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Inhibitory role of REV-ERBα in the expression of bone morphogenetic protein gene family in rat uterus endometrium stromal cells

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1Department of Animal and Marine Bioresources Sciences, Graduate School of Agriculture, Kyushu University, Fukuoka, Japan; 2Department of Anatomy and Neurobiology, Kinki University School of Medicine, Osaka, Japan; and 3Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Submitted 30 June 2014; accepted in final form 5 January 2015

Tasaki H, Zhao L, Isayama K, Chen H, Yamauchi N, Shigeyoshi Y, Hashimoto S, Hattori MA. Inhibitory role of REV-ERBα in the expression of bone morphogenetic protein gene family in rat uterus endometrium stromal cells. Am J Physiol Cell Physiol 308: C528–C538, 2015. First published January 14, 2015; doi:10.1152/ajpcell.00220.2014.—Uterus circadian rhythms have been implicated in the gestation processes of mammals through entraining of the clock proteins to numerous downstream genes. Bone morphogenetic proteins (BMPs), having clock-controlled regulatory sites in their gene promoters, are expressed in the uterus during decidualization, but the regulation of the Bmp gene expression is poorly understood. The present study was designed to dissect the physiological roles of the uterus oscillators in the Bmp expression using the uterus endometrial stromal cells (UESCs) isolated from Per2-dLuc transgenic rats on day 4.5 of gestation. The in vitro decidualization of UESCs was induced by medroxyprogesterone acetate and 2-O-dibutyryl cAMP. A significant decline of Per2-dLuc bioluminescence activity was induced in decidual cells, and concomitantly, the expression of canonical clock genes was downregulated. Conversely, the expression of the core Bmp genes Bmp2, Bmp4, Bmp6, and Bmp7 was upregulated. In UESCs transfected with Bmal1-specific siRNA, in which Rev-erbα expression was downregulated, Bmp genes, such as Bmp2, Bmp4, and Bmp6 were upregulated. However, Bmp1, Bmp7, and Bmp8a were not significantly affected by Bmal1 silencing. The expression of all Bmp genes was enhanced after treatment with the REV-ERBα antagonist (SR8278), although their rhythmic profiles were different from each other. The binding of REV-ERBα to the proximal regions of the Bmp2 and Bmp4 promoters was revealed by chromatin immunoprecipitation-PCR analysis. Collectively, these results indicate that the Bmp genes are upregulated by the attenuation of the cellular circadian clock; in particular, its core component REV-ERBα functions as a transcriptional silencer in the Bmp gene family.

BONE MORPHOGENETIC PROTEINS (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily and are involved in a variety of cellular functions, such as proliferation, differentiation, and remodeling (51). The expression of BMPs in reproductive tissues, including the uterus is well known (17, 29, 34, 42, 54, 57, 61). The BMP family, including BMP2, BMP4, BMP6, and BMP7, is spatiotemporally expressed in the mouse uterus during the progressive phases of implantation; these BMP family members may have specific functions in each of these phases (43, 68). BMP2 is highly expressed within the decidual area surrounding the site of blastocyst attachment (36, 67) and plays a critical role in decidualization (36). In addition, deletion of Bmp2 in the uterus causes specific gene changes, including the disruption of the Wnt signaling, the disruption of progesterone receptor signaling, and the induction of Pigs2 (14, 34, 36). BMP2 failed to be expressed in the decidual uterus treated with the progesterone receptor antagonist, RU486 (7), suggesting that progesterone has a critical role in Bmp2 expression. However, the mechanism underlying the regulation of Bmp gene expression in uterus endometrial stromal cells (UESCs) remains poorly understood.

There are many E-box and ROR/REV-ERB response elements (RORE), which are the circadian-clock-controlled cis-regulatory elements, in upstream regions from transcriptional start sites of the Bmp genes. A growing number of studies provide evidence that numerous peripheral circadian clock systems are partially self-operative and independent in their responses to external and internal stimuli other than the stimuli originating from the suprachiasmatic nucleus (SCN), known as the central pacemaker of the circadian clock (21, 56, 62, 63). The molecular mechanism of the mammalian circadian clock involves a primary conservative interlocked transcriptional-translational feedback loop (28). This loop comprises a core group of clock genes and their protein products. The transcriptional activators BMAL1 and CLOCK form a heterodimer, which drives the expression of the Per1–3 and Cry1–2 genes by recognizing E-box cis-elements in their promoters (18, 26, 60). In turn, the heterodimer of PER and CRY are formed and repress CLOCK-BMAL1 activity to inhibit their own transcription (1, 32, 49). Further adding to the complexity, CLOCK-BMAL1 heterodimer induce expression of the nuclear receptor, REV-ERBα, resulting in the repression of the transcription of Bmal1 through direct binding to the RORE located in the Bmal1 promoter (4, 9). Furthermore, other accessory proteins, including the orphan receptor (ROR) and albumin D-box binding protein (DBP), constitute additional loops that make the oscillator robust and tunable (20, 37, 44, 64). In addition to regulating each other to sustain oscillations, the core clock proteins also entrain the rhythmic expression of numerous downstream genes through binding to an E-box, RORE, and D-box at their promoters. These downstream genes
have been named clock-controlled genes (CCGs) and form a large family. Existing evidence shows that the rhythmic expression of putative CCGs constitutes 8–10% of expressed transcripts in some tissues (2, 55). Most of these genes are involved in organ functions and show tissue-specific expression.

BMAL1, an important core clock protein, is indispensable in maintaining the integrity of the circadian feedback loop and the homeostasis of numerous behaviors and physiological processes (6, 19, 31, 47, 52, 69). Recent genetic studies also strongly suggest an important role of Bmal1 in mammalian reproductive physiology (8, 38, 47). Bmal1−/− mice are infertile with profound reproductive defects, such as impaired steroidogenesis, disrupted estrous cyclicity, and implantation failure. However, the specific physiological events, such as implantation and placentation, which are regulated by uterus oscillators, remain poorly understood.

The transcriptional-translational feedback loop consisting of clock genes elicits the circadian rhythms in differentiated somatic cells (50). Bmal1-deficient embryonic fibroblast cells and Bmal1-knockdown 3T3-L1 cells fail to differentiate into adipocytes (52). The expression of Rev-erba, also known as Nr1d1, is transcriptionally upregulated during adipocyte differentiation (10). The circadian system, consisting of clock genes, is also disrupted in differentiating cells of rat ovaries and uteri (22). Several recent studies have demonstrated that circadian clock genes are rhythmically expressed in the uterus (3, 16, 22, 25, 35, 40). In rodents and humans, the UESCs undergo proliferation and differentiation into decidual cells in response to ovarian steroids and blastocyst implantation at the early stage of pregnancy (13, 15, 68). Decidualization is the response to ovarian steroids and blastocyst implantation at the early stage of pregnancy (13, 15, 68). Decidualization is a process that is critical to the establishment of fetal-maternal communication and the progression of implantation, and this process ultimately results in the formation of the placenta. We recently proved that the Per2 expression is down-regulated in the UESCs during decidualization; this downregulation influences Vegfα expression (59). Deregulation of the circadian clock may attenuate or disrupt the expression of CCGs and can have a profound influence on organ functions.

On the basis of the aforementioned evidence, we hypothesized that dysfunction of the circadian clockwork during the stage of decidualization may require upregulation or downregulation of the expression of numerous CCGs related to the formation of the placenta, including the Bmp gene family. Therefore, we analyzed the core clock genes and the Bmp gene family of UESCs prepared from Per2-dLuc transgenic rats by inducing in vitro decidualization. In addition, Bmal1 interference and REV-ERBα antagonist treatment were performed to probe the critical physiological function of the uterus oscillators. Hence, the present study provides novel evidence that uterus oscillators transcriptionally control the Bmp gene family.

MATERIALS AND METHODS

Animals. All of the experiments were carried out in strict accordance with the recommendations in the Guide for Animal Experiments in the Faculty of Medicine, Kyushu University, and Law No. 105 and Notification No. 6 of the Government of Japan. All procedures were reviewed and approved by the Committee on the Ethics of Animal Experiments of the Kyushu University (permit no.: A24-054-2). Per2-dLuc transgenic rats were obtained from our breeding colony. In this transgenic rat, the mouse Per2 promoter region, which is sufficient for circadian oscillation, was fused to a dLuc reporter gene (60). Animals were maintained on 12:12-h light-dark cycles (zeitgeber time, ZT0: 0800 light on; ZT12: 2000 light off) and ad libitum feeding throughout all experiments.

Preparation and culture of UESCs. The UESCs were isolated from Per2-dLuc transgenic rats on day 4.5 of gestation, as reported previously (22, 39, 41). The uterine lumens were filled with PBS containing 0.1% collagenase and incubated at 37°C for 1 h in a shaking water bath. The harvested cells were washed thrice with fresh DMEM/F12, and seeded onto 35-mm collagen-coated dishes at the density of 2 × 10⁵ cells/dish with 2 ml of culture medium (phenol red-free DMEM/F12 supplemented with 10% charcoal-treated FBS and 1× PS). The culture medium was replaced at 15 min after cell seeding to remove epithelial cells. Cultured stromal cells were positively immunostained for vimentin, a marker protein of stromal cells, but negatively for cytokeratin, a marker of epithelial cells. The staining result revealed a purity of ~95%. Cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C for 2 days. Then, cells were cultured in serum-free medium supplemented with 1× antibiotic-antimycotic (AA; Nacalai Tesque, Kyoto, Japan), 1× insulin-transferrin-selenium (ITS, Life Technologies, Carlsbad, CA), 0.1% BSA (Sigma-Aldrich, St. Louis, MO), and 100 nM progesterone (P₄; Sigma-Aldrich) for additional 2 days prior to other treatments.

In vitro decidualization. Confluent UESCs were further cultured for 4.5 days in DMEM/F12 supplemented with 0.1 mM medroxyprogesterone acetate (MPA; Sigma-Aldrich), 0.5 mM 2-O-dibutyryl cAMP (db-cAMP; Sigma-Aldrich), 1× AA, 1× ITS, 0.1% BSA, as previously described (39). The differentiating status was revealed by expression of the Prlβ2 gene (27).

Real-time monitoring of Per2-dLuc oscillations. The cultured UESCs were synchronized with 100 nM dexamethasone (DXM; Sigma-Aldrich) for 2 h in the serum-free medium containing 1× AA. Then, cells were given the serum-free medium DMEM/F12 supplemented with 15 mM HEPES, 0.1 mM luciferin (Wako, Tokyo, Japan), 0.1% BSA, 1× AA, and 1× ITS, and subjected to luminescence determination. Luciferase activity was chronologically monitored at 37°C with a Kronos Dio AB-2550 luminometer (ATTO, Tokyo, Japan) interfaced to a computer for continuous data acquisition (22–24). In some experiments, confluent UESCs were cultured for 24 h with 10 nM db-cAMP (Sigma-Aldrich) or 0.1% DMSO (vehicle control), synchronized with DXM, and then monitoring was performed in the presence of SR8278 or DMSO. The data are presented as photon counts per minute. Bioluminescence data were detrended by subtracting the 24-h running average from the raw data. Detrended data sets were smoothed by taking 2-h running averages. The amplitude and period of Per2-dLuc oscillations were documented by the single cosinor method using Timing Series Single 6.3 (Expert Soft Tech Solution, Richelieu, France).

Microarray analysis. RNA samples isolated from cultured UESCs at 30, 36, 42, and 48 h after synchronization with DXM were used for microarray analysis using the Whole Rat Genome Microarray 4x44K ver. 3.0 (Agilent Technologies, Santa Clara, CA) representing 30,367 probe sets. Bioinformatics analysis was performed using Agilent future extraction software (Agilent Technologies). The data were filtered for signal intensity values (P ≤ 0.05, detectable), which allowed removing very low signal values (57). The ratio of signal intensity values was calculated for the Bmp gene family.

Bmal1 siRNA transfection. Three sequences targeting the Bmal1 mRNA were designed to silence Bmal1 in UESCs. siRNA transfection was performed using Lipo2000 (Life Technologies), according to the manufacturer’s protocol.
DXM for monitoring of luciferase activity. pregnant rats on day 4.5 of gestation. The pieces of uterine tissue were collected from pregnant rats on day 4.5 (ZT4) of gestation. The PCR reaction was performed in 10 μl of 1× PCR buffer, 0.2 mM each of dNTP, 0.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), 0.2 μM each of the synthetic primer sets (Table 2), and 20 ng of cDNA. The amplification was performed in 42 cycles, and resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels.

Quantitative RT-PCR. Cultured cells were harvested at indicated time points. Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. RNA samples were treated with RNase-free DNase (Qiagen). The RNA extraction and RT-PCR. Cultured cells were harvested at indicated time points. Total RNA was isolated using an RNeasy mini kit (Qiagen) and cDNAs were generated by RT with Oligo (dT)15 and random primers, as described above. qRT-PCR was performed in a 50-μl volume containing a 20-ng cDNA sample in GoTaq qPCR Master Mix and 250-nM specific primers listed in Table 2, with the Mx3000P real-time qPCR System (Agilent Technologies) using the parameters as described in our previous report (11). All reactions were performed in triplicate and displayed amplification efficiency between 80% and 120%. Relative quantification of each mRNA was performed using the comparative quantity (copies) method, creating standard curves. The quantity for each sample was normalized to Gapdh.

Chromatin immunoprecipitation. Confluent EUSCs were synchronized with DXM and then harvested at 48 h and 3.0 × 10⁶ cells were used per one IP reaction. Chromatin immunoprecipitation (ChIP) assay was performed by using SimpleChIP Plus enzymatic chromatin IP kit (Cell Signaling Technology, Beverly, MA), as instructed by the manufacturer’s protocol. Briefly, cells were fixed with 1.0% formaldehyde for 10 min to cross-link proteins to DNA, and the cross-linking was stopped with glycine. The extracted genome DNA was digested for 20 min with 0.5 μl micrococcal nuclease. After centrifugation at 13,000 rpm for 1 min, the nuclear pellet was suspended in the ChIP buffer containing protease inhibitors and lysed with sonication (3 pulses, 20 s). After determining DNA concentration, the cross-linked chromatin (2 μg) was incubated overnight at 4°C with an REV-ERBα antibody (5 μg/500 μl; Cell Signaling Technology) as the negative control and incubated with Protein G agarose beads for 2 h. The chromatin was eluted from the agarose beads by incubating for 30 min at 65°C. Apg 2 h. Purified DNA was amplified by PCR with specific primer set as follows: Bmp2: forward 5’-GAAGAGTGATCGG-ACC-3’ and reverse 5’-GTGAGCAGCTTTTGTGA-3’ (AC_000071.1); Bmp4: forward 5’-GTGAGCAGCTTTTGTGA-AATTCCTGGAATA-3’ and reverse 5’-GGGTACCCTGGCCTGGGAAAT-3’.

F, forward; R, reverse.

Table 1. siRNA sequences targeting Bmal1 mRNA

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<th>Target Sequence 5’-3’</th>
<th>siRNA Sequence 5’-3’</th>
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<tr>
<td>siRNA1</td>
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<td>F: GAAAAAGCCCUCGGGACAATTTT</td>
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<tr>
<td>siRNA2</td>
<td>CAGUAAAGUGGAGAIAAA (1358–1376)</td>
<td>R: GAAAAAGCCCUCGGGACAATTTT</td>
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<tr>
<td>siRNA3</td>
<td>GAGAAAAGAICAGACGAC (1775–1793)</td>
<td>R: GAAAAAGCCCUCGGGACAATTTT</td>
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<tr>
<td>Nonsilencing RNA (control)</td>
<td></td>
<td>R: GAAAAAGCCCUCGGGACAATTTT</td>
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Table 2. Primer sequences for the targeted genes in real-time-quantitative PCR and RT-PCR

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<th>Gene</th>
<th>Accession No.</th>
<th>Primer Sequence 5’-3’</th>
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<td>Bmal1</td>
<td>NC_005100</td>
<td>GACCTGGACCAAGGAAATGAGA</td>
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<tr>
<td>Rev-erba</td>
<td>NM_031313</td>
<td>GACAGTCGACACCACCCAGACTC</td>
</tr>
<tr>
<td>Per2</td>
<td>NM_031678</td>
<td>CACCCCTCACTGGATAGTGA</td>
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<td>Bmp1</td>
<td>NM_031323</td>
<td>TGAGGTGAATGCGGTGAAGG</td>
</tr>
<tr>
<td>Bmp2</td>
<td>NM_017178</td>
<td>GTGTCTGTTAGGCTGTGAC</td>
</tr>
<tr>
<td>Bmp4</td>
<td>NM_012827</td>
<td>GAGGCCCTGCTCATTTTTGG</td>
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<td>Bmp6</td>
<td>NM_013107</td>
<td>AATTCGAGGCACCTTTCTTTA</td>
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<td>Bmp7</td>
<td>NM_001191856</td>
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<td>Bmp8a</td>
<td>NM_001109432</td>
<td>GGAGGCAGCTTGCAGTTCAG</td>
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<td>NM_022846</td>
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<td>Gapdh</td>
<td>NM_017008</td>
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Amplicon, bp

97 102 90 118 147 124 78 154 141 178 122

doi:10.1152/ajpcell.00220.2014 • www.ajpcell.org
Differences were considered significant at \( P < 0.05 \) using SigmaPlot software (Ver. 11.2; Systat Software, San Jose, CA).

RESULTS

Expression levels of the Bmp genes in UESCs and whole uterus tissues, as revealed by DNA microarray and RT-PCR.

To gain insight into the cellular clocks of UESCs and the circadian regulation of physiology, the global expression of the Bmp genes, which are vital for decidualized uterus function, were analyzed by DNA microarray (58). The results are shown in Fig. 1A. The expression levels of Bmp1, Bmp2, Bmp4, Bmp6, Bmp7, and Bmp8a, designated as core Bmp genes, were relatively high, while the expression levels of Bmp3, Bmp5, Bmp10, and Bmp15 were undetectable. Of the core Bmp genes, alterations of Bmp2, Bmp4, and Bmp7 transcripts were significant \((P < 0.01)\). We also confirmed the detection of the transcripts of core Bmp genes in the uteri of E4.5 of pregnant rats by RT-PCR (Fig. 1B). The core Bmp genes were analyzed in the following experiments.

Downregulation of Per2 oscillation and core clock gene expression in UESCs undergoing decidualization. To downregulate the circadian clock in UESCs, the in vitro decidualization of UESCs was induced by MPA and db-cAMP. The differentiating status was revealed by high expression of the Prl8a2 gene, a useful marker of decidualization (Fig. 2A). A marked expression of Prl8a2 was detected during the period of the second phase \((P < 0.01)\). The subsequent bioluminescent monitoring displayed a pattern of Per2-dLuc bioluminescence in the decidual cells that was distinct from that in the control cells. A significant decline of Per2-dLuc bioluminescence and a significantly decreased oscillation amplitude \([P < 0.05, \text{ vs control (CONT)}]\) were observed in the decidual cells. However, there was no significant effect of decidualization on the period of the second phase. In addition, we evaluated the expression of canonical clock genes. As the results are shown in Fig. 2B, Bmal1 and Rev-erba expression displayed an antiphase rhythmic profile in the control cells. The Per2 transcript rhythms were also out of phase with the Bmal1 rhythm.

Expression levels of core bmp genes in decidual UESCs. To gain understanding of the physiological function of the cellular circadian clockwork under the regulation of circadian oscillators, we evaluated the expression profiles of core Bmp genes in decidual UESCs induced by MPA and db-cAMP. The expression levels of the core Bmp genes (Bmp2, Bmp4, Bmp6, and Bmp7) were significantly increased in the decidual cells compared with those in the control cells \((P < 0.01)\) (Fig. 3). The Bmp8a transcript displayed a large oscillation but was not significantly altered compared with the control cells. In contrast, the expression level of Bmp1 was not significantly affected by decidualization. The transcripts of Bmp6, Bmp7, and Bmp8a displayed similar rhythmic patterns.
mic profiles, with a peak of expression at ~35 h after synchronization. Conversely, Bmp2 expression displayed a circadian pattern with phases that opposed those in Bmp6, Bmp7, and Bmp8a that peaked at about 40 h.

Effect of Bmal1 knockdown on the expression of core bmp genes in UESCs. Given the critical role of Bmal1 in sustaining the cellular circadian rhythm, Per2-dLuc oscillations were investigated using UESCs transfected with the presence of
**Bmal1** siRNA (siRNA) or nonsilencing RNA (CONT). The results are shown in Fig. 4. Both the cell groups generated several Per2-dLuc oscillations. A decline of Per2-dLuc bioluminescence generation and a decreased oscillation amplitude (*P* < 0.05, vs. CONT) in siRNA-treated cells were observed (Fig. 4A). However, no significant expression of Prl8a2 was increased in siRNA-treated cells at 46 h after synchronization (Fig. 4B). This result suggests that no decidualization was induced by Bmal1 knockdown. The transcript levels of canonical clock genes (Bmal1 and Rev-erba) were analyzed in UESCs treated with siRNA (Fig. 4C). After transfection with siRNA, the Bmal1 transcript level was reduced by 50% compared with that in the CONT group (*P* < 0.01). Concomitantly, Rev-erba was significantly downregulated by Bmal1 silencing and displayed no rhythmic profile, as revealed by cosinor analysis. We next evaluated the expression of core Bmp genes in UESCs with or without siRNA treatment. The transcript levels of Bmp genes, such as Bmp2, Bmp4, and Bmp6 were significantly increased in the siRNA treatment group compared with those in the CONT group (*P* < 0.01) (Fig. 5). The Bmp7 transcript level was slightly increased in the siRNA treatment group, but this increase was not significant. Conversely, the transcript levels of the other Bmp genes (Bmp1 and Bmp8a) were not affected by Bmal1 silencing.
Effect of the antagonist SR8278 treatment on the expression of core bmp genes in UESCs. To further understand the physiological function of the UESC circadian clockwork and to detect whether core Bmp genes are controlled under the regulation of REV-ERBa, we evaluated the expression of canonical core clock genes with or without the antagonist (SR8278) treatment. The results are shown in Fig. 6. A decline of Per2-dLuc bioluminescence expression and significantly decreased oscillation amplitude were observed in SR8278-treated cells (P < 0.05, vs. CONT) (Fig. 6A). However, no significant expression level of Prl8a2 was increased by the antagonist treatment compared with those in the CONT group (Fig. 6B). This result indicates that no decidualization was induced after the antagonist treatment. The transcript levels of the Bmal1 gene were significantly enhanced after treatment with SR8278 (P < 0.05) (Fig. 6B). Conversely, the Rev-erba transcript level was significantly downregulated in the SR8278-treated cells (P < 0.05). Interestingly, the transcript levels of all of the core Bmp genes examined were greatly enhanced in the SR8278 treatment group compared with those in the CONT group (P < 0.01) (Fig. 7). The transcripts of Bmp4, Bmp6, and Bmp7 showed parallel rhythmic profiles with a peak of expression at
In rodents and humans, the UESCs undergo proliferation and differentiation into decidual cells, and the placenta is ultimately formed. The UESCs isolated from pregnant rats on day 4.5 of gestation are proliferative in culture. In vitro decidualization of the UESCs induced by MPA and db-cAMP results in attenuation of the rhythmic expression of canonical clock genes (Bmal1, Rev-erba, and Per2), as well as the generation of Per2-dLuc oscillations (Fig. 2). The antiphasic expression patterns of clock genes in the UESCs indicate that uterus oscillators share a similar functional molecular mechanism with the suprachiasmatic nucleus. In consequence, the present data support the previous finding that the normal circadian clockwork is disturbed during cellular differentiation (5, 22).

Despite the clear evidence that canonical clock genes are expressed in UESCs, the impact of these genes on uterus function remains obscure. Therefore, it is valuable to elucidate the Bmp gene family as putative CCGs under the direct regulation of uterus oscillators. It is well known that the BMP gene family is spatiotemporally expressed in the mouse uterus during the progressive phases of implantation (43, 67). The Bmp2 gene is expressed within the decidual area surrounding the site of blastocyst attachment (36, 67) and plays a critical role in decidualization (36). It was reported that progesterone has a critical role in Bmp2 expression (7). However, the mechanism underlying the regulation of Bmp gene expression in UESCs remains largely unclear. To screen the Bmp gene family implicated by uterus oscillators, we listed a series of genes from the DNA microarray data for the Bmp genes (Fig. 1) (58) and

$$-\text{35 h after synchronization. Conversely, Bmp1 and Bmp2 showed parallel rhythmic patterns with a peak of expression at } \sim 40\text{ h.}$$

**REV-ERBα binding to the putative RORE sites in upstream regions from transcriptional start sites of Bmp2 and Bmp4.** ChIP-PCR analysis was performed to confirm that REV-ERBα exerted an inhibitory role in Bmps expression through direct binding to the RORE sites. We searched putative RORE-half elements within 3,000 bp upstream regions from transcriptional start sites of Bmps (Table 3). Of them, Bmp2 and Bmp4 were selected as representative genes, because these were highly responsive to SR8278 treatment and putative RORE sites located at their proximal regions (Fig. 8A). We prepared the protein/DNA cross-linked chromatin and immunoprecipitated with a specific REV-ERBα antibody and normal rabbit IgG (negative control). ChIP-PCR analysis revealed that specific bands were detected in both Bmp2 and Bmp4 samples precipitated with anti-REV-ERBα antibody (Fig. 8B). However, a trace amount of PCR product was detected in the Bmp2 sample precipitated with normal rabbit IgG, probably due to its nonspecific amplification. These results indicated that REV-ERBα directly bound to at least AGGTCA-429/-434 in the Bmp2 promoter and TGACCT-714/-709 and ACTGGA-643/-638 in the Bmp4 promoter.

**DISCUSSION**

A growing number of studies have shown that peripheral circadian oscillators orchestrate and coordinate integral and unique physiological processes in each of their specific tissues (45, 53). However, the specific physiological functions that are regulated by uterus oscillators remain to be determined. In the present study, we attempted to unravel the implicated physiological roles of uterus oscillators and focused on putative CCGs, namely the Bmp gene family, under the direct regulation of uterus oscillators using UESCs. The present study demonstrated that core clock genes, such as Bmal1, Rev-erba, and Per2 are downregulated in UESCs during the in vitro decidualization induced by MPA and db-cAMP. Concomitantly, the expression levels of core Bmp genes, including Bmp2, Bmp4, Bmp6, Bmp7, and Bmp8α were significantly increased in the decidual cells. Bmal1 silencing induced significant increases in the expression levels of Bmp2, Bmp4, Bmp6, and Bmp7. In addition, we demonstrated that the clock protein REV-ERBα negatively regulates the expression of core Bmp gene family members, including Bmp1, Bmp2, Bmp4, Bmp6, Bmp7, and Bmp8α.

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**Table 3. Number of putative clock-controlled elements E-box and RORE at the 5′-upstream of the Bmp genes**

<table>
<thead>
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<th>Accession No.</th>
<th>Protein</th>
<th>No. of sites (to −3000)*</th>
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<td>Bmp1</td>
<td>NC_005114.3</td>
<td>bone morphogenetic protein 1</td>
<td>3</td>
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<td>Bmp2</td>
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<td>Bmp4</td>
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<td>Bmp6</td>
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<td>3</td>
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<td>Bmp8α</td>
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*E-box, 5′-(A/C)ACGT(G/T)-3′; RORE, 5′-(A/G)GGTCA-3′; 5′-TGACCT(C/T)-3′.

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Fig. 8. ChIP-PCR analysis of the putative RORE sites in upstream regions from transcriptional start sites of Bmp2 and Bmp4. A: putative RORE-half elements located in upstream regions from transcriptional start sites of Bmp2 and Bmp4 are listed. B: confluent UESCs (3.8 × 10⁶ cells) were synchronized with DXM and then harvested at 48 h and fixed with formaldehyde to cross-link proteins to DNA, as described in MATERIALS AND METHODS. The extracted genome DNA was digested with micrococcal nuclease into fragments. After DNA purification, the cross-linked chromatin (2 μg) was used for immunoprecipitation using an REV-ERBα antibody and normal rabbit IgG (negative control). Purified DNA was amplified with specific primer sets for Bmp2 and Bmp4. Input, input sample; IgG, negative control; Ab, anti-REV-ERBα antibody.
confirmed their transcript expression in whole uterus samples at E4.5 of pregnant rats. On the basis of microarray and RT-PCR analysis, Bmp1, Bmp2, Bmp4, Bmp6, Bmp7, and Bmp8α were designated as core Bmp genes in UESCs. Of the core Bmp genes, Bmp2, Bmp4, and Bmp7, but not Bmp1, Bmp6, and Bmp8α, displayed significant changes. This finding suggests that differential regulation of the core Bmp genes occurs in UESCs. In addition, core Bmp genes displayed rhythmic expression and increased amplitude of their mRNA levels in the decidual uterus except for Bmp1. In consideration of the above-mentioned reports and our findings, it was expected that Bmal1 knockdown in cultured UESCs, which impairs the uterus oscillators, would provide evidence concerning the expression of a series of Bmp genes as putative CCGs.

Bmal1 is indispensable in sustaining the transcriptional-translational feedback loop. Recent genetic studies strongly suggest an important role of Bmal1 in mammalian reproductive physiology (8, 38, 46). Bmal1−/− mice are infertile with implantation failure and impaired steroidogenesis. However, it remains elusive how the uterus oscillator coordinates and regulates uterus physiological events such as implantation and placentation.

The present results revealed that Bmal1 knockdown with siRNA significantly diminishes the expression level of a core oscillator gene (Rev-erba), as well as the amplitude of Per2-dLuc oscillations (Fig. 4). Our results are consistent with those of a previous study, which found that Rev-erba mRNA reaches baseline levels over 24 h at diestrus or across the estrus cycle in the ovaries of Bmal1−/− mice (8). In the present study, it is noteworthy that Bmal1 silencing significantly enhanced Bmp2, Bmp4, Bmp6, and Bmp7 mRNA expression (Fig. 5), providing further evidence that cellular oscillators negatively regulate these genes. In contrast, Bmal1 silencing did not have an impact on altering Bmp8α mRNA expression, which is not consistent with their increased expression in decidual cells (Fig. 3). One possible explanation for this inconsistency is that the transcriptional activation of Bmal1 and Clock heterodimer is weak or their expression is regulated by other clock components. More recently, it was reported that Bmp4 is negatively regulated through upregulation of the transcriptional factor and clock protein Nfil3/E4BP4 in osteoblasts (24).

REV-ERBα is an important nuclear receptor in the regulation of cell physiology. REV-ERBα was recruited to the target genes promoter by its binding to the agonist heme (45, 66). Recent studies have consistently shown that REV-ERBα is a transcriptional silencer (20, 44, 64). Conversely, the REV-ERBα antagonist SR8278 can increase the transcription of REV-ERBα target genes rather than the repression (30). Here, we analyzed the Bmp gene family expression in cultured UESCs using the antagonist SR8278. This synthetic antagonist stimulates the expression of REV-ERBα target genes involved in gluconeogenesis (30). In the present study, Bmal1 expression was significantly increased by SR8278 (Fig. 6) due to the decreased negative feedback potency of REV-ERBα. Similarly, expression of the entire core Bmp genes was increased in UESCs cultured with SR8278 (Fig. 7). This upregulation may result from the decreased potency of REV-ERBα through competitive inhibition of its heme binding, but not the increased expression of Bmal1. Actually, many putative RORE [5′- (A/G)GGTCA-3′ or 5′-TGACC(C/T)-3′] sites commonly exist at the 5′-upstream region of all the Bmp genes family, while there are a few canonical E-box (5′-CACGTG-3′ or 5′-CACGTT-3′) sites in Bmp1, Bmp6, and Bmp8α (Table 3). Inhibition of REV-ERBα binding to these putative RORE sites may induce the upregulation of the entire core Bmp genes. The majority of Bmp gene family members (Bmp1, Bmp2, Bmp4, Bmp6, and Bmp7) displayed rhythmic transcript levels as revealed by cosinor analysis, indicating that uterus oscillators may regulate the decidualization process through negative regulation of Bmp gene expression. However, Bmal1 silencing and SR8278 treatment did not induce the differentiation of stromal cells into decidual cells. No induced decidualization may result, at least in part, from the short treatment duration, but this issue remains to be investigated. In addition, from the results of highly expressed Bmp genes in the uterus and UESCs treated with the REV-ERBα antagonist, we finally analyzed the binding of REV-ERBα to their upstream regions from the transcriptional start sites of Bmp2 and Bmp4. ChIP-PCR analysis revealed that Bmp2 and Bmp4 have the binding sites of REV-ERBα on the proximal regions (Fig. 8). The present study indicates that REV-ERBα is recruited to the Bmp genes promoter in cultured UESCs and is directly bound to, at least, AG-GTCA−429/−434 in the Bmp2 promoter and TGACCT−714/−709 and ACTGGA−643/−638 in the Bmp4 promoter, located in the proximal regions from transcriptional start sites. Consequently, REV-ERBα functions as a transcriptional silencer in the Bmp gene expression.

In conclusion, our results provide new evidence regarding the physiological function of uterus oscillators. To the best of our knowledge, the present study is the first to probe uterus
CCGs in pregnant rat UESCs in vitro with a combination of Bmal1 interference and the REV-ERβ antagonist SR8278. Our findings suggest that the core Bmp genes are controlled under cellular circadian clocks, and especially REV-ERβ suppresses their transcriptions by binding to the RORE sites (Fig. 9). Also, the transcriptional activation of the BMAL1 and CLOCK heterodimer still works. The attenuation of uterus expression and, in turn, be a trigger for the increased expression of core Bmp genes and decidualization.

REFERENCES


