Biochemical and Biophysical Research Communications 412 (2011) 132–135

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Circadian *Clock* genes *Per2* and *clock* regulate steroid production, cell proliferation, and luteinizing hormone receptor transcription in ovarian granulosa cells

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ARTICLE INFO

Article history:
Received 12 July 2011
Available online 27 July 2011

Keywords:
Circadian *Clock*
Steroid hormone
Granulosa cell
Ovary

ABSTRACT

Circadian *Clock* genes are associated with the estrous cycle in female animals. Treatment with *Per2* and *Clock* siRNAs decreased the number of granulosa cells and *LHr* expression in follicle-stimulating hormone FSH-treated granulosa cells. *Per2* siRNA treatment did not stimulate the production of estradiol and expression of *P450arom*, whereas *Clock* siRNA treatment inhibited the production of estradiol and expression of *P450arom* mRNA. *Per2* and *Clock* siRNA treatment increased and unchanged, respectively, progesterone production in FSH-treated granulosa cells. Similarly, expression of *StAR* mRNA was increased by *Per2* siRNA and unchanged by *Clock* siRNA. Our data provide a new insight that *Per2* and *Clock* have different action on ovarian granulosa cell functions.

1. Introduction

Circadian rhythms are associated with reproductive processes in mammals. In mammals, a group of circadian genes that encode transcription regulation factors (*Clock, Cry, Bmal1, Per1* and *Per2*) is expressed in most organs, tissues, and cells. *Per1, Per2*, and *Bmal1* are expressed rhythmically in the rat ovary [1–3]. The rhythmic expression of these genes was observed in both granulosa and theca cells within follicles and corpora lutea [2,3]. In circadian *Clock* gene knockout (KO) mice, mice without *Bmal1* (*Bmal1* KO) completely lack circadian rhythms [4]. Although these studies focused on the relationship between circadian rhythmicity and the ovarian estrous cycle, the effect of circadian *Clock* genes on ovarian cell functions such as steroid production associated with follicular development is still unknown. Since circadian *Clock* genes function as transcription factor [5], we hypothesized that circadian *Clock* genes regulate granulosa cell function to promote follicular development.

In the present study, we examined the functional roles of circadian *Clock* genes *Per2* and *Clock* on bovine granulosa cell functions by using siRNAs knockdown for *Per2* and *Clock*. Here, we report a new insight that *Per2* and *Clock* have different action to granulosa cell function.

2. Materials and methods

2.1. Granulosa cell culture

Ovaries were obtained from cows and heifers at a local abattoir. Granulosa cells from small follicle (<5 mm in diameter) were collected as described previously [6]. Cells were cultured in the culture medium containing 10% FCS for 24 h at 37 °C in 5% CO_2 and then culture dishes (35 mm, Corning®, CellBIND®, Corning Inc Co., USA) were washed with PBS to remove unattached cells. The culture medium was replaced with a medium supplemented with 2% FCS, 1 ng/ml FSH, 0.1 μM androstenedione, and 1 μg/ml insulin. The culture was then maintained for 96 h. Granulosa cell viability was determined by trypan blue exclusion.

2.2. Depletion of endogenous Per2 and Clock using Per2 and Clock siRNAs

The following target sequences were designed using software by B-Bridge International, Inc. (CA, USA): 5′-CCAGAAACCUUUGGCCUAA-3′ (Accession No. XM001254267, nt. 232–252) for the *Per2* gene, and 5′-GGACAAAUCUACUGUUCUA-3′ (Accession No. XM589710, nt. 204–224) for the *Clock* gene. For *Per2* and *Clock* depletion, granulosa cells (1.5×10^5 cells) were first plated in 35 mm culture dishes. After 24 h in culture, the medium was removed, and half of the culture dishes were transfected with *Per2* or *Clock* siRNAs. The remaining dishes were transfected with scrambled siRNAs for *Per2*

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doi:10.1016/j.bbrc.2011.07.058

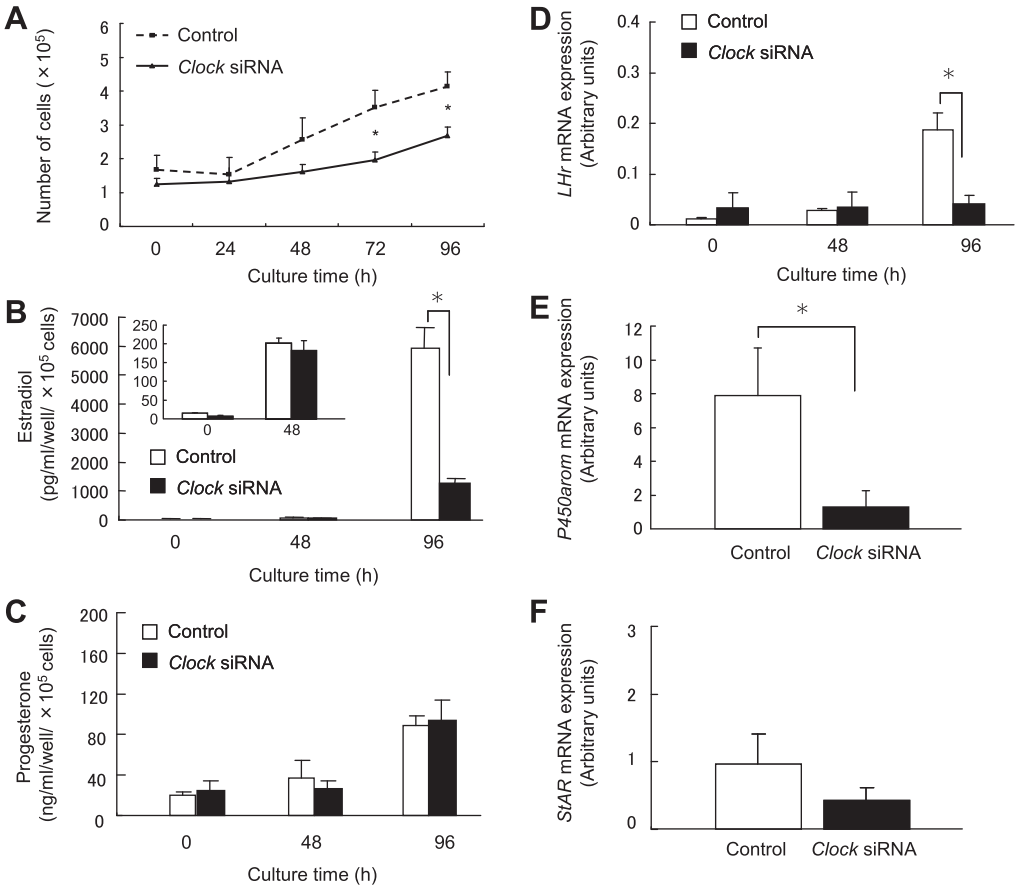

Fig. 1. Role of *Clock* on granulosa cell function. Cell proliferation (A), production of estradiol (B) and progesterone (C), and expression of *LHr* (D) in FSH-treated granulosa cells from follicles (<5 mm in diameter) was determined at the indicated time points (h) of culture. Expression of *P450arom* (E) and *StAR* (F) mRNAs was determined at 96 h after FSH treatment. Data are expressed as mean ± S. E. M. of three separate experiments from triplicate samples at each time point. An asterisk denotes a significant difference at $p < 0.05$. Scramble siRNA is shown as control.

(5′-auccgcguaccauauaacgTT-3′) and *Clock* (5′-cgucaauuaucauaguacgTT-3′) as control. All transfections were performed using the Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. *Per2, Clock*, and scrambled siRNAs were used at final concentrations of 50 nM, 25 nM, and 25 nM, respectively.

2.3. RNA extraction, reverse transcriptase (RT) and quantitative PCR

Total RNA from the cultured granulosa cells were extracted with TRIZOL® reagent (Life Technologies, Inc.) following the method provided by the manufacturer and frozen at −80 °C. Before the RT reaction, all samples (theca tissues and cultured theca cells) were treated with DNase using a commercial kit (SV Total RNA Isolation System). Single-strand cDNA was reverse-transcribed from total RNA using a First Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics Co., Indianapolis, IN, USA) and random primers. The mRNA expression levels of *LHr, P450arom, StAR* and *ß-actin* were quantified by real-time PCR using an iQ5 Cycler (Bio-Rad Laboratories, CA, USA) using a commercial kit (QuantiTectTM SYBR® Green PCR, QIAGEN GmbH, Hilden, Germany). The primer sequences were as follows: *LHr*, forward; 5′-aagaaaatgcacgcctggag-3′ and reverse 5′-gtggcatccaggaggttggt-3′; *P450arom*, forward; 5′-ttgcaaagcatccccaggtt-3′ and reverse 5′-aggtccacaacgggctggta-3′; *StAR*, forward; 5′-gtggattttgccaatcacct-3′ and reverse 5′-ttattgaaaacgtgccacca-3′; *ß-actin*, forward; 5′-ccaaggccaaccgtgagaagat-3′ and reverse 5′-ccacgttccgtgaggatcttca-3′. The amplification program consisted of an initial 15-min activation at 95 °C followed by 40 cycles of PCR (Each cycle consisting of 15 s of denaturation at 94 °C, 30 s of annealing at 58 °C and 20 s of extension at 72 °C). The values were normalized using *β-actin* as the internal standard.

2.4. Hormone assay

Estradiol and progesterone in culture medium were measured by enzyme immunoassay (EIA) after extraction with diethyl ether as described by Miyamoto et al. [7]. The extraction efficiency was 85%. Ranges of the standard curves were 2–2000 pg/ml for E2 and 0.05–50 ng/ml for P4. The intra- and interassay coefficients of variation averaged 6.2% and 8.5% for E2, and 4.5% and 7.4% for P4, respectively.

2.5. Data analysis

All data are presented as mean ± S.E.M. The statistical differences of *Clock* genes in cultured granulosa cells were determined by Student's *t*-test. Differences were considered significant at $p < 0.05$ or less.

3. Results and discussion

We examined the involvement of *Clock* and *Per2* in the function of granulosa cells treated with FSH by using siRNA knockdown. Our data demonstrate that siRNA knockdown of *Per2* and *Clock* suppressed the proliferation of granulosa cells treated with FSH (Figs. 1 and 2A). Loss of *mPER2* function was associated with

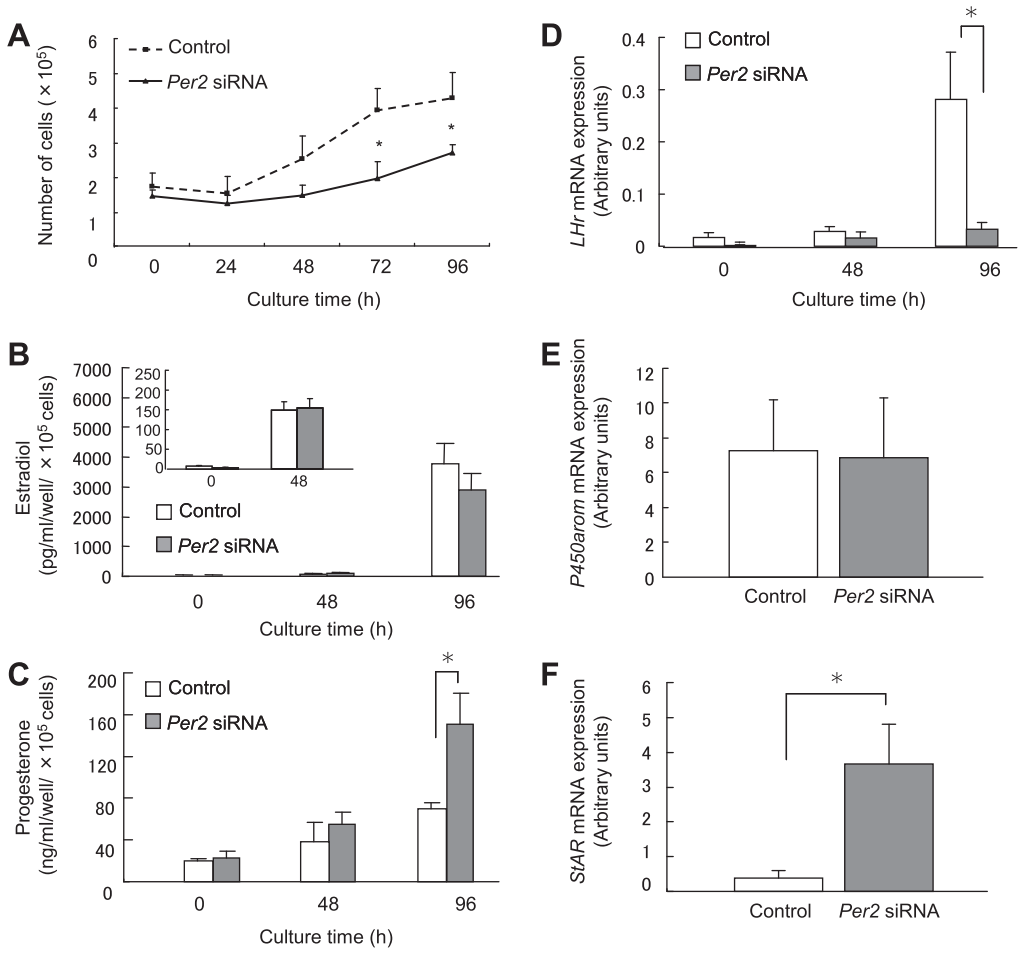

Fig. 2. Role of *Per2* on granulosa cell function. Cell proliferation (A), production of estradiol (B) and progesterone (C), and expression of *LHr* (D) in FSH-treated granulosa cells from follicles (<5 mm in diameter) was determined at the indicated time points (h) of culture. Expression of *P450arom* (E) and *StAR* (F) mRNAs was determined at 96 h after FSH treatment. Data are expressed as mean ± S. E. M. of three separate experiments from triplicate samples at each time point. An asterisk denotes a significant difference at $p < 0.05$. Scramble siRNA is shown as a control.

deregulated expression levels of *c-Myc* which regulates the G0 to G1 transition in the cell cycle [8]. The expression of genes involved in cell cycle regulation, such as cyclin D1, cyclin A, and p53, is deregulated in many tissues of $Per2^{m/m}$ mice [9]. Moreover, the kinase Wee1, a critical regulator of the G_2/M transition during cell division, was proposed to be a direct CLOCK:BMAL1 target, mediating the circadian gating of mitosis in the liver [10]. The clock Δ19 mutation was also reported to reduce the proliferation of embryonic fibroblasts [11]. Collectively, these studies suggest that the circadian *Clock* genes *Per2* and *Clock* can control cell proliferation at multiple levels including the G_1 phase, G_2/M transition and regulation of expression of the genes involved in cell cycle regulation. Therefore, our data suggest that *Per2* and *Clock* are involved in the proliferation of granulosa cells by regulating the cell cycle.

Estradiol that is produced by granulosa cells plays an important role in mammalian ovarian cycle. Our data demonstrated that siRNA knockdown of *Clock* inhibited estradiol production (Fig. 1B), and the expression of *LHr* (Fig. 1D) and *P450arom* mRNAs (Fig. 1E) in FSH-treated granulosa cells. These results suggest that *Clock* is associated with the inductions of estradiol production and expression of *LHr* and *P450arom* mRNAs in granulosa cells. *Clock* genes function as transcription factors in several cells and tissues [5]. CLOCK and BMAL1 dimerize and directly and indirectly activate transcription of the *Per* and *Cry* genes through E-box elements (5′-CACGTG-3′) [12,13]. Accumulated PER and CRY proteins in the cytosol inhibit the activity of CLOCK:BMAL1 [14]. The transcriptional regulation network of these genes forms a circadian *Clock* oscillator, which is known to control output genes and to affect physiological and metabolic processes [14,15]. Thus, the CLOCK:BMAL1 complex regulates expression of *Clock* genes via the E-box. The E-box site exists in the promoter region of the bovine *P450arom* gene [16], whereas this transcription site does not exist in the promoter region of the *LHr* gene. Thus, the CLOCK:BMAL1 complex may bind to the E-box and thus be associated with transcriptional regulation of *P450arom*.

A study performed using *Per2* mutant mice demonstrated that *Per2* is an important factor associated with the reproductive process and estrous cycle in female animals [17]. However, the role of *Per2* in ovarian functions such as follicular development and steroid production is still unknown. Our data show that siRNA knockdown of *Per2* increased the progesterone production (Fig. 2C) and expression of *StAR* mRNA (Fig. 2F), and decreased the expression of *LHr* mRNA (Fig. 2D), in FSH-treated granulosa cells. These results suggest that *Per2* is involved in the suppression of progesterone production and *StAR* mRNA expression and in the induction of *LHr* mRNA expression. Analysis of the promoter region of the mouse *StAR* gene revealed four putative E-boxes within the 2.5-kb region upstream of the transcription start site [18]. A recent study reported that the simultaneous expression of CLOCK and BMAL1 increased *StAR* promoter activity and that this induction was attenuated by the coexpression of negative regulators such as PER and CRY [18]. Thus, *StAR* transcription is regulated by the CLOCK:BMAL1 heterodimer via the distal E-box elements and is

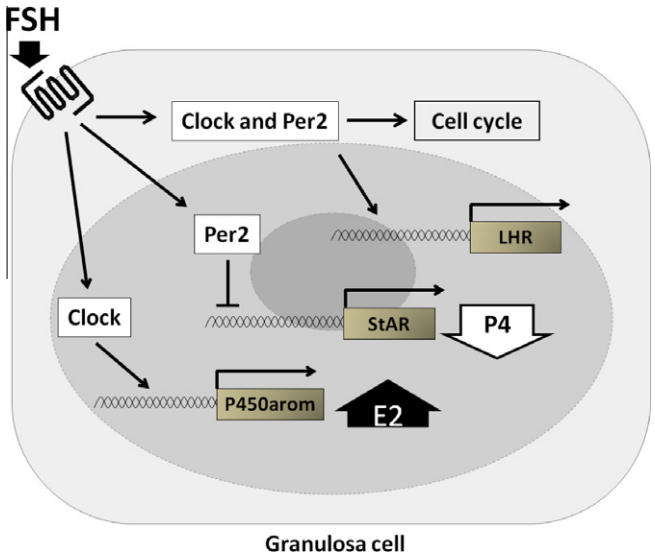

Fig. 3. The function of *Per2* and *Clock* in granulosa cells. *Clock* enhances estradiol production by increasing the expression of *P450arom*, and *Per2* inhibits progesterone production by decreasing the expression of *StAR*. In addition, both *Clock* and *Per2* regulate cell proliferation and expression of *LHr* mRNA in FSH-treated granulosa cells. Thus, *Clock* and *Per2* have different roles in determining granulosa cell function during follicular development. *Clock* and *Per2* might contribute to follicular development by being involved in FSH-inducible maturation of the granulosa cell.

inhibited by negative regulators (PER and CRY). Therefore, in the present study *Per2* may inhibit *StAR* expression by suppressing the CLOCK:BMAL1 heterodimer.

In conclusion, our results provide a new insight that *Per2* and *Clock* may control granulosa cell function such as cell proliferation, steroid production and *LHr* gene expression (Fig. 3). Specifically, different actions of *Per2* and *Clock* may be associated with follicular selection and the recruitment of the follicle that is destined to become the dominant follicle in bovine and human ovaries. We propose that a circadian clock function, as well as oscillation or cyclicity, plays an important role in the ovarian physiology.

Conflict of interest

The authors declare that no conflicts of interest exist that would prejudice the impartiality of this study.

Acknowledgments

The authors thank Dr. K. Okuda of Okayama University, Japan, for donating the progesterone antibodies. This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), Japan.

References

[1] J. Fahrenkrug, B. Georg, J. Hannibal, P. Hindersson, S. Gras, Diurnal rhythmicity of the *Clock* genes *Per1* and *Per2* in the rat ovary, Endocrinology 147 (2006) 3769–3776.
[2] B.N. Karman, S.A. Tischkau, Circadian *Clock* gene expression in the ovary: effects of luteinizing hormone, Biology of Reproduction 75 (2006) 624–632.
[3] P.J. He, M. Hirata, N. Yamauchi, S. Hashimoto, M.A. Hattori, Molecular Cellular Biochemistry 302 (2007) 111–118.
[4] M.K. Bunger, L.D. Wilsbacher, S.M. Moran, C. Clendenin, L.A. Radcliffe, J.B. Hogenesch, et al., Mop3 is an essential component of the master circadian pacemaker in mammals, Cell 103 (2000) 1009–1017.
[5] H. Ukai, R.H. Ueda, Systems biology of mammalian circadian *Clocks*, Annual Review of Physiology 72 (2010) 579–603.
[6] H. Yamashita, C. Murayama, R. Takasugi, A. Miyamoto, T. Shimizu, Bone morphogenetic protein (BMP)-4 suppresses progesterone production by inhibiting histone H3 acetylation of *StAR* in bovine granulosa cells in vitro, Molecular and Cellular Biochemistry 348 (2011) 183–190.
[7] A. Miyamoto, K. Okuda, F.J. Schweigert, D. Schams, Effects of basic fibroblast growth factor, transforming growth factor-beta and nerve growth factor on the secretory function of the bovine corpus luteum in vitro, Journal of Endocrinology 135 (1992) 103–114.
[8] C.C. Lee, Tumor suppression by the mammalian *Period* genes, Cancer Causes and Control 17 (2006) 525–530.
[9] L. Fu, H. Pelicano, J. Liu, P. Huang, C. Lee, The circadian gene *Period2* plays an important role in tumor suppression and DNA damage response in vivo, Cell 111 (2002) 41–50.
[10] T. Matsuo, S. Yamaguchi, S. Mitsui, A. Emi, F. Shimoda, H. Okamura, Control mechanism of the circadian *Clock* for timing of cell division in vivo, Science 302 (2003) 255–259.
[11] B.H. Miller, E.L. McDearmon, S. Panda, K.R. Hayes, J. Zhang, J.L. Andrews, et al., Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation, Proceedings of the National Academy of Sciences of the United States of America 104 (2007) 3342–3347.
[12] N. Gekakis, D. Staknis, H.B. Nguyen, F.C. Davis, L.D. Wilsbacher, D.P. King, et al., Role of the CLOCK protein in the mammalian circadian mechanism, Science 280 (1998) 1564–1569.
[13] K. Kume, M.J. Zylka, S. Sriram, L.P. Shearman, D.R. Weaver, X. Jin, et al., mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop, Cell 98 (1999) 193–205.
[14] S.M. Reppert, D.R. Weaver, Coordination of circadian timing in mammals, Nature 418 (2002) 935–941.
[15] S. Panda, J.B. Hogenesch, S.A. Kay, Circadian rhythms from flies to human, Nature 417 (2002) 329–335.
[16] J. Vanselow, R. Furbass, A. Zsolnai, C. Kalbe, H.M. Said, M. Schwerin, Expression of the aromatase cytochrome *P450* encoding gene in cattle and sheep, Journal of Steroid Biochemistry and Molecular Biology 79 (2001) 279–288.
[17] V. Pilorz, S. Steinlechner, Low reproductive success in *Per1* and *Per2* mutant mouse females due to accelerated ageing?, Reproduction 135 (2008) 559–568
[18] G.H. Son, S. Chung, H.K. Choe, H.D. Kim, S.M. Baik, H. Lee, et al., Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production, Proceedings of the National Academy of Sciences of the United States of America 105 (2008) 20970–20975.