Core clock gene Bmal1 deprivation impairs steroidogenesis in mice luteinized follicle cells

Yizi Wang, Ph.D\textsuperscript{1,3}, Minghui Chen, Ph.D\textsuperscript{1,3}, Jian Xu, Ph.D\textsuperscript{1,2}, Xinyan Liu, Ph.D\textsuperscript{1,3}, Yuwei Duan, MD\textsuperscript{1,3}, Canquan Zhou, Ph.D\textsuperscript{1,3}, Yanwen Xu, Ph.D\textsuperscript{1,3}\textsuperscript{*}

\textsuperscript{1} Reproductive Medicine Center, the First Affiliated Hospital of Sun Yat-sen University, Guangdong, Guangzhou, China
\textsuperscript{2} Reproductive Medicine Center, Guangzhou Women and Children’s Medical Center, Guangzhou Medical University, Guangzhou, China
\textsuperscript{3} Guangdong Provincial Key Laboratory of Reproductive Medicine, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

*Corresponding author: xuyanwen@live.cn

CORRESPONDENCE:

ADDRESS: Reproductive Medical Center, The First Affiliated Hospital of Sun Yat-sen University, Zhongshan Er Road No. 1, Yuexiu District, Guangzhou, Guangdong 510080, China. EMAIL: xuyanwen@live.cn. Tel: +86 13682213000

AUTHORS EMAIL:
Wang Yizi: wangyizi1000@126.com
Chen Minghui: doctorcmh@aliyun.com
Xu Jian: 284374434@qq.com
Liu Xinyan: liuxinyan36@yeah.net
Duan Yuwei: yuweiduan@126.com
Zhou Canquan: zhoucanquan@mail.sysu.edu.cn

Word count of the manuscript text: 6376
Abstract

Luteinization is the event of corpus luteum formation, a way of follicle cells transformation and a process of steroidogenesis alteration. As the core clock gene, Bmal1 was involved in regulation of ovulation process and luteal function afterwards. Till now, the underlying roles of luteinization played by Bmal1 remain unknown. To explore the unique role of Bmal1 in luteal steroidogenesis and its underlying pathway, we investigated luteal hormone synthesis profile in Bmal1 knockout female mice. We found that luteal hormone synthesis was notably impaired, and phosphorylation of PI3K/NfκB pathway was significantly activated. Then, the results were verified in in vitro cultured cells, including isolated Bmal1 interference granulosa cells (GCs) and theca cells (TCs) respectively. Hormones levels of supernatant culture media and mRNA expressions of steroidogenesis-associated genes (star, Hsd3β2, cyp19a1 in GCs, Lhcr, star, Hsd3β2, cyp17a1 in TCs) were mutually decreased, while the phosphorylation of PI3K/NfκB was promoted during in vitro luteinization. After PI3K specific-inhibitor LY294002 intervention, mRNA expressions of Lhcr and Hsd3β2 were partially-rescued in Bmal1 interference TCs, together with significantly increased androstenedione and T synthesis. Further exploration in TCs demonstrated BMAL1 interacted directly but negatively with NfκB p65 (RelA), a subunit which was supposed as a mediator in Bmal1-governed PI3K signaling regulation. Taken together, we verified the novel role of Bmal1 in luteal steroidogenesis, achieving by negative interplay with RelA mediated PI3K/NfκB pathway.

Key words: circadian gene Bmal1, luteal steroidogenesis, theca cells, NFκB/PI3K pathway, proliferation
Introduction

*Brain muscle arnt-like 1 (Bmal1)* is the core component of the internal circadian clock system, which has been proved to be indispensable to maintain the mammalian circadian rhythms [1, 2]. *Bmal1* deprivation could bring about endogenous or entrainable oscillation alteration [3]. Up to 10% of transcriptome in human was expressed in circadian manner [4]. However, rhythmicity of subordinate clocks was not completely governed by single master pacemaker but showed tissue-specific characteristics [5, 6]. Recently, extensive non-circadian regulation patterns of *Bmal1* were confirmed in many peripheral biological processes [7]. For example, germline *Bmal1* loss results in an acceleration of aging but adult-life inducible knockout ones do not have such gross effects, while both are deficient in the circadian clock [8].

In ovary, luteinization is a transient process orchestrated by endocrine, paracrine and autocrine signals in a timely manner after ovulation. Both granulosa and theca cells in the ovulated follicle undergo luteinization to form corpus luteum, which are responsible for synthesis of progesterone and estrogen required for maintaining early pregnancy before placenta establishment. Evidences from previous studies have implicated that *Bmal1* was involved in regulation of ovulation process and luteal function afterwards [9-11]. Epidemiologically, shift on sleep-wake schedules might lead to spotting or other abnormalities in luteal phase [12, 13]. Physiologically, verified by immunohistochemistry, BMAL1 expression significantly increased during corpus luteum formation [14]. Female mice with *Bmal1* knockout were inspected with impaired steroidogenesis, corpus luteum defect, and consequentially increased risks of implantation failure [1, 3, 15]. Furthermore, serum progesterone concentrations were at a comparative lower levels at the
3.5 day of gestation in Bmal1-/- pregnant mice [15], a primary pregnancy stage when endogenous hormones were maintained mainly by corpus luteum prior to placenta formation.

The defects in ovulation and luteum function in Bmal1-/- mice were mainly considered to be due to abolishment of circadian regulation on HPO (hypothalamic-pituitary-ovarian) axis. However, peripheral roles of Bmal1 in ovary could not be ignored. In the mouse model with Bmal1 specifically abolished in ovaries generated in the study of Liu et al, ovarian-targeted Bmal1 deprivation evoked embryonic implantation impairment and simultaneously compromised serum progesterone concentrations [16]. Subsequently, ovarian cell type-specific Bmal1 knockout mice were generated by Mereness et al. Analogic phenotype as premature primordial follicle aggregation observed in Bmal1-/- mice was merely detected in mice with targeted deletion of the Bmal1 locus in theca cells (TCs) (TCKO), but not in those females with granulosa cells (GCs) Bmal1 knockout (GCKO)[17]. When concerning luteinization, speculations were raised whether regulations governed by Bmal1 were achieved mainly through ovarian TCs other than GCs, although the majority studies to date had tended to emphasis GCs’ roles rather than TCs’.

Till now, molecular mechanisms of Bmal1 in ovary underlying early luteinization after ovulation still remain elusive and definitely deserve further exploration. By sequence comparison to classic circadian clock controlled cir-regulatory elements (E-box, D-box and RORE)[18], several steroidogenesis associated genes expressed in both GCs and TCs (Star, Lhcgr, Hsd3β2, and Ptgs2), exclusively in GCs (Fshr, Cyp19a1), or exclusively in TCs (Cyp17a1) were detected in presence with one or more aforementioned elements in their promoters. In consideration of the aforementioned evidences, the implicated hypothesis about whether Bmal1 affected luteal hormone synthesis through
entraining steroidogenic-associated genes with clock controlled promoters directly or via other cellular mechanisms still need to be unraveled.

Given GCs and TCs’ critical involvement in steroidogenesis and their cell-specific characteristics during luteinization, it is imperative to further explore the roles of Bmal1 in these two kinds of cells separately. Therefore, the main purpose of the present study was to investigate the assigned roles of Bmal1 in cultured GCs and TCs respectively during luteinization, and to explore relevant cellular mechanism using both Bmal1 knockout mouse model and Bmal1-knockdown cultured cells in vitro.

Method
Animals

Heterozygous Bmal1 knockout mice on C57BL/6J background was purchased from Nanjing Biomedical Research Institute of Nanjing University. Age-matched wild-type C57BL/6J mice were purchased from the Guangdong Province Laboratory Animal Center. Female Bmal1-/- offspring were developed by heterozygous pairs mating, whose genotype were determined as previously described[19]. The feeding conditions and Zeitgeber time definition were described in our published paper[20], as from 6 a.m. (Zeitgeber time 0, ZT 0) light on to 6 p.m. (ZT 12) light off. All experimental protocols including animals were approved by the Ethical Committee of the First Affiliated Hospital of Sun Yat-Sen University.

Serum Hormone Measurements

The estrous of 8-week-old female mice were synchronized as previous described[21]. One week later, females received an intraperitoneal injection (i.p.) of 15IU pregnant mare serum gonadotrophin (PMSG; Ningbo Second
Hormone Factory, Ningbo, China) at 8:00 (ZT 2) followed by 10IU human chorionic gonadotropin (HCG, sigma) after 48 hours. Twenty-four, 36, 48 hours (functional stage of luteal phase) and 72, 96 hours (regression stage) later after PMSG/HCG injection[21], whole blood samples were over time collected by cardiac puncture just after sacrificed by cervical dislocation. Before centrifugation, blood was allowed to coagulate at room temperature for 1 h. Serum was separated according to manufacture protocol and stored at −80 °C until assayed. Estradiol and progesterone levels were measured analyzed by RIA using commercial iodine $^{125}$I RIA Kits (Beijing North Biotechnology Research Institute). The sensitivity of the progesterone and estradiol RIA assays was 20 ng/mL. The intra-assay error and inter-assay error were <10 and <15%, respectively.

**Isolation and Identification of Granulosa cells and Theca interstitial cells**

Four- to 5-week-old SPF Wild-type female mice at proestrus stage were super-ovulated by an intraperitoneal injection (i.p.) of 15IU pregnant mare serum gonadotrophin (PMSG; Ningbo Second Hormone Factory, Ningbo, China) at 8:00 (ZT 2). Mice were humanely sacrificed by cervical dislocation 48 h after injection.

By puncturing follicles from the isolated ovaries with a 26 gauge needle immediately, granulosa cells were liberated and collected into precooled DMEM-F12 media. Residual tissue was reserved for TCs collection. GCs suspensions were filtered through 40 μm nylon filter and then centrifuged at 1200rpm for 5 minutes. Sediments were collected and centrifugation procedure was repeated twice. Supernatant was discarded and the cells seeded into a 24-well culture plated ($2 \times 10^5$ cells/well). Every 2 to 3 days, half of the cultured DMEM/F-12 (1:1) media (containing 100U/mL penicillin, 100 U/mL
streptomycin, 15% fetal bovine serum and 10ng/mL EGF) was replaced with fresh one. TCs isolation was performed according to the method previously described [22] with slight modification as follows: 4mL of 33% Percoll was layered on top of the 45% Percoll solution, on top of which the dispersed cells were gently layered, and tubes were centrifuged at 1600rpm for 15 minutes. Then by aspiration inside the 33% Percoll layer followed by centrifugation at 1600rpm for 5 minutes, the sediments were collected. The final pellet of TCs was resuspended in the reported culture media volume after washing twice with McCoy’s 5a medium.

Isolated cells were counted with a hemacytometer and a trypan blue staining was applied to test its viability. FSHR (follicle stimulating hormone receptor) and CYP17A1 antibody, as specific marker enzyme expressed in GCs and TCs respectively, was utilized to verify the purity and cell types according to a previous report by immunofluorescence staining [23]. Briefly, after 4% paraformaldehyde fixation and 0.1% Triton X-100 penetrating cell membrane, cells were incubated overnight at 4 °C with rabbit anti-mouse FSHR antibody (1:200 dilution, Novus) and rabbit anti-mouse CYP17A1 (1:200 dilution, Santa Cruz). After washing in PBS, the cells were incubated for 1 h at 37 °C with a secondary biotinylated donkey anti-rabbit IgG antibody (dilution 1:300, Santa Cruz). The cells were then washed in PBS and incubated for 10 min at 37 °C with DAPI dye liquor. The staining of FSHR was recorded with a laser confocal microscope (Olympus CKX41). A positive staining was evaluated by a green fluorescence for CYP17A1 (1:200 dilution, Santa Cruz), and blue fluorescence represented FSHR (1:200 dilution, Novus).

MTS
Ovarian cell viability was evaluated based on MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method by applying MTS assay kit (Abcam, ab197017) according to the manufacturer's instructions. In brief, 48-hour post Bmal1 siRNA transfection (as zero point at horizontal axis), 20 μL/well of the MTS was added to cultured TCs and GCs (5 × 10^4 cells/well in 180 μL fresh medium in 96-well plates) for an additional 4 hours of incubation under 37°C respectively at 24-, 48- and 72-hour point in time. Untransfected cells were served as the control. Absorbance at OD=490 nm was measured using microplate reader (Daojin UV-2450), and results were expressed as a percentage of the untreated control.

**siRNA transfection**

Three systemic siRNA targeting Bmal1 with fluorescence-FITC labeling for mice were delivered into primary theca cells using Lipofectamine 3000 transfection kit (Invitrogen Corp) one day after cell plating when the cell density reached 60%. The planted density was 5×10^5 at each well in 6-well plant with 5 microlitres (μL)/well FITC siRNA-Lipofectamine 3000 dilution medium without serum each well to a final concentration of 50nM. Meanwhile, the non-silencing RNA was transferred in the same condition with merely lipo complex (Mock) and PBS (NC) as controls (All of the siRNA were synthesized by RuiBo Biotechnology Company, Guangzhou). The process was lucifugal. Candidate sequences of RNA oligos were listed in Table 1. Among them, the first one with the highest transfection effect was finally chosen for the subsequent experiment. Transfection was processed according to the manufacturer’s instructions. Visible green FITC-siRNA fluorescence and quantitative Bmal1 mRNA extraction were prepared to test the efficacy of
siRNA 48 hours later. Cells were harvested at the indicated time points and processed for further analysis. After transfection, cells were re-suspended in regular culture medium and plated. Proteins of target pathway were analyzed by Western blot analysis using specific antibodies. Nf-κBp65 siRNA was a commercial product (CST, 6337S).

**Measuring hormone synthesis and sensitivity to LH stimulation in vitro**

Culture supernatants from GCs or TCs monolayers were taken from individual wells to detect hormone levels 48 h after siRNA transfection with additional 12-hour LH co-culture. After centrifugation at 2500g at 4°C for 15 minutes, the supernatant was stored at -80°C for hormone measurement. The concentrations of Estradiol (E$_2$, KGE014, R&D Systems, Minneapolis, MN), testosterone (T, RnD, KGE010), progesterone (P$_4$, EA, Merck-Millipor, STTHMAG-21K-02) and androstenedione (AND, LSBio, ELISAKit-LS-F39181) were determined by ELISA using kits according to the manufacturer's protocols respectively. A reported physiological concentration (2.5ng/mL) of luteinizing hormone (LH) was added for 12 hours to induce and maintain hormone secretion. Intra- and inter-assay precisions as described by coefficients of variations were T, $\leq$3.1% and $\leq$6.3%; AND, $<$8% and 10%. The detection limits of E$_2$, T, P$_4$ and AND were 12.3 - 3000 pg/mL, 0-10 ng/mL, 0.156-10 ng/mL, and 0.156–10 ng/mL respectively.

**LY294002 Treatment**

Post siRNA transfection to Bmal1 impairment, LY294002 (Sigma, L9908-LMG) as a PI3K inhibitor, was (200 µM) co-cultured with cells for 2 hours at 37°C to suppress the phosphorylation of PI3K pathway.
Quantitative real-time PCR analysis

GCs and TCs were harvested and RNA was extracted using Trizol reagents according to manufacturer instructions (Invitrogen, USA). Quality and concentration of total RNA was checked using NanoDrop (Thermo Fisher Scientific). Samples were sequentially treated with RNase-free DNase I (TaKaRa, Japan) and Superscript II reverse transcriptase (Invitrogen, USA) to remove contaminating genomic DNA and reverse transcribed into cDNA. qRT-PCR were performed in triplicate according to protocols[24]. The sequences of the primers for core circadian genes (Per1, Per2, Cry1, Cry2, Clock, Bmal1) and hormone synthesis related genes (Fshr, Lhcgr, Star, Cyp11a1, Hsd3β2, Cyp19a1 and Cyp17a1) were listed in Table 2. Transcript levels were normalized with that of the housekeeping gene Gapdh.

Western blot analysis

After centrifugation (12000 rpm, 15min at 4°C), the supernatants were collected for protein analysis. The protein concentration was determined by Bradford protein assay. Western blot analysis was performed as previously described. Briefly, 50 µg proteins from each sample were loaded onto an SDS polyacrylamide gel for electrophoresis and subsequently transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat dry milk in PBS for an hour and incubated with a primary antibody against BMAL1 (1:200 dilution), AKT (1:1000 dilution), p-AKT (Thr 308) (1:2000 dilution), mTORC1 (1:1000 dilution), p-mTORC1 (Ser2481) (1:2000 dilution), S6K1 (1:1000 dilution), p70-S6K1(Thr389) (1:2000 dilution), NfκBp65(dilution 1:250 dilution), p-NfκB (Ser536) (1:250 dilution) overnight at 4°C (BMAL1,
mTORC1, p-mTORC1, NfκBp65 and p-NfκB were purchased from Abcam, Cambridge, UK; AKT, p-AKT, S6K1S and p70-S6K1 were purchased from CST Danvers, USA). Samples were then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, USA) (1:5000 dilution). Band intensities were visualized with chemiluminescence reagent (Millipore Corp., Bedford, MA) by the BioMax film (Kodak) and then analyzed with Gel-Pro analyzer 4.0 software. β-Actin (Santa Cruz, USA) protein was utilized as a loading control.

**Bmal1 overexpression in theca cells**

A lentivirus overexpression vector with the Bmal1 and enhanced green fluorescent protein gene (eGFP) insert (LV5-eGFP-Bmal1, GenePharma, Shanghai, China) was constructed successfully and packaged into high-titer lentiviruses. The primary mice TCs were transfected with recombinant virosonome or empty vector when the cell density was approximately 50%. Expression of eGFP protein in each group was observed under an inverted fluorescence microscope at five days after transfection. The infection efficiency was confirmed by qRT-PCR.

**Immunoprecipitation (IP)**

All steps were performed at 4°C or on ice for immunoprecipitation after overexpression of BMAL1 in the primary theca cells.

Cells were washed twice with ice-cold PBS after gentle aspiration of culture media and lysed with lysis buffer containing protease inhibitor (Roche) for 10 minutes. Whole-cell protein lysates were next centrifuged at 16000x g for 10 minutes and normalized against a standard curve of BSA by Bradford assay.
One miligram of total protein was washed with DynaBeads Protein G magnetic beads (Invitrogen) for 15 minutes. Supernatants were transferred and incubated with 3µg of mice anti-BMAL1 antibody (Abcam, Cambridge, UK)/µg of total protein at 4°C overnight with constant agitation, or mice IgG (Abcam, Cambridge, UK) for negative control. A total of 30µL of pre-washed magnetic beads were added to per lysates for half an hour with constant agitation to capture the BMAL1-combined protein complex. Immobilized fractions were washed at least three times with ice-cold lysis buffer including protease and phosphatase inhibitors. All lysis buffer was instantly centrifuged at 16000x g for 5 seconds. The supernatant was collected, then 35µL of 2x SDS sample buffer was added, followed by boiling for 5 minutes at 95°C.

The complex was separated on an SDS-PAGE gel, and transferred to PVDF membrane. Anti-BMAL1 input with use of anti-BMAL1 as the probe was showed in Lane 1 (BMAL1 + cell samples). The enrichment in Panel 1 was sampling alone, and residual proteins in Panel 2 and 3 were jointly sampled. Lane 2 was performed with use of the anti-IgG antibody (Lane 2, IgG + cell samples) and western blot of mere antibody IgG (Lane 3, mere IgG). Content in Panel 4 (of Lane 2) was sampled alone, and Panel 5 and 6 were sampled together, while the residuals in Lane 2 were sampled as whole. Proteins appearing in both Lane 2 and Lane 3 were identified as non-specific. After rolling out the non-specific ones from those proteins appeared at Lane 1, the specific proteins that direct interacted with BMAL1 were obtained. Abundance of membranes from BMAL1 immunoprecipitates were determined and categorized by mass spectrometric analysis (Q Exactive mass spectrometer, Thermo Fisher, USA).

Data analysis
Results from Western blot were quantified with the ImageJ software. Statistical analysis was performed by applying GraphPad Prism (version 5.0) and Student’s t-test. For comparison between two groups, a one-way ANOVA was performed followed by Dunnett’s correction. Values of $P < 0.05$ were considered significant and indicated by asterisks in the figures.

Results

Deprivation of $Bmal1$ impaired luteal function in vivo

We firstly investigated the change of progesterone levels at different time points post PMSG/HCG injection in $Bmal1$ knockout mice (Fig.1.a). Serum progesterone level peak was shown at 36 h following PMSG/HCG treatment in $Bmal1$-/- females, evidently advanced to the wide-type females which showed the progesterone peak twelve hours later. The peak progesterone level was statistically reduced to $18.4 \pm 5.6$ ng/mL of $Bmal1$ knockout females as compared to $36.2 \pm 7.7$ ng/mL of wide-type ones ($P<0.01$). Serum estradiol level declined progressively overtime after luteinization within 72 hours in the two genotypes (Fig.1.b). The estradiol concentrations were $37.3 \pm 8.0$ pg/ml and $18.9 \pm 6.1$ pg/ml of $Bmal1$-/- mice at 24, 48 h respectively, significantly lower than $56.1 \pm 10.3$ pg/ml and $30.7 \pm 11.0$ pg/ml of wide types at the same time points ($P=0.01$, $P=0.03$). Thereafter, serum $E_2$ rose in advance in $Bmal1$-/- ones, reaching to $28.4 \pm 4.4$ pg/ml at 96 h, while the $E_2$ remained at a lower level of $17.2 \pm 5.7$ pg/ml in wild types ($P=0.03$).

$Bmal1$ knockdown promoted in vitro follicle cell proliferation

TCs exhibited fibroblast-like, long fusiform or anomalistic triangular shapes, while GCs looked like cobblestones with polygonal or cuboidal shapes (Fig.2.a). Cells was further identified by Cytochrome P450, family 17, subfamily A,
polypeptide 1 (CYP17A1), known to be specifically expressed in TCs, and the GCs-specific marker Follicle Stimulating Hormone Receptor (FSHR). As shown in Fig.2.b, the majority isolated TCs remained CYP17A1 positive and FSHR negative, while GCs showed the opposite results. Additionally, total protein from the isolated TCs and GCs were extracted respectively for western blotting of CYP17A1 and FSHR. As shown in Fig.2.c, FSHR was expressed at very low levels in the TCs, while CYP17A1 was expressed at high levels. Conversely, FHSR, but not CYP17Aa was expressed abundantly in the GCs.

*Bmal1* transcription rates were decreased to 27.34 ± 5.92% in TCs and 32.21 ± 4.40% in GCs respectively after siRNA transfection when compared to the controls by qRT-PCR (*P* < 0.01) (Fig.2.b). The transfection efficiency was confirmed by western blot (Fig.2.d for TCs and Fig.2.e for GCs).

MTS assay was conducted to evaluate cells proliferation 72h post-transfection. Growth curve was drawn according to OD value. As presented in Fig.2.f, cell growth of both TCs and GCs significantly accelerated after *Bmal1* siRNA transfection, as compared to the controls.

Considering feedback loops and compensated interactions between *Bmal1* and other clock genes, transcriptional levels of core circadian genes such as *Cry1, Cry2, Per1, Per2*, and *Clock* were tested in the ovaries of *Bmal1*−/− mice. As shown in Fig.4.a, the mRNA expression of *Cry2* was significantly decreased, while *Cry1* was significantly increased in ovaries from *Bmal1* knockout mice. However, transcriptional expressions of *clock, Per1*, and *Per2* were unaffected (Fig.2.g).

**Suppression of Bmal1 impaired secretion of steroid hormones**

To further investigate the role of *Bmal1* on luteal hormone secretion, we measured steroid hormone concentrations in the culture supernatants of cells
subjected to LH stimulation for 12 hours. Cells transfected with empty vector consisted of the sham control group.

In TCs, both AND and T concentrations were significantly lower in the Bmal1-siRNA group (AND $5.51 \pm 1.39$ ng/10^5 cell, T $4.41 \pm 1.87$ ng/10^5 cell) than those in the controls (AND $7.85 \pm 1.46$ ng/10^5 cell, T $7.15 \pm 1.10$ ng/10^5 cell) ($P=0.006$ and 0.033 respectively). Meanwhile, P₄ levels were presented with down trend post transfection (Fig.3.a). As presented in Fig.3.b, suppression of Bmal1 impaired E₂ and P₄ synthesis in GCs (E₂: $1.48 \pm 0.56$ vs. $3.02 \pm 0.47$ pg/10^5 cell, $P = 0.009$; P₄: $5.82 \pm 1.01$ vs. $2.02 \pm 0.73$ ng/10^5 cell, $P = 0.003$).

A subset of genes related to steroidogenesis was further investigated after Bmal1 siRNA transfection. In the Bmal1-siRNA group, the transcriptional level of Lhcg in TCs significantly decreased (Fig.3.c Left), while no change was detected in GCs (Fig.3.d Left). However, the differences of Lhcg levels in TCs between Bmal1-siRNA group and the control were vanished after LH stimulation, which raised comparability within groups (Fig.3.c Left). For other steroidogenesis-associated genes, in TCs, the mRNA levels of Star, Hsd3β₂, and Cyp17a1 in the Bmal1-siRNA group were significantly lower than control group after LH stimulation, except Cyp11a1 (Fig.3.c Right). As in GCs, the mRNA levels of Star, Hsd3β₂, and Cyp19a1, except FSHR, in the transfected group were significantly lower than the control. (Fig.3.d Right). The reduced transcriptional levels of Star, Cyp19a1, Hsd3 β₂, and Cyp17a1 were in accordance with decreased secretion of E₂ and down trend of P₄.
Bmal1 interference activated phosphorylation of PI3K/NfκB pathway

Since it has been reported that Bmal1 knockout activated phosphorylation of the PI3K/AKT/mTORC1 pathway[25, 26], we supposed that it may work as an attractive candidate closely involved in steroidogenic regulation. NfκB, recently proved as a mediator of inducible transcriptional response to circadian signaling, was also reported to have the ability to cross-talk with the PI3K/AKT/mTORC1[27, 28]. Based on these evidences, we hypothesized that Bmal1 might orchestrate luteal steroidogenesis through PI3K/AKT/mTORC1/NfκB pathway. To prove our hypothesis, we examined the phosphorylated proteins of the implicative pathway under the condition of Bmal1 complete loss in vivo and partially knockdown in vitro. Our results showed that global Bmal1 deletion in vivo resulted in activation of PI3K/AKT/mTORC1 phosphorylation pathway and simultaneously enhanced NfκB phosphorylation at Ser536 in integrated ovary (Fig.4.a).

To strength our findings, phosphorylation of Akt (Thr 308), mTORC1 (Ser 2481), downstream S6K1 at Thr389 together with NfκB at Ser 536 were all detected in both isolated TCs and GCs after Bmal1-siRNA transfection. Our results showed that phosphorylation of both pathway PI3K/AKT/mTORC1 and NfκB was activated, which was in accordance with the changes observed in vivo when Bmal1 was completely deprived. These results confirmed that Bmal1 impairment leads to over-activation of PI3K/NfκB signaling phosphorylation in luteinized TCs and GCs (Fig.4.b).

PI3K/NfκB interacted with negative feedback to BMAL1 on in isolated TCs

To further explore the mechanism under PI3K pathway, isolated TCs was furtherly exposed to LY294002, a selective PI3K inhibitor, post Bmal1-siRNA transfection with LH stimulation. Cellular transcription levels and
phosphorylation of NfkB as well as supernatant hormone levels of cultured TCs were examined.

As shown in Fig. 5.a, LY294002 intervention significantly rescued AND and T synthesis, together with the elevated trend of Pa level, under conditions post Bmal1 interference. In accordance with hormonal findings, expression levels of Hsd3β2 and Lhgr rose with significant differences post LY294002 intervention while Star remained unaffected. Paradoxically, Cyp17a1 was furtherly reduced. (Fig. 5.b)

Meanwhile, Bmal1 exerted a negative feedback on mRNA transcription of NfkB (Fig. 5.c). Blockade of PI3K signaling suppressed total protein levels which were enhanced by Bmal1 knockdown, as well as phosphorylation of NfkB p65 at Ser536 (Fig. 5.d).

Evidences of direct negative interaction between BMAL1 and NfkB

To explore clues of direct interaction between BMAL1, mTOC and NfkB, we characterized proteins in nucleus and cytosol of unsynchronized TCs that co-precipitated with BMAL1 through SDS-PAGE (Fig. 6.a) followed with mass spectrometry (MS). Primary comparison was conducted between panels from IgG + cell samples and IgG to identify and roll out duplicated proteins, after which the exclusive proteins that co-precipitated with BMAL1 were retrieved from Lane 1. Among candidate proteins, only NfkB p65 (RelA), a subunit of NfkB, was identified. Other retrieved peptides directly interacted with BMAL1 corresponding to annotated mouse proteins were summarized together in Supplementary Excel.

Next, we verified the direct interaction between Bmal1 and NfkB p65 by Bmal1 enhancement experiment in TCs. When Bmal1 was over-expressed, NfkB mRNA transcription, corresponding translated proteins and its subunit
RelA’ phosphorylation were consistently decreased (Fig.6.b and Fig.6.c). Combined with our previous results from Bmal1 knock-down and knock-out experiments (Fig.4.a and Fig.4.b), which showed protein levels of phosphorylated NfκB p65 was up-regulated, we proved the negative feedback between Bmal1 and NfκB p65. It was further verified in NfκB p65 knockdown experiment, as an obvious increase of BMAL1 protein levels was detected when NfκB p65 was interfered (Fig.6.d). Additionally, to confirm mediated role of NfκB RelA regarding Bmal1-induced AKT phosphorylation, western blotting experiments explored the levels of p-AKT. As expected, protein levels of p-AKT was presented to be inhibited in accordance with the changes of NfκB p65 (Fig.6.d). The cellular mechanism regarding Bmal1 governed NFκB/PI3K pathway in steroidogenesis regulation in TCs was summarized in the schematic diagram (Supplementary Fig.1)

**DISCUSSION**

During the past decades, substantial evidences have come to light that ovarian clock orchestrates and synchronizes reproductive physiological events such as follicle aggregation, ovulation and implantation. However, the impact of cellular self-sustaining endogenous circadian rhythms on luteal function is less well studied. The present study showed that deprivation of Bmal1, as a molecular-switch of the circadian oscillation, altered luteal phase profile in Bmal1 null mice. Correspondingly, luteal steroidogenesis was attenuated after Bmal1 interference, as shown in isolated GCs and TCs respectively. Molecularly, a novel role of Bmal1, which negatively interplayed with PI3K/NfκB in theca cells with respect to regulation of luteal steroidogenesis, was proclaimed.

Bmal1 exerts its function mainly through central clock, which was proved in Bmal1 knockout mice. However, previous studies have demonstrated its
peripheral role in ovary, for ovarian-specific knockout of Bmal1 compromised progesterone synthesis, and in vitro study using Bmal1 down-regulation GCs further confirmed the result [16, 29]. However, change of luteal phase profile after Bmal1 deprivation remained obscure. Here, we showed that global Bmal1 deprivation could evidently alter mice luteal phase profile, characterized by an advanced peak of P₄ and minor amplitude of E₂, indicating possible luteal phase defect. It was noteworthy that an advanced E₂ level rose at 96 h, a time-point corresponding to the late stage of luteinization followed by a new round of estrus. This metestrus E₂ priming let us reason that it might be a foreboding of early recruitment of antral follicles for the next round of ovulation, leading to early-onset of ovarian aging detected from female mice with impaired or null Bmal1 expression[16]. Considering endocrine function of corpus luteum involving not only Bmal1 but also Per2 [30, 31], another core clock gene which was proved to interact with Bmal1 in a transcription-translation feedback loop but in a displacement-type manner [32], we tested the transcriptional level of Per2. Nevertheless, no change of Per2 expression was found in ovaries of Bmal1 knockout female mice, which excluded the possible impact of Per2 in the current study.

To clarify peripheral role of Bmal1 in the ovary, we used Isolated TCs and GCs, each has independent characteristics with respect to steroidogenesis. Meanwhile, in vitro culture created an environment with no more pulsatile and rhythmic endocrine regulation from hypothalamus and pituitary. Although a bulk of evidences has proved that Bmal1 was involved in hormone synthesis of ovarian steroid cells, majority focused on GCs [11, 33]. Given GCs and TCs were born to be with distinctive cellular characteristics and governed underneath diverse endocrine cues, we furtherly explored cell-specific effects raised from Bmal1 on luteal steroidogenesis. As shown here, evidently impaired AND and T
synthesis with a downward trend of P₄ secretion was detected in TCs. Meanwhile, GCs encountered a significant decreased level of E₂ secretion, accompanied by a fall of P₄ in supernatant after partial Bmal1 loss.

The underlying mechanism of how Bmal1 modulate the steroidogenesis during luteinization in different type of luteal cells remains unclear. Acting through ovarian clock controlled genes were proved to be an effective way [34], since different clock-controlled elements were detected at the promoters of genes involved in steroidogenesis [29]. In the present study, siRNA-mediated knockdown of Bmal1 caused downregulation of Lhcgr in TCs, which supported existing viewpoint that Lhcgr was regulated by circadian clock [35, 36]. Interestingly, exogenous LH stimulation could completely rescue Lhcgr expression in isolated Bmal1-interferred TCs. In this respect, it might answer why TCKO mice presented with an indistinctive phase shift and abolished rhythm of Lhcgr but not a whole loss of its expression, since compensation in TCs might be achieved from endogenous LH secretion in vivo [17]. However, this impaired-rescued process only worked in TCs other than GCs, as our finding showed that Lhcgr of GCs was left impervious after Bmal1 partially deprivation, consistently with the reported phenotype that GCKO mice persisted with Lhcgr diurnal circadian rhythm [17]. This phenomenon might be attributed to cell-specific characteristics, although we could not exclude the possibility due to incomplete deprivation of Bmal1 transcriptions, whose residuals were capable enough to sustain considerable sensitivity to LH stimulation. Physiologically, TCs interact directly with ovarian stroma and receiving signals straightforward from peripheral circulation system and the master suprachiasmatic nucleus (SCN) of the hypothalamus [37]. Since GCs locate under the surrounding of TCs, it is reasonable to speculate that intimate cellular interactions worked between GCs and TCs in in vivo models may
compensate the deficiency of clock function in GCs, leading to the negative result in GCKO mice.

It is worthy to point out that Bmal1 may exert non-circadian related functions [38], which is regarded as unique cellular functions distinct or independent from its role in maintaining circadian oscillation [39]. Although the exact mechanism remains unclear, certain evidences have addressed its non-circadian functions [8], for example, Bmal1 knockout mice are characterized with aging properties and metabolic abnormalities, while knockout mice of other clock genes in the core circadian feedback loops, such as Cry1/Cry2 or Per1/Per2 double knockouts, did not present with the same phenotype [40].

As shown in our study, Bmal1 knockdown activated phosphorylation of PI3K/NfκB pathway, reducing androgen biosynthesis and transcriptional levels of Hsd3β2 and Lhcgr. Blockage of PI3K/NfκB by selective PI3K inhibitor rescued expressions of Hsd3β2 and Lhcgr but exerted no impact on Star levels, which sustained the existed perspective that Star might be strictly and directly under circadian control rather than PI3k/NfκB signaling. As for Cyp17α1, other pathway might be participated under Bmal1 control, which deserved further exploration [41].

It remains elusive how Bmal1 acted on PI3K pathway. By mass spectrometry in our study, no protein subunit related to mTOC and PI3K was found, indicating that none direct intermediary anchored BMAL1 to mTOC and PI3K, which was in accordance with the results of Wu et al. [42]. However, a subunit of NfκB, RelA (also known as NFκB p65) was screened out, which was proposed to anchor as a mediator where BMAL1 exerted regulation to PI3K pathway in a direct manner. The NfκB complex was inclined to be activated
during aging process[43]. Till now, only RelA and RelB from NFκB family have been proved to have direct interactions with BMAL1. Concerning how Bmal1 interacted with NfκB, to date, very limited evidences were found and none was involved in follicle cells. Here, we proved a negative feedback loop between BMAL1 and NfκB p65, as indicated by converse changes of one to another in the gain and loss experiments, which was consistent with the findings in human aortic endothelial cells by Mengru et al. [44]. However, in mouse embryo fibroblasts (MEFs), the BMAL1-CLOCK complex was detected to directly combine with NFkB p65 subunit, and transcriptionally active form of this specific RelA subunit was proved to be consisted with the dimer overexpression, especially CLOCK dependence[45], which elucidated a converse change to our findings. Another subunit RelB of NFκB was found to physically interact with the circadian activator BMAL1 in the presence of CLOCK to repress targeted circadian gene expression[46].

Previously findings suggested that NFκB was a downstream of PI3K mediated by AKT [47], however, latest studies pointed out that there should be a cross-talk between these two cellular signaling [48]. In our study, by interference the subunit RelA of NFκB and application of PI3K specific inhibitor LY294002, we originally proved a positive cross-talk between NFkB p65 and PI3K/AKT pathway in ovarian TCs mechanistically and functionally, as the luteal steroidogenesis of TCs was correspondingly explored. The precise mechanism on what kind of molecular interaction or modification between Bmal1-induced RelA and PI3K/AKT requires further study.

This study has several limitations. First, we only did Bmal1 knockdow experiment using siRNA. TCs with Bmal1 knockout by Crisp-cas9 may give us more convincing results, although technically it is difficult as no stabilized-passaged TC line strain was available. Moreover, if a rescue experiment
covering Bmal1 recover was conducted *in vitro*, our conclusion might be more conclusive. Lastly, we could not differentiate whether phenotypic changes of TCs were due to disruption of circadian rhythms or the alterations of non-circadian related function of Bmal1 in the current study, since the rhythm of Bmal1 in isolated cells was merely weakened but not completely abolished.

To sum up, our study elucidated the significant role of the core circadian gene Bmal1 in luteal steroidogenesis, mainly interacting negatively but directly with PI3K/NfκB pathway. Our results highlighted the importance of circadian control on reproductive function, which definitely deserves more profound studies in the future.

**Competing interests**
The authors declare that they have no competing interests.

**Authors’ contributions**
Xu Yanwen, Wang Yizi and Zhou Canquan designed the research. Wang Yizi, Chen Minghui, Xu Jian, Liu Xinyan and Duan Yuwei performed the specific research. Xu Jian, Liu Xinyan and Duan Yuwei analyzed the data. Xu Yanwen and Wang Yizi wrote the paper. All authors read and approved the final manuscript.

**Acknowledgements**
The research team would like to thank Pro. Guo Jinhu who helped with experiment design and provided for research support. We also thank Pro. Xu Ying for her help in providing latest associated biological references.

**Funding statement:**
This work was supported by National Natural Science Foundation of China (Grant No. 81771588, Youth Program: Grant No. 81801412), Guangdong Basic and Applied Basic Research Foundation (2019A1515010991) and the National Basic Research Program of China (973 Program, Grant No. 2012CB947604).

Data availability statement:
The data used to support the findings of this study are included within the article.

REFERENCES

1. Sellix MT: Circadian clock function in the mammalian ovary. J Biol Rhythms 2015, 30(1):7-19.
2. Huang N, Chelliah Y, Shan Y, Taylor CA, Yoo SH, Partch C, Green CB, Zhang H, Takahashi JS: Crystal structure of the heterodimeric CLOCK:BMAL1 transcriptional activator complex. Science (New York, NY) 2012, 337(6091):189-194.
3. Boden MJ, Varcoe TJ, Voultsios A, Kennaway DJ: Reproductive biology of female Bmal1 null mice. Reproduction (Cambridge, England) 2010, 139(6):1077-1090.
4. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB: Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 2002, 109(3):307-320.
5. McDearmon EL, Patel KN, Ko CH, Walisser JA, Schook AC, Chong JL, Wilsbacher LD, Song EJ, Hong HK, Bradfield CA et al: Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice. Science (New York, NY) 2006, 314(5803):1304-1308.
6. Zhang R, Lahens NF, Ballance HI, Hughes ME, Hogenesch JB: A circadian gene expression atlas in mammals: implications for biology and medicine. Proceedings of the National Academy of Sciences of the United States of America 2014, 111(45):16219-16224.
7. Alvarez JD, Chen D, Storer E, Sehgal A: Non-cyclic and developmental stage-specific expression of circadian clock proteins during murine spermatogenesis. Biol Reprod 2003, 69(1):81-91.
8. Yang G, Chen L, Grant GR, Paschos G, Song W-L, Musiek ES, Lee V, McLoughlin SC, Grosser T, Cotsarelis G et al: Timing of expression of the core clock gene Bmal1 influences its effects on aging and survival. Sci Transl Med 2016, 8(324):324ra316.
9. Boden MJ, Varcoe TJ, Voultsios A, Kennaway DJ: Reproductive biology of female Bmal1 null mice. Reproduction (Cambridge, England) 2010, 139(6):1077-1090.
10. Pan X, Taylor MJ, Cohen E, Hanna N, Mota S: Circadian Clock, Time-Restricted Feeding and Reproduction. International journal of molecular sciences 2020, 21(3):E831.
11. Sen A, Hoffmann HM: Role of core circadian clock genes in hormone release and target tissue sensitivity in the reproductive axis. Mol Cell Endocrinol 2020, 501:110655.
12. Wang Y, Gu F, Deng M, Guo L, Lu C, Zhou C, Chen S, Xu Y: Rotating shift work and menstrual characteristics in a cohort of Chinese nurses. *BMC women's health* 2016, 16:24.

13. Shibui K, Uchiyama M, Okawa M, Kudo Y, Kim K, Liu X, Kamei Y, Hayakawa T, Akamatsu T, Ohta K *et al*: Diurnal fluctuation of sleep propensity and hormonal secretion across the menstrual cycle. *Biol Psychiatry* 2000, 48(11):1062-1068.

14. Wiggins G, Legge M: Cyclic Variation of Cellular Clock Proteins in the Mouse Estrous Ovary. *J Reprod Infertil* 2016, 17(4):192-198.

15. Ratajczak CK, Boehle KL, Muglia LJ: Impaired steroidogenesis and implantation failure in Bmal1/- mice. *Endocrinology* 2009, 150(4):1879-1885.

16. Liu Y, Johnson BP, Shen AL, Wallisser JA, Krentz KJ, Moran SM, Sullivan R, Glover E, Parlow AF, Drinkwater NR *et al*: Loss of BAM1 in ovarian steroidogenic cells results in implantation failure in female mice. *Proc Natl Acad Sci U S A* 2014, 111(39):14295-14300.

17. Mereness AL, Murphy ZC, Forrestel AC, Butler S, Ko C, Richards JS, Sellix MT: Conditional Deletion of Bmal1 in Ovarian Theca Cells Disrupts Ovulation in Female Mice. *Endocrinology* 2016, 157(2):913-927.

18. Takahashi JS: Transcriptional architecture of the mammalian circadian clock. *Nat Rev Genet* 2017, 18(3):164-179.

19. Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA: Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 2000, 103(7):1009-1017.

20. Xu J, Li Y, Wang Y, Xu Y, Zhou C: Loss of Bmal1 decreases oocyte fertilization, early embryo development and implantation potential in female mice. *Zygote (Cambridge, England)* 2016, 24(5):760-767.

21. Park HJ, Park SJ, Koo DB, Lee SR, Kong IK, Ryoo JW, Park YI, Chang KT, Lee DS: Progesterone production is affected by unfolded protein response (UPR) signaling during the luteal phase in mice. *Life sciences* 2014, 113(1-2):60-67.

22. Tian Y, Shen W, Lai Z, Shi L, Yang S, Ding T, Wang S, Luo A: Isolation and identification of ovarian theca-interstitial cells and granulose cells of immature female mice. *Cell biology international* 2015, 39(5):584-590.

23. Chen MH, Li T, Ding CH, Xu YW, Guo LY, Zhou CQ: Growth differential factor-9 inhibits testosterone production in mouse theca interstitial cells. *Fertility and sterility* 2013, 100(5):1444-1450.

24. Xu J, Xu Y, Miao B, Deng M, Wang Y, Xiang P, Zhou C: Influence of menstrual cycle on the expression of clock genes in peripheral blood mononuclear cells in Macaca fascicularis. *European journal of obstetrics, gynecology, and reproductive biology* 2015, 186:54-58.

25. Guo Z, Yu Q: Role of mTOR Signaling in Female Reproduction. *Front Endocrinol (Lausanne)* 2019, 10:692.

26. Acosta-Martínez M: PI3K: An Attractive Candidate for the Central Integration of Metabolism and Reproduction. *Front Endocrinol (Lausanne)* 2011, 2:110.

27. Meng F, Liu L, Chin PC, D'Mello SR: Akt is a downstream target of NF-kappa B. *The Journal of biological chemistry* 2002, 277(33):29674-29680.

28. Ghoneum A, Said N: PI3K-AKT-mTOR and NFkB Pathways in Ovarian Cancer: Implications for Targeted Therapeutics. *Cancers* 2019, 11(7).
29. Chen H, Zhao L, Kumazawa M, Yamauchi N, Shigeyoshi Y, Hashimoto S, Hattori MA: Downregulation of core clock gene Bmal1 attenuates expression of progesterone and prostaglandin biosynthesis-related genes in rat luteinizing granulosa cells. *American journal of physiology Cell physiology* 2013, **304**(12):C1131-1140.

30. Fahrenkrug J, Georg B, Hannibal J, Hindersson P, Gras S: Diurnal rhythmicity of the clock genes Per1 and Per2 in the rat ovary. *Endocrinology* 2006, **147**(8):3769-3776.

31. Shimizu T, Hirai Y, Murayama C, Miyamoto A, Miyazaki H, Miyazaki K: Circadian Clock genes Per2 and clock regulate steroid production, cell proliferation, and luteinizing hormone receptor transcription in ovarian granulosa cells. *Biochemical and biophysical research communications* 2011, **412**(1):132-135.

32. Chiou YY, Yang Y, Rashid N, Ye R, Selby CP, Sancar A: Mammalian Period represses and de-represses transcription by displacing CLOCK-BMAL1 from promoters in a Cryptochrome-dependent manner. *Proceedings of the National Academy of Sciences of the United States of America* 2016, **113**(41):E6072-e6079.

33. Alvarez JD, Hansen A, Ord T, Bebas P, Chappell PE, Giebultowicz JM, Williams C, Moss S, Seagal A: The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. *J Biol Rhythms* 2008, **23**(1):26-36.

34. Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH et al: Interacting molecular loops in the mammalian circadian clock. *Science (New York, NY)* 2000, **288**(5468):1013-1019.

35. Wang W, Yin L, Bai L, Ma G, Zhao C, Xiang A, Pang W, Yang G, Chu G: Bmal1 interference impairs hormone synthesis and promotes apoptosis in porcine granulosa cells. *Theriogenology* 2017, **99**:63-68.

36. Lisa Gilioli MM, Manuela Simoni & Livio Casarini: The regulation of LHCGR-dependent signaling is linked to circadian gene expression. *Endocrine Abstracts* 2017, **49**:EP1131.

37. Young JM, McNeilly AS: Theca: the forgotten cell of the ovarian follicle. *Reproduction (Cambridge, England)* 2010, **140**(4):489-504.

38. Liang X, Cheng S, Jiang X, He X, Wang Y, Jiang Z, Hou W, Li S, Liu Y, Wang Z: The noncircadian function of the circadian Clock gene in the regulation of male fertility. *J Biol Rhythms* 2013, **28**(3):208-217.

39. Musiek ES, Lim MM, Yang G, Bauer AQ, Qi L, Lee Y, Roh JH, Ortiz-Gonzalez X, Dearborn JT, Culver JP et al: Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration. *The Journal of clinical investigation* 2013, **123**(12):5389-5400.

40. Dierickx P, Van Laake LW, Geijsen N: Circadian clocks: from stem cells to tissue homeostasis and regeneration. *EMBO reports* 2018, **19**(1):18-28.

41. Baburski AZ, Sokanovic SJ, Bjelic MM, Radovic SM, Andric SA, Kostic TS: Circadian rhythm of the Leydig cells endocrine function is attenuated during aging. *Exp Gerontol* 2016, **73**:5-13.

42. Wu R, Dang F, Li P, Wang P, Xu Q, Liu Z, Li Y, Wu Y, Chen Y, Liu Y: The Circadian Protein Period2 Suppresses mTORC1 Activity via Recruiting Tsc1 to mTORC1 Complex. *Cell metabolism* 2018.

43. Salminen A, Huuskonen J, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T: Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflam-maging. *Ageing research reviews* 2008, **7**(2):83-105.
44. Xie M, Tang Q, Nie J, Zhang C, Zhou X, Yu S, Sun J, Cheng X, Dong N, Hu Y et al: BMAL1-Downregulation Aggravates Porphyromonas Gingivalis-Induced Atherosclerosis by Encouraging Oxidative Stress. Circulation research 2020, 126(6):e15-e29.

45. Spengler ML, Kuropatwinski KK, Comas M, Gasparian AV, Fedtsova N, Gleiberman AS, Gitlin, II, Artemicheva NM, Deluca KA, Gudkov AV et al: Core circadian protein CLOCK is a positive regulator of NF-kappaB-mediated transcription. Proceedings of the National Academy of Sciences of the United States of America 2012, 109(37):E2457-2465.

46. Bellet MM, Zocchi L, Sassone-Corsi P: The RelB subunit of NFkappaB acts as a negative regulator of circadian gene expression. Cell cycle (Georgetown, Tex) 2012, 11(17):3304-3311.

47. Bai D, Ueno L, Vogt PK: Akt-mediated regulation of NFkappaB and the essentialness of NFkappaB for the oncogenicity of PI3K and Akt. International journal of cancer 2009, 125(12):2863-2870.

48. Hussain AR, Ahmed SO, Ahmed M, Khan OS, Al Abdulmohsen S, Platanias LC, Al-Kuraya KS, Uddin S: Cross-talk between NFkB and the PI3-kinase/AKT pathway can be targeted in primary effusion lymphoma (PEL) cell lines for efficient apoptosis. PloS one 2012, 7(6):e39945.

LEGENDS:

Figure Legends:
Figure 1. Fig.1. Luteal profiles of serum hormone levels through out 4 days post PMSG/HCG injection. Hormones were measured by ELISA kits. Each value represents the mean ± SD. (a) Progesterone levels. # peak value comparison between Bmal1-/- and wild-type female mice with P < 0.05 or less. (b) Estradiol levels. * Comparison between Bmal1-/- and wild-types at corresponding labelled time point with P < 0.05 or less. n=3 females at 8 weeks old per time point in each group.

Fig.2. Follicle cells proliferation and transcriptions of other core circadian genes post siRNA-Bmal1 transfection. (a) Cells morphology. Fusiform-aligned TCs and cobblestone-like GCs (200x, Scale Bar=100μm). (b) Immunofluorescence staining of CYP17A1 and FSHR observed under fluorescence microscope (400x, Scale Bar = 50μm). Most isolated TCs remained CYP17A1 positive and FSHR negative, while immunofluorescence staining of CYP17A1 and FHSR showed opposite in GCs. (c) Relative protein levels of CYP17A1 and FSHR by western blot (left) and corresponding band intensities (right). (d) and (e) Transfection efficiency of TCs (d) and GCs (e). Transfected cells were signaled by green fluorescence (upper) and knockdown efficiency in isolated cells (below ) was ascertained by qRT-PCR analysis of Bmal1 mRNA. Both Mock (nonsiliencing) and si-Neg (empty vector transfected) were controls. *, P < 0.05 vs. the si-Neg group. (f) Growth curve according to OD value after siRNA-Bmal1 transfection detected by MTS assays. Bmal1 interference accelerated both isolated GCs and TCs growth significantly. (g). mRNA expression of core circadian genes (Cry1, Cry2, Per1, Per2, clock) in mice ovaries quantified by qRT-PCRT, wild type mice. Bmal1-/-, mice genotyped with whole bmal1 knockout. *, P < 0.05 or less. Data are
presented mean ± SD of 3 independent determinations. *, P < 0.05 or less vs. the Neg (control) group. TCs, theca cell. GCs, granulosa cells.

Fig.3. Bmal1 downregulation decreased luteal hormones synthesis and impaired mRNA transcriptions of steroidogenesis-associated genes in lutenized follicle cells. (a). Androgen and progesterone concentrations in TCs’ supernatants. (b). Progesterone and estradiol levels in GCs’ supernatants. Bmal1 interference deteriorated P_4 and E_2 production. (c). Left, TCs’ Lhcgr mRNA levels. Right, mRNA transcriptions of other targeted steroidogenesis-associated genes in TCs. (d). Left, GCs’ Lhcgr mRNA levels. Right, mRNA transcriptions of other targeted steroidogenesis-associated genes in GCs. The relative mRNA expression was normalized to Gapdh and expressed as relative to CONT. siRNA, group of cells transfected with siRNA against Bmal1. CONT, cells transfected with siRNA with empty vector as controls. LH, cell cultured with LH supply additionally for 12 hours. DMEM, cells cultured with regular culture media containing DMEM. mRNA levels of target genes in cells were quantified by qRT-PCR using their specific primers. Data are presented mean ± SD of 5 independent determinations. *, P < 0.05 or less vs. the control group.

Fig.4. Bmal1 loss activated phosphorylation of NFκB/PI3K pathway pathway. W. (a). Ovarian protein levels and quantitation assessment of Bmal1-knockout mice by Western blot. Left, a typical Western-blot. Right, bands were densitometrically qualified and the intensities shown were normalized to β-Actin, relatively to WT control. PD 5, post natal 5 days. 12 w, mice at 12 weeks old. WT, wild type mice. Bmal1-/-, mice genotyped with whole bmal1 knockout. *, P < 0.05 or less vs. PD5 WT; #, P < 0.05 or less vs. 12w WT. Data are presented mean ± SD of 3 independent experiments. (b). Protein levels and quantitation assessment of Bmal1-knockdown GCs and TCs with siRNA transfection. Left, a typical Western-blot. Right, bands were densitometrically qualified and the intensities shown were normalized to β-actin, relatively to CONT of corresponding cell types. GC, granulosa cell. TC, theca cell. siRNA, group of cells transfected with siRNA knockdown Bmal1 transcriptions. CONT, cells transfected with siRNA with empty vector as controls. *, P < 0.05 or less vs. GC CONT. #, P < 0.05 or less vs. TC CONT. All data were the mean ± SD of 3 independent experiments where all the samples were repeated in thrice.

Fig.5. Effects of PI3K specific inhibitor LY294002 on hormone synthesis and underlying NFκB pathway of isolated ovarian theca cells. (a) Androgen and progesterone concentrations in supernatant culture media. (b) mRNA levels of target genes after Bmal1 siRNA (siRNA) transfection normalized to Gapdh. siRNA, group of cells transfected with siRNA against Bmal1 with intact solvent co-cultured as control. mRNA levels of target genes in cells were quantified by qRT-PCR using their specific primers. Data are presented mean ± SD of 5 independent determinations. *, P < 0.05 or less vs. siRNA. (c). Bmal1 downregulation enhanced mRNA levels of NFκB. (d). Bmal1 knockdown enhanced total NFκB p65 protein expression and activated phosphorylation of NFκB p65 at Serine-536, which could be suppressed by LY 294002. Left, a typical Western-blot. Right, bands were densitometrically qualified and the intensities were normalized to β-Actin, relatively to WT control. *, P < 0.05 or less vs. corresponding protein in siBmal1-/LY294002-. #, P < 0.05 or less vs. corresponding protein in siBmal1+/ LY294002-. siBmal1-, cells transfected with empty vector without Bmal1 interference. siBmal1+, siRNA transfection to interfere Bmal1. LY 294002, a PI3K
specific inhibitor. LY 294002 -, cells treated with mere solvent. LY 294002 +, cells co-cultured with solvent dissolved with LY 294002 50 μM for a 2-hour incubation at 37°C. Bars represented as mean ± SD of 3 independent determinations.

Fig. 6. Immunoprecipitations and feedback loops verification of interactions between BMAL1 and NFκB. (a). Immunoprecipitations to characterize proteins that co-precipitated with BMAL1 followed by mass spectrometry in primary-cultured mice theca cells. (b) and (c). Effect of Bmal1 over-expression on the transcription and protein level of NFκB in mice theca cells. (b). Expression of NFκB quantified by qRT-PCR. (c). Left, a typical Western-blot. Right, bands were densitometrically qualified and the intensities shown were normalized to β-Actin, relatively to Vec-BMAL1 as control. *, P < 0.05 or less vs. corresponding protein in Vec. Neg, cells without transfection as negative control. Vec, cells transfected with empty vector as sham control. Vec-Bmal1, transfected with vector to overexpress Bmal1. Bars represented as mean ± SD of 3 independent determinations. (d). Effects of NfκB p65 knockdown on BMAL1/PI3K pathway in theca cell. Left, a typical Western-blot. Right, bands were densitometrically qualified and the intensities shown were normalized to β-Actin, relatively to NfκB p65-siRNA – as control. *, P < 0.05 or less vs. corresponding protein in NfκB p65-siRNA – . NfκB p65-siRNA – = CONT, cells transfected with empty vector without NfκB p65 interference. NfκB p65-siRNA + = siRNA , siRNA transfection to interfere NFκB p65 subunit expression. Bars represented as mean ± SD of 3 independent determinations.

Supplementary Figure
Fig. 1. Schematic diagram summarizing the Bmal1 regulated NFκB/PI3K pathway in hormone synthesis modulation in mice theca cells.
Table 1. siRNA sequences targeting *Bmal1* mRNA

| Gene | siRNA Target Sequence (5’-3’) |
|------|------------------------------|
| *Bmal1* | CCAAGGAAGTTGAATACAT |
| 1 | GCTCTTTCTTCTGTAGAAT |
| 2 | GCAAACTACAAGCCAACAT |

siRNA, small interfering RNA.
Table 2. Primer sequences for mice targeted steroidogenesis-associated genes and circadian clock genes.

| Gene   | Primers Sequence (5’-3’) | Forward | Reverse |
|--------|--------------------------|---------|---------|
| Bmal1  | CCGTGGACCAAGGAAGTGA      | CTGTGAGCTGTGGGAAGGTT |
| Star   | GGGTGGATGGTCAAGTTC       | AGCACCAGGTCGTTCAATA  |
| Cyp11a1| GTCCCACTCCTCAAAGCCAG     | GAAGCACCAGGTCGTTCAATA |
| Hsd3β2 | GCCCCTACTGTACTGGCTTG     | TCCCGATCCACTCTGGAGGTT |
| Cyp17a1| GCCCAAGTCAAAGACACCTAAT   | GCCCAAGTCAAAGACACCTAAT |
| Cyp19a1| ACCTGGAGTGGAGCTTTAACCTGC| CAGGTTCTGTGACGCGTTG  |
| Fshr   | TGAGTCTGGCTATGCTGCTCA    | CACCTCAACAGCCAAAC   |
| Lhcgr  | GAAACGCTTTTATTTCTGCTATT | CAGGGATTGAAAGACATCTG |
| NFxB   | TGATCCACATCAGAATGCA      | CAGGAAGGATATGGAAGCA |
| Clock  | GGACTCTCAACACCACACAG     | GCCACGTGAAAGAAAGCAC |
| Per1   | AGATCAAACCTCAGAGACAGC    | AGATCAACTGCTGGAGACAGC |
| Per2   | TGCCCTACATCTTCTACTGT     | CAACACTGACAGCCGAGAA |
| Cry1   | CCATCCGCTCCTCATTATATCC   | GAAGCAAAAATCAGCCACCTG |
| Cry2   | AATTCCCTCAGCAGCCACAGC    | TTCTGCACACAGGAGTTGTC |
| Gapdh  | F: TGTAGACCATTAGTGAGGTCA | AGGTCGCTGTGAACGAGATTT |

Downloaded from Bioscientifica.com at 10/31/2020 08:09:41AM via free access
Fig. 1. Luteal profiles of serum hormone levels throughout 4 days post PMSG/HCG injection. Hormones were measured by ELISA kits. Each value represents the mean ± SD. (a) Progesterone levels. # peak value comparison between Bmal1/- and wild-type female mice with $P < 0.05$ or less. (b) Estradiol levels. * Comparison between Bmal1/- and wild-types at corresponding labelled time point with $P < 0.05$ or less. n=3 females at 8 weeks old per time point in each group.
**Figure 4d**

Expression of proteins (NFκB p65, BMAL1, p-AKT, and β-Actin) under control (CONT) and NfκB p65-siRNA treatment conditions. The relative expression levels are shown in the bar graph. The data indicates a significant change in expression with siRNA treatment compared to control conditions.
Supplementary Figure
BMAL1 LOSS

PI3K
AKT
mTORC1
S6K1

LHR

NFκB
RelA

Lhcgr

Cholesterol

P540cc
Star

Pregnenolone

CYP17A1

17α-Hydroxypregnenolone

DHEA

Androstenediol

3β-HSD

Androstenediol

Testosterone

3β-HSD

Copyright © 2019 Society of Reproduction and Fertility
Fig. 1. Schematic diagram summarizing the *Bmal1* regulated NFκB/PI3K pathway in hormone synthesis modulation in mice theca cells.
| Accession Number | Description |
|------------------|-------------|
| MEF2D_MOUSE      | Pre-mRNA-processing factor 19 OS=Mus musculus GN=Prpf19 PE=1 SV=1 |
| 51539 (8) 4 (1)  | 0.06 Mice:specific enhancer factor 20 O5=Mus musculus GN=Maf2l PE=1 SV=2 |
| 48026 (0) 5 (1)  | 0.07 Act-related protein 38 O5=Mus musculus GN=Arctb PE=1 SV=2 |
| 22913 (8) 0 (1)  | 0.32 ribosomal protein 79 O5=Mus musculus GN=Rps9 PE=1 SV=1 |
| 52010 (8) 0 (1)  | 0.46 Engagement of cell free O5=Mus musculus GN=Ube2c PE=1 SV=2 |
| 102765 (4) 3 (1) | 0.52 Kbtbd13 O5=Mus musculus GN=Kbtbd13 PE=2 SV=2 |
| 15141 (1) 0 (1)  | 0.53 Probable ATP-dependent RNA helicase DDX41 O5=Mus musculus GN=Ddx41 PE=1 SV=2 |
| 43176 (25) 0 (1) | 0.54 Dimethylaniline monooxygenase [N-oxide-forming] 4 O5=Mus musculus GN=Fmo4 PE=2 SV=3 |
| 106065 (14) 0 (1)| 0.55 Histone H2A1H O5=Mus musculus GN=H2a1h PE=1 SV=1 |
| 26479 (26) 0 (1) | 0.56 Peptidyl-prolyl cis-trans isomerase-like 4 O5=Mus musculus GN=Ppil4 PE=1 SV=2 |
| 13681 (19) 0 (1)| 0.57 Heterogeneous nuclear ribonucleoprotein M O5=Mus musculus GN=Hnrnpm PE=1 SV=3 |
| 35405 (9) 0 (1)  | 0.59 pre-mRNA 3' end processing protein WDR33 O5=Mus musculus GN=Wdr33 PE=1 SV=1 |
| 13151 (57) 0 (1)| 0.60 PC4 and SFRS1-interacting protein O5=Mus musculus GN=Psip1 PE=1 SV=1 |
| 77378 (32) 0 (1)| 0.61 Keratin, type I cytoskeletal 42 O5=Mus musculus GN=Krt42 PE=1 SV=1 |
| 11638 (12) 0 (1)| 0.62 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 15103 (13) 0 (1)| 0.63 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 215758 (255) 0 (1)| 0.64 Ubiquitin carboxyl-terminal hydrolase 26 O5=Mus musculus GN=Usp2 PE=2 SV=2 |
| 35466 (19) 0 (1)| 0.65 Histone-lysine-methyltransferase ASH1L O5=Mus musculus GN=Ash1l PE=1 SV=1 |
| 72780 (35) 0 (1)| 0.66 Insulin-like growth factor 2 receptor O5=Mus musculus GN=Igf1r PE=1 SV=1 |
| 28137 (25) 0 (1)| 0.67 GHK2 O5=Mus musculus GN=Ghk2 PE=1 SV=1 |
| 17105 (20) 0 (1)| 0.68 Ghk2 O5=Mus musculus GN=Ghk2 PE=1 SV=1 |
| 14444 (15) 0 (1)| 0.69 General transcription factor 3C polypeptide 5 O5=Mus musculus GN=Gtf3c5 PE=2 SV=2 |
| 13212 (19) 0 (1)| 0.70 Fidgetin-like protein 1 O5=Mus musculus GN=Fignl1 PE=2 SV=1 |
| 27577 (15) 0 (1)| 0.71 General transcription factor 3C polypeptide 5 O5=Mus musculus GN=Gtf3c5 PE=2 SV=2 |
| 5789 (21) 0 (1) | 0.72 Keratin, type I cytoskeletal 36 O5=Mus musculus GN=Krt36 PE=1 SV=1 |
| 10418 (12) 0 (1)| 0.73 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 32638 (57) 0 (1)| 0.74 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 5317 (6) 0 (1)  | 0.75 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 13794 (9) 0 (1)  | 0.76 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 16898 (32) 0 (1)| 0.77 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 16596 (23) 0 (1)| 0.78 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 7940 (20) 0 (1) | 0.79 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 76896 (11) 0 (1)| 0.80 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 39789 (20) 0 (1)| 0.81 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 10437 (14) 0 (1)| 0.82 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 54912 (8) 0 (1) | 0.83 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 23332 (20) 0 (1)| 0.84 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 76924 (11) 0 (1)| 0.85 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77267 (11) 0 (1)| 0.86 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.87 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.88 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.89 Keratin, type II cytoskeletal 5 O5=Musmusculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.90 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.91 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.92 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.93 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.94 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.95 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.96 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.97 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.98 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |
| 77056 (11) 0 (1)| 0.99 Keratin, type II cytoskeletal 5 O5=Mus musculus GN=Krt5 PE=1 SV=1 |

**Note:** The above table contains a subset of the data from the original publication, focusing on specific domains or subunits as indicated by the accessions. The full dataset includes a larger number of proteins and interactions.
| ENSMUSG00000011385 | 0.14 | 60S ribosomal protein L4 OS=Mus musculus GN=Rpl4 PE=2 SV=1 | 12 (2) |
| ENSMUSG00000003613 | 0.07 | Elongation factor 1-gamma OS=Mus musculus GN=Eef1g PE=1 SV=3 | 1 (1) |
| ENSMUSG00000001051 | 0.23 | Bola-like protein 1 OS=Mus musculus GN=Bola1 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000001057 | 0.05 | Uncharacterized protein C2orf22 homolog OS=Mus musculus PE=2 SV=1 | 1 (1) |
| ENSMUSG00000010617 | 0.06 | UAF60 small nuclear ribonucleoprotein Prp4 OS=Mus musculus GN=Prpf4 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000006866 | 0.01 | GON-4-like protein OS=Mus musculus GN=Gon4 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000010617 | 0.08 | p21-activated protein kinase-interacting protein 1 OS=Mus musculus GN=Pak1p1 PE=2 SV=2 | 1 (1) |
| ENSMUSG00000008719 | 0.00 | Oxidized low-density lipoprotein receptor 1 OS=Mus musculus GN=Olr1 PE=2 SV=2 | 1 (1) |
| ENSMUSG00000003940 | 0.05 | Peptaxid holomog OS=Mus musculus GN=Pe1 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000009698 | 0.03 | AF4/FMR2 family member 4 OS=Mus musculus GN=Af4 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000005788 | 0.03 | General transcription factor II-I OS=Mus musculus GN=Gtf2i PE=1 SV=3 | 1 (1) |
| ENSMUSG00000003298 | 0.16 | Seleneocyte-specific elongation factor OS=Mus musculus GN=Esf2c PE=2 SV=2 | 1 (1) |
| ENSMUSG00000007840 | 0.04 | Diintegrin and metalloproteinase domain-containing protein 21 OS=Mus musculus GN=Adam21 PE=2 SV=1 | 1 (1) |
| ENSMUSG00000008236 | 0.01 | SERTA-domain containing protein 2 OS=Mus musculus GN=Sertad2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000003098 | 0.3 | Thymoproph embryonic factor OS=Mus musculus GN=Tel PE=2 SV=1 | 1 (1) |
| ENSMUSG00000007844 | 0.16 | Keratin, type I cytoskeletal 17 OS=Mus musculus GN=Krt17 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000007844 | 0.04 | Microtubule-actin cross-linking factor 1 OS=Mus musculus GN=Mact1 PE=1 SV=2 | 1 (1) |
| ENSMUSG00000008506 | 0.15 | Class A basic helix-loop-helix protein 15 OS=Mus musculus GN=Bhlha15 PE=2 SV=2 | 1 (1) |
| ENSMUSG00000008506 | 0.07 | Dual homolog subfamily A member 2 OS=Mus musculus GN=Dnaja2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.08 | F-box only protein 16 OS=Mus musculus GN=Fax16 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.02 | CoBagen alpha-3(iii) chain OS=Mus musculus GN=Cobag3 Pe=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.01 | Peroxisomal material 1 protein OS=Mus musculus GN=Pm1 PE=1 SV=2 | 1 (1) |
| ENSMUSG00000008506 | 0.16 | MCG15085 OS=Mus musculus GN=Try4 PE=2 SV=2 | 1 (1) |
| ENSMUSG00000008506 | 0.09 | Metastasis-associated protein MTA2 OS=Mus musculus GN=Mta2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.38 | Aryl hydrocarbon receptor nuclear translocator-like protein 1 OS=Mus musculus GN=Arntl PE=1 SV=2 | 1 (1) |
| ENSMUSG00000008506 | 0.19 | Caveolin-2 OS=Mus musculus GN=Cav2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.08 | DNAJ homolog subfamily A member 2 OS=Mus musculus GN=Dnaja2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.08 | Protein Slc8a2 OS=Mus musculus GN=Slc8a2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.05 | Protein Cilp2 OS=Mus musculus GN=Cilp2 PE=4 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.15 | Microtubule-associated protein UXT OS=Mus musculus GN=Uxt PE=2 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.01 | General transcription factor II-I OS=Mus musculus GN=Gtf2i PE=1 SV=3 | 1 (1) |
| ENSMUSG00000008506 | 0.09 | Caveolin-2 OS=Mus musculus GN=Cav2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.04 | CO4A3 OS=Mus musculus GN=Co4a3 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.08 | Methionyl-tRNA synthetase homolog (fragment) OS=Mus musculus GN=Mtr4 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.00 | Metastasis-associated protein MTA2 OS=Mus musculus GN=Mta2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.38 | Arginyl hydrocarbon receptor nuclear translocator-like protein 1 OS=Mus musculus GN=Arnti PE=1 SV=2 | 1 (1) |
| ENSMUSG00000008506 | 0.19 | Caveolin-2 OS=Mus musculus GN=Cav2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.08 | DNAJ homolog subfamily A member 2 OS=Mus musculus GN=Dnaja2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.08 | Protein Slc8a2 OS=Mus musculus GN=Slc8a2 PE=1 SV=1 | 1 (1) |
| ENSMUSG00000008506 | 0.15 | Microtubule-associated protein UXT OS=Mus musculus GN=Uxt PE=2 SV=1 | 1 (1) |
molecular_function

- RNA binding: 38%, 20%
- enzyme binding: 49%, 13%
- ion binding: 47%, 13%
- DNA binding: 46%, 12%
- structural molecule activity: 24%, 6%
- cytoskeletal protein binding: 26%, 7%
- structural constituent of ribosome: 24%, 6%
- ATPase activity: 18%, 5%
- transcription factor activity, protein binding: 17%, 5%
- transcription factor binding: 17%, 5%
