Circadian miR-218-5p targets gene CA2 to regulate uterine carbonic anhydrase activity during egg shell calcification

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ABSTRACT MicroRNAs (**miRNAs**) are involved in regulating the circadian clock. In our previous work, miR-218-5p was found to be a circadian miRNA in the chicken uterus, but its role in the eggshell formation process was not clear. In the present study, we found that the expression levels of miR-218-5p and two 2 predicted target genes carbonic anhydrase 2 (**CA2**) and neuronal PAS domain protein 2 (**NPAS2**) were oscillated in the chicken uterus. The results of dual-luciferase reporter gene assays in the present study demonstrated that miR-218-5p directly targeted the 3' untranslated regions of CA2 and NPAS2. miR-218-5p showed an opposite expression profile to CA2 within a 24 h cycle in the chicken uterus. Moreover, over-expression of miR-218-5p reduced the mRNA and protein expression of CA2, while miR-218-5p knockdown increased CA2 mRNA and protein expression. Overexpression of CA2 also significantly increased the activity of carbonic anhydrase Ⅱ ($P < 0.05$), whereas knockdown of CA2 decreased the activity of carbonic anhydrase Ⅱ. miR-218-5p influenced carbonic anhydrase activity via regulating the expression of CA2. These results demonstrated that clock-controlled miR-218-5p regulates carbonic anhydrase activity in the chicken uterus by targeting CA2 during eggshell formation.

Key words: chicken uterus, circadian, miR-218-5p, carbonic anhydrase, eggshell formation

INTRODUCTION

Circadian clocks regulate the physiology and behavior of organisms to adapt to the day-night cycle. The endogenous central circadian clock is located in the hypothalamic suprachiasmatic nucleus (**SCN**) (Dunlap, 1999). In a core oscillator, CLOCK: BMAL1 or NPAS2: BMAL1 heterodimers bind directly to E-box elements located in the *Per* and *Cry* genes and activate their transcription (Welsh et al., 1995; Reppert and Weaver, 2002; Bell-Pedersen et al., 2005; Pevet and Challet, 2011; Mohawk et al., 2012). Neuronal PAS domain protein 2 (**NPAS2**), a core circadian molecule, has been reported to compensate for CLOCK. Therefore, NPAS2

is essential for normal functioning of the biological rhythmic order (Debruyne et al., 2006; Debruyne et al., 2007; Englund et al., 2009; Landgraf et al., 2016). Regulation of the circadian clock involves post-transcriptional, translational, and post-translational mechanisms. In recent years, microRNAs (**miRNAs**) have been recognized to play an important role in regulating various aspects of biological processes (Mehta and Cheng, 2013). miRNAs are evolutionarily conserved small non-coding RNAs that silence gene expression through translational repression or mRNA degradation (Gallego and Virshup, 2007; Lim and Allada, 2013; Anna and Kannan, 2021). miRNAs bind to the target 3' untranslated regions (**3'UTR**) of mRNA by specific sequences and regulate mRNA stability or translation efficiency (Fabian et al., 2010). miRNAs are involved in various physiological processes by targeting most protein-coding genes. In particular, global deletion of Dicer, an enzyme that encodes pre-miRNA cleavage to generate miRNA, significantly shortens the circadian rhythm

cycle, providing genetic evidence that miRNAs play a regulatory role in clock function (Chen et al., 2013). Several studies have identified specific miRNAs that regulate the expression of core clock components (Kojima et al., 2010; Luo and Sehgal, 2012; Shende et al., 2013).

Our previous study found that circadian clocks are also involved in the function of the poultry reproductive system, especially in the chicken uterus (Zhang et al., 2016; Cui et al., 2021; Zhang et al., 2022). Some rhythmic miRNAs have been identified by 24 h high-throughput sequencing of the chicken uterus and we found that circadian miR-449c-5p regulates uterine Ca^{2+} transport during eggshell calcification in chickens (Cui et al., 2021). In our previous study, miR-218-5p was identified as one of the rhythmic miRNAs, and carbonic anhydrase 2 (**CA2**) and *NPAS2* were predicted to be 2 target genes of miR-218-5p. CA2 is reported to catalyze HCO_3^- formation (Annan et al., 2019), which is one of the main elements of calcium carbonate (**CaCO_3**). We hypothesize that circadian miR-218-5p is involved in eggshell formation. Thus, we investigated the role of miR-218-5p in chicken uterus and its function in uterine tubular gland cells.

MATERIALS AND METHODS

Animals and Sample Collections

All animal experiment procedures and sample collection followed the rulers approved by the Institutional Animal Care and Use Committees of Sichuan Agricultural University (Certification No. YCS-B2018102013).

All birds were raised at the Sichuan Agricultural 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 the Poultry Breeding Group of Sichuan Agricultural University) were caged individually and had access to feed and water ad libitum. The feeding procedures were consistent with those described in our previous study (Zhang et al., 2022).

For sample collection, zeitgeber time 0 (**ZT0**) was defined as the moment when the light was turned on (06:00 Beijing Time), and subsequent light simulation times were denoted as ZT4 (10:00 Beijing Time), ZT8 (14:00 Beijing Time), ZT12 (18:00 Beijing Time), ZT16 (22:00 Beijing Time), and ZT20 (02:00 Beijing Time), respectively. 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 tissue samples were harvested from those birds within 24 h (each of the 3 birds at 6 time points). The first sampling time was 10:00 a.m. in Beijing time (ZT4). The samples were collected every 4 h until 6:00 a.m. the next morning (ZT0/ZT24). The uterine tissues were sampled and quickly frozen in liquid nitrogen and stored at −80°C for RNA extraction.

Bioinformatics Analysis

The potential target genes of miR-218-5p were predicted using TargetScan (http://www.targetscan.org/vert_72/) and miRDB (http://mirdb.org/index.html) databases. Genes that appeared in both databases were selected as target genes of miR-218-5p. The target genes that were negatively correlated with miR-218-5p were subjected to Gene ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) enrichment analysis using OmicShare tools, a free online platform for data analysis (www.omicshare.com/tools). An FDR < 0.05 was defined as significantly enriched.

Immunohistochemistry Assay

The uterine tissues were collected at time points ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24, and washed 3 times with sterile PBS. Then fixed in 4% paraformaldehyde at room temperature (**RT**) for 20 min, and then treated with 3% hydrogen peroxide, the endogenous enzyme was inactivated. Subsequently, the sample was washed in PBS solution for 5 min, then added with blocking agent (goat serum), incubated for 20 min at RT, and incubated with anti-CA2 (Abcam, Cambridge, UK) overnight at 4°C. After incubation, the samples were washed and incubated with fluorescent-labeled secondary antibodies for 30 min under RT. After the second incubation, the samples were washed with PBS, and then incubated with peroxidase (**POD**) labeled streptavidin (DyLight 488) for 30 min at RT. DAB kit (BBI, Canada) under RT color for 5 to 30 min. An optical microscope (Nikon Eclipse E100, Japan) and an imaging system (Nikon DS-U3, Japan) were used to obtain microscopic photographs. Image-Pro Plus software (version 6.0; Media Cybernetics, Rockville, MD) was used to analyze the obtained images.

Cell Culture, Identification, and Transfection

Uterine tubular gland cells were isolated from the uterus of the Rohman layers during the peak laying period according to previous studies (Moll et al., 1982; Huang et al., 2005). The tubal uterus was dissected and removed after the chickens were humanely euthanatized by cervical dislocation. The two ends of the uterus were tied with a fine thread to prevent contamination. The uterus was repeatedly cleaned with PBS containing 100 U of penicillin-streptomycin-glutamine for several times, and then the redundant tissue (vascular and tissue membrane, etc.) were removed with sterile scissors in biosafety cabinets, and the endometrium was cut with surgical scissors into $0.1 \text{ cm}^2 \times 0.1 \text{ cm}^2$ tissue block. Using 1 mg/mL type I collagenase digestive tissue block at 37°C for 60 min and then 0.25% pancreatin digest for 10 min at 37°C, and then centrifuged, after which the supernatant was discarded. Resuspending the cells in a growth medium containing F12 (Hyclone, South Logan, UT) + 10% fetal bovine serum (Gibco, Middleton, WI) + 0.1% penicillin/streptomycin (Invitrogen,

Carlsbad, CA). Planked with the density of 2×10^6/mL and cultured in growth medium, and maintained in a humidified atmosphere with 5% (v/v) CO_2 at 37°C.

For cell type identification, cells were plated on glass cover slides in culture medium, washed with PBS after the culture medium was removed, and fixed with 4% paraformaldehyde for 10 min. The cells were then washed 3 times with PBS and permeabilized with 0.2% Triton X-100 for 10 min. After washing with PBS, the cells were incubated with primary antibody rabbit anti-Cytokertin 18 (Bioss, Beijing, China) at 4°C overnight. The cells were washed and incubated with fluorescent secondary antibodies at room temperature for 1 h in the dark. The cells were then washed thrice times with TBST and the fluorescence intensity was observed using a fluorescence microscope (DP80; Olympus, Japan). According to manufacturer's instructions, lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) was used for cell transfection after cell coverage reached 70 to 80%. The inhibitor and mimic of miR-218b-5p were synthesized by GenePharma Co., Ltd. (Shanghai, China) and CA2 small interfering RNA (**siRNA**) was designed by Sangon Biotech Co., Ltd. (Shanghai, China).

RNA Preparation and Quantitative Real-Time PCR

Total RNA from uterine tissues and cells was extracted using Trizol (Invitrogen, Cincinnati, OH) according to the manufacturer's protocol (Rio et al., 2010). The RNA concentration and purity were estimated by determining the A260/A280 absorbance ratio, and the 18 S and 28 S bands in a 1% agarose gel. RNA integrity was detected using a Bioanalyzer 2100 system (Agilent Technologies, China). Primers for qRT-PCR were designed using Primer Premier 5 (Table 1). qRT-PCR was performed as previously described (Cui et al., 2021). Chicken GAPDH and U6 were used as internal controls.

Luciferase Assay

DF-1 cells were seeded in 48-well plates and cultured in a growth medium containing F12 (Hyclone, UT) and 10% fetal bovine serum (Gibco, Langley, OK). Plasmids (wild type or mutant type of CA2-3′-UTR) were co-transfected with mimic negative control (**NC**) and miR-218-5p, respectively until the confluence reached 60 to 70%. After 48 h of transfection, luciferase activity was detected using a luciferase reporter kit (Promega, Madison, WI) according to the manufacturer's instructions.

Western Blot Assay

Chicken uterine gland cells were seeded in 6-well dishes and cultured in growth medium, and were transfected when the confluence reached 70 to 80%. After 48 h of transfection, total protein was extracted using a total protein extraction kit (Solarbio, Beijing, China) and the protein concentration was determined using a BCA protein determination kit (BestBio, Shanghai, China) according to the manufacturer's instructions. Equivalent amounts of total proteins from the different treatments were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with QuickBlockTM blocking buffer (Beyotime, Shanghai, China) for 2 h at room temperature. The membranes were incubated with specific primary antibodies at 4°C overnight, including anti-CA2 and anti-β-tubulin (Abcam, Cambridge, UK; diluted 1: 1,000). The next day, the PVDF membranes with proteins were incubated with a specific secondary antibody (ZenBio, Chengdu, China; diluted 1:5,000). Finally, enhanced chemiluminescence (**ECL**) luminous fluid (Beyotime) was used for detection. The relative gray scales of CA2 were detected using ImageJ software, and 3 independent replicates were set for each treatment.

Enzymed-Linked Immunosorbent Assay

The cells and cultured medium were harvested after repeated freeze-thaw cycles 3 times and centrifuged at 2,000 r/min for 20 min to discard the precipitate, and the supernatant samples from each treatment group were collected. The carbonic anhydrase 2 (CA2) activity of each sample was determined using specific kits according to the manufacturer's protocol (Mlbio, Shanghai, China). Purified chicken CA2 antibody was coated on a microporous plate to prepare a solid phase antibody. All samples were successively added to the microporous coated plate, and then combined with HRP labeled detection antibodies to form an antibody-antigen enzyme-conjugated antibody complex. After thorough washing, TMB was added as a substrate for color rendering. TMB was converted to blue under catalysis by the HRP enzyme, and to the final yellow under the action of acid. Color depth was positively correlated with chicken CA2 in the samples. The absorbance was measured at 450 nm using a microplate reader, and the

Table 1. Primers used for qRT-PCR.

Target gene	FForward sense (5′ - 3′)	Reverse sense (5′ - 3′)
CA2	GTACGACAGCCACAACGGAC	CTTGCAGCACTGACTTGTCG
NPAS2	TGCCACCTATGCAACTCCAG	TGATACCCCATATTTCCTTGCTGT
GAPDH	TCCTCCACCTTTGATGCG	GTGCCTGGCTCACTCCTT
gga-miR-218-5p	ACACTCCAGCTGGGGTTGTGCTTGATCTAA	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACATGGTT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

content of chicken CA2 in each sample was calculated according to the standard curve.

Statistical Analysis

Experimental data are presented as the average ± standard deviation (**SD**) of a minimum of 3 biological replicates. The Student's two-tailed unpaired t test was used to determine the statistical significance of in vitro experiments. All statistical tests were two-sided, and a P value of less than 0.05 was considered statistically significant.

RESULTS

Morphological Changes of Chicken Oviduct

In this study, we observed morphological changes in the chicken oviducts at 6 time points within 24 h. The results showed that the eggs were in the magnum at ZT4, in the isthmus at ZT8, and all the other 4 time points were in the uterus (Figure 1), indicating that the eggshell was formed mainly at night.

Bioinformatics Analysis and Target Genes' Biological Functions of miR-218-5p

We performed sequence alignment and found that the seed sequence of chicken miR-218-5p was conserved in mammals (Figure 2A), and found the stem-loop of pre-gga-miR-218-5p (Figure 2B). GO and KEGG pathway analyses were performed on the 270 target genes (Figure 2C). The most significantly enriched GO items obtained in the biological process (**BP**) group were cellular processes, biological regulation, metabolic processes, and responses to stimuli. Meanwhile, in the cellular component group, the cell parts, organelles and membranes were significantly enriched. In the molecular function group, the binding, catalytic activity, transcription regulator activity, and transporter activity were significantly enriched (Figure 2D). KEGG analysis showed that these target genes were enriched in 2-oxo-carboxylic metabolism, mRNA surveillance pathway, cell adhesion molecules, focal adhesion, endocytosis, tight junction, ECM-receptor interaction, adherens junction, apelin signaling pathway, and erbB signaling pathway (Figure 2E).

CA2 and NPAS2 are the Target Genes of miR-218-5p

First, using TargetScan online software to predict the target genes of *miR-218-5p*, we found that the 3′-UTR of chicken *CA2* and *NPAS2* mRNAs had potential binding sites for *miR-218-5p* (Figures 3D, 3E and 3G). Moreover, after determining the expression levels of *miR-218-5p, CA2* and *NPAS2* at 6 time points within a 24 h cycle in uterine tissues, we found that the expression level of *miR-218-5p* increased from ZT16 to ZT0 (ZT24), reaching its highest level at ZT0; however, it eventually decreased from ZT0 to ZT8. Reciprocally, the mRNA expression of *CA2* and *NPAS2* decreased, and then increased sharply (Figures 3A−3C). The dual-luciferase reporter assay results showed that the luciferase activities of the CA2 and NPAS2 wild-type reporter vectors decreased significantly in response to the miR-218-5p mimic whereas no dramatic changes were observed in their mutant vectors (Figures 3F and 3H). These results indicate that CA2 and NPAS2 are target genes of miR-218-5p.

ZT0   ZT4   ZT8

ZT12   ZT16   ZT20

Figure 1. Morphological changes in chicken uterus at 6 time points within 24 h. Eggs were in the magnum at ZT4, in the isthmus at ZT8, and all the other four time points (ZT0, ZT12, ZT16, and ZT20) were in the uterus.
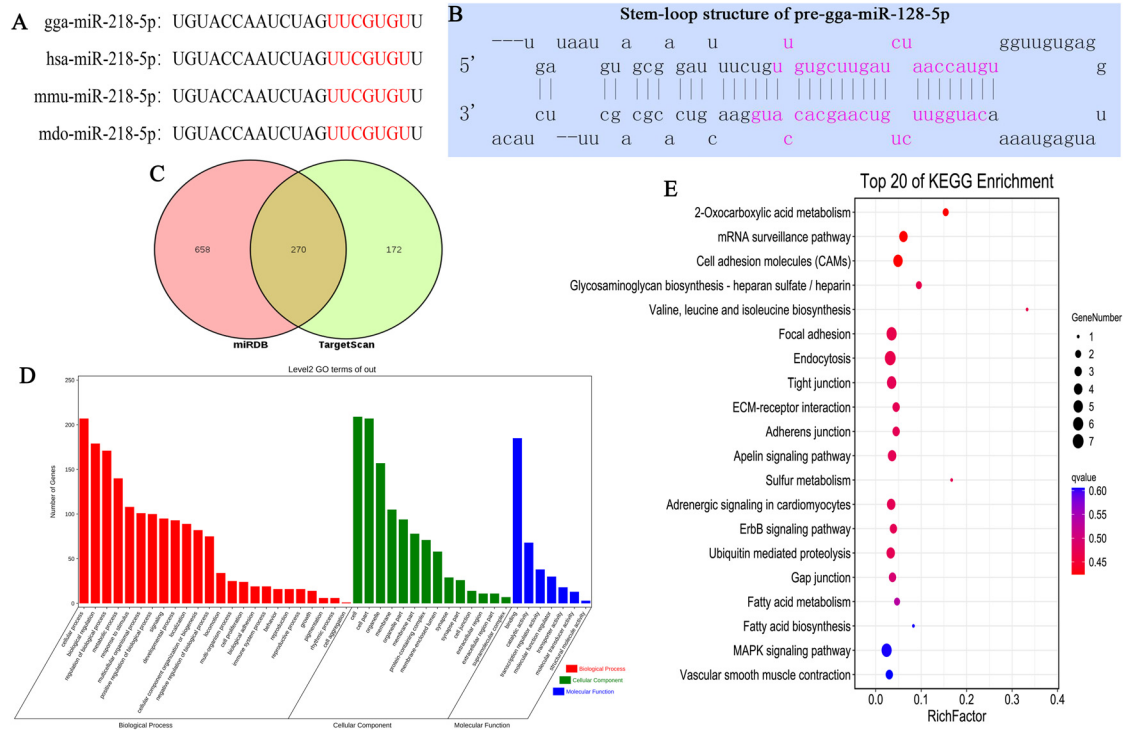
Figure 2. Sequencing characteristics and biological functions of target genes of miR-218-5p. (A) Mature sequence (red characters) of miR-218-5p among different animal species: gga, chicken; hsa, homo sapiens; mmu, mus musculus; mdo, Monodelphis domestica. (B) Stem-loop structure of pre-gga-miR-128-5p. (C) Venn diagram showing the target genes of miR-218-5p using Targetscan and miRDB. (D) GO enrichment analysis of 270 target genes. (E) TOP 20 significantly enriched pathways associated with 270 target genes.

Clock-Controlled miR-218-5p Regulates the mRNA and Protein Expression of CA2

Immunofluorescence analysis revealed that the isolated and cultured cells were correct (Figure 4A). miR-218-5p expression increased significantly after transfection with the miR-218-5p mimic ($P < 0.05$; Figure 4B), but decreased after transfection with the miR-218-5p inhibitor (Figure 4C). miR-218-5p overexpression inhibited the mRNA and protein expression levels of CA2 (Figures 4D−4F), and the inhibition of miR-218-5p promoted the mRNA and protein expression levels of CA2 (Figures 4G−4I). Therefore, these results indicate that clock-controlled miR-218-5p modulates CA2 expression. Immunohistochemistry showed that the protein expression of CA2 in the uterus first increased and then gradually decreased from ZT0 to ZT20 with the highest expression at ZT12 (Figures 5A and 5B), which was similar to the trend of the mRNA expression of CA2.

CA2 Regulated Carbonic Anhydrase Activity in Uterine Tubular Gland Cells

The mRNA and protein levels of CA2 were determined after transfection with pcDNA3.1-CA2 and pcDNA3.1 empty plasmid, or Si-CA2-1, si-CA2-2, and Si-NC. Compared with the pcDNA3.1 group, the mRNA and protein levels of CA2 were significantly increased in the pcDNA3.1-CA2 group (Figures 6A −6C). Meanwhile, the Si-CA2-1 and si-CA2-2 groups

had lower mRNA and protein levels of CA2 than the Si-NC group (Figures 6D−6F). Compared to the control group (mimic NC), overexpression of miR-218-5p significantly decreased the concentration of CA2 after transfection for 48 h, indicating a reduction in intracellular carbonic anhydrase activity (Figure 6G). The knockdown of miR-218-5p increased the concentration of CA2 (Figure 6H). Moreover, CA2 overexpression significantly increased the concentration of CA2 after transfection 48 h (Figure 6I). These results were similar to the downregulation of miR-218-5p, but contrary to the knockdown of CA2 (Figure 6J). These results indicate that CA2 regulates uterine carbonic anhydrase activity.

DISCUSSION

Circadian clock can be divided into 2 groups: central and peripheral. The central clock is located in the hypothalamic supra chiasmatic nucleus (SCN), which is stimulated by light and produces master clock signals transmitted through humoral and neuroendocrine pathways to the peripheral tissue to coordinate the peripheral clock (Fahrenkrug et al., 2006; Olcese et al., 2006; Resuehr et al., 2007). Eggshell calcification in the uterus of laying hens involves multiple cellular and molecular processes, including calcium carbonate secretion, transport and biological mineralization (Sah et al., 2018; Khan et al., 2019). It has been reported that circadian clock is a major component and significantly controls cuticle deposition in the uterus of laying hens (Poyatos
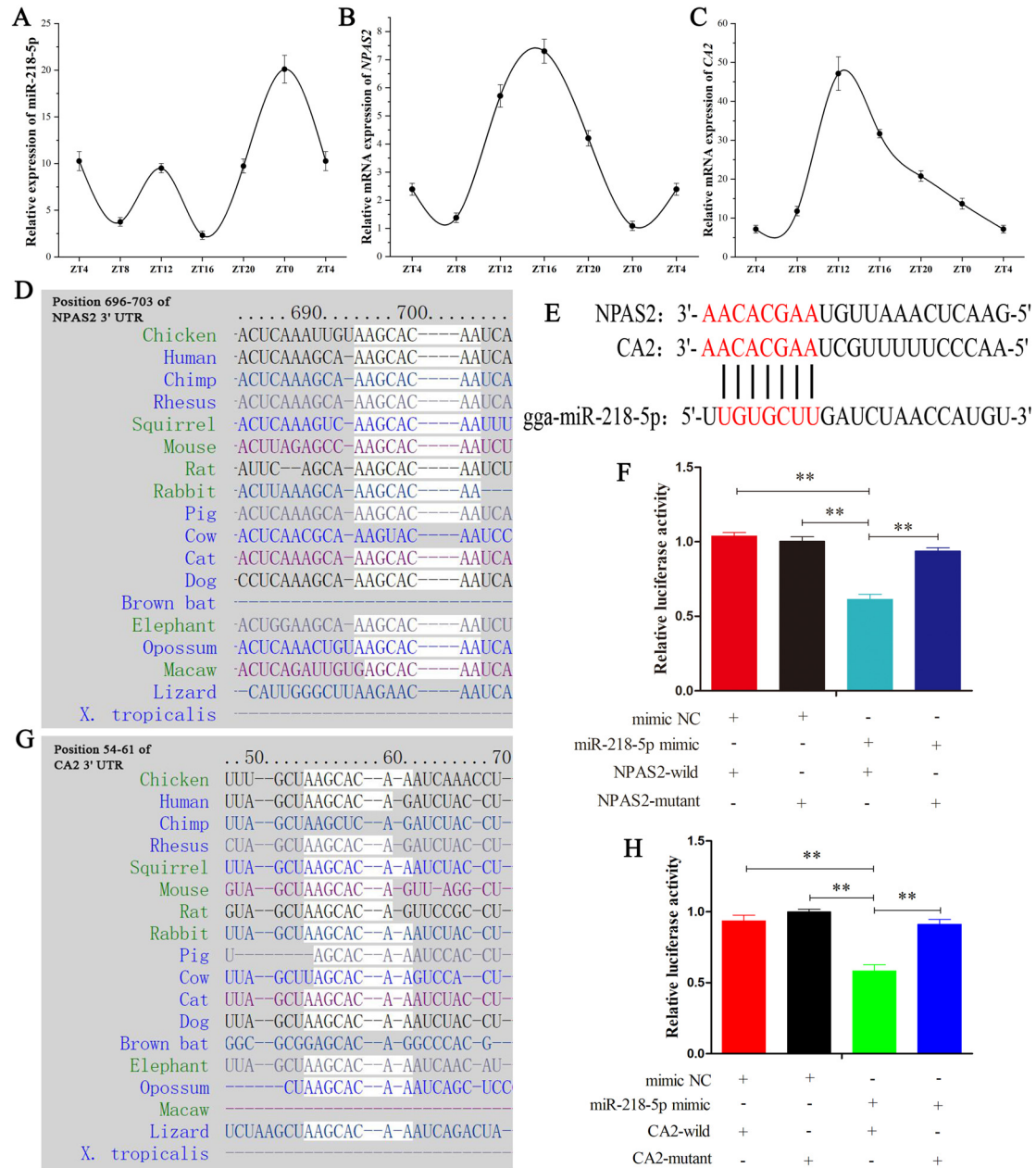
Figure 3. CA2 and NPAS2 are target genes of miR-218-5p. (A–C) Relative expression of miR-218-5p, CA2, and NPAS2 at the time point of ZT0 (ZT24), ZT4, ZT8, ZT12, ZT16, and ZT20 in the chicken uterus, respectively. (D, G) The target position of the miR-218-5p seed sequence on the CA2 and NPAS2-3′UTR sequences of different animal species was predicted using TargetScan software. (E) Target position of the miR-218-5p seed sequence in the CA2 and NPAS2-3′UTR sequences (red characters). (F, H) Chicken DF-1 cells were co-transfected with CA2 and NPAS2-3′UTR wild-type or mutant dual-luciferase vector and the miR-218-5p mimic or mimic-NC. The relative luciferase activity was assayed 48 h later. Replications = 3. Data are presented as mean ± SD; *$P < 0.05$ and **$P < 0.01$.

et al., 2020). We found functional clocks in the chicken infundibulum and uterus (Zhang et al., 2016; Zhang et al., 2017). Moreover, clock and ion transport related genes showed cosine expression patterns in the oviduct (Zhang et al., 2022). However, there are few studies on the role of clock-controlled miRNAs in eggshell formation in chicken eggshell gland (**ESG**). Therefore, we identified specific circadian miRNAs in the chicken uterus in our previous study including miR-218-5p (Cui et al., 2021). However, whether miR-218-5p plays a role in the regulation of eggshell formation has not yet been reported. The present study demonstrated an interesting function of miR-218-5p in the regulation of chicken uterus. One of the major findings of the present study was that miR-218-5p and CA2 exhibited reciprocal regulation: miR-218-5p bound to the 3′-UTR of CA2 mRNA and obviously suppressed the expression of CA2. Another finding is that NPAS2 is also the target gene of miR-218-5p, and the levels of miR-218-5p, CA2, and NPAS2 in uterine samples all showed a pattern of cosine expression. These results indicated that the circadian clock modulates miR-218-5p.

Carbonic anhydrase (**CA**), a zinc metal enzyme, catalyzes the reversible hydration of CO2 to form HCO_3^-
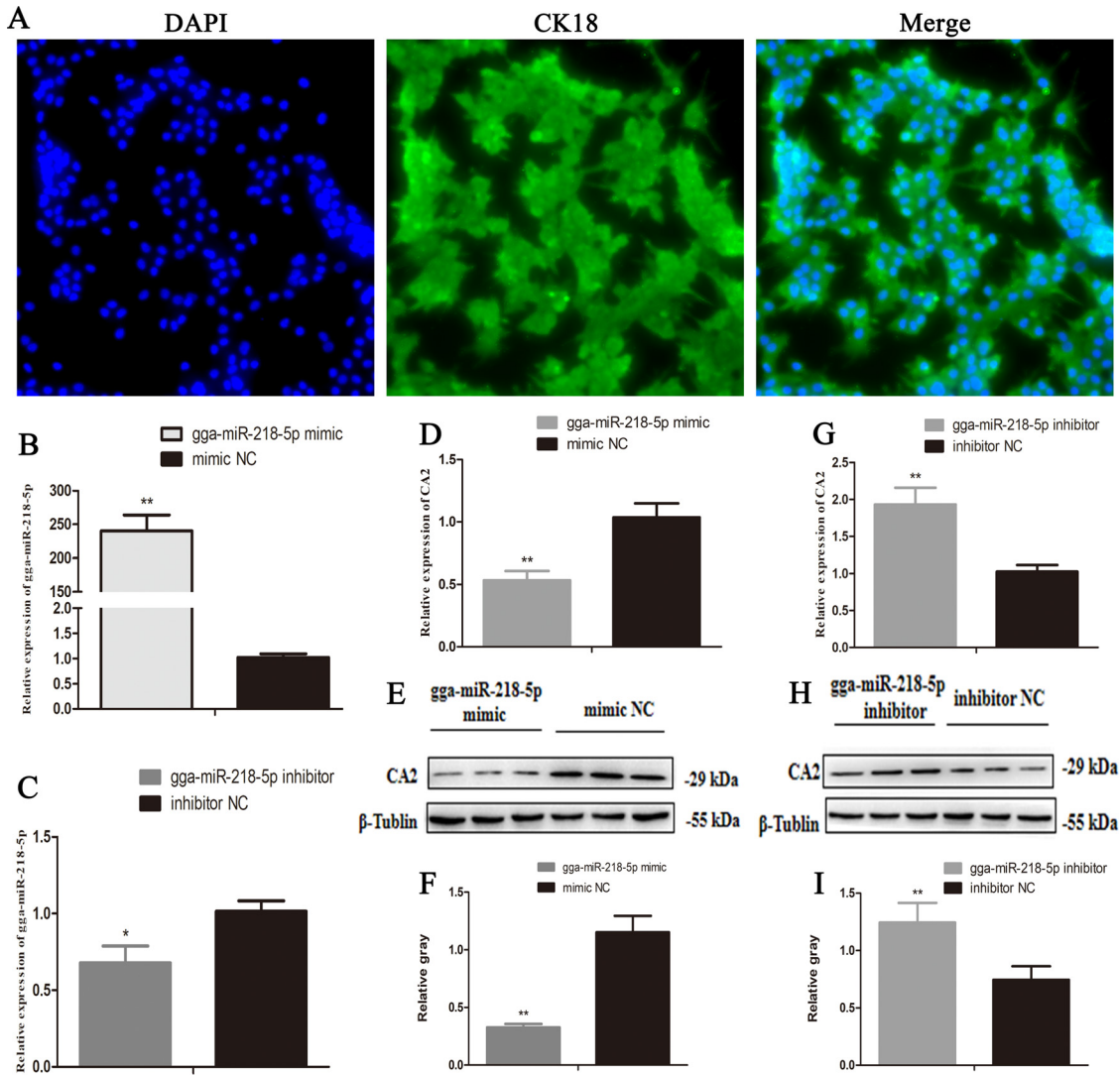
Figure 4. Clock-controlled miR-218-5p modulated CA2 mRNA and protein levels. (A) Immunofluorescence analysis was performed to identify uterine tubular gland cells. (B, C) qRT-PCR was used to determine miR-218-5p expression levels after transfection with miR-218-5p overexpression and miR-218-5p inhibition plasmids. (D, G) mRNA expression of CA2 in chicken uterine tubular gland cells was detected by qRT-PCR after the overexpression and inhibition of miR-218-5p, respectively. (E, F) Protein expression of CA2 in chicken uterine tubular gland cells was detected by western blot analysis after overexpression of miR-218-5p. (H, I) Protein expression of CA2 in chicken uterine tubular gland cells was detected by western blot analysis after miR-218-5p inhibition. β-Tubulin was used as the reference gene. Replications = 3. Data are presented as mean $\pm$ SD; *$P < 0.05$ and **$P < 0.01$.
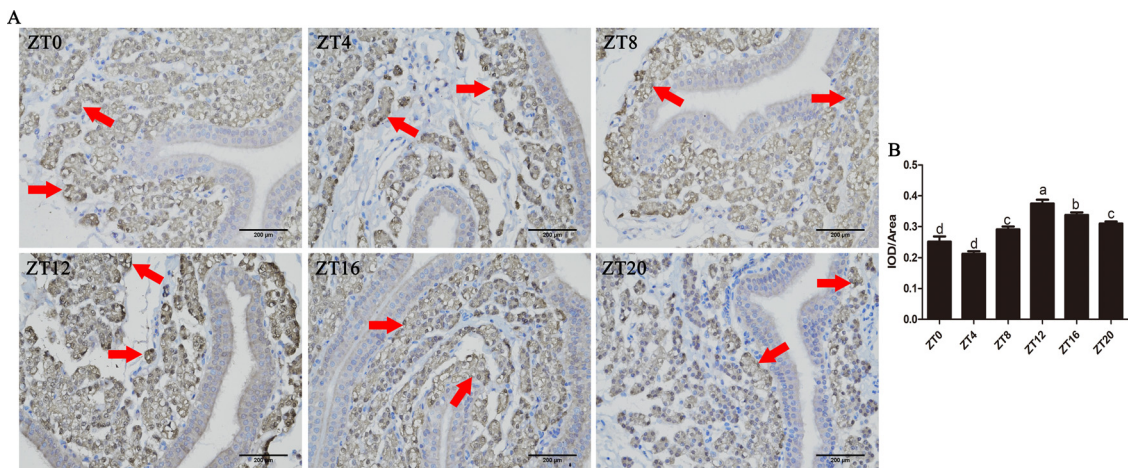

Figure 5. CA2 immunohistochemistry in the chicken uterus. (A) Immunohistochemical staining with the CA2 antibody visualized using the chromogen diaminobenzene (brown staining) in the chicken uterus. Arrows indicate relative areas of positive staining. (B) Digital conversion histogram; each point represents mean $\pm$ SD. Different lowercase letters indicate significant differences among groups ($P < 0.05$).
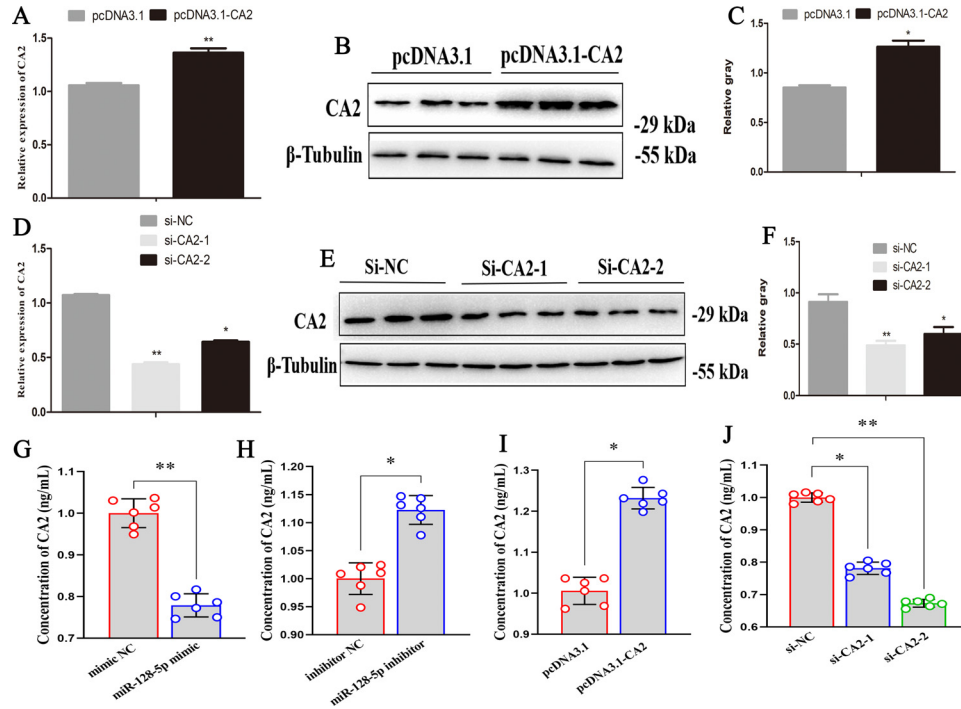
Figure 6. CA2 regulated carbonic anhydrase activity in uterine tubular gland cells. (A−C) mRNA and protein expressions of CA2 were detected after transfection of overexpression plasmid (pcDNA3.1-CA2) and empty pcDNA3.1 vector. (D−F) mRNA and protein expressions of CA2 were detected after transfection with small interfering RNA (Si-CA2-1, Si-CA2-2) and siRNA negative control (Si-NC). (G and H) The concentration of carbonic anhydrase ‖ was measured using a microplate reader after the overexpression and inhibition of miR-218-5p. (I and J) The concentration of carbonic anhydrase ‖ was measured using a microplate reader after the overexpression and inhibition of CA2. Replications = 6. Data are presented as mean $\pm$ SD; $*P < 0.05$ and $**P < 0.01$.

and protons (Annan et al., 2019). CA is abundant in most cells and plays a role in several processes, including ion transport, gas exchange, bone resorption and calcification, extracellular and intracellular pH control and vascular regulation (Nishita et al., 2011; Koç et al., 2014; Jakubowski et al., 2018). At least 16 carbonic anhydrase isozymes have different molecular structures (Esbaugh and Tufts, 2006). The major isoenzymes of CA in human erythrocytes are CA-Ⅰ and CA-Ⅱ (Zolfaghari et al., 2016). Chicken CA has attracted much

attention for a long time because of its important role in eggshell formation. Large amounts of Ca^{2+} and CO_3^{2-} are eventually deposited into $CaCO_3$ on eggs shells (Gutowska and Mitchell, 1945; Hodges and Lorcher, 1967). The present study showed that there was a rhythmic expression of the mRNA and protein levels of CA2 and our systematic experiment confirmed that miR-218-5p directly targeted CA2. The results indicated that that expression of miR-218-5p was opposite to that of CA2 and NPAS2 within the 24 h cycle in chicken uterus,
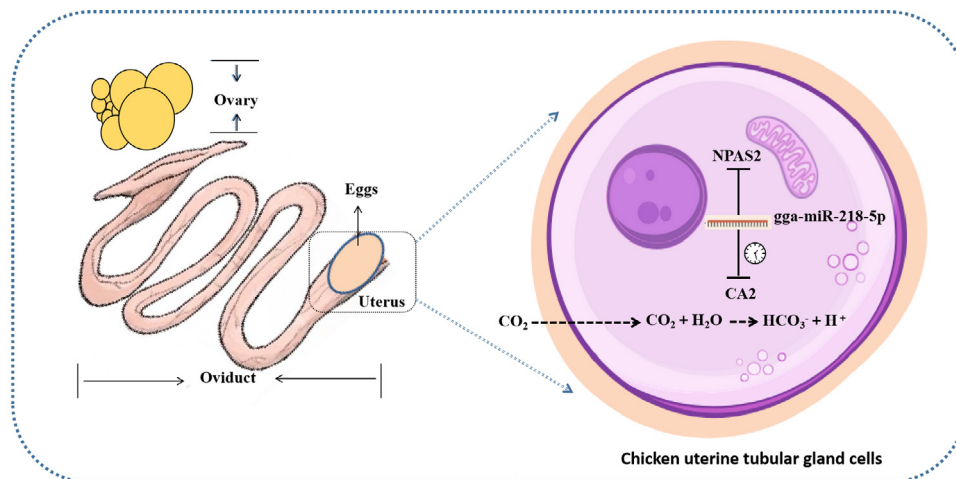

Figure 7. Schematic model of gga-miR-218-5p mediated activity of carbonic anhydrase ‖ in chicken uterine tubular gland cells. NPAS2, one of the core clock gene is the target gene of gga-miR-218-5p, and clock-controlled gga-miR-218-5p in the uterus of chickens regulates activity of carbonic anhydrase ‖ by targeting CA2 during eggshell calcification.

and it also demonstrated that miR-218-5p inhibited mRNA and protein levels of CA2 in uterine gland cells.

Eggshells are highly ordered structures with unique mechanical properties. It contains 95% calcium carbonate in its calcitic polymorph and 3.5% organic macromolecules (Jonchère et al., 2012; Rodríguez-Navarro et al., 2015). Therefore, eggshell formation depends upon numerous physiological adaptations and processes by uterine cells, which exhibit the capacity to transfer large amounts of Ca^{2+} and HCO_3^-. CA-II supplies HCO_3^- substrate for transport and removes HCO_3^- following transport (Everaert et al., 2010). CA activity in the uteri of laying quails was twice as high as that in the uterus of shelled quails in the uterus of non-laying quails (Pearson et al., 1977). It has been suggested that CA activity in the quail uterus may be related to uterine calcium secretion. Our previous study showed that the number of tubular gland cells increased as well as were neatly arranged and expression of ATP2B4 reached the peak time point at ZT12 (Cui et al., 2021), which is the time point for the secretion of uterine fluid and rapid mineralization of eggshells (Marie et al., 2014). From the results of mRNA detection and immunohistochemistry, the expression level of CA2 also reached its highest at ZT12, which was similar to the trend of the expression level of ATB2B4. We further explored the role of CA2 in carbonic anhydrase activity in the uterine tubular gland cells and found that *CA2* overexpression significantly increased the CA2 concentration but significantly decreased it with the transfection of *CA2* knockdown, indicating that CA2 promotes carbonic anhydrase activity. Moreover, overexpression of miR-218-5p showed similar changes in CA2 concentration as knockdown of *CA2,* which was contrary to the result of CA2 overexpression.

CONCLUSIONS

In summary, our study demonstrated that circadian miR-218-5p in the chicken uterus showed a pattern of cosine expression. *CA2* and *NPAS2* are target genes of miR-218-5p. In addition, clock-controlled miR-218-5p regulates the activity of carbonic anhydrase ‖ via its target CA2 in uterine tubular gland cells. These findings revealed a new miRNA-mediated pathway for chicken eggshell formation.

ACKNOWLEGMENTS

DISCLOSURES

The authors have no conflicts of interest to report.

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