Loss of BMAL1 in ovarian steroidogenic cells results in implantation failure in female mice

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The circadian clock plays a significant role in many aspects of female reproductive biology, including estrous cycling, ovulation, embryonic implantation, onset of puberty, and parturition. In an effort to link cell-specific circadian clocks to their specific roles in female reproduction, we used the promoter that controls expression of Steroidogenic Factor-1 (SF1) to drive Cre-recombinase–mediated deletion of the brain muscle arnt-like 1 (Bmal1) gene, known to encode an essential component of the circadian clock (SF1\textsuperscript{−/−}Bmal1\textsuperscript{−/−}). The resultant SF1\textsuperscript{−/−}Bmal1−/− females display embryonic implantation failure, which is rescued by progesterone supplementation, or bilateral or unilateral transplantation of wild-type ovaries into SF1\textsuperscript{−/−}Bmal1\textsuperscript{−/−} dams. The observation that the central clock, and many other peripheral clocks, are fully functional in this model allows the assignment of the implantation phenotype to the clock in ovarian steroidogenic cells and distinguishes it from more general circadian related systemic pathology (e.g., early onset arthropathy, premature aging, ovulation, late onset of puberty, and abnormal estrous cycle). Our ovarian transcriptome analysis reveals that deletion of ovarian Bmal1 disrupts expression of transcripts associated with the circadian machinery and also genes critical for regulation of progesterone production, such as steroidogenic acute regulatory factor (Star). Overall, these data provide a powerful model to probe the interlocking and synergistic network of the circadian clock and reproductive systems.

ovary | circadian rhythm | fertility | steroidogenesis

\textbf{Significance}

This work demonstrates that specific peripheral clocks play unique and discrete roles in specific aspects of reproductive biology. Our use of a cell-specific conditional knockout model, in coordination with ovary transplant technology, permits examination of a peripheral clock without the impacts of off-target deletions that might indirectly impact reproductive function. In this case, we show that the molecular circadian clock, found in ovarian steroidogenic cells, is crucial for normal female reproduction, specifically embryonic implantation. The observation that implantation can be rescued by a single ovary with normal molecular clock machinery (i.e., brain muscle arnt-like 1 (Bmal1)) may provide direction for clinical intervention strategies when aberrant circadian oscillations are influencing fertility.


The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE48758).

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molecular clock in the steroidogenic cells of the ovary. To this end, we used the Cre transgene driven by the promoter of steroidogenic factor 1 (i.e., Cre^{sf1}) (20), which deletes Bmal1 in steroidogenic cells of the gonads and adrenal glands and gonadotrophic cells of the pituitary (SF1-Cre). To isolate the effects of our Cre^{sf1} deletion to the ovary, we used ovary transplantation.

**Results**

**Conditional Bmal1 Knockout Mice.** The Bmal1^{sf1} allele was generated by inserting lox-P sites to flank the basic helix–loop–helix encoding exon of murine Bmal1 (Fig. S14). (8, 9). To disrupt the circadian clock in SF1-Cre cells, mice harboring the Bmal1^{sf1} allele were bred to mice expressing a Cre transgene driven by the promoter of the Nr5a1 gene, which controls expression of the SF1 protein (this transgene is hereafter designated Cre^{sf1}) (20). In these studies, mice homozygous for the “floxed” allele (fx) and hemizygous for Cre were used as the experimental group (i.e., Bmal1^{sf1};Cre^{sf1}, designated as SF1-Bmal1^{sf1/fx}). Littermates that were negative for the Cre transgene (i.e., Bmal1^{sf1/fx}, or Bmal1^{+/+}) or littermates that were positive for Cre while harboring a wild-type allele (i.e., Bmal1^{sf1/+} Cre^{sf1/−}) were used as control groups.

To examine the specificity of excision mediated by Cre^{sf1}, we analyzed DNA from various tissues for the presence of the Bmal1^{sf1}-excised and Bmal1^{sf1}-unexcised alleles. As shown in Fig. S1B, the Cre^{sf1} transgene leads to excision of the Bmal1^{sf1} allele in brain, pituitary, adrenal gland, and ovaries, but not in liver, muscle, uterus, and ovoduct.

SF1-Bmal1^{sf1/fx} Females Display Behaviors Consistent with a Normally Functioning Central Circadian Clock. Wheel running analysis of SF1-Bmal1^{sf1/fx} females revealed normal circadian rhythms of locomotor activity, with no significant differences from controls for free running period, circadian rhythm amplitude, or activity levels (Fig. 1). Consistent with previous reports (6), global Bmal1^{−/−} females showed a significantly lower circadian rhythm amplitude and a significantly lower activity level in both light-dark situations and constant darkness (P < 0.05). Further evidence of normal circadian rhythms of behavior in SF1-Bmal1^{sf1/fx} females came from studies of feeding behavior, where control females and SF1-Bmal1^{−/−} females displayed normal circadian feeding rhythms, consuming more food in dark phase than in light phase (Fig. S2A). In contrast, global Bmal1^{−/−} females consumed similar amounts of food in light phase and dark phase (P > 0.05). Taken in sum, these analyses are consistent with the idea that SF1-Bmal1^{sf1/fx} females have normal circadian rhythms of behavior due to a normally functioning circadian clock within the SCN (21).

SF1-Bmal1^{sf1/fx} Females Lack Arthropathy and Early Aging Phenotypes. Global deletion of Bmal1 leads to an early onset arthropathy and premature aging phenotypes such as body weight reduction and organ shrinkage (22, 23). Unlike global Bmal1^{−/−} mice, the hind limbs of 6-mo-old SF1-Bmal1^{−/−} mice, stained with alizarin red (to detect calcification of ligaments and tendons; ref. 22), exhibited no inappropriate ossification (Fig. S2B). Additional evidence of normal aging includes the observation that, unlike Bmal1^{−/−} animals, SF1-Bmal1^{sf1/fx} mice appeared outwardly healthy and displayed normal body weights as late as 300 d of age (Fig. S2C). Furthermore, SF1-Bmal1^{sf1/fx} females also display normal weights of whole body, ovaries, uterus, spleen, heart, kidney, and liver at 100 and 150 d (Fig. S2 D–J).

SF1-Bmal1^{sf1/fx} Females Are Unable to Deliver Offspring Despite Normal Onset of Puberty and Normal Estrous Cycles. Consistent with a previous report (11), we observed that Bmal1^{−/−} females exhibit a 5-6 d delay in a classical marker of female puberty, vaginal opening, than do control mice (Fig. 2A). Importantly, this delay in vaginal opening is not observed in SF1-Bmal1^{sf1/fx} females, indicating normal onset of puberty in these mice. Unlike global Bmal1^{−/−} females (11), the SF1-Bmal1^{sf1/fx} females also exhibited normal estrous cycling that was indistinguishable from wild-type controls (Fig. 2B and C and Fig. S3A). No significant difference was found in estrous cycle length or proportion of each stage between control and SF1-Bmal1^{−/−} mice (P > 0.05, Wilcoxon rank sum test).

To further assess female reproductive potential, females were mated to proven wild-type fertile males. Females were scored for ability to deliver pups over a 3-mo period. As shown in Table S1, ~95% of control females gave birth to pups. In contrast, none of the Bmal1^{−/−} (0/13) or SF1-Bmal1^{−/−} (0/12) females delivered pups, although copulation plugs appeared regularly every 6–8 d in SF1-Bmal1^{−/−} females but not in global Bmal1^{−/−} females. In contrast to the control females, SF1-Bmal1^{−/−} displayed wide vaginal openings and cornified cells in vaginal smears at approximately 6.5 d postcoitum (dpc), characteristic of early pregnancy loss and reentry into estrus (Fig. S3B).

SF1-Bmal1^{−/−} Females Display Implantation Failure. To understand reproductive failure in the SF1-Bmal1^{−/−} females, we paired females with wild-type fertile males and examined them for evidence of mating, ovulation, and implantation. As shown in Table 1, SF1-Bmal1^{−/−} females exhibited rates of copulation and frequency of ovulation that were indistinguishable from controls. Normal ovulation was also demonstrated by histological analysis of the SF1-Bmal1^{−/−} ovaries at 3.5 dpc, showing that SF1-Bmal1^{−/−} females displayed similar numbers of corpora lutea (4.6 ± 0.5 per section, n = 13) compared with wild-type controls (5.0 ± 0.7 per section, n = 6) (Fig. 2D). In contrast, the global Bmal1^{−/−} displayed
observation that classical clock outputs such as Dbp, Bmal1, Nr1d2, Rora, and Per2 mRNAs displayed rhythmic expression in wild-type ovaries from ZT12, 2.5 dpc, to ZT12, 3.5 dpc [cosine wave-optimization algorithm (COSOPT), pMMIC-β<0.05] (24). When pathway analysis (25) was used to examine the pathways of differentially expressed genes between SF1-Bmal1<sup>−/−</sup> and Bmal1<sup>+/−</sup> based on the microarray data, the top 10 most significant processes included “lipid biosynthetic and metabolic processes,” “steroid metabolic process,” “cholesterol biosynthetic process,” and “circadian rhythm” (Table S2).

Additional support for disruption of the circadian clock and steroid biosynthesis in ovaries of SF1-Bmal1<sup>−/−</sup> mice came from two observations. First, microarray and qPCR revealed that three of the most sensitive outputs of the clock, Dhp, Nr1d2, and Rora, were significantly dysregulated in both Bmal1<sup>−/−</sup> or SF1-Bmal1<sup>−/−</sup> ovaries at ZT0 and ZT12 (Fig. 3 and Table S2). Although a transcript from the excised Bmal1 allele is generated, genomic analysis indicates no functional protein product is produced from the excised allele (6, 8, 9). Second, microarray demonstrated that the top three most down-regulated (based on fold change) transcripts in the ovaries of SF1-Bmal1<sup>−/−</sup> mice were the products of the Aldob, Star, and Ms4a10 loci (20-, 10-, and ninefold down-regulated, respectively). The Star gene product, steroidodogenic acute regulatory protein, is a rate limiting enzyme in steroidogenesis. The ~10-fold down-regulation of Star mRNA in SF1-Bmal1<sup>−/−</sup> ovaries recapitulates what has been reported for ovaries from mice with a global deletion of Bmal1 (10, 11), which we also reproduce here (Fig. 3). Further examination of the gene list also revealed a down-regulation of the Lhcgr gene product (Fig. 3; P < 0.05, two-way ANOVA). This locus encodes the receptor for luteinizing hormone and is thought to play a significant role in Star expression (26).

Transplantation of Wild-Type Ovaries into SF1-Bmal1<sup>−/−</sup> Females Rescues Implantation Failure. To isolate the effects of our SF1 deletion to the ovarian compartment, as opposed to the adrenal or pituitary, we performed ovary transplantation experiments (Table 2). First, we performed bilateral replacement of SF1-Bmal1<sup>−/−</sup> ovaries with Bmal1<sup>+/+</sup> ovaries. We observed this transplantation led to a 100% rescue (n = 12) of implantation and resulted in a normal number of live births. In the reverse transplantation, we replaced the ovaries of Bmal1<sup>+/+</sup> females with SF1-Bmal1<sup>−/−</sup> ovaries. Despite the regular appearance of copulation plugs in all 11 dams, only 4 of 11 recipients gave birth to pups. To evaluate the relative contributions of the ovary and hypothalamus-pituitary axis and test the hypothesis that a wild-type ovary

<table>
<thead>
<tr>
<th>Gene</th>
<th>SF1-Bmal1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Bmal1&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>SF1-control</th>
<th>% implantation (10.5 dpc) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% implantation (6.5 dpc) (n)</td>
<td>86 (28)</td>
<td>0 (16)*</td>
<td>83 (23)</td>
<td>6 (17)*</td>
</tr>
<tr>
<td>% implantation (10.5 dpc) (n)</td>
<td>—</td>
<td>—</td>
<td>0 (4)</td>
<td></td>
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</table>

The females were paired with proven males. The copulation rate was calculated as the number of females with vaginal plugs divided by the total number of female mice examined. The ovulation rate was calculated as the number of females with embryos at 3.5 dpc divided by the number of plugged females examined. The implantation rate was calculated as the number of females with detectable decidual swellings divided by the total number of 6.5 dpc or 10.5 dpc plugged females examined. Numbers in parentheses = number of mice tested. SF1-control includes Bmal1<sup>+/+</sup>Cre<sup>+/−</sup> and Bmal1<sup>+/+</sup> females. *Significantly different between mutant and controls (P < 0.001, χ² test).
Effects of Progesterone or Prolactin Supplementation on Implantation in SF1-Bmal1<sup>+/−</sup> Females. Given that progesterone secretion by the corpus luteum is essential for implantation and maintenance of pregnancy (27, 28), we examined the levels of this hormone in SF1-Bmal1<sup>+/−</sup> mice. We observed that both Bmal1<sup>−/−</sup> and SF1-Bmal1<sup>−/−</sup> females had significantly reduced serum progesterone levels compared with controls at both 3.5 dpc and 6.5 dpc (Fig. 4 A and B, respectively). Interestingly, at both times, progesterone was still detectable in SF1-Bmal1<sup>+/−</sup> mice, suggesting incomplete excision within the ovarian compartment of Bmal1 using SF1-Cre. We also tested progesterone levels in the bilaterally transplanted females on 3.5 dpc (Fig. S3D). Prolactone levels cosegregated with the genotype of the donor ovaries, with recipients receiving SF1-Bmal1<sup>+/−</sup> ovaries displaying lower progesterone level than those who received Bmal1<sup>−/−</sup> ovaries (P < 0.05).

To determine whether progesterone biosynthesis was related to implantation failure in SF1-Bmal1<sup>+/−</sup> females, we asked whether progesterone supplementation could rescue implantation. As shown in Table 3 and Fig. 5, on 6.5 dpc, five of eight SF1-Bmal1<sup>+/−</sup> females supplemented with progesterone exhibited successful implantation (Fig. 5C, 1 and 2) (P < 0.001 vs. SF1-Bmal1<sup>−/−</sup> females with vehicle control), with similar numbers of implantation sites compared with controls (P > 0.2 vs. wild-type females) (Table 3 and Fig. 5A, 1 and 2). None of nine vehicle-treated SF1-Bmal1<sup>+/−</sup> females (Fig. 5B, 1 and 2) showed evidence of implantation at 6.5 dpc. To test whether progesterone-rescued pregnancies could be sustained beyond 6.5 dpc, we injected five females with progesterone until 10.5 dpc. Of these five females, three displayed histologically normal embryos (Table S3 and Fig. 5G, 1 and 2), with numbers of implantation sites and overall embryo development similar to that of wild-type controls (Fig. 5E, 1 and 2).

Prolactin (PRL) is a pituitary hormone that also supports function of the corpus luteum during the perimplantation period (29). Because SF1-Bmal1<sup>+/−</sup> females showed lower PRL levels than wild-type controls on 3.5 dpc but not 6.5 dpc (Fig. 4 C and D), we evaluated the effects of supplemental PRL by using established protocols. PRL supplementation of SF1-Bmal1<sup>+/−</sup> females at 6.5 dpc resulted in small uterine swellings in four of nine females. Staining of these uteri revealed abnormal vasculature (Fig. 5D, 1), indicating decreased vascular permeability compared with wild-type or progesterone-rescued SF1-Bmal1<sup>+/−</sup> females (Fig. 5C, 1 and 2). Examination of histological sections confirmed that PRL supplementation increased uterine cell density, but decidual tissue (Fig. 5D, 2) was not as fully developed as wild-type or progesterone-rescued implantation sites (Fig. 5C, 2). None of the four SF1-Bmal1<sup>−/−</sup> females supplemented with PRL until 10.5 dpc displayed uterine swellings (Table S3 and Fig. 5H). In summary, unlike progesterone, supplemental PRL only partially rescued implantation in SF1-Bmal1<sup>+/−</sup> females.

**Table 2. Effect of ovary transplantation on fertility outcome**

<table>
<thead>
<tr>
<th>Donor ovary genotype</th>
<th>Recipient ovary genotype</th>
<th>Ovary transplant</th>
<th>% of recipients with successful parturition (n)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmal1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>SF1-Bmal1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Bilateral</td>
<td>100 (12)</td>
<td>9e−4&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF1-Bmal1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Bmal1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Bilateral</td>
<td>36 (11)</td>
<td></td>
</tr>
<tr>
<td>Bmal1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>SF1-Bmal1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Unilateral</td>
<td>100 (3)</td>
<td></td>
</tr>
</tbody>
</table>

Donor and recipient genotypes are indicated. Numbers in parentheses are number of mice transplanted.

*The ratio of successful parturition is significantly higher in recipients with wild type ovaries than SF1-Bmal1<sup>−/−</sup> ovaries (χ² test).
SF1-Bmal1<sup>−/−</sup> pituitaries (Fig. S4 and Table S4), suggesting normal functioning of the molecular clock in this tissue (30). No significant gene expression differences were detected between control and SF1-Bmal1<sup>−/−</sup> pituitaries.

**Discussion**

The importance of the central clock in reproductive biology is supported by the observation that deletion of the central clock, through SCN lesions or global deletion of Bmal1, corresponds to defects in estrous cycling and ovulation (11, 17, 18). The SCN is also known to regulate PRL release and, thus, supports luteal maintenance of pregnancy (31, 32). Infertility in Bmal1<sup>−/−</sup> females has been attributed to many factors, including gonadotropin hormone release, oocyte production, corpus luteum growth and development, impaired steroidogenesis at undefined sites, and defects in embryo development and implantation (11). The majority of these events require complex intraorgan communication among the nervous system, multiple components of the endocrine reproductive axis, and their targets. Understanding the interplay between the central clock and peripheral clocks and their involvement in reproductive outcome is made difficult by the complicated interlocking nature of the reproductive and circadian clock systems (33). Our results demonstrate that the molecular clock within steroidogenic compartment of the ovary plays a crucial role in one aspect of fertility, implantation.

An advantage of the SF1-Bmal1<sup>−/−</sup> mouse model used here is that through cell-specific Cre-excision, deletion of a “steroidogenic cell clock” can occur without disruption of the biology driven by the central clock or many other peripheral clocks (Figs. 1 and 3 and Fig. S1). As evidence, we show that SF1-Bmal1<sup>−/−</sup> mice, with the exception of reproductive failure, are phenotypically similar to wild-type mice and do not display many of the significant pathologies described for the global Bmal1<sup>−/−</sup> mice that could indirectly influence fertility including early onset arthropathy (22), abnormal estrous cycle, late onset of puberty as defined by vaginal opening (Fig. 2 and Fig. S2) (11), and premature early aging as defined by organ shrinkage (23). Using the SF1-Bmal1<sup>−/−</sup> model, we show that deletion of Bmal1 in SF1-Cre cells leads to implantation failure associated with low progesterone levels (Fig. S3, Fig. 4, and Table 1). Thus, our model isolates the Bmal1<sup>−/−</sup> implantation failure to the steroidogenic compartments of the pituitary (i.e., gonadotrophs), adrenal, or ovary. Importantly, the implantation failure observed in this model recapitulates the progesterone-dependent implantation failure that is observed in global Bmal1<sup>−/−</sup> females (10).

By incorporating ovarian transplantation into this model system, we are able to isolate the BMAL1-dependent implantation failure to the ovarian steroidogenic compartment (Table 2). Moreover, we were able to use transplantation to demonstrate that the presence of a single wild-type ovary is sufficient to completely rescue implantation failure in SF1-Bmal1<sup>−/−</sup> females. This observation

**Table 3. Effect of progesterone or PRL on implantation sites in SF1-Bmal1<sup>−/−</sup> females at 6.5 dpc**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>% with IM. (n)</th>
<th>I.S./mouse*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF1-control</td>
<td>Corn oil</td>
<td>91 (11)</td>
<td>9.4 ± 0.4</td>
</tr>
<tr>
<td>SF1-control</td>
<td>Progesterone</td>
<td>75 (12)</td>
<td>8.9 ± 0.4</td>
</tr>
<tr>
<td>SF1-Bmal1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Corn oil</td>
<td>0 (9)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>SF1-Bmal1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Progesterone</td>
<td>62.5 (8)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>7.2 ± 1.2</td>
</tr>
<tr>
<td>SF1-control</td>
<td>Saline</td>
<td>91 (11)</td>
<td>8.8 ± 0.33</td>
</tr>
<tr>
<td>SF1-Bmal1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Saline</td>
<td>0 (6)&lt;sup&gt;§&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>SF1-Bmal1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>PRL</td>
<td>44 (9)&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>8 ± 0.91</td>
</tr>
</tbody>
</table>

Numbers in parentheses are number of mice tested. IM., implantation; I.S., implantation sites.

*The number of implantation sites in mice with successful implantation.

Values are mean ± SEM.

<sup>†</sup>P < 0.005 vs. SF1-control (Bmal1<sup>fx/fx</sup>Cre<sup>ff</sup> and Bmal1<sup>ff/ff</sup>) mice treated with corn oil (χ<sup>2</sup> test).

<sup>‡</sup>P < 0.005 vs. SF1-Bmal1<sup>−/−</sup> mice treated with corn oil (χ<sup>2</sup> test).

<sup>‡‡</sup>P < 0.001 vs. SF1-control mice treated with corn oil (χ<sup>2</sup> test).

<sup>§</sup>P < 0.01 vs. SF1-control mice treated with saline (χ<sup>2</sup> test).

<sup>‡‡</sup>P = 0.06 vs. SF1-Bmal1<sup>−/−</sup> mice treated with saline (χ<sup>2</sup> test).

Chicago sky blue staining did not permeate the implantation sites.

Fig. 4. Effects of Bmal1 deletion on serum progesterone and PRL levels. Each point represents a single animal. Progesterone levels in Bmal1<sup>−/−</sup> and SF1-Bmal1<sup>−/−</sup> females at 3.5 dpc (A) and 6.5 dpc (B) and PRL levels in SF1-Bmal1<sup>−/−</sup> females at 3.5 dpc (C) and 6.5 dpc (D) are shown. SF1-control includes Bmal1<sup>ff/ff</sup> and Bmal1<sup>fx/fx</sup>Cre<sup>ff</sup> females. P values, by Wilcoxon sum test, are indicated.

Fig. 5. Effects of progesterone or PRL treatment on implantation at 6.5 dpc or 10.5 dpc. Representative whole uteri (1) and H&E-stained sections (2) are shown. SF1-Bmal1<sup>−/−</sup> females (B–D and F–H) and wild-type controls (A and E) received corn oil (A, B, and F), progesterone (C and G), or PRL (D and H) until 6.5 dpc (A–D) or 10.5 dpc (E–H). (Scale bars: 0.5 mm.)
suggestions that functioning of the gonadotropic or adrenal clocks in SFI-Bmal1−/− females is permissive for normal ovulation, fertilization, implantation, and parturition. Although the observation of complete implantation rescue after transplant of wild-type ovaries into SFI-Bmal1−/− mice implicates the ovarian clock, interpretation of the reverse transplantation experiment is complicated by the possibility that our surgical procedure results in the presence of enough residual ovarian tissue to support implantation. In support of this idea, publications from other laboratories suggest between 3 and 36% of litters obtained from such grafted animals may be derived from remaining ovarian host fragments that are difficult to remove during surgery (34, 35). Another possibility is that the wild-type hypothalamic-pituitary axis can support the transplanted SFI-Bmal1−/− ovaries. Nevertheless, the lower frequency of implantation suggests that this level of support is largely insufficient. Again, these results lead us to conclude that the ovarian clock is the primary determinant of implantation.

These data and those reported by others (10, 11) suggest that BMAL1 plays a role in the intrinsic molecular clock of ovarian steroidogenic cells, and that this clock plays an important role in the production of progesterone through the enzyme STAR. Importantly, the mechanism by which BMAL1 regulates Star is worthy of further investigation. Although Star expression has been reported to oscillate in F1 follicles of chicken ovaries or rat mature granulose cells (36, 37), we did not observe circadian oscillation of this mRNA in whole ovaries from 2.5 dpc to 3.5 dpc (Fig. 3). Moreover, although previous chromatin immunoprecipitation analysis in mouse adrenal indicates that Bmal1 binds to E-boxes in the Star promoter, other studies in liver have not shown evidence of direct interaction (38, 39). Although there is still much to be learned about the roles of the circadian clock in many aspects of fertility, these data support a model of female reproduction where regulation of progesterone by molecular clock machinery within steroidogenic cells of the ovary is a crucial factor in one critical aspect of fertility, implantation. Of equal importance is the conclusion that circadian clocks at additional sites appear to regulate other important aspects of fertility, such as onset of puberty, estrous cycling, and ovulation.

Materials and Methods

Ovary transplantation was carried out bilaterally or unilaterally as indicated (40). Briefly, mice were anesthetized by inhalation through a nose cone of 2% (vol/vol) isoflurane (Halocarbon Products) mixed with oxygen using a V7276 anesthesia machine (Surgivet Veterinary Surgical Products). The mice received a s.c. injection of buprenorphine (0.05–0.1 mg/kg, Sigma) and were placed on a heated disinfected pad. Recipient ovaries were surgically removed through a tiny incision of the bursa, and donor ovaries were inserted into the bursa of the recipient under a dissecting microscope. All females recovered normal movement 1 h after the surgery. Females were mated to wild-type fertile males 21 d after the surgery. For additional information, see SI Materials and Methods and Table S5.

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