Impaired Steroidogenesis and Implantation Failure in *Bmal1*$^{-/-}$ Mice

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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 *Bmal1*. *Bmal1*$^{-/-}$ females are infertile. They exhibit progression through the estrous cycle, although these cycles are prolonged. Normal follicular development occurs in *Bmal1*$^{-/-}$ females, and healthy embryos of the expected developmental stage are found in the reproductive tract of *Bmal1*$^{-/-}$ females 3.5 d after mating to wild-type males. However, serum progesterone levels are significantly lower in *Bmal1*$^{-/-}$ *vs.* *Bmal1*$^{+/\pm}$ females on d 3.5 of gestation. Low progesterone levels in *Bmal1*$^{-/-}$ females are accompanied by decreased expression of steroidogenic acute regulatory protein in corpora lutea of *Bmal1*$^{-/-}$ *vs.* *Bmal1*$^{+/\pm}$ females. Whereas implantation of embryos is not observed in untreated or vehicle-treated *Bmal1*$^{-/-}$ females, exogenous administration of progesterone to *Bmal1*$^{-/-}$ females is able to reinstitute implantation. These data suggest that implantation failure due to impaired steroidogenesis causes infertility of *Bmal1*$^{-/-}$ females. **(*Endocrinology* 150: 1879–1885, 2009)**

Infertility affects 8% of females aged 19–26 yr and 18% of females aged 35–39 yr (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 shift workers. 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 of the hypothalamus, which is considered the master clock 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 shift workers 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 suprachiasmatic nucleus leads to irregular estrous 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*$^{\Delta 19}$ mutant. Decreased fertility of *Clock*$^{\Delta 19}$ mutant females is noted by several groups (9, 13, 14) but refuted in one known report (6). *Clock*$^{\Delta 19}$ mutant females reportedly experience irregular estrous 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 nonproductive 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-

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Abbreviations: StAR, Steroidogenic acute regulatory protein; ZT, Zeitgeber time.

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 estrous 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*$^{+/\pm}$ mice and are less likely to begin running within 0.5 h of lights off than *Bmal1*$^{+/\pm}$ 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 estrous 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, Madison, WI) and maintained by heterozygote $F_1 \times F_1$ 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, whereas 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 d for continuously mated *Bmal1*$^{+/+}$ females and 39.25 ± 4.64 d 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 12-h light, 12-h dark cycle. Females were checked for presence of a copulatory plug the morning after mating. Morning of the plug [1000 h, Zeitgeber time (ZT) 4] was noted as d 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 Animal Studies Committee.

Estrus

Estrous cycle stage was determined in females aged 2–6 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 estrous stage.

Histological analysis

Ovaries were collected from virgin females 14 wk of age. Implantation sites were collected from gravid d 10.5 females (ZT 4). Tissues were fixed in 4% paraformaldehyde overnight, processed, and embedded in paraffin using standard protocols. Six- to 8-μm sections were cut. For ovaries, every 10th section was kept. For implantation sites, every fifth 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 Pedersen 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 (Carl Zeiss Micro Imaging, Inc. Thornwood, NY). Statistical analysis was by two-way ANOVA.

Oocyte and embryo recovery

Females were mated to wild-type males. At ZT 4 on d 3.5 of gestation, females were killed 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 retroorbital 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-RIAs according to manufacturer's protocol (Diagnostic Products Corp., Los Angeles, CA). Statistical analysis was by *t* test for comparisons at a single time point and two-way ANOVA for comparisons at multiple time points.

In situ hybridization

In situ hybridization was performed as previously described (34). Briefly, ovaries were fixed in 4% paraformaldehyde overnight, followed by 3 d in 10% sucrose for cryopreservation. Ovaries were then embedded in OCT (Sakuva Finetek USA, Torrance, CA), and 14-μm sections were cut on a cryostat. *Bmal1* and steroidogenic acute regulatory protein (*StAR*) riboprobes were generated from GenBank NM_007489 nucleotides 2324-2637 and GenBank NM_011485 nucleotides 541-703, respectively. Sense and antisense [α-^{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 (Bethesda, MD). 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.

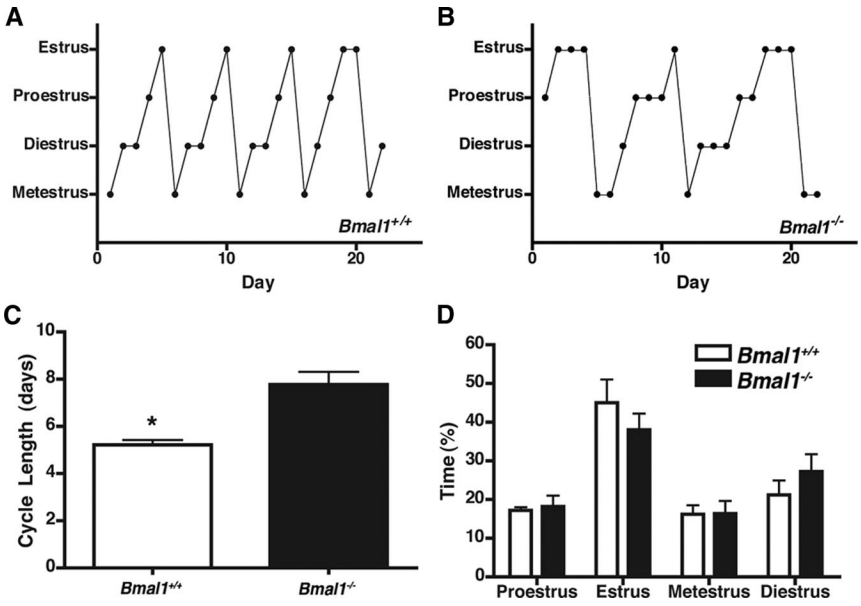

FIG. 1. Prolonged estrous cycles in *Bmal1*$^{-/-}$ females. Estrous cycle was assessed by histological analysis of vaginal smears. Progression through the stages of the estrous cycle in representative *Bmal1*$^{+/+}$ (A) and *Bmal1*$^{-/-}$ (B) females is shown. Mean cycle length (C) and proportion of time spent in each estrous stage (D) (n = 5 for each group) is also shown. Values are means ± SEM. *, $P < 0.001$.

Hormone supplementation

Females were mated to wild-type males. Starting on d 3.5 of gestation, females were injected sc with 2 mg progesterone (Sigma, St. Louis, MO) 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 RIA (data not shown). At ZT 4 on d 10.5, females were killed 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$^{-/-}$ females are infertile with prolonged estrous cycles

To establish that *Bmal1*$^{-/-}$ females are infertile, we mated *Bmal1*$^{-/-}$ females to *Bmal1*$^{+/+}$ males. No litters ever resulted from these matings, although plugs were detected a total of 11 times among five mated *Bmal1*$^{-/-}$ females. This is in agreement with previous reports indicating that *Bmal1*$^{-/-}$ females are infertile (26). To establish whether these females progress through the estrous cycle, stage of estrus of sexually mature females was tracked for 22 consecutive days by histological analysis of vaginal smears. Five of five analyzed *Bmal1*$^{+/+}$ mice and five of six *Bmal1*$^{-/-}$ females were found to be cycling. Although the majority of *Bmal1*$^{-/-}$ females were found to be cycling, the length of the cycle was 49% longer in *Bmal1*$^{-/-}$ females compared with *Bmal1*$^{+/+}$ 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 estrous cycle stage between *Bmal1*$^{-/-}$ and *Bmal1*$^{+/+}$ females (Fig. 1D).

Histological analysis of ovaries from 14-wk-old females

Ovaries from 14-wk-old females were examined to determine whether normal follicular development occurs in the infertile *Bmal1*$^{-/-}$ females. In the ovaries of both *Bmal1*$^{+/\pm}$ and *Bmal1*$^{-/-}$ 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*$^{-/-}$ *vs.* *Bmal1*$^{+/\pm}$ ovaries (n = 4, *Bmal1*$^{+/\pm}$; n = 3, *Bmal1*$^{-/-}$) (Fig. 2B).

Normal preimplantation embryos present in *Bmal1*$^{-/-}$ females

To determine whether *Bmal1*$^{-/-}$ females ovulate, and, if so, whether oocytes of *Bmal1*$^{-/-}$ females can be fertilized and undergo normal preimplantation development, oocytes and embryos were flushed from the reproductive tract of *Bmal1*$^{+/\pm}$ and *Bmal1*$^{-/-}$ females successfully mated to *Bmal1*$^{+/+}$ males at ZT 4 on d 3.5 of gestation and examined. Oocytes or embryos were present in the repro-

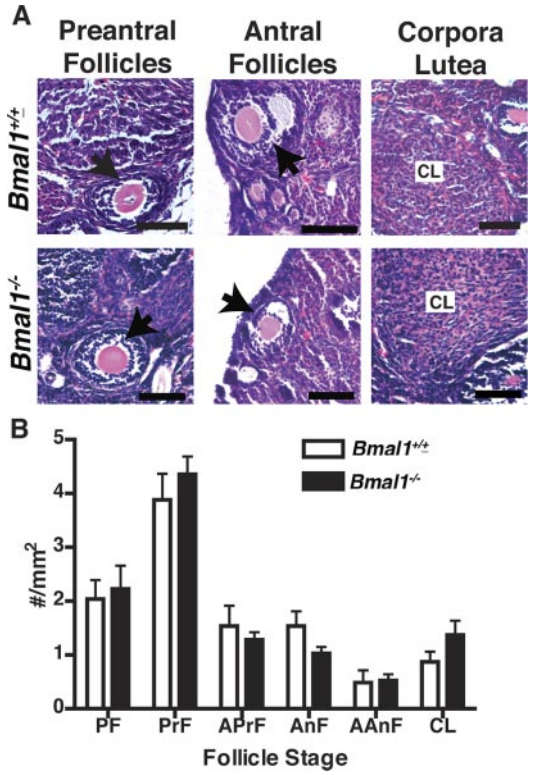

FIG. 2. Normal follicular development in *Bmal1*$^{-/-}$ females. Follicles were counted on sections of hematoxylin and eosin-stained ovary. A, Normal preantral follicles (indicated by *arrows*), antral follicles (indicated by *arrows*), and corpora lutea in *Bmal1*$^{+/\pm}$ and *Bmal1*$^{-/-}$ ovaries. *Black bars*, 50 μm. B, Abundance of follicles in different stages of development. Values are means ± SEM (n = 4, *Bmal1*$^{+/\pm}$; 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.

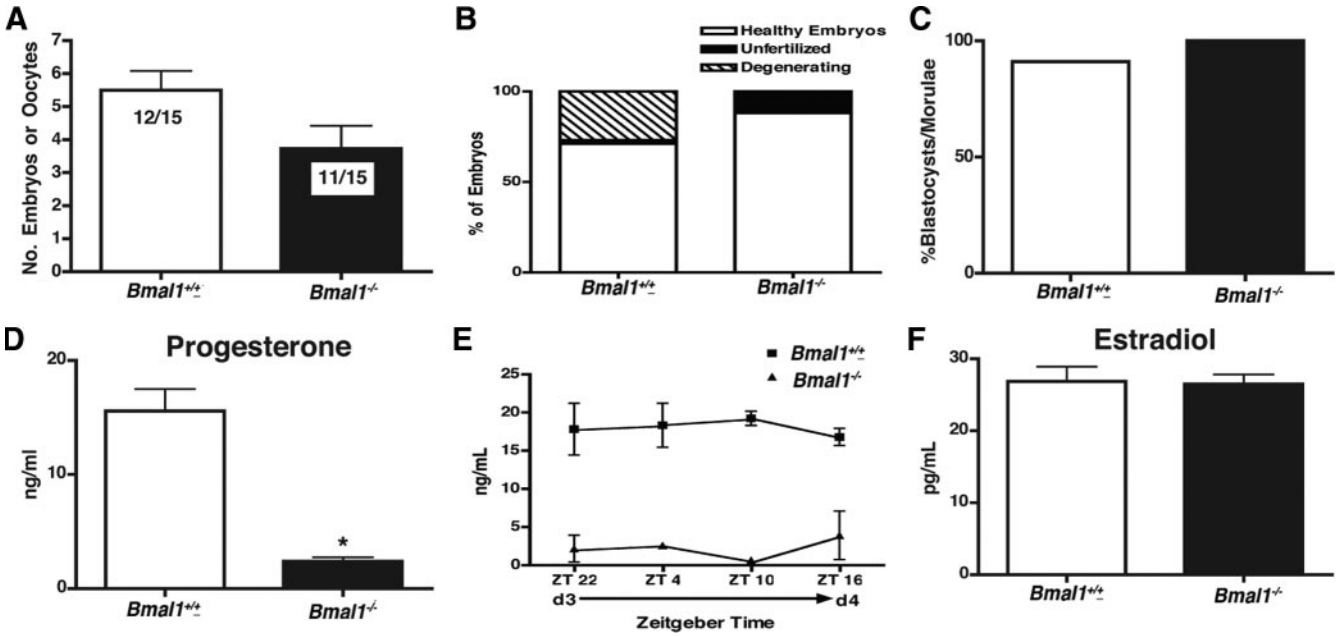

FIG. 3. Healthy preimplantation embryos but low serum progesterone levels in *Bmal1*$^{-/-}$ females at d 3.5 of gestation. Oocytes and embryos were flushed from the reproductive tract at d 3.5 of gestation and analyzed under a light microscope. Serum progesterone and estradiol at d 3.5 of gestation were assayed by solid-phase ^{125}I-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/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 d 3.5 (n = 14, *Bmal1*$^{+/\pm}$; n = 15, *Bmal1*$^{-/-}$). Values are means ± SEM. *, $P <$ 0.0001. E, Circadian serum progesterone throughout d 3.5 (n = 3, *Bmal1*$^{+/\pm}$; n = 3–4, *Bmal1*$^{-/-}$ per time point). Values are means ± SEM ($P < 0.0001$ with respect to genotype). F, Serum estradiol (n = 12, *Bmal1*$^{+/\pm}$; n = 15, *Bmal1*$^{-/-}$). Values are means ± SEM.

ductive tract of 12 of 15 *Bmal1*$^{+/\pm}$ females and 11 of 15 *Bmal1*$^{-/-}$ females analyzed. Slightly fewer oocytes or embryos were isolated from each of the *Bmal1*$^{-/-}$ females than from *Bmal1*$^{+/\pm}$ females examined, but this was not statistically significant ($P = 0.062$; n = 12, *Bmal1*$^{+/\pm}$; n = 11, *Bmal1*$^{-/-}$) (Fig. 3A). In the case of both *Bmal1*$^{-/-}$ and *Bmal1*$^{+/\pm}$ 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 d 3.5 *Bmal1*$^{-/-}$ females

Appropriate regulation of progesterone and estradiol levels is important for the process of implantation on d 3.5 of gestation in mice (36). Progesterone levels are increased at the time of implantation and remain elevated throughout gestation, whereas a spike in estrogen levels occurs on the day of implantation. To determine whether the normal hormonal milieu is present in *Bmal1*$^{-/-}$ females to support implantation, serum progesterone and estradiol levels were assayed at ZT 4 on d 3.5 of gestation. Serum progesterone levels were approximately 6.5-fold higher in *Bmal1*$^{+/\pm}$ females than *Bmal1*$^{-/-}$ females at ZT 4 ($P < 0.0001$; n = 14, *Bmal1*$^{+/\pm}$; n = 15, *Bmal1*$^{-/-}$) (Fig. 3D). Progesterone levels in *Bmal1*$^{+/\pm}$ and *Bmal1*$^{-/-}$ females were assessed over the circadian day to determine whether up-regulation of progesterone is merely delayed in the *Bmal1*$^{-/-}$ females. Serum was collected from animals killed at 6-h intervals beginning 6 h before the d 3.5 ZT 4 time point at ZT 22 on d 3 of gestation. Progesterone levels were not affected by time of day. Higher progesterone levels in *Bmal1*$^{+/\pm}$ females *vs.* *Bmal1*$^{-/-}$ females persisted at all time points ($P < 0.0001$; n = 3–4 *Bmal1*$^{+/\pm}$ or *Bmal1*$^{-/-}$ females per time point) (Fig. 3E). There was no difference in serum estradiol levels of *Bmal1*$^{+/\pm}$ and *Bmal1*$^{-/-}$ females at ZT 4 on d 3.5 of gestation (n = 12, *Bmal1*$^{+/\pm}$; n = 15, *Bmal1*$^{-/-}$) (Fig. 3F).

StAR expression is decreased in d 3.5 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 d 3.5 *Bmal1*$^{-/-}$ females compared with *Bmal1*$^{+/\pm}$ females, *Bmal1*$^{-/-}$ females were analyzed for potential luteal defects. *Bmal1* mRNA expression was detected in the corpora lutea of ovaries from *Bmal1*$^{+/+}$ females at ZT 4 on d 3.5 (Fig. 4, A and 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 h. Expression of *StAR*, the enzyme responsible for catalyzing the rate-limiting step of steroidogenesis, was assessed in d 3.5 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*$^{+/\pm}$ *vs.* *Bmal1*$^{-/-}$ females throughout d 3.5 at all time points analyzed ($P < 0.0001$, n = 3–7 *Bmal1*$^{+/\pm}$ or *Bmal1*$^{-/-}$ females per time point) (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 supplemental Fig. 1, published as supplemental data on

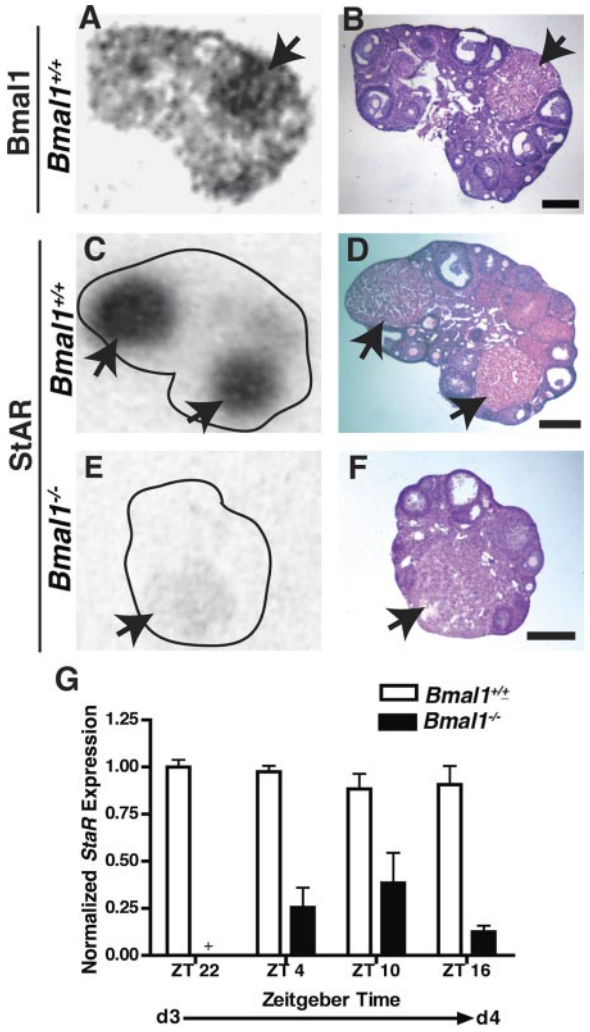

FIG. 4. *Bmal1* and *StAR* are expressed in corpora lutea of d 3.5 *Bmal1*$^{+/\pm}$ females. Ovary sections were subjected to *in situ* hybridization with radiolabeled antisense *Bmal1* or *StAR* probes and then hematoxylin and eosin stained for histological analysis. A, Autoradiograph from *in situ* hybridization for *Bmal1* at ZT 4 d 3.5. B, Hematoxylin and eosin staining of the same section. *Arrows* point to corpora lutea. *Black bar*, 200 μm. C and E, Representative autoradiographs from *in situ* hybridization for *StAR* at ZT 4 d 3.5. D and F, Hematoxylin and eosin staining of the same sections. *Arrows* point to corpora lutea. *Black bars*, 200 μm (n = 3, *Bmal1*$^{+/\pm}$; n = 3, *Bmal1*$^{-/-}$). G, Circadian *StAR* expression throughout d 3.5. Values are means ± SEM (n = 3, *Bmal1*$^{+/\pm}$; n = 3–7, *Bmal1*$^{-/-}$ per time point). +, No corpora lutea observed in any mice examined at this time point ($P < 0.0001$ with respect to genotype).

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Progesterone administration restores implantation in *Bmal1*$^{-/-}$ females

In mice, d 3.5 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 with *Bmal1*$^{+/\pm}$ females (Fig. 3, D and E). Therefore, implantation was evaluated. At d 10.5 of gestation, implantation sites were present in uteri of 75% (six of eight) of untreated *Bmal1*$^{+/\pm}$ females, 50% (six of 12) of *Bmal1*$^{+/\pm}$ females receiving daily injections of vehicle starting at d 3.5, and 83% (five of six) of *Bmal1*$^{+/\pm}$ females receiving daily injections of progesterone starting at d 3.5 (Table 1). Implantation sites were never found in untreated *Bmal1*$^{-/-}$ females (none of six) or vehicle-treated *Bmal1*$^{-/-}$ females (none of six) on d 10.5 of gestation ($P < 0.05$ compared with untreated *Bmal1*$^{+/\pm}$ females). However, when *Bmal1*$^{-/-}$ females received daily injections of progesterone starting at d 3.5, 38% (five of 13) displayed implantation sites at d 10.5. Significantly fewer implantation sites were observed in individual gravid *Bmal1*$^{-/-}$ progesterone-treated females *vs.* untreated *Bmal1*$^{+/\pm}$ females ($P < 0.05$, n = 6 *Bmal1*$^{+/\pm}$ females, n = 5 *Bmal1*$^{-/-}$ females). Whereas implantation sites in progesterone-treated *Bmal1*$^{-/-}$ females had a smaller diameter than those in each *Bmal1*$^{+/\pm}$ female group ($P < 0.001$, n = 3 implantation sites from each of three to four females per group) (Fig 5E), they were histologically normal (Fig. 5, A–D).

TABLE 1. Implantations in d 10.5 females

Maternal genotype	d 10.5 IS, %	Average no. of IS
Bmal1$^{+/\pm}$ (untreated)	75% (6/8)	7.5
Bmal1$^{+/\pm}$+vehicle	50% (6/12)	8.5
Bmal1$^{+/\pm}$+P4	83% (5/6)	8
Bmal1$^{-/-}$ (untreated)	0% (0/6)[a]	N/A
Bmal1$^{-/-}$+vehicle	0% (0/6)[a]	N/A
Bmal1$^{-/-}$+P4	38% (5/13)	5.6[b]

Females were mated to wild-type males. Vehicle and P4-treated females were injected sc daily starting at d 3.5 of gestation. IS, Implantation site; P4, progesterone.

[a] $P < 0.05$ compared with percent with IS for *Bmal*$^{+/\pm}$ (untreated).

[b] $P < 0.05$ compared with average number of IS for *Bmal1*$^{+/\pm}$ (untreated). P4 dose was 2 mg/d.

Discussion

Although *Bmal1*$^{-/-}$ 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*$^{-/-}$ females (Fig. 2) and confirmed by the presence of oocytes and embryos in the reproductive tract of *Bmal1*$^{-/-}$ females at d 3.5 of gestation (Fig. 3A). Whereas ovulation occurs in the *Bmal1*$^{-/-}$ females, steroid hormone deficiency appears to hinder later stages of reproduction. Under normal conditions, progesterone levels are robustly increased at d 3.5, 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*$^{-/-}$ dams at the time of implantation (Fig. 3, D and 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

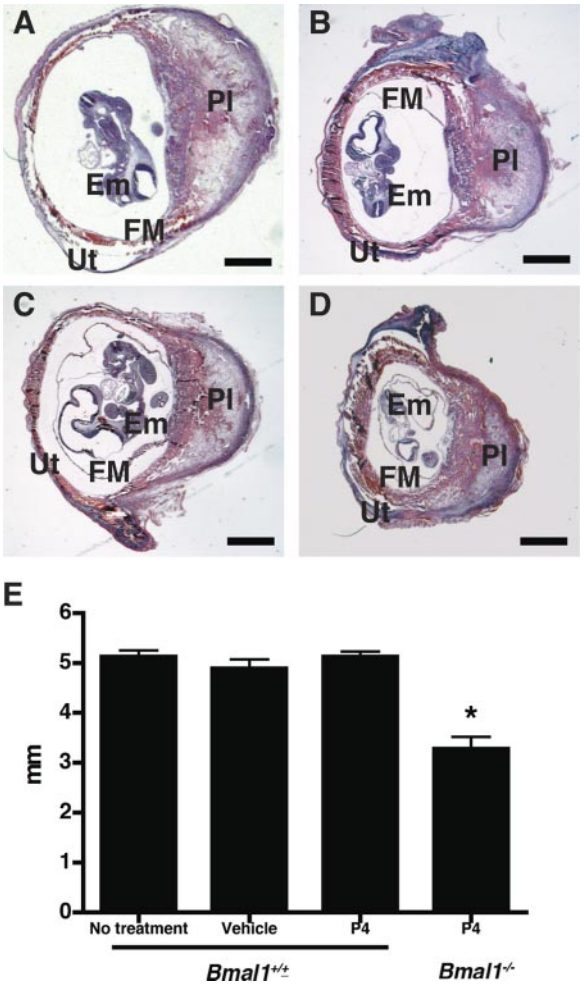

FIG. 5. Implantation sites found in progesterone-supplemented *Bmal1*$^{-/-}$ females are small but histologically normal. Paraffin sections of implantation sites were hematoxylin and eosin stained. Diameter of whole implantation sites was measured under a light microscope. Representative implantation sites from untreated *Bmal1*$^{+/\pm}$ females (A), vehicle-treated *Bmal1*$^{+/\pm}$ females (B), progesterone-treated *Bmal1*$^{+/\pm}$ females (C), and progesterone-treated *Bmal1*$^{-/-}$ females (D) are shown. *Black bar*, 1 mm. Em, Embryo; Pl, placenta; FM, fetal membranes; Ut, uterus. E, Implantation site diameter (n = 3 implantation sites from each of three to four females per group). *, $P < 0.001$ compared with each of the other groups. P4, Progesterone.

Bmal1$^{-/-}$ females compared with *Bmal1*$^{+/\pm}$ females (Fig. 3F). Similarly, a significant decrease in levels of progesterone but not estradiol has been reported for *StAR*$^{-/-}$ 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 d 3.5 *Bmal1*$^{+/\pm}$ females (12 of 12) *vs.* d 3.5 *Bmal1*$^{-/-}$ females (six of 16) ($P < 0.0005$), indicating a luteinization defect in the *Bmal1*$^{-/-}$ females. Expression of *Bmal1* mRNA in the corpora lutea of d 3.5 *Bmal1*$^{+/+}$ females (Fig. 4, A and B) indicates a potential role for *Bmal1* in this organ. On d 3.5, the corpora lutea of *Bmal1*$^{-/-}$ females fail to express *StAR*, an enzyme required for steroidogenesis to the levels observed in *Bmal1*$^{+/\pm}$ females (Fig. 4, C–G). A similar defect exists in the *Bmal1*$^{-/-}$ male. Alvarez *et al.* (26) reported that *Bmal1*$^{-/-}$ males have decreased levels of testosterone and a corresponding decreased expression of *StAR* mRNA in the testis. 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). Because *Bmal1* mRNA is expressed in d 3.5 corpora lutea of *Bmal1*$^{+/+}$ females, BMAL1 may be a direct regulator of *StAR* expression in the corpora lutea of *Bmal1*$^{+/\pm}$ 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 d 3.5 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 d 3.5 (Fig. 3A) and a lower number of implantation sites present in the uterus at d 10.5 after progesterone treatment (Table 1) than *Bmal1*$^{+/\pm}$ 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.

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