Expression of Ski in the Corpus Luteum in the Rat Ovary

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ABSTRACT

Sloan-Kettering virus gene product of a cellular protooncogene *c-Ski* is an unique nuclear pro-oncoprotein and belongs to the Ski/Sno proto-oncogene family. Ski plays multiple roles in a variety of cell types, it can induce both oncogenic transformation and terminal muscle differentiation when expressed at high levels. Ski protein is implicated in proliferation/differentiation in a variety of 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 this study was, by means of immunohistochemical methods, to locate Ski protein in the rat ovaries during ovulation and corpora lutea (CL) formation to predict the possible involvement of Ski in luteinization. In addition, we performed 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 vivo* models. In order to examine the expression pattern of Ski protein along with the progress of luteinization, follicular growth was induced by administration of equine chorionic gonadtropin to immature female rat, and luteinization was induced by human chorionic gonadtropin treatment to mimic luteinizing hormone (LH) surge. While no Ski-positive granulosa cells were present in preovulatory follicle, Ski protein expression was induced in response to LH surge, and was maintained after the formation of corpus luteum (CL). These results indicate that Ski is profoundly expressed in the luteinized granulosa cells and luteal cells of CL during luteinization, and suggest that Ski may play a role in luteinization of granulosa cells.

(Key words : Ski, luteinization, granulosa cells)

INTRODUCTION

The proto-oncogene, *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 retroviruses, 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; Boyer *et al*., 1993; Sleeman and Laskey, 1993). Ski protein is a nuclear transcription factor that does not bind DNA directly (Baker and Harland, 1997; Heldin *et al*., 1997; Akiyoshi *et al*., 1999; Kawabata *et al*., 1999; Luo *et al*., 1999; Sun *et al*., 1999). Due to its unique binding properties with multiple factors, Ski could posses various roles in the regulation of both cellular proliferation and differentiation (Tarapore *et al*., 1997; Nicol and Stavnezer, 1998). Therefore, *c-Ski* gene product (Ski) has been implicated to have dual roles in both regulating proliferation and differentiation of these cells. However, little is known about the pathways through which Ski exerts its actions. Previous studies have identified *c-Ski* expression among the tissues (Lyons *et al*., 1994), including in ovary. Among the tissues where *c-Ski* expression has been identified, ovary is one of the tissues in which *c-Ski* expression has been identified, but the role of this gene in the ovary remains unknown. However, the expression of *c-Ski* gene does not necessarily indicate the presence of Ski protein, since degradation of Ski protein has been implicated as one of the regulatory mechanisms of Ski function.

In response to the LH surge, a preovulatory follicle embarks on a terminal differentiation pathway (luteinization) that trans-

forms granulosa and theca cells of a preovulatory follicle into luteal cells to form a CL. Luteinizing follicular cells then undergo specific morphological changes (e.g. compact, nuclear-dominant cells to become loosely connected hypertrophic cells) as well as physiological alterations in their transition to luteal cells. In addition, the LH surge induces specific transcription factors that regulate the expression of a myriad of genes in periovulatory follicles to bring about ovulation and luteinization (Richards and Zahniser, 2009; Friberg *et al.*, 2010).

The aim of present study was, by means of immunohistochemical techniques, to locate Ski protein in the rat ovaries during ovulation and subsequent CL formation using the eCG/hCG-primed rat model in order to predict the possible involvement of Ski in luteinization.

MATERIALS AND METHODS

1. Animals

The adult male and female (8- to 14-week-old) and the immature female (25-d-old) Wistar-Imamichi rats were purchased from the Imamichi Institute of Animal Reproduction (Ibaraki, Japan). Rats were housed under controlled light condition (12h light: light on 07:00~19:00), and food and water were given *ad libitum*. All animals received humane care according to the Guide for the Care and Use of Animals of The University of Tokyo.

2. Animal Models

1) Adult Animals

Estrous cycles were monitored every day by observation of vaginal smears, and only those animals showing consecutive regular 4-day estrous cycles were used. Ovarian follicles were obtained at estrus. Rats were killed by decapitation and ovaries were collected and quickly frozen in liquid nitrogen for embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) for immunohistochemical detection of Ski, PCNA and for TUNEL, respectively. The obtained samples were stored at −80℃ until use.

2) Immature Animals

Synchronized folliculogenesis was initiated in immature (age 25 days) rats by administration of of eCG (40 IU/rat, s.c.) followed by hCG (15 IU/rat, s.c.) to induce ovulaton and luteinization (Bell and Lunn, 1968). In this luteinization model, ovulatory rupture occurs 12~16 h after hCG injection (No-

thnick and Curry, 1996). To evaluate Ski expression in the granulosa, luteinizating granulosa cells and CL, rats were sacrificed by cervical dislocation and ovaries were collected during follicular development, preovulatory phase (0, 24, and 48 h after eCG injected) and during the ovulatory phase and postovulatory phase respectively (3, 6, 12, 24, and 48 h after hCG injected; n = 3~4 animals per time point). After removal of connective tissues, ovaries were weighed, and embedded in OCT compound for immunohistochemical detection of Ski and for TUNEL. The obtained samples were stored at −80℃ until use. At each time point, Blood to blood samples were also collected, and after serum were separated by centrifugation, they were stored at −20℃ until assayed for estradiol concentration.

3. Immunohistochemical Analysis of Ski and PCNA

Frozen tissue sections (5 μm thick) were prepared from the OCT-embedded rat ovary tissues, mounted on glass slides, air-dried and fixed in 4% paraform aldehyde (PFA) in phosphate buffered-saline (PBS) for 20 min, followed by incubation in 0.1% Triton X-100 in PBS for 15 min. After several washes with PBS, endogenous peroxidase activity was inactivated by incubation in 0.3% hydrogen peroxide in methanol for 30 min. Then the sections were immersed in blocking solution (8% skim milk in PBS) for 30 min. Then, the primary rabbit antibody specific for Ski [Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100 with 5% normal goat serum (NGS) in PBS] or mouse monoclonal antibody specific for PCNA (Santa Cruz Biotechnology; dilution 1:200 with 5% NGS in PBS) was applied and incubated for 60 min, respectively. After several washes with PBS, the sections were incubated with the simple stain MAX-PO (R; for rabbit primary antibody; Nichirei, Tokyo, Japan) or the MAX-PO (M; for mouse primary antibody; Nichirei), which is a horse-radish peroxidase conjugated secondary antibody, for 60 min, and then positive signals were developed with 3,3'-diaminobenzidine (DAB; Dojindo, Kumamoto, Japan), respectively. The sections were counterstained with hematoxylin to visualize nuclei.

4. Determination of Apoptotic Cells by TUNEL Method

Frozen sections of ovaries (5 μm thick) were air-dried and fixed in 4% PFA in PBS for 20 min. To detect the DNA fragmentation, TUNEL was performed using commercial kit (*In Situ* Cell Detection Kit, POD; Roche, Penzberg, Germany). All experiments were performed according to the manufacturer's instructions. The slides were rinsed with 3% hydrogen peroxide

in methanol for 30 min and incubated with permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. After several washes with PBS, the sections were incubated with TdT and detection buffer conjugated with horse-radish peroxidase (Converter-POD) for 60 min at 37 C. Positive signals were developed with DAB, and the sections were counterstained with hematoxylin to visualize nuclei.

5. Hormone Assay

Serum concentrations of estradiol were measured by estradiol enzyme-immunoassay kits (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's protocol.

6. Statistical Analysis

Statistical analyses were conducted using StatView (version J5, Abacus Concepts, Berkley, CA, USA). One-way analysis of variance and Dunnett's test were used to determine differences betweeneCG/hCG treated and control groups. Differences were considered statistically significant at $p<0.05$.

RESULTS

1. Localization of Ski Protein in the Corpora Lutea (CL)

To locate the Ski protein expression in the corpora lutea (CL), immunohistochemical analysis was performed. As shown in Fig. 1, Ski was localized in most of the cells within CL and widely distributed throughout the CL.

2. PCNA- and TUNEL-Positive Cells in the CL

In order to determine whether the presence of PCNA- and TUNEL-positive cells within the same CL is exclusive, immunohistochemical analysis of PCNA and TUNEL staining were performed. As shown in Fig. 2, both PCNA- and TUNEL-positive cells are present within the same CL. This indicates that cell proliferation and cell death can occur simultaneously within the CL.

3. Overlapping of Ski-Positive and TUNEL-Positive Cells

In order to determine if Ski-positive cells of the CL are overlapped with TUNEL-positive cells and are exclusive to PCNA-positive cells as was observed in follicles, double-staining experiments were performed. As shown in Fig. 3, proliferating PCNA-positive cells were not co-localized with Ski-positive cells. On the other hand, Ski was present in TUNEL-positive cells. These results suggest that Ski is involved in luteolysis.
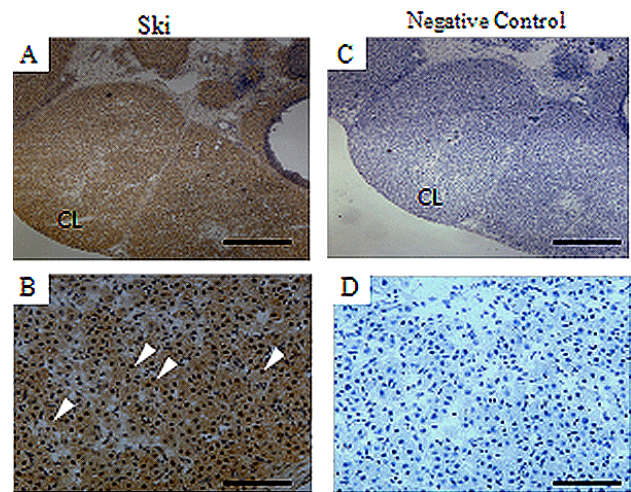

Fig. 1. Immunohistochemical localization of Ski in the corpus luteum of an adult female rat at estrous. A (B) and C (D) are adjacent sections showing Ski-staining and negative control, respectively. CL, corpus luteum. Ski-positive cells (white arrowheads in B) are widespread throughout in the corpus luteum. B and D, high magnification of A and C. Scale bars = 400 μm in A and B 100 μm in B and D.
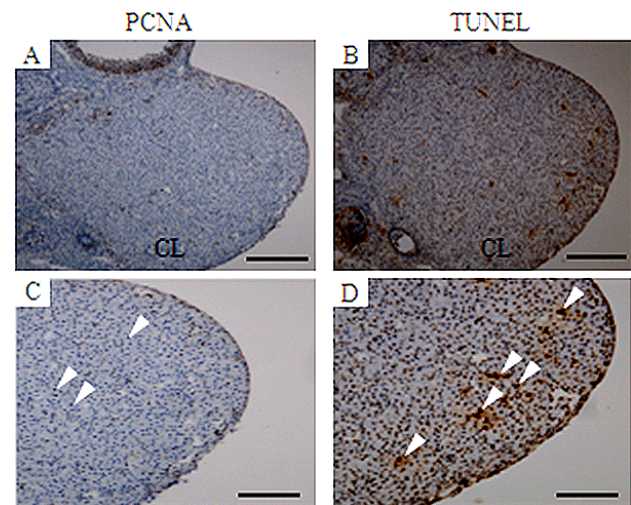

Fig. 2. Immunohistochemical analyses of PCNA and TUNEL in the corpus luteum of the adult rat. A (C) and B (D) show PCNA and TUNEL stainings, respectively. CL, corpus luteum. Note the presence of PCNA and TUNEL positive luteal cells (white arrowheads in C and D). C and D, high magnification of A (B). Scale bars = 400 μm in A and B, 100 μm in C and D.

4. Expression of Ski Protein during Luteinization

To investigate the possible involvement of Ski during luteinization, immunohistochemical analysis of Ski was performed on ovarian sections obtained from rats having single genera-
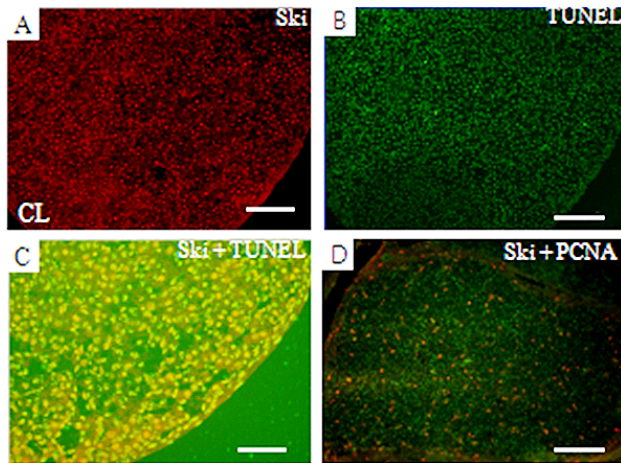
Fig. 3. Immunofluorescent double-staining of Ski (A) and TUNEL (B) in the corpus luteum of adult female rat. Note that Ski-positive luteal cells (red) and TUNEL-positive luteal cells (green) are mostly overlapped (yellow). C, merged photos of A and B. Note that Ski-positive cells (green) and PCNA positive cells (red) are not overlapped (D). CL, corpus luteum. Scale bars = 200 μm in A, B, C and D.

Fig. 4. Changes in ovarian weights (A) and serum estradiol-17β concentrations (B). CTRL, control (without hormonal treatment). The data are means ± SE (n = 3). *, $P<0.01$ vs. CTRL

Fig. 5. Immunohistochemistry of Ski (A) Time (h) indicates hours after hCG injection. Note the absence of Ski-positive granulosa cells in preovulatory follicle (0 h). Scale bar = 400 μm. (B) Quantitative analyses of Ski-positive cells. The data are expressed as proportions of Ski positive cells. The data are means ± SE (n = 6). *, $P< 0.01$ vs. hCG 3, 6 and 12 h.

tion of CL. For this purpose, follicular growth was induced with eCG, then followed by hCG to induce subsequent luteinization and ovulation. Consistent with previous reports (Bell and Lunn, 1968), ovarian weights were significantly higher in the rats treated with eCG only and primed with eCG/hCG than in the control rats, indicating that eCG and hCG treatment successfully induced follicular growth, ovulation and luteinization (Fig. 4A). Serum estradiol concentrations were higher in the rats treated with eCG only and primed with eCG/hCG than in the control rats until 12h after hCG injection (Fig. 4B). Thereafter, estradiol concentrations were decreased to a level comparable to that in control rats, representing the occurrence of initiation of luteinization (Fig. 2∼6). These results indicate the successful treatment with eCG and hCG to establish a rat model having single generation of CL. Then, we performed immunohistochemical analyses of Ski on ovarian sections from eCG/hCG treated rats. As shown in Fig. 5, Ski was expressed in luteinizing granulosa cells at 6 and 12 h after hCG injection. Ski expression was persisted after the formation of CL (48 and 96 h after hCG). Quantitative analysis revealed that the proportion of Ski-positive cells at 24 h after hCG injection was significantly higher than that of the previous time points (before 12 h). Since ovulation in this rat model is expected to occur between 12 and 24 h, this result suggests that the number of Ski-positive cells increases after ovulation.
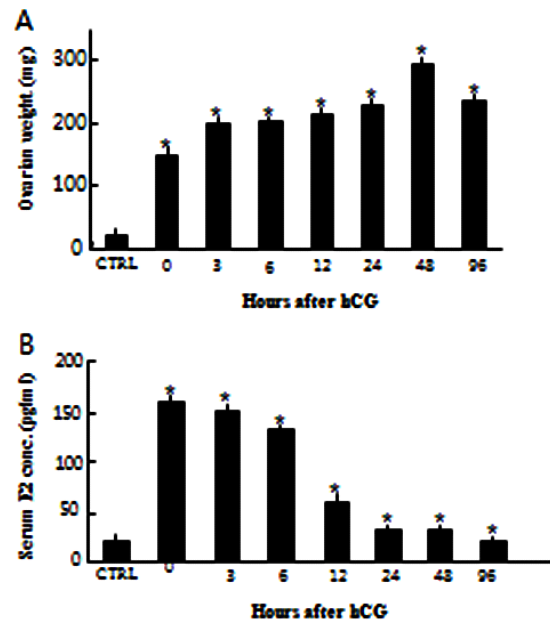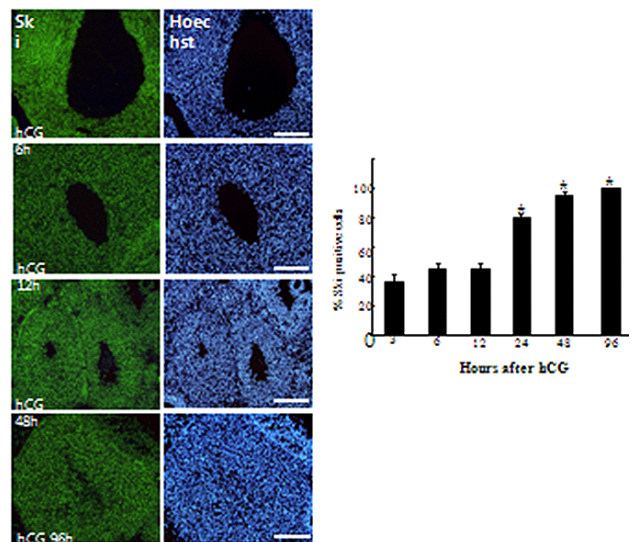
To exclude the possibility that apoptotic cell death is taking place during CL formation in eCG/hCG treated rats, TUNEL was performed. No apoptotic cell death was detected during

luteinization whereas Ski was present (Fig. 6). On the other hand, a numerous number of TUNEL-positive cells were observed in atretic follicle containing Ski-positive cells. These results clearly exclude the possible presence of Ski-positive cells that are dying during CL formation. In Table 1, the results of immunohistochemistry of Ski and TUNEL staining were summarized. Taken together, the results suggested that Ski expression is induced in granulosa cells by the effect of hCG (LH) and the number of Ski-positive cells increases after ovulation.

DISCUSSION

The presence of Ski has been demonstrated in a variety of tissues (Lyons *et al*., 1994; Yamanouchi *et al*., 1997), as was shown in this study, and is suggested to play multiple roles in
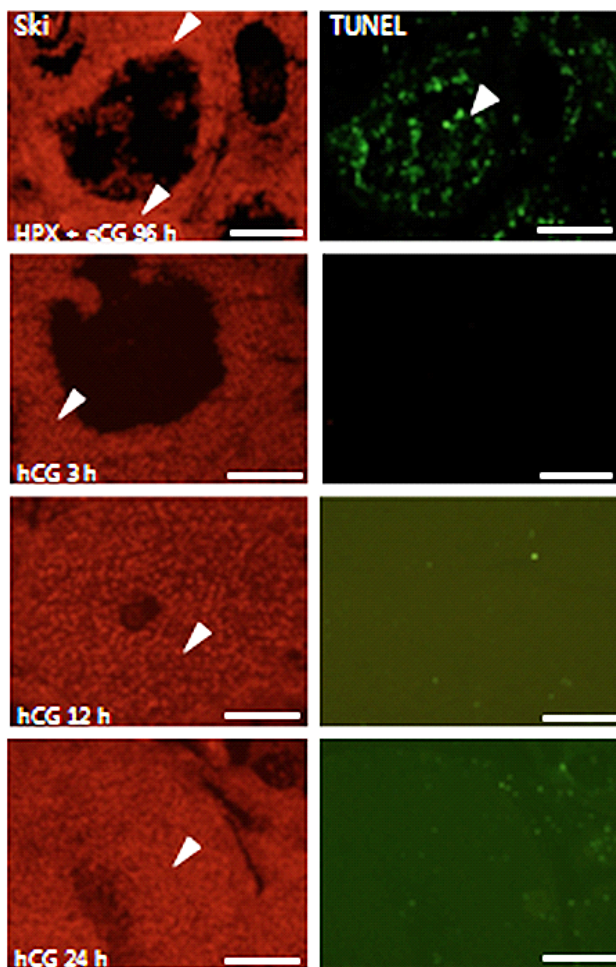

Fig. 6. Immunohistochemistry of Ski and TUNEL staining. Note the presence of Ski and TUNEL double-positive cells only in atretic follicle (HPX+eCG 96 h). Scale bar = 400 μm.

Table 1. Summary of TUNEL and immunostaining of Ski in luteinizating granulosa cells and luteal cells during luteinization

	Luteinization (After hCG treatment)						
	0	3	6	12	24	48	96
Ski	−	+	+	+	++	++	+++
TUNEL	−	−	−	−	−	−	−

Signal intensity: −, weak; +, moderate; ++, intense; +++, very intense

a variety of cell types (Colmenares and Stavnezer, 1989; Ambrose *et al*., 1995; Heyman *et al*., 1997). For example, Ski is expressed in proliferating myoblast (Soeta *et al*., 2001) and uterine epithelial cells (Yamanouchi *et al*., 1999). In the uterus, endometrial *c-Ski* gene expression has been induced by estrogen treatment, which is known to induce proliferation of uterine epithelial cells (Yamanouchi *et al*., 1999), and progesterone treatment eliminated estrogen-induced *c-Ski* expression (Yamanouchi *et al*., 2000). In addition, *c-Ski* is known to induce myogenic differentiation of quail embryonic cells (Colmenares and Stavnezer, 1989). Thus, most studies to date have indicated that Ski mediates cell proliferation and differentiation (Liu *et al*., 2001; Luo, 2003; Medrano, 2003).

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. During luteinization, the steroidogenic follicular granulosa and theca cells differentiate to luteal steroidogenic cells, and simultaneously other non-steroidogenic cell populations such as fibroblast, endothelial, blood and immune cells migrate extensively into the newly formed CL (Naito and Takahashi, 1988; Nelson *et al*.,1992; Brännström *et al*., 1994; Matsuyama, 1995). Therefore, the Ski-positive cells observed in the CL may consist of both steroidogenic and non-steroidogenic cells. Since these non-steroidogenic cells are essential for luteinization (Naito and Takahashi, 1988; Nelson *et al*., 1992; Brännström *et al*., 1994; Matsuyama, 1995), this suggests the other novel role of Ski in non-steroidpgenic cells in the CL.

In conclusion, the results of this study demonstrated, as far as we 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. The mechanism underlying the Ski expression of CL, if present, is currently unknown. Ski, as a nuclear protein, has been shown to be associated with a variety of other cellular proteins (Akiyoshi *et al.*, 1999; Wu *et al.*, 2002), and it is believed that such a unique property of Ski enables it to express multiple functions (Colmenares and Stavnezer, 1989; Ambrose *et al.*, 1995; Yamanouchi *et al.*, 2000). In this regard, the search for novel Ski-interacting proteins in granulosa cells would be of interest to further clarify the uncovered function of Ski. Further studies are still required to reveal the molecular mechanisms regulating Ski protein levels and activity in the ovary.

ACKNOWLEDGEMENTS

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