UNIVERSITY OF CALIFORNIA SAN DIEGO

Characterization of BMAL1 in the Mouse Pre-Implantation Embryo

A thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

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Professor James Cooke

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The Thesis of Ryan Dang Nguyen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABBREVIATIONS

2pn	2-pronuclear
Bmal1-mKO	maternal knockout of *Bmal1*
BSA	bovine serum albumin
CO_2	carbon dioxide
DAB	3,3'-diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
HCG	human chorionic gonadotropin
IHC	immunohistochemistry
MZT	maternal-to-zygotic transition
PBS	phosphate-buffered solution
PFA	paraformaldehyde
PMSG	pregnant mare serum gonadotropin
WT	wildtype

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ABSTRACT OF THE THESIS

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Pre-implantation development depends on several processes, including the deposition of maternal factors into the oocyte during oogenesis, the expression of maternal mRNAs, and the degradation of maternal mRNA in tandem with zygotic genome activation during the maternal-to-zygotic transition (MZT). These processes ultimately determine whether an embryo implants into the uterine lining, which is necessary for pregnancy.

It is known that mice with whole body knockouts of *Bmal1* (hereafter referred to as *Bmal1*$^{-/-}$ mice) are infertile. Previous studies have indicated that this infertility deficit occurs in pre-implantation development. Furthermore, other studies found the presence of *Bmal1* mRNAs

in mouse pre-implantation development. With this, I hypothesized that the maternal expression of *Bmal1* is necessary for pre-implantation development.

To investigate this hypothesis, I performed immunostaining on embryos from the MZT, and whole ovary sections from wildtype (WT) mice to characterize the expression of BMAL1. I also quantified the number of metaphase II oocytes obtained from WT and *Bmal1*$^{-/-}$ mice, to look for deficits in pre-implantation development. I found a higher percentage of abnormal oocytes from *Bmal1*$^{-/-}$ mothers. Fluorescent imaging of these embryos reveals fluorescence of BMAL1 in the cytoplasm and nucleus of embryos throughout the MZT. Immunostaining of whole ovary sections revealed the presence of both BMAL1 and CLOCK protein within developing oocytes and the surrounding ovarian tissue. Now that BMAL1 expression has been characterized, further investigation can begin on the molecular roles of BMAL1 in mouse pre-implantation development.

INTRODUCTION

The common definition of pregnancy specifies its beginning at implantation, the burrowing of an embryo into the uterine lining. The success of implantation hinges upon the success of developmental processes prior, such as oogenesis, fertilization, and embryogenesis. Oogenesis is the development of primordial germ cells into oocytes, the female gametes. Fertilization is the fusion of the female and male gametes to form a zygote. After fertilization, the zygote undergoes rapid cell cycles to divide into many cells, in a process called cleavage, and eventually develops into a blastocyst, which implants into the uterine lining. This overall process of cellular division is called embryogenesis, and the specific stages of embryogenesis are outlined below in Figure 1B.

In all members of the animal kingdom [1], the development of the embryo is first guided by maternal gene expression. mRNAs and proteins from the mother's ovarian cells are deposited into the oocyte during oogenesis, and the mRNAs are translated by the oocyte's cellular machinery to express vital protein products. During this process, the oocyte's genome is transcriptionally silenced, leaving the oocyte dependent on maternal gene expression to develop [2].

As development progresses, the maternal transcriptome and proteome eventually degrades. The degradation of maternal mRNA and proteins generally begins moments before fertilization [2, 3], and the clearance of these maternal factors varies between animals. In mammals such as mice, for example, degradation completes as early as the beginning of the 2-cell stage [2]. Similarly, in humans, it completes between the 2-cell stage and the 4-cell stage [4]. While the maternal transcriptome and proteome degrades, the zygote's genome activates, thereby shifting developmental control from the mother to the zygote. This period of drastic

transcriptomic change, visualized in Figure 1A, is called the maternal-to-zygotic transition (MZT).

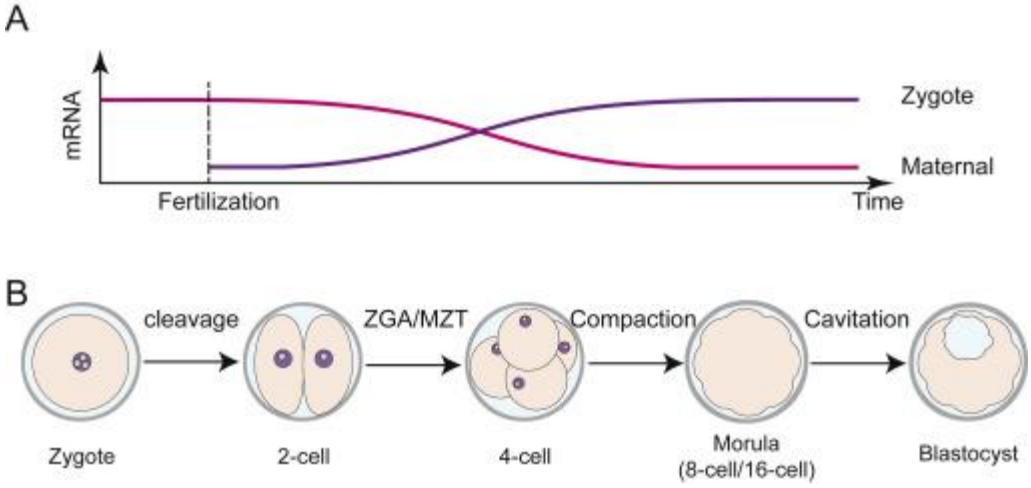

Figure 1: The MZT and embryogenesis. Panel A shows the relative levels of maternal mRNA and zygotic mRNA as development progresses. Starting from fertilization, maternal mRNA levels decrease due to programmed degradation, while zygotic mRNA levels increase due to zygotic transcription. Panel B shows the stages of embryogenesis in mammals. Not shown before the zygotic stage is the 2-pronuclei (2pn) stage; it is the stage at which the genetic material of the parents has not yet fused into a single nucleus. In mammals, the MZT ends at the 4-cell stage, when the presence of zygotic mRNAs is high, but the presence of maternal mRNA is little to none. This figure has been reproduced with the permission of Yao et. al. [5].

Despite their transience, the deposition and expression of maternal mRNAs and proteins are vital to embryogenesis. The egg is reliant on maternal gene expression to develop properly, and genes that are expressed maternally in this manner are called maternal effect genes. Mutations in such genes can cause embryonic lethality, even if the paternal copy is intact. The impact of mutations in maternal effect genes is often observed as a deficit during pre-implantation development. For example, AGO2 is a protein involved in maternal mRNA degradation, and AGO2-null mice produce embryos that do not survive beyond the 4-cell stage [6].

A study by Xu, *et. al.* [7], has revealed the possibility that the gene *Bmal1* in mice may be a maternal effect gene, based on the pre-implantation deficits they observed. For the sake of brevity, mice with whole body knockouts of *Bmal1* are referred to as *Bmal1*$^{-/-}$ mice, and oocytes and embryos from female *Bmal1*$^{-/-}$ mice will be called *Bmal1*-mKO, with “mKO” meaning

"maternal knockout." Xu, *et. al.*, found that significantly lower numbers of *Bmal1*-mKO embryos developed to the blastocyst stage, compared to WT embryos [7]. These *Bmal1*-mKO blastocysts also had significantly lower implantation rates compared to WT blastocysts. This is true even when *Bmal1*-mKO embryos were implanted into pseudopregnant wildtype females, suggesting that the deficit is within the embryo, not in the intrauterine environment [7]. Finally, *Bmal1*-mKO embryos exhibited a greater number of embryonic irregularities that are not found as often in WT embryos [7].

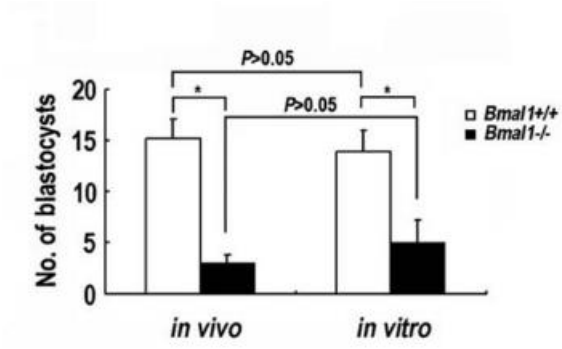

Figure 2: Impaired development to blastocyst stage seen in *Bmal1*-mKO embryos. Xu, *et. al.*, superovulated WT and *Bmal1*$^{-/-}$ mice to study the progression of embryos to blastocyst stage. In the *in vivo* conditions, the authors harvested and counted the blastocysts 3 days after observation of a vaginal plug. In the *in vitro* conditions, cumulus-oocyte complexes were harvested from WT and *Bmal1*$^{-/-}$ mice, the oocytes were fertilized, and the zygotes were allowed to develop to the blastocyst stage in embryo culturing media. In both conditions, the authors found significantly lower numbers of *Bmal1*-mKO embryos that developed to the blastocyst stage, compared to WT embryos [7].

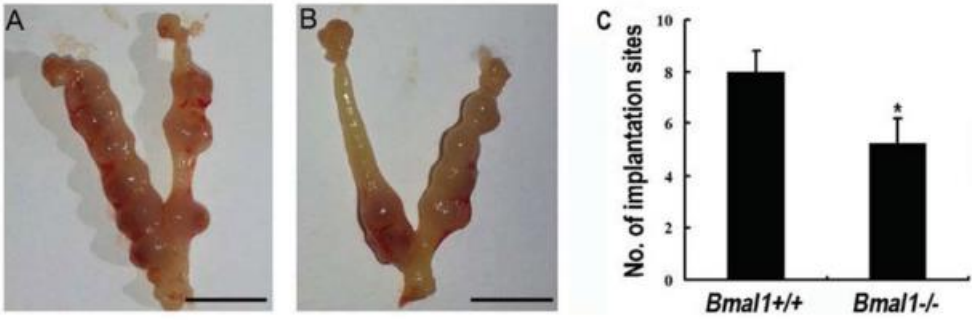

Figure 3: Impaired implantation seen in Bmal1-mKO embryos. *Bmal1*-mKO embryos which did survive to the blastocyst stage were implanted into pseudopregnant WT mice. Implantation rates of *Bmal1*-mKO blastocysts was significantly impaired [7].

Two other bioinformatics studies, one investigating mouse embryos only [8] and the other investigating both mouse and human embryos [9], have also identified *BMAL1* as a gene whose transcripts were present from the oocyte stage, a stage that precedes the MZT. Furthermore, *BMAL1* transcripts were detected throughout preimplantation development, and evaded complete degradation. These studies suggest that *Bmal1* is a gene that is necessary for pre-implantation development in the mouse, and that the human homolog *BMAL1* may be necessary for pre-implantation development in humans as well.

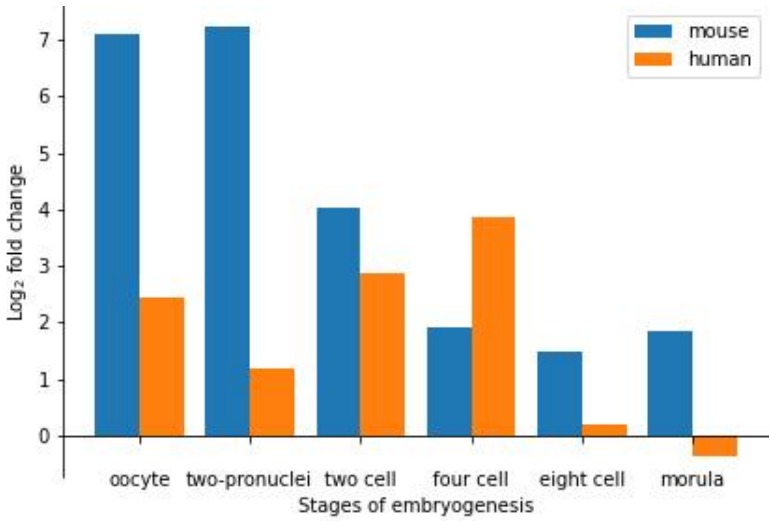

Figure 4: BMAL1 transcripts in mouse and human embryos. The data from Xue, *et. al.* [9], showing the $\log_2$ fold changes in the levels of mRNA transcripts of *BMAL1* in the embryos of mice and humans from the 2pn stage to the morula stage. The graph was rendered with the "matplotlib" package of Python.

BMAL1 has been historically known as a master regulator of circadian rhythms [10]. In this function, BMAL1 dimerizes with CLOCK to regulate the rhythmic transcription of other genes by binding to E-box motifs within the genome [11]. Interestingly, however, rhythmicity of *Bmal1* mRNA has not been identified before the differentiation of blastocyst cells in the inner cell mass [12]. This suggests that BMAL1 might have non-circadian functions in the pre-implantation embryo. A number of studies have identified other functions of BMAL1 in addition to regulating circadian rhythms, including: promoting cap-dependent translation [13], dimerizing with CLOCK to increase the efficiency of histone acetylation [14], and regulating

m^6A modification and/or the m^6A reader YTHDF2, which targets mRNA for degradation [15]. It is possible that BMAL1 may be serving these functions within the oocyte or the pre-implantation embryo because successful pre-implantation development requires each of these functions: the translation of maternal mRNAs, the acetylation of histones in the zygotic genome, and the proper degradation of the maternal transcriptome.

However, there remain two gaps of knowledge concerning the role of *BMAL1* in pre-implantation development. The first gap in knowledge is the presence of BMAL1 protein within the pre-implantation embryo. While the presence of *Bmal1* mRNA was revealed in the developing mouse embryo from the 2pn stage to the 2-cell stage and beyond [8, 9], extensive characterization of BMAL1 protein within the different stages of embryogenesis is lacking. This is especially true for the stages of the MZT. Broadening scientific knowledge about *BMAL1* in embryogenesis requires solid proof that BMAL1 is expressed as a protein, not just as a maternal RNA, since RNAs themselves can serve cellular functions without being translated. The second gap in knowledge pertains to the research of Xu, *et al*. [7]. Though they reported that embryos from female *Bmal1*$^{-/-}$ mice do not survive past the blastocyst stage, they did not investigate the stages between fertilization and blastocyst, or the quality of the oocyte. Thus, it is still unknown at which stage the embryos are lost.

The goal of my project was to fill in these two gaps in knowledge. The first gap was addressed by harvesting wildtype and *Bmal1*-mKO metaphase II oocytes and comparing their overall quality. The second gap was addressed by using immunohistochemical (IHC) techniques to visualize the BMAL1 protein in wildtype mouse ovaries and wildtype mouse embryos at each stage of the murine MZT. Because I believe that the deficits are maternal, all female mice regardless of their *Bmal1* genotype were mated with WT mice. The findings of this project will expand the breadth of knowledge on pre-implantation development and *Bmal1*, and it is possible that these findings will have novel implications for the basis of infertility.

RESULTS

In this work, I sought to characterize the expression of BMAL1 in WT embryos and identify the specific deficit observed in embryos generated from female *Bmal1*$^{-/-}$ mice. For the sake of brevity, I refer to oocytes and embryos generated from female *Bmal1*$^{-/-}$ mice as *Bmal1*-mKO oocytes and embryos. To accomplish these aforementioned goals, my experiments took two main directions.

First, I sought to pinpoint the specific timepoint at which *Bmal1*-mKO embryos exhibited a deficit. I started my investigation at the metaphase II oocyte stage, the stage at which the egg is first ovulated.

Second, I used IHC to characterize the expression of BMAL1 in developing oocytes within the ovary and in wildtype embryos, to determine at which stage *Bmal1* mRNA is translated, and where in the developing embryos BMAL1 protein is localized. CLOCK was also immunostained because its molecular role is heavily intertwined with that of BMAL1. The function of *Bmal1* in pre-implantation development, which is outside the scope of my thesis, will be heavily informed by the results I introduce in the following paragraphs. Such information will be utilized by members of my laboratory for subsequent studies.

Comparison of oocyte quality between WT and Bmal1-*mKO oocytes*

My first experiment examined oocyte quality. I superovulated both female WT mice and female *Bmal1*$^{-/-}$ mice to look for possible deficits in oogenesis that might be caused by deficiency of BMAL1. After superovulation without mating, I expected to see oocytes paused in metaphase II, with one polar body and one germinal vesicle. For signs of deficiencies, I looked for oocytes that exhibited blebbing, abnormally large polar bodies, or cell division (Figure 4). After one superovulation run, I found that there was a higher percentage of normal WT oocytes compared to *Bmal1*-mKO oocytes (Figure 5). Though these results look promising, more superovulation runs are needed to substantiate these results.

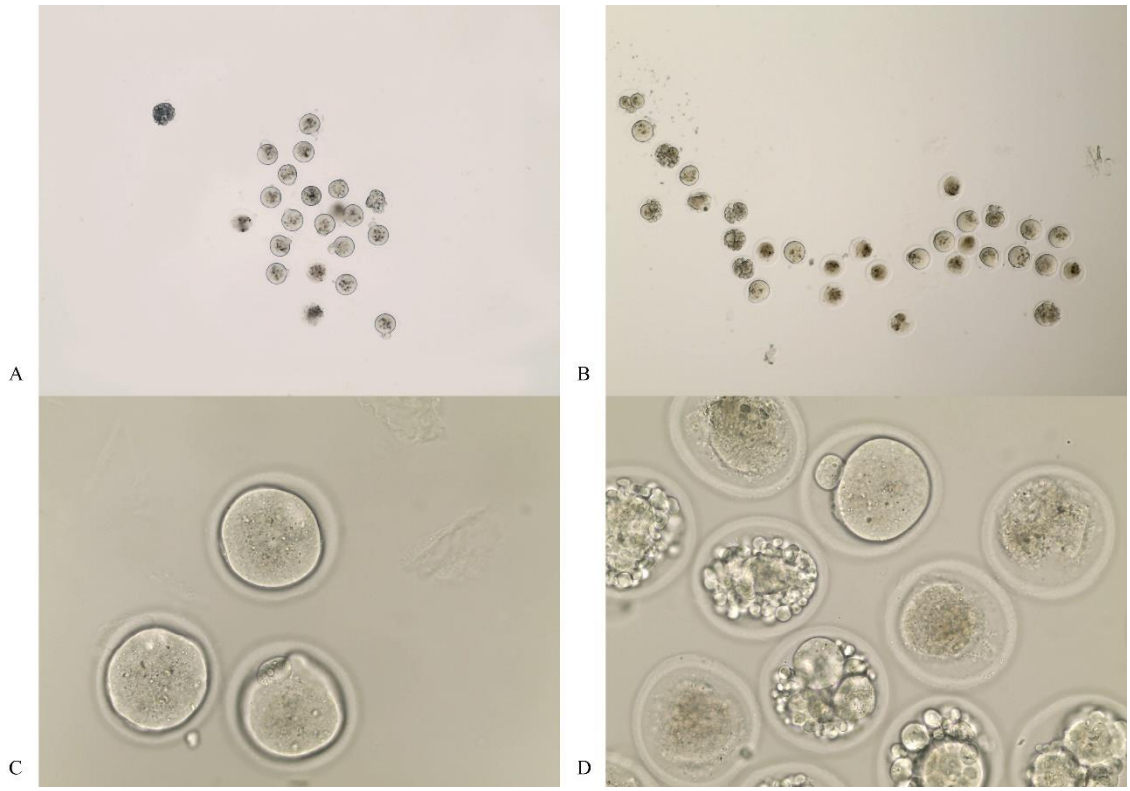

Figure 5: Morphology of WT and *Bmal1-mKO* oocytes. (A,C) WT oocytes. (B,D) *Bmal1*-mKO oocytes. Figures 6A and 6B were taken in brightfield at 10x magnification, while Figures 6C and 6D are zoomed on oocytes from Figures 6A and 6B respectively, at 40x magnification.

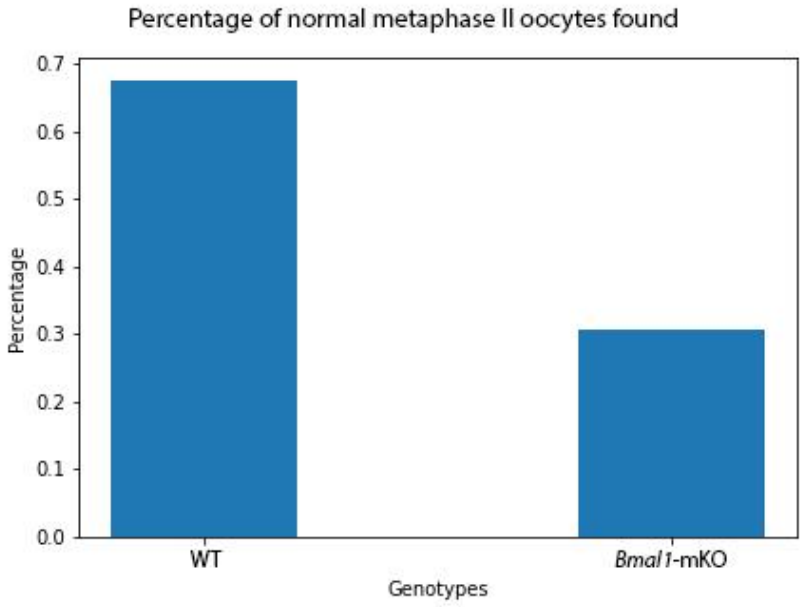

Figure 6: Percentage of normal WT and *Bmal1*-mKO metaphase II oocytes. The percentage of normal WT oocytes found was 67.6%, and the percentage of normal *Bmal1*-mKO oocytes found was 30.8%.

The presence of* Bmal1 *in the ovary and the immature oocyte

In this second experiment, I sought to elucidate the expression of BMAL1 and CLOCK proteins inside the immature oocyte, prior to ovulation and fertilization. Knowing the presence of these two proteins in the immature oocyte would help us determine whether BMAL1 and CLOCK might be acting in early oocyte development. I collected ovaries from WT mice and then fixed them for preservation. After fixation, the ovaries were processed, embedded, and sectioned for IHC with DAB staining to visualize BMAL1 and CLOCK in the ovarian tissue and in the immature oocytes within the ovarian follicles. In Figure 7A, BMAL1 is heavily expressed in all tissues of the ovary, including the connective tissue, the granulosa cells, and the oocyte. CLOCK is expressed in the connective tissue and oocyte at lesser levels but (Figure 7B), but is expressed at higher levels in the granulosa cells.

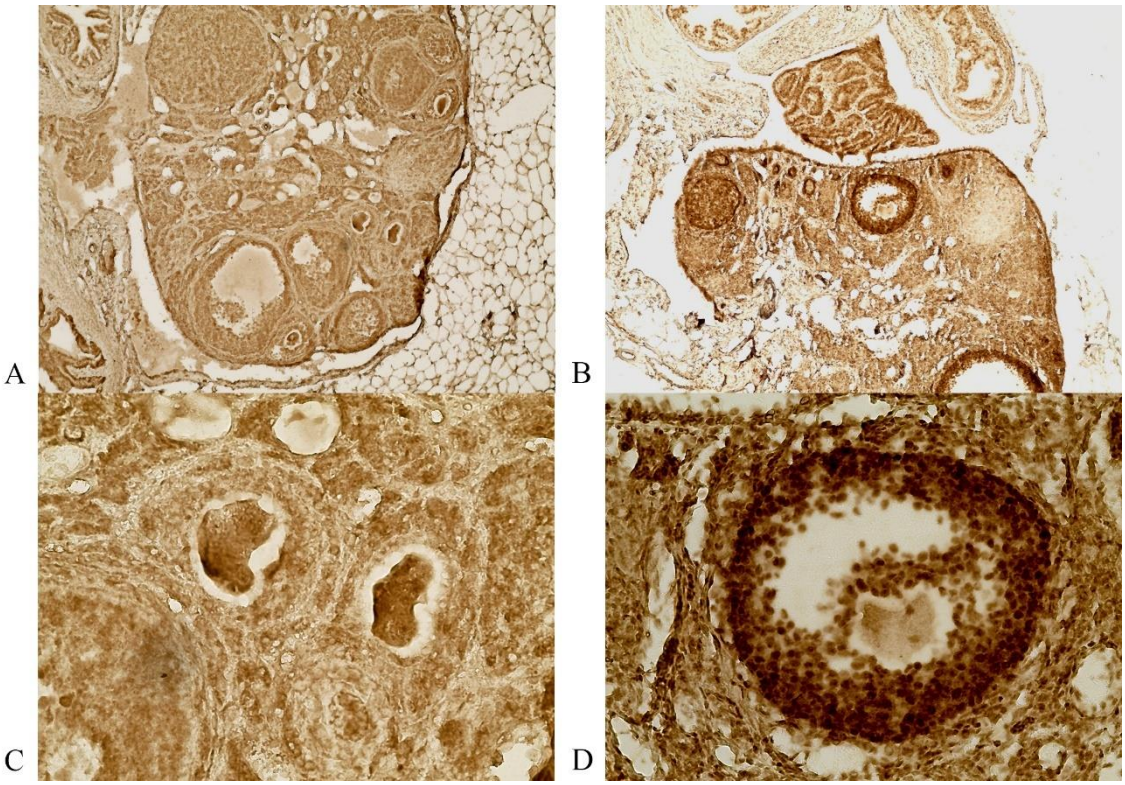

Figure 7: Immunohistochemical staining of intact, adult murine ovarian tissue. Figure 7C is an image zoomed on a secondary follicle from Figure 7A, while Figure 7D is an image zoomed on a tertiary follicle from Figure 7B. (A, C) α-BMAL1, (B, D), α-CLOCK. (A,B) at 4x

magnification, (C,D) at 40x magnification. Visualization for both was performed using DAB staining.

Characterization of BMAL1 in WT pre-implantation embryos

Lastly, I aimed to characterize the spatiotemporal expression of BMAL1 and CLOCK protein in wildtype mouse embryos within the MZT, to confirm its expression. Wildtype mice were superovulated, mated, and then harvested at specific time points to yield embryos at the 2pn stage, the 2-cell stage, and the 4-cell stage. Then, these embryos were fixed and immunostained for BMAL1 and CLOCK. As multiple possibilities for BMAL1 function in the early embryo involve interactions with CLOCK, I chose to immunostain for the presence of both proteins. DAPI was also added to stain the nucleus. Finally, a few embryos were stained with the secondary antibodies but no primary antibodies, to control for non-specific binding of secondary antibodies and autofluorescence.

As mentioned previously, two bioinformatics studies found that *BMAL1* and *CLOCK* mRNAs were found in mouse and human embryos throughout preimplantation development [8, 9]. As shown in Figure 8, both BMAL1 and CLOCK were present both within the nucleus and the cytoplasm for all stages of the MZT, which confirms my predictions from the bioinformatics studies that BMAL1 and CLOCK are expressed as proteins throughout preimplantation development.

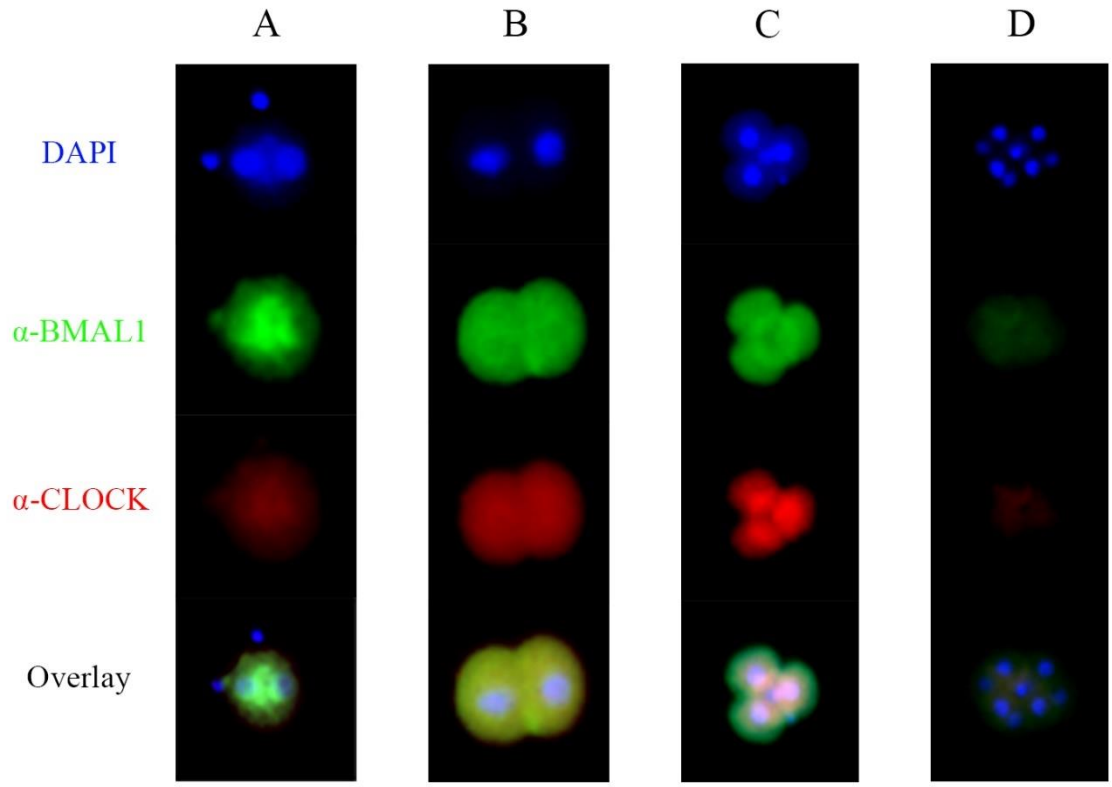

Figure 8: Immunohistochemical staining of WT embryos in the MZT. (A) 2pn, (B) 2-cell, (C) 4-cell, (D) control for non-specific binding of secondary antibodies and autofluorescence, using 8-cell embryos. Pictures were taken with Keyance microscope at 10X.

DISCUSSION

My study shows that both BMAL1 and CLOCK are expressed throughout pre-implantation development, starting from oogenesis (Figure 7) to the end of the MZT (Figure 8). *Bmal1*-mKO had higher numbers of abnormal oocytes than did WT embryos (Figures 5 and 6), pointing toward a deficit that occurs at some point during oogenesis. In order to determine at which stage in oogenesis BMAL1 is required, further analysis of ovarian follicles during maturation is required. In particular, the ovaries harvested from WT and Bmal1-mKO mice could be fixed and analyzed for oocytes counts at each stage of oogenesis. Another related experiment would look forward in time instead of backwards: WT and *Bmal1*-mKO embryos at the 2pn stage would be harvested and allowed to grow in embryo culturing media through each stage of embryogenesis, in order to track and record the survival rates for each genotype.

Figure 7 shows the expression of both BMAL1 and CLOCK within the immature oocyte in the ovarian follicle. In addition, Figure 8 shows that both BMAL1 and CLOCK continue to be expressed in the developing embryo at each stage of the MZT. It is important to note that BMAL1 and CLOCK are within both the nucleus and the cytoplasm in all stages of the MZT, which means it is possible that BMAL1 and CLOCK are dimerizing together to accomplish a biological function. However, further experiments are needed to make definitive conclusions about BMAL1 and CLOCK's molecular roles. To add to the complexity, BMAL1 and CLOCK can shuttle in and out of the nucleus, and it is possible that my experiment might have caught both proteins in transit from one cellular compartment to another in a rhythmic fashion [11]. One experiment that could address this would be to take the embryos of one stage at regularly spaced intervals of time and immunostain them for BMAL1 and CLOCK. This experiment would produce a series of images of BMAL1 and CLOCK fluorescence over time for a particular embryonic stage, and this series could prove or disprove the hypothesis that BMAL1 and CLOCK are shuttling in and out of the nucleus in a rhythmic fashion.

As mentioned earlier, BMAL1 and CLOCK dimerize to migrate to the nucleus and regulate transcription by binding to E-box motifs, thereby generating circadian rhythmicity [11]. However, it is known that embryos do not have circadian rhythms before the blastocyst stage [12]. Therefore, BMAL1 and CLOCK may be serving functions outside of circadian rhythms. One possibility is that the BMAL1::CLOCK heterodimer still serves as a transcription factor for other genes in a non-rhythmic fashion. Other possibilities of BMAL1's molecular roles include binding to CLOCK to increase the efficiency of histone acetylation [14], increasing the efficiency of cap-dependent translation [13], and regulating m^6A modifications and the activity of the m^6A reader YTHDF2 [15]. Although outside the scope of this project, there are a number of experiments that could be performed by members of my laboratory in the future to investigate these molecular roles. For example, one could utilize an antibody that targets phosphorylated BMAL1, which would test the hypothesis that BMAL1 is promoting cap-dependent translation in the embryo. In addition, one could perform chromatin immunoprecipitation for BMAL1 and CLOCK to evaluate whether BMAL1::CLOCK is increasing the efficiency of histone acetylation. Next-generation sequencing technologies could aid future members of the laboratory determine other functions of BMAL1. ChIP-Seq, for example, could help one determine if BMAL1::CLOCK is acting as a transcription factor for non-circadian rhythm genes in the zygotic genome. RIP-Seq, another example, could help one determine if BMAL1 might be regulating m^6A modifications in maternal mRNAs.

MATERIALS AND METHODS

Mice

C57BL6/J mice were group-housed by sex on a 12:12 light/dark cycle with an *ad libitum* standard chow diet and water in a temperature-controlled room. Generation of heterozygous *Bmal1* knockout mice was achieved by crossing a *Bmal1*-floxed mouse obtained from The Jackson Laboratory with a ZP3-Cre mouse, resulting in a heterozygous, full-body knockout of *Bmal1*, as described and validated in Schoeller *et. al.* [16]. Generation of the homozygous *Bmal1* knockout mice, the *Bmal1*$^{-/-}$ mice, was achieved by crossing two heterozygous mice for the *Bmal1* knockout allele. All experimental procedures were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Obtaining metaphase II oocytes, 2-pronuclei embryos, 2-cell embryos, and 4-cell embryos

Female wildtype C57BL6/J mice between the ages of 3 weeks to 9 weeks were stimulated with intraperitoneal injection of 6 IU PMSG (ProSpec, Cat. No. hor-272-a), and then 46 hours later another intraperitoneal injection of 6 IU hCG (Sigma Aldrich, Cat. No. C1063-1VL). To generate embryos, female mice were mated overnight in a 1:1 breeding scheme with WT males. To harvest oocytes and embryos at specific developmental periods of time, mice were euthanized according to the schedule described below. All mice were euthanized with CO_2, followed by cervical dislocation prior to collection of oocytes or embryos.

Metaphase II oocytes were obtained by collecting them from the oviduct 18 hours after the hCG injection. Mice from which metaphase II oocytes were collected were not mated. For harvesting 2-pronuclei embryos and older, mice were immediately paired after hCG injection and left together overnight. 2pn embryos were obtained 18 hours after mating. 2-cell embryos were obtained 42 hours after mating. 4-cell embryos were obtained 54 hours after mating.

The harvesting of metaphase II oocytes and 2-pronuclei embryos commenced with the bursting of the oviduct's ampulla within a solution of 0.03% hyaluronidase in M2 media (Sigma

Aldrich, Cat. No. M7167), which was utilized to detach the cumulus cells. The harvesting of 2-cell and 4-cell embryos commenced with the cutting of the oviduct within only M2 media. All oocytes and embryos were washed three times in M2 media to rid them of cellular debris.

Immunofluorescence of Bmal1, Clock in pre-implantation embryos

First, the zona pellucida was removed in acidified Tyrode's solution, followed by three washes in PBS with 0.1% BSA. The oocytes and embryos were fixed with 4% PFA in PBS for 1 hour at room temperature, followed by three washes in PBS with 0.1% Tween (PBS-T).

Embryos were permeabilized by incubation in PBS-T for 30 minutes at room temperature, followed by a block with 4% normal goat serum in PBS-T for 30 minutes at room temperature. Then, embryos were incubated with the primary antibodies in 1% normal goat serum overnight at 4°C. α-BMAL1 (EMD Millipore, Cat. No. AB2204, lot no. 3722056) was used at a dilution of 1:2000, while α-CLOCK (Sigma, Cat. No. HPA001867, lot no. A00940) was used at a dilution of 1:100. A few embryos were excluded from incubation with primary antibodies, in order to control for non-specific binding of secondary fluorescent antibodies.

After overnight incubation, the embryos were washed three times with PBS-T. Then, the embryos were incubated with secondary antibodies for 1 hour at room temperature. Alexa Fluor 488 goat anti-guinea pig IgG (Invitrogen, Cat. No. A11073) was utilized to visualize α-BMAL1, while Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Cat. No. A11012) was used to visualize α-CLOCK. Both secondary antibodies were utilized at a dilution of 1:2000. The oocytes and embryos were washed three times with PBS-T again, and mounted on slides using a 70% dilution of Prolong Gold antifade medium with DAPI (Invitrogen, Cat. No. P36930). The fluorescent signal was detected using the 10x objective of the KEYENCE fluorescent microscope.

Harvesting and immunohistochemistry of mouse ovaries

Mice were euthanized with CO_2, followed by cervical dislocation. One ovary was dissected and fixed in a solution of 4% PFA, and then paraffin embedded with the assistance of

Reveal Biosciences, Inc. Serial sections of thickness 10 µm were collected from the ovary using a microtome.

After deparaffinization, antigen retrieval, and quenching of endogenous peroxidase, ovary sections were blocked in goat serum and incubated with the antibodies against BMAL1 and CLOCK as described above. The following day, the ovary sections were washed and incubated with two antibodies (Vector Laboratories): biotinylated goat anti-guinea pig IgG (Cat. No. BA-7000-1.5) and biotinylated goat anti-rabbit IgG (Cat. No. BA-1000-1.5). This was followed with treatment with avidin-biotin-horseradish peroxidase (Vector Laboratories, Cat #: PK-4000) for 1 hour. Finally, ovary sections were stained with DAB reagent (Vector Laboratories, Cat. No. SK-4105) for approximately 3-5 minutes to visualize BMAL1 and CLOCK. The ovary sections were washed in PBS-T, followed by 5 minutes in Milli-Q water to quench the staining reaction. Slides were coverslipped using VectaMount permanent mounting medium (Vector, Cat. No. H-5000).

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