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The Role of the Clock Gene Bmal1 in Female Fertility and Parturition

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The Role of the Clock Gene Bmal1 in Female Fertility and Parturition

By

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A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University
in partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Successful reproduction is essential for survival of species. For humans, reproductive problems can cause large emotional and medical burdens. Evidence in both humans and rodents indicates that circadian rhythmicity is important for supporting reproductive function. A molecular clock orchestrates circadian rhythmicity. Impairment in fertility and parturition are observed in female mice expressing a mutant form of Clock, suggesting critical roles for clock genes in reproduction. Since the clock gene Bmal1, but not Clock, is necessary for the generation of circadian rhythmicity, exploring the role of Bmal1 in reproduction may yield a better understanding of the importance of the molecular clock in this process. We characterized the reproductive phenotype of Bmal1−/− females. These females were infertile, and our data suggests that implantation failure due to impaired steroidogenesis is a major contributor to this infertility. Expression of Bmal1 and other clock genes has been demonstrated in such tissues important for the early stages of the reproductive process as the ovary, oviduct, GnRH neurons, and non-gravid uterus. However, the expression of clock genes in tissues involved in the later stages of
gestation has been largely unexplored. We determined that Bmal1 and other core clock genes are expressed in the gravid uterus, placenta, and fetal membranes of wild-type mice during the last third of gestation. Many of these genes were expressed in a rhythmic fashion throughout the circadian day suggesting the presence of operating peripheral molecular clocks in these tissues. The study of the role of Bmal1 in the late stages of the reproductive process is complicated by the infertility experienced by Bmal1−/− mice. To circumvent the problem of infertility and to examine the role of peripheral tissue Bmal1 expression in late gestation, we generated conditional Bmal1 knockout mice. These mice were used in concert with Telokin-Cre mice to disrupt Bmal1 specifically in the myometrium, the muscle portion of the uterus. Myometrial Bmal1 disruption was found to result in a disregulation of labor timing and altered expression of contractile-associated proteins. Results presented here demonstrate an importance of the core clock gene Bmal1 in both early and late stages of the reproductive process. These studies may lead to a better understanding of the roles of clock genes in supporting normal reproductive function in women.
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CHAPTER ONE

Introduction
Successful reproduction is essential for survival of species. For humans, reproductive problems can cause large emotional and medical burdens. Infertility affects 8% of females aged 19-26 years and 18% of females aged 35 to 39 years (1). In many cases, assisted reproductive technologies have given new choices to couples experiencing infertility. However, the success rates of such procedures are somewhat disappointing, with 44% of procedures resulting in a pregnancy and 36% resulting in a live birth (2). Furthermore, such procedures come at a great financial cost. In the United States, the cost per live birth using assisted reproductive technologies is estimated at $41,132 (3). Reproductive challenges are also often faced at the end of pregnancy in the form of preterm birth. In the United States, 12.7% of births are preterm (4). In recent decades, the rate of preterm birth has increased (5). Preterm birth is associated with both increased risk of neonatal mortality and chronic sequelae such as respiratory illness, cerebral palsy, and vision and hearing impairment (6), making it a major public health concern. Given the importance of the reproductive process and the problems associated with malfunctions therein, it is necessary to gain a better understanding of the molecular basis of how pregnancies are established, maintained, and resolved.

Several lines of evidence indicate an importance of circadian rhythmicity in reproduction. In women, disruption of circadian rhythmicity may lead to reproductive difficulties. Studies of female shiftworkers demonstrate that this population of women has an increased incidence of altered menstrual function (7), subfertility (8), low birth weight babies (9), and preterm labor (9). Furthermore, mammalian species exhibit a tendency to begin labor and deliver at a characteristic time of day. Epidemiological data from two separate studies indicate that humans tend to labor in the early morning hours.
Mice deliver in the dark phase of a light:dark cycle, while rats and hamsters deliver during the light phase. Little is known however about how rhythmicity may be affecting reproduction. The work embodied in this thesis seeks to elucidate the mechanistic basis for circadian effects on reproduction.

**The Molecular Clock**

Circadian rhythmicity is generated by the oscillating expression levels of clock genes (12) (Figure 1). Transcriptional feedback loops help drive these oscillations. The CLOCK:BMAL1 heterodimer induces transcription of genes including the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes by binding to E-box enhancers. PER and CRY complex and inhibit the ability of the CLOCK:BMAL1 heterodimer to bind E-boxes, thus shutting down their own transcription (12). Post-translational modification, notably phosphorylation, of clock proteins also plays a role in driving the molecular clock (12). The clock genes are expressed not only in the suprachiasmatic nucleus (SCN), which is regarded as the masterclock of the organism, but also in numerous other peripheral tissues that have been examined.

Rhythmic expression of core clock genes has been noted in tissues important for fertility including the ovary (13), oviduct (14), and uterus (15) of non-gravid female rodents. Rhythmic expression of clock genes in testis has been reported (16), but other studies indicate that this tissue is nonrhythmic (17, 18). One study examining clock gene expression in Leydig cells of the testis found rhythmic expression of *Bmal1* specifically in this cell type (19). Additionally, rhythmic clock gene expression has been reported for epididymis and prostate, while the seminal vesicle is reportedly nonrhythmic (20).

Little information is available regarding the existence of fetal peripheral clocks.
Previous studies on rhythmic clock gene expression in the fetus have focused on the fetal SCN. The mouse fetal SCN develops between days 12 and 15 of gestation (21), and exhibits circadian oscillation of Per1 transcript by day 17 of gestation (22). One study using in vivo bioluminescent imaging of pregnant Per1::luciferase rats indicated expression of Per1 in the fetus by day 10 of gestation and diurnal fluctuation in the expression of Per1 by day 12 (23). However, in this study it was not possible to determine the fetal tissues that contributed to the gene expression observed.

The Molecular Clock and Reproduction: Rodent Studies

SCN-lesioning studies in rats support an importance of circadian rhythmicity for normal reproductive phenotype. Lesions of the SCN result in a loss of rhythmicity of locomotor activity and drinking behavior (24). SCN-lesioned female rats manifest irregular estrus cycles (25), inhibition of the preovulatory surge in luteinizing hormone (LH) (26), and failed ovulation (27). In addition, lesioning of the SCN on day 19 of gestation in pregnant female rats has been shown to change the circadian gating of parturition. Rats normally deliver during a 36 hour time window with two peak frequencies of parturition on days 21 and 22 of gestation. These peaks occur 24 hours apart. However, lesioned rats show one peak in parturition frequency at a time in between those of the two normal peaks (28). These studies indicate the importance of the central molecular clock in supporting female fertility and dictating the normal timing of birth.

Characterization of genetically-altered mice with clock gene deficiencies have also indicated the importance of the molecular clock for normal female reproductive phenotype. Of these genetically-altered mice, reproductive phenotype has been most extensively described in the ClockΔ19/ClockΔ19 females. ClockΔ19/ClockΔ19 mice have a 51
amino acid deletion in the transcriptional activation domain of *Clock*. The mutant protein maintains its ability to heterodimerize with BMAL1, but is unable to activate transcription at E-box elements (29, 30). This mouse strain exhibits a prolonged circadian period (~27.3 hours) and a breakdown of rhythmicity in constant darkness (31). The *ClockΔ19/* 
*ClockΔ19* mice have irregular estrus (15, 32, 33), a lack of responsiveness to superovulation (34), and an absence of the preovulatory LH surge (33). Additionally, transient expression of the *ClockΔ19* protein in GT1-7 cells, a mouse hypothalamic cell line, causes a change in the pattern of gonadotropin releasing hormone (GnRH) secretion (32). Decreased fertility of the *ClockΔ19/* *ClockΔ19* mice has been reported by several groups (32, 34, 35) but refuted in one published report (15). Further aberrant phenotypes have been reported after the establishment of pregnancy in *ClockΔ19/* *ClockΔ19* mice. A high incidence of midgestational fetal resorption has been noted for pregnant *ClockΔ19/* *ClockΔ19* dams (33). In addition, increased rates of prolonged, but nonproductive parturition have been noted for laboring *ClockΔ19/* *ClockΔ19* females (15, 33). Differing parturition phenotypes between SCN lesioned and *ClockΔ19/* *ClockΔ19* rodents indicate the possibility for different roles of the molecular clock in the SCN versus peripheral tissues in supporting normal labor.

Reproductive abnormalities of *Bmal1* null female mice have been commented on, but have not been analyzed to the depth that they have in *ClockΔ19/* *ClockΔ19* females. *Bmal1* null mice have their basic helix-loop-helix (bHLH) domain disrupted with a neomycin cassette. The bHLH domain is important for heterodimerization of BMAL1 and CLOCK as well as binding of the heterodimer to DNA. The *Bmal1* null mouse exhibits a loss of circadian rhythmicity in constant darkness and an abrogation of
rhythmicity of fellow clock genes \emph{mPer1} and \emph{mPer2} (36). \emph{Bmal1} null females have been noted to have a delayed onset of puberty (37), but reports as to the regularity of their estrus cycle are conflicting (37, 38). \emph{Bmal1} null females are reportedly infertile (19, 38). However, embryos have been noted to be present in the reproductive tract of \emph{Bmal1} null females at day 3.5 of gestation that have been superovulated and mated (37).

The reproductive phenotype of the \emph{Bmal1} null male has been more thoroughly explored. \emph{Bmal1} null males are infertile (19). These males have low levels of serum testosterone and expression of genes involved in steroidogenesis is reduced in the testis (19). Of the steroidogenesis related genes found to be downregulated in the \emph{Bmal1} null testis, the greatest reduction is in \emph{steroidogenic acute regulatory protein (StAR)} (19). This enzyme regulates the rate-limiting step of steroidogenesis. Interestingly, \emph{StAR} expression is reduced in other steroidogenic tissues of \emph{Bmal1} null mice (19). In addition, \emph{BMAL1} was found to increase \emph{StAR} transcription in a Leydig cell line, indicating a direct effect of \emph{BMAL1} on \emph{StAR} expression (19).

\textbf{Roles of Peripheral Clocks}

Although the SCN largely orchestrates circadian rhythmicity and entrains the molecular clocks present in other tissues (12), the importance of the peripheral clocks is emerging. Some exogenous factors such as stress (39) and food availability (40) have been shown to alter rhythmicity in peripheral clocks independent of the SCN. Furthermore, peripheral clocks have been identified as having some roles independent of the central clock in the SCN. A study by McDearmon and colleagues demonstrated differential roles of the clock gene \emph{Bmal1} in the SCN and muscle (41). In addition to losing circadian rhythmicity in constant darkness, \emph{Bmal1} null mice have decreased activity levels and body weight (41).
When *Bmal1* expression is restored specifically in the SCN of *Bmal1* null mice, circadian rhythmicity in constant darkness is reinstated, but activity levels and body weight are still below normal (41). Conversely, when *Bmal1* expression is restored specifically in muscle of *Bmal1* null mice, normal activity levels and body weight are observed, but the animals are arrhythmic in constant darkness (41). The role of the molecular clock in the retina has also been explored (42). The retinas of *Bmal1* null mice have an abnormal electrical and transcriptional response to light. Using a conditional *Bmal1* null mouse, Storch and colleagues demonstrated that mice deficient for *Bmal1* in retina only had the same aberrant retinal response to light as conventional knockout animals (42). This indicates a discrete physiological role for the molecular clock in the retina.

**Mice As Model Organisms**

Because most human genes have a murine counterpart, much can be learned about human development and disease by studying the mouse. Gene-targeting by homologous recombination in embryonic stem cells has facilitated the deletion of specific genes of interest in mice. The conventional knockout mice produced using this technology have been invaluable in elucidating the function of many genes. However, in some cases an early developmental role or multiple adult roles of a gene make it difficult or impossible to study all functions of a gene of interest using the conventional knockout system. In such cases, it is often beneficial to inactivate a gene of interest in a tissue-specific manner. The bacteriophage P1-derived *Cre*-LoxP system has been used for this purpose. *Cre* recombinase induces site-specific genetic recombination at 34 base pair loxP sites. LoxP sites are introduced around areas of a gene necessary for functionality using homologous recombination into embryonic stem cells, resulting in a “floxed” allele. Mice
with a floxed allele are then bred to transgenic mice with Cre under the control of a tissue or cell-type specific promoter. In progeny conditional knockout mice, the gene of interest is inactivated only in the targeted area.

Mice have been used extensively to examine both early and late stages of reproduction. Convenient for reproductive studies, mice have a short duration of gestation. Although the mouse has many advantages as a research tool, its relevance for increasing the understanding of human parturition has been questioned due to known differences in the parturition cascades of mice and humans. In both species, high levels of progesterone are produced during pregnancy. Progesterone is necessary for implantation and for maintaining uterine quiescence during gestation. In the mouse, the corpora lutea are responsible for steroid hormone production throughout gestation, while in humans this responsibility shifts from the corpora lutea to the placenta as gestation progresses. At the onset of normal murine parturition, prostaglandins trigger luteolysis, the structural and functional degradation of the corpora lutea, and a decrease in circulating progesterone (43) (Fig. 2). Humans exhibit no such progesterone withdrawal at term (44). A functional progesterone withdrawal, mediated by a decrease in the progesterone receptor’s transcriptional activity, may be a part of normal human parturition (45). Other important components of the parturition cascade are known to be shared between mice and humans. For instance, in each species transcript for contractile associated proteins (CAPs), such as oxytocin receptor (Oxtr), prostaglandin F₂α receptor (FP), and connexin43 (Cx43), is upregulated in the uterus at term (46-49).

In the studies described herein, conventional and conditional Bmal1 knockout mice are used to assess the importance of clock genes in female reproductive function.
Animals deficient in \textit{Bmal1} were chosen for these studies because of the robust circadian phenotype of the \textit{Bmal1} conventional knockout mouse. The \textit{Bmal1} null mouse loses circadian rhythmicity in constant darkness \cite{50}, indicating that \textit{Bmal1} is necessary for circadian function. While the \textit{Clock}^{Δ19}/\textit{Clock}^{Δ19} mouse, whose reproductive phenotype has been extensively characterized, exhibits a prolonged circadian period (~27.3 hours) and a breakdown of rhythmicity in constant darkness \cite{31}, the \textit{Clock} conventional knockout mouse has a more subtle circadian phenotype. In the \textit{Clock} null mouse, exons 5 and 6 which contain the bHLH domain are deleted. In these mice, no CLOCK protein is detectable via an antibody to the C-terminus. This \textit{Clock} knockout mouse exhibits a slightly shorter circadian period than wild type controls but maintenance of circadian rhythmicity in constant darkness. This finding indicates that CLOCK is not necessary for circadian function \cite{51}. Therefore, the study of mice deficient in \textit{Bmal1} may yield a better understanding of the role of the molecular clock and circadian rhythmicity in reproduction than previous studies using animals with altered \textit{Clock}.

The studies depicted in this thesis use conventional \textit{Bmal1} null mice to examine the impact of this gene on the early stages of the reproductive process. Because \textit{Bmal1} null females are infertile, conditional \textit{Bmal1} knockout mice are used to examine the role of \textit{Bmal1} in the later stages of the reproductive process. Through the use of conditional knockout mice, we also aim to better understand the specific roles of peripheral tissue \textit{Bmal1} in the reproductive process.
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**Figure 1.** Molecular clock. CLOCK/BMAL1 drives transcription of the *Period (Per)* and *Cryptocrome (Cry)* genes by binding E-box enhancers. The PER/CRY complex blocks induction of transcription by CLOCK/BMAL1.
Figure 2. Parturition cascade in mice. Membrane phospholipids are converted to prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) by the sequential action of cytoplasmic phospholipase A$_2$ (cPLA$_2$), cyclooxygenase-1 (COX-1), and PGF synthase. Action of PGF$_{2\alpha}$ on PGF$_{2\alpha}$ receptors (FP) in the corpus luteum results in involution of this organ and a decrease in serum progesterone levels. Decreased progesterone levels induce expression of contractile associated proteins (CAPs) like *connexin43 (Cx43)*, *oxytocin receptor (Oxtr)*, and *FP* in the myometrium. Uterine contractility is increased and labor proceeds.
CHAPTER TWO

Impaired Steroidogenesis and Implantation Failure in Bmal1^- Mice

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Abstract

Evidence in humans and rodents suggests that normal circadian rhythmicity is important for supporting reproductive function. A molecular clock underlies circadian rhythmicity. Impaired fertility is observed in some genetically altered mice with deficiencies in genes of the molecular clock, suggesting a critical role for these genes in reproduction. Here, we systematically characterize the reproductive phenotype of females deficient in the clock gene \textit{Bmal1}. \textit{Bmal1}\textsuperscript{-/-} females are infertile. They exhibit progression through the estrus cycle, although these cycles are prolonged. Normal follicular development occurs in \textit{Bmal1}\textsuperscript{-/-} females, and healthy embryos of the expected developmental stage are found in the reproductive tract of \textit{Bmal1}\textsuperscript{+/-} females 3.5 days after mating to wild-type males. However, serum progesterone levels are significantly lower in \textit{Bmal1}\textsuperscript{-/-} versus \textit{Bmal1}\textsuperscript{+/-} females on day 3.5 of gestation. Low progesterone levels in \textit{Bmal1}\textsuperscript{-/-} females are accompanied by decreased expression of steroidogenic acute regulatory protein (StAR) in corpora lutea of \textit{Bmal1}\textsuperscript{-/-} versus \textit{Bmal1}\textsuperscript{+/-} females. While implantation of embryos is not observed in untreated or vehicle treated \textit{Bmal1}\textsuperscript{-/-} females, exogenous administration of progesterone to \textit{Bmal1}\textsuperscript{-/-} females is able to reinstitute implantation. This data suggests that implantation failure due to impaired steroidogenesis causes infertility of \textit{Bmal1}\textsuperscript{-/-} females.
Introduction

Infertility affects 8% of females aged 19-26 and 18% of females aged 35-39 (1). Given these statistics, a greater understanding of normal fertility and causes of infertility is warranted. Disruption of circadian rhythmicity may lead to reproductive difficulties in women. Altered menstrual function (2), subfertility (3), and increased incidence of miscarriage (4) have been reported in female shiftworkers. Circadian rhythmicity is driven by a group of core clock genes, including Clock, Bmal1, Per1/2, and Cry1/2 (5). These genes are expressed in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is considered the masterclock of the organism, as well as in many peripheral tissues. Expression of clock genes has been noted in the uterus (6), oviduct (7), ovary (8), and GnRH neurons (9), indicating a potential role for these genes in supporting normal reproduction.

Although it is difficult to determine whether the reproductive difficulties noted in female shiftworkers are related to stressful lifestyle or specifically a disruption of circadian rhythmicity, there is evidence from rodent studies to suggest that disruption of circadian rhythmicity can lead to aberrant reproductive phenotypes. In female rats, ablation of the SCN leads to irregular estrus cyclicity (10) and an inhibition of the LH surge (11) and ovulation (12). In addition, the phenotypes of genetically altered mice with clock gene deficiencies indicate a role for these rhythm generating genes in supporting normal female reproductive phenotype. The most thoroughly characterized clock gene deficient mouse is the Clock^{Δ19} mutant. Decreased fertility of Clock^{Δ19} mutant females is noted by several groups (9, 13, 14), but refuted in one known report (6). Clock^{Δ19} mutant females reportedly experience irregular estrus cycles (6, 9, 15), and no
coordinated LH surge on the day of proestrus (15). In addition, these females have low progesterone levels at midgestation and a high incidence of midgestational fetal resorptions and extended but non-productive labor (15).

The Clock$^{\Delta 19}$ mutants studied for reproductive phenotype have a deletion in Clock’s transcriptional activation domain which results in dominant-negative, antimorphic activity (16-18). These Clock$^{\Delta 19}$ mutants have a breakdown of wheel-running rhythmicity in constant darkness (19). However in light:dark cycles, Clock$^{\Delta 19}$ mutants are reported to exhibit nearly normal wheel-running, with running concentrated in the dark period (19, 20). In addition, Clock$^{\Delta 19}$ mutants selectively bred for a capacity to synthesize melatonin produce the hormone rhythmically in both light:dark and dark:dark cycles (20, 21). This suggests that central rhythmicity is maintained in these mutants and may facilitate their estrus cycle progression and ovulation.

The more recently generated Clock$^{-/-}$ mice maintain circadian rhythmicity in constant darkness (22). This indicates that despite the previously held view in the circadian field, Clock is not required for the generation of circadian rhythmicity (22). Bmal1$^{-/-}$ mice have a much more robust circadian phenotype than Clock$^{-/-}$ mice. In light:dark cycles, Bmal1$^{-/-}$ mice spend more time running in the light phase than do Bmal1$^{+/+}$ mice and are less likely to begin running within 0.5 hours of lights off than Bmal1$^{+/+}$ mice (23). Bmal1$^{-/-}$ mice also experience a complete loss of circadian rhythmicity in constant darkness (24). Therefore, Bmal1$^{-/-}$ mice may be valuable in examining roles for genes of the molecular clock in addition to generation of circadian rhythmicity. Through use of Bmal1$^{-/-}$ mice, effects of Bmal1 on processes seemingly unrelated to circadian rhythmicity such as hair growth (25) and ossification of ligaments
and tendons (23) have been demonstrated. Infertility of both male and female Bmal1−/− mice has been reported (26). Bmal1−/− males have been demonstrated to be deficient in the production of testosterone, suggesting a role for Bmal1 in normal steroidogenesis (26). It has been suggested that Bmal1−/− females have delayed puberty, irregular estrus cycles, and smaller ovaries and uteri, but are able to ovulate (27, 28). However, there has been no thorough examination of the reproductive phenotype of these females.

In the present report, we characterize reproductive function in female Bmal1−/− mice. We find these mice to be infertile, with normal ovulation, but a defect in steroidogenesis that results in implantation failure. Our data indicate that Bmal1−/− females are unable to generate the high levels of progesterone necessary for successfully maintaining gestation.
Materials and Methods

Animals

*Bmal1*−/− outbred mice were provided by C. Bradfield (University of Wisconsin) and maintained by heterozygote F1 x F1 crosses (24). Genotyping was performed by PCR as described (24). Wild-type and heterozygous littermates were used as controls unless otherwise noted. Continuous matings of *Bmal1*+/+ females and *Bmal1*+/− females were analyzed. Average litter size of mated *Bmal1*+/+ females was 7.33±0.33 pups while average litter size of mated *Bmal1*+/− females was 8.0±1.0 (P=0.4770; n=6, *Bmal1*+/+ litters; n=4, *Bmal1*+/− litters). Time between litters was 34.20±6.34 days for continuously mated *Bmal1*+/+ females and 39.25±4.64 days for continuously mated *Bmal1*+/− females (P=0.5607; n=8, *Bmal1*+/+ litters; n=7, *Bmal1*+/− litters). These interpregnancy intervals and litter sizes are similar to those reported by other investigators for control animals (29). Animals were maintained on a 12h:12h light:dark cycle. Females were checked for presence of a copulatory plug the morning after mating. Morning of the plug (10 am, ZT 4) was noted as day 0.5 of gestation. All animal experimentation described was conducted in accordance with accepted standards of humane animal care and was approved by the Washington University in St. Louis animal studies committee.

Estrus

Estrus cycle stage was determined in females aged 2 to 6 months by histological analysis of vaginal smears for 22 consecutive days. Vaginal smears were taken daily and cell morphology was analyzed to determine cycle stage (30). Cycle length was defined as time between onsets of estrus. Statistical analysis was by t-test for cycle length and two-way ANOVA for proportion of time in each estrus stage.
Histological Analysis

Ovaries were collected from virgin females 14 weeks of age. Implantation sites were collected from gravid d10.5 females (ZT 4). Tissues were fixed in 4% paraformaldehyde overnight, processed, and embedded in paraffin using standard protocols. 6-8 µm sections were cut. For ovaries, every 10th section was kept. For implantation sites, every 5th section was kept. Sections were stained with hematoxylin and eosin and examined with a light microscope.

Follicle Development

Ovarian follicle classification was based on the scheme of Pederson and Peters (31). As previously described, follicles were counted on five of the largest hematoxylin and eosin-stained sections from each ovary and standardized to total area of the section (32). Counts and area were determined using AxioVision software. Statistical analysis was by two-way ANOVA.

Oocyte and Embryo Recovery

Females were mated to wild-type males. At ZT 4 on d3.5 of gestation, females were sacrificed by cervical dislocation and oocytes and embryos were harvested (33). Briefly, the reproductive tract was dissected out and oocytes and embryos were flushed out of the fallopian tubes and uterus with PBS. Morphology of oocytes and embryos recovered was examined under a microscope. The examiner was blinded to genotype of the mouse. Statistical analysis was by t-test for number of oocytes and embryos recovered.

Hormone Measurements

Mice were anesthetized with 2.5% avertin and blood was collected by retro-orbital sinus sampling using heparinized capillary tubes. Serum was separated by centrifugation and
stored at -80°C until assayed. Estradiol and progesterone levels were assayed using solid-phase $^{125}$I-radioimmunoassays according to manufacturer’s protocol (Diagnostic Products Corp., Los Angeles, CA). Statistical analysis was by t-test for comparisons at a single timepoint and two-way ANOVA for comparisons at multiple timepoints.

**In Situ Hybridization**

*In situ* hybridization was performed as previously described (34). Briefly, ovaries were fixed in 4% paraformaldehyde overnight, followed by three days in 10% sucrose for cryopreservation. Ovaries were then embedded in OCT and 14 μm sections were cut on a cryostat. *Bmal1* and *StAR* riboprobes were generated from Gen Bank NM_007489 nucleotides 2324-2637 and Gen Bank NM_011485 nucleotides 541-703 respectively. Sense and antisense [$\alpha^-{33}$P]-labeled cRNA probes were generated from these templates using appropriate polymerases and hybridized to slides. After washing, slides were exposed to autoradiographic film and scanned at high resolution. Densitometric analysis was performed using National Institutes of Health Image software. Statistical analysis was by two-way ANOVA. To assess ovarian histology, hybridized slides were stained with hematoxylin and eosin and examined with a light microscope.

**Hormone Supplementation**

Females were mated to wild-type males. Starting on d3.5 of gestation, females were injected s.c. with 2 mg progesterone (Sigma) in sesame oil daily or 200 μL of sesame oil as a vehicle control at ZT 4 as described (35). Serum progesterone levels in hormone supplemented mice were confirmed to be at or above levels observed in wild-type gravid mice by radioimmunoassay (data not shown). At ZT 4 on d10.5, females were sacrificed by cervical dislocation and their uteri examined for the presence of implantation sites.
Diameter of whole implantation sites was measured under a light microscope. Frequency of implantation was analyzed by Marascuillo procedure. Diameter length and number of implantation sites were analyzed by one-way ANOVA.
Results

**Bmal1<sup>-/-</sup> Females Are Infertile With Prolonged Estrus Cycles**

To establish that *Bmal1<sup>-/-</sup>* females are infertile, we mated *Bmal1<sup>-/-</sup>* females to *Bmal1<sup>+/+</sup>* males. No litters ever resulted from these matings although plugs were detected a total of eleven times amongst five mated *Bmal1<sup>-/-</sup>* females. This is in agreement with previous reports indicating that *Bmal1<sup>-/-</sup>* females are infertile (26). To establish whether or not these females progress through the estrus cycle, stage of estrus of sexually mature females was tracked for 22 consecutive days by histological analysis of vaginal smears. 5 out of 5 analyzed *Bmal1<sup>+/+</sup>* mice and 5 out of 6 *Bmal1<sup>-/-</sup>* females were found to be cycling. Although the majority of *Bmal1<sup>-/-</sup>* females were found to be cycling, the length of the cycle was 49% longer in *Bmal1<sup>-/-</sup>* females compared to *Bmal1<sup>+/+</sup>* females (*P*<0.0001, n=5 for each genotype) (Fig. 1, A-C). However, there was no difference in the proportion of time spent in any estrus cycle stage between *Bmal1<sup>-/-</sup>* and *Bmal1<sup>+/+</sup>* females (Fig. 1D).

**Histological Analysis of Ovaries from 14-Week-Old Females**

Ovaries from 14-week-old females were examined to determine if normal follicular development occurs in the infertile *Bmal1<sup>-/-</sup>* females. In the ovaries of both *Bmal1<sup>+/+</sup>* and *Bmal1<sup>-/-</sup>* females, normal, healthy follicles in all stages of development and corpora lutea, were observed (Fig. 2A). There were no differences in the abundance of follicles in any stage of development in *Bmal1<sup>-/-</sup>* versus *Bmal1<sup>+/+</sup>* ovaries (n=4, *Bmal1<sup>+/+</sup>*; n=3, *Bmal1<sup>-/-</sup>*) (Fig. 2B).

**Normal Preimplantation Embryos Present in Bmal1<sup>-/-</sup> Females**

In order to determine whether *Bmal1<sup>-/-</sup>* females ovulate, and, if so, whether oocytes of *Bmal1<sup>-/-</sup>* females can be fertilized and undergo normal preimplantation development,
oocytes and embryos were flushed from the reproductive tract of Bmal1\(^{+/+}\) and Bmal1\(^{-/-}\) females successfully mated to Bmal1\(^{+/+}\) males at ZT 4 on d3.5 of gestation and examined. Oocytes or embryos were present in the reproductive tract of 12 of 15 Bmal1\(^{+/+}\) females and 11 of 15 Bmal1\(^{-/-}\) females analyzed. Slightly fewer oocytes or embryos were isolated from each of the Bmal1\(^{-/-}\) females than from Bmal1\(^{+/+}\) females examined, but this was not statistically significant (\(P=0.062; n=12, \text{Bmal1}^{+/+}; n=11, \text{Bmal1}^{-/-}\)) (Fig. 3A). In the case of both Bmal1\(^{-/-}\) and Bmal1\(^{+/+}\) females, the majority of embryos isolated were fertilized and healthy (Fig. 3B). Furthermore, in each group, the embryos isolated were predominantly in the morula or blastocyst stage as would be expected at this time of gestation (Fig. 3C).

**Decreased Serum Progesterone, but Not Estradiol in d3.5 Bmal1\(^{-/-}\) Females**

Appropriate regulation of progesterone and estradiol levels is important for the process of implantation on d3.5 of gestation in mice (36). Progesterone levels are increased at the time of implantation and remain elevated throughout gestation, while a spike in estrogen levels occurs on the day of implantation. To determine if the normal hormonal milieu is present in Bmal1\(^{-/-}\) females to support implantation, serum progesterone and estradiol levels were assayed at ZT 4 on d3.5 of gestation. Serum progesterone levels were approximately 6.5 fold higher in Bmal1\(^{+/+}\) females than Bmal1\(^{-/-}\) females at ZT 4 (\(P<0.0001; n=14, \text{Bmal1}^{+/+}; n=15, \text{Bmal1}^{-/-}\)) (Fig. 3D). Progesterone levels in Bmal1\(^{+/+}\) and Bmal1\(^{-/-}\) females were assessed over the circadian day to determine whether upregulation of progesterone is merely delayed in the Bmal1\(^{-/-}\) females. Serum was collected from animals sacrificed at 6 hour intervals beginning 6 hours prior to the d3.5 ZT 4 timepoint at ZT 22 on d3 of gestation. Progesterone levels were not affected by time
of day. Higher progesterone levels in $Bmal1^{+/\pm}$ females versus $Bmal1^{-/-}$ females persisted at all timepoints ($P<0.0001$; n=3-4 $Bmal1^{+/\pm}$ or $Bmal1^{-/-}$ females per timepoint) (Fig. 3E). There was no difference in serum estradiol levels of $Bmal1^{+/\pm}$ and $Bmal1^{-/-}$ females at ZT 4 on d3.5 of gestation (n=12, $Bmal1^{+/\pm}$; n=15, $Bmal1^{-/-}$) (Fig. 3F).

**StAR Expression is Decreased in d3.5 Corpora Lutea of Bmal1−/− Females**

In mice, the corpus luteum is responsible for production of steroid hormones throughout gestation. Because serum progesterone levels are significantly lower in d3.5 $Bmal1^{-/-}$ females compared to $Bmal1^{+/\pm}$ females, $Bmal1^{-/-}$ females were analyzed for potential luteal defects. $Bmal1$ mRNA expression was detected in the corpora lutea of ovaries from $Bmal1^{+/\pm}$ females at ZT 4 on d3.5 (Fig. 4, A&B). Because $Bmal1$ transcript expression does not change in rat corpora lutea over the circadian day (8), expression was not analyzed over the course of 24 hours. Expression of steroidogenic acute regulatory protein (StAR), the enzyme responsible for catalyzing the rate-limiting step of steroidogenesis, was assessed in d3.5 corpora lutea of $Bmal1^{-/-}$ and $Bmal1^{+/\pm}$ females. StAR mRNA was robustly expressed in the corpora lutea of $Bmal1^{+/\pm}$ females but nearly undetectable in the corpora lutea of $Bmal1^{-/-}$ females (Fig. 4, C-F). StAR expression was significantly higher in corpora lutea of $Bmal1^{+/\pm}$ versus $Bmal1^{-/-}$ females throughout d3.5 at all timepoints analyzed ($P<0.0001$, n=3-7 $Bmal1^{+/\pm}$ or $Bmal1^{-/-}$ females per timepoint) (Fig. 4G). There were no differences in StAR expression in $Bmal1^{+/\pm}$ or $Bmal1^{-/-}$ females with respect to time of day. $Bmal1$ and StAR sense *in situ* are shown in Supplementary Figure 1.

**Progesterone Administration Restores Implantation in Bmal1−/− Females**
In mice, d3.5 is the start of the period of uterine receptivity to implantation (36). At this time, serum progesterone levels were found to be significantly lower in Bmal1⁻/⁻ females compared to Bmal1⁺/⁻ females (Fig. 3D&E). Therefore, implantation was evaluated. At d10.5 of gestation, implantation sites were present in uteri of 75% (6/8) of untreated Bmal1⁺/⁻ females, 50% (6/12) of Bmal1⁺/⁻ females receiving daily injections of vehicle starting at d3.5, and 83% (5/6) of Bmal1⁺/⁻ females receiving daily injections of progesterone starting at d3.5 (Table 1). Implantation sites were never found in untreated Bmal1⁻/⁻ females (0/6) or vehicle treated Bmal1⁻/⁻ females (0/6) on d10.5 of gestation (P<0.05 compared to untreated Bmal1⁺/⁻ females). However, when Bmal1⁻/⁻ females received daily injections of progesterone starting at d3.5, 38% (5/13) displayed implantation sites at d10.5. Significantly fewer implantation sites were observed in individual gravid Bmal1⁻/⁻ progesterone treated females versus untreated Bmal1⁺/⁻ females (P<0.05, n=6 Bmal1⁺/⁻ females, n=5 Bmal1⁻/⁻ females). While implantation sites in progesterone treated Bmal1⁻/⁻ females had a smaller diameter than those in each Bmal1⁺/⁻ female group (P<0.001, n=3 implantation sites from each of 3-4 females per group) (Fig 5E), they were histologically normal (Fig. 5A-D).
Discussion

Although *Bmal1<sup>−/−</sup>* females have previously been noted to be infertile (26), this report marks the first systematic characterization of their reproductive phenotype. Because clock genes have a described role in regulating GnRH pulsatility (9), and circadian regulation of the LH surge has been demonstrated (37), impaired ovulation seemed a likely cause of this infertility. However, failed ovulation is not observed in these females. Intact ovulation is suggested by the presence of corpora lutea in virgin *Bmal1<sup>−/−</sup>* females (Fig. 2) and confirmed by the presence of oocytes and embryos in the reproductive tract of *Bmal1<sup>−/−</sup>* females at d3.5 of gestation (Fig. 3A). While ovulation occurs in the *Bmal1<sup>−/−</sup>* females, steroid hormone deficiency appears to hinder later stages of reproduction. Under normal conditions, progesterone levels are robustly increased at d3.5, the time of implantation, and remain high throughout gestation. Progesterone-responsive genes such as *Hoxa10* and *Hoxa11* (38) have roles in uterine receptivity, implantation, and decidualization (36). After implantation, high levels of progesterone are responsible for maintaining uterine quiescence. Progesterone deficiency is observed in *Bmal1<sup>−/−</sup>* dams at the time of implantation (Fig. 3D&E), and this deficiency persists at midgestation (data not shown). Regulation of estradiol levels is also important for coordinating implantation. However, there is no difference in estradiol levels in *Bmal1<sup>−/−</sup>* females compared to *Bmal1<sup>+/+</sup>* females (Fig. 3F). Similarly, a significant decrease in levels of progesterone but not estradiol has been reported for *StAR<sup>−/−</sup>* females (39). During gestation, estradiol is present at considerably lower levels than progesterone. Therefore, a defect in steroidogenesis may have a greater effect on progesterone levels than estradiol levels.
In mice, the corpora lutea are responsible for steroid hormone production throughout gestation. Interestingly, corpora lutea of pregnancy were observed significantly less frequently in d3.5 $Bmal1^{+/+}$ females (12/12) versus d3.5 $Bmal1^{-/-}$ females (6/16) ($P<0.0005$), indicating a luteinization defect in the $Bmal1^{-/-}$ females. Expression of $Bmal1$ mRNA in the corpora lutea of d3.5 $Bmal1^{+/+}$ females (Fig. 4, A&B) indicates a potential role for $Bmal1$ in this organ. On d3.5, the corpora lutea of $Bmal1^{-/-}$ females fail to express $StAR$, an enzyme required for steroidogenesis, to the levels observed in $Bmal1^{+/+}$ females (Fig. 4, C-G). A similar defect exists in the $Bmal1^{-/-}$ male. Alvarez et al. reported that $Bmal1^{-/-}$ males have decreased levels of testosterone and a corresponding decreased expression of $StAR$ mRNA in the testis (26). This group also demonstrated the ability of BMAL1 to increase $StAR$ expression in the MA-10 Leydig cell line (26). The CLOCK/BMAL1 heterodimer has also been shown to increase $StAR$ expression in cultured chicken granulosa cells (40). As $Bmal1$ mRNA is expressed in d3.5 corpora lutea of $Bmal1^{+/+}$ females, BMAL1 may be a direct regulator of $StAR$ expression in the corpora lutea of $Bmal1^{+/+}$ females. Interestingly, because levels of serum progesterone and $StAR$ expression do not change with respect to time of day, the observed steroidogenesis defect appears to result from a clock-independent role of $Bmal1$.

Implantation was never observed in either untreated or vehicle treated mated $Bmal1^{-/-}$ females. However, progesterone supplementation starting on d3.5 was able to reinstitute implantation in these females (Table 1). The observation of histologically normal implantation sites in these progesterone supplemented $Bmal1^{-/-}$ females (Fig. 5D)
indicates that insufficient progesterone is a major factor in the observed implantation defect.

In addition to indicating that impaired steroidogenesis accounts for implantation failure in Bmal1−/− mice, this study reveals the existence of multiple reproductive defects in Bmal1−/− females. Bmal1−/− females had a lower number of oocytes and embryos present in the reproductive tract at d3.5 (Fig. 3A) and a lower number of implantation sites present in the uterus at d10.5 after progesterone treatment (Table 1) than Bmal1+/− females, suggesting potential irregularity of the hormonal milieu necessary for precipitating ovulation. In addition, the smaller size of the implantation sites in progesterone supplemented Bmal1−/− females suggests that Bmal1−/− females may have uterine abnormalities preventing normal maintenance of gestation. Further exploration of the many roles of Bmal1 in female reproduction is warranted. The data presented here indicate an important role for Bmal1 in steroidogenesis in the gonad and suggest that Bmal1 may have a similar function in other heretofore unanalyzed steroidogenic tissues.
Acknowledgements

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Figure 1. Prolonged estrus cycles in Bmal1−/− females. Estrus cycle was assessed by histological analysis of vaginal smears. Progression through the stages of the estrus cycle in representative Bmal1+/+ (A) and Bmal1−/− (B) females. Mean cycle length (C) and proportion of time spent in each estrus stage (D). n=5 for each group. Values are means +/-SEM. *p<0.001.
Figure 2. Normal follicular development in Bmal1−/− females. Follicles were counted on sections of hematoxylin and eosin stained ovary. A, Normal preantral follicles (indicated by arrows), antral follicles (indicated by arrows), and corpora lutea in Bmal1+/+ and Bmal1−/− ovaries. Black bars=50μm. B, Abundance of follicles in different stages of development. Values are means +/- SEM. n=4, Bmal1+/+; n=3, Bmal1−/−. PF, primordial and primary follicles; PrF, preantral follicles; APrF, atretic preantral follicles; AnF, antral follicles; AAnF, atretic antral follicles; CL, corpora lutea.
Figure 3. Healthy preimplantation embryos, but low serum progesterone levels in Bmal1+/ females at d3.5 of gestation. Oocytes and embryos were flushed from the reproductive tract at d3.5 of gestation and analyzed under a light microscope. Serum progesterone and estradiol at d3.5 of gestation were assayed by solid-phase ¹²⁵I-RIA.

A, Average number of oocytes/embryos isolated from each gravid female. Values are means +/- SEM. Numbers in bars indicate the number of females with oocytes or embryos/the total number of females analyzed. B, Percentage of embryos isolated identified as healthy, unfertilized, and degenerating. C, Percentage of healthy embryos isolated in the blastocyst or morula stage. D, Serum progesterone at ZT 4 on d3.5. n=14, Bmal1+/-; n=15, Bmal1-. Values are means +/- SEM. *p<0.0001. E, Circadian serum progesterone throughout d3.5. n=3, Bmal1+/-; n=3-4, Bmal1- per timepoint. Values are means +/- SEM. p<0.0001 with respect to genotype. F, Serum estradiol. n=12, Bmal1+/-; n=15, Bmal1-. Values are means +/- SEM.
Figure 4. Bmal1 and StAR are expressed in corpora lutea of d3.5 Bmal1+/− females. Ovary sections were subjected to in situ hybridization with radiolabeled antisense Bmal1 or StAR probes and then hematoxylin and eosin stained for histological analysis. A, Autoradiograph from in situ hybridization for Bmal1 at ZT 4 d3.5. B, Hematoxylin and eosin staining of the same section. Arrows point to corpora lutea. Black bar=200μm. C and E, Representative autoradiographs from in situ hybridization for StAR at ZT 4 d3.5. D and F, Hematoxylin and eosin staining of the same sections. Arrows point to corpora lutea. Black bars=200μm. n=3, Bmal1+/−; n=3, Bmal1−/−. G, Circadian StAR expression throughout d3.5. Values are means +/- SEM. n=3, Bmal1+/−; n=3-7, Bmal1−/− per timepoint. + denotes no corpora lutea observed in any mice examined at this timepoint. p<0.0001 with respect to genotype.
Figure 5. Implantation sites found in progesterone supplemented Bmal1-/- females are small, but histologically normal. Paraffin sections of implantation sites were hematoxylin and eosin stained. Diameter of whole implantation sites was measured under a light microscope. Representative implantation sites from untreated Bmal1+/+ females (A), vehicle treated Bmal1+/+ females (B), progesterone treated Bmal1+/+ females (C), and progesterone treated Bmal1-/- females (D). Black bar=1 mm. Em, embryo; Pl, placenta; FM, fetal membranes; Ut, uterus. E, Implantation site diameter. n=3 implantation sites from each of 3-4 females per group. * denotes p<0.001 compared to each of the other groups.
Supplementary Figure 1. Bmal1 and StAR sense in situ. Sequential ovary sections were subjected to in situ hybridization with radiolabeled sense Bmal1 or StAR probes and then hematoxylin and eosin stained for histological analysis. Autoradiographs from in situ hybridization for Bmal1 (A) and StAR (B) at ZT 4 d3.5, and hematoxylin and eosin staining of an adjacent section (C).
Table 1. Implantations in d10.5 females

<table>
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<th>Maternal Genotype</th>
<th>% with d10.5 IS</th>
<th>Ave. No. of IS</th>
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<tr>
<td>Bmal1&lt;sup&gt;+/−&lt;/sup&gt; (untreated)</td>
<td>75% (6/8)</td>
<td>7.5</td>
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<tr>
<td>Bmal1&lt;sup&gt;+/−&lt;/sup&gt; + vehicle</td>
<td>50% (6/12)</td>
<td>8.5</td>
</tr>
<tr>
<td>Bmal1&lt;sup&gt;+/−&lt;/sup&gt; + P4</td>
<td>83% (5/6)</td>
<td>8</td>
</tr>
<tr>
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<td>0% * (0/6)</td>
<td>N/A</td>
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<tr>
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<td>0% * (0/6)</td>
<td>N/A</td>
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<tr>
<td>Bmal1&lt;sup&gt;−/−&lt;/sup&gt; + P4</td>
<td>38% (5/13)</td>
<td>5.6 *</td>
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</tbody>
</table>

Females were mated to wild-type males. Vehicle and P4 treated females were injected s.c. daily starting at d3.5 of gestation. * denotes P<0.05 compared to % with IS for Bmal1<sup>+/−</sup> (untreated). * denotes P<0.05 compared to ave. no. IS for Bmal1<sup>+/−</sup> (untreated). P4 dose was 2 mg per day. IS, implantation site; P4, progesterone.
CHAPTER THREE

Clock Gene Expression in Gravid Uterus and Extra-Embryonic Tissues During Late Gestation in the Mouse

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Abstract

Evidence in humans and rodents suggests the importance of circadian rhythmicity in
parturition. A molecular clock underlies the generation of circadian rhythmicity. While
this molecular clock has been identified in numerous tissues, the expression and regulation
of clock genes in tissues relevant to parturition is largely undefined. Here, we examine the
expression and regulation of the clock genes Bmal1, Clock, Cry(Cryptochrome)1/2, and
Per(Period)1/2 in the murine gravid uterus, placenta, and fetal membranes during late
gestation. All clock genes examined were expressed in the tissues of interest throughout
the last third of gestation. Upregulation of a subset of these clock genes was observed in
each of these tissues in the final two days of gestation. Oscillating expression of mRNA
for a subset of the examined clock genes was detected in the gravid uterus, placenta, and
fetal membranes. Furthermore, bioluminescence recording on explants from gravid
Per2::luciferase mice indicated rhythmic expression of PER2 protein in these tissues.
These data demonstrate expression and rhythmicity of clock genes in tissues relevant to
parturition indicating a potential contribution of peripheral molecular clocks to this
process.
Introduction

Successful parturition is necessary for survival of the species. Mammalian species exhibit a tendency to begin labor and deliver at a characteristic time of day. Moreover, the study of female shiftworkers indicates that disruption of circadian rhythmicity may lead to preterm labor and low birth weight (1). These observations suggest that parturition may be a circadian controlled process.

Circadian rhythmicity is generated by the oscillating expression levels of clock genes (2). Transcriptional feedback loops help drive these oscillations. The CLOCK:BMAL1 heterodimer drives transcription of a group of genes including the *Period (Per)* and *Cryptochrome (Cry)* genes by binding to E-box enhancers. PER and CRY complex and inhibit the ability of the CLOCK:BMAL1 heterodimer to act as an enhancer, thus shutting down their own transcription (2). Post-translational modification, notably phosphorylation, of clock proteins also plays a role in driving the molecular clock (2).

The clock genes are expressed not only in the suprachiasmatic nucleus (SCN), which is regarded as the masterclock of the organism, but also in numerous other peripheral tissues. Clock genes in the retina (3) and skeletal muscle (4) have been shown to have roles independent of clock genes expressed in the SCN. Rhythmic expression of clock genes has been noted in female reproductive tissues including the ovary (5), oviduct (6), and uterus (7) of non-gravid rodents (reviewed in (8)). Little information exists on the presence of the molecular clock in tissues involved in the resolution of pregnancy such as the gravid uterus and specific tissues of the conceptus.

Previous studies on rhythmic clock gene expression in the conceptus have focused on
the fetal SCN. The mouse fetal SCN develops between days 12 and 15 of gestation (9), and exhibits circadian oscillation of Per1 transcript by day 17 of gestation (10). One study using in vivo bioluminescent imaging of pregnant Per1::luciferase rats indicated expression of Per1 in the fetus by day 10 of gestation and diurnal fluctuation in the expression of Per1 by day 12 (11). However, in this study it was not possible to determine the fetal tissues that contributed to the gene expression observed.

Rodent studies indicate a role for clock genes in the resolution of pregnancy. An impact of maternal SCN-lesioning on the timing of labor in rats has been demonstrated. For normal rats, the onset of labor occurs with two peaks of frequency 24 hours apart on days 21 and 22 of gestation. SCN-lesioned rats, however, labor during this same time period with a single peak in frequency midway between the two normal peak times (12). This indicates a role in parturition initiation for the central molecular clock located in the SCN. ClockΔ19 mutant mice, which have a deletion in the transcriptional activation domain of Clock resulting in dominant-negative function in all tissues, display high incidences of midgestational fetal resorption and extended but nonproductive labor (13). These studies suggest a potential role for genes of the molecular clock in regulating the onset and progression of parturition.

The presence of peripheral molecular clocks in the gravid uterus and non-SCN tissues of the conceptus has not previously been described. In the present report, we examine the expression of clock genes in the gravid mouse uterus, placenta, and fetal membranes during late gestation. We find that the core clock genes are expressed throughout the final third of gestation in these tissues. In many cases, expression of clock genes is rhythmic and regulated with respect to progression through gestation. Therefore,
peripheral molecular clocks exist in tissues relevant to parturition.
Materials and Methods

Experimental Animals

Male and female C57BL/6/SvJ/129 mixed, C57BL/6, and knock-in mice (congenic on C57Bl/6J) expressing a mPeriod2::luciferase (Per2::Luc) fusion protein (14) were fed ad libitum and maintained on a 12 h light/dark cycle. Lights on was designated as ZT 0. Females in C57BL/6/SvJ/129 x C57BL/6/SvJ/129, C57BL/6 x C57BL/6, and Per2::Luc x Per2::Luc matings were checked for presence of a copulatory plug the morning after mating. Morning of the plug (1000 h, ZT 4) was noted as 0.5 days post-coitum (dpc) and the female was removed from the cage with the male. While for late gestational profiling and PER2 bioluminescence studies mating was continuous until plug detection, for circadian profiling females were mated to males for a limited time window of four hours nightly. This was done in order to facilitate detection of small fold change differences in transcript over the circadian day in tissues from these females. All animal experimentation described was conducted in accordance with accepted standards of humane animal care and was approved by the Washington University Animal Studies Committee.

Real-time qPCR

Uterus, placenta, and fetal membranes (amnion/chorion) were harvested from gravid C57BL/6/SvJ129 females for late gestational profiling and C57BL/6 females for circadian profiling, snap frozen in liquid nitrogen, and stored at -80°C until processing. For late gestational profiling, tissues were collected at ZT 4 (.5 dpc samples) or ZT 12 (.0 dpc samples) (n=3 animals per timepoint). For circadian day profiling, samples were collected at specified ZTs (n=3 animals per timepoint). Total RNA was extracted from
tissue samples using TriZol reagent (Invitrogen). RNA from individual samples was converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen), which includes a step for elimination of genomic DNA. Previously reported forward and reverse primers for Bmal1, Clock, Cry1, Cry2, Per2 (15), Per1 (16), Dbp (17), and Actg(Actin) (18) were used. The amplification efficiency of these primers was verified as described by the system manufacturer (Applied Biosystems, Foster City, CA). Forward and reverse Gapdh(glyceraldehyde 3-phosphate dehydrogenase) primers were designed using Primer Express software. These primers were validated for use with real-time qPCR by determining amplification efficiency and optimal primer conditions as described by the system manufacturer (Applied Biosystems, Foster City, CA). Gapdh was used for standardization in all experiments except for the late gestational profiling of clock gene expression in the fetal membranes because this gene was upregulated in this tissue on the final days of gestation. In these studies, Actg primers were used for standardization. All primer sequences are reported in Table 1. Reactions were completed on the Applied Biosystems 7500 Fast Real-Time PCR System. PCR conditions were as follows: one cycle at 50°C for 2 minutes, one cycle at 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for one minute. A subsequent step to generate a dissociation curve was used to verify that a single amplicon was generated. Reactions were prepared using Power SYBR Green PCR Master Mix (Applied Biosystems) and 500 nM each of forward and reverse Dbp primers, 900 nM each of forward and reverse Gapdh primers, or forward and reverse primers for other genes at concentrations previously described (15, 16, 18). Sequence Detection Software (v1.3.1) was used to determine the $C_t$ value for each reaction. The $\Delta\Delta C_t$ method was used for quantification (19). Statistical analysis was by one-way ANOVA, followed by Bonferroni’s post hoc test. Comparisons were considered statistically significant when $p$ values were <0.05.
**Tissue Explant Culture and Bioluminescence Recording**

Uterus, placenta, and fetal membranes (amnion/chorion) were harvested from each of three gravid *Per2::luc* females at ZT2-4 on the morning of 16.5 dpc (n=3 mice; 5-6 fetuses). Tissue explants were cultured in 1 ml of DMEM (Invitrogen) supplemented with 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.1 mM beetle luciferin (Promega, Madison, WI) according to published methods (20). Placenta explants were placed on Millicell membranes (Millipore, Bedford, MA). Each explant was sealed in a 35 mm Petri dish with a coverslip and vacuum grease and maintained at 36°C in darkness. Bioluminescence was continuously monitored with a photomultiplier tube (Hamamatsu, Shizouka, Japan) for 4-6 days. Data sets were detrended by subtracting the 24-h running average from the raw data. We then added the mean of the raw bioluminescence to each trace and plotted this as detrended bioluminescence.
Results

Expression and regulation of clock genes in the final third of gestation

To determine whether clock genes are expressed and regulated in parturition-relevant tissues as gestation progresses, expression of *Bmal1, Clock, Cry1/2, and Per1/2* was evaluated in uterus, placenta, and fetal membranes from females 14.5 to 19.0 dpc by real-time qPCR (n=3 mice per timepoint). Parturition was expected to occur at approximately 19.5 dpc. Tissues were harvested at ZT 4 (.5 dpc timepoints) or ZT12 (.0 dpc timepoints).

In the uterus, expression of all examined genes was noted by 14.5 dpc and persisted through 19.0 dpc (Figure 1A-F). In the uterus, *Cry1* was the only gene to undergo significant changes over the final third of gestation (*p*<0.05; one-way ANOVA), with an increase at 18.0 dpc (*p*<0.05 vs. 14.5, 15.5, 16.5, 17.5, 18.5, and 19.0 dpc) (Figure 1C).

Expression and significant changes in each of the genes examined was observed over the final third of gestation in the placenta (*p*<0.05; one-way ANOVA) (Figure 2A-F). An increase in *Bmal1* expression was observed at 18.5 dpc (*p*<0.05 vs. 14.5, 15.5, 16.0, 16.5, 17.0, 17.5, and 18.0 dpc) (Figure 2A). *Clock* expression was increased at 18.5 dpc (*p*<0.05 vs. 16.0) and 19.0 dpc (*p*<0.05 vs. 14.5, 15.5, 16.0, 16.5, and 18.0 dpc) (Figure 2B). *Cry1* expression was increased at 18.0 dpc (*p*<0.05 vs. 14.5 dpc) (Figure 2C). *Cry2* expression was increased at 19.0 dpc (*p*<0.05 vs. 14.5, 15.5, 16.0, and 16.5 dpc) (Figure 2D). *Per1* expression was increased at 18.5 dpc (*p*<0.05 vs. 14.5, 15.5, 16.0, 16.5, and 17.0 dpc) (Figure 2E). *Per2* expression was increased at 19.0 dpc (*p*<0.05 vs. 14.5, 15.5, 16.0, 16.5, and 17.0 dpc) (Figure 2F).

Likewise, expression of all examined clock genes was observed in the fetal membranes by 14.5 dpc and persisted through 19.0 dpc (Figure 3A-F). Significant changes in expression over the final third of gestation were noted only in *Bmal1* (*p*<0.05; one-way ANOVA). *Bmal1* expression was increased at 17.5 dpc (*p*<0.05 vs. 14.5, 16.0, and 16.5 dpc) and 19.0 dpc (*p*<0.05 vs. 14.5 dpc) (Figure 3A).

Circadian oscillation of clock gene transcripts during late gestation

To determine whether clock gene transcript expression is rhythmic in uterus, placenta, and
fetal membranes during late gestation, expression of Bmal1, Clock, Cry1/2, and Per1/2 was evaluated at 4-hour intervals during day 16 of gestation by real-time qPCR (n=3 mice per timepoint).

In the uterus, significant circadian changes were noted only in expression of Cry1 (p<0.05; one-way ANOVA), with changes in Bmal1 approaching significance (p=0.053; one-way ANOVA) (Figure 4A-C). In placenta, significant changes in Cry1 and Per1 were noted over the course of the day (p<0.05; one-way ANOVA), with changes in Cry2 approaching significance (p=0.053; one-way ANOVA) (Figure 4D-F). For Cry1, the observed maxima at ZT 16 is significantly different from the minima at ZT 0 and ZT 4 (p<0.05). Likewise, the Per1 maxima at ZT 12 is significantly different from the minima at ZT 4 (p<0.05). In the fetal membranes, changes in Bmal1, Cry2, and Per1 were observed with respect to time of day (p<0.05; one-way ANOVA) (Figure 4G-I).

The transcript expression of the clock-regulated gene Dbp was also examined in the uterus, placenta, and fetal membranes during day 16 of gestation. Dbp transcription is known to be controlled by binding of the CLOCK:BMAL1 heterodimer to E-box enhancers in the gene (21); therefore oscillations in Dbp support that the molecular clock is functioning. Significant differences (p<0.05; one-way ANOVA) in Dbp expression over the circadian day were observed in uterus and placenta (Figure 5A&B). In uterus, the maxima at ZT 12 is significantly different from the minima at ZT 0 (p<0.001). In placenta, the maxima at ZT 12 is significantly different from the minima at ZT 20 (p<0.05). Changes observed over the circadian day in the fetal membranes were not significant (Figure 5C).

**Circadian oscillation of PER2 protein during late gestation**

The circadian oscillations of clock gene mRNA detected in late gestation may or may not translate to oscillation in protein. To examine potential circadian oscillations in PER2, tissue explants from three homozygous Per2::luc dams at 16.5 dpc were subjected to bioluminescence recording. Uterus, placenta, and fetal membranes all showed damped circadian bioluminescence indicating rhythmic expression of PER2 protein (Figure 6).
Using the daily peak bioluminescence as a phase marker, we found the periods of uterine (21.4 +/- 5.2 h, n=4 explants), fetal membranes (21.2 +/- 2.3 h, n=5), and placental explants (19.5 +/- 1.8 h, n=4) did not differ (p>0.05, One-way ANOVA). These tissues reached their first peak of bioluminescence between 0.7 and 3.5 h after explantation, around the time of projected dusk.
Discussion

Given the known circadian gating to the timing of birth and disturbances to the progression of labor in ClockΔ19 females (13), we hypothesized that peripheral molecular clocks exist in tissues relevant to parturition. This report indicates that clock genes are expressed in the murine gravid uterus, placenta, and fetal membranes. In many cases clock gene expression is rhythmic and regulated with respect to progression through gestation.

Here we report, for the first time, evidence that the gravid uterus, placenta, and fetal membranes express genes of the molecular clock. Rhythmic expression of transcript for some, but not all, of the examined clock genes was observed in uterus, placenta, and fetal membranes over day 16 of gestation (Figure 4). While rhythmic expression of transcript was in many cases detected, it is possible that either our 4-hour intervals missed a peak in expression or our real-time qPCR conditions were not sensitive enough to detect lower amplitude rhythms of transcripts that appeared nonrhythmic. Alternatively, there may be a reliance on translational or post-translational control to generate rhythmicity of some of these genes in these tissues at this time. Rhythmic expression of PER2 in each of the examined tissues indicates that rhythmicity exists at the protein level (Figure 6). In uterus and placenta, rhythmic expression of transcript for Dbp, a clock-regulated gene, further indicates the presence of molecular clock function (Figure 5A&B).

In the uterus, circadian regulation of Cry1 transcript was observed (Figure 4B), with regulation of Bmal1 in antiphase to Cry1 approaching significance (Figure 4A). This pattern of expression is expected since in most tissues examined previously, Bmal1 cycles antiphase to negative regulators Cry and Per. Circadian expression of Bmal1, Cry1, and Per2 transcript has previously been examined in non-gravid mouse uterus (7). The Cry1 profile of gravid uterus reported here is similar in magnitude and time of peak and nadir to data previously reported for non-gravid uterus (7). However, some discrepancies exist between the previously reported non-gravid uterus data and the gravid uterus data presented here. The Bmal1 profile for gravid uterus has similar time of peak and nadir but smaller
magnitude of change compared to that reported for non-gravid uterus (7). While a circadian change in expression of Per2 transcript has been reported for non-gravid uterus (7), we do not observe such a change in gravid uterus here. Given the numerous physiological and gene expression changes occurring in the uterus during gestation, such differences in regulation of clock genes is not unexpected.

In the placenta, circadian oscillation of Cry1 and Per1 was observed, with regulation of Cry2 approaching significance (Figure 4E&F). The oscillation observed in each of these genes in the placenta is in phase with one another as expected. The changes in expression observed in the placenta are of a similar magnitude as those observed in the uterus.

In the fetal membranes, oscillation in expression of Bmal1, Cry2, and Per2 were observed with respect to time of day (Figure 4G-I). These changes are similar in magnitude to gene expression changes observed in uterus and placenta. The changes in Bmal1 are antiphase to those seen in Cry2 and Per2.

Bioluminescent imaging of tissue explants from gravid Per2::luc females indicates oscillating expression of PER2 protein in gravid uterus, placenta, and fetal membranes (Figure 6). Significant oscillations in Per2 transcript were detected in the placenta, but not in the gravid uterus or fetal membranes. The observed oscillations of PER2 in the uterus and fetal membranes may therefore be a result of regulation at the translational or post-translational level. Oscillating clock protein levels in the presence of nonrhythmic transcript expression has been demonstrated previously (22).

Regulation of clock genes as parturition approaches may support a role for these genes in the initiation of labor. Upregulation in transcript for some or all of the examined clock genes was observed over the final two days of gestation in the current study. In the uterus, Cry1 expression increases at day 18.0 (Figure 1). In the placenta, expression of each of the examined clock genes is observed between days 18.0 and 19.0 (Figure 2). In the fetal membranes, Bmal1 expression increases significantly at days 17.5 and 19.0 (Figure 3). The timing of these inductions is consistent with a possible role for these genes in
regulating the cascade of events controlling the initiation of parturition. These genes could be impacting parturition through either conventional clock effects or a previously undetermined nonconventional effect. Because clock gene conventional knockout and mutant mice are subfertile or infertile (23-27), conditional clock gene knockout mice will be important in ascertaining the role of clock genes in parturition.

Roles of molecular clock genes specific to peripheral tissues have been demonstrated (3, 4). We report here that molecular clocks exist in tissues important to parturition. Clock genes thus have the potential to impact parturition. Dissecting the roles of clock genes in peripheral tissues may lead to new insights into this critical but poorly understood process.
Funding

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Acknowledgements

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Figure 1. Clock gene expression in uterus during late gestation. Pregnant female mice were sacrificed at 9 timepoints during the final third of gestation starting with 14.5 dpc and ending on 19.0 dpc. Tissues were collected from three females per timepoint and analyzed for mRNA expression by real-time qPCR. Expression is normalized to Gapdh. Expression of A) Bmal1, B) Clock, C) Cry1 (p<0.05; one-way ANOVA), D) Cry2, E) Per1, and F) Per2 mRNA. Values are means +/- SEM. *denotes timepoints significantly different from others by Bonferroni's post hoc test, p<0.05.
Figure 2. Clock gene expression in placenta during late gestation. Pregnant female mice were sacrificed at 9 timepoints during the final third of gestation starting with 14.5 dpc and ending on 19.0 dpc. Tissues were collected from three females per timepoint and analyzed for mRNA expression by real-time qPCR. Expression is normalized to Gapdh. Expression of A) Bmal1, B) Clock, C) Cry1 (p<0.05; one-way ANOVA), D) Cry2, E) Per1, and F) Per2 mRNA. Values are means +/- SEM. *denotes timepoints significantly different from others by Bonferroni's post hoc test, p<0.05.
Figure 3. Clock gene expression in fetal membranes during late gestation. Pregnant female mice were sacrificed at 9 timepoints during the final third of gestation starting with 14.5 dpc and ending on 19.0 dpc. Tissues were collected from three females per timepoint and analyzed for mRNA expression by real-time qPCR. Expression is normalized to Actg. Expression of A) Bmal1, B) Clock, C) Cry1 (p<0.05; one-way ANOVA), D) Cry2, E) Per1, and F) Per2 mRNA. Values are means +/- SEM. *denotes timepoints significantly different from others by Bonferroni's post hoc test, p<0.05.
Figure 4. Clock gene expression in gravid uterus, placenta, and fetal membranes from 16.0-17.0 dpc. Pregnant female mice were sacrificed at 4-hour intervals during day 16 of gestation at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20. Tissues were collected from three females per timepoint and analyzed for mRNA expression by real-time qPCR. Expression is normalized to Gapdh. Expression of A) Bmal1 (p=0.053; one-way ANOVA) and Clock, B) Cry1 (p<0.05; one-way ANOVA) and Cry2, and C) Per1 and Per2 in uterus. Expression of D) Bmal1 and Clock, E) Cry1 (p<0.05; one-way ANOVA) and Cry2 (p=0.053; one-way ANOVA), and F) Per1 (p<0.05; one-way ANOVA) and Per2 in placenta. Expression of G) Bmal1 (p<0.05; one-way ANOVA) and Clock, H) Cry1 and Cry2 (p<0.05; one-way ANOVA), and I) Per1 (p<0.05; one-way ANOVA) and Per2 in fetal membranes. Values are means +/- SEM. *denotes peaks significantly different from nadirs by Bonferroni’s post hoc test, p<0.05.
**Figure 5.** *Dbp* expression in gravid uterus, placenta, and fetal membranes from 16.0-17.0 dpc. Pregnant female mice were sacrificed at 4-hour intervals during day 16 of gestation at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20. Tissues were collected from three females per timepoint and analyzed for mRNA expression by real-time qPCR. Expression is normalized to *Gapdh*. Expression of *Dbp* in A) uterus (*p*<0.05; one-way ANOVA), B) placenta (*p*<0.05; one-way ANOVA), and C) fetal membranes. Values are means +/- SEM. * denotes peaks significantly different from nadirs by Bonferroni's post hoc test, *p*<0.05.
Figure 6. PER2 rhythms in gravid uterus, placenta, and fetal membranes. Representative Per2::luc bioluminescence recordings from cultured A) uterus, B) placenta, and C) fetal membranes. Recordings were taken from tissues harvested from each of three pregnant Per2::luc females.
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CHAPTER FOUR

Disruption of Myometrial Bmal1 Alters Parturition Timing in Mice

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Abstract

Human and rodent studies indicate a role for circadian rhythmicity and associated clock gene expression in supporting normal parturition. The importance of clock gene expression in tissues outside the suprachiasmatic nucleus is emerging. Here, we use a Bmal1 conditional knockout mouse line with a novel Cre transgenic line to examine the role of myometrial Bmal1 in parturition. Females with disrupted myometrial Bmal1 are less likely to labor during the dark phase of normal light:dark cycles than their littermate controls. Furthermore, these females have altered expression levels of contractile-associated proteins in the uterus during late gestation. These data indicate a specific role for myometrial Bmal1 in supporting normal parturition.
**Introduction**

Although crucial for successful mammalian reproduction, the process of parturition is at best incompletely understood. Parturition appears to be a circadian-regulated process. Mammalian species exhibit a tendency to begin labor and deliver at a characteristic time of day. Epidemiological data from two separate studies indicate that humans tend to labor in the early morning hours (1, 2). Mice deliver in the dark phase of a light:dark cycle, while rats and hamsters deliver during the light phase. Furthermore, female shiftworkers have increased incidences of preterm labor and low birth weight babies (3), suggesting that disruption of circadian rhythmicity may result in parturition difficulties. Circadian rhythmicity is driven by a group of core clock genes, including *Clock, Bmal1, Cry1/2*, and *Per1/2* (4). These genes are expressed in the suprachiasmatic nucleus (SCN) of the hypothalamus, which serves as the master clock of the organism, as well as in many peripheral tissues. In mice, expression of clock genes has been shown in gravid uterus, fetal membranes, and placenta (5), suggesting a role for clock gene expression in these tissues in supporting normal parturition.

Rodent studies further support an importance of circadian rhythmicity and associated clock genes for normal parturition. Lesions of the SCN on day 19 of gestation in pregnant female rats has been shown to change the circadian gating of parturition. Rats normally deliver during a 36-hour time window with two peaks in frequency of parturition on days 21 and 22 of gestation. These peaks occur 24 hours apart. However, SCN-lesioned rats show one peak in parturition frequency at a time in between those of the two normal peaks (6). Abnormal phenotypes have also been noted for *ClockΔ19/ ClockΔ19* mice during the end stages of reproduction. These females have a high incidence
of midgestational fetal resorption (7) and increased rates of prolonged, but nonproductive parturition (7, 8).

Clock^{Δ19/}/ Clock^{Δ19} mice have a deletion in Clock’s transcriptional activation domain resulting in antimorphic activity of the protein (9-11). While these mice have a breakdown of rhythmicity in constant darkness (12), the more recently generated Clock^{−/−} mice maintain rhythmicity in constant darkness (13). Since Bmal1^{−/−} mice lose circadian rhythmicity in constant darkness (14), it seems that Bmal1 but not Clock is necessary for generation of circadian rhythmicity. Therefore, studying reproductive phenotype in Bmal1^{−/−} mice may yield a better understanding of the impact of circadian rhythmicity and the molecular clock on reproduction. While the early stages of the reproductive process have been studied in Bmal1^{−/−} females (15), examination of the late stages has not been possible due to infertility in these mice (15, 16).

The SCN orchestrates circadian rhythmicity and entrains the molecular clocks present in peripheral tissues (4). The importance of these peripheral clocks is emerging. Tissue-specific roles for expression of Bmal1 in muscle (17) and retina (18) have been identified. Deletion of Bmal1 in specific peripheral tissues can circumvent the problem of infertility in conventional Bmal1^{−/−} mice and facilitate the study of the role of Bmal1 and the importance of peripheral clocks in parturition.

In the present report, we explore the role of Bmal1 in the myometrium for supporting successful parturition using a conditional Bmal1 knockout mouse line and a new myometrium and bladder specific Cre transgenic. We find that Bmal1 expression in the myometrium is important for timing of parturition and normal expression of contractile-associated proteins at the end of gestation.
Materials and Methods

Animals

Animals were maintained on a 12-h light, 12-h dark cycle with food and water available ad libitum. All animals used for experiments were 2-4 months of age. Females were mated to wild-type males. Morning of the plug was noted as d0.5 of gestation and the female was removed from the cage containing the male. On the C57BL/6 background, normal parturition occurs at d19.5 in our colony. Mice are known to deliver in the dark phase of the light:dark cycle. Therefore, we began monitoring parturition by checking the female’s cage for pups before lights out on d19.0 of gestation (ZT 11). The female’s cage was again checked after lights on at d19.5 of gestation (ZT 3). If parturition was not complete at this time, the female was checked a minimum of three times daily. Parturitions resulting in litters of 5-10 pups were included in analysis by chi-square test. \( P<0.05 \) was considered statistically significant. All animal experimentation described was conducted in accordance with accepted standards of humane animal care and was approved by the Washington University Animal Studies Committee/Vanderbilt University Institutional Animal Care and Use Committee.

Generation of conditional Bmal1 mice

A bacterial artificial chromosome (BACPAC Resources, Children’s Hospital Oakland Research Institute) encompassing the Bmal1 gene was digested with EcoRV and BssHII and an 8.7 kb fragment containing the Bmal1 locus was subcloned into EcoRV/BssHII-digested pBluescriptII\(^+\). A loxP site was inserted upstream of exon 2 into a unique AvrII site. A new BstEII site was created during this insertion. A cassette containing neomycin acetyltransferase (neo) flanked by two FRT sites and an additional loxP site was inserted into a unique BamHI site downstream of exon 4 to create the final construct. The linearized construct was electroporated into B6/Blu-1 ES cells. DNA from 167 G418
resistant ES cell clones was analyzed by Southern blot. Briefly, the DNAs were digested with EagI and BstEII and analyzed with probes flanking the region within the targeting vector. Four clones positive for targeted homologous recombination were identified. Karyotype analysis revealed an absence of chromosomal abnormalities in two of these clones. These clones were injected into C57BL/6 blastocysts using standard methods. After germline transmission was verified by Southern blot on tail DNAs, the neo cassette was removed by mating to transgenic mice expressing Flp recombinase under control of the Actin promoter (The Jackson Laboratory). Genotyping for presence of the conditional Bmal1 allele was performed using PCR with primers F: 5’-GAACACAGAGGCTGGAGCTC-3’ and R: 5’-CTGTGATCCTCATACCCACG-3’.

Conditional Bmal1 animals were bred to Actin-Cre mice (The Jackson Laboratory) for validation of function of the allele. In order to assess the efficiency of Bmal1 deletion, uterine and brain genomic DNAs were subjected to Southern blot hybridization.

Generation of Telokin-Cre transgenic mice

The construct used to generate Telokin-Cre transgenic mice was made from a 150 kb bacterial artificial chromosome encompassing the Telokin gene. An internal ribosome entry site followed by an eGFPcre FRT-flanked kanamycin cassette was inserted into the 3’ UTR of the Telokin gene by homologous recombination. Positive kanamycin selection was used to identify incorporation of the Cre-containing cassette. The kanamycin cassette was then deleted by transformation of arabinose-inducible Flp-expressing EL250 bacteria (19). A final round of homologous recombination was used to delete a loxP site present in the sequence of the bacterial artificial chromosome. The DNA construct was then injected into C57BL/6 oocytes by standard methods. Two founders were obtained and
one was characterized extensively for expression of the transgene. *Telokin-Cre*+ mice were crossed with ROSA26 mice (The Jackson Laboratory) and *Cre* expression was evaluated by β-galactosidase staining on tissue sections following standard methods. Genotyping of *Telokin-Cre* mice was performed by PCR with primers 5’-ATAGCTGGCTGGTGAGAT-3’ and 5’-TGCTTATAAACACCCTGTACG-3’.

**Real-time qPCR**

For analysis of *Bmal1* deletion, uterus was harvested from non-gravid, estrus stage females at ZT 4 (n=6-7 animals per genotype), snap frozen in liquid nitrogen, and stored at -80°C until processing. Total RNA was extracted from tissue samples using TriZol reagent (Invitrogen). RNA from individual samples was converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen), which includes a step for elimination of genomic DNA. *Bmal1* and *Gapdh* primers and real-time qPCR methods used were previously described (5). *Gapdh* was used for standardization. The ΔΔCt method was used for quantification (20). Statistical analysis was by Student’s t-test. Comparisons were considered statistically significant when *P* values were <0.05.

**Histological analysis**

Uterus was collected from non-gravid virgin females determined to be in estrus at ZT 13. Implantation sites were collected from gravid d16.0 females at ZT 13. Tissues were fixed in 4% paraformaldehyde overnight, processed, and embedded in paraffin using standard protocols. Seven μm sections were cut. Sections were stained with hematoxylin and eosin or trichrome blue and examined with a light microscope.

**Progesterone measurements**

Non-gravid females determined to be in estrus and gravid females at d16.0 and d19.0 were euthanized with CO₂ at ZT 13 and blood was collected by cardiac puncture. Serum was separated by centrifugation in heparanized tubes and stored at −80°C. Progesterone levels
were then assayed using solid-phase $^{125}$I-RIA according to manufacturer’s protocol (Diagnostic Products Corp., Los Angeles, CA). Statistical analysis was by two-way ANOVA. $P<0.05$ was considered statistically significant.

**Northern Hybridization Analyses**

In order to validate of disruption of Bmal1 in Bmal1 flox homozygous mice with global Cre expression, animals were sacrificed at ZT 4 and heart and brain were collected. For parturition studies, non-gravid estrus stage, d16.0, and d19.0 females were sacrificed at ZT 13 and uterus was collected. Tissue was snap frozen in liquid N$_2$ and stored at -80°C until processing. RNA was prepared using Trizol reagent (Invitrogen). Ten µg of total RNA for each sample were subjected to electrophoresis through agarose-formaldehyde gels and transferred to nitrocellulose membranes. Two Bmal1 riboprobes, one targeting the region of the gene floxed in the conditional allele and one targeting the 3’ region of the gene, were generated from GenBank NM_007489 nucleotides 734-1093 and 2324-2637, respectively. The Oxytocin receptor (Oxtr) riboprobe was generated from GenBank D86599 nucleotides 432-1461. A plasmid containing a 1150 bp fragment of the Connexin43 (Cx43) coding region was a gift of Dr. Eric Beyer (University of Chicago) and was used to generate the Cx43 riboprobe. Antisense $[^{32}\text{P}]-$labeled cRNA probes were generated from these templates using appropriate polymerases and hybridized to membranes. After washing, membranes were exposed to autoradiographic film and scanned at high resolution. Densitometric analysis was performed using National Institutes of Health Image software (Bethesda, MD). Each mRNA hybridization signal was corrected for loading and recovery by normalization to Cyclophilin A mRNA hybridization on the same filter. Statistical analysis was by two-way ANOVA and Student’s t-test. $P<0.05$ was considered statistically significant.
Results

Myometrial-Specific Deletion of Bmal1

To determine the role of Bmal1 in the myometrium during parturition, we generated a conditional Bmal1 knockout mouse. Exons 2-4 of Bmal1 are flanked by loxP sites (“floxed”) in this animal (Figure 1A). Exons 3 and 4 of Bmal1 encode the basic helix-loop-helix (bHLH) domain which is necessary for heterodimerization of Bmal1 with Clock and DNA binding. In the presence of Cre recombinase this critical region is deleted in the conditional knockout animal. Targeted insertion of the floxed allele in the conditional Bmal1 knockout mice was confirmed by Southern blot (Figure 1B).

In order to verify that Bmal1 is disrupted in the presence of Cre in our floxed Bmal1 mice, we generated animals homozygous for the floxed allele and positive for ubiquitously acting Actin-Cre. In these animals, Cre recombinase should excise the floxed region of Bmal1 in every tissue and the phenotype should resemble that of the conventional Bmal1−/− mice previously reported (14). Northern blot analysis was performed on heart and brain RNA from Bmal1−/− animals generated with the ubiquitously acting Cre and wild-type littermate controls. Two separate riboprobes were used. One riboprobe targets Bmal1 in the region floxed in our conditional knockout allele (Figure 2A) and a second riboprobe targets the 3’ end of the Bmal1 transcript which is downstream of the floxed region in our allele (Figure 2B). Northern blot analysis using each of these Bmal1 riboprobes demonstrated efficient knockdown of Bmal1 transcript in Bmal1−/− animals generated using ubiquitously acting Cre. The efficacy of the conditional knockout allele was further validated by monitoring wheel-running activity in Bmal1 flox homozygous animals with or without Actin-Cre. In the absence of Actin-Cre, Bmal1 flox
homozygous animals were rhythmic in light:dark and dark:dark (Figure 2C). Mice homozygous for the floxed Bmal1 allele and positive for Actin-Cre were arrhythmic in dark:dark and displayed a lower level of activity overall (Figure 2D). This is similar to the wheel-running activity reported for previously generated Bmal1−/− mice (14).

Expression of Telokin, the carboxy terminus of the smooth muscle myosin light chain kinase, is restricted to myometrium and other visceral smooth muscle sites (21, 22). Homologous recombination was used to generate Telokin-Cre transgenic mice. β-galactosidase staining on sections from ROSA26 heterozygous; Telokin-Cre+ animals demonstrated robust expression of the transgene in myometrial layer of the uterus, but not in other tissues such as heart (Figure 3A). Expression was also noted in bladder, but absent in brain, kidney, liver, ovary, spleen, and skeletal muscle (data not shown).

Having confirmed that our conditional knockout allele can be used to effectively delete Bmal1 and that the Telokin-Cre mice express Cre specifically in the myometrium, we generated mice homozygous for the floxed Bmal1 allele and positive for the Telokin-Cre transgene. Southern blot analysis on uterus from these mice showed disruption of Bmal1 (Figure 3). The band representing the unrecombined allele which remains in uterus from mice of this genotype represents other cell types present in the uterus such as epithelium and stroma, where expression of the Telokin-Cre transgene is not expected. Analysis by real-time qPCR shows a 20% reduction in Bmal1 transcript in uterus of Bmal1 flox homo; Telokin-Cre+ mice versus Bmal1 flox homo; Telokin-Cre− littermates (P<0.05) (Figure 3), consistent with deletion of transcript in the myometrial portion of the uterus. The deletion of Bmal1 in the myometrium was not found to result in any gross anatomical changes in the uterus of either non-gravid estrus stage females (Figure 4A) or
pregnant females (Figure 4B). Differences in uterine thickness observed were within a normal range of variability. Furthermore, Bmal1 flox homo; Telokin-Cre+ mice were found to have normal fertility.

**Impaired parturition in females with disrupted myometrial Bmal1**

We monitored parturition in Bmal1 flox homo; Telokin-Cre+ females and littermate controls (Table 1). 93% of control females but only 64% of Bmal1 flox homo; Telokin-Cre+ females completed parturition during the dark phase between d19.0 and d19.5 of gestation (P<0.05). One Bmal1 flox homo; Telokin-Cre+ female did not begin parturition until d22 of gestation and was sacrificed due to distress.

**Normal progesterone decline but altered contractile-associated protein expression at late gestation in females with disrupted myometrial Bmal1**

High levels of serum progesterone throughout gestation and a precipitous decline before the onset of parturition are characteristic of murine pregnancy (23). Serum progesterone levels were assayed in non-gravid estrus phase, gravid d16.0, and gravid d19.0 Bmal1 flox heterozygous; Telokin-Cre+ and Bmal1 flox homo; Telokin-Cre- littermate controls. A normal increase in serum progesterone at d16.0 and withdrawal at d19.0 were observed in the Bmal1 flox heterozygous; Telokin-Cre+ females (Figure 5A). There were no significant differences in progesterone levels between Bmal1 flox heterozygous; Telokin-Cre+ and Bmal1 flox homo; Telokin-Cre- littermate controls. Prior to parturition, the expression level of a cadre of contractile-associated proteins increases in the mouse and human myometrium (23). Expression of contractile-associated proteins Oxtr and Cx43 transcript were evaluated in uterus of non-gravid estrus phase, gravid d16.0, and gravid d19.0 Bmal1 flox heterozygous; Telokin-Cre+ and Bmal1 flox homo Telokin-Cre- littermate controls. Expression of
Cx43 differed based on genotype ($P<0.01$, two-way ANOVA). Cx43 was significantly higher at d16.0 in Bmal1 flox homo; Telokin-Cre$^+$ versus control uterus ($P<0.05$; Student’s $t$-test). Increased Cx43 expression in d19.0 Bmal1 flox homo; Telokin-Cre$^+$ versus control uterus approached significance ($P=0.08$; Student’s $t$-test). No significant differences in uterine Oxtr expression between Bmal1 flox homo; Telokin-Cre$^+$ and controls were observed. However, differences in Oxtr expression at d19.0 approached significance ($P=0.14$; Student’s $t$-test).
Discussion

The importance of circadian rhythmicity and clock gene expression for normal resolution of pregnancy has been indicated by both human and rodent studies (1-3, 6, 7). Dissecting the roles of clock genes during late gestation is complicated by subfertility or infertility observed in genetically altered mice with clock gene deficiencies (15, 16, 24-26). Here, we use mice with a myometrium-specific disruption of Bmal1 to circumvent the problem of Bmal1-/- infertility and examine the role of Bmal1 in a tissue that plays a key role in parturition. We find that timing of labor and expression of contractile-associated proteins is altered in these females.

While other conditional Bmal1 lines have been previously reported (18), we describe here for the first time utilization of a novel Telokin-Cre transgenic line. We demonstrate expression of this transgene specifically in the myometrial layer of the uterus (Figure 3A). Expression of the Telokin-Cre transgene was noted in bladder, but deletion in this tissue is not likely to affect the reproductive system. Deletion of uterine Bmal1 in Bmal1 flox homo; Telokin-Cre+ was demonstrated at the DNA level by Southern blot (Figure 3B) and at the RNA level by real-time qPCR (Figure 3C). These methods indicated a reduction and not an elimination of uterine Bmal1. This is expected due to the many different cell types besides myometrium found in the uterus. We attempted to demonstrate myometrium-specific deletion of BMAL1 on uterine sections from Bmal1 flox homo; Telokin-Cre+ females with multiple commercially available BMAL1 antibodies, but the antibodies were not suitably specific for this purpose. However, since β-galactosidase staining on uterine sections from ROSA26 heterozygous; Telokin-Cre+
mice indicates that transgene expression is specific to the myometrial layer of the uterus (Figure 3A), deletion of Bmal1 should be restricted to the myometrium.

All mammalian species examined demonstrate a propensity for delivery at a specific time of day. Mice deliver during the dark phase of a normal light:dark cycle, shortly before lights on. Females with disruption of myometrial Bmal1 were more likely than their littermate controls to labor outside of this normal time window, during the light phase ($P<0.05$). Bmal1 is a transcription factor (4) and many changes in gene expression in the myometrium are observed as parturition approaches (23). Therefore, it seemed likely that deletion of Bmal1 in the myometrium would result in altered expression of genes involved in parturition. We examined the expression profiles of known contractile-associated proteins Cx43 and Oxtr in uterus of non-gravid, d16.0, and d19.0 Bmal1 flox homo; Telokin-Cre$^+$ and Bmal1 flox homo; Telokin-Cre$^-$ littermate controls (Figure 5B&C). Uterine Cx43 expression was found to differ by genotype ($P<0.01$; two-way ANOVA), with a significant increase in Cx43 at d16.0 ($P<0.05$; Student’s $t$-test) and a trend towards increased expression at d19.0 ($P=0.08$; Student’s $t$-test) in females with disruption of myometrial Bmal1. There was also a trend toward increased expression of Oxtr at d19.0 ($P=0.14$; Student’s $t$-test) in females with disruption of myometrial Bmal1. These increases in expression of Cx43 and Oxtr may contribute to the increased incidence of early start of parturition in myometrial Bmal1 disrupted females. The effect of myometrial Bmal1 disruption on expression of these two genes was examined because of the genes’ known importance to parturition and myometrium-localized expression. Disruption of myometrial Bmal1 likely also alters expression of additional genes that contribute to the high incidence of both early and late labor.
This report indicates an importance of myometrium-specific expression of *Bmal1* in the timing of parturition. While altered expression patterns of *Cx43* and *Oxtr* likely contribute to this phenotype, it is expected that changes in expression of additional genes downstream of *Bmal1* also plays a role. Conditional *Bmal1* mice can be used to explore possible roles of *Bmal1* expression in additional peripheral tissues such as placenta and fetal membranes (5) in supporting the normal resolution of pregnancy. Furthermore, in this report we introduce the *Telokin-Cre* line which expresses *Cre* in the myometrial layer of the uterus. We expect that this line will be valuable for increasing the understanding of uterine biology in future studies.
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Figure 1. Generation of a floxed Bmal1 allele. A) Targeting vector and knockout strategy. B) Southern blot of tail DNA from mice of indicated genotypes. +, wild type; flox, conditional allele.
Figure 2. Validation of floxed *Bmal1* allele. Animals were sacrificed at ZT 4 and heart and brain were harvested. Northern blot used two different riboprobes targeting *Bmal1* transcript. *Bmal1* expression was analyzed using riboprobes targeting A) the floxed region and B) the 3’ end of the *Bmal1* gene. *Cyclophilin A* expression on the same membranes is shown. +/-, wild-type allele; -/-, *Bmal1*/- from ubiquitously expressed Cre. Wheel-running activity was monitored in animals released into dark:dark after 19 days of monitoring in light:dark. Representative double-plotted actograms of *Bmal1* flox homozygous animals that are C) negative or D) positive for *Actin-Cre*. n=3 animals per genotype.
Figure 3. Specific disruption of Bmal1 using myometrium-specific Cre transgenic mouse. A) β-galactosidase staining of uterus and heart sections from ROSA26+/− mice that are negative (top) and positive (bottom) for Telokin-Cre. e, endometrium; m, myometrium. B) Southern blot on uterus and brain from mice of indicated genotypes. +, wild-type; −, disrupted allele. C) Bmal1 transcript expression in uterus by real-time qPCR. Expression is normalized to Gapdh. Values are means ±SEM. *P<0.05; Student’s t-test.
Figure 4. Normal uterine histology in females with myometrial Bmal1 disrupted. Paraffin sections of non-gravid uterus and whole implantation sites were stained with trichrome blue or hematoxylin and eosin and examined with a light microscope. A) Sections of non-gravid uterus from Bmal1 Flox Homo; Telokin-Cre<sup>−</sup> (top) and Bmal1 Flox Homo; Telokin-Cre<sup>+</sup> (bottom) females. Panels on left are at 63X. Boxed area is shown to the right at 144X. Arrows indicate myometrial layers. e, endometrium; m, myometrium. B) Sections of whole implantation site from Bmal1 Flox Homo; Telokin-Cre<sup>−</sup> (top) and Bmal1 Flox Homo; Telokin-Cre<sup>+</sup> (bottom) females. Implantation site panels on left are at 7X. Boxed area of uterus is shown on the right at 144X. Arrows indicate myometrial layers. p, placenta; b, fetal brain; l, fetal liver.
Figure 5. Normal progesterone levels, but altered contractile-associated protein expression in females with disrupted myometrial Bmal1. Estrus stage non-gravid females and females of the day of gestation specified were sacrificed at ZT 13. Progesterone was assayed by solid-phase 125I-RIA. Cx43 and Oxtr expression were evaluated by Northern blot and standardized to Cyclophilin A expression.

A) Serum progesterone levels (n=3, non-gravid and d19.0 for each genotype; n=7-8, d16.0 for each genotype). Values are means ±SEM. B) Cx43 expression (n=3-4 per genotype per timepoint). Values are means ±SEM. P<0.01 with respect to genotype; 2-way ANOVA. *P<0.05 vs. d16.0 Bmal1 Flox Homo, Telokin-Cre+; Student’s t-test. C) Oxtr expression (n=3-4 per genotype per timepoint). Values are means ±SEM.
Table 1. Parturition timing.

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<th>Early</th>
<th>Late</th>
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<td>Bmal1 Flox Homo; Tel-Cre*</td>
<td>27</td>
<td>25 (93%)</td>
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<td>1</td>
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<tr>
<td>Bmal1 Flox Homo; Tel-Cre*</td>
<td>22</td>
<td>14 * (64%)</td>
<td>4</td>
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*P<0.05 vs control normal labors by Chi-square test.
CHAPTER FIVE

Summary and Conclusions
The studies described in this thesis aimed to identify the role of the clock gene \textit{Bmal1} in the female reproductive system. The data demonstrate roles for \textit{Bmal1} in early and late stages of the reproductive process in females and suggest interesting areas for future research.

The work presented in Chapter Two explores the role of \textit{Bmal1} in female fertility. \textit{Bmal1} null mice are infertile. While follicle development, ovulation, fertilization, and preimplantation embryo development are observed in these females, implantation never occurs. This implantation defect results from an inability of the \textit{Bmal1} null females to produce the high levels of progesterone necessary at this stage of gestation. Low levels of the steroidogenesis enzyme \textit{StAR} in corpora lutea of \textit{Bmal1} null females accompany this progesterone deficiency.

In addition to demonstrating implantation failure caused by impaired steroidogenesis in \textit{Bmal1} null females, these studies suggest the possibility of other fertility defects in these animals. Fewer oocytes appear to be ovulated in \textit{Bmal1} null females versus controls and fewer implantation sites per female are observed in progesterone supplemented \textit{Bmal1} null females versus control females. These observations may indicate abnormalities in the hormonal milieu that precipitates ovulation. Furthermore, while progesterone supplementation reinstitutes implantation in some \textit{Bmal1} null females, the incidence of implantation in supplemented \textit{Bmal1} null females does not reach that in unsupplemented controls. This may reflect roles for uterine \textit{Bmal1} in inducing expression of implantation related genes. Lastly, the implantation sites observed in progesterone supplemented \textit{Bmal1} null females have a significantly smaller diameter than those found in control females. This may indicate that implantation is
delayed in the progesterone supplemented $Bmal1$ null females and thus that these embryos are at an earlier stage of development, or that $Bmal1$ expression in the uterus is necessary for creating a normal environment for embryo growth. Further examination of these observed abnormalities may reveal additional roles for $Bmal1$ in the early stages of the reproductive process.

In addition to identifying a role for $Bmal1$ in female fertility, the work presented in Chapter Two adds to the emerging body of data indicating $Bmal1$ as an important regulator of steroidogenesis. $Bmal1$ has previously been demonstrated to regulate steroid hormone production through modulation of $StAR$ expression in murine testis (1) and adrenal gland (2) as well as in preovulatory follicles of domestic poultry (3). Here, $Bmal1$ is shown to be an important regulator of steroidogenesis in murine corpora lutea.

The work presented in Chapter Three aims to characterize clock gene expression in peripheral tissues that are key players in the later stages of reproduction. Recent studies indicate that clock gene expression in peripheral tissues can have important functions independent of SCN clock gene expression (4, 5). The data presented indicates the presence of the core clock genes in murine uterus, placenta, and fetal membranes during the last third of gestation. Interestingly, the expression of a subset of these genes increases in the final two days of gestation in each of these tissues. The timing of this induction is consistent with a possible role for these genes in regulating gene expression changes known to occur as parturition approaches. A subset of the examined clock genes display rhythmic transcript expression throughout the circadian day and bioluminescence studies indicate rhythmicity of PER2 protein in these tissues during day 16 of gestation.
This data suggests that functional peripheral molecular clocks are present in these tissues during late gestation.

These gene expression studies identify three peripheral tissues where clock genes may exert an impact on late gestation. Bmal1 conditional knockout mice were generated to explore possible roles for Bmal1 in these tissues during the late stages of the reproductive process. In Chapter Four, the role of myometrial Bmal1 expression in parturition is examined using these mice. The myometrium was selected for exploration because BMAL1 functions as a transcription factor and transcript of contractile associated proteins such as Prostaglandin F$_{2\alpha}$Receptor, Oxtr, and Cx43 is upregulated in the myometrium as parturition approaches (6). Mice normally deliver during the dark phase of the light:dark cycle; however, myometrial Bmal1 deficient females are more likely to deliver outside of the dark phase of the light:dark cycle than control females. This randomization of birth timing is accompanied by a disregulation of contractile associated protein expression. Increased expression of Cx43 and Oxtr in uterus of myometrial Bmal1 deficient females versus controls is noted during late gestation.

Further investigation of the cause of randomization of birth timing in myometrial Bmal1 deficient females is likely to reveal other gene expression changes that contribute to the high incidence in both early and late labor observed. In addition, the Bmal1 conditional knockout mice generated for use in these studies will be useful for future examination of the roles of Bmal1 in placenta, fetal membranes, or other portions of the uterus during late gestation.

The identification of the importance of Bmal1 for mammalian reproduction suggests that mutations in this gene may contribute to reproductive difficulties in women.
It may prove informative to determine whether women experiencing reproductive difficulties have a high incidence of mutations in *Bmal1* or other clock genes. Furthermore, women affected by familial advanced sleep phase syndrome (FASPS) may be a relevant population for study. Men and women with FASPS experience early wake times and early sleep times (7). Mutations in *Per2* and *Casein kinase I δ*, which phosphorylates PER2, have been shown to underlie this syndrome (7). Since *Per2* is a regulator of *Bmal1*, it would be interesting to determine whether these females have a high incidence of reproductive difficulties.

A select group of regulatory mechanisms function at multiple points during the reproductive process. For example, cyclooxygenases and prostaglandins are critical for both implantation (8) and parturition (9). Furthermore, a luteotrophic role of oxytocin has been noted in mice along with its classically known role in parturition (10). Progesterone regulation functions in implantation (8) and is crucial for parturition in many organisms, possibly including humans (11). The work presented in this thesis adds *Bmal1* to this list of regulators functioning at multiple points in the reproductive process. The conservation of other such regulators amongst species suggests the importance of *Bmal1* and circadian rhythmicity to human reproduction.
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