The effects of estrus cycle on the expression of ovarian biological clock-related genes of Xiang pigs

Liang-Ting Tang, Shihui Huang, Xi Niu, Sheng Li, Jiafu Wang, Xue-Qin Ran

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ABSTRACT

The biological clock has been studied to play a critical role in the reproductive system of various living organisms like swine. To examine the effects of estrus cycle on the expression of ovarian biological clock-related gene in Xiang pig, in this study, we analyzed the expression and alternative splicing of biological clock-related genes in ovary during estrus and diestrous periods. In total, we detected 90 clock-related genes expressed in the ovaries of the Xiang pigs and found 33 clock-related genes differentially expressed between estrous and diestrous stages. We identified 44 differential splicing events from the transcripts of 34 biological clock-related genes. Furthermore, we also found 20 genes including the core clock components, *arntl* and *cry1* were differentially regulated only at AS level and 14 genes, including *per1* and *clock*, were differentially regulated at both expression and AS levels. We also proved that the core clock genes *per1, cry1, clock* and *arntl* and the clock-related genes, *ppp1cb* and *ntrk1* were rhythmically expressed in Xiang pig ovaries by RT-qPCR experiments. The results demonstrated that the biological clock in the ovaries of Xiang pigs might play an important role in regulating the ovarian physiological functions by the transcriptional and post-transcriptional regulation.

Keywords: *Circadian rhythm; Xiang pig; Ovary; Estrus cycle; Alternative splicing*

INTRODUCTION

The circadian rhythm is the regular change of many functional activities of the biological organism to adapt to the alternation of day and night in the environment. It is endogenous and persists even without external synchronizing factors, for example, in constant darkness. Circadian rhythm is controlled by central biological clocks related to hypothalamic Suprachiasmatic Nucleus (SCN) and peripheral biological clocks distributed in various tissues [1]. SCN cells modulate the activity of Gonadotropin-Releasing Hormone (GnRH) neurons by direct innervation or other hypothalamic nucleus, which are necessary for the occurrence of ovulation [2-3]. It is known that the molecular model of mammals circadian clock including human is a multi-feedback loop for transcription and translation including transcriptional regulation and post-transcriptional regulation [4]. It consists of Positive Regulatory Components (*Bmal1* and *Clock*) and Negative Regulatory Components (*Per 1/2* and *Cry 1/2*) [5]. The positive and the negative regulatory elements also participate in the circadian regulation of Clock Controlled Genes (CCG). The clock-controlled genes act as specific regulators of rhythmic physiological functions in cells and tissues [6]. For example, the function of ovary is regulated by the biological clock, including the estrus cycle, Luteinizing Hormone (LH) levels, ovulation and embryo implantation [7,8]. Ovary is an important reproductive organ of female mammals. In each estrus cycle, the ovaries undergo a series of morphological, hormonal and biochemical changes, which directly affect and/or determine the litter size of female animals [9]. The realization of ovarian function is a very complex biological process, involving the transcriptional regulation of a large number of genes [10]. Alternative Splicing (AS) is also an important way of gene expression regulation, which occurs widely in various tissues and cells of almost all organisms [11-12]. Many studies demonstrate that the biological clock existed in the ovary [13-16]. The expression of clock genes have been described in the ovary of rat, mice and ruminant [17-19]. It indicates that clock genes are involved in the process of follicle formation, ovulation, oocyte maturation and steroid hormone synthesis [20]. The lack of circadian clock might be related with varieties of reproductive disorders. For instance, mice with *per1* and *per2* knockout present a significant decrease in reproductive rate due to the irregular and non-cyclical estrus cycle [21,22]. Although mice with bmal1 knockout are capable of ovulation, they exhibit delayed puberty, irregular estrus cycles, and small ovaries and uterus [23-25]. Loss of *bmal1* also negatively affects progesterone level, leading to embryo implantation failure

Institute of Agro-Bioengineering, College of Animal Science, Guizhou University, Guiyang, China

Correspondence: Liang-Ting Tang, Institute of Agro-Bioengineering, College of Animal Science, Guizhou University, Guiyang, China, e-mail xqran@gzu.edu.cn
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[25, 23]. Mice mutant with 51 amino acid deletions in the transcription activation domain of clock protein show an irregular estrus cycle, and the normal LH surges disappear on the day of estrus [26]. In addition, genes of ovarian granulosa cells that are important for follicular development have been shown to be controlled by clock genes, such as LH receptor (*lhcgr*), prostaglandin synthase (*ptgs2*), steroid-producing enzymes (steroid-producing acute regulatory protein, *cyp11a1*, aromatase, etc.), and gap junction protein *Connexin-43* [27-29]. Estrus is a sign of reproductive maturity. Moreover, it has been confirmed that the central biological clock located in the SCN was the core of maintaining normal reproductive function and the estrus cycle [30,31]. However, the function of ovarian biological clock during estrus period is still needed to be clear.

The Xiang pig is a small local breed in China with some unique biological characteristics, such as small size, precocious puberty, low birth rate and insignificant estrous behavior [32]. In order to understand more in-depth views of the influence of estrus on biological clock and further explore the physiological functions of clock genes in porcine ovary, we analyzed the expression of biological clock-related genes and alternative splicing of transcripts in the ovaries of Xiang pigs during estrus and diestrous period based on the whole transcriptome sequencing analysis.

MATERIALS AND METHODS

Materials

About 40 sows of Xiang pig were selected for observation of estrus performance. They were reared in a standard manner that the pigs drank freely and fed timely under the normal condition. The average light intensity was about 200 lux, the ambient temperature was maintained at 15°C-18°C, and the humidity was in the range of 50% ± 10%. The estrus detection was carried out 2 times to 3 times a day. Redness and swelling of the vulva and a positive standing reflex test of the boar were defined as estrus manifestations. Ovary samples of 14 Xiang pig sows were taken, 7 of them were in the diestrous period, and the others were subjected to routine castration surgery on the third day of estrus (the sows showed strong redness and swelling of the vulva). The sampling day was at 8 am-9 am at March 15 to 30, 2022, with the same light and temperature conditions as previously mentioned, and fasting before ovariectomy surgery. All samples were immediately immersed in liquid nitrogen and stored at -80°C for RNA extraction. The animal procedures were approved by the guidelines of Guizhou University Subcommittee of Experimental Animal Ethics with No. of EAE-GZU-2020-P002.

Total RNA extraction

Total RNA from 14 ovarian samples were extracted using TRIzol reagent kit (Invitrogen, USA) according to the instructions. The quantity and completeness of total RNA were evaluated by the Bio-analyzer system (Agilent 2100, Agilent Technologies, and USA). The total RNA was stored at -80°C for subsequent analysis. The same samples were used for sequencing and PCR analysis.

Library construction and sequencing

Total of 14 cDNA libraries were constructed using RNA as templates from ovarian tissues. The library construction was carried out according to the standard procedures of BGI (Shenzhen Huada Gene Co., Ltd). After DNase I enzyme treatment, magnetic beads with *oligo* (dT) were used to isolate mRNA. Under appropriate temperature conditions, the purified mRNA was fragmented by adding fragmentation buffer, and then cDNA was synthesized using random primers and mRNA fragment templates. After 3'end adenylation, the DNA fragments were ligated to the linker adaptors. cDNA fragments of 100 bp-200 bp in length were selected for PCR amplification to generate a cDNA library. The libraries were sequenced on the Illumina HiSeqTM 2500 sequencing platform, and paired-end sequences with 150 bp in length were generated.

Sequencing data analysis

The original sequences generated by the Illumina platform were saved in fastq format. The clean reads were obtained by removing low-quality sequences, reads (more than 5% unknown nucleotides), and sequencing adapters. The clean reads should be evaluated through FastaQC software (Q20<20%). The reference genome sequence (ssc11.1 version) and annotation files of pig genomes were downloaded from the Ensembl database. Used HISAT2 software (v2.1.0) to map the clean reads to the pig reference genome. HISAT2 is an efficient alignment tool for RNA-seq experiments. It uses an indexing framework based on the BWT and Ferragina-Manzini (FM) index algorithms for alignment and normalization. The Subread feature Counts software (v2.0.0) was used to count the number of reads mapped to the gene. Subread feature Counts applies the read summarization tool to analyze the expression level from RNA-seq, which applies efficient chromosome hash algorithm and feature block technology for statistics and data standardization.

Analysis of differential expression

The expressed level of each gene were calculated by CPM values (counts per million mapped reads) in all samples, and added 0.001 to CPM value as the gene expression level. Then we used Bioconductor software package Limma and DESeq2 to analyze Differentially Expressed Genes (DEGs) between two groups with biological replicates. The FDR ≤ 0.1 and $|\log2FC| \geq 0.5$ were used as the thresholds for determining the significance of gene expression difference.

Identification of differential alternative splicing events

The rMATS software (v4.0.2) was used to analyze differentially alternative splicing events between two groups. The rMATS recognizes five alternative splicing events, which are Skipped Exon (SE), alternative 5' splice site (A5SS), Alternative 3' Splice Site (A3SS), Mutually Exclusive Exons (MXE) and Retained Intron (RI) [33]. Also, FDR≤0.05 was used as a threshold to detect the differential significance of alternative splicing.

Validation

The same RNA samples from the ovarian for RNA-seq experiment were used to validate the differential expression by qRT-PCR and to validate the AS events by RT-PCR method. The primers were

designed using Primer 6.0, which was listed. G*APDH* and *actin* genes were taken as internal controls. The PCR efficiency of the primers was controlled within 100% ± 10%. All qPCR reactions were performed on the CFX98TM real-time system (Bio-rad co.) according to the manufacturer's instructions and recommended cycling conditions. Three replicate reaction tests were performed using a volume of 10 μl (containing 5 μl of 2 × Super Real Pre Mix Plus), 0.3 μl of each of the forward and reverse primers (10 pM / μl) and 1 μL cDNA. The qPCR conditions were as following: initial denaturation at 95°C for 3 minutes, then denaturation at 94°C for 10 seconds in 40 cycles, and annealing and extension at 60°C for 30 seconds. The relative expression level of target gene utilized the method of 2(-μCt) as reported by Livak. The different level of gene expression between two groups was tested by software SPSS (v21.0) taking the P<0.05 as threshold of significant difference. The results were presented as mean ± standard deviation. The 8 AS events were randomly selected from 8 genes to validate by RT-PCR method. The RT-PCR annealing temperature was 55°C for 30 seconds, and the extension temperature was 72°C for 60 seconds by 34 cycles.

To verify the rhythmic expression of circadian rhythm-related genes in Xiang pig ovary, six genes, *per1, cry1, clock, arntl, ppp1cb and ntrk1,* were selected to detected quantitatively by RT-qPCR method at six time points (08:00, 12:00, 16:00, 20:00, 24:00, 04:00). Three ovary samples of Xiang pigs were collected at each time point, and RNA was extracted from the ovarian samples for qPCR verification of genes. The amplification conditions were the same as the previous RT-qPCR method.

RESULT

Summary of sequencing data

The 14 cDNA libraries from ovaries in estrus and diestrous stages were constructed and sequenced respectively, and the sequencing data were filtered and normalized, which were then mapped to the reference genome with HISAT2. It generated 75002160-77100634 read pairs, and 93.57%-96.26% of reads were aligned to the reference genome (Table 1). The only matching reads were performed the following statistical analysis.

TABLE 1

RNA-Seq data description

	Samples	Input Read Pairs	Both Surviving	Forward Only Surviving	Reverse Only Surviving	Dropped	Percentage of mapped reads (%)
Diestrous	D1	77089626	74382355 (96.49%)	2471254 (3.21%)	164408 (0.21%)	69380 (0.09%)	96.01%
	D2	77040960	74260107 (96.39%)	2550481 (3.31%)	160145 (0.21%)	70227 (0.09)	95.88%
	D3	76170080	73520243 (96.52%)	2408511 (3.16%)	167409 (0.22%)	73917 (0.10%)	95.93%
	D4	77100634	74147772 (96.17%)	2713372 (3.52%)	165857 (0.22%)	73633 (0.10%)	96.26%
	D5	76860177	74164234 (96.49%)	2449821 (3.19%)	177911 (0.23%)	68211 (0.09)	96.06%
	D6	76903234	74486606 (96.86%)	2179432 (2.83%)	173980 (0.23%)	63216 (0.08%)	96.11%
	D7	77054008	74411266 (96.57%)	2408339 (3.13%)	163511 (0.21%)	70892 (0.09%)	95.95%
Estrus	E1	75005968	74960964 (99.94%)	45004 (0.06%)	0 (0.00%)	0 (0.00%)	95.98%
	E2	74990854	74960858 (99.96%)	29996 (0.04%)	0 (0.00%)	0 (0.00%)	95.90%
	E3	75206715	75176632 (99.96%)	30083 (0.04%)	0 (0.00%)	0 (0.00%)	95.93%
	E4	75187643	75135012 (99.93%)	52631 (0.07%)	31 (0.00%)	0 (0.00%)	96.24%
	E5	75184086	75154012 (99.96%)	30074 (0.04%)	31 (0.00%)	1 (0.00%)	96.15%
	E6	76109159	74099877 (97.36%)	1682012 (2.21%)	243549 (0.32%)	83720 (0.11%)	94.13%
	E7	75002160	73149607 (97.53%)	1545044 (2.06%)	225006 (0.30%)	82502 (0.11%)	93.57%

Expression of biological clock-related genes in Xiang pig ovary After mapping and annotating based on the pig reference genome assembly Sscrofa11.1, the RNA-seq data of circadian rhythms and clock genes were picked up from the genome-scale data sets (Table 1). Taking threshold value of 0.1 CPM, 90 rhythms genes were determined, in which 81 were expressed in the estrus ovaries and 83 were detected from the diestrous ovaries. The expression levels of those genes were ranged from 867.2 CPM in the highest abundant transcripts to the cut off value of 0.1 CPM (Table 1). Compared with the diestrous ovary, there were more genes (55.6% in estrus, 42.2% in diestrous) at the medium expression level (10 CPM-100 CPM), and fewer genes at the high (>100 CPM) or low (0.1-10 CPM) expression level in the estrus ovary (Figure 1). The top expressed genes were *nono* and *sfpq* with 455.6 and 446.1 CPM in the estrus ovary, and *sfpq* and *top2a* with 532.6 and 512.1 CPM in the diestrous ovary, respectively.

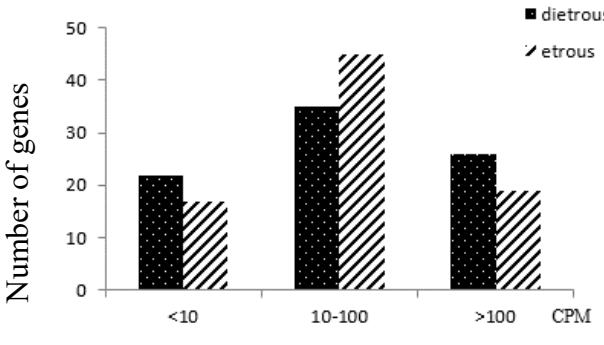

Figure 1) *Expression frequency of circadian rhythm genes in the ovaries of Xiang pigs in estrus and diestrous period (x-axis: CPM Value; y-axis: Number of genes)*

Analysis of differential expression genes

We used R package Limma and DESeq2 to analyze Differentially Expressed Genes (DEGs) between estrus and diestrous ovaries. Also, the P-value ≤ 0.01 and absolute value of log2FC ≥ 0.5 were used as the threshold for determining the significant difference of gene expression. The results showed that 33 DEGs in the ovaries between estrus and diestrous cycles, comprising 13 down-regulated genes (*top2a, nrip1, prkdc, setx, ncoa2, pparg, pspc1, per3, sirt1, timeless, ezh2, ppara,* and *ppp1ca*) and 8 up-regulated genes (*atf4, csnk1d, klf10, id2, bhlhe40, id4, ass1,* and *ntrk1*) in at least 2-fold at estrus stage. The fold changes for DEGs were ranged from 0.17 to 26. Furthermore, we found that 5 genes (*ppp1cb, id3, per1, klf9,* and *nampt*) were at least 1.5-2 fold upregulated and 7 genes (*ep300, ube3a, dyrk1a, sin3a, clock, creb1,* and *magel2*) were at least 1.5 fold-2 fold down regulated in estrus ovary (Table 1). A lot of the genes were expressed in ovaries at medium or high levels (48.9% and 27.4%), including the core clock components, *arntl, clock*, *per1/2/3*, and *cry1/2*.

Detection of differential splicing genes

The rMATS was used to analyze Differentially Alternative Splicing Genes (DSGs) between estrus and diestrous ovaries. Overall, 44 differential splicing events were identified from the transcripts of 34 circadian rhythms genes (P<0.05) including 3 A5SS, 12 RI, 14 SE and 15 MXE events (Table 1). There were multiple genes with complex alternative splicing patterns. Of those, nono presented the most abundant differential alternative splicing events, which included four differential splicing types: A5SS, RI, SE, and MXE. Also, *ppp1cb* harbored three differential splicing types (RI, SE and MXE). Then, ENSSSCG00000000107, *per1, clock, ube3a* and *fbxw11* each contained two differential alternative splicing types. It was found that 20 genes comprised the core clock components, *arntl* and *cry1* were differentially regulated only at AS level. At least 14 genes, such as *per1* and *clock*, were differentially regulated in both expression and AS level in ovaries between estrus and diestrous.

Validation of DEGs by RT-qPCR method

To validate the results from RNA-seq, we used RT-qPCR method to further examine the differentially expressed genes using the same RNA samples from the ovaries (Figure 2). It confirmed that *ppp1cb, csnk1d, per1, klf9, nampt, bhlhe40, id4,* and *ass1* were significantly high expressed in estrus samples, which were in accordance with the RNA-seq analysis (Table 1). The expression of atf4 and ntrk1 in estrus and diestrous ovaries is not significantly different. Moreover, 8 AS events, selected randomly from 8 genes, were detected by RT-PCR and consistent with the RNA-Seq results (Figure 1).The primer sequences were shown in Table 1.

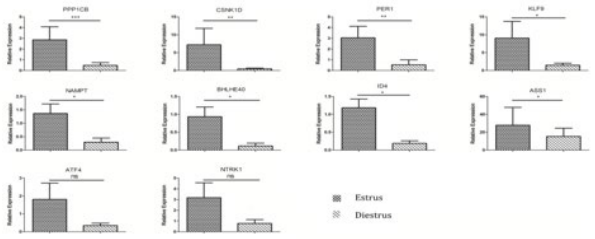

Figure 2) *The verification of differential expression by RT-qPCR. (The gene ppp1cb, csnk1d, per1, klf9, nampt, bhlhe40, id4, and ass1 were significantly high expressed in estrus samples. These data were in accordance with the RNA-seq results and indicated that RNA-Seq results were reliable.)*

Validation of rhythmic expression of clock-related genes

To verify whether the expression of clock-related genes in Xiang pig ovaries was rhythmic, we further examined the expression patterns of *per1, cry1, clock, arntl, ppp1cb and ntrk1* at six time points (08:00, 12:00, 16:00, 20:00, 24:00, 04:00) using RT-qPCR. The samples at each time point were obtained from 3 Xiang pig ovaries collected within one hour. Among them, the expression of per1 and ppp1cb peaked at 24:00 midnight. However, the expression patterns of *clock* and *arntl* were opposite, in which the peaks presented at 8:00 am and it fell to the lowest at 24:00. The maximum values was appeared at 20:00 and 24:00 pm for gene *cry1*, and it was at 16:00 pm for gene *ntrk1*. It illustrated that the core clock genes *per1*, *cry1, clock* and *arntl* and the clock-related genes *ppp1cb* and *ntrk1* were rhythmic expressed in Xiang pig ovaries (Figure 3).

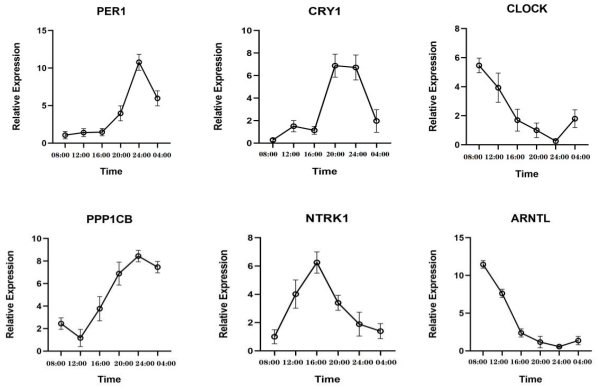

Figure 3) *qPCR.RNA was extracted from ovarian samples for qPCR validation of clock-related genes, and data were shown as mean ± SE (n = 3). GraphPad Prism was used to plot line chart, and independent samples t-test and One-way ANOVA were used for statistical analysis of the corresponding test data. P <0.05 was considered statistically significant.*

DISCUSSION

The mammalian biological clock system has a multi-level structure, including the main clock located in the suprachiasmatic nucleus and the sub-clocks of peripheral organs and tissues [1]. The ovary is a pivotal reproductive organ in female animal, and the expression of the biological clock gene has been found in the ovaries of many organisms [20]. In this study, we analyzed the expression profile and alternative splicing of the biological clock gene in Xiang pig ovaries in estrous cycle from the RNA-seq data. The study found out a total of 90 rhythms genes that were expressed in ovaries. The expression

abundance of these genes was ranging from 0.1 CPM to 532.6 CPM. A lot of the genes were expressed in ovaries at medium or high levels (48.9% and 27.4%), including the core clock components, *arntl, clock, per1/2/3, and cry1/2*. Furthermore, 33 genes were detected to undergo differentially expression and 34 genes were detected to undergo differential alternative splicing between estrous and diestrous ovaries. The DEGs and DSGs related with rhythms might have a connection with the regulation of estrus process in Xiang pig.

Transcription and translation of core clock components genes (*clock* or *npas2, arntl/bmal1,* or *arntl2/bmal2, per1/2/3,* and *cry1/2*) play a critical role in rhythm generation process. *Clock* and *Bmal1* heterodimerize to activate transcription of circadian target genes including *per1/2/3* and *cry1/2*. *Per* and *cry* interact and conversely inhibit transcription of *bmal1* and *clock* genes. These genes and their protein products are organized into interlocking positive and negative transcriptional and translational feedback loops, which regulates circadian rhythm generation in the brain Suprachiasmatic Nucleus (SCN) and peripheral organs [33,34]. In this study, we found that three core clock genes were differentially expressed between estrus and diestrous ovaries. *Clock* and *per3* were down-regulated and *per1* was up-regulated in the estrous ovaries. *Clock* plays a key role in maintaining the circadian rhythm and activating downstream elements. Inhibition of *clock* can inhibit cell growth and increase the rate of apoptosis [35]. *In vivo* experiments show that female mice is injected with *CLOCK-shRNA* have fewer oocytes, fewer litters, and a higher rate of apoptosis. The results indicate that clock plays an important role in fertility, and down-regulation of clock leads to cell apoptosis and decrease reproductive capacity [35]. It was proposed that the down-regulation of clock in the ovary at estrus stage might be related to the low litter size of the Xiang pig. The core clock gene period 1 (*per1*) may be a prolific gene in Drosophila [19]. Female mice with the *per1* mutation showed a normal number of implantation sites but reduced litter size [22]. *Per1* mRNA locates in the secondary oocytes and follicles of ruminants (sheep, cattle) and found that there was no relationship between its transcription level and prolificacy, and this gene did not map to the known QTL region of ovulation rate in cattle [19]. Treatment with Progesterone for 1 hour could induce the expression of *per1* mRNA in MCF-7 cells [36]. In fact, the up-regulation of *per1* was a common feature of many tissues in response to certain types of hormonal stress (For example; luteinising hormone) [37-39]. This expression pattern indicated that *per1* may be the most sensitive effector in the biological clock system [40]. Some studies have found that estrus leads to changes in the expression time and amplitude of the biological clock genes [41]. Rising estrogen cause female animal to go into estrous stage [42].The estrus in Xiang pig might be speculated to reset the biological clock by up-regulating the *per1* gene to response the stimulus of steroid hormone. In this study, *per3* was down-regulated in the ovaries of estrus pigs. There is little information on the function of per3 gene in reproduction except for brain development [43]. In contrast, the expression patterns of clock genes in other pig breeds are much different from that in Xiang pig. In the ovarian follicles of Large White pigs and Mi pigs, the core circadian clock genes were all down-regulated and not differentially expressed between estrus and diestrous periods [44].Compared with Large White pigs and Mi pigs, the characteristics of Xiang pig are specific, such as low birth rate and insignificant estrous behavior. It would be interesting to prove whether the expression profiles of core biological clock genes in ovary is a reason for the special reproductive traits such as estrus performance and litter size in different pig breeds.

The biological clock system consists of an input pathway, a core oscillator and an output pathway. The post-translational modification of clock proteins is essential to maintain the accuracy and robustness of the evolutionarily conserved circadian clock [45]. Post-translational modification and degradation of the clock proteins are key steps in determining the length of the circadian clock cycle [46]. Our research found that the products of most differentially expressed genes were related to the post-translational modification of the core clock protein. For example, *Ppp1Cb* and *Ppp1Ca* can reduce the phosphorylation of *Per2*, and affect the nuclear localization of the protein *Per2*, which may at least partially change the cycle and phase shift characteristics of the biological clock [47]. Also, highlighted *DYRK1A* is the enzyme responsible for the phosphorylation of *Cry2* at Ser557 and plays a key role in regulating the protein level of Cry2 [48]. The casein kinases *CSNK1D* and *CSNK1E* phosphorylate the *PER* protein and provide a marker for subsequent degradation [46]. The *UBE3A* binds and degrades *Bmal1* in an ubiquitin ligase-dependent manner [45]. Furthermore, *MAGEL2* regulates the ubiquitination and stability of *Cry1,* and changes its nuclear and cytoplasmic distribution [49]. Lastly, *SIRT1* deacetylates *Per2* and *Bmal1*, thereby participates in biological rhythm regulation [50]. The previous reports illustrate that the estrus cycle affects the localization and degradation of the core clock protein by changing their post-translational modification. And transcription and post-transcriptional regulation are the basis of clock system component activities, and post-transcriptional mechanisms accounts for more than half of the regulatory network [51]. The results of this study further strengthened the view that the post-translational mechanism participated in the circadian gene regulatory network. In addition, we detected some differentially expressed genes from Xiang pig ovaries that might affect the expression of core clock genes at the transcriptional level (Table 1). For example, *Top2A* binds to the unique GC-rich open chromatin structure of the bmal1 promoter region, indicating that *Top2* on the bmal1 promoter affects transcription of *bmal1* [52]. Also, CBP/P300 and tissue-specific cofactors regulate clock /*Bmal1* transcription positively or negatively [53]. Accordingly, ID (DNA binding inhibitor) is an important transcription repressor. Each ID protein contains a helix-loop-helix domain through which it can interact with *bHLH* protein. However, ID lacks the basic domain that allows binding to the E-box element, which leads to the ID being able to modify the transactivation of *clock* genes and CCG by interfering with the *clock-Bmal1* heterodimer, bHLH orange factor and other bHLH factors [54]. These data indicate that the estrus cycle promoted the time change of the biological clock in reproductive tissues at the level of transcription and post-translational modifications (Figure 4).

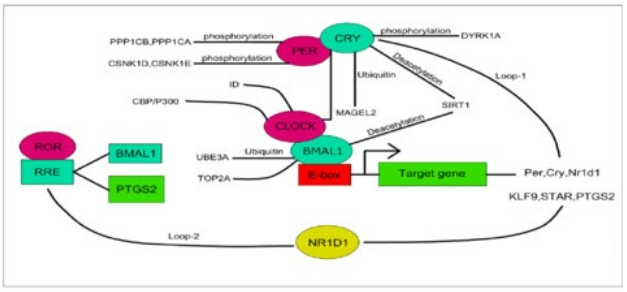

Figure 4) *The Effects of estrus cycle on ovarian circadian rhythm genes of Xiang pigs (This figure showcases the differentially expressed gene products in the estrus and diestrous ovaries are related to the post-translational modification of the core clock protein, which may lead to changes in the expression of core clock genes and clock-controlled genes).*

In addition, we detected several genes controlled by the circadian rhythm, which were differentially expressed in the ovaries of the two periods including *klf9, star, ptgs2*. Other studies have shown that *klf9* is a clock output gene, and *CLOCK* and *BMAL1* complexes can bind and activate transcription at the 5' flanking region of *klf9*, which is blocked by the co-expression of *PER1*. Also, *KLF9* may play a role in regulating the effects of *CLOCK/BMAL1* and in the expression of DBP, other clock and clock output genes, thereby changing the timing and amplitude of the circadian oscillations of gene transcription [55]. The previous studies have found that Steroidogenic Enzymes (STAR), Prostaglandin Synthase (PTGS2), which are related to ovarian progesterone synthesis, are Clock-Controlled Genes (CCGs). The E-box enhancer exists in the 5'-flanking region of star, which binds to the *CLOCK-BMAL1* heterodimer and activates the transcription of gene star [56]. Co-expression of the negative regulators *PER* and *CRY* attenuates this activation [57]. The promoter of *ptgs2* has an E-box element and a REV-ERBα/ ROR*response* Element (RORE). The secretion of *PGF2* can be balanced by the inhibition or stimulation of transcriptional regulation on REV-ERB and *BMAL1/CLOCK*, respectively [58]. It is thus inferred that the peripheral circadian oscillator, such as ovary, could play an essential role in synchronizing local physiology through regulation of the expression of clock-controlled genes [59]. In this study, *star* and *ptgs2* in the ovaries of the estrus pigs were up-regulated by 6.42 times and 5.55 times, respectively. It might be possible that the core clock genes might regulate the production of steroid hormones by controlling the ovarian-specific CCG, thereby affecting the estrus cycle of Xiang pigs.

Pre-mRNA splicing is a basic biological process through which introns of nascent RNA are removed and exons are merged to form mature RNA, which is then translated into protein [60]. Through Alternative Splicing (AS), different proteins are produced, resulting in a wider variety of cellular functions [61]. Except for transcription or post-translational controls, alternative splicing also plays a key role in circadian rhythms in a cell-type-specific manner [62]. Recent research reports several cases, in which AS is involved in the regulation of biological clocks in plants, mice, and fruit flies [63-67]. In this study, *Arntl* only underwent differential alternative splicing, while *clock* and *per1* experienced both of differential alternative splicing and differential expression. The differential alternative splicing event occurred in *per1* was the retention of introns [14-15]. This event would result in the deletion of amino acid residues from positions 545 to 1278 in the encoded protein, and the deletion site was located in the core domain to activate the protein. This intron retention event occurred more frequently in the ovaries of Xiang pigs in the diestrous period, and the active *PER1* protein decreased. During the estrus period, the occurrence of the intron retention event decreased, and the active *PER1* protein level increased, which further increased the expression of *PER1*. It indicated that the core clock component *PER1* may respond to the changes of sterol hormones in the ovary through transcriptional regulation and post-transcriptional regulation. *Clock* underwent the differential exon 17 skipping event. When this event occurred, the encoded protein would be deleted amino acid residues from positions 484 to 513. The deleted amino acids were not located in the core domain and the influence on the protein activity was still unclear. These findings suggested that AS might directly change the structure of core clock components to regulate the ovarian biological clock, and the AS regulation of biological clock genes might be independent in the gene expression. However, more evidence needs to be accumulated whether the AS events in these biological clock genes would turn on and turn off the circadian rhythm in pig.

CONCLUSION

In this study, we examined 90 clock-related genes expressed in Xiang pigs' ovaries and found that 33 clock-related genes were differentially expressed between estrus and diestrous stages. We identified 44 differential splicing events from transcripts of 34 circadian clock-related genes. In addition, we also found that 20 genes including core clock components *arntl* and *cry1* were differentially regulated at AS level only, and 14 genes including *per1* and *clock* were differentially regulated at both expression and AS level. We also demonstrated by RT-qPCR experiments that the core clock genes *per1, cry, clock* and *arntl* and the clock-related genes *ppp1cb* and *ntrk1* were rhythmically expressed in ovaries of Xiang pig. The results suggested that the circadian clock in Xiang pig ovaries might play a key role in regulating ovarian physiological functions through transcriptional and post-transcriptional regulation.

AUTHOR CONTRIBUTION

LTT, JFW, and XQR conceived and designed the experiments; LTT performed the experiments; LTT and XN analyzed the data; LTT, XN, SL, and SHH contributed to samples and data analysis; and LTT wrote the paper. All authors reviewed the manuscript.

ETHICS APPROVAL

All animal procedures were approved by the Institutional Animal Care and Use Committee of Guizhou University (Approval number GZU-201709). The procedures were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

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CONFLICTS OF INTEREST

All the authors have no conflict of interest with anyone about this manuscript. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted. No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. The work described has not been published previously, and not been submitted to any other journal, in whole or in part. All the authors listed have approved the manuscript that is enclosed**.**

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