Induction of Ski Protein Expression upon Luteinization in Rat Granulosa Cells

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ABSTRACT

Ski protein is implicated in proliferation/differentiation in a variety of cells. We had previously reported that Ski protein is present in granulosa cells of atretic follicles, but not in preovulatory follicles, suggesting that Ski has a role in apoptosis of granulosa cells. The alternative fate of granulosa cells other than apoptosis is to differentiate to luteal cells, however, it is unknown whether Ski is expressed and has a role in granulosa cells undergoing luteinization. Thus, the aim of the present study was to examine whether the initiation of luteinization with luteinizing hormone (LH) directly regulates expression of Ski in the luteinized granulosa and luteal cells after ovulation by *in vitro* models. RT-PCR and real time PCR analysis respectively revealed that LH had no effect on *c-Ski* mRNA expression in the cultured granulosa cells regardless of LH treatment. Though Ski protein is absent in granulosa cells of preovulatory follicle, its mRNA (*c-Ski*) was expressed and the level was unchanged even after LH surge. Taken together, these results demonstrated that Ski protein expression is induced in granulosa cells upon luteinization, and suggested that its expression is regulated post-transcriptionally. Moreover, expression of mRNA of *Arkadia*, an E3 ubiquitin ligases, in luteinizing granulosa cells *in vivo* was assessed by realtime-PCR. The levels of *Arkadia* mRNA expression were unchanged during follicular growth and postovulatory luteinization. These findings suggest that Ski protein level may be regulated during luteinization at translational and/or post-translational level but not by Arkadia.

(Key words : Arkadia, granulosa cells, luteinization, Ski)

INTRODUCTION

Ovarian folliculogenesis and luteinization in mammals is a complex series of events regulated by endocrine and paracrine/autocrine factors. Amongst the endocrine factors, the role of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), in regulating folliculogenesis and luteinization is well established. Gonadotropin is an important survival factor for developing follicles in escaping atresia and stimulating early antral follicles to complete their final differentiation, reaching the preovulatory follicle stage. In response to the LH surge, a preovulatory follicle embarks on a terminal differentiation pathway, called "luteinization", that transforms granulosa and theca cells of a preovulatory follicle into luteal cells to form corpus luteum (CL). Luteinizing follicular cells undergo specific morphological and biochemical changes as well as endocrinological alterations in their transition to luteal cells (Roy, 1994; Smith *et al*., 1994). Many genes that are highly expressed in growing preovulatory follicles are turned off by the LH surge, whereas the expression of numerous genes that are involved in ovulation and luteinization increases dramatically during the periovulatory period (Espey and Richards, 2002; Richards *et al*., 2005).

The effects of gonadotropins are mediated or regulated by a number of paracrine/autocrine growth factors (Nilsson and Skinner, 2002; Nilsson and Skinner, 2003). For example, members of the transforming growth factor-b (TGF-β) family are expressed by oocytes, granulosa cells, and theca cells in a developmental-stage dependent manner and play roles in proliferation/atresia of granulosa/theca cells, steroidogenesis, oocyte maturation, ovulation, and luteinization (Pehlivan *et al*., 2001; Juengel and McNatty, 2005; Knight and Glister, 2006). The members of downstream signaling molecules (Smads) and binding proteins (follistatin) of TGF-β family are also expressed

† This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.
‡ This work received grant suuport from the Agenda Program (No. PJ0082402011), Rural Development Adminstration, Republic of Korea.
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in the ovary, and function in follicular development and luteinization (Drummond *et al*., 2002; Xu *et al*., 2002).

The signaling pathway of TGF-β family is shown to be regulated by Ski, the protein encoded by proto-oncogene *c-Ski* (Li *et al*., 1986; Stavnezer *et al*., 1986; Nomura *et al*., 1989; Stavnezer *et al*., 1989; Sutrave and Hughes, 1989), via its interactions with Smad proteins (Liu *et al*., 2001; Luo, 2003; Luo, 2004). The *c-Ski*, has been identified as the cellular homologue of *v-Ski*, that was originally identified as the transforming gene of the avian Sloan-Kettering viruses, which transform chicken embryonic fibroblasts, leading to their morphological transformation and anchorage-independent growth (Li *et al*., 1986; Stavnezer *et al*., 1986; Nomura *et al*., 1989; Stavnezer *et al*., 1989; Sutrave and Hughes, 1989). In addition to its transforming activity, *c-Ski* is known to induce myogenic differentiation of quail embryonic cells (Li *et al*., 1986). Thus, *c-Ski* has been implicated to have dual roles in both regulating transformation (proliferation) and differentiation of cells.

On the other hand, closely related to *c-Ski is* TGF-β, which is a ubiquitous cytokine that regulates cell differentiation, proliferation, apoptosis and morphogenesis (Stroschein *et al*., 1999 ; Sun *et al*., 1999; Stroschein *et al*., 2001 ; Shi and Massague., 2003). Previous studies indicate that Ski is a relatively unstable protein whose expression levels can be regulated by ubiquitin-mediated proteolysis (Colmenares *et al*., 1991; Boyer *et al*., 1993). In general, ubiquitin-dependent protein degradation plays critical roles in a wide variety of biological processes, including signal transduction, cell-cycle progression and transcriptional regulation (Hershko and Ciechanover, 1998). The protein ubiquitination is catalyzed by a cascade of enzymes, including an ubiquitin-activating enzyme E1, an ubiquitin-conjugating enzyme E2 and an ubiquitin ligase E3. Recently, several groups have reported that Arkadia, a RING-type E3 ubiquitin ligase, is not only widely expressed throughout mammalian tissues but also induces the ubiquitination and degradation of corepressors, *c-Ski* to enhance TGF-β family signaling (Koinuma *et al*., 2003; Nagano *et al*., 2007).

Ski can act either as a positive or negative transcriptional regulator, depending on the physiological context of co-factors. In agreement with this, expression of *c-Ski* is not tissue-restricted (Sutrave and Hughes, 1989; Colmenares and Stavnezer, 1990; Yamanouchi *et al*., 1999), and Ski's biological activity likely depends on not only its expression levels but also its required association with other transcription factors (Nagase *et al*., 1990; Baker and Harland, 1997; Heldin *et al*., 1997; Akiyoshi *et al*., 1999). Due to its unique binding properties with multiple factors, Ski could posses various roles in both the regulation of cellular proliferation and differentiation (Tarapore *et al*., 1997; Nicol and Stavnezer, 1998). On the other hand, the activity and expression of Ski can be regulated at the level of transcriptional activation, posttranslational modification, protein stability, as well as intracellular localization.

We had previously shown that Ski is present in granulosa cells of atretic follicles in the rat ovary, while it is absent in those of preovulatory follicles (Kim *et al*., 2006). Since the TGF-β family play important roles during luteinization, it is highly possible that Ski is involved in this process. Thus, the aim of the present study was to examine whether the initiation of luteinization with luteinizing hormone (LH) directly regulates expression of Ski in the luteinized granulosa and luteal cells after ovulation by *in vitro* models.

MATERIALS AND METHODS

1. Animals

The immature (25 day old) female Wistar-Imamichi rats were purchased from the Imamichi Institute of Animal Reproduction (Ibaraki, Japan). Synchronized folliculogenesis was initiated by administration of of eCG (40 IU, s.c.) followed by hCG (15 IU, s.c.) to induce ovulation and subsequent luteinization (Bell and Lunn, 1968). In this model, ovulation occurs at around 12 h after hCG injection (Nothnick and Curry, 1996). The rats were killed by cervical dislocation and ovaries were collected at the time points indicated (n = 3～4 animals per time point). In the experiment where gene expression was examined by real time PCR, granulosa cells were collected from the ovary and immediately used for RNA isolation. All animals received humane care according to the Guide for the Care and Use of Animals of The University of Tokyo.

2. Preovulation Follicle Isolation and Culture

Granulosa cells were obtained by puncturing the large preovulatory follicles 48 h after injection of eCG with a 27-gauge needle, (70 μm). The granulosa cells were collected in PBS and aliquots of the cell suspension were assessed for cell viability by trypan blue exclusion. After (15 mM HEPES, 50 U/ml penicillin, 50 mg/ml streptomycin sulfate) /DMEM:F12 (1:1) at adensity of 2×10^5 24-well plates Granulosa cells were incubated in the presence and absence of LH (ovine) 10 ng/ overnight and then used for isolation of RNA.

3. Extraction of Total RNA

Granulosa cells were obtained at 0, 3, 6 and 12 h after hCG-injection by puncturing the large preovulatory follicles with a 27-gauge needle. The cells were washed with PBS and immediately used for RNA isolation.

Briefly, Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, 200 μl of chloroform per 1 ml TRIzol reagent was added to the 24-well plates and they were vortexed for a few seconds. After incubated at room temperature (RT) for 3 min, they were centrifuged at 15,000 rpm for 15 min at 4℃. After centrifugation, to the supernatants, equal volume of isopropylacohol (0.5 ml per 1 ml of TRIzol reagent) was added and they were vortexed for a few seconds. After incubating for 10 min at RT, they were centrifuged at 15,000 rpm for 15 min at 4℃. The supernatant was discarded, and the resulting RNA pellet was rinsed with 75% ethanol, dried up and dissolved in 30 ml diethylpyrocarbonate-treated water. The RNA concentrations were measured spectrophotometrically.

4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

First-strand cDNA was synthesized by using SuperScript II (Invitrogen) with oligo d(T)16 primers (500 mg/2.5 mM) as described in manufacturer's instruction. In brief, 2 mg of total RNA and 1 ml oligo d(T)16 primers were mixed and heat-denatured for 15 min at 65℃, then placed on ice at 1 min. Then, 4 μl of 5 × RT-buffer for SuperScript II (Invitrogen), 5 μl of dNTP (RNase free, 2 mM), 2 ml of dithiothreitol, 2 μl of RNase inhibitor, 1 μl of SuperScript II (Invitrogen) were added (total volume, 20 μl), and incubated at 42℃ for 60 min. The reaction was terminated by incubating for 15 min at 70℃. PCR was carried out using αTaq polymerase (Bionex, Seoul, Korea) according to the manufacturer's recommended protocol. Briefly, Total volume, 50 μl of the reaction mixture contained 2 μl of cDNA samples, 0.25 μl of α-Taq (5 units/ml), 5 μl of 10 × PCR buffer, 4 μl of dNTPs (2.5 mM), 3.75 μl of 10 × Enhancer, 33 μl of sterilized water for PCR and 1 ml of each primers (10 pmol). The primer set used for amplification of the partial rat *c-Ski* cDNA fragment consisted of the forward primer, 5'-ACC ATC TCG TGC TTC GTG GTG GGA–3' and the reverse primer, 5'-CTC CTT GCC CGT GTA ATC CTG GCT-3', was designed on the basis of the DNA sequences of the mouse and human *c-Ski* gene published in the literature (Yamanouchi *et al*., 1997). The predicted PCR amplified size of *c-Ski* was 561 bp. After PCR, an aliquot of the reaction mixture was electrophoresed on 1% agarose gel and the products were visualized with ethidium bromide staining.

5. qPCR (Real Time PCR)

Total RNA was isolated using Trizol reagent (Invitrogen), and cDNA was synthesized by SuperScript II (200 U/ml) with oilgo-dT 16 primer. qPCR was done using with LightCycler (Roche Diagonostics GmbH, Mannheim, Germany) nd LightCycler FastStart Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) according to the manufacturer' instructions. The primer sequences used were as follows: rat c-ski (forward primer; 5' CAGCAGATCAACTCGGTGTG-3' reverse primer; 5'AGGAT GCCCATGACTTTGAG-3', rat arkadia (forward primer; 5' CGTGAGGAGAACTGCATCAA-3' reverse primer; 5' GGAT GTGCTAATGCATGCTG -3' and rat HPRT (forward primer; 5'GACCGGTTCTGTCATGTCG-3' reverse primer; 5'ACCTG GTTCATCATCACTAATCAC -3'. Rat-specific primers were designed using PRIMER3 software (available at http://fokker.wi.mit.edu/primer3/), and the specificity for each primer set was confirmed by both electrophoresis of the PCR products and analyzing the melting (dissociation) curve after each qPCR. Twenty microlitter of the reaction solution consisted of 2 μl of the template (appropriate dilution was determined by gene), 10 μl of LightCycler FastStart Thunderbird SYBR qPCR Mix, 1 μl of 10 μM of each primer and 3 μl of diethylpyrocarbonate-treated water. PCR amplification was performed as follows: pre-denature for one cycle at 95℃ for 15 min and 45 cycles at 95℃ for 15 sec, 59℃ for 20 sec and 72℃ for 30 sec. Melting curve analysis was performed at 65～95℃ with 0.1℃/sec temperature transition.

RESULTS

The results obtained above suggested the expression of Ski in granulosa cells is induced by hCG (LH) treatment *in vivo*. In order to examine whether LH directly induces *c-Ski* expression, RT-PCR and qPCR were performed on the RNA samples from granulosa cells cultured in the presence or absence of LH. As shown in Fig. 1A, *c-Ski* mRNA was expressed in granulosa cells regardless of LH treatment. qPCR analysis further revealed that there was no difference in the expression level of *c-Ski* mRNA between the cells treated with and without LH (Fig. 1B). These results indicated that *c-Ski* mRNA is expressed in granulosa cells even without the effect of LH,

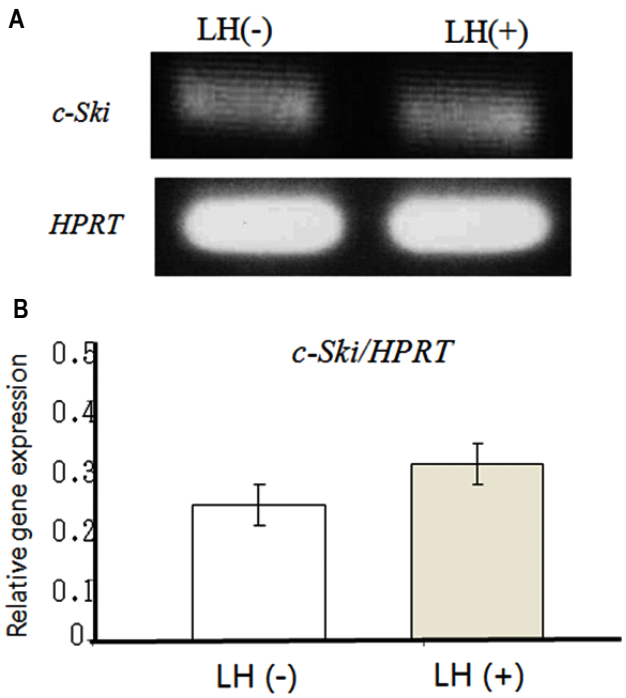

Fig. 1. Levels of expression of *c-Ski* mRNA in immature rat ovaries with or without (control) hCG treatment for 24 h, normalized for *HPRT* mRNA expression. Expression of *c-Ski* mRNA in the cultured granulosa cells was confirmed using RT-PCR (A) and qPCR (B) (mean ± SE; n = 4).

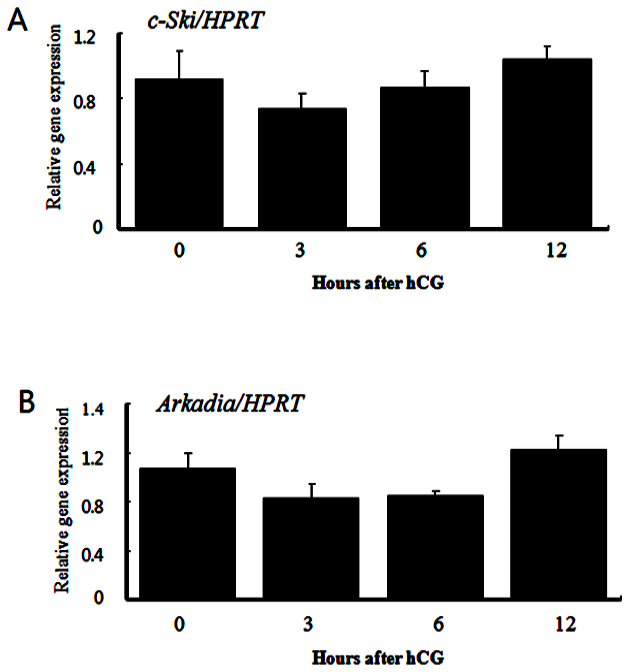

Fig. 2. Granulosa cells were obtained at 0, 3, 6 and 12 h after hCG-injection by puncturing the large preovulatory follicles with a 27-gauge needle. qPCR analyses of *c-ski* (A) and *Arkadia* (B). Relative expression levels to HPRT were calculated and graphed. The data are means ± SE (n = 3~4).

and LH does not directly regulate c-*Ski* mRNA expression.

In order to examine if *c-Ski* expression is under the control of LH, luteinizing granulosa cells were obtained from eCG/hCG-primed rats before ovulation, and their expression of *c-Ski* mRNA was determined by qPCR. Unexpectedly, *c-Ski* mRNA was present in granulosa cells even before hCG injection, and its expression level was unchanged after hCG injection (Fig. 2A), indicating that *c-Ski* mRNA expression in granulosa cells is not regulated during luteinization. This raises the possibility that the amount of Ski protein is regulated at the translational and/or post-translational, not transcriptional, level during luteinization.

Recently, Nagano *et al*. (Nagano *et al*., 2010) reported that knockdown of *Arkadia*, an E3 ubiquitin ligase, abrogated TGF-β-induced degradation of Ski protein, demonstrating that *Arkadia* is responsible for the degradation of Ski protein. Thus, in order to examine the possible involvement of *Arkadia* in the regulation of Ski protein, the expression level of *Arkadia* mRNA during luteinization of granulosa cells was assessed by qPCR. As shown in Fig. 2B, the level of *Arkadia* mRNA expression was unchanged during luteinization of granulosa cells, suggesting that contribution of Arkadia on the changes of Ski protein level may be little if any.

DISCUSSION

The results of the present study demonstrated the presence of Ski protein in luteinizing granulosa cells and cells within the CL, suggesting the possible involvement of Ski during luteinization and in the maintenance of CL function.

The process of luteinization is regarded to be under the control of luteinization inhibitors, which are thought to act to prevent rapid luteinization and suppress progesterone synthesis until the oocyte is released at ovulation (Knight *et al*., 2006). Granulosa cells secrete activin, which stimulates granulosa cell proliferation in preantral/early antral follicles (Zhao *et al*., 2001), and up-regulates FSH receptors and FSH-induced aromatase activity (Xiao *et al*., 1992a; Xiao *et al*., 1992b). Besides activin, BMP-4, BMP-7, BMP-6, BMP-15, and GDF-9 (Nilsson and Skinner, 2002; Nilsson and Skinner, 2003; McNatty *et al*., 2005) enhance estradiol and inhibin secretion by granulosa cells

while suppressing progesterone secretion. After ovulation and during CL formation, inhibin/activin subunit expression is down-regulated in most species. A recent study also showed that follicular expressions of BMP-2, BMP-3, BMP-4, BMP-6, and BMP-7 are profoundly reduced upon ovulation (Erickson and Shimasaki, 2003). These reports suggest that these TGF-β family proteins have roles in delaying follicular atresia and/or luteinization. Thus, considering that Ski negatively regulates the activity of TGF-β family proteins, it is possible that Ski, whose expression is up-regulated in granulosa cells, may favor luteinization by bioneutralizing intrafollicular activity of these proteins.

Several studies indicated that transcripts of Ski, *c-Ski*, are ubiquitously expressed in many tissues and mRNA levels are relatively constant during cell cycle, differentiation, and embryogenesis (Grimes *et al*., 1993; Ambrose *et al*., 1995). The result of the present study that *c-Ski* expression was unchanged during luteinization of granulosa cells is in agreement with these reports. Thus, we speculated that regulated ubiquitin-proteasome, proteolysis of Ski is one of the important ways to control its activity during luteinization of granulosa cells. For this reason, we picked up Arkadia as a candidate that could mediate degradation of Ski protein. As a result, this was not the case. However, this result does not exclude the possibility that other proteolytic enzyme is involved in degradation of Ski. For instance, several ubiquitin ligases, including Smurf1, Smurf2, and the APC/Cdh1 complex other than Arkadia, may induce the proteasome degradation of Ski during luteinization. Based on the recent findings that degradation of Ski protein is mediated by an E3 ubiquitin ligase, *Arkadia* (Nagano *et al*., 2007), we examined if *Arkadia* expression is changed during luteinization of granulosa cells. The result demonstrated that *Arkadia* is indeed expressed in grannulosa cells but its expression is unchanged. However, this does not exclude the possibility that Arkadia is involved in regulating Ski protein during luteinization of granulosa cells since we currently have no data on the Arkadia protein expression due to an unavailability of rat Arkadia-specific antibody. Alternatively, it is also possible that other ubiquitin ligases, including Smad ubiquitin regulatory factors, Smurf1 and Smurf2, and the APC/Cdh1 complex (Kavsak *et al*., 2000; Ebisawa *et al*., 2001; Episkopou *et al*., 2001; Hanyu *et al*., 2001), are the candidates to regulate Ski protein level in granulosa cells. Therefore, the issue regarding the regulatory mechanism of Ski protein expression level awaits for further investigation.

The results of this study demonstrated the presence of Ski protein in luteinizing granulosa cells and cells within the CL, suggesting the possible involvement of Ski during luteinization and the maintenance of CL function. The present results, however, did not support the notion that LH regulates the amount of Ski protein at transcriptional level. Thus, the author, at present, concluded that the up-regulation of Ski protein expression *in vivo* in granulosa cells may be resulted from the indirect effect of LH. However, technical limitation of using garnulosa cells could not exclude other possibilities. One possibility is that this *in vitro* experimental model using isolated granulosa cells does not necessarily recapitulate *in vivo* environment, i.e., three-dimensional structure of the follicle. Another possibility is naturally occurring luteinization, since it has been reported that once granulosa cells were isolated, they initiate spontaneous luteinization (Espey and Richards, 2002; Stouffer *et al*., 2007; Richards and Zahniser, 2009). To exclude these possibilities, it will be necessary to re-examine the effect of LH on *c-Ski* expression using follicle culture system.

RUNX1/2 as a transcriptional regulator have been shown to play an essential role in the differentiation of various cell types including to thymus, bonemarrow, and various hematopoietic cell lines (Taniuchi *et al*., 2002). Recently, Park (2010) have demonstrated that *RUNX1* and *RUNX2* are involved in differentiation of luteinizing granulosa cells, by regulating the expression of cell cycle inhibitors (e.g. Cdkn1a and Cdknlb) and LH-induced key mediators, such as progesterone receptors, EGF-like peptides, PTGS2, and C/EBPβ (Natraj and Richards, 1993; Roy and Harris, 1994; Morris *et al*., 1995; Sterneck *et al*., 1997; Robker and Richards, 1998). From these results, it is conceivable that transcriptional regulators induced in periovulatory follicles after the LH surge play a crucial role in controlling the transcriptional activation/repression of periovulatory genes. This suggests the possible relation of Ski to other specific transcriptional factors during the LH-induced terminal differentiation of granulosa cells (luteinization) in the ovary.

In conclusion, the results of the present study demonstrated, as far as the author knows, for the first time that the high levels of Ski are expressed in luteinizing granulosa cells during ovulation and luteinization, suggesting that Ski is involved in luteinization and in the maintenance of CL function. In addition, it was suggested that the amount of Ski protein is regulated at the post-transcriptional level. Further studies are still required to reveal the molecular mechanisms regulating Ski protein le-

vels and activity in the ovary.

ACKNOWLEDGEMENTS

The critical reading of this manuscript by Drs. Sakae and Akihiro Ikeda is greatly acknowledged.

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(접수: 2011. 8. 9 / 심사: 2011. 8. 10 / 채택: 2011. 11. 18)