27]. Cerny et al.[28] reported that LPS affected 49 of inflammatory mRNAs and approximately 51 inflammatory-associated miRNAs in the mouse oviduct. Tang et al. [29] also reported that the LPS-induced inflammatory response is regulated by miRNA (microRNA-29a) in Maurine macrophages. These findings support the involvement of miRNAs in inflammatory responses. Most miRNAs identified in the current study were also reported in other cell studies. Some miRNAs were up-regulated under LPS treatment, while others were down-regulated, such as bta-miR-99b, which was up-regulated following LPS treatment while bta-miR-19b was down-regulated. Zheng et al. [26] reported the expression of bta-miR-99b and bta-miR-19b in Holstein cattle and found that bta-miR-19b is highly enhanced under heat stress, while bta-miR-99b showed low enhancement. miR-99b has also been reported to regulate the host immune system against infection diseases in murine dendritic cells [30]. Our results demonstrated that bta-miR-20a in bovine endometrial cells was down-regulated by LPS. Corresponding to these results, miR-20a has been reported to be involved in inflammatory responses in murine alveolar macrophages induced by LPS [31]. Lu et al. [32] reported that miR-375 is down-regulated in human epithelial cells after stimulation by IL-13, while in the current study bta-miR-375 was up-regulated in LPS-inoculated cells, indicating its role in the inflammation response. Some miRNAs such as let-7c, which was previously reported to be associated with an anti-inflammatory role in LPS-treated alveolar macrophages [33], was also significantly enhanced in the current study.

To gain insight into the biological mechanism involved in endometrial cell inflammation, GO analysis was conducted. The results of GO analysis revealed that LPS increased different GO terms. Most GO terms were significantly enhanced in biological processes under LPS treatment than in the cellular component and molecular functions. Notable results included the significant enhancement of the cellular response to LPS, cellular response to biotic stimulus, and regulation response

to an external stimulus in biological processes (Supplementary Fig. A). Cytokine receptor binding and cytokine activity were also significantly enhanced in molecular function (Supplementary Fig. A). These findings generally agree with those of previous studies showing that LPS increases the levels of inflammatory molecules such as cytokines and chemokines in response to pathogens that cause various infectious diseases in cattle [34-36]. LPS is also crucial for activating the defense against bacterial infections by stimulating chemokines to initiate an inflammatory response [37, 38]. These reports are also in agreement with results from the current study showing that LPS significantly enriched some of biological processes such as cell chemotaxis, regulation of inflammatory response, regulation of immune system process, cellular response to molecule of bacterial origin, and response to external stimulus. Cario et al. [39] also stated that LPS induces a pro-inflammatory response by activating distinct pathways in intestinal epithelial cells and regulating mRNA expression. The role of LPS in the inflammatory response and its role in biological processes was also reported by Noleto et al. [40], who indicated that inhibiting glycolysis compromises the inflammatory responses to LPS in endometrial tissues, leading to reduced accumulation of inflammatory cytokines by at least half (50%).

The results of KEGG analysis indicated that LPS significantly increased several pathways such as the AMPK signaling pathway, TNF signaling, pertussis, and TLR signaling pathway (Supplementary Fig. B). Chen et al. [41] reported that phosphorylation of p38 MAPK and ERK was down-regulated upon inoculation of LPS in murine macrophages. This phenomenon was also observed in the current study. The MAPK signaling pathway can promote the production of inflammatory cytokines such as TNF-α [42, 43]. It has been reported that LPS treatment up-regulated the mRNA expression of TNF-α and TLR4 in bovine endometrium epithelial cells [44]. Bromfield et al. [45] reported significant expression of TLR4 in response to LPS in bovine granulosa cells, which agrees with the findings of the current study. Wen et al. [46] reported that LPS significantly stimulated dynamic TLR genes changes at the protein level in pufferfish. This suggest a pivotal role of the TLR pathway in the immune system, particularly in inflammatory responses, which generally agrees with

the findings of the current study.

Although the current study revealed differentially-expressed miRNAs and mRNAs and their KEGG pathways in bovine endometrial cells in response to LPS, further studies are required to determine the exact mechanism of the miRNAs and their target genes in the inflammatory response of bovine endometrial cells under LPS treatment.

5. Conclusion

Our study provides insight into miRNAs and their target mRNAs in the inflammatory response in bovine uterus endometrial cells induced by LPS. The predicted 116 miRNA-target gene pairs and their GO and KEGG enrichment are of critical importance for understanding the role of miRNA involvement in inflammatory responses.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Table 1. Primers used for qRT-PCR of selected genes

mRNA	Sequence (5'-3')	Product size(bp)	Annealing temp.(□)	Accession No.
LYPLA2	F: GGATCCCTGTGACACTCAAC R: GGTACGTCTTGAACTGGACC	435	52	NM_001080362.1
TLR4	F: CCGACAACATCCCCATATCAA R: GGGTTTCCCGTCAGTATCAA	193	51	NM_174198.6
TSPAN7	F: CTCCTTCGTCTTCTGGATCACT R: AACAGGCCAAACACGACGAT	159	52	NM_001076384.1
C3	F: GAAAAGAACCGCTGGGAGGA R: AGGAGGCGTAGTGTCGTAGT	108	53	NM_001040469.2
CYB5R1	F: GGCTTCGTCTCTGTGGACAT R: AAGCGCATCTTTGGCGAGTA	146	53	NM_001034518.1
YEATS2	F: GACAAGCCAGGGCAGGTTAT R: TGGGCAAAGAGGTGGCATAG	189	55	NM_001105367.1
CXCL3	F: GCTGCCATTGCCTGCAAACTT R: TGGGAGCTTCAGGGTTGAGAC	150	57	NM_001046513.2
BET1	F: CCATTGAAATAGGGCATGAAG R: CGTTTGGCTTCCTCTGGATA	134	50	NM_001099157.1
JUN	F: CAAGAACTCCGACCTCCTCAC R: GATATGCCCGTTGCTGGACT	100	56	NM_001077827.1
β-actin	F:CTTCCTGGGCATGGAATCCT R:TTGATCTTCATTGTGCTGGGTG	189	54	NM_173979.3

Table 2. Primers used for qRT-PCR of selected miRNA

MiRNA	Sequence (5'-3')	Annealing temp. (℃)
bta-miR-375	F:CGGCAATTTTGTTCGTTCGGCTC R:ATCCAGTGCAGGGTCCGAGG	62
bta-miR-744	F:CGTATGCGGGGCTAGGGCTA R:ATCCAGTGCAGGGTCCGAGG	64
bta-miR-135a	F:CGGATTCGCTATGGCTTTTTATTCCT R:ATCCAGTGCAGGGTCCGAGG	61
bta-miR-197	F:CGATACGTTCACCACCTTCTCCA R:ATCCAGTGCAGGGTCCGAGG	62
bta-miR-19b	F:CGGTCCTGTGCAAATCCATGCAA R:ATCCAGTGCAGGGTCCGAGG	62
bta-miR-365-3P	F:CGGCCGGTAATGCCCCTAAAAAT R:ATCCAGTGCAGGGTCCGAGG	62
bta-miR-183	F:GGCTGGTATGGCACTGGTAGAAT R:ATCCAGTGCAGGGTCCGAGG	61
bta-miR-20a	F:CGGCGGTAAAGTGCTTATAGTGC R:ATCCAGTGCAGGGTCCGAGG	60
U6	F:GCTTCGGCAGCACATATACTAAAAT R:CGCTTCACGAATTTGCGTGTCAT	60

Table 3 Categorization of reads of small RNAs at different groups

Sample	control	LPS
raw reads	7344770	7237639
N% >10%	12	15
Low quality	8009	6627
5 adapter	141	177
3 adapter	94784	92500
With ployA/T/G/C	5642	5565
clean reads	7236182(98.52%)	7132755(98.55%)
exon	85895	107446
intron	367852	463136
Known miRNA	906	882
Novel miRNA	13	16

Table 4 Categorization of reads of mRNAs at different groups

Sample	control	LPS
raw reads	43276962	37003950
Clean reads	41263672	35251970
Q20 (%)	95.32	95.35
Q30 (%)	88.9	89
GC content (%)	50.74	49.99
Total mapped	37896748	32494500
Uniquely mapped	37389034	32125482
Reads map to“+”	19295836	16558117
Reads map to“-”	18600912	15936383
Error%	0.0168	0.0166

Table 5 Differentially expressed miRNAs in LPS compared with the control

miRNA	q value	Direction
bta-miR-99b	0	+
bta-miR-183	6.87E-169	+
bta-miR-222	2.58E-80	+
bta-miR-504	3.45E-40	+
bta-miR-375	4.08E-36	+
bta-miR-1468	4.52E-29	+
bta-let-7c	1.63E-11	+
novel_12	4.47E-08	+
bta-miR-543	6.21E-06	+
bta-miR-296-3p	0.0004191	+
bta-miR-744	0.0097056	+
bta-miR-193b	2.20E-19	—
bta-miR-495	1.42E-09	—
bta-miR-342	1.68E-07	—
bta-miR-197	4.07E-05	—
bta-miR-365-3p	5.07E-05	—
bta-miR-20a	0.000772	—
bta-miR-19b	0.003055	—
bta-miR-362-5p	0.003055	—
bta-miR-135a	0.009706	—

“+”and“-”mean refers to sense strands and anti-sense strand, respectively.

Table 6 Differentially expressed mRNAs in LPS compared with the control

mRNA	p value	Direction
ADAMTS5	5.00E-05	+
ZIP14	0.00015	+
A20	2.00E-04	+
ST2	5.00E-05	+
IL1A	0.00015	+
COX1	1.00E-04	+
CD141	5.00E-05	+
BIRC2_3	5.00E-05	+
……	……	……
CDH13	3.00E-04	—
DES	5.00E-05	—
FLT1	5.00E-05	—
CSPG4	5.00E-05	—
SLC6A6S	5.00E-05	—
SLC14A	2.00E-04	—
NF66	0.00025	—

Fig. 1 Length distribution and abundance of the miRNA libraries

Fig. 2 The data quality of the mRNA sequences. (A) The data quality of control group;(B) The data quality of LPS group;(a) Base percentage composition along reads;(b) Distribution of mean error;(c)Distribution of qualities.

Fig. 3 Differentially expressed profiles of mRNA. (A) Heatmaps and hierarchical cluster of mRNA expression. (B) qRT-PCR results of selected differentially expressed mRNAs. (C) Scatter plot of mRNA in control and LPS groups. (D) Volcano plot of mRNA in control and LPS groups. $^{**}P<0.01$

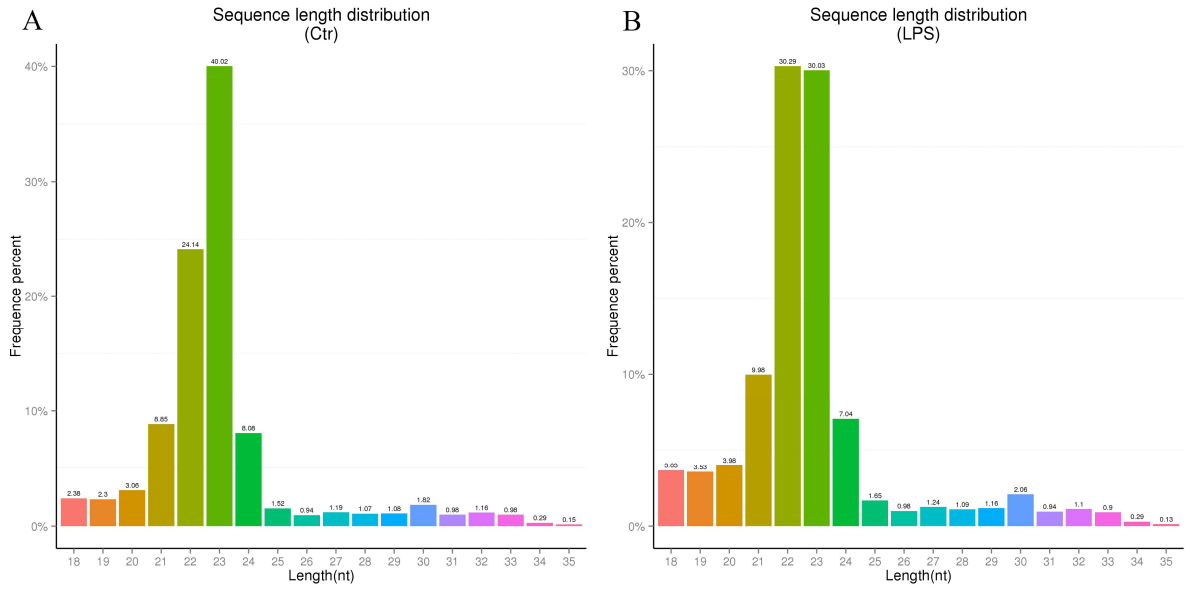
Fig. 4 Differentially expressed profiles of miRNA. (A) Heatmaps and hierarchical cluster of miRNA expression. (B) qRT-PCR results of selected differentially expressed miRNAs. (C) Scatter plot of mRNA in control and LPS groups. (D) Volcano plot of mRNA in control and LPS groups. $^{**}P<0.01$

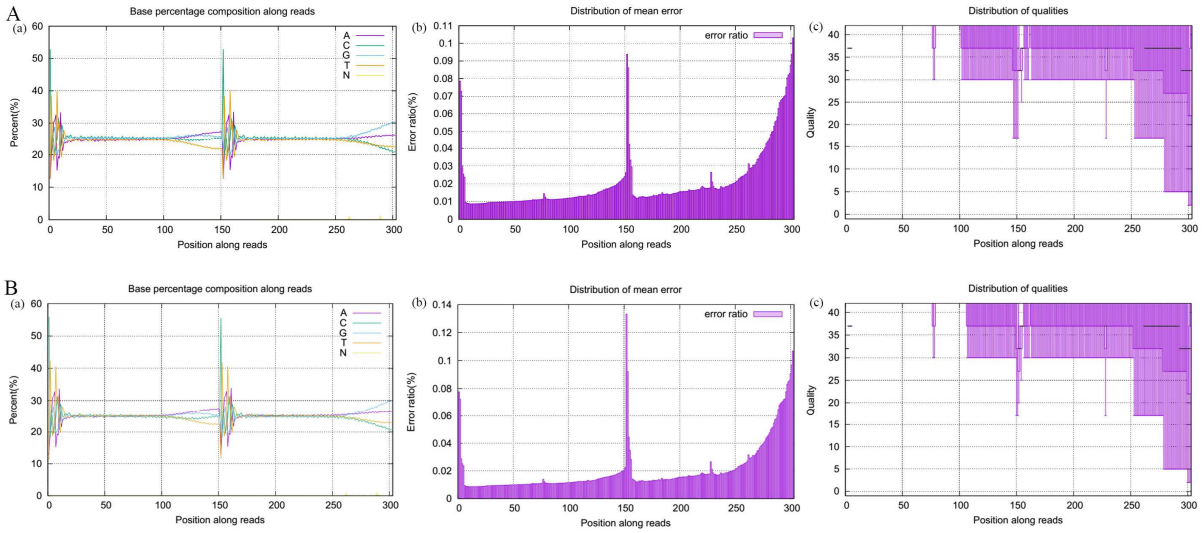
Fig.5 The results of GO and KEGG enrichment analysis of differentially expressed miRNAs and target genes. (A) The 30 GO terms from GO enrichment analysis. (B) The number of enriched KEGG pathways is 20.

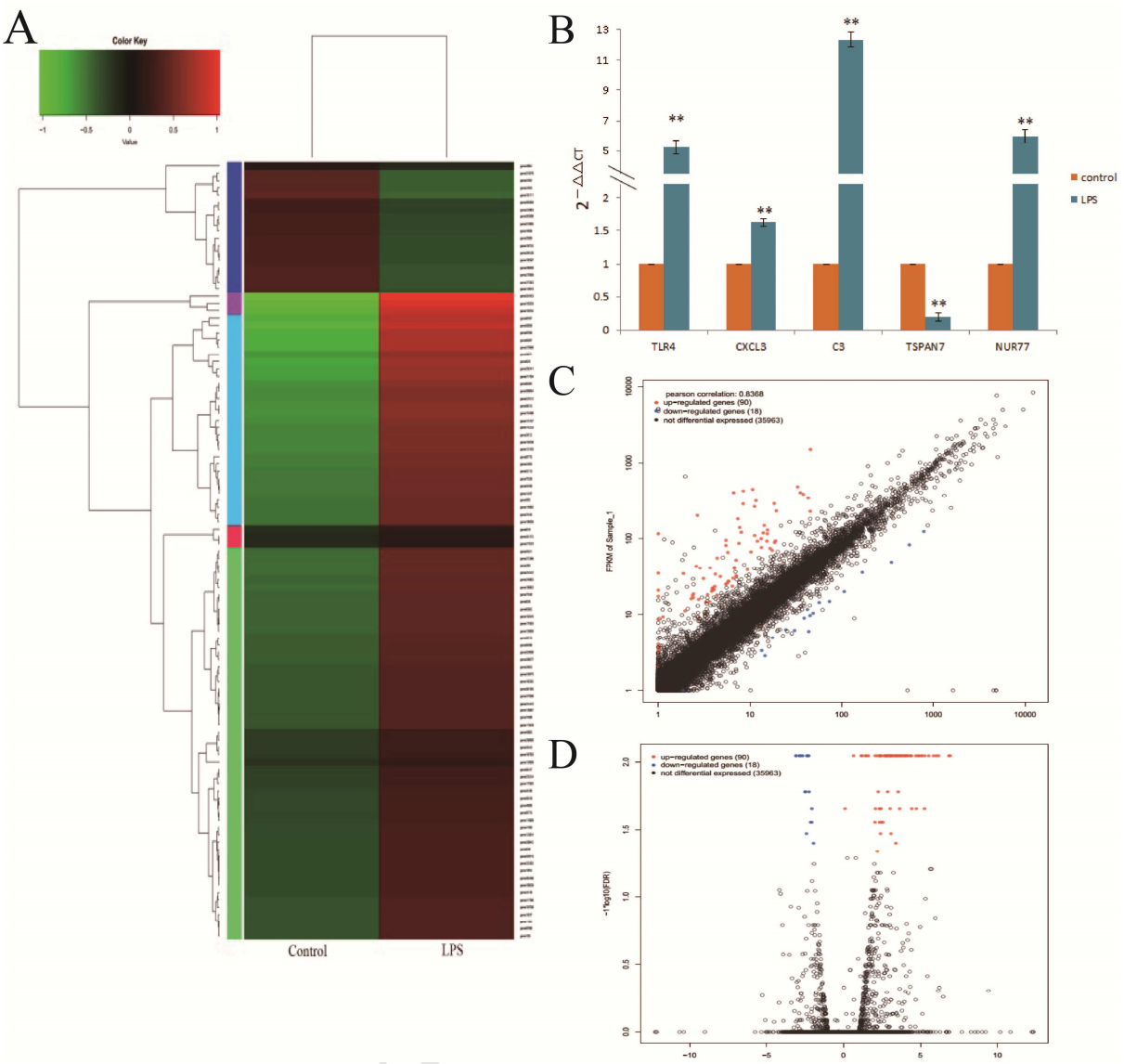
Fig. 6 The interaction between miRNAs and target genes. (A) Differentially expressed miRNA-mRNA pairs and regulatory network between control and LPS groups. miRNA and mRNA expression level in LPS and control by qRT-PCR. (B) The expression level of bta-miR-19b, bta-miR-135a and YEAST2; (C) The expression level of bta-miR-197 and YEAST2, BET1, CYB5R1. (D) The expression level of bta-miR-744 and LYPLA2. $^{**}P<0.01$; * $P<0.05$

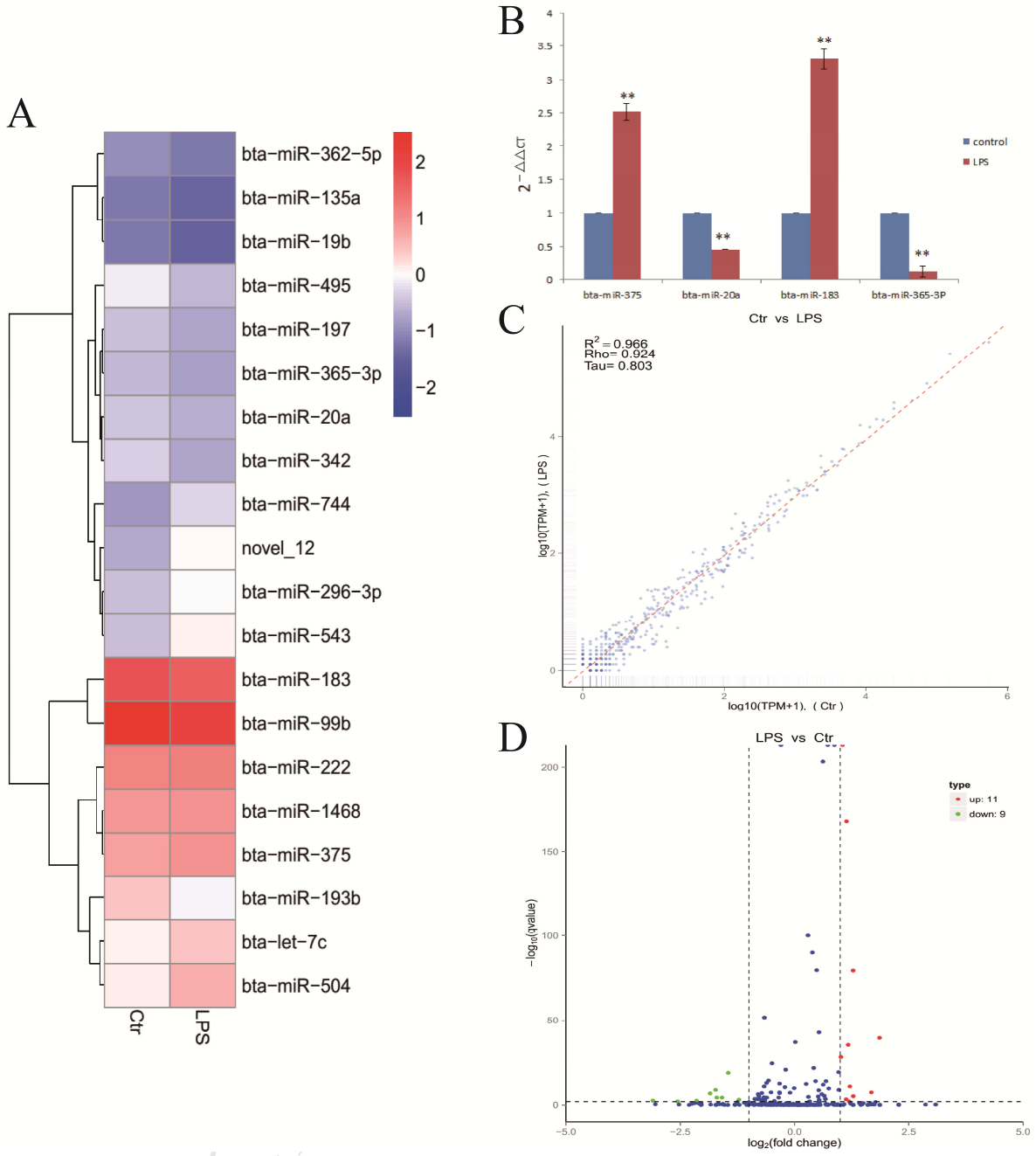
Supplementary Fig. A. The results of GO enrichment analysis. The 80 significantly enhanced GO terms from GO enrichment analysis.
$^{**}P<0.01$; * $P<0.05$

Supplementary Fig. B. The results of KEGG enrichment analysis of 66 significantly enriched KEGG pathways
$^{**}P<0.01$; * $P<0.05$





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bta-miR-342
bta-miR-744
novel_12
bta-miR-296-3p
bta-miR-543
bta-miR-183
bta-miR-99b
bta-miR-222
bta-miR-1468
bta-miR-375
bta-miR-193b
bta-let-7c
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control
LPS
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bta-miR-20a
bta-miR-183
bta-miR-365-3P
Ctr vs LPS
C
R^2 = 0.966
Rho= 0.924
Tau= 0.803
log10(TPM+1), (LPS)
log10(TPM+1), (Ctr)
D
LPS vs Ctr
type
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down: 9
$-\log_{10}$(qvalue)
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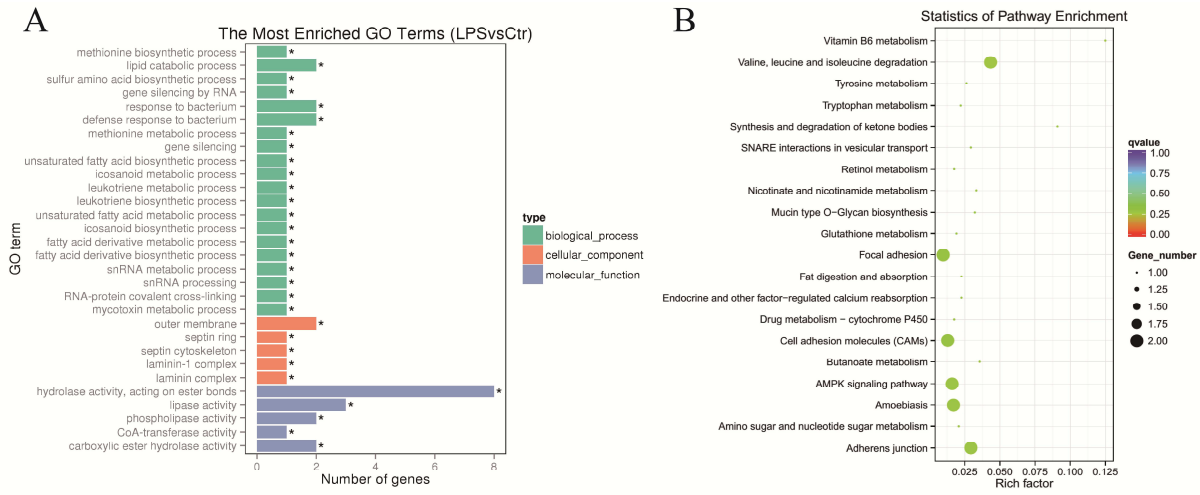

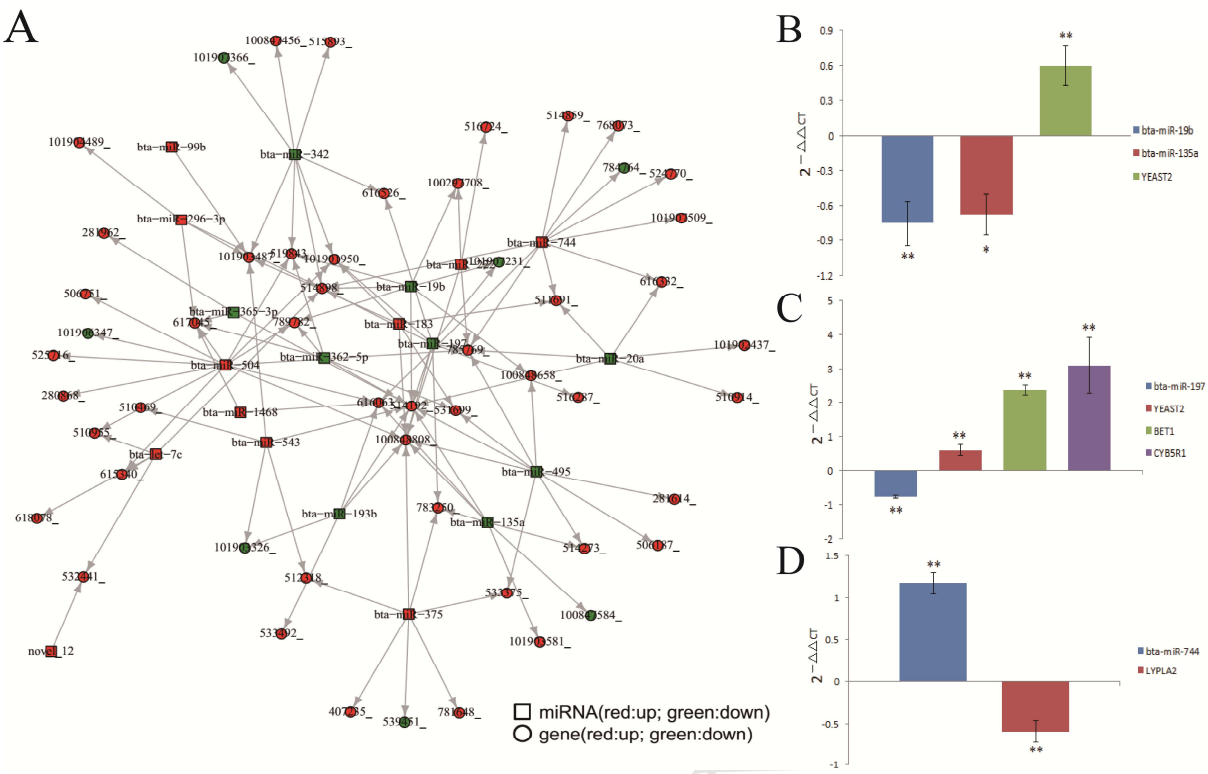

Highlights

1. miRNA and mRNA expression profiling of bovine endometrial cells treated with LPS
2. Integrating analysis of miRNA and mRNA expression profiles of bovine endometrial cells treated with LPS