Cyclical DNA Methyltransferase 3a Expression Is a Seasonal and Estrus Timer in Reproductive Tissues  
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It is becoming clear that epigenetic modifications such as DNA methylation can be dynamic and, in many cases, reversible. Here we investigated the photoperiod and hormone regulation of DNA methylation in testes, ovaries, and uterine tissue across multiple time scales. We hypothesized that DNA methyltransferase 3a (dnmt3a) is driven by photoperiodic treatment and exhibits natural variation across the female reproductive cycle and that melatonin increases whereas estrogen reduces DNA methylation. We used Siberian hamsters (Phodopus sungorus) due to their robust changes in reproductive physiology across seasonal and estrus time scales. Our findings indicate that short-day (SD) winter-like conditions significantly increased global DNA methylation and dnmt3a expression in the testes. Using immunohistochemistry, we confirm that increased dnmt3a expression was primarily localized to spermatogonium. Conversely, the ovaries did not exhibit variation in DNA methylation or dnmt3a/3b expression. However, exposure to SD significantly increased uterine dnmt3a expression. We then determined that dnmt3a was significantly decreased during the estrus stage. Next, we ovariectomized females and subsequently identified that a single estrogen/progesterone injection was sufficient to rapidly inhibit dnmt3a and dnmt3b expression. Finally, we demonstrate that treatment of human embryonic kidney-293 cells with melatonin significantly increased both dnmt3a and dnmt3b expression, suggesting that long-duration nocturnal signaling in SD may be involved in the regulation of DNA methylation in both sexes. Overall, our data indicate that dnmt3a shows marked photoperiod and estrus plasticity that likely has broad downstream effects on the timing of the genomic control of reproductive function. (Endocrinology 157: 2469–2478, 2016)

Biological rhythms in reproductive physiology are common across vertebrates: from fish and reptiles to bird and mammalian species (1–3). Our understanding of a role for epigenetic modifications, such as DNA methylation, in regulating biological rhythms is in its infancy. Daily rhythms in metabolism and food intake are strongly associated with cyclical changes in histone acetylation (4, 5). Moreover, daily changes in the amount of DNA methylation in a number of gene promoter regions are involved in timing circadian locomotor behavior (6). Despite these advances, the role of epigenetic rhythms during reproductive cycles is not well described. In seasonally breeding species such as the Siberian hamster (Phodopus sungorus), the hypothalamus exhibits photoperiod-dependent reduction in global DNA methylation and enzymes involved in the methylation of DNA (7). Whether similar changes in DNA methylation occur in the timing of reproductive physiology in peripheral tissues, such as the testes, ovary, and/or uterus, is poorly understood.

In mammals, the key enzymes that catalyze the methylation of DNA consist of three distinct isoforms: DNA methyltransferase 1 (dnmt1), 3a (dnmt3a) and 3b (dnmt3b). dnmt1 is critical for the maintenance methylation of DNA during cell division; whereas both dnmt3a and dnmt3b are involved in de novo methylation primarily in postmeiotic cells (8, 9). dnmt1, dnmt3a, and dnmt3b enzymes have

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been identified in testes, ovary, and uterine tissue. *dnmt1*, *dnmt3a*, and *dnmt3b* are predominantly localized in the spermatogonia (10), epithelial layer in ovaries (11), and endometrium cells in the uterus (12). The localization of *dnmts* in peripheral reproductive tissues indicates the potential for timing cyclical changes within molecular pathways involved in fertility (13). Indeed the mechanisms that regulate the functional role of methyltransferases are well described in germline cells and during development (reviewed in reference 14). The objective of this paper was to examine photoperiod- and hormone-dependent changes in DNA methylation and *dnmt1*, *dnmt3a*, and *dnmt3b* mRNA expression in adult testes, ovary, and uterine tissues.

Given the massive seasonal and estrus changes in testicular and uterine tissue in Siberian hamsters, we tested the hypotheses that peripheral reproductive tissues exhibit significant variation in global gonadal DNA methylation and DNA methyltransferase expression. Here we investigated the photoperiod and hormonal regulation of gonadal DNA methylation and *dnmt1*, *dnmt3a*, and *dnmt3b* expression in testicular, ovarian, and uterine tissue and cell culture. Using adult male and female Siberian hamsters, we identified marked naturally occurring plasticity in *dnmt3a* methyltransferase expression that is regulated by photoperiod, melatonin, and ovarian hormones. Increased *dnmt3a* in short day (SD) testes results in a substantial accumulation of global DNA methylation. The findings reported herein reveal robust plasticity in key DNA methylation enzymes and indicated epigenetic reorganization within peripheral reproductive tissues across multiple time scales. Overall, this work has significant implications for reproductive timing and fertility in mammalian species.

Materials and Methods

Animals

Adult male and female Siberian hamsters (total n = 100; 3–8 mo old) were randomly selected from a colony maintained at the University of Aberdeen. Adulthood in hamsters occurs between the age of 3 and 9 months (15). Hamsters are classified as aged at 14 months (16), and reproductive decline does not occur in females until after 9 months (15). Hamsters were housed in polypropylene cages in a long day (LD) photoperiod (15L:9D). Food and water were provided ad libitum, and hamsters were provided cotton-nesting material. All procedures were approved by the Animal Welfare and Ethics Review Board at the University of Aberdeen and conducted under the Home Office license (70/7917).

Experimental designs

Photoperiod regulation of reproductive physiology and gonadal DNA methylation

Thirty-six adult male and female hamsters (3–8 mo) were used in this study. Male (n = 8) and female (n = 8) hamsters were group housed in long day (15L:9D) conditions prior to the experiment. Baseline measures of body weight were recorded and measured for the duration of the experiment. A group of males (n = 10) and females (n = 10) were transferred from LD to short day cabinets (Arrowright; SD 9D:15L) for 8 weeks. At the termination of the study, animals were killed by cervical dislocation and testes, ovary, and uterine mass was determined using ADAM scales (Adam Equipment PGL2002) and measured to ±0.1 g. Tissues were frozen in powdered dry ice and stored at −80°C until global gonadal DNA methylation and RNA expression analyses (see below).

Naturally occurring changes in uterine DNA methyltransferase enzymes across the estrous cycle

Female hamsters (n = 33) were group housed and maintained in LD. On the final day of the experiment, animals were killed from 3:00 PM to 5:00 PM to capture the proestrus surge in prolactin (17). Females were lightly anesthetized with isofluorane gas (4%), and 500 µL whole blood was collected via the right retroorbital sinus using Natelson tubes coated with sodium heparin. The blood samples were kept on ice and then centrifuged at 9000 rpm (3622 g) in 4°C for 20 minutes. Plasma was removed and stored at −20°C until prolactin levels were determined by an ELISA assay (see below). Females were killed by cervical dislocation and uterine mass was measured and subsequently frozen in powdered dry ice. Samples were kept at −80°C until RNA extraction.

Unlike mice and rats, female hamsters do not exhibit marked cyclical changes in vaginal cell types, resulting in the inability for external tracking of the estrus stages. To determine the stage of the estrus cycle, we took advantage of a well-described method that uses convergent measures consisting of uterine mass and plasma prolactin concentrations (17). The combinations of reproductive measures permit the identification of diestrus (low prolactin; small uteri), proestrus (high prolactin; intermediate uteri), and estrus (low prolactin; engorged uteri) stages of the female cycle. Plasma prolactin concentrations were determined using a hamster prolactin ELISA (2BScientific Ltd). Samples were assayed in duplicate and compared with a standard curve. The intraassay coefficient was 4.4%. Analyses of the uterine weight and plasma prolactin values resulted in the identification of diestrus (n = 13), proestrus (n = 12), and estrus (n = 8) females.

The sufficiency of ovarian steroids to regulate DNA methylation enzymes

To assess the sufficiency of ovarian hormones on uterine DNA methyltransferase expression, females were ovarioectomized (n = 21) and maintained in LD for 8 weeks to reduce the circulating levels of gonadal steroids. In brief, ovariectomies were conducted while hamsters were under deep anesthesia (5% isofluorane gas). The ovaries are externalized via bilateral incisions to the dorsum (lateral to the spine, caudal to the ribcage).
The ovary was localized at the distal end of the uterine horn and ligated with sterile sutures (4-0, nonabsorbable monofilament nylon). The ovary was then excised and repeated for the other ovary. The abdominal wall and skin were closed separately with sterile sutures (5-0 nonabsorbable and 4-0 nonabsorbable, respectively, monofilament nylon). After ovariectomy, female body mass decreased on average 7.5 g (±0.9 SEM), a reliable long-term indicator of reduced ovarian steroids (18). Estrogen and progesterone (E2P4) injections were prepared by dissolving diethylstilbestrol (Sigma-Aldrich) and progesterone (P4; Sigma-Aldrich) in sterile vegetable oil to a final concentration of 5 μg estrogen and 500 μg progesterone in 100 μL sterile vegetable oil (OIL). These values were selected based on previous work in female hamsters (19). Females received an ip injection at 5:00 PM with 100 μL of the hormone cocktail. Control hamsters were injected with 100 μL of OIL. The following day hamsters were killed at 12 (n = 6) and 24 hours (n = 6) after the injection by cervical dislocation, and uterine weights were measured and frozen in powdered dry ice. We selected 12- and 24-hour time points to control for potential daily variation in estrous study were assayed in duplicate. Quantitative PCR products in 2.5% agarose gel. A melting curve analysis was performed. All cDNA tissue samples were run in triplicate; annealing dependent on target mRNA (see Supplemental Table 1) for 30 seconds, and 4) an extension at 72°C for 30 seconds. The specificity of select samples was established by resolving PCR products in 2.5% agarose gel. A melting curve analysis was added to determine the quality and specificity of each reaction. Quantification of mRNA expression levels was accomplished with iQ Sybr Green Supermix (Bio-Rad Laboratories). We used PCR Miner (20) to calculate the reaction efficiencies and cycle thresholds. According to the MIQE guidelines, samples that had efficiency values below 0.8 or above 1.2 were excluded from analyses (21). The expression of each target gene of interest was measured in relation the average cycling time for two reference targets: glyceraldehyde 3-phosphate dehydrogenase (gapdh) (7, 22) and 18S ribosomal RNA (18s) (23) and calculated using 2-(ΔΔCt).

Assessment of global gonadal DNA methylation

DNA was extracted from tissues using DEasy kits (QIAGEN) following the manufacturer’s directions. One microgram of DNA was digested using nuclease P1 (5 U; Sigma-Aldrich) and then incubated at 70°C for 30 minutes. One microliter of alkaline phosphatase (5 U; Sigma-Aldrich) was added and the samples were incubated at 37°C for 30 minutes. The samples were then transferred to 65°C for 15 minutes and then placed at -20°C until assayed. Global DNA methylation levels were measured using a 5-methyl-2'-deoxycytidine quantitation ELISA kit (Cell Biolabs Inc). The kit is a competitive assay used for the quantification of 5-methyl-2'-deoxycytidine and has previously been used in Siberian hamsters (7). Samples were run in duplicates and the intraassay coefficient of variation was 10%.

Quantification of RNA expression

RNA was extracted from tissues using Trizol (ThermoFisher Scientific). Nucleic acid concentration and quality were determined by spectrophotometer (Nanodrop; ThermoFisher Scientific). cDNA was synthesized using Superscript III (Invitrogen) and cDNA was stored at -80°C until a quantitative PCR was performed. All cDNA tissue samples were run in triplicate; cDNA from human embryonic kidney-293 (HEK293) cell culture and estrus study were assayed in duplicate. Quantitative PCRs were performed using a Bio-Rad Laboratories CFX96 system using the following steps: 1) an initial denaturation at 95°C for 30 seconds and then 39 cycles of 2) 95°C for 10 seconds, 3) annealing dependent on target mRNA (see Table 1) for 30 seconds, and 4) an extension at 72°C for 30 seconds.

### Table 1. Antibody Table

<table>
<thead>
<tr>
<th>Peptide/Protein Target</th>
<th>Antigen Sequence (If Known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody</th>
<th>Species Raised (Monoclonal or Polyclonal)</th>
<th>Dilution Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT3a</td>
<td>AA 10-118 of human DNMT3a</td>
<td>Anti-DNA methyltransferase 3a antibody</td>
<td>ThermoFisher/Invitrogen, PA3-16557</td>
<td>Rabbit, polyclonal</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Abbreviation: AA, amino acid.
cases, the immunoreactivity signal was abolished. Preadsorption of the primary antibody with 5 μg or 10 μg blocking peptide (3227BP; Cambridge Bioscience Ltd) for 2 hours resulted in a dose-dependent decrease in staining intensity (P < .05 and P < .001, respectively). Overall, these data indicate that the primary antibody used here is specific for the endogenous DNMT3a antigen.

**Sufficiency of melatonin to drive DNA methylation enzymes**

To assess the potential direct effects of melatonin on dnmt3a and dnmt3b expression, we conducted a melatonin dose-dependent study using cell culture. Given the low levels of DNA methylation and absence of dnmt3a/b plasticity in the ovary (see Results below), we selected HEK293 cells to examine the role of melatonin dependent regulation of dnmt expression because these cells are known to express melatonin receptor 1a (26). Cells of the HEK293 cell line were grown in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and sodium pyruvate (complete medium) in a T75 flask in a humidified atmosphere at 37°C. HEK293 cells were selected for melatonin assay because these cells express dnmt3a and dnmt3b as well as the melatonin receptor involved in the neural control of the seasonal photoperiodic response (27, 28). When confluent, HEK293 cells were plated in 24-well plates as follows. Media were removed and cells were rinsed with PBS (at 37°C), and 5 mL of trypsin was added to detach cells. Two milliliters of media were added before pipetting up and down to mix and transferring to a 15-mL tube to pellet cells by centrifugation. The supernatant was removed and cells resuspended in 7 mL complete medium (at 37°C). One hundred fifty microliters of media were added before pipetting up and down to mix and transferring to a 15-mL tube to pellet cells by centrifugation. The supernatant was removed and cells were resuspended in 7 mL complete medium (at 37°C) for 2 hours. The immunoreactivity signal was abolished. Preadsorption of the primary antibody with 5 μg or 10 μg blocking peptide (3227BP; Cambridge Bioscience Ltd) for 2 hours resulted in a dose-dependent decrease in staining intensity (P < .05 and P < .001, respectively). Overall, these data indicate that the primary antibody used here is specific for the endogenous DNMT3a antigen.

### Results

**SD-induced gonadal involution facilitated testicular DNA methylation**

Exposure to SD significantly reduced testes mass (t = 14.33; P < .001; Figure 1A). Regressed testes were observed to have a robust and significant effect on global DNA methylation levels (t = 3.17; P < .005; Figure 1B), indicating that the timing of testicular involution may be controlled by increased DNA methylation. Next, we assessed the levels of DNA methyltransferase expression to identify the enzymes involved in increased DNA methylation in regressed testes. dnmt1 expression was found to have significantly greater levels in LD compared with the SD conditions (t = 2.77; P < .01; Supplemental Figure 1A). Increased dnmt1 in LD testes may be due to the production of sperm during the breeding periods. dnmt3a expression was observed to exhibit the predicted increase in SD testes, and regressed testes had significantly greater

![Figure 1](https://academic.oup.com/endo/article-abstract/157/6/2469/2422617)
levels compared with LD ($t = 2.80; P < .01$; Figure 1C). 

\textit{dnmt3b} expression was found to remain constant across photoperiodic conditions ($t = 0.79; P = .22$; Figure 1D).

**Regressed testes have more DNMT3a-expressing cells**

A $t$ test was conducted to evaluate the effect of SD on the number of DNMT3a-expressing cells in the testes. SD significantly reduced testes mass ($t = 9.05; P < .001$; Figure 2A). There was a significant increase in the number of DNMT3a cells in the SD compared with LD testes ($t = 2.159; P < .05$; Figure 2, B–D). The SD increase in DNMT3a appears to be localized to spermatogonium (Supplemental Figure 2).

**Ovarian DNA methylation remains constant across photoperiodic conditions**

Ovary mass showed a relatively small yet significant decrease in SD compared with LD hamsters ($t = 2.17; P < .05$; Figure 3A). Unlike the testes, there was no significant photoperiodic effect on ovarian global DNA methylation ($t = 0.81; P = .21$; Figure 3B). Not surprisingly, there was no significant difference between LD and SD levels of \textit{dnmt1} ($t = 1.39; P = .09$; Figure 1B), \textit{dnmt3a} ($t = 0.27; P = .39$; Figure 3C), or \textit{dnmt3b} expression ($t = 0.62; P < .27$; Figure 3D).

**SD significantly increased uterine \textit{dnmt3a} and \textit{dnmt3b}**

Exposure to SD significantly reduced uterine mass ($t = 3.388; P < .005$; Figure 4A). The photoperiodic condition did not have a significant effect on \textit{dnmt1} expression ($t = 0.95; P = .18$; Figure 1C). The decrease in uterine mass was paralleled by a significant increase in \textit{dnmt3a} expression ($t = 3.103; P < .05$; Figure 4B) and \textit{dnmt3b} expression ($t = 10.0; P < .01$; Figure 4C). Histological analyses indicate that DNMT3a expression in SD shows a robust immunoreactive signal in the endometrium layer in the uterus (Supplemental Figure 3) (12).

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**Figure 2.** SD increase DNMT3a expression in the testes. A, SD significantly reduced testicular mass. B, The number of DNMT3a-expressing cells (summed across 10 seminiferous tubules) in SD-regressed testes exhibited a significant increase compared with LD controls. C and D, Exemplar photomicrographs of LD and SD DNMT3a expression in testicular tissue. Note the large decrease in testicular lumen in SD compared with LD samples. White scale bar represents 3 mm.

**Figure 3.** Ovarian tissue lacks photoperiod-dependent changes in DNA methylation. A, SD hamsters were observed to display a slight decrease in ovarian mass. Unlike the testes, the ovary did not exhibit a significant difference in global DNA methylation (B). Moreover, both \textit{dnmt3a} (C) and \textit{dnmt3b} (D) relative expression remained constant across LD and SD conditions.
d\textit{mnt}3a expression is reduced during estrus

As previously established (Dodge et al [17]), the estrus cycle in female hamsters can be determined using the combined uterine mass and plasma prolactin measures. A one-way ANOVA indicated that uterine mass exhibits significant variation across the cycle ($F = 15.623; P < .001$; Figure 5A). LSD post hoc analyses confirmed that diestrus females have significantly lower uterine mass compared with the proestrus ($P < .01$) and estrus ($P < .001$) stages. Furthermore, the uterine mass during estrus was significantly engorged and greater compared with proestrus ($P < .005$). Plasma prolactin exhibited significant variation across the estrus cycle ($F = 24.202; P < .001$; Figure 5A). LSD analyses indicated that plasma prolactin concentrations significantly increased from diestrus to proestrus ($P < .001$). Plasma prolactin concentrations then decreased during the estrus phase ($P < .001$). Estrus females were found to have slightly higher levels of plasma prolactin compared with diestrus females ($P < .05$).

A one-way ANOVA revealed a significant difference in $d\text{mnt}3a$ ($F = 3.53; P < .05$; Figure 3B) expression across the estrus cycle. LSD analyses indicated that $d\text{mnt}3a$ expression significantly decreased during the transition from proestrus to estrus ($P = .01$). Diestrus females had intermediate levels because $d\text{mnt}3a$ levels were not significantly different compared with estrus ($P = .44$) or proestrus ($P = .06$) hamsters. There was no significant variation in $d\text{mnt}3b$ expression observed across diestrus, proestrus, or estrus phases ($F = 2.22; P = .33$; Figure 5C). There was no significant change in $d\text{mnt}1$ expression across the estrus cycle ($F = 0.26; P = .77$; Figures 1D).

E2P4 is sufficient to inhibit DNA methyltransferase expression

A Kruskal-Wallis ANOVA revealed that a single bolus injection of E2P4 was sufficient to significantly increase uterine mass ($H = 8.34; P < .05$; Figure 6A). Dunnett’s method identified that uterine mass was significantly greater than OIL-treated controls 12 hours ($P < .05$) and 24 hours ($P < .05$) after the injection. These data confirm that E2P4 was capable of inducing engorged uterine and estrus within 24 hours. A one-way ANOVA revealed a

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**Figure 4.** Photoperiod-induced variation in relative $d\text{mnt}3a$ and $d\text{mnt}3b$ uterine expression. Female hamsters transferred to SD exhibit a significant decrease in uterine mass (A). SD uterine expression had significantly more relative $d\text{mnt}3a$ (B) and $d\text{mnt}3b$ (C) expression compared with LD controls. ***, Significance at $P < .001$; **, significance at $P < .01$; *, significance at $P < .05$.  

**Figure 5.** Estrus significantly decreased relative $d\text{mnt}3a$ expression. A. Combined uterine mass and plasma prolactin reliably indicate diestrus (DI), proestrus (PRO), and estrus phases (EST). Uterine mass is low in diestrus and significantly increased during proestrus and again during estrus. Plasma prolactin is low in diestrus, significantly increased during proestrus, and then decreases during estrus. B. The levels of relative $d\text{mnt}3a$ expression are significantly reduced during estrus. C. There was a nonsignificant decrease in relative $d\text{mnt}3b$ expression during estrus. White, gray, and black bars indicate DI, PRO, and EST stages, respectively. ***, Significance at $P < .001$; **, significance at $P < .01$; *, significance at $P < .05$.  

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significant difference in dnmt3a expression after administration of E2P4 (F = 13.57; *P < .001; Figure 6B). The Dunnett’s method indicated that E2P4 induced a rapid inhibition in dnmt3a expression, with a significant reduction after 12 hours (*P < .001) and 24 hours (*P < .001) compared with OIL-treated females. Similarly, there was a significant difference in dnmt3b across treatment groups (F = 32.35; *P < .001; Figure 6C). E2P4 significantly reduced dnmt3b expression in uterine tissue 12 (*P < .001) and 24 hours (*P < .001) after administration. dnmt1 expression was also found to be significantly reduced by E2P4 treatment (F = 8.79; *P < .005; Figure 1E). The Dunnett’s method revealed that dnmt1 expression was significantly lower 24 hours after the injection (*P < .005) but not after 12 hours (*P = .51).

Melatonin is sufficient to increase dnmt3a and dnmt3b

Melatonin treatments categorically increased dnmt3a and dnmt3b expression in HEK293 cells. A one-way ANOVA revealed a significant difference in dnmt3a expression across treatment groups (F = 17.207; *P < .001; Figure 7A). The Dunnett’s method for post hoc analyses established that all doses of melatonin induced a significant increase in dnmt3a compared with control cells (P < .001), but dnmt3a expression was similar across all melatonin concentrations (P > .05). Similarly, the one-way ANOVA revealed a significant difference in dnmt3b expression (F = 39.207; *P < .001; Figure 7B). All doses of melatonin were observed to have significantly greater dnmt3b expression compared with controls (P < .001).

Discussion

In this paper, we show marked photoperiod-dependent regulation of DNA methylation in testes. The increased methylation appears to be driven by dnmt3a. One potential driver for the SD-induced increase in DNA methylation may be a lengthening of nocturnal melatonin duration. Incubation of HEK293 cells with various concentrations of melatonin was sufficient to elicit a categorical increase in both dnmt3a and dnmt3b expression. Surprisingly, the ovary failed to show photoperiodic variation in DNA methylation, indicating a marked gonadal difference in the role of DNA methylation across the seasonal reproductive cycle. Instead, seasonal variation in DNA methylation may act in the uterus for reproductive timing.

Further examination of dnmt3a and dnmt3b expression revealed significant plasticity during the estrus cycle, with inhibition during the estrus stage due to the increased secretion of estrogen and progesterone. We conclude that seasonal and estrus variation in testicular and uterine dnmt3a expression enhanced DNA methylation, triggered reproductive involutions, and reduced fertility.

Cyclical patterns in epigenetic modifications are gradually being uncovered. Recent work has identified marked daily (6) and seasonal (7) changes in DNA methylation. In the hypothalamus, there is a decrease in global DNA methylation and dnmt3b expression in adult Siberian hamsters after prolonged exposure to SD compared with LD (7). Here we show SD stimulated an increase in DNA methylation and dnmt3a and dnmt3b expression in peripheral tissues (ie, testes and uterus). These findings suggest that DNA methylation patterns show opposite cyclic changes.
in the central nervous system (7) compared with peripheral systems, such as reproductive tissue and immune cells (21). DNMT3b expression in the brain is widely distributed and located in several hypothalamic nuclei (7). Peripheral tissues (eg, testes, ovary) consist of a relatively homogenous cell population compared with the complex networks and diverse cells located in the hypothalamus. The increased DNMT3b expression in LD hypothalamus likely reflects the outcome of multiple localized changes and not the result of a single brain region. A greater resolution of anatomically localized changes in DNMT3b expression in the hypothalamus will help resolve the opposite patterns observed in neuroendocrine nuclei and peripheral reproductive tissues. It is clear that melatonin and ovarian hormones are involved in the regulation of dnmt3a and dnmt3b expression. Given the categorical increase in dnmt3a and dnmt3b after exposure to melatonin and the rapid change in response to a single bolus of E2P4, it is likely that these hormones could be acting directly on promoter regions or in the recruitment of transcription binding factors. Altered hormonal regulation of cell autonomous timing of DNA methylation may be one potential molecular mechanism that underlies the seasonal disruption in animal health (29).

Epigenetic modifications during gamete development are well described (30). Conditional knockout dnmt3a mice exhibit severe reproductive deficits; males exhibit impaired spermatogenesis and lack DNA methylation in parentally imprinted genomic regions (31). In females, conditional knockouts of dnmt3a are lethal and also have an absence of DNA methylation at parentally imprinted genomic regions (31). These data support a model in which dnmt3a signaling in males and females is vital for the generation of viable gametes and, ultimately, fertility. In our study, we observed relatively low levels of dnmt3a in testes and ovaries in LD compared with nonbreeding, SD conditions. The increase in dnmt3a expression in the regressed testes likely provides an inhibitory signal that arrests spermatogenesis. Whether enhanced melatonin or reduced gonadal steroids (ie, E2P4) provide a hormonal signal that permits the greater dnmt3a expression requires further exploration. Nevertheless, the molecular outcome was a massive increase in DNA methylation that results in broad methylation across the entire genome, resulting in reproductive involution. Given that the seasonal pattern in DNA methylation occurs over multiple annual oscillations, we propose that cyclical DNA methylation in reproductive tissues provides a single trigger with broad implications for the timing of gene transcription that enables yearly switches in gamete development and fertility.

DNMT3a/b has a high enzymatic activity and can rapidly methylate cytosine residues (eg, 3 h) (32). In this paper, we have shown that melatonin can increase dnmt3a and dnmt3b expression in cell culture within 4 hours, and a single bolus of E2P4 can inhibit uterine levels within 12 hours. These data indicate that key seasonal and reproductive hormones can have a significant impact on dnmt3a/b expression and ultimately lead to a lasting effect on the epigenomic landscape. It is important to note that caution should be exercised when extrapolating the melatonin-dependent increase of dnmt3a/b in HEK293 results to seasonal regulation of DNA methylation in hamster reproductive tissues. Given the large variation in gene transcription during spermatogenesis (33), seasonal and estrus patterns in dnmt3a likely function to secure the inhibition of select genes leading to the successful timing of RNA expression required for optimal fertility. It is likely that several other hormones with links to reproduction function (eg, leptin) will also impact the probability of dnmt3a/b expression. Overall, the rapid and long-term effects of melatonin and ovarian steroids reveal a novel and robust effect on methyltransferase expression and illustrate that hormone-driven changes in the epigenomic landscape are probably more common than previously thought.

The comparison of ovarian and testicular DNA methylation permitted the identification of significant sex differences in the levels of dnmt1 and dnmt3a expression. The higher levels of dnmt1 expression in testes is likely attributable to gamete production (ie, spermatogenesis) (34). Several testicular genes exhibit reversible, seasonal variation in expression, and these changes are proposed to enhance fertility during the breeding periods (35). Because seasonal variations in sperm parameters are common across mammalian species, including humans (36, 37), the patterns in dnmt1 and dnmt3a may represent an evolutionarily ancient molecular signaling mechanism for the timing of reproduction. A role for dnmt1 in the timing of reproductive physiology in the uterus is less clear (Figure 1). dnmt3a has been shown to be important for decidualization, exhibiting a transient estrogen-dependent decrease (38). Similarly, we found that E2P4 significantly reduced dnmt3a and dnmt3b. The specific role of reduced dnmt3a during decidualization is unknown but may permit stromal vascularity and/or glandular epithelial secretion.

In conclusion, we present novel and robust findings that dnmt3a expression is dynamic and propose that variation in dnmt3a is involved in the local timing of reproductive physiology in key tissues. These data have significant implications for our understanding of the potential effects of DNA methylation for fertility in a rodent species. One particularly important finding was the significant increase in global DNA methylation in the male testes during

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reproductive involution. Future work that includes alternative methods, such as chromatin immunoprecipitation for DNMT3a, will be important to confirm the large photoperiodic variation in DNA methylation and identify the genomic regions targeted in both testicular and uterine tissue. Uncovering the mechanism that underlies this natural pattern could have a significant impact for developing alternative methods for contraceptives. Moreover, these data provide further evidence that epigenetic modifications exhibit dynamic and cyclical patterns in expression and indicate DNA methylation is a key characteristic of timing biological rhythms.

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Author contributions included the following: T.J.S. conceived the project, designed the experiments, analyzed the data, and wrote the manuscript. E.W.J.L. conducted the experiments and analyzed the data. C.S.C. conducted the immunocytochemistry. M.L. conducted the HEK293 cell culture assays. E.M.C. and A.S.B. provided technical assistance.

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