Original Article

Roles of NR5A1 and NR5A2 in the regulation of steroidogenesis by Clock gene and bone morphogenetic proteins by human granulosa cells.

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Abbreviations:

BMP, bone morphogenetic protein
Cry, cryptochrome
FSH, follicle-stimulating hormone
FSHR, FSH receptor
FSK, forskolin
GDF, growth differentiation factor
LRH-1, liver receptor homolog-1
Per, period
SF-1, steroidogenic factor-1
StAR, steroidogenic acute regulatory protein
P450acc, steroid side-chain cleavage enzyme
3βHSD, 3β-hydroxysteroid dehydrogenase
P450arom, aromatase

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Abstract

The functional role of the transcription factors NR5A1 and NR5A2 and their interaction with Clock gene and bone morphogenetic proteins (BMPs) were investigated in human granulosa KGN cells. Treatment with BMP-15 and GDF-9 suppressed forskolin (FSK)-induced steroidogenesis as shown by the mRNA expression levels of StAR and P450scc but not the mRNA expression level of P450arom. Of interest, treatment with BMP-15 and GDF-9 also suppressed FSK-induced NR5A2 mRNA expression. Treatment with BMP-15 suppressed NR5A2 mRNA and protein expression but increased Clock mRNA and protein expression levels by granulosa cells. The mRNA expression levels of NR5A1, but not those of NR5A2, were positively correlated with the levels of Clock mRNA, while the mRNA levels of Id-1, the target gene of BMP signaling, were positively correlated with those of NR5A1 but not with those of NR5A2. It was also demonstrated that the mRNA expression levels of NR5A1 were positively correlated with those of P450arom and 3βHSD, whereas the mRNA expression level of NR5A2 was correlated with those of StAR and P450scc. Furthermore, inhibition of Clock gene expression by siRNA attenuated the expression of NR5A1, and the mRNA

58 levels of Clock gene were significantly correlated with those of NR5A1. Collectively,
59 the results suggested a novel mechanism by which Clock gene expression induced by
60 BMP-15 is functionally linked to the expression of NR5A1, whereas NR5A2 expression
61 is suppressed by BMP-15 in granulosa cells. The interaction between Clock
62 NR5A1/NR5A2 and BMP-15 is likely to be involved in the fine-tuning of steroidogenesis
63 by ovarian granulosa cells (250 words).
64

Introduction

The NR5A nuclear orphan receptors are crucial regulators for the endocrine and reproductive systems [1]. Despite the similarity of the structures of molecules of NR5A1 and NR5A2, these factors have diverged effects on the reproductive system. These two factors, NR5A1, which is also known as steroidogenic factor-1 (SF-1), and NR5A2, which is also called liver receptor homolog-1 (LRH-1), can recognize the same DNA binding sites, but they elicit diverged effects depending on the target tissues and cells [2]. NR5A1 is mainly expressed in steroidogenic tissues, while NR5A2 is expressed in tissues of endodermal origin and also in the gonads, in which both receptors regulate homeostasis of cholesterol and steroidogenesis, cell proliferation, and stem cell pluripotency accompanying the cofactor interactions [1, 3].

As for the Clock genes in the mammalian reproductive system, the expression profile of Clock genes has been demonstrated in tissues comprising the hypothalamic-pituitary-gonadal axis [4-6]. Despite central roles of the hypothalamus in reproduction control, the functional synchronicity to Clock-related genes expressed in the peripheral tissues has remained unclear. Clock genes are expressed in granulosa and theca cells,

oocytes and stromal cells in developing follicles [7-9]. Defects of Clock gene expression in the ovary and dysfunction or dysregulation of the synchronicity between central and peripheral clocks cause various reproductive abnormalities. In this regard, we have reported that the expression levels of Clock genes are functionally linked to the expression of steroidogenetic factors and enzymes in human granulosa cells [10]. However, the downstream molecules of the Clock action and the functional interaction with NR5A nuclear receptors in the regulation of ovarian steroidogenesis have yet to be clarified.

Recently, it has been shown that local growth factors expressed in the ovary play indispensable roles in female fertility via an autocrine and/or paracrine mechanism in mammals [11, 12]. The activity of local factors such as bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), and activins/inhibins, by cooperating with gonadotropins and steroids, is critical for the growth and maturation of ovarian follicles. The ovarian BMP system mainly regulates follicle-stimulating hormone (FSH)-receptor (FSHR) activity in granulosa cells, which can be linked to the fine-tuning of folliculogenesis through the inhibition of luteinization [11-14].

In the present study, we attempted to elucidate the roles of the transcription factors NR5A1 and NR5A2, which play key roles in reproductive function as well as steroidogenesis, by focusing on the functional link between Clock genes and BMP signaling using human granulosa cells.

Materials and Methods

Reagents and cell culture

Forskolin (FSK) was purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO) and recombinant human proteins of BMP-6, -9, -15 and GDF-9 were obtained from R&D Systems Inc. (Minneapolis, MN). Human granulosa KGN cells, originating from a human ovarian granulosa-like tumor cell line [15-17], were cultured in DMEM/F12 containing 10% FCS at 37°C in a condition with 5% CO_2.

RNA extraction and quantitative RT-PCR

KGN cells (1×10^5 cells/ml) were treated with FSK (1 μM) or BMPs/GDFs (final concentration of 30 ng/ml) in 12-well plates containing serum-free DMEM/F12 for the

indicated periods. Concentrations of FSK and BMP/GDF ligands used in the experiments were chosen on the basis of our earlier data obtained from the same *in vitro* experiments [17-20]. Total cellular RNA was extracted using TRI Reagent® (Cosmo Bio Co., Ltd., Tokyo, Japan) and the concentration of extracted RNA was determined by a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Primer pairs for detecting and amplifying genes for steroidogenic acute regulatory protein (StAR), steroid side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD), and aromatase (P450arom) and a housekeeping gene, ribosomal protein L19 (RPL19), were utilized as we reported previously [16, 18-22]. Other primer sequences were similarly determined from different exons to eliminate PCR products originating from chromosomal DNA as follows: 1503-1522 and 1692-1712 for Clock (AF011568), 801-820 and 1016-1035 for NR5A1 (NM_004959), and 821-840 and 1067-1086 for NR5A2 (NM_205860). Reverse transcription using ReverTra Ace® (TOYOBO CO., LTD., Osaka, Japan) was applied for real-time PCR using the LightCycler® Nano real-time PCR system and LightCycler® 96 System (Roche Diagnostic Co., Tokyo, Japan) after optimizing each annealing condition and the

amplification efficiency [20]. The target gene mRNA level was determined 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. The mRNA level of each target gene was individually normalized by RPL19, calculated as $2^{-(\Delta\Delta Ct)}$, and the data were shown as the ratios of target gene mRNA to RPL19 mRNA.

Transient transfection and quantitative PCR

KGN granulosa cells (1×10^5 cells) were cultured in DMEM/F12 containing 10% FCS without antibiotics in 12-well plates. Either Clock-specific siRNA or control siRNA duplex (10 μM; 30 pmol/well) was transiently transfected to the cells for 12 h following the manufacturer's protocol using transfection reagents (Santa Cruz Biotechnology, Santa Cruz, CA) [23]. The cells were subsequently cultured in serum-free DMEM/F12 in the presence or absence of FSK (1 μM). After 24-h culture, the culture medium was removed and total cellular RNA was extracted, and the isolated RNA was subjected to real-time RT-PCR for the quantification of mRNA levels of Clock, NR5A1, NR5A2 and P450arom as described in the former section.

145

146 *Western immunoblotting*

147 KGN cells (1×10^5 cells/ml) were treated with FSK (1 μM) and BMP-15 (30 ng/ml) in
148 serum-free DMEM/F12 for 24 h and 48 h. RIPA lysis buffer (Upstate Biotechnology,
149 Lake Placid, NY), which contains 1 mM Na_3VO_4, 1 mM NaF, 2% SDS and 4% β-
150 mercaptoethanol, was used to collect the cell lysates. The collected lysates were applied
151 for SDS-PAGE/immunoblotting analysis by using antibodies against Clock (sc-271603:
152 Santa Cruz Biotechnology), NR5A2 (Cat No. GTX106024: GeneTex, Inc. Irvine, CA)
153 and actin (A2066: Sigma-Aldrich Co. Ltd.). The signal density of each band was
154 analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE). The ratios
155 of the signal intensities for the target protein levels of Clock normalized by actin levels
156 were calculated to evaluate the changes of target protein levels.

157

158 *Statistical analysis*

159 All of the results are shown as means ± SEM based on data from at least three independent
160 experiments with sample triplication. The results were statistically analyzed by

ANOVA with Fisher's protected least significant difference (PLSD), unpaired *t*-test and linear regression analysis (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). *P* values <0.05 were accepted as statistically significant.

Results

First of all, we examined the effects of BMPs/GDFs on FSK-induced steroidogenesis. Since the expression level of functional FSHR is known to be very low in KGN cells [16], FSK, instead of FSH, was used to investigate the effect of Clock gene expression on steroidogenesis. As shown in **Fig. 1A**, FSK (1 μM) treatment for 24 h induced mRNA expression of steroidogenic factors and enzymes including P450arom, StAR, P450scc and 3βHSD by KGN cells in a serum-free condition. Treatment with BMP-15 (30 ng/ml) and treatment with GDF-9 (30 ng/ml) suppressed mRNA expression of StAR and P450scc, but not expression of P450arom or 3βHSD, induced by FSK (1 μM). As shown in **Fig. 1B**, the expression of NR5A2 mRNA was upregulated by FSK (1 μM) treatment for 24 h and co-treatment with either BMP-15 (30 ng/ml) or GDF-9 (30 ng/ml) significantly suppressed FSK-induced NR5A2 mRNA expression for 24-h culture.

177 On the other hand, the expression level of NR5A1 mRNA was not significantly altered
178 by treatment with FSK in combination with BMP-15 or GDF-9 (**Fig. 1B**).

179 Next, to investigate the effects of BMP-15 on the expressional changes of
180 NR5A1, NR5A2 and Clock, KGN cells were treated with BMP-15 in a serum-free
181 condition without adding FSK. As shown in **Fig. 2,** the mRNA level of NR5A2, but not
182 that of NR5A1, was significantly reduced by treatment with BMP-15 (30 ng/ml) for 24 h
183 (**Fig. 2A**). Furthermore, the protein level of NR5A2 reached a significant reduction by
184 48-h treatment with BMP-15 (30 ng/ml) (**Fig. 2A**). It was also revealed that Clock
185 mRNA and protein levels were significantly increased by treatment with BMP-15 (30
186 ng/ml) for 24 h (**Fig. 2B**).

187 In addition, the interrelationships between the expression levels of Clock, the
188 target gene of BMP signaling Id-1, and NR5A1 or NR5A2 were examined (**Fig. 2C**, **2D**).
189 KGN cells were treated with FSK (1 μM) in combination with each BMP/GDF ligand
190 (30 ng/ml) including BMP-6, -9, -15 and GDF-9 for 24 h, and then extracted RNA was
191 subject to quantitative RT-PCR for determining NR5A1, NR5A2, Clock and Id-1 mRNA
192 levels. As shown in **Fig. 2C**, the mRNA expression level of NR5A1 (R^2=0.26; *P<0.05:

193 n=16), but not that of NR5A2 (R^2=0.058; P=0.35: n=17), was positively correlated with
194 the level of Clock mRNA. Of note, Id-1 mRNA levels were positively correlated with
195 mRNA levels of NR5A1 (R^2=0.39; *P<0.05: n=16), while Id-1 mRNA levels showed a
196 weakly negative correlation with NR5A2 mRNA levels (R^2=0.022; P=0.57: n=17) (**Fig.**
197 **2D**).

198 The interrelationships between expression levels of NR5A1/NR5A2 and
199 steroidogenetic factors/enzymes were also assessed (**Fig. 3**). KGN cells were treated
200 with FSK (1 μM) in combination with each BMP/GDF ligand (30 ng/ml) including BMP-
201 6, -9, -15 and GDF-9 for 24 h, and then quantitative PCR was performed to evaluate the
202 interrelationships between mRNA expression levels of NR5A1/NR5A2 and mRNA
203 expression levels of P450arom, StAR, 3βHSD and P450scc. As shown in **Fig. 3A**,
204 mRNA expression levels of NR5A1 showed strictly positive correlations with mRNA
205 expression levels of P450arom (R^2=0.75; **P<0.01: n=17) and 3βHSD (R^2=0.69;
206 **P<0.01: n=17) and a weak correlation with mRNA expression levels of StAR (R^2=0.31;
207 *P<0.05: n=17). On the other hand, mRNA expression levels of NR5A2 were
208 correlated with those of StAR (R^2=0.60; **P<0.01: n=17) and P450scc (R^2=0.37;

209 $^{**}P<0.01$: $n=17$) (**Fig. 3B**).

210 We next performed knockdown experiments of Clock gene expression by using
211 siRNA in KGN cells (**Fig. 4**). Transfection of Clock-specific siRNA resulted in an
212 average 36% reduction of Clock gene expression compared with that in cells transfected
213 with control-siRNA (**Fig. 4A**). Under the condition of suppressed expression of Clock
214 gene in KGN cells, mRNA levels of NR5A1 were significantly reduced in the presence
215 of FSK (1 μM) for 24 h (**Fig. 4A**). However, the Clock gene suppression did not affect
216 NR5A2 mRNA levels regardless of the treatment with FSK (1 μM). Of interest, the
217 expression level of Clock gene showed a significant positive correlation ($R^2=0.28$;
218 $^{*}P<0.05$: $n=18$) with the mRNA level of NR5A1 rather than P450arom ($R^2=0.26$; $P=0.09$:
219 $n=12$) (**Fig. 4B**).

220

221 **Discussion**

222 In the present study, new roles of the transcription factors NR5A1 and NR5A2
223 in the regulation of ovarian steroidogenesis were demonstrated (**Fig. 4C**). It was notable
224 that the expression level of NR5A1, but not that of NR5A2, was positively correlated

with the expression levels of Clock gene and the BMP-target gene Id-1. The results also suggested a novel mechanism by which Clock expression induced by BMP-15 is functionally linked to the expression of NR5A1, whereas NR5A2 expression is suppressed by BMP-15 in granulosa cells (**Fig. 4C**). The functional interaction among Clock, NR5A1/NR5A2 and BMP-15 is likely to be involved in the fine-tuning of steroidogenesis by ovarian follicles.

In the presence of gonadotropin actions, NR5A receptors act to enhance the activity of various steroidogenic genes encoding StAR, P450scc, P45011b, P450c17 and 3βHSD [24]. NR5A receptors have also been shown to affect the expression of ferrodoxin 1 , which is an electron donor for catalytic activity by P450scc, in granulosa cells [25]. Although it has been reported that NR5A2 plays a critical role in the transcriptional regulation of P450arom as the rate-limiting enzyme for estrogen biosynthesis in the placenta and ovary [26], the functional divergence of NR5A factors was uncovered in the present study; namely, NR5A1 expression was shown to be linked to the activities of P450arom and 3βHSD, while NR5A2 expression was shown to be related to the induction of StAR and P450scc by human granulosa KGN cells.

There have been increasing findings suggestive of interactions between Clock genes and female reproduction. The importance of Clock genes for maintenance of reproductive functions has been indicated by the results of experiments using mutant mice with disruptions of various Clock genes [27]. Bmal1-knockout mice showed attenuated progesterone secretion [28] and deletion of the Bmal1 gene in the ovary also resulted in decreased progesterone synthesis and implantation failure [29]. In cultured human luteinized granulosa cells, it was shown that the expression of Per2, but not that of Clock or Bmal1, displayed oscillating patterns similar to those of StAR expression [30]. A recent clinical study also demonstrated that, among the various circadian genes expressed in cultured human luteinized granulosa cells, only Per1 and Clock had significant trends of decreasing expression with the aging process [31].

In our earlier study using human granulosa KGN cells [10], a functional link between Clock gene expression and ovarian steroidogenesis was uncovered. Of note, Clock mRNA had a strongly positive correlation with P450arom expression [10], and suppression of Clock gene expression induced by siRNA transfection decreased FSK-induced estradiol production by downregulation of P450arom. In the present study,

257 inhibition of Clock gene expression caused by siRNA resulted in attenuated expression
258 of NR5A1 mRNA in the presence of FSK. Of interest, the expression level of Clock
259 gene showed a significant positive correlation with the mRNA level of NR5A1 rather
260 than P450arom, suggesting that NR5A1 is a functional key to regulate ovarian
261 steroidogenesis, especially for estrogen synthesis, under the influence of Clock genes.

262 Accumulating evidence has shown that the BMP system in the ovary plays
263 important roles in female fertility as an autocrine/paracrine factor in mammals [11-13].
264 BMPs are known to regulate FSH-induced steroidogenesis by granulosa cells in a ligand-
265 dependent manner. As the mechanism by which BMP-15 regulates ovarian
266 steroidogenesis, it has been shown that BMP-15 suppresses FSH-induced progesterone
267 production by inhibiting FSHR expression in rat primary granulosa cells [32, 33]. In the
268 present study using human KGN cells, it was found that BMP-15 suppressed mRNA
269 expression of StAR and P450scc induced by FSK, rather than that of 3βHSD and
270 P450arom. Thus, it is most likely that the suppression of NR5A2 expression by BMP-
271 15 led to subsequent reduction of StAR and P450scc expression by human granulosa cells.
272 Moreover, BMP-15 was found to upregulate Clock gene expression, which is directly

linked to the expression of NR5A1, being followed by induction of the expression of P450arom and 3βHSD, by human granulosa cells. In this regard, we recently reported the biological roles of Clock genes in adrenocortical steroidogenesis in cooperation with the adrenal BMP-6 and activin system [34]. Taken together, it is possible that the local BMP system controls the expression of Clock genes that is functionally linked to steroidogenic regulation.

In the present study, all the findings were solely based on the characteristics of KGN cells originated from human granulosa tumor cells. Although KGN cells mimic the characteristics of immature granulosa cells in terms of aromatase activity [15], the cells show much lower expression of functional FSHR and/or weaker FSHR signaling activation compared with those in primary granulosa cells. On the other hand, based on our previous study on this cell line [16], the characteristics of steroidogenesis and its BMP responsiveness have been well preserved. However, further *in vivo* study would be necessary to delineate the molecular interaction between the activities of NR5As, Clock-related molecules and BMPs.

Collectively, the results of the present study suggested a novel mechanism by

289 which Clock expression induced by BMP-15 is functionally linked to the expression of
290 NR5A1, whereas NR5A2 expression leading to StAR and P450scc transcription is
291 suppressed by BMP-15 in granulosa cells. The interaction between Clock,
292 NR5A1/NR5A2 and BMP-15 is likely to be involved in the fine-tuning of steroidogenesis
293 by ovarian follicles (**Fig. 4C**). Given the possibility that BMP-15 regulates other
294 important genes, transcription factors and epigenetic changes, genome-wide analysis
295 would be performed to determine in detail the effects of BMP-15 in ovarian
296 steroidogenesis. Considering that NR5A1/NR5A2 are essential modulators of ovarian
297 function in mammals, this interaction could be a new therapeutic target for treatment of
298 infertility.

299

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Figure Legends:

Fig. 1: Effects of BMP-15 on forskolin (FSK)-induced expression of steroidgenetic enzymes and NR5A1/NR5A2 by human granulosa KGN cells. Human granulosa KGN cells were treated with FSK (1 μM), BMP-15 (30 ng/ml) or GDF-9 (30 ng/ml) in serum-free DMEM/F12 in 12-well plates for 24 h. Total cellular RNAs were extracted and the expression levels of **A**) steroidogenic factors and enzymes and **B**) NR5A1/NR5A2 were determined by real-time qPCR. The target gene mRNA levels were standardized by RPL19 levels and expressed as fold changes. Results in all panels are shown as means ± SEM of data from at least three individual experiments with triplicated samples. The results were analyzed by ANOVA with Fisher's PLSD test. Values indicated with different superscript letters show significant difference at $P < 0.05$.

Fig. 2: Effects of BMP-15 on NR5A1/NR5A2 and Clock expression and the interrelationships between expression levels of NR5A1/NR5A2, Clock and Id-1 mRNAs in granulosa cells. **A**, **B**) Cells were treated with BMP-15 (30 ng/ml) in serum-free DMEM/F12 for 24 h. Total cellular RNAs were extracted and the expression

levels of (**A**) NR5A1/NR5A2 and (**B**) Clock mRNAs were determined by real-time qPCR as mentioned above. The cell lysates were also extracted and subjected to immunoblotting for (**A**) NR5A2 and (**B**) Clock with actin analysis after 24-h and 48-h treatment with BMP-15 (30 ng/ml). The relative integrated density of each protein band was digitized and the density of the Clock band was standardized by that of actin. Results in all panels are shown as means ± SEM of data from at least three individual experiments with triplicated samples. The results were analyzed by the unpaired *t*-test. $*P < 0.05$ and $**P < 0.01$ vs. control groups. **C**, **D**) KGN cells were treated with BMP/GDF ligands (30 ng/ml) and with FSK (1 μM) in serum-free DMEM/F12 for 24 h. Total cellular RNAs were extracted and the expression levels of Clock, Id-1 and NR5A1/NR5A2 mRNAs were determined by real-time qPCR. The target gene mRNA levels were standardized by RPL19 levels and expressed as fold changes. Then linear regression analysis was performed for mRNA expression levels of (**C**) Clock and (**D**) Id-1 genes and NR5A1/NR5A2. $*P < 0.05$ of the significant correlations.

Fig. 3: Interrelationships between expression levels of NR5A1/NR5A2 and

steroidogenetic enzyme mRNAs in granulosa cells. KGN cells were treated with BMP/GDF ligands (30 ng/ml) and with FSK (1 μM) in serum-free DMEM/F12 for 24 h. Total cellular RNAs were extracted and the expression levels of NR5A1/NR5A2, steroidogenic factor and enzyme mRNAs were determined by real-time qPCR. The target gene mRNA levels were standardized by RPL19 levels and expressed as fold changes. Then linear regression analysis was performed for mRNA expression levels between either **A**) NR5A1 or **B**) NR5A2 and steroidogenic factors and enzymes. **P* < 0.05 and ***P* < 0.01 of the significant correlations.

Fig. 4: Inhibitory effects of Clock gene expression on NR5A1/NR5A2 and steroidogenetic enzyme expression and functional interaction of Clock, NR5A1/NR5A2 and BMP-15 by granulosa cells. KGN cells were transiently transfected with siRNAs for Clock or control gene in the presence or absence of FSK (1 μM) for 24 h. **A**) Total cellular RNAs were extracted and the mRNA levels of Clock and NR5A1/NR5A2 were examined. The target gene mRNA levels were standardized by RPL19 levels and expressed as fold changes. Results in all panels are shown as

means ± SEM of data from at least three individual experiments with triplicated samples. The results were analyzed by the unpaired *t*-test and ANOVA with Fisher's PLSD test. Values indicated with different superscript letters show significant difference at $P < 0.05$; and $*P < 0.05$ vs. control groups. **B**) Then linear regression analysis was performed for mRNA expression levels between Clock and either NR5A1 or P450arom. $*P < 0.05$ of the significant correlations. **C**) The results suggested a novel mechanism by which Clock expression induced by BMP-15 is functionally linked to the expression of NR5A1, whereas NR5A2 expression is suppressed by BMP-15 in granulosa cells. The expression level of NR5A1 is correlated with that of P450arom and 3βHSD, while the expression level of NR5A2 is linked to that of StAR and P450scc. These interactions are likely to be involved in the fine-tuning of ovarian steroidogenesis by granulosa cells.