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—Research Note—

Expression of Pin1, a Peptidyl-Prolyl Isomerase, in the Ovaries of eCG/hCG-Treated Immature Female Mice

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Abstract. Protein phosphorylation on certain serine or threonine residues preceding proline (Ser/Thr-Pro) is a pivotal signaling mechanism in diverse cellular processes. Pin1 is a highly conserved enzyme that isomerizes only the phosphorylated Ser/Thr-Pro bonds in certain proteins, thereby inducing conformational changes. Although much protein is phosphorylated in the ovary, the role of Pin1 in the ovary is still unknown. The purpose of this study is to investigate the effects of gonadotropins on protein and mRNA expression of Pin1 in mice ovaries. Quantitative PCR analysis showed that the expression of Pin1 mRNA significantly increased in the ovaries of equine chorionic gonadotropin (eCG)-treated mice compared with those of untreated mice ($P<0.05$). However, human chorionic gonadotropin (hCG) attenuated the expression of Pin1 mRNA increased by eCG. The protein level of Pin1 showed the same tendency as the expression of mRNA. The mRNA expression of E2F transcription factor, which controlled the expression of Pin1, was significantly decreased in the eCG-treated ovaries compared with the controls ($P<0.05$). These observations suggest that gonadotropins may regulate the expression of Pin1 without E2F transcription factor, indicating that Pin1 might be an important factor for protein signal transduction during follicular development.
Key words: E2F, Follicle development, Gonadotropin, Ovary, Pin1

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A peptidyl-prolyl isomerase, Pin1, catalyses the cis-trans isomerization of phosphorylated serine/threonine-proline bonds in phosphoproteins, thereby altering conformation leading to a change in protein stability or function [1–3]. For the gonad, Pin1 is highly expressed in the testis, and Pin-null mice develop age-dependent testicular atrophy and fertility defects [4]. Recent analyses confirmed the crucial role of Pin1 in maintaining spermatogonia in the adult testis [5] and revealed the importance of this enzyme in the proliferation of primordial germ cells during embryonic development [6]. In contrast, very few follicles were observed in the ovaries of Pin1-null mice [6].

The growth of ovarian follicles and ovulation are complex processes that involve dramatic changes in granulosa cell function. These changes are sequential and are dictated by specific, tightly regulated responses to gonadotropins, steroids, and growth factors [7–9]. One of the most dramatic changes in granulosa cell function is the rapid switch from the high proliferation stage that characterizes the granulosa cells of preovulatory follicles to the nonproliferative, terminally

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differentiated phase of luteal cells. Cell cycle progression and proliferation are controlled by the balance of positive and negative regulators converging on cell cycle kinase cascades [10–12]. Pin1 was originally identified by its ability to physically and functionally interact with a mitotic kinase [13]. A study using various model systems showed that manipulating Pin1 function affects progression of the cell cycle [1]. Furthermore, Pin1 directly interacts with regulators of the cell cycle. Although gonadotropins activate the cell cycle of granulosa cells, it is still unknown whether gonadotropins regulate Pin1 expression during follicular development in the ovary.

The cell cycle is regulated by a complex system of kinases and transcription factors, which are substrates for the kinases. This system is subject to very precise control. One of the most important factor families in this system is the E2F-family. Pin1 expression is induced by growth signals through E2F transcription factors, with protein levels fluctuating during the cell cycle in normal, but not cancerous, cells [14–16]. The aim of this study was to investigate the involvement of Pin1 and E2F during follicular development in gonadotropin-treated immature female mice.

Materials and Methods

Animals and treatment

Immature female ICR mice were treated according to the experimental protocols described below. On day 21 post partum, the animals were i.p administered 5 IU of equine chorionic gonadotropin (eCG, Teikoku Zohki Pharmaceutical Co., Tokyo, Japan) to promote follicular development, followed 48 hrs later by administration of 5 IU of hCG (Sankyo Co., Ltd., Tokyo, Japan). Ovaries were removed 0 (with full grown follicles) and 6 (preovulatory follicles) hours after hCG treatment, and were obtained on day 23 in the control mice without hormone injection.

RNA extraction, reverse transcription, and real-time PCR

The ovaries collected from the treated and untreated mice were put into RNase-free tubes, immediately frozen in liquid nitrogen and stored at –80 C until total RNA extraction. Total RNA was extracted using Trizol reagent (Life Technologies, Tokyo, Japan) following the method provided by the manufacturer and was quantified using an ultraviolet (UV)-visible recording spectrophotometer (UV-160; Shimadzu Corporation, Tokyo, Japan). Total RNA was stored at –20 C until cDNA synthesis. For cDNA synthesis, 1 μg of total RNA from each sample and 1 μl of oligo (deoxythymidine)$_{12–18}$ primers (0.5 μg/μl) were diluted to a final volume of 8 μl in DEPC-treated water, and heated at 65 C for 5 min to denature the higher-order structure of the RNA. Then, 4 μl of 5 × RT buffer, 1 μl of RNAguard™ RNase inhibitor (Amersham Biosciences Corp., USA), 4 μl of 2.5 mM dNTPs mix, and 2 μl of 0.1 M DTT were added to each mixture. After pre-incubation at 42 C for 5 min, 1 μl of Superscript II reverse transcriptase (Invitrogen USA) was added. The mixtures were incubated at 42 C for 50 min to synthesize cDNA and then heated at 70 C for 15 min to stop cDNA synthesis and to ferminate deoxyribonuclease activity. The product was diluted to a final volume of 80 μl with sterilized-distilled water and stored at –20 C until PCR analysis. Quantitative PCR was performed using the LightCycler Detection System (Roche) and SYBR Green interaction dye. PCR was performed using the following primers: Pin1, 5′-GGA GAG GAA GAC TTT GAA TCT CTG G-3′ and 5′-TGG TTT CTG CAT CTG ACC TCT G-3′; E2F, 5′-CTA CAA CAG ACT GAG GAC CAG TTC C-3′ and 5′-TCC ATG CCC CAC AGG TAT TC-3′; and GAPDH, 5′-CTT TGT CAA GCT CAT TTC CTG GTA T-3′ and 5′-TCC AGG GTT TCT TAC TCC TTG G-3′. The PCR conditions were 2 min at 95 C, followed by 40 cycles of 30 s at 95 C, 30 s at 58 C, and 45 s at 72 C. The amount of product at each step was monitored in real time.

Western blot analysis

The ovary tissues were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol) without bromophenol blue and 2-mercaptoethanol, and sonicated for 10 sec. The resulting lysates were cleared by centrifugation at 15,000 rpm for 10 min. After the protein concentration was determined by BCA kit (Pierce, Rockford, IL), 5% 2-mercaptoethanol (final concentration) was added. Aliquots of 20 μg cell lysate were heated at 95 C for 5 min and subjected to 15% SDS-PAGE. The proteins were transferred to a PVDF Membrane (BIORAD). The membrane

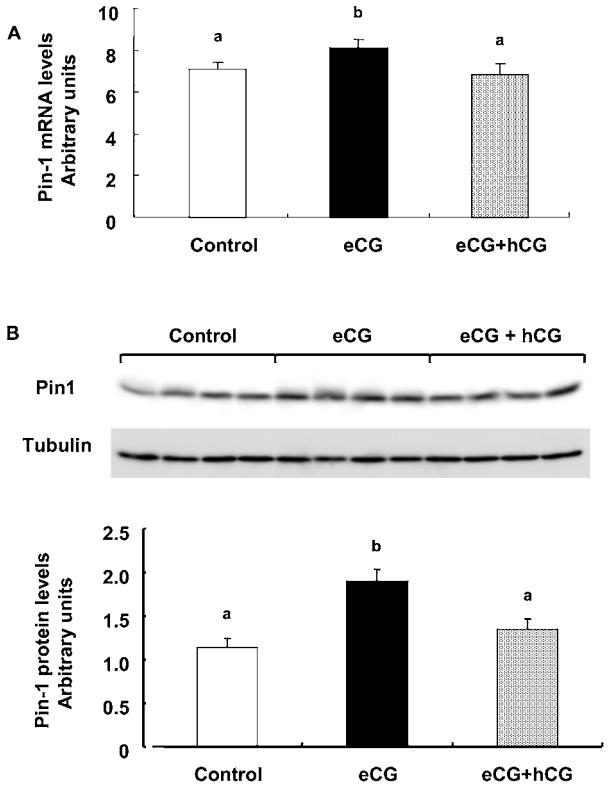

Fig. 1. Expression of Pin1 mRNA (A) and protein (B) in the eCG and hCG treated-mice ovaries. Different superscripts denote significantly different values (P<0.05). Data are represented as means ± SEM. Significant differences were analyzed by ANOVA followed by the Fisher's LSD test as a multiple comparison test.

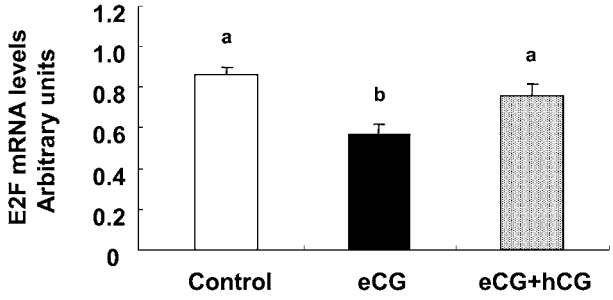

Fig. 2. Expression of E2F mRNA in the eCG and hCG treated-mice ovaries. Different superscripts denote significantly different values (P<0.05). Data are represented as means ± SEM. Significant differences were analyzed by ANOVA followed by the Fisher's LSD test as a multiple comparison test.

was blocked in TBST (20 mM Tris-HCl, pH 7.4-buffered saline, and 0.02% Tween 20) containing 3% non-fat dry milk for 1 h at room temperature and incubated overnight at 4 C with anti-Pin1 antibody (1:2000 dilution, Calbiochem) or anti-tubulin antibody (1:2000 dilution, Sigma). After washing 3 times with TBST, the membrane was incubated with anti-mouse IgG conjugated with horseradish peroxidase (1:5000 dilution, Sigma). Signals were visualized using an ECL system (Amersham Bioscience) according to the manufacturer's protocol. Densitometric analysis was performed using a LAS-3000 Image Analyzer (FujiFilm, Tokyo, Japan).

Statistical analysis

All data are presented as means ± S.E.M. Expression of Pin1 and E2F among the controls, eCG alone, and eCG plus hCG groups was analyzed by ANOVA, followed by the Fisher's LSD test as a multiple comparison test. Differences were considered significant at P<0.05 or less.

Results

Expression of Pin1 mRNA was significantly increased (P<0.05) in the eCG-treated ovaries compared with the control ovaries but decreased to the same level as the controls when hCG was added in addition to eCG (Fig. 1A). The protein level of Pin1 showed the same tendency as the expression of mRNA (Fig. 1B). Pin1 expression is induced by growth signals through E2F transcription factor. E2F mRNA levels decreased in the eCG-treated ovaries compared with the controls. However, treatment with hCG resulted in the expression of E2F mRNA recovering to the same level as the controls (Fig. 2).

Discussion

Our data is the first evidence to demonstrate that eCG increases the level of Pin1 mRNA and protein, and that hCG attenuates the increased level in mice ovaries. In addition, the mRNA level of E2F, which regulates Pin1 expression, decreased in the eCG-treated mice ovaries and recovered to the same level as the controls as a result of treatment with hCG.

Pin1 directly interacts with regulators of the cell

cycle, especially a large number of mitosis-specific phosphoproteins [17]. Activated Cdc2 and MAPK phosphorylate a large number of proteins [18, 19]. Many proteins phosphorylated by Cdc2 and MAPK are Pin1 substrates, and are distributed in different mitotic structures [20–22]. Pin1 deletion leads to mitotic catastrophe [13, 23]. As folliculogenesis proceeds to the preantral and preovulatory stages, granulosa cells become responsive to gonadotropins, produce steroid and peptide factors, and undergo a transient phase of rapid cell proliferation [24, 25]. FSH promotes activation of the p42/p44 MAPK/extracellular signal-regulated kinase (ERK) pathway [26] and activates the p38 MAPK pathway [27]. The present study indicates that the levels of protein and mRNA of Pin1 increased in the eCG-treated ovaries, suggesting that Pin1 may be controlled by the proteins that are phosphorylated by the MAPK pathway during follicular development. In contrast, hCG inhibited the Pin1 expression induced by eCG. Since granulosa cell division ends abruptly at the LH surge [25], the decreased expression of Pin1 mRNA and protein may indicate the stop of the follicular growth in eCG-treated ovaries.

Pin1 expression is induced by growth signals through E2F transcription factors [14–16]. The present study demonstrated that the level of E2F mRNA decreased in the eCG-treated ovaries, indicating that expression of Pin1 did not parallel expression of E2F. The transcriptional activity known as E2F comprises several members (E2F and DP protein members) that, in various heterodimeric configurations, bind DNA and regulate the expression of a multitude of genes involved in many aspects of cell growth and proliferation [28, 29]. E2F components regulate transcription of their target genes when bound to their promoters as dimers with a DP protein. E2F family members can be functionally grouped into activators (E2F1, E2F2, and E2F3a) or repressors (E2F3b, E2F4, E2F5, E2F6, and E2F7) of transcription. A detailed experiment at the cell level is necessary to determine the relationship between gonadotropins and E2F family members.

In conclusion, the results from this study provide the first evidence that expression of Pin1 is regulated in the ovary by gonadotropins and that the expression is not controlled by E2F transcription factor. In the future, it will be interesting to examine how Pin1 expression is regulated in the ovary.

References

1. **Lu KP, Liou YC, Zhou XZ**. Pinning down proline-directed phosphorylation signaling. *Trends Cell Biol* 2002; 12: 164 –172.
2. **Stukenberg PT, Kirschner MW**. Pin1 acts catalytically to promote a conformational change in Cdc25. *Mol Cell* 2001; 7: 1071–1083.
3. **Yaffe MB, Schutkowski M, Shen M, Zhou XZ, Stukenberg PT, Rahfeld JU, Xu J, Kuang J, Kirschner MW, Fischer G, Cantley LC, Lu KP**. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. *Science* 1997; 278: 1957–1960.
4. **Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, Fujimori F, Uchida T, Hunter T, Lu KP**. Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. *Proc Natl Acad Sci USA* 2002; 99: 1335–1340.
5. **Atchison FW, Means AR**. Spermatogonial Depletion in Adult Pin1-Deficient Mice. *Biol Reprod* 2003; 69: 1989–1997.
6. **Atchison FW, Capel B, Means AR**. Pin1 regulates the timing of mammalian primordial germ cell proliferation. *Development* 2003; 130: 3579–3586.
7. **Hirshfield AN**. Development of follicles in the mammalian ovary. *Int Rev Cytol* 1991; 124: 43–101.
8. **Pederson T**. Follicular kinetics in the ovary of the cyclic mouse. *Acta Endocrinol* 1970; 64: 304–323.
9. **Richards JS**. Hormonal control of follicular growth and maturation in mammals. In: Jones RE (ed.), The Vertebrate Ovary: Comparative Biology and Evolution. New York: Plenum Press; 1978; 331–360.
10. **Sherr CJ**. Cancer cell cycles. *Science* 1996; 274:1672–1677.
11. **Sherr CJ**. G1 progression: cycling on cue. *Cell* 1994; 79: 551–555.
12. **Sherr CJ, Roberts JM**. Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* 1995; 9: 1149–1163.
13. **Lu KP, Hanes SD, Hunter T**. A human peptidyl-prolyl isomerase essential for regulation of mitosis. *Nature* 1996; 380: 544–547.
14. **Shen M, Stukenberg PT, Kirschner MW, Lu KP**. The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phospho-proteins. *Genes Dev* 1998; 12: 706–720.
15. **Ryo A, Liou YC, Wulf G, Nakamura M, Lee SW, Lu**

KP. Pin1 is an E2F target gene essential for the Neu/Ras-induced transformation of mammary epithelial cells. *Mol Cell Biol* 2002; 22: 5281–5295.

16. **You H, Zheng H, Murray SA, Yu Q, Uchida T, Fan D, Xio ZXJ**. IGF-1 induces Pin1 expression in promoting cell cycle S-phase entry. *J Cell Biochem* 2002; 84: 211–216.
17. **He J, Xu J, Xu XX, Hall RA**. Cell cycle-dependent phosphorylation of disabled-2 by cdc2. *Oncogene* 2003; 22: 4524–4530.
18. **Ubersax JA, Woodbury EL, Quang PN, Paraz M, Blethrow JD, Shah K, Shokat KM, Morgan DO**. Targets of the cyclin-dependent kinase Cdk1. *Nature* 2003; 425: 859–864.
19. **Roux PP, Blenis J**. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol. *Mol Biol Rev* 2004; 68: 320–344.
20. **Lu PJ, Zhou XZ, Liou YC, Noel JP, Lu KP**. Critical role of WW domain phosphorylation in regulating its phosphoserine-binding activity and the Pin1 function. *J Biol Chem* 2002; 277: 2381–2384.
21. **Xu YX, Hirose Y, Zhou XZ, Lu KP, Manley JL**. Pin1 modulates the structure and function of human RNA polymerase II. *Genes Dev* 2003; 17: 2765–2776.
22. **Palancade B, Marshall NF, Tremeau-Bravard A, Bensaude O, Dahmus ME, Dubois MF**. Dephosphorylation of RNA polymerase II by CTD-phosphatase FCP1 is inhibited by phospho-CTD associating proteins. *J Mol Biol* 2004; 335: 415–424.
23. **Lu PJ, Wulf G, Xiao ZZ, Davies P, Kun PL**. The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein. *Nature* 1999; 399: 784–788.
24. **Greenwald GS, Roy SK**. Follicular development and its control. In: Knobil E, Neill J (eds.), The Physiology of Reproduction, ed 2. New York: Raven Press; 1994; 1: 629–724.
25. **Robker RL, Richards JS**. Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and $p27^{KIP1}$. *Mol Endocrinol* 1998; 12: 924–940.
26. **Das S, Maizels ET, DeManno D, St. Clair E, Adam SA, Hunzicker-Dunn M**. A stimulatory role of cyclic adenosine 3′,5′-monophosphate in follicle-stimulating hormone-activated mitogen-activated protein kinase signaling pathway in rat ovarian granulosa cells. *Endocrinology* 1996; 137: 967–974.
27. **Maizels ET, Cottom J, Jones JR, Hunzicker-Dunn M**. Follicle stimulating hormone (FSH) activates the p38 mitogen-activated protein kinase pathway, inducing small heat shock protein phosphorylation and cell rounding in immature rat ovarian granulosa cells. *Endocrinology* 1998; 139: 3353–3356.
28. **Cam H, Dynlacht BD**. Emerging roles for E2F: beyond the G1/S transition and DNA replication. *Cancer Cell* 2003; 3: 311–316.
29. **Trimarchi JM, Lees JA**. Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* 2002; 3: 11–20.