Relationship between Sloan-Kettering Virus Expression and Granulosa Cells of Atretic Follicles 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. The aim of the present study was to locate Ski protein in rat ovaries in order to predict the possible involvement of Ski in follicular development and atresia. First, expression of *c-Ski* mRNA in the ovaries of adult female rats was confirmed by RT-PCR. Then, ovaries obtained on the day of estrus were subjected to immunohistochemical analysis for Ski and proliferating cell nuclear antigen (PCNA) in combination with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL). Ski was expressed in granulosa cells that were positive for TUNEL, but negative for PCNA, regardless of the shape and size of follicles. Expression of Ski in TUNEL-positive granulosa cells, but not in PCNA-positive granulosa cells, was also verified in immature hypophysectomized rats having a single generation of developing and atretic follicles by treatment with equine chorionic gonadotropin (eCG). These results indicate that Ski is profoundly expressed in the granulosa cells of atretic follicles, but not in growing follicles, and suggest that Ski plays a role in apoptosis of granulosa cells during follicular atresia.

(Key words : Ski, Ovary, Granulosa cell, Follicular atresia, Apotosis)

INTRODUCTION

Cellular protooncogene *c-Ski* was originally shown to induce myogenesis when transfected to non-myogenic quail embryo cells *in vitro* (Colmenares *et al.*, 1989). Transgenic mice expressing the *c-Ski* gene under the control of a murine sarcoma virus long terminal repeat show large increases in their skeletal muscle mass (Sutrave *et al.*, 1990), while *c-Ski*-deficient mice have defects in their skeletal muscle development (Berk *et al.*, 1997). In addition, the mice lacking *c-Ski* show perinatal lethality due to defects in neurulation and craniofacial patterning as well as skeletal muscle development, and excessive apoptosis has been detected in *c-Ski* deficient mouse embryos (Berk *et al.*, 1997; Shinagawa *et al.*, 2001), supporting the idea that Ski can act as an anti-apoptotic factor (Soeta *et al.*, 2001). It has been also suggested that *c-Ski* is involved in mediating proliferative effect of 17β-estradiol in uterine epithelial cells (Yamanouchi *et al.*, 1999).

Ski protein is a nuclear transcription factor that does not bind DNA directly (Nagase *et al.*, 1990; Baker *et al.*, 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. Therefore, *c-Ski* gene product (Ski) has been implicated to have dual roles in both regulating proliferation and differentiation of theses 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 the ovary. However, the role of this gene in the ovary remains unknown.

Within the mammalian ovary, follicles are the functional unites. Each follicle contains an oocyte surrounded by one or several layers of somatic (granulosa and theca) cells (McNatty *et al.*, 2005). Follicles progress through a serious of complex processes from the resting primordial stage to the point of ovulation, however, only very few follicles reach the ovulatory stage with most undergoing atresia (Knight *et al.*, 2006). The rat

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has an incomplete 4~5 days estrous cycle, and their ovaries contain follicles at various stages of development (growing follicles, preovulatory follicles, and atretic follicles) during the estrous cycle. Thus, the rat ovaries may be useful to predict the role of Ski protein by immunohistochemical analyses. In addition, several experimental models that represent specific stages of follicular development (Dhanasekaran *et al.*, 1989; Shi *et al.*, 2003), and follicular atresia (Boone *et al.*, 1997) have been well established in the rat.

The aim of the present study was, by means of immunohistochemical techniques, to locate the Ski protein expression in the rat ovaries during the estrous cycles and in ovaries with a having single generation of developing and atretic follicles in order to predict the possible involvement of Ski in follicular development and atresia.

MATERIALS AND METHODS

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 (12 h 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.

Animal Models

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 from 8- to 14-week-old rats at estrus. Rats were sacrificed by decapitation and cerebrum, cerebellum, small intestine, lung, heart, skeletal muscle, spleen, kidney, liver, uterus, ovary, fat, prostate gland and testis were collected and quickly frozen in liquid nitrogen for RT-PCR or embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) for immunohistochemical detection of Ski, proliferating cell nuclear antigen (PCNA) and for TdT-mediated dUTP biotin nick end labeling (TUNEL) respectively. The obtained samples were stored at −80℃ until use.

Immature Animals

Immature female Wistar-Imamichi hypophysectomized (Hypox) rats (at the age of 25 days) were purchased and housed as described above. As shown in, Scheme 5, Hypox rats were treated subcutaneously with equine chorionic gonadotropin (eCG; 40 IU/rat in 200 ml saline) at the age of 26 days are known to exhibit ovarian follicular development of a single generation of follicles and subsequent follicular atresia due to lack of hypophyseal LH surge (Boone *et al.*, 1997). Rats were sacrificed by cervical dislocation at 0, 2 and 4 days after eCG treatment, and ovaries were excised. After removal of connective tissues, ovaries were weighed, embedded in OCT compound for immunohistochemical detection of Ski, PCNA and for TUNEL, respectively. The obtained samples were stored at −80℃ until use. At each time point, Blood samples were also collected, and after serum were separated by centrifugation, they were stored at −20℃ until assayed for estradiol concentration.

Extraction of Total RNA

Briefly, one hundred mg of the tissue was homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with Digital Homogenizer (Iuchi, Osaka, Japan). Then, 200 μl of chloroform per 1 ml TRIzol reagent was added to the homogenates 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 μl diethylpyrocarbonate-treated water. The RNA concentrations were measured spectrophotometrically.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

First-strand cDNA was synthesized by using SuperScript II (Invitrogen) with oligo d(T)16 primers (500 μg /2.5 μM) 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 μM), 2 μl of dithiothreitol, 2 μl of RNase inhibitor, 1 ml 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 aTaq 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 a-Taq (5 units/μl), 5 μl of 10 × PCR buffer, 4 μl of dNTPs (2.5 μM), 3.75 μl of 10 × Enhancer, 33 μl of sterilized water for PCR and 1 μl 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 (Nomura *et al.*, 1989; Namciu *et al.*, 1995; Yamanouchi *et al.*, 1997). Rat-specific glyceraldehyde-3-phosphatedehydrogenase (GAPDH) primers were designed using Primer3 software (available at http://fokker.wi.mit.edu/primer3/) and the primers were: forward primer: 5′-CAT TGT TGC CAT CAA CGA CCC CTT-3′ and reverse primer: 5′-ACT CAG CAC CAG CAT CAC CCC ATT-3′), respectively. The *c-Ski* genes were amplified under following conditions: 1cycle of 94℃ for 5 min; 30 cycles of 94℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min; 1 cycle of 72℃ for 10min. The *GAPDH* genes were amplified under following conditions: 1 cycle of 94℃ for 5 min; 35 cycles of 94℃ for 1 min, 60℃ for 1 min, 72℃ for 1 min; 1 cycle of 72℃ for 10 min, respectively. The predicted PCR amplified size of *c-Ski* and *GAPDH* were 561 and 194 bp, respectively. After PCR, an aliquot of the reaction mixture was electrophoresed on 1% agarose gel and the products were visualized with ethidium bromide staining.

Immunohistochemical Analysis of Ski and PCNA

Frozen tissue sections (5 μm thick) were prepared from the OCT-embdedded 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.

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℃ Positive signals were developed with DAB, and the sections were counterstained with hematoxylin to visualize nuclei.

Hormone Assay

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

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 between eCG-treated and control groups. Differences were considered statistically significantat $p<0.05$.

RESULTS

Expression of *c-Ski* Gene in Various Tissues

To analyze the tissue distributions of the *c-Ski* gene in the rat, RT-PCR analysis was performed. As shown in Fig.1, the *c-Ski* gene was expressed in all the tissues examined, which was in agreement with the previous findings made in mice by Lyons *et al.* (1994) and in equine by Yamanouchi *et al.* (1997). The ovary was one of the tissues that intensively expressed the *c-Ski* gene.

Localization of Ski Protein in the Follicles of Adult Female Rats

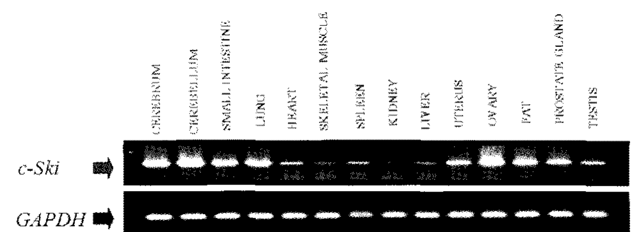

Fig. 1. RT-PCR analysis of *c-Ski* (upper) and *GAPDH* (lower) genes in various tissue from adult male and female rats.

To locate Ski protein expression in the ovaries of adult females, immunohistochemical analysis was performed. As shown in Fig. 2, the granulosa cells of follicles exhibited both positive and negative staining for Ski protein regardless of the size of follicles and stage of follicular development. On the other hand, almost all theca cells showed intensely positive staining.

Ski Protein Expression in Granulosa Cells of Atretic Follicles

Since Ski protein has been implicated to have roles in cell proliferation (Yamanouchi *et al.*, 1999; Shinagawa *et al.*, 2001; Soeta *et al.*, 2001) and predicted to act as an anti-apoptotic factor (Berk *et al.*, 1997), the above results showing a distinct Ski protein expression pattern in granulosa cells of the follicles led us to examine the relationship between follicular growth and/or apoptosis (atresia) and Ski protein expression. For this purpose, serial ovarian sections of adult rats were subjected to immunohistochemical analyses of Ski and PCNA and to TUNEL staining, respectively. As shown in Fig.3, apparently Ski-positive granulosa cells were seen in atretic (apoptosis) follicles judged by TUNEL staining where no PCNA-positive granulosa cells were seen. On the other hand, granulosa cells of healthy (growing) follicles exhibited intense PCNA immunoreactivity, and were neither positive for Ski nor TUNEL. These results suggest that Ski protein expressed in granulosa cells of atretic follicles may have apoptosis-related function in these cells.

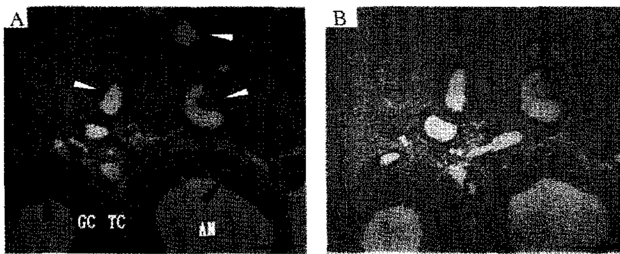

Fig. 2. Immunohistochemical analysis of Ski in the ovarian follicles of an adult female rat at estrus. A and B are adjacent sections showing Ski staining and negative control, respectively. GC, granulosa cells; TC, theca cells; AN, follicular antrum. Black and white arrowheads indicate Ski-positive and Ski-negative granulosa and thecal cells, respectively. Scale bars=400 mm.

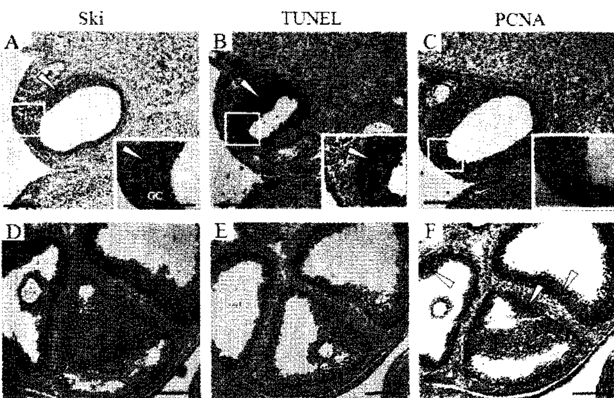

Fig. 3. Immunohistochemical analyses of Ski, TUNEL and PCNA in the follicle of the adult rat. A (D), B (E) and C (F) show Ski, TUNEL and PCNA stainings, respectively. GC, granulosa cells; AN, follicular antrum. Note that granulosa cells which are positive for Ski, TUNEL and PCNA (white arrowheads in A, B and F), but negative for Ski, TUNEL and PCNA, respectively. The inset is a higher magnification of one of the follicles (Scale bars=100 mm). Scale bars =400 μm in A, B and C, 200 mm in D, E and F.

Localization of Ski Protein in the Follicles of Single Generation in eCG-Primed Immature Hypophysectomized Rats

Gonadotropin is an important survival factor for the developing follicles to escape from atresia and to reach the preovulatory follicle stage (Byskov *et al.*, 1978; Byskov *et al.*, 1979; Greenwald *et al.*, 1988; Hirshfield *et al.*, 1988; Hirshfield *et al.*, 1991; Hsueh*etal.*, 1994). Moreover, preovulatory follicles that are not exposed to hypophyseal LH surge at appropriate period undergo atresia (Hori *et al.*, 1969; Mahesh. 1985; Tebar *et al.*, 1995; Donath *et al.*, 2000; Asai *et al.*, 2002). As shown in Fig. 4, Immature hypophysectomized rats primed with eCG is known to exhibit development of the follicles of single generation and subsequent follicular atresia due to the lack of hypophyseal LH surge (Boone *et al.*, 1997).

To examine if the correlations between Ski expression and PCNA expression as well as those between Ski expression and TUNEL staining are also observed in this immature rat model, immunohistochmical and TUNEL analyses were performed in the ovary having follicles of single generation. Serum estradiol concentrations as well as ovarian weights on day 0, 2 and 4 days after eCG administration were measured. As shown in

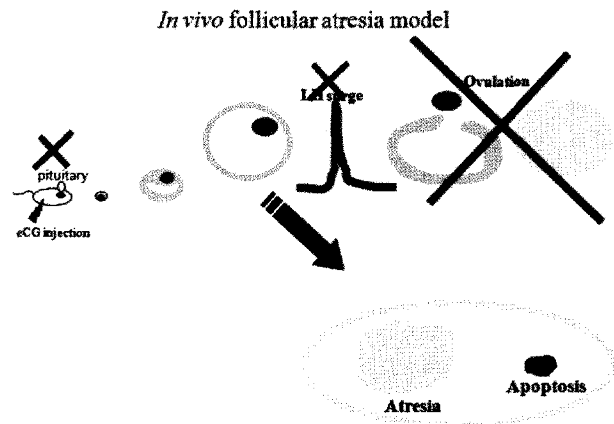

Fig. 4. Immature hypophysectomized rats primed with eCG are known to exhibit development of a single generation of follicles and subsequent follicular atresia due to lack of an LH surge (Boone *et al.*, 1997).

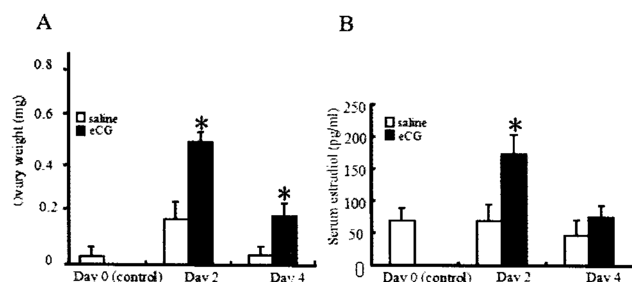

Fig. 5. Changes in ovarian weights (A) and serum estradiol-17β concentrations (B). CTRL, control (without hormonal treatment). The data are means±SE (n=6). *, $p<0.05$ vs. CTRL

Fig. 5A and B, both serum estradiol concentrations and ovarian weights were significantly higher in eCG-primed rats than in control rats on 2 day after administration, indicating that eCG treatment successfully induced follicular growth. However, on day 4 after treatment, neither estradiol concentrations (Fig. 5A) nor ovarian weights (Fig. 5B) were different between control and eCG-primed rats, indicating the occurrence of follicular atresia.

As shown in Fig. 6, on day 0, some follicles exhibited positive staining of either PCNA or TUNEL in granulosa cells but they were exclusive. In early proliferating granulosa cells so-called early antral follicles,

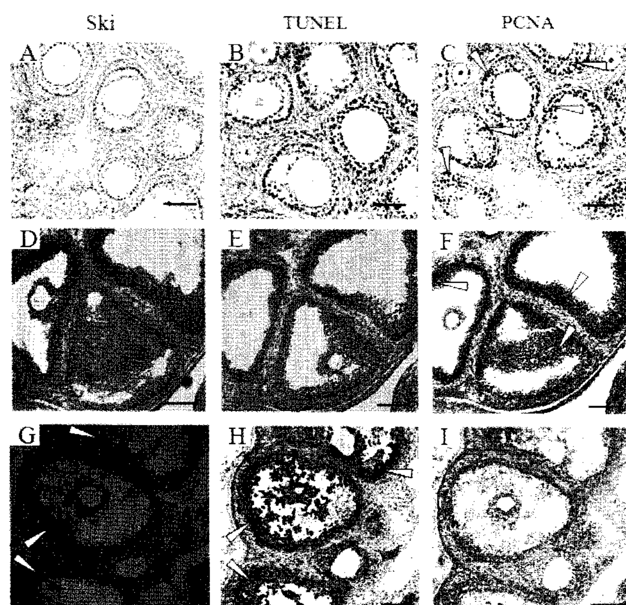

Fig. 6. Immunohistochemical analyses of ovarian sections from eCG-primed immature hypophysectomized rats. Panels A, B and C are adjacent sections showing the Ski, TUNEL and PCNA signals, respectively, 0 days after eCG treatment. D, E and F are adjacent sections showing the Ski, TUNEL and PCNA signals, respectively, 2 days after eCG treatment. G, H and I are adjacent sections showing the Ski, TUNEL and PCNA signals, respectively, 4 days after eCG treatment. Arrowheads indicate positive signals for PCNA (C and F), Ski (G) and TUNEL (H) in granulosa cells. Scale bars =400 μm.

no Ski-positive and TUNEL-positive granulosa cells were observed while they were mostly positive for PCNA. As shown in Fig. 6, on day 2 after eCG administration, more follicles with PCNA positive granulosa cells became evident indicating the increased number of growing follicles but still no Ski-positive and TUNEL-positive granulosa cells were observed in late antral follicles. However, as shown in Fig. 6, on day 4 after eCG administration, a numerous number of follicles with TUNEL-positive granulosa cells appeared indicating these follicles are undergoing to atresia and granulosa cells in these atretic follicles were positive for Ski and negative for PCNA. Thus, these results confirm that Ski protein is expressed in granulosa cells undergoing to apoptosis.

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 a variety of cell types (Colmenares *et al.*, 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 *et al.*, 1989). Thus, most studies to date have indicated that Ski mediates cell proliferation and differentiation (Liu *et al.*, 2001; Luo *et al.*, 2003; Medrano *et al.*, 2003). Actually, the initial observation showing distinct expression of Ski in granulosa cells in the follicles lead the author to predict that Ski might be expressed in growing follicles. However later experiments revealed that this was not the case, and Ski was unexpectedly found to be expressed in apoptotic granulosa cells rather than proliferating granulosa cells in the ovary. In relation to apoptosis, Ski has been implicated to have a role as anti-apoptotic factor. For examples, mice deficient for *c-Ski* show excessive apoptosis (Berk *et al.*, 1997; Shinagawa *et al.*, 2001) and overexpression of antisense *c-Ski* in L6 myoblasts causes apoptosis of the cells (Soeta *et al.*, 2001). Thus, the present notion that Ski may be involved in apoptosis proposes a new concept regarding the Ski function.

Many apoptosis related genes have been discovered in various tissues and organs including the ovary. It has been reported that p53 up-regulates several apoptosis-related proteins, such as Bax (Miyashita *et al.*, 1995),

cyclin G (Okamoto *et al.*, 1994), p21 (El-Diery *et al.*, 1993), IGFBP-3 (Buckbinder *et al.*, 1995), Gadd45 (Kastan *et al.*, 1992), mdm-2 (Barak *et al.*, 1993), and Fas (Muller *et al.*, 1997). Though the precise role of Ski in mediating apoptosis of granulosa cells in the ovary is unknown so far, Ski may induce some downstream apoptosis-related gene in these cells during apoptosis.

The mechanism underlying the Ski-induced apoptosis of granulosa cells, 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 *et al.*, 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.

PAL31 (proliferation-associated leucine-rich protein) is a nuclear protein expressed by various cell types (Sun *et al.*, 2001). Analysis of PAL3 1 mRNA in the adult tissues revealed that it is expressed at a high level in the spleen, testis, thymus and ovary but at a low level in the adult brain (Mutai *et al.*, 2000). PAL31 has been shown to be required for cell cycle progression (Sun *et al.*, 2001) and colocalized with PCNA in neural progenitor cells in rat brain and PC12 cells (Mutai *et al.*, 2000). PAL31 also has been shown to function as a caspase-3 inhibitor (Sun *et al.*, 2006). These results indicate that PAL31 acts not only in cell cycle progression but also as a cell survival factor. This was quite contrast to the possible function of Ski in granulosa cells as suggested in this Chapter, and lead the author to suspect possible relation of Ski to PAL31 during follicular development and atresia in the ovary. This issue would be further studied in the future.

In conclusion, the results of present study demonstrated, as far as the author knows, for the first time the cellular localization of Ski in the ovary and that high levels of Ski are expressed in atretic follicles, but not in healthy follicles. Based on the present findings, it was suggested that Ski is involved in inducing apoptosis of granulosa cells and plays a key role in follicular selection. Further studies are needed to clarify its function, interaction, and correlation with other molecules in rat ovaries.

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