Cyclic Variation of Cellular Clock Proteins in the Mouse Estrous Ovary

George Wiggins, Michael Legge *

- Department of Biochemistry, University of Otago, Dunedin, New Zealand

Abstract

Background: The mammalian ovary is controlled by a number of biological rhythms, which regulate the recruitment and release of mature oocytes. The main objective of this study was to investigate the role of cellular clock proteins during follicle maturation in the mouse estrous ovary.

Methods: Immunohistochemical (IHC) studies were performed on ovaries from 50 estrous staged mice culled at two time points of 09:00 [day] and 01:00 [mid-point of the dark cycle]. Six antibodies were used to identify the expression of core cellular clock proteins (BMAL1, CLOCK, CRY1, CRY2, PER1 and PER2) within the ovary and four follicle stages, primordial, primary, antral and corpus lutea. IHC data was scored using the Allred protocol and significance determined by Mann-Whitney tests. Differences were considered significant at p<0.05.

Results: All four follicle stages presented greater BMAL1 and CLOCK protein scores during the day and up regulation of CRY1-2 and PER1-2 at night. In primordial follicles, BMAL1 and CLOCK increases were significant (p<0.05) and CRY-1 and PER-1 were highly significant (p<0.001), and CRY-2 did not reach significance. Primary follicles demonstrated a similar response with BMAL1 and CLOCK, and CRY-1, PER-1-2 all reaching significant expression (p<0.05; p<0.001; p<0.001 respectively). CRY-2 expression was not significant. Antral follicles did not show significant BMAL1 or CLOCK expression, CRY-1 and PER-1 were highly significant (p<0.001) and CRY-2 had a small but significant increase (p<0.05). Corpus lutea demonstrated significant BMAL1 increase but CLOCK had no significant variation. CRY-1, PER-1-2 increases were highly significant (p<0.001) and CRY-2 was up regulated but failed to reach significance.

Conclusion: The ovary demonstrated a cellular clock response to the light: dark cycle and in addition, as the ovarian follicles mature changes in the positive and negative arms of both clock responsive proteins were observed.

Keywords: Cellular clock, Ovarian follicles, Ovary.

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development of an antral follicle to a Graafian fol-
licle and the LH surge causing follicular rupture
and release of the oocyte for fertilization (5-7).
Despite the hormonal control of follicle develop-
ment being well understood, the pre-antral develop-
ment still has many complexities which remain
unresolved particularly the recruitment and selection
of primordial follicles to progress to maturity
(8). Two observations suggest a circadian cellular
clock made be implicated in this development.
First-
ly in animals, the photoperiod or the light: dark
cycle directly effects the timing and efficiency of
ovulation. Hamsters have been shown to vary the
timing of ovulation dependent on the ratio of light:
dark, with the exposure to constant light disrupt-
ing regularity in ovulation (9). Furthermore, rats
cease ovulation after 60 days of constant light and
ovulation is lost along with a decrease in LH (10).
In addition to animal studies, there is substantial
evidence that disruption of the cellular clocks in
humans also perturbs reproductive cycles includ-
ing regularity in ovulation (11). Secondly, two proteins involved
in the cellular clock (CLOCK and PER-1) have
been identified in both the testis and ovaries, how-
ever, they differ in their expression between these
organs. The testis, which can continually produce
gametes, shows a lack of oscillation of these pro-
teins, which is normally considered critical to the
function of the cellular clock (12), whereas the
ovary has an oscillating cellular clock as demon-
strated by mRNA studies (13, 14).

The cellular clock has been identified in the ma-
ajority of mammalian peripheral tissues after the
original discovery in the suprachiasmatic nucleus
(SCN). The core of the cellular clock is estab-
lished by a transcriptional/translational feedback
loop set up by the oscillation of clock genes and
their respective proteins (15). The positive arm
and the negative arm are diurnally expressed with
the peaks of their expression approximately 12 hr
out of synchronization (16). The mechanism is de-
pendent on the control over the formation of the
heterodimer BMAL1: CLOCK (17, 18), through a
basic helix-loop-helix (bHLH) domain. The het-
erodimer is able to bind to the E-box regulatory
element where it recruits the cellular machinery
involved in the transcriptional up regulation of
downstream genes (19). The E-box promoted genes
can be separated into two classes; genes involved
in the negative regulation of the cellular clock
(PER1-3 and CRY1-2) and genes that have physi-
ological function (clock output genes). The nega-
tive clock genes are translated and form a protein
complex (CRY: PER) which is able to translocate
into the nucleus where it inhibits the promotion of
E-box transcription via BMAL1: CLOCK, thus
ultimately reducing their Per and Cry transcription
and completing the cellular clock. A critical fea-
ture of a circadian clock is that it can function au-
tonomously, without external input. However, it
can be modified or entrained to the local cellular
environment (20), thereby providing a model mech-
anism where the cellular clock is able to control
cellular activities including metabolic homeosta-
sis, cell cycle control and hormone oscillations by
altering the transcription of the clock output genes.

Although previous work has focused on cellular
clock mRNA expression, it is established that
mRNA expression may not predict the cellular
protein level for a number of proteins and should
not be used as a surrogate to demonstrate the cor-
responding cellular protein expression and alter-
native techniques such as immunohistochemistry
should be used to provide definitive identification
and localization of the tissue specific protein (21).
In this study, immunohistochemistry was used to
investigate cellular clock protein expression and it
was demonstrated that the mouse estrous ovary
expresses all six of the core cellular clock proteins
and that they are responsive to the light:dark cy-
cle.

Methods

Animals: All mice used were sexually mature
female C57BL/6J, 6 to 10 weeks old and were
maintained in the University of Otago Animal
Facility. Mice were provided food and water ab
libitum and maintained on a 12:12 hour light dark
cycle (Lights onset at 0700/zeitgeber time (ZT)
0). Tissues were obtained at two different chrono-
logical time points, (ZT2/0900 and ZT18/0100 [mid-
point of the dark cycle]), and within each time
point estrous was determined representing hor-
mone responsive ovaries at ovulation. In addition
to ovaries, kidney and liver were obtained as con-
control tissues, which demonstrate circadian clock
protein expression.

Animal procedures were under the approval of
the University of Otago Animal Welfare Office
[AEC: 39/08].

Estrous staging: For estrous staging, vaginal
smears were obtained and stained with 1% meth-
ylene blue for 10 min (22). Cell type and frequen-
cy were assessed where no/low epithelial cells
suggested metestrous and high epithelial cell
numbers, indicating mice in estrous. Estrous was
determined for mice at 09:00 hr and 01:00 hr. A total of 50 mice were scored as being in estrous with 40 mice in estrous during the day and 10:00 at night.

**Immunohistochemistry:** The ovaries were fixed in buffered formal saline and processed for histology. Serial sections were cut from each ovary and tissue sections were deparaffinized in xylene and progressively rehydrated through graded alcohols and a final water wash. Heat antigen retrieval was performed in citrate buffer pH=6.0 for 25 min in a KOS multifunctional microwave tissue processor (Milestone, Italy). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and non-specific binding was blocked using 3% bovine serum albumin (Invitrogen Life Sciences, USA). Tissues were incubated with each of the six clock protein primary antibodies (AbCam, UK) using optimized dilutions as shown in table 1. Following primary antibody incubation and washes, the sections were incubated with an anti-rabbit secondary antibody conjugated to biotin (AbCam, UK). Vectorstain ABC elite kits (Vector Laboratories, USA) were then used to visualize the bound primary antibody. Binding was located using commercial 3,3’-diaminobezidine hydrochloride (DAKO, Australia) and the sections were then washed and counterstained with haematoxylin, dehydrated through graded alcohols and cleared in xylene before mounting in Entellan (Merk Chemicals, Germany).

**Cell scoring:** All sections were examined microscopically by two observers and imaging data stored for analysis using an Olympus 1X71 microscope and DP control imaging system (Shinjuku, Japan). Scoring for staining location and intensity was undertaken using Allred scoring protocol (23, 24), which combines the cellular staining intensity with nuclear localization. Sections were imaged and given a score out of eight, which is derived from a staining intensity score (0-3) and a nuclear positivity score (0-5). Staining intensity included 0=negative, 1=weak, 2=moderate, and 3=intense stain. Nuclear positivity was scored by a cell count where 0%=0, 1-9%=1°, 10-32%=2°, 33-65%=3°, 66-90%=4°, 90-100%=5°. For follicles large enough, a minimum of 100 cells per follicle were counted across a minimum of 10 objective fields to ensure a representative observation and when possible 20 follicles were counted for each stage.

**Statistics:** Allred scores are presented as the mean ±S.E.M. Statistical differences were determined by Mann-Whitney tests. Differences were considered significant at p<0.05 or less (SPSS Inc, Chicago, IL, USA).

## Results

Mean Allred positivity scores (±SEM) for each respective clock protein are shown in figure 1. For each of the four follicle stages assessed, all presented with greater BMAL1 and CLOCK protein scores in the day (09:00 hr) ovarian sections. Conversely, the four negative arm clock proteins (CRY1-2 and PER 1-2) were up regulated in the night ovarian sections (01:00 hr). Of the four negative clock proteins, only CRY1 had a statistically significant difference with time for all follicle stages (primordial follicles, p<0.05; primary follicles, p<0.001; antral follicles, p<0.001; corpus lutea, p<0.05).

### Primordial follicle cellular clock

All sections for primordial follicle staining (Figure 1A and figure 2) exhibited a light:dark response with greater BMAL1 and CLOCK expression during the day. However, although BAM1 demonstrated a significant decrease at night (p<0.05), CLOCK demonstrated little variation between day and night. CRY-1 and PER-1 and 2 demonstrated highly significant increases (p<0.001) at night but CRY-2 did not increase.

### Primary follicle cellular clock

All sections for primary follicle staining (Figure 1B and figure 3) exhibited a light dark response similar to that identified in the primordial follicles for both BMAL1 and CLOCK in daytime. However, BAM1 did not decrease at night whereas CLOCK decreased at night in a similar response identified in primordial follicles. Three of the four negative arm clock proteins, CRY-1, PER-1, and 2, demonstrated highly significant increases at night (p<0.001) with

### Table 1. Primary clock related optimized antibody dilutions and incubation times

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution factor</th>
<th>Concentration (µg/ml)</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLOCK</td>
<td>1.1000</td>
<td>1.0</td>
<td>1 hr at RT</td>
</tr>
<tr>
<td>BMAL1</td>
<td>1.2000</td>
<td>5</td>
<td>ON at 4 °C</td>
</tr>
<tr>
<td>CRY1</td>
<td>1.1000</td>
<td>1</td>
<td>ON at 4 °C</td>
</tr>
<tr>
<td>CRY2</td>
<td>1.1000</td>
<td>2.5</td>
<td>ON at 4 °C</td>
</tr>
<tr>
<td>PER1</td>
<td>1.500</td>
<td>2</td>
<td>1 hr at RT</td>
</tr>
<tr>
<td>PER2</td>
<td>1.500</td>
<td>1.1</td>
<td>1 hr at RT</td>
</tr>
<tr>
<td>SIRT1</td>
<td>1.2000</td>
<td>0.55</td>
<td>1 hr at RT</td>
</tr>
</tbody>
</table>

RT: Room Temperature; ON: Overnight
only CRY-2 not increasing significantly.

**Antral follicle cellular clock:** No significant variation for either BMAL1 or CLOCK expression was demonstrated between day and night (Figure 1C and Figure 4). However, Cry-1 and PER-1 demonstrated highly significant increases at night (p<0.001) and CRY-2 had a smaller but significant increase (p<0.05) at night. Only PER-2 failed to show a significant increase at night (p<0.05).

**Corpus lutea cellular clock:** Whilst BMAL1 had a significant daytime increase in expression (p<0.05), CLOCK had little variation between day and night.

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**Figure 1.** Mean Allred positivity scores for each respective clock related proteins. The four follicle stages all display a clock where the positive clock-proteins (BMAL1 and CLOCK) presented greater scores in day sections. Conversely, the four negative clock-related proteins were upregulated in night sections. A: Positivity of clock-related proteins expressed in primordial follicles. Only CRY1 displayed a statistically significant difference across the time. B: Positivity of clock-related proteins expressed in primary follicles. C: Positivity of clock-related proteins expressed in antral follicles. D: Positivity of clock-related proteins expressed in corpus lutea. All scores are mean Allred scores±S.E.M. Mann-Whitney statistics was performed on Allred data.

*p<0.05, **p<0.01, ***p<0.0001

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**Figure 2.** Representative primordial follicle histology sections with the six clock proteins expression indicated by brown staining. Box encloses BAML1 and CLOCK (positive arm) proteins

**Figure 3.** Representative primary follicle histology sections with the six clock proteins expression indicated by brown staining. Box encloses the BAML1 and CLOCK (positive arm) proteins
expression. All four of the negative arm clock proteins were up regulated at night (Figure 1D and figure 5) with PER-2 demonstrating the greatest increase \((p<0.001)\). Although CRY-2 expression was also up regulated, it failed to reach statistical significance.

**Discussion**

The function of the ovary is the production and release of oocytes for fertilization at regular intervals and the provision of hormones for initiating and maintaining early pregnancy, which requires regular time keeping over a reproductive life time in a finely controlled process. The ovary maintains a steady periodic course of maturing follicles despite the diminishing pool of available follicles and it is generally considered that this fine control occurs at the pre-antral (hormone unresponsive) stage of ovarian follicle development. Despite this very well regulated organ, to date, little is known about the peripheral clock mechanism of the ovary and in particular the transition from primordial follicles to hormone responsive follicles prepared for ovulation.

The process of primordial recruitment has been vigorously studied with numerous factors implicated in this process (25-27). Anti-mullerian hormone (AMH) is a promising target as it appears to control the amount of primordial follicles able to proceed through development, acting as a regulatory to this development and impeding primordial recruitment (28). However, AMH does not provide an explanation to the complex process of selection of why does one follicle develop over another? Although this current study could not definitively answer this either, it did propose a mechanism that can be further investigated.

The central function of peripheral cellular clocks is the ability to form heterodimers between both the positive arm proteins (BAML1 and CLOCK) and the negative arm proteins (CRY 1 and 2, and PER 1 and 2) and for the heterodimers to translocate to the nucleus to control gene expression. From these data, both the primordial and primary follicles appear to have a similar positive/negative arm feedback loop. However, as follicles enter the hormone responsive antral stage cyclicity of the positive arm appears to be lost between day and night with little change in expression of BAML1 and CLOCK but the negative arm still retains the rhythm of elevated expression at night and lower expression during the day. This pattern continues in the corpus lutea. Previous research has indicated that mRNA expression of Per 2 in the SCN is increased by estrogen exposure (29), whereas Per 2 expression period is shortened in the uterus (30, 31). Furthermore, ovarian BAML1 mRNA expression has been reported to increase at pro-estrous indicating that it may well be estradiol responsive (13). Overall, there appears to be a switch in clock gene responsiveness as follicles move in to the antral stage.
Conclusion

Though the understanding of the role of peripheral cellular clocks is not fully established, there are clear indications that the switching between the positive and negative arms of the clock is strongly implicated in cellular metabolism (31), such as the control of glucose homeostasis, gluconeogenesis and intra-cellular redox (32-35). Given that each follicle stage of development may well have differing metabolic requirements, variable signals from the ovarian cellular clock may coordinate follicle responsiveness both for cell signaling within the ovary and hormone responsiveness. For the present study, this could not be determined but clock protein expression rhymicity in the ovary at estrous was established indicating that the ovary, unlike the testis, has a regulated peripheral cellular clock consistent with many other body tissues and the light: dark cycle is critical in regulating the clock. Further work is required to determine the functional relationship between other stages of the estrous cycle, time, clock protein expression and changes in ovarian metabolic activity.

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Conflict of Interest

This work was supported by a grant from Lottery Health, New Zealand. Authors declare no conflict of interest.

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