A transcriptome analysis for 24-hour continuous sampled uterus reveals circadian regulation of the key pathways involved in eggshell formation of chicken

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ABSTRACT Circadian timing system controlled the rhythmic events, for example, ovulation and oviposition in chickens. However, how biological clock mediates eggshell formation remains obscure. Here, A 24-h mRNA transcriptome analysis was carried out in the uterus of 18 chickens with similar oviposition time points to identify the rhythmic genes and to reveal critical genes and biological pathways involved in the eggshell biomineralization. JTK_CYCLE analysis and real-time PCR revealed a total of 1,793 genes from the sequencing database with 23,513 genes (FPKM>1) were rhythmic genes regulating the rhythmic system and the expression of typical clock genes *Per2, Cry1, Bmal1, Clock, Per3*, and *Rev-erbβ* were rhythmically expressed, which suggested that endogenous clock in uterus might control the eggshell mineralization. Time of peak expression of the rhythmic genes was analyzed based on their acrophase. The main phases clustered at the periods from Zeitgeber time 0 (**ZT0**) to ZT4 (6:00−10:00) and from ZT10 to ZT14 (16:00-20:00). The rhythmic genes were annotated to the following Gene Ontology terms rhythmic process, lyase, ATP binding, cell membrane component. KEGG pathway enrichment analysis revealed the top 15 rhythmic genes were involved in vital biological pathways, including syndecan (1, 2, 3)-mediated signaling, post-translational regulation of adheres junction stability and disassembly, FoxO family signaling, TGF-β receptor and transport of small molecular pathways. 166 of total 1,235 genes (13.4%) were defined as rhythmic transfer factors (**TFs**) and they were investigated expression time distribution of cis-elements of circadian clock system D-box, E-box, B-site, and Y-Box within 24 h. Results indicated that rhythmic TFs at each phase are potential drivers of their circadian transcription activities. Compared with the control, the expression abundances of ion transport elements *SCNN1G, CA2, SPP1*, and *ATP1B1* were significantly decreased after the interference of *Bmal1* gene in synchronized uterine tubular gland cells. Clock genes changed their expression along with the eggshell formation, indicating that there is circadian clock in the uterus of chicken and it regulates the expression of eggshell formation genes.

Key words: chicken, eggshell, uterus, rhythm, clock gene

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INTRODUCTION

Nearly all animal organisms possess a circadian clock, which leads to oscillations in many physiological processes allowed for the adaptation and response to predictable daily changes in the environment. Under natural daylight conditions, many birds exhibit a diurnal rhythm in oviposition (egg-laying). For most hens, oviposition occurs in the morning (Lillpers, 1991; Zakaria et al., 2005), few occur in the afternoon. Oviposition happens continuously in a couple of days, but it will stop 24 h if this happens after certain time point in the afternoon. Then the next egg will be laid in the morning again, such a series of eggs laid on successive days was named a "sequence" or "clutch" of eggs (Silver, 1986).

Egg formation needs 25 to 26 h as its complex physiological processes. After ovulation, the yolk enters the oviduct where albumen, eggshell membranes, and the

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eggshell are sequentially deposited in the different segments of the oviduct. Approximately 5 to 6 g of calcium carbonate is deposited into the chicken eggshell during its formation, the whole process needs 17 to 20 h and happened at the uterus, where specially named eggshell gland (**ESG**). Generally, the mineralization process of eggshell in uterus can be divided into 3 stages, including initial stage (5−10 h after ovulation), rapid deposition stage (10−20 h after ovulation), and final mineralization stage (20−24 h after ovulation) (Jeong et al., 2012; Bailey and Silver, 2014), which indicates that the eggshell formation has daily rhythm. The papillary layer and palisade layer of eggshell were mainly formed in the rapid deposition stage, which could deposit 0.33 g calcium carbonate per hour. This deposition process ended 2 h before laying (Gautron et al., 1997). Intriguingly, studies showed that time point of oviposition was strongly correlated with egg weight and shell quality (Samara et al., 1996). In commercial layers, the first clutch of eggs laid in the morning had greater egg weight and shell thickness, compared with the subsequent sequential eggs laid in the afternoon (Massaro and Davis, 2010). Based on previous studies, we hypothesis that eggshell formation may be controlled by clock-driven diurnal rhythm of mineralization.

Precise timing and rhythmicity in hormone release and tissue sensitivity in the HPG axis is regulated by circadian clocks located at hypothalamus, pituitary (Olcese et al., 2006; Resuehr et al., 2007), and ovary (Fahrenkrug et al., 2006; Karman and Tischkau, 2006; Zhang et al., 2017). There are few studies on the role of endogenous clock in eggshell formation in the ESG. Expressing rhythms of clock gene and circadian-controlled genes (**CCG**) in the rat oviduct were reported 10 yr ago (Kennaway et al., 2003), and the functions of clock in the mouse and rat uterus were tightly related with the processes of implantation and decidualization (He et al., 2007; Ratajczak et al., 2010). In our previous study, we detected the presence of functional clocks in chicken infundibulum and uterus (Zhang et al., 2016). However, their functions in this tissue remain unclear.

Recent finding reported that endogenous clock is a major component and significantly controls cuticle deposition in the uterus of laying hens (Poyatos et al., 2020). To clearly understand the control mechanisms of uterine clock exerted on CCGs regarding the phases in a day, a systemic understanding of the dynamic transcriptional regulations of the circadian clock is necessary. The aim of this study is to identify the key candidate genes regulated by clock genes during eggshell formation via transcriptome analysis, so we carried 24-h mRNA transcriptional analysis in the chicken uteruses with similar oviposition time points and detected the expression abundances of the ion transport elements involved in uterine mineralization in original uterine tubular gland cells, and the regulatory relationships between core clock genes and ion transport genes were determined.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were approved by the Committee on Experimental Animal Management of Sichuan Agriculture University (Certification No. YCS-B2018102013), and carried out in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the State Council of the People's Republic of China.

Animal Managements

All experimental animals were raised at the Sichuan Agriculture University Poultry Breeding farm (Ya'an, China). Five hundred 30-wk-old laying hens (BH-01, a dual-purpose line with black-shank and yellow-dotted feather cultivated by Poultry Breeding Group of Sichuan Agriculture University) were caged individually and accessed to the feed and water ad libitum. Chickens were inoculated with Avian Influenza (H9 and H5 subtype) and Newcastle Disease (**NDV**) on d 8, 10, and 24, respectively. Light was provided for 24 h during the first 3 d after hatching, and gradually decreased to 12 h from d 4 to 28 and suspended at this level for 13 wk. Then light was increased from 12 h to 16 h from d 120 to 190 and kept 16-h light every day until d 500. Continuous light was provided from 6:00 of Beijing time each day. From d 210, the oviposition time for each chicken was recorded every half an hour from 6:00 to 18:00 and lasted 30 d. Schematic representation of the experimental strategy has been shown in Figure 1A.

Sample Collections

For sample collections, Zeitgeber time 0 (**ZT0**) is defined as the moment of light turned on. After 4 h, the moment was named ZT4. Eighteen birds with consistent laying records between ZT2 and ZT2.5 and laying eggs within 30 d from 500 individuals were selected for sample collection. Uterine tissues were harvested from those birds within 24 h (each of 3 birds for 6 time points). The first sampling time was 10:00 a. m. of Beijing time (ZT4). Samples were taken every 4 h until 6:00 am in the next morning (ZT0/ZT24). Uterine tissue samples were collected and quickly frozen in liquid nitrogen and stored at -80°C for RNA extraction.

Primer Design

The list of ion transporters was established based on our transcriptomic data and the chicken genome database (GRCg6a, GenBank assembly accession: GCA_000002315.5). The novel transporters in chicken were identified using human orthologue in Swiss-Prot/TrEMBL and RefSeq databases. Primers were designed with Primer 5.0 and Oligo 6.0 and further verified by

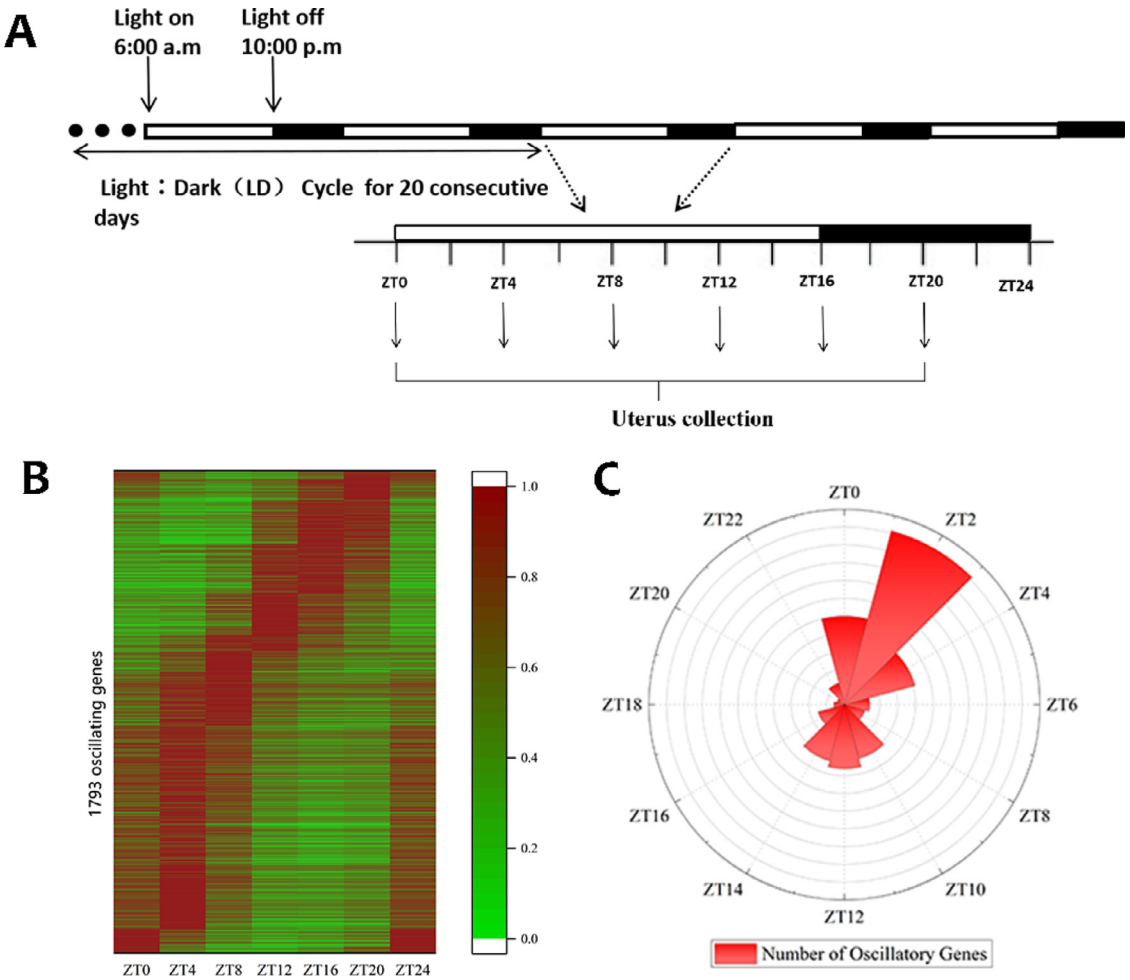

Figure 1. Circadian transcription in chicken uterus. (A) Schematic representation of the experimental strategy used in this study. Cells and tissues were cultured outside the body (ex vivo) and synchronized by a single DEX pulse to evaluate their rhythmicity (Light/Dark cycle [LD]; black and white bars show light-off and light-on, respectively). (B) Heat map of the relative transcription of 1,793 oscillating genes sorted by oscillation phase. (C) Rose diagram showing the distribution of peak time of rhythmic expression gene.

NCBI BLAST for dimerization and specificity using Amplify 3X software. Detail was shown in Table 1.

Real-Time qPCR and RNA-Sequencing

Total RNA was extracted from frozen tissue with Trizol reagent (Takara, Tokyo, Japan) according to the manufacturer's procedure. RNA concentrations were measured at 260 nm using a NanodropND 2000 (Thermo Fischer, Wilmington, DE). The integrity of the RNA was evaluated in 2% agarose gel and with an Agilent 2100 Bioanalyser (Agilent Technologies, Massy, France). Only RNA samples with a 28S/18S ratio > 1.3 were considered for RT-PCR and qRT-PCR experiments. A total amount of 3 μg RNA per sample was used for the RNA-seq. Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Inc., Ipswich, MA). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After the cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated.

Bioinformatics Analyzing

The rhythmic genes were screened using JTK_CYCLE R package and then were classified according to their peak of expression (Hughes et al., 2010). The ggplot2 package in R software was used to make a rose diagram. For each wedge, the corresponding area is proportional to the number in the group. Gene ontology (**GO**) and Kyoto Encyclopaedia of Genes and Genomes (**KEGG**) enrichment were performed with an online software David Functional Annotation Tool (https://david.ncifcrf.gov/tools.jsp). Ensemble gene IDs of the rhythmic genes were uploaded for analysis. Heatmaps were plotted using R version 3.2.4. The promoter sequences of the JTK-positive rhythmic genes were obtained using Gene2Promoter (Genomatix software package used for retrieval and analysis of promoters). Then the "overrepresented transcription factor binding sites" were analyzed using Genomatix software and the calculated z-scores. JASPAR (http://jaspar.

Table 1. Primers used in qRT-PCR.

Gene	Primers (5'-3')	Product length (bp)	Accession number
Baml1	F: CTGTGACAGAGGGAAGATACTGT R: TGGCAATGTCTTTCGGATGAAGG	119	NM_001001463
Clock	F: TGAGCTCCATTGCAGACAGAA R: TCCATCTTCCGAGCATTACCT	179	NM_001289834
Per2	F: CCAACTTTGTTGTGTGCTCCTTGC R: CTGGAACAAACAGGTTGGTGTGTG	115	NM_204262
Per3	F: CACAGGTTCAGCAGCATCAG R: ATCATCACCCAGGCTGACTC	219	NM_001289779
Cry1	F: CCGGGAAACGCCCAAA R: TGCTCTGCCGCTGGACTT	66	NM_204245
Cry2	F: ACAAGCCACCCCTCACCTAC R: GATGGGACCCCATACACATC	150	NM_204244
Rev-erbβ	F: TGTTGCTGGCTTGACGAAAG R: CGACACTGCTGGCATCTGTT	234	NM_205205
CALB1	F: CAGGGTGTCAAAATGTGTGC R: GCCAGTTCTGCTCGGTAAAG	215	NM_205513
SCNN1G	F: CAAAAGGCACTTCACCCGTTTC R: GGACAATGATCTTGGCTCCTGTC	191	XM_414880
CA2	F: ATCGTCAACAACGGGCACTCCTTC R: TGCACCAACCTGTAGACTCCATCC	101	NM_205317
CLCN2	F: CCTGGACACCAATGTGATGCTG R: CACGAAGGTCTTCAGGGTGAGATAC	184	XM_423073
CLCN5	F: CGATTGGAGGAGTGCTCTTTAGTC R: CAAAAGGATTGATGGAACGCAG	123	XM_420265
ATP1B1	F: CACCAACCTGACCTATGACG R: TCTTTGTCGCTGTACTGGATG	78	NM_205520
SPP1	F: CACAGACTTCCCCACAGAG R: CATCCTCAATGAGCTTCCTGG	152	NM_205535
NKAIN4	F: GACGCTGCACCTTGATTTTC R: AGGAAGTTGGCTAGGATAGGG	109	XM_015296647
ATP6V1C2	F: GGCCAATGGAGTTGACCTGA R: TCCGAGTCAGCAAGTTTCCC	218	XM_025148941
KCNJ15	F: ACCCCGTGTGATGTCCAAAA R: CTCCCGCTTTGAGGTGAACT	238	XM_425554
SLC41A3	F: GGTGGGAATCCTGCTCTACG R: GATGAGCCAAACCAGACGGA	157	XM_015293362

genereg.net) used to search and identify transcription factors (**TFs**) and motifs common to differentially regulated genes.

Cell Culture and Treatment

Uterine tubular gland cells were collected from the uterus of 210 d BH-01 chickens, adding type *y* collagenase at 37°C to digest the tissue for 60 min and then 0.25% pancreatin to digest for 10 min at 37°C. Cells were planked with the density of $2 \times l0^6$ /mL and cultured in Dulbecco's modified Eagle medium (**DMEM**/F12) supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA) and a mixture of 100 U of penicillin-streptomycin-glutamine, and maintained in a humidified atmosphere with 5% (v/v) CO_2 at 37°C. When the cells reached 80 to 90% confluence, they were treated with *Bmal1* siRNA interference. The cells from the interference group and the control group were treated with RNA fragment and empty RNA fragment for 24 h, respectively, and then treated with dexamethasone (**DEX**) for 1h.

Statistical Analysis

Gene expression data were analyzed and processed by $2^{-\Delta\Delta Ct}$ method. The results were expressed by mean ± standard error (**SE**). Significant differences were determined via the Tukey-Kramer test at *P*-value < 0.05.

RESULTS

Circadian Transcriptome Analysis

RNA-seq was finished based on the tissues collected at 6 time points within 24-h with 3 replications at each time point. Data quality was summarized in Table 2. At each time point, samples contained an average of 8.0 Gb clean bases. Their sequencing error rate was 0.02%, and GC content was close to 50%. The proportions of Q20 and Q30 values were high, meanwhile the proportion of redundant data was small. The clean reads were mapped to the chicken genome (GRCg6a) with a reference annotation containing 28,568 genes listed in the Ensembl database. At a threshold of 0.1 FPKM (Fragments Per Kilobase of transcript per Million mapped reads), 23,513 genes were expressed at least at one time point and were used for further analysis. JTK_CYCLE analysis was performed as previously described method and identify 1,793 cyclical genes (7.63%) within a period between 21 and 24 h ($21 \leq$ period (τ) ≤ 24 h), and the acrophase (peak expression time, in ZT) for genes that are rhythmically expressed (Figure 1B). The times of peak

Table 2. Summary of data quality.

Samples	Raw reads	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
ZT4_1	55787378	53879924	8.08G	0.02	96.49	92.06	50.87
ZT4_2	56894518	54047194	8.11G	0.02	96.25	91.70	50.36
ZT4_3	48748188	47504690	7.13G	0.02	96.28	91.61	49.85
ZT8_1	69480856	66365884	9.95G	0.02	96.40	91.90	51.47
ZT8_2	55839196	54365064	8.15G	0.02	96.34	91.72	49.98
ZT8_3	50551240	49387862	7.41G	0.02	96.71	92.42	48.44
ZT12_1	51047042	49645380	7.45G	0.02	96.45	92.04	51.14
ZT12_2	57708442	55418418	8.31G	0.02	96.73	92.53	50.53
ZT12_3	50322534	48556788	7.28G	0.02	96.34	91.81	51.10
ZT16_1	49675246	48304160	7.25G	0.02	96.53	92.12	50.77
ZT16_2	57426188	54806808	8.22G	0.02	96.34	91.76	50.24
ZT16_3	68679710	64503502	9.68G	0.02	96.23	91.65	51.67
ZT20_1	56610476	55246764	8.29G	0.02	96.56	92.20	51.33
ZT20_2	55784806	53303722	8G	0.02	96.44	92.01	50.91
ZT20_3	62079102	59149464	8.87G	0.02	96.28	91.67	51.16
ZT0_1	54947760	52025888	7.8G	0.02	96.51	92.07	50.66
ZT0_2	52529050	50351454	7.55G	0.02	96.53	92.13	51.72
ZT0_3	56568338	53947214	8.09G	0.02	96.38	91.97	50.69

expression of the rhythmic genes were analyzed based on their acrophase. The phases were not equally distributed throughout the 24-h day, and the circadian genes were separated into 2 defined groups with a bimodal distribution of peak expression. As shown in Figure 1C, the main phase cluster was located at early morning, showing 963 genes having expression peak at the period from ZT0 to ZT4, while a distinct trough appeared at mid-afternoon (132 genes having expression peak at the period from ZT6 to ZT8). Another phase clusters showed 502 and 196 genes had expression peaks at the periods from ZT10 to 14 and ZT16 to 22 (at night), respectively. Among those rhythmic genes, 514 genes lacked gene annotation. Thus total 1235 unique genes were kept in the database after the correction and removal of genes for redundancy (n = 44) (Table S1).

The mRNA expression profiles of core circadian genes *Clock, Bmal1, Per2, Per3*, and *Cry1* and CCGs *Rev-erbβ* detected via real-time PCR were in accordance with the counterpart profile detected via RNA-seq (Figure 2), which proved the data reliability obtained via transcriptome sequencing.

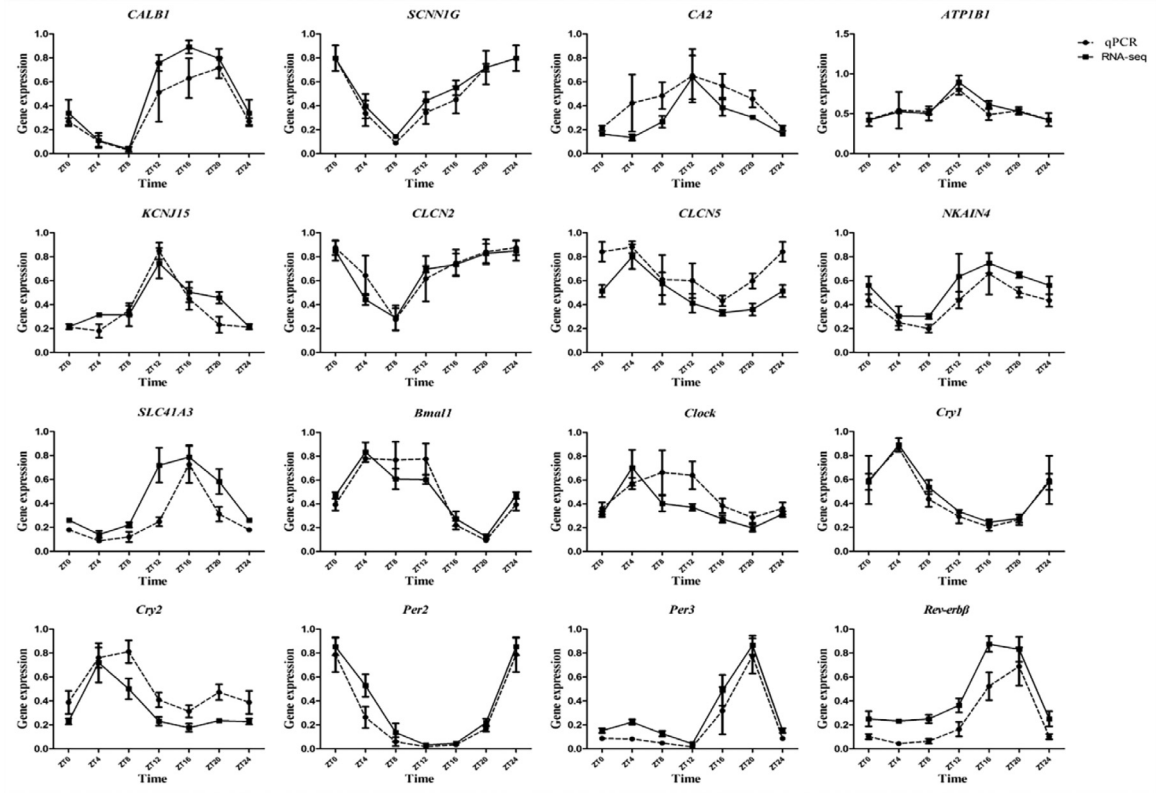

Figure 2. Relative expression of the clock genes and eggshell formation related genes determined by RNA-seq and RT-qPCR, respectively. Data are double plotted for better visualization. RT-qPCR data are expressed as the mean ± SE (n = 3 per time point) and normalized to the maximum expression of the day.

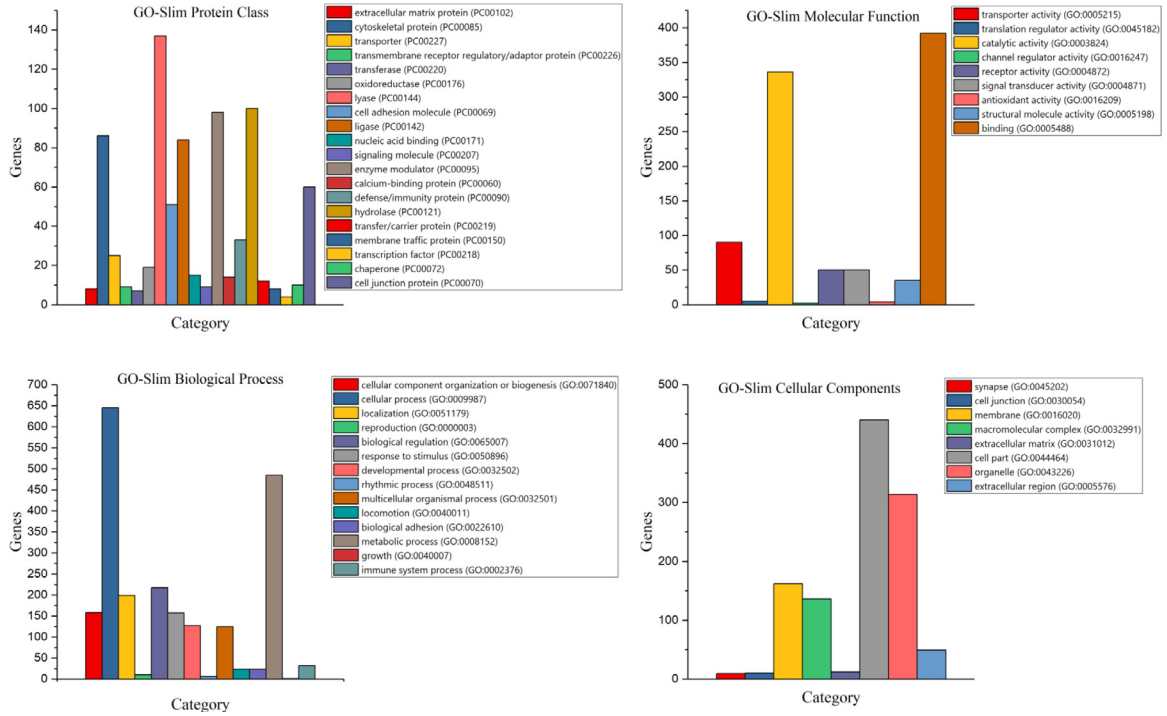

Figure 3. GO analysis of rhythmic genes in the uterus of layers. (A) GO-slim protein class. (B) GO-slim molecular function. (C) GO slim biologic process. (D) GO-slim cellular components. All the GO terms with a modified Fisher Exact P-value < 0.05 and threshold gene count of 3 were considered enriched.

Biological Functions of the Rhythmic Expression Genes

GO and KEGG analyses revealed the biological functions of the rhythmic genes (Figure 3). The most significantly enriched GO items obtained in the protein group were lyase, oxidoreductase, hydrolase, and cytoskeletal protein. Meanwhile, in the molecular function group, the catalytic activity, binding and transporter activity were significantly enriched. Similarly, in the biology process group, cellular process, metabolic process, biological regulation, and localization were enriched significantly, and those of cell constituent were cell part, organelle, membrane and macromolecular complex. The top 15 pathways-based networks were composed of the circadian clock and circadian rhythm pathways, syndecan (1, 2, 3)-mediated signaling events, post-translational regulation of adherens junction stability and disassembly, androgen receptor, FoxO family signaling, and TGF-β receptor (Figure 4).

Analysis of Transcription Factors and Binding Sites

The transcription factors (TFs) which rhythmically expressed were identified to define their regulatory networks. 166 genes from total 1,235 genes (13.4%) were defined as rhythmical TFs. A motif analysis of rhythmic TFs at different circadian phases revealed that there was phase-specific enrichment of transcription factor motifs and 4 sets of motifs were enriched at different

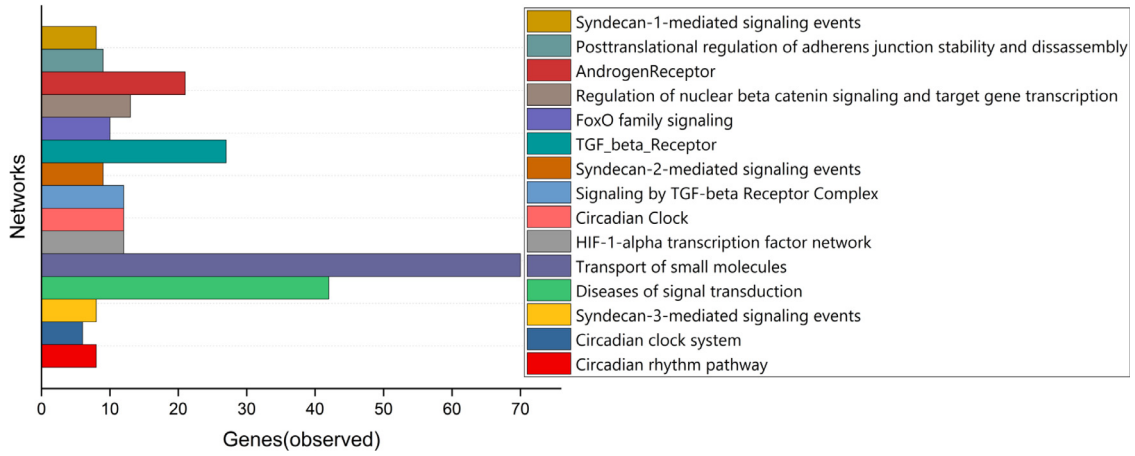

Figure 4. The top 15 KEGG pathway enrichment analysis of rhythmic genes in the uterus of layers. All the pathways with a modified Fisher Exact P-value < 0.05 and threshold gene count of 3 were considered enriched.

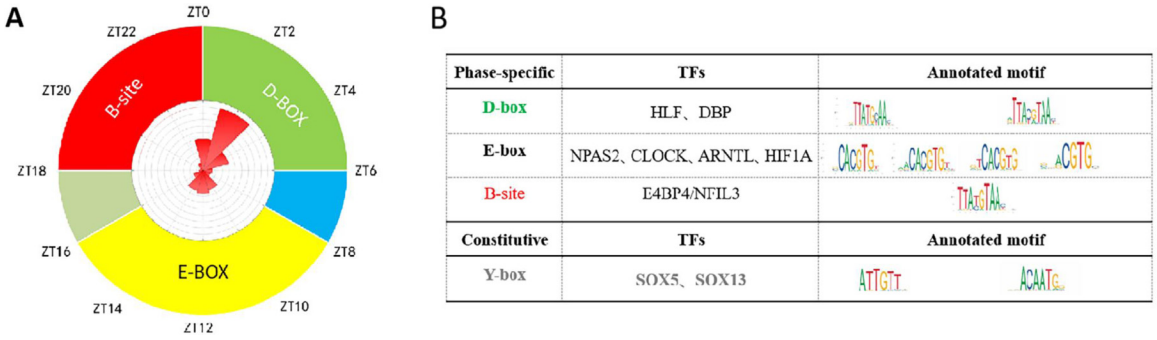

Figure 5. Motifs specifically enriched in each eRNA group. (A) Motifs specifically enriched in each phase are labeled in the clock diagram. (B) Each motif and its transcription factors in assigned groups are shown in the table.

time points (Figure 5). Four E-box motifs were enriched at ZT8 and ZT16, respectively, while 2 D-box motifs were enriched at ZT0 and ZT6, respectively, and a B-site motif was enriched at ZT18 and ZT24, respectively.

Effect of Bmal1 Knockdown on the Expression of Ion Transport Genes

To better understand the physiological function of the uterus circadian clockwork and detect the specific CCGs under the regulation of uterine circadian oscillators, we evaluated the expression of a subset of genes including canonical core clock genes and ion transport genes in the uterine epithelial cells with *Bmal1* siRNA treatment. As shown in Figure 6, after DEX treatment, the clock genes *Baml1, Per2*, and *Rev-erbβ* and ion transport genes *SCNNIG, CA2, SPP1,* and *ATP1B1* expressed rhythmically. These ion transport genes were essential substances of eggshell composition and involved in sodium ion transport, bicarbonate enzyme catalytic production process, protein composition of eggshell organic matter, and inorganic calcium ion transport in the uterus. Figure 7 indicated the expression abundances of core clock genes and ion transport genes in uterine epithelial cells with *Bmal1* siRNA treatment. The mRNA expression of *Bmal1* was inhibited significantly after being transfected with *Bmal1* siRNA ($P < 0.01$). *Rev-erbβ* was regarded as the core clock gene with the regulation of BMAL1-CLOCK heterodimer through E-box elements located at their promoter regions. Thus, *Rev-erbβ* transcript profile was significantly downregulated in *Bmal1* siRNA cells ($P < 0.01$). Meanwhile, mRNA expression levels of several ion transport genes *SCNNIG, CA2, ATP1B1,* and *SPP1* were significantly inhibited in the *Bmal1* siRNA treatment group, compared with those in the control ($P < 0.01$). Their interference efficiencies were 37.6%, 23.7%, 30.6%, 42.5%, and 25.4%, respectively. These results indicated that the clock gene regulated the expression of eggshell forming genes.

DISCUSSION

As far as we know, the present study is the first deep-sequencing of the laying hens' uterus within 24 h cycle,

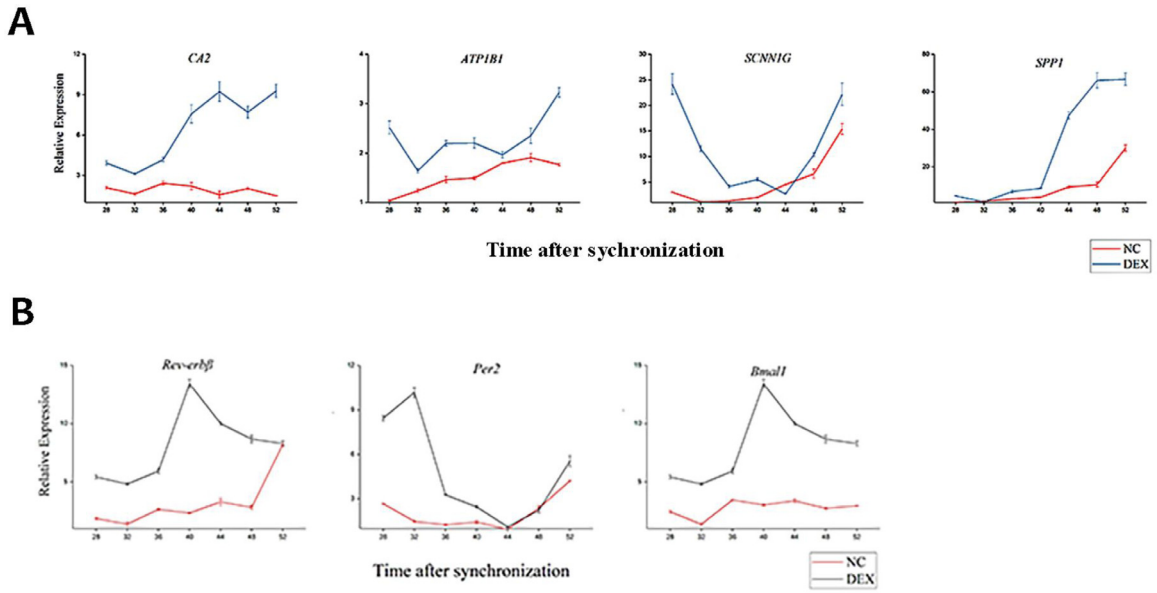

Figure 6. Expression of core clock genes and ion transport genes in uterine tubular gland cells after dexamethasone (DEX) treatment. (A) The expression changes of the clock-controlled-genes *CA2, ATP1B1, SCNNIG,* and *SPP1* after DEX treatment. (B) The expression changes of the clock genes *Rev-erbβ, Per2,* and *Baml1* after DEX treatment. Data were expressed as the mean ± SE (n = 3).

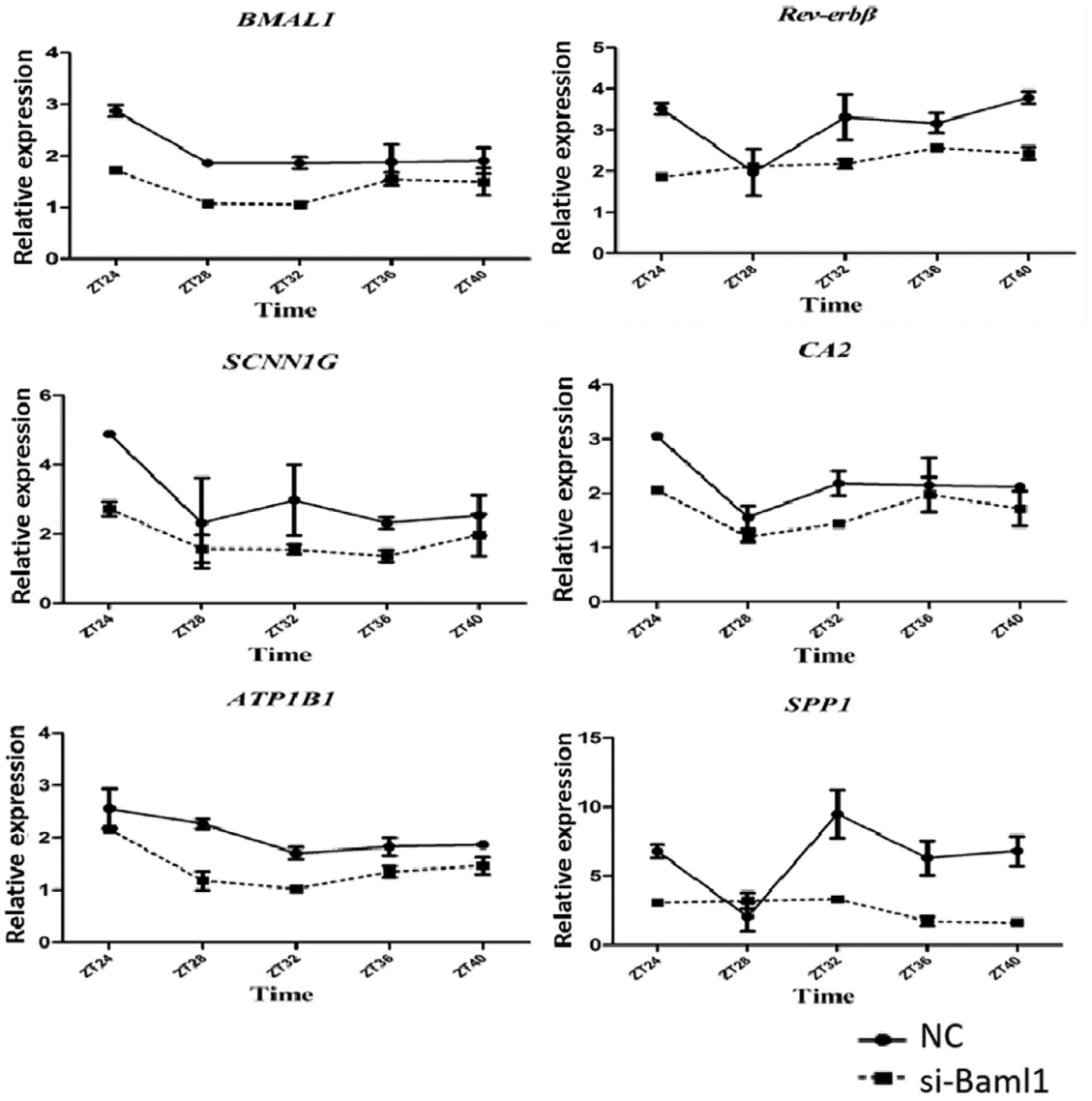

Figure 7. Expression of core clock genes and ion transport genes in uterine tubular gland cells with or without Bmal1 siRNA treatment. (A) The expression changes of the clock genes *Baml1* and *Rev-erbβ* in uterine tubular gland cells with or without *Bmal1* siRNA treatment. (B) The expression changes of the clock genes *SCNNIG, CA2, ATP1B1,* and *SPP1* in uterine tubular gland cells with or without *Bmal1* siRNA treatment. Data were expressed as the mean ± SE (n = 3).

and we hypothesis the periodic regulation of eggshell formation from biological rhythm perspective. The rhythmic genes identified in this study differed greatly from previous studies that compared the transcriptome of the uterus with an egg or not (Brionne et al., 2014; Khan et al., 2019; Yin et al., 2020). The present study captured some unique rhythmic events, including the eggshell formation process, especially the ion transport process. The physiological changes of the hens with continuous laying ability tend to be stable, which makes the high-throughput sequencing data more scientific. In the previous study (Zhang et al., 2016), we noticed that if the oviposition time of layers fluctuated around a fixed period, their continuous laying performances were usually perfect. Obviously, their physiological and biochemical status were stable. Thus, the samples we collected with similar oviposition time are suitable for studying the relationship between circadian rhythm and eggshell formation.

High throughput sequencing can detect gene transcription on a large scale. Also, the expansion of data scale brings challenges to scan the rhythmic genes. Therefore, analytical methods are needed to determine the period, phase, and amplitude of gene expression (Hughes et al., 2010). There are various ways to analyze gene expression rhythm, including cosine, double cosine, cosine weakening, cosine peak, cosine linear trend, cosine index trend, and so on (Deckard et al., 2013). Different analysis methods have different advantages in screening a specific cosine expression. De Lichtenberg (**DL**) method was used to analyze yeast cell rhythm (de Lichtenberg et al., 2005), which is beneficial for screening genes with reduced cosine expression. LS, as a Fourier method, was originated from the field of astrophysics and known to combines 2 analytical methods established by Lomb and Scargle (LOMB, 1976; Scargle, 1982). JTK_CYCLE combines Jonckheere-Terpstra and Kendall's tau methods for screening rhythmic

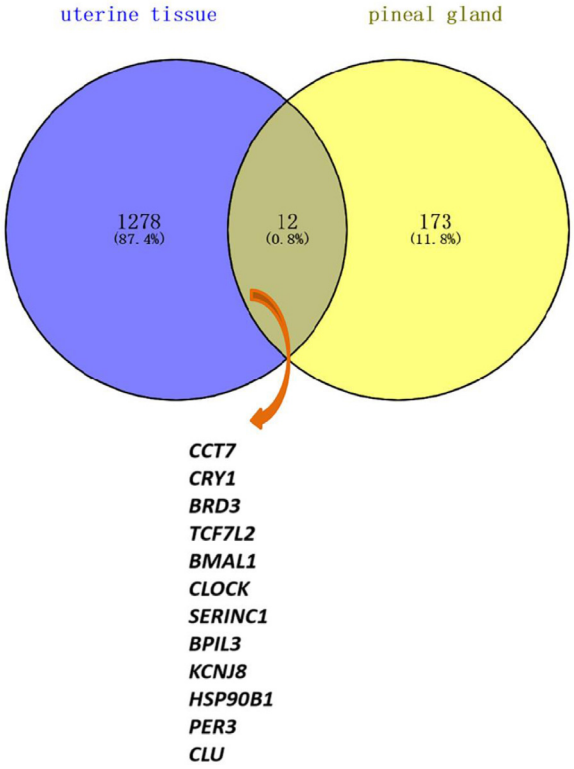

Figure 8. Veen figure for the overlap of genes from the chicken pineal gland and uterine.

components in genome-scale data. JTK_CYCLE and LS can screen genes with cosine expression. The uterus clock gene showed cosine expression. Therefore, our present study focuses on cosine expression genes. JTK can use r script to call data to facilitate researchers' calculation. Also, JTK can screen genes with cosine peak, cosine linear trend, and cosine index trend, which is more suitable than LS function. According to the purpose of this study, JTK_CYCLE is the most suitable method to screen the rhythmic genes.

The estimate of ~8% ESG transcriptome exhibits circadian rhythm which is close to the value estimated in previous studies (Sherratt et al., 2019). However, only a few studies were performed to identify the circadian transcriptome in chicken tissues. When we compared the identified rhythmic genes with the result of previous microarray study performed in chicken pineal gland (Bailey et al., 2003), we found that 12 genes *CCT7, CRY1, BRD3, TCF7L2, BMAL1, CLOCK, SERINC1, BPIL3, KCNJ8, HSP90B1, PER3*, and *CLU* overlapped between the 2 tissues (Figure 8). Meanwhile, 4 typical clock genes are also involved, which supports the view that rhythmic transcriptome is tissue-specific (Mure et al., 2018). Additionally, transcription of known core clock components showed the classic sinusoidal circadian profile ($q < 0.05$) with a zenith and nadir at the expected time points. This verifies our previous study which indicated the presence of functional clock in the ESG (Zhang et al., 2017). Together, these results demonstrate the robustness of our data.

According to GO annotation and KEGG pathway analyses of the rhythmic genes, 8 genes were annotated as rhythmic process in the BP results of GO annotation. Along with 4 core clock genes (*Clock, Bmal1, Cry1,* and *Per3), Npas2* (neural PAS domain protein 2), *Bhlhe40* (basic helix loop helix family member E40), *Top1* (toposomerase I), and *Sirt1* (sirtuin 1) genes were included. *Npas2* is a transcription factor of bHLH-PAS family, whose function is similar to the clock genes. It forms NPAS2/BMAL1 protein heterodimer, which combines with the E-box of the downstream genes to enhances the expression of *Per* and *Cry* clock genes. With the accumulation of PER/CRY protein dimer, the activity of NPAS2/BMAL1 is inhibited and a feedback loop is formed (McNamara et al., 2001). Deletion or mutation of *Npas2* will lead to biological rhythm disorder (Englund et al., 2009). Crumbley et al. (2010) found that the biological rhythm of mouse was not greatly affected by the mutation of clock gene, which may be due to the replacement of *Clock* function by *Npas2.* The rhythmic expression of *Npas2* gene in the uterus enriches the regulatory pathway of clock gene. *Bhlhe40* can bind to E-box to regulate the expression of other genes (Asanoma et al., 2015). The encoded protein interacts with BMAL1 (Xiong et al., 2016) and binds to the E-box located in the upstream regulatory region of *PER1* and inhibits the transcription of *PER1* activated by CLOCK/BMAL1 (Pisanu et al., 2018). The present study indicates that more clock genes show rhythmic expression, and provides a basis for exploring the downstream functional genes of biological clock regulation in chicken uterus.

Links to the circadian clock were previously described for several networks. A role for FoxOs in regulation of circadian rhythms upon incoming metabolic cues has been reported (Zheng et al., 2007; Chaves et al., 2014). TGF-β has been implicated in lung fibrogenesis. In mammals, TGF-β is able to up-regulate BMAL1 expression in both lung epithelial cells and normal lung fibroblasts (Dong et al., 2016). Furthermore, except for the several pathways described above, the significantly enriched pathways we have identified in this study include circadian clock and circadian rhythm pathway. Therefore, these pathways may be influenced via the circadian clock, or via the circadian clock controlled transcription of one or more several key regulatory factors, or via the circadian clock of post transcriptional mechanisms, such as protein degradation and phosphorylation.

Circadian clocks exert their functions via a transcriptional and translational autoregulatory feedback loops. In addition to controlling clock genes' expression, these oscillators also drive rhythmic expression of thousands of target genes by binding to cis-regulatory sites in their promoter region (Resuehr et al., 2007). Recent work has explored the genome-wide cis-acting targets of several circadian transcription factors, and has identified multiple circadian clock regulatory elements (**CCREs**), such as E/E'-box (CACGT[G/T]/CANNTG), DBP binding elements (D-box) (TTATG[T/C]AA), and RevErbA/ROR binding elements (RRE) ([A/T]A[A/T]NT[A/G]GGTCA) (Ueda et al., 2002; Ueda et al., 2005). These factors are regulated by the core components of the circadian clock feedback loop, and show obvious circadian

rhythm alternation. Interestingly, the phase distribution of circadian transcription factors in our study is uneven distribution; those peaking at ZT0−ZT6, ZT8−ZT16, and ZT18−ZT24 were enriched for D-box, E-box, and B-site motifs, respectively. Four E-box motifs (NPAS2, CLOCK, ARNTL, and HIF1A) were mostly enriched from phase ZT8 to ZT16, which coincided with the peak of BMAL1 binding to the genome (Rey et al., 2011; Koike et al., 2012). We also discovered that the 2 D-box motifs, recognized by PAR-bZIP proteins including DBP and HLF (Cowell et al., 1992; Li and Hunger, 2001; Mitsui et al., 2001), were mostly enriched from phase ZT0 to ZT6, which coincided with the phase of known target genes for these TFs (Gachon et al., 2006). Moreover, E4BP4 and NFIL3 emerged as key regulators of the ZT18−ZT22 B-site, implying a potential role of B-site proteins in the circadian regulation of transcripts with this phase. In addition to these phase-specific motifs, constitutively enriched motifs in all phases were identified, most prominently the SOX5 and SOX13 motifs. Thus, those TFs bounded specifically at each phase are potential drivers of their circadian transcription activities.

Eggshell formation takes place daily in the hen ESG and requires highly active mechanisms of ion transfer to secrete the calcium and bicarbonate (carbonate). We presumed the rhythmic genes contained the genes that associated with eggshell formation. We compared the rhythmic genes from the data set with the over-expressed genes (302 genes) in chicken ESG when the shell is mineralizing (Brionne et al., 2014). Ninety-one overlaps included many known eggshell calcification markers, which reflected the possibility that uterine clock might play an important role in eggshell mineralization. Many of the rhythmic genes we screened are involved in eggshell ion transport (Gachon et al., 2006; Khan et al., 2019). Furthermore, the largest number of rhythmic genes (70 genes) were enriched in the "transport of small molecules" pathway. The results of the in-vitro verification of eggshell formation genes were consistent with the transcriptome sequencing results, indicating that eggshell formation genes were expressed in a rhythmic manner. The regulation of clock genes initially revealed the molecular regulatory mechanism of periodic eggshell formation. The previous studies compared the uterus transcriptome with or without egg, and found some high expression genes when the egg was present in the uterus, for example, *SPP1* and *CA2* (Brionne et al., 2014; Yin et al., 2020). Therefore, these high expression genes related to ion transport may be also controlled by clock gene.

Some promising candidates as regulators of uterine circadian rhythms are the sex steroids estrogen (Nakamura et al., 2008) and progesterone (Rubel et al., 2012), which directly modulate the circadian clock in the uterus (He et al., 2007). What drives the change in circadian period in the uterus during pregnancy is unknown; and more studies are required to understand how the uterine sensitivity to hormones change works in both the nonpregnant and pregnant states.

CONCLUSION

According to the mRNA transcriptome sequencing, 23,513 genes were expressed in the uterus of chicken, and 1,793 rhythmic genes were screened by JTK_CYCLE, and 39 items were annotated by GO, among which rhythmic process, lyase, ATP binding, cell membrane component were consistent with egg shell cycle formation. In-vitro cell experiments showed that the rhythmic expressions of eggshell forming genes were regulated by clock gene. Generally, our findings reveal that the circadian clock exists in the uterus of chicken and regulates the expression of genes related to ion transportation, which directly affects the eggshell formation in chickens.

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DISCLOSURES

The authors declare no conflicts of interest.

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