Interaction of ovarian steroidogenesis and clock gene expression modulated by bone morphogenetic protein-7 in human granulosa cells

Satoko Nagao1), Nahoko Iwata1), Yoshiaki Soejima1), Takaaki Takiguchi1), Tamami Aokage1), Yuka Kozato1), Yasuhiro Nakano1), Takahiro Nada1), Toru Hasegawa2) and Fumio Otsuka1)

1) Department of General Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Kitaku, Okayama 700-8558, Japan
2) Department of Obstetrics and Gynecology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Kitaku, Okayama 700-8558, Japan

Abstract. A functional link between clock gene expression and ovarian steroidogenesis was studied using human granulosa KGN cells. Similarities between changes in the mRNA and protein expression levels of Bmal1 and Clock and those of Per2 and Cry1 were found in KGN cells after treatment with forskolin. Among the interrelationships between the expression levels of clock and steroidogenic factors, Clock mRNA had a strongly positive correlation with P450arom and a negative correlation with 3βHSD. Knockdown of Clock gene by siRNA resulted in a significant reduction of estradiol production by inhibiting P450arom expression, while it induced a significant increase of progesterone production by upregulating 3βHSD in KGN cells treated with forskolin. Moreover, BMP-7 had an enhancing effect on the expression of Clock mRNA and protein in KGN cells. Thus, the expression levels of Clock, being upregulated by forskolin and BMP-7, were functionally linked to estradiol production and progesterone suppression by human granulosa cells.

Key words: Bone morphogenetic protein (BMP), Clock, Granulosa cells, Steroidogenesis

IN MAMMALS, the expression of clock-related genes has been demonstrated in tissues composing the axis of the hypothalamic-pituitary-gonadal system [1-3]. Functional roles of the hypothalamus in the biological timings and rhythms for control of the reproductive axis have been gradually recognized. However, the significance of clock-related genes expressed in ovarian follicles and granulosa cells has remained unclear.

Results of recent studies have shown that clock genes are expressed in the ovary, and accumulated findings have demonstrated a significant interrelationship between clock expression and follicular development [1, 2, 4]. It has been reported that expression changes of clock genes are undetectable in primordial and preantral follicles but can be detected at the timing of early antral stages of follicles and then become clearer at the late antral stages to preovulatory follicles [5-7]. In the ovarian follicles, clock genes are expressed in granulosa cells, theca cells, oocytes and stromal cells composing the developing follicles [5-7]. The alteration of clock gene expression in ovarian follicles may contribute to setting up the timing of ovulation. Dysfunction of clock gene expression in the ovary and desynchronicity between ovarian and extra-ovarian clocks may cause various reproductive abnormalities. However, the regulatory mechanism and physiological roles of clock-related genes expressed in the ovary have remained uncertain.

There are a variety of reproductive regulators such as growth factors, cytokines and hormones that can be locally activated in the ovary. In mammals, it has been shown that various growth factors expressed in the ovary play important roles in female fertility via an autocrine and/or paracrine mechanism [8, 9]. The activity of local factors such as bone morphogenetic proteins (BMPs),
growth differentiation factors (GDFs), and activins and inhibins, by cooperating with gonadotropins, is critical for growth and maturation of the follicles. The molecules of BMP system comprised of functional units of BMP ligands and their receptors are expressed in growing follicles in a cell-specific manner. The BMP system mainly regulates follicle-stimulating hormone (FSH)-receptor activity in granulosa cells, which can be linked to the fine-tuning of ovarian folliculogenesis [8-11].

In the present study, we investigated the biologic effects and functional roles of clock genes, including Bmal, Clock, Period (Per) and Cryptochrome (Cry), in the process for regulating folliculogenesis and steroidogenesis in cooperation with ovarian BMPs.

Materials and Methods

Reagents and cell culture

Forskolin (FSK) and 4-androstene-3,17-dione were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO); and recombinant proteins of human BMPs were from R&D Systems Inc. (Minneapolis, MN). Human granulosa KGN cells, originating from a human ovarian granulosa-like tumor cell line [12-14], were cultured in DMEM/F12 containing 10% FCS at 37°C in a condition with 5% CO₂.

Quantitative RT-PCR analysis

KGN cells (1 × 10⁵ cells/mL) were treated with forskolin (1 μM) or BMPs (100 ng/mL) in 12-well plates containing serum-free DMEM/F12 for the indicated periods. Concentrations of forskolin and BMP ligands used in the current experiments were selected based on our earlier data obtained from the same in vitro experiments [14-17]. Total cellular RNA was extracted using TRI Reagent® (Cosmo Bio Co., Ltd., Tokyo, Japan) and the concentration of extracted RNA were determined by NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Primer pairs for detecting the following genes: steroidogenic acute regulatory protein (StAR), steroid side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD), aromatase (P450arom), and a housekeeping gene, ribosomal protein L19 (RPL19), were utilized as we earlier reported [13, 15-19]. Other primers were also selected from different exons to eliminate PCR products originated from chromosomal DNA as follows: 996–985 and 1,099–1,118 for Bmal1 (from GenBank accession #AB000812); 2,072–2,091 and 2,262–2,281 for Clock (NM_001267843); 1,614–1,633 and 1,870–1,889 for Per2 (NM_0022817); and 2,203–2,222 and 2,461–2,480 for Cry1 (NM_004075). Reverse transcription using ReverTra Ace® (TOYOBO CO., LTD., Osaka, Japan) was performed and proceeded to real-time PCR using the LightCycler® Nano real-time PCR system and LightCycler® 96 System (Roche Diagnostic Co., Tokyo, Japan) after optimizing annealing conditions and amplification efficiency [17]. Determination of target gene mRNA level was performed by the method using Δ threshold cycle (Ct), in which subtraction of the Ct value of RPL19 from that of the target genes was utilized to calculate the ΔCt value. Each target gene mRNA level, normalized by RPL19, was calculated as 2⁻(ΔCt), and the computed data were shown as the ratios of target gene to RPL19 mRNA.

Transient transfection and steroid assay

KGN cells (1 × 10⁵ cells) were cultured in DMEM/F12 containing 10% FCS without antibiotics in 12-well plates. Clock-specific siRNA and control siRNA duplex (10 μM; 30 pmol/well) were transiently transfected to KGN cells for 12 h using the transfection reagents following the manufacturer’s protocol (Santa Cruz Biotechnology, Santa Cruz, CA) [20]. The cells were then incubated with androstenedione (100 nM), a substrate for aromatase, in serum-free DMEM/F12 in the presence or absence of forskolin (1 μM). After 24-h culture, the culture medium was collected and cellular RNA was also isolated. Estradiol and progesterone levels were determined in the supernatant of the collected medium. The RNA was subjected to real-time RT-PCR for the quantification of mRNA levels of Clock, P450arom and 3βHSD as described. The concentrations of estradiol and progesterone were determined in the collected medium using Architect individual kits (Cayman Chemical Co., Ann Arbor, MI, USA) by a chemiluminescent immunoassay (CLIA). In each cell-free culture medium, steroids were undetectable (progesterone content of <10 pg/mL and estradiol content of <15 pg/mL).

Western immunoblotting

KGN cells (1 × 10⁵ cells/mL) were pretreated with the indicated concentrations of forskolin or BMP-7 in serum-free DMEM/F12 for 24 h. RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY), which contains 1 mM Na₃VO₄, 1 mM NaF, 2% SDS and 4% β-mercaptoethanol, was used to collect the cell lysates. The collected lysates were applied for SDS-PAGE/immunoblotting analysis by using antibodies against Clock, Bmal1, Per2, Cry1 (Santa Cruz Biotechnology, Santa Cruz, CA), and actin (Sigma-Aldrich Co. Ltd.). The C-DiGit® Blot Scanner System (LI-COR Biosciences, NE) was utilized to analyze the signal density of each band. The ratios of the signal intensities for the target protein levels normalized by actin levels were shown to evaluate the changes of target protein levels.
Statistical analysis

All the results are shown as means ± SEM based on the data from more than three independent experiments with sample triplication. The results were statistically analyzed by ANOVA with Fisher’s protected least significant difference (PLSD) or unpaired $t$-test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). $P$ values <0.05 were accepted as statistically significant.

Results

We first examined the changes of clock gene expression after stimulation of human granulosa KGN cells with forskolin in serum-free conditions for 24 h. As shown in Fig. 1A, the expression patterns of Bmal1 and Clock mRNA were similar with a peak increase of mRNA at 6 h after forskolin (1 μM) stimulation. The mRNA changes of Per2 and Cry1 were also similar, but the mRNA levels were decreased 1–3 h after treatment with forskolin (1 μM; Fig. 1A). The changes in protein levels for Clock and Bmal1 and those for Per2 and Cry1 were also found to be similar, with Clock and Bmal1 protein levels being increased and Per2 and Cry1 protein levels being decreased by forskolin (1 μM) treatment (Fig. 1B).

We next examined the expression correlation between steroidogenic enzymes and clock genes in human granulosa KGN cells. The mRNA levels of StAR, P450scc, 3βHSD and P450arom and clock genes, including Clock, Bmal1, Per2, and Cry1, were examined in KGN cells serially cultured for 24 h in the presence of forskolin (1 μM). As shown in Fig. 2, among these factors, linear regression analysis showed significant correlations between the mRNA expression levels of P450arom and Clock ($R^2 = 0.53$, **$p < 0.01$: $n = 18$) and between the mRNA levels of 3βHSD and Clock ($R^2 = 0.63$, **$p < 0.01$: $n = 13$) in forskolin-treated KGN cells.

To clarify the functional role of Clock expression in granulosa cells, steroidogenetic activity was evaluated in KGN cells in which Clock expression was repressed by the siRNA-targeting method. As shown in Fig. 3A, knockdown of Clock expression by siRNA significantly reduced Clock mRNA levels by 62% and 32% in basal and forskolin (1 μM)-induced conditions, respectively,
after 24-h treatment. The Clock suppression resulted in significant reduction of forskolin-induced P450arom mRNA by 32%, while it resulted in significant enhancement of forskolin-induced 3βHSD mRNA by 270% in KGN cells, suggesting that Clock expression is positively correlated with aromatase expression and negatively related to 3βHSD expression (Fig. 3A). In accordance with the results for mRNA levels, forskolin-induced estradiol production was suppressed and forskolin-induced progesterone production was in turn augmented in the culture medium of KGN cells treated with Clock-siRNA (Fig. 3B).

To know the functional link between the ovarian BMP system and Clock induction, we examined the effects of BMPs (100 ng/mL) on Clock mRNA expression by KGN cells for 24-h culture. As shown in Fig. 4A, BMP-2, -4, -6 and -7 moderately increased Clock mRNA levels, whereas BMP-9 had no effect on Clock mRNA levels. BMP-7 induced a significant increase in Clock mRNA expression, the effect being time-dependently observed during 24-h culture (Fig. 4B). As shown in Fig. 4C, the protein level of Clock expression was also increased by treatment with BMP-7 (100 ng/mL) for 24 h.

**Discussion**

The functional role of the molecular clock in the ovary is known to be associated with steroidogenesis, folliculogenesis, cellular differentiation, responsiveness to gonadotropins, and ovulation [4, 21]. In granulosa cells, the existence of an autonomous function of the clock and the expression of various related genes for follicular development including FSH-receptor signaling, luteinizing hormone (LH) receptor and steroidogenic enzymes have been reported [22-24]. For instance, knockout of Bmal1 was reported to severely attenuate progesterone secretion in mice [25]. Conditional deletion of the Bmal1 gene in steroid hormone-producing cells of the ovary also resulted in decreased progesterone synthesis and implantation failure in female mice [26], suggesting a role of Bmal1 expression in progesterone induction from luteinized granulosa cells and maintenance of the post-ovulatory progesterone surge.

On the other hand, shRNA-mediated knockdown of Clock in the ovary was shown to lead to reduced litter size and oocyte release in female mice [27]. Clock and Per2 genes are also expressed in dominant antral follicles
in the human ovary [28] and, of interest, testosterone can induce expressional changes of Per2 and StAR. This implies a potential relationship between hyperandrogenemia and a disorder of steroidogenesis in granulosa cells leading to the development of polycystic ovary syndrome (PCOS) via alteration of the expression pattern of clock genes [28, 29]. It has also been reported that Per2 and Clock differentially affect granulosa cell functions, including cell proliferation, steroid production and LH receptor expression, that are associated with follicular selection and recruitment of follicles [30].

In the present study, a functional link between clock gene expression and ovarian steroidogenesis was uncovered using human granulosa KGN cells (Fig. 4D). Since the expression level of functional FSH receptor is known to be very low in KGN cells [13], forskolin, instead of FSH, was utilized to investigate the effect of clock gene expression on steroidogenesis. Of note, Clock mRNA had a strongly positive correlation with P450arom expression, whereas it showed a negative correlation with 3βHSD expression. Inhibition of Clock gene expression by siRNA transfection suppressed estradiol production by impairment of P450arom expression, while it enhanced progesterone production by upregulation of 3βHSD in KGN cells treated with forskolin. Moreover, it was shown that BMP-7 treatment induced Clock expression in KGN cells. Thus, the expression level of Clock, being upregulated by forskolin and BMP-7, was revealed to functionally contribute to estradiol production as well as progesterone reduction by human granulosa cells (Fig. 4D).

In this regard, the accumulating evidence has shown that BMPs play key roles in female fertility for mammals as autocrine/paracrine factor [8-10]. BMP ligands expressed in ovarian follicles differentially regulate FSH-induced steroidogenesis by granulosa cells in a ligand-dependent manner. Among the BMP ligands that reside in the ovary, BMP-4 and -7 expressed in theca cells can regulate steroidogenesis by increasing FSH-induced estradiol production and suppressing progesterone production by granulosa cells [8-10], in which BMP-7 also promotes the recruitment process of primordial follicles into the growing follicular pool [31]. BMP-6 inhibits progesterone synthesis by suppressing adenylyl cyclase activity induced by FSH [32], although it has no mitotic activity on granulosa cells. BMP-7 actions on FSH-induced estradiol production occur via suppression of the ERK1/2 pathway induced by FSH.

Fig. 3 Inhibitory effects of Clock gene expression by siRNAs on steroidogenesis by granulosa cells. KGN cells (1 × 10^5 cells/mL) were transiently transfected with siRNAs (10 μM) for Clock or control gene and incubated with androstenedione (100 nM) in the presence or absence of FSK (1 μM) for 24 h. A) Total cellular RNAs were then extracted and the mRNA levels of P450arom, 3βHSD and Clock were examined. B) Estradiol and progesterone levels were determined in the supernatant of the collected medium. Results are shown as means ± SEM. The results were analyzed by ANOVA. *p < 0.05 vs. control groups and between the indicated groups.
BMP-2 and -4 stimulate FSH-induced p38-MAPK signaling, leading to an increase of estradiol synthesis [33]. Not only the local BMP ligands expressed in the ovary but also circulating BMP-9 are similarly involved in the suppression of progesterone synthesis in granulosa cells [19].

In the present study, we found that, among the BMP ligands examined, BMP-7 significantly upregulated the expression of Clock mRNA and protein by human granulosa KGN cells. Given that Clock expression is functionally linked to enhancement of estradiol production and reduction of progesterone production by human granulosa cells, the regulatory effects of BMP-7 on ovarian steroidogenesis [33] might be, at least in part, involved in the upregulation of endogenous Clock gene expression in granulosa cells. The mechanism by which BMP-7 regulates ovarian steroidogenesis was previously studied by using rat primary granulosa cells, in which BMP-7 was found to suppress FSH-induced progesterone synthesis by inhibiting StAR expression and to increase FSH-induced estradiol synthesis by upregulating P450arom expression [31, 33]. In the present experiments, it was found that BMP-7 affected the levels of 3βHSD expression rather than StAR, though this discrepancy could be
due to the characteristic differences between rat primary granulosa cells and human KGN cells. As other humoral modulators related to ovarian steroidogenesis, androgens [15], incretins [16], orexins [17] and melatonin [35], which can affect endogenous BMP-Smad signaling activity in granulosa cells [36, 37], may also be involved in the modulation of Clock gene expression in the ovary. Further studies are necessary to determine the functional interaction between the activities of Clock-related molecules and ovarian steroidogenesis.

Collectively, the results have revealed a new link between clock gene expression and ovarian steroidogenesis induced by forskolin, in which Clock mRNA has a positive correlation with P450arom and a negative correlation with 3βHSD (Fig. 4D). The endogenous activity of Clock could be a clue for determining the steroidogenic property in granulosa cells in the presence of gonadotropins and/or growth factors such as BMP-7.

Acknowledgements

The present work was supported by Grants-in-Aid for Scientific Research (No. 15K09434 and 18K08479), The Uehara Memorial Foundation (Japan), Ryobi Teien Memory Foundation Award, and Forum on Growth Hormone Research (FGHR) Award.

Disclosure Statement

The authors have nothing to disclose.

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