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ORIGINAL ARTICLE

Relationship between Sloan-Kettering virus expression and mouse follicular development

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Abstract Sloan-Kettering virus gene product (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/SnoN are important transcription regulators of the transforming growth factor-β (TGF-β) superfamily and function mainly through heterodimers. Since TGF-β superfamily are key regulators of follicle development and it has been previously shown that SnoN is also vital to follicle development, this research was conducted to clarify the relationship between Ski expression and mouse follicular development, in ovaries of neonatal and gonadotropin-induced immature mice by immunohistochemical and real-time PCR techniques. In postnatal mice, positive staining for Ski was highly detected in oocyte nuclei at postnatal day 1. With follicular development, the localization moved gradually from oocyte nuclei to perinuclear space and the total levels decreased. During the estrous cycle, Ski expression was apparent at proestrus and estrus, faint at metestrus, highest at diestrus. After injection of gonadotropin, Ski was found in perinuclear space and weak in oocyte nuclei. Following the initiation of luteinization, the expression of Ski was found in corpus luteum. Real-time PCR results also showed that *Ski* mRNA expression was opposite to ovulation-related genes during the cumulus expansion, with the development of the follicles, its expression level decreased. Ski is expressed in a specific manner during follicle development, ovulation and luteinization. So Ski might play essential roles in these processes especially during early follicular development.

Keywords Ski · TGF-β · Ovary · Follicular development · Oocyte

Introduction

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 [1]. 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 and subsequent the corpus luteum (CL) formation, with most undergoing atresia [2]. Progression during follicle development requires effective communication between both the oocyte and granulosa cells, and between granulosa and theca cells [3]; it also requires pituitary gonadotrophins, endocrine signals, and metabolic hormones, which interact with local autocrine/paracrine signaling pathways [4].

Many of the factors in this dialog belong to the transforming growth factor-β (TGF-β) superfamily. The TGF-β superfamily include three mammalian isoforms of TGF-β (TGF-$\beta_{1,2,3}$), anti-mullerian hormone (AMH), two inhibins (A, B), three activins (A, B, and AB), bone morphogenetic proteins (BMP-1-BMP-20), and at least nine growth differentiation factors (GDF-1-GDF-9). Members of the TGF-β superfamily have wide ranging influences on many tissue and organ systems including the ovary signaling via

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the TGF-β signaling pathway: First, TGF-β combines with its receptor and induces phosphorylation of the receptor-related Smad (R-Smad) proteins, including Smad2 and Smad3; the phosphorylated R-Smads then form heteromeric complexes with a common mediator Smad (Co-Smad), Smad4, and translocate into the nucleus to activate the transcription of downstream genes. Members of the TGF-β superfamily are critical regulators of follicle development, especially GDF-9 and BMP-15, which are expressed in an oocyte-specific manner from a very early stage and play a key role in promoting follicle growth beyond the primary stage [5]. TGF-β superfamily members are not the only factors to be expressed by oocytes, granulosa cells, and theca cells in a developmental stage related manner, functioning as intraovarian regulatory molecules vital to follicle recruitment, granulosa and theca cell proliferation/atresia, steroidogenesis, oocyte maturation, ovulation, and luteinization [6, 7]. TGF-β superfamily receptors, ligands, and binding proteins are also expressed in a developmental stage manner in different ovarian compartments [2, 8–10].

Sloan-Kettering virus gene product (Ski), an unique nuclear protein, is a member of the *ski/sno* proto-oncogene family. Ski is ubiquitously expressed in all adult and embryonic tissues, but in low levels [11, 12]. Upregulation of Ski and SnoN expression is detected only in specific tissues and cell types during defined stages of embryonic development and in some human cancer cells [13, 14]. Ski has been revealed to be a significant negative regulator of TGF-β pathways by interacting with Smad proteins (Smad2, Smad3, and Smad4) which are critical components of the signaling pathway [11, 12, 15–17]. This interaction suppresses the ability of the Smads to activate TGF-β target genes and subsequent downstream genes [9, 15]. Furthermore, overexpression of Ski can induce morphological transformation and anchorage-independent growth of chicken embryo fibroblasts [18, 19], and can block TGF-β-induced growth arrest in TGF-β responsive cells [9]. As a transcription factor, Ski can form homodimers and heterodimers with SnoN and the Ski–SnoN heterodimers have been shown to have more dominant activity [20].

Ski/SnoN are negative regulators of TGF-β pathways via their interaction with Smad proteins, and since TGF-β signaling plays an important role in follicular development, SnoN is also vital to follicle development [10]. Ski may also have an important role in follicular development. Recent studies have demonstrated that Ski is detected in the granulosa cells of atretic follicles, and might be one of the key molecules involved in follicular atresia in rat ovaries [6]. The aim of this study was to investigate the roles of Ski in mouse follicular development using immunohistochemical and real-time PCR techniques.

Materials and methods

Animals, tissue collection

Male and female mature Kunming white strain mice (8 weeks old) were obtained from the Experimental Animal Center of Shandong University (Jinan, Shandong, China). Animals were bred in a pathogen-free mouse room with a constant photoperiod (light/dark cycle, 12 h/12 h) with water and food ad libitum. All animals were treated according to the Guidelines of Shandong Normal University for the Care and Use of Laboratory Animals.

For estrous cycle analysis, vaginal smears were collected on glass slides daily (8:00 and 9:00 am). Only mice exhibiting normal cycles ($n = 10$, at least three consecutive cycles) were used for the following experiments.

Mature male and female mice (8 weeks old) were mated to produce newborn mice. Immature female mice at postnatal day (PD) 21 ($n = 20$) were treated with a subcutaneous (SC) injection of pregnant mare serum gonadotropin (PMSG, 10 IU) and 48 h later with human chorionic gonadotropin (hCG, 10 IU) to obtain PMSG 24 h, hCG 0, 4, 11, 24 h, and day 23 (without PMSG/hCG treatment) ovaries.

Ovaries were collected and quickly frozen in liquid nitrogen for subsequent real-time PCR analysis or embedded in O.C.T compound (Sakura Finetek Inc., Torrance, CA, USA) for immunohistochemical studies.

RNA extraction and real-time PCR

The RNA was extracted using Trizol reagent (Invitrogen Co, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) was treated with RNase-free DNase (Promega Co, Madison, WI, USA). Moloney murine leukemia virus (MMLV) reverse-transcriptase (Promega Co, Madison, WI, USA) was used for synthesizing single stranded complementary DNA (cDNA).

Real-time PCR was performed using SYBR green master mix in ABI Prism7300 machine (Applied Biosystems, Foster City, CA). The sequences of the primers used were as follows:

Ski (forward):	5′-CTG GAG GCA GAG TTG GAG CAC-3′
(reverse):	5′-CAT CTT CTT CTC GTT TTC AGC-3′
Ptgs2 (forward):	5′-CTC TAT CAC TGG CAC CCC CTG-3′
(reverse):	5′-GAA GCG TTT GCG GTA CTC ATT-3′
β-actin (forward):	5′-TGA GAC CTT CAA CAC CCC AGC-3′

(reverse):	5′-GAT GTC ACG CAC GAT TTC CCT-3′
Tnfaip6 (forward):	5′-AGG TCT GCT ACT GGC ACA TTC-3′
(reverse):	5′-GGC TTG ACT GGA TTT AGA TGC-3′
Has2 (forward):	5′-ACC CTG CCT CAT CTG TGG AGA-3′
(reverse):	5′-TGT TGG TAA GGT GCC TGT CGT-3′
Cd44 (forward):	5′-TCT TGC TAC TGA CTC CAA CGT-3′
(reverse):	5′-TGC CCA CAC CTT CTC CTA CTA-3′
C1qbp (forward):	5′-ACA GGG GCA GAA GGC TGA AGA-3′
(reverse):	5′-GGT CCG CAA GGA AAT CCA TTA-3′

The PCR was carried under the following conditions (3 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C). Melting-curve analyses were checked to verify product identity. PCR was run in triplicate and was expressed relative to the levels of endogenous β-actin in the same sample [21]. PCR amplification efficiency was analyzed using LinRegPCR 11.0 software (Academic Medical Center, Amsterdam, the Netherlands) and data were calculated according to a calibrator sample using $\Delta\Delta$Ct method [22].

Immunohistochemistry

For immunohistochemistry, sections (8 μm thick) were fixed in −20°C acetone for 10 min, then incubated in 0.3% (v/v) Triton X-100 in PBS (pH 7.2) for 20 min. Endogenous peroxidase activity was quenched by incubating samples in 0.3% (v/v) hydrogen peroxide (H_2O_2) for 30 min followed by washing with PBS. The sections were blocked in 5% (w/v) bovine serum albumin (BSA) (Sigma-aldrich Co, Santa Clara, CA, USA) for 30 min at 37°C. After blocking, sections were incubated with Ski antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, SC-9589, 1:50 dilution) overnight at 4°C. Samples were then incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG secondary antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, SC-2768, 1:200 dilution) for 60 min at 37°C before developing with a diaminobenzidine (DAB) kit (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, SC-24982). Negative controls were incubated with preimmune serum instead of the primary antibody. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted. The expression of Ski protein was examined and photographed (Olympus Co, Tokyo, Japan).

Statistical analysis

All numerical data are means ± SEM. Multiple comparisons between groups were tested by one-way analysis of variance (one-way ANOVA) with post-hoc least significant difference (LSD) test. Values were considered significantly different if $P \leq 0.05$. Different letters on the bars indicate significant differences. All data were analyzed by SPSS statistical software (SPSS10 Inc, Chicago, IL, USA).

Results

Expression of Ski protein during postnatal ovarian follicular development

To examine the relationship between Ski expression and postnatal follicular development, we collected ovaries from neonatal mice at PD 1, 4, 6, 10, and 16. Ski was strongly expressed in the oocyte nuclei of germ cell cysts and primordial follicles, and weakly in the perinuclear space at PD 1 (Fig. 1a, b). During ovarian development, the location of Ski expression changed. The expression level in oocyte nuclei gradually decreased, while its level increased around the oocyte nuclei at PD 4 (primordial and primary follicles) and PD 6 (primordial, primary, and secondary follicles) (Fig. 1c, d). When theca cells appeared in the ovary, Ski expression remained detectable in oocyte nuclei and perinuclear space in PD 10 and 16 (early antral stages, Fig. 1e, f), although the total level was lower compared to those at PD 1, 4, and 6.

Expression of Ski protein during the estrous cycle

Immunohistochemical analysis was performed to elucidate whether Ski was expressed in ovaries during estrous cycle. We found that the spatial location of Ski protein at various stages of estrous cycle was not changed. As shown in Fig. 2, Ski protein was detected in the oocytes throughout the estrous cycle. Ski expression was the highest at diestrus (Fig. 2a, b), apparent at proestrus (Fig. 2c, d) and estrus (Fig. 2e, f), and faint at metoestrus (Fig. 2g, h). Also we found that Ski was strongly expressed in the oocytes of small antral follicles but weak in those of large antral follicles and CL. No positive staining for Ski was detected in granulosa, thecal, and interstitial cells in the ovaries of mature mice during estrous cycle.

Ski expression in the ovary of gonadotropin-treated mice

We also explored whether the levels of Ski expression correlated with gonadotropin-induced follicular development

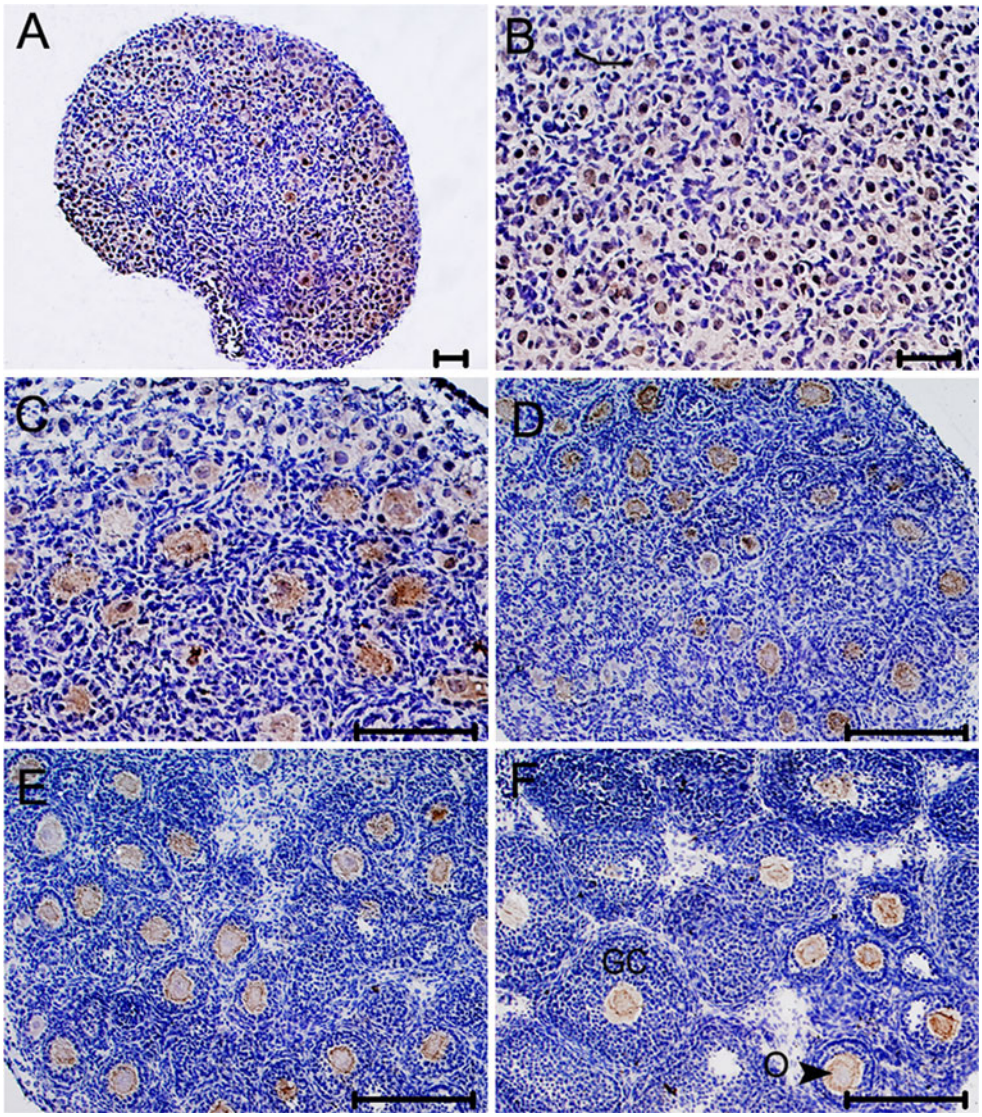

Fig. 1 Immunohistochemical localization of Ski protein in the ovaries of neonatal and immature mice. Ovaries were collected from mice at (**a**, **b**) postnatal day (PD) 1, (**c**) day 4, (**d**) day 6, (**e**) day 10, (**f**) day 16. Positive immunostaining was detected in oocyte nucleus (arrowhead). Positive staining for Ski was first detected in oocyte nucleus and perinuclear space at PD1 (**a**). Higher magnification views Ski immunoreactivity at PD 1 (**b**). During early stages of follicular development Ski immunoreactivity was detected obviously (**c**, **d**). Following ovarian development, staining of Ski decreased gradually, faint at PD 10, 16 (**e**, **f**, respectively). *GC* granulosa cells, *O* oocyte. Scale bars 25 μm (**a**), 50 μm (**b**) and 100 μm (**c**–**f**)

in immature mice. Follicles proceeded to grow to antral, preovulatory, and CL stages after PMSG and hCG stimulation. During follicular growth, Ski immunoreactivity was still present in the oocytes, more strongly in the perinuclear space compared to oocyte nuclei, but decreased in intensity as follicular development progressed (Fig. 3a–c). The Ski protein signal was faint in follicles showing cumulus expansion (4 h post-hCG, Fig. 3d) and hardly detected in preovulatory follicles (11 h post-hCG, Fig. 3e). However, it was expressed in CL (24 h post-hCG, Fig. 3f). Ski protein immunoreactivity was absent in stroma, thecal tissue, and granulosa cells.

Expression of *Ski* mRNA and ovulation-related genes in gonadotropin-induced immature mice

Since the expression level of Ski protein was found to decrease during oocyte development, real-time PCR was used in an attempt to quantify the relationship between *Ski* mRNA expression and ovulation-related genes during the cumulus expansion in follicles of gonadotropin-primed immature mice (Fig. 4a–f). *Ptgs2* and its target *Tnfaip6* are essential for cumulus expansion. The HA pathway, including hyaluran synthase 2 (*Has2*), HA-receptor, *Cd44*, and an HA-interacting protein, *C1qbp* is equally vital

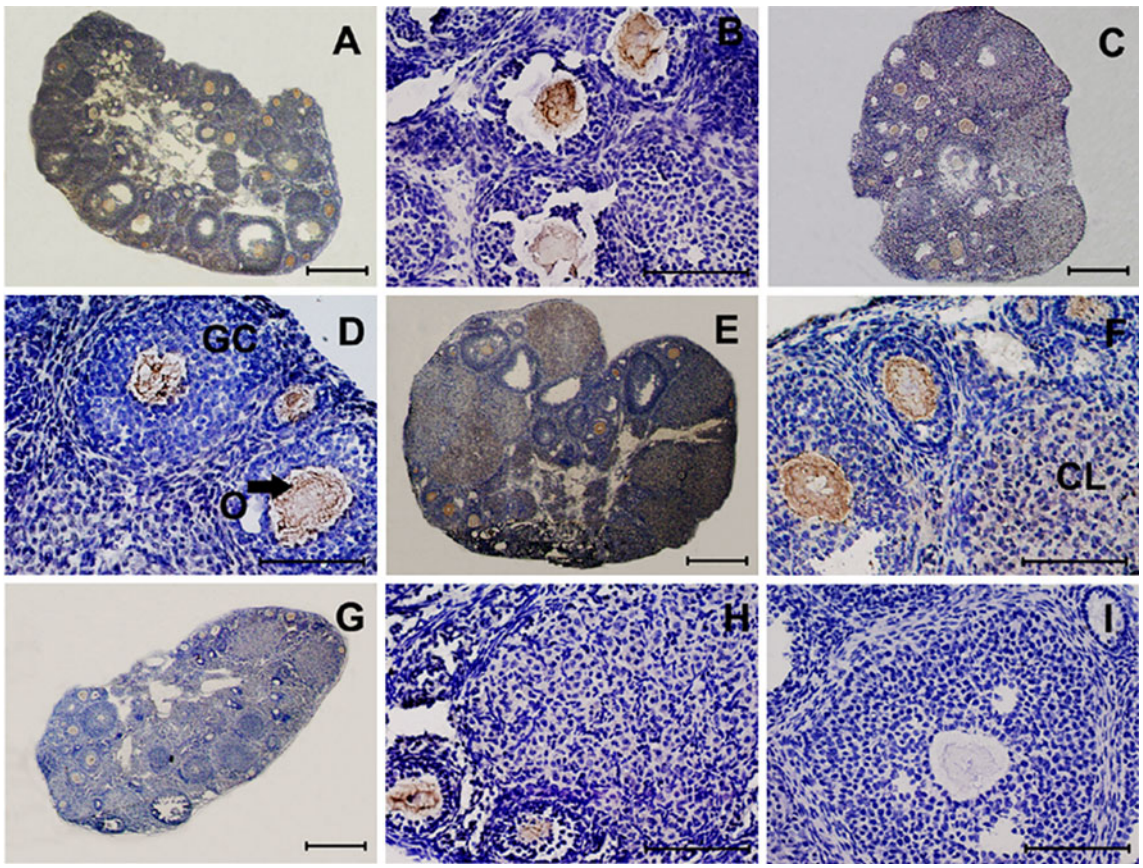

Fig. 2 Localization of Ski protein in mouse ovaries during the estrous cycle. Ski expression was the highest at diestrus (**a**, **b**), apparent at proestrus (**c**, **d**), and estrus (**e**, **f**), and faint at metoestrus (**g**, **h**). Negative control using preimmune serum instead of primary antibody (**i**). *CL* corpus luteum, *GC* granulosa cells, *O* oocyte. *Scale bars* 50 μm (**a**, **c**, **e**, and **g**) and 100 μm (**b**, **d**, **f**, **h**, and **i**)

for cumulus expansion. Strikingly, we found the levels of *Tnfaip6* (Fig. 4b), *ptgs2* (Fig. 4c), and *Has2* (Fig. 4d) [23–25] mRNA expression increased significantly at 4 h post-hCG, when most of follicles are in the stage of cumulus expansion; they decreased sharply at the stage of ovulation (11 h post-hCG) and were low at all other stages. Conversely *Ski* mRNA (Fig. 4a) expression was opposite to the above genes, with the highest expression seen at PD 23, and decreased at other stages. *Cd44* (Fig. 4e) and *C1qbp* (Fig. 4f) mRNA expression hardly changed throughout.

Discussion

In this study, we examined Ski expression in both neonatal and gonadotropin-treated immature mice and throughout their estrous cycle for the first time. The results showed that Ski expression in ovary is regulated in a highly cell-specific manner. We found that Ski is highly expressed in oocyte nuclei in small follicles. With follicular development, the localization moved gradually from oocyte nuclei to perinuclear space and the expression level of Ski decreased. Ski immunoreactivity was not detected in interstitial cells, granulosa cells, or theca cells. Interestingly, positive staining was detected in CL. The highly regulated mode of localization might imply an important role of Ski in ovarian functions such as early follicular development.

We observed the localization of Ski in oocyte nuclei and the perinuclear space. The presence of Ski decreased gradually as follicles progressed from small antral to large antral, suggesting that Ski is down-regulated during oocyte maturation, and Ski may serve as an important intraovarian factor involved in early follicular development and have a close relationship with oocyte growth.

The oocyte plays an important role during follicular development, and its health is vital to ensure successful fertilization. During follicular growth and antrum formation, the oocytes and oocyte-secreted factors (OSFs) play a crucial role in regulating oocyte growth, granulosa cell differentiation, and follicular development [3, 19]. Three OSFs, which belong to TGF-β superfamily, GDF-9, BMP-15 (also known as GDF 9B), and BMP-6 have been found to be selectively expressed by oocytes from primary

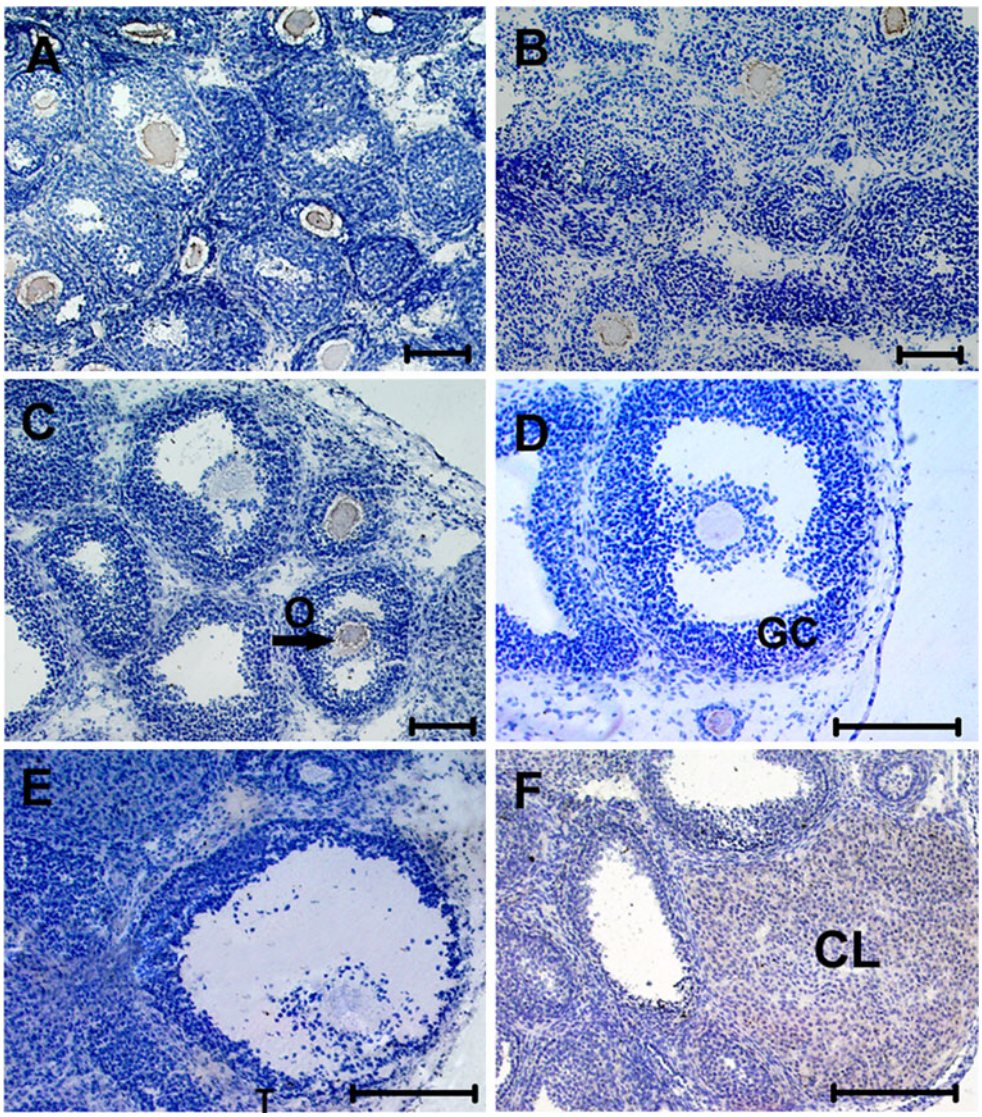

Fig. 3 Ovarian localization of Ski protein during gonadotropin-induced follicular development in immature mouse ovary. With development of the follicles, positive staining was detected in oocyte nucleus and perinuclear space (*arrowhead*) with gradually reduced intensity. PD 23 (**a**), 24 h post-PMSG (**b**), in large follicles at 0 h post-hCG (**c**), in cumulus expansion follicles at 4 h post- hCG (**d**), in preovulatory follicles at 11 h post-hCG (**e**) and in CL at 24 h post-hCG (**f**). *CL* corpus luteum, *GC* granulosa cells, *T* theca cells, *O* oocyte. *Scale bars* 50 μm (**a**–**c**) and 100 μm (**d**–**f**)

follicles in rodents [26], and have been shown to be essential for promoting follicle growth beyond the primary stage [7, 27–29]. Mice with a null mutation in the G*df-9* gene are infertile and show arrested follicular development at the primary stage, indicating that oocyte-derived GDF-9 is essential for further follicle progression [30]. The expression of GDF-9 in mice usually begins at the primary follicular stage and is absent in primordial follicles [26]. BMP-15 mature protein was originally undetected in oocytes of mice treated with PMSG alone for 48 h or followed by hCG for 2 or 5 h. However, production of BMP-15 greatly increased in oocytes of mice treated with PMSG followed by hCG for 9 h [31]. Unlike BMP-15 and GDF-9 which are produced exclusively by oocytes, BMP-6 is produced by oocytes and granulosa cells, though the oocytes appear to be the primary source of follicular BMP6 in many species [7, 32]. In rats, mRNA encoding BMP-6 was first observed in the oocytes and granulosa cells of preantral follicles but was not observed in primordial or primary follicles [32]. *Bmp-15* or *Bmp-6* gene knockout females are subfertile because of decreased ovulation and fertilization rates [33, 34]. Our observation is that the expression of Ski is opposite to that of GDF-9, BMP-6, and BMP-15, Ski was found to be most highly expressed in germ cell cysts and primordial follicles, decreasing after the primary stage. Since activation of TGF-β signaling

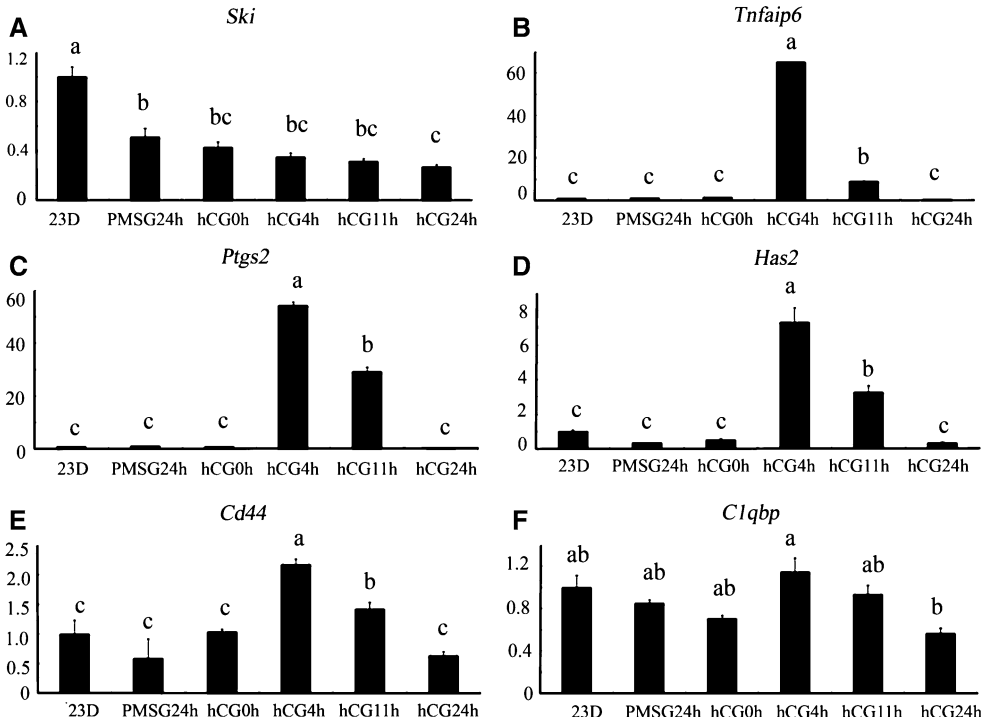

Fig. 4 Real-time quantitative PCR analysis of *Ski* mRNA (**a**) and cumulus expansion-related genes *Tnfaip6* (**b**), *Ptgs2* (**c**), *Has2* (**d**), *Cd44* (**e**), and *C1qbp* (**f**) mRNA expression in gonadotropin-primed immature mice. Gene expression level was normalized to *β-actin* in the same sample. Data were collected from three animals. *Bar* represents mean ± SEM. Means without a common letter differ significantly, $P < 0.05$

could lead to rapid degradation of Ski, which is mediated by cellular proteasomes [17, 35], the initiation of GDF-9, BMP-6, and BMP-15 expression in oocytes resulting in activation of TGF-β signaling, may result in the gradual decline in Ski expression. The progressive decreasing expression of Ski during follicle growth might also suggest its potential roles in the recruitment of follicles at very early stage of follicular development.

We also examined whether gonadotropins could affect the expression of Ski. We found that Ski expression is also gradually decreased by treatment with PMSG and hCG. It is well known that gonadotropins are important survival factors for developing follicles in escaping atresia and reaching the preovulatory stage. Ski expression in gonadotropin-primed immature mice may be correlated with its ability to negatively modulate the signal transduction of the TGF-β superfamily by directly associating with Smad proteins and blocking the ability of the Smads to activate expression of many if not all TGF-β-responsive genes [36].

Our real-time PCR data showed that the amount of Ski present decreased with time especially during the cumulus expansion phase. The pattern of Ski expression during cumulus expansion may be inhibited by components of the TGF-β signaling pathway, enabling other OSFs, GDF-9, and BMP-15 to be expressed at a higher level during cumulus expansion.

Estrous cycles, as examined by vaginal cytology, reflected the function of the hypothalamic–pituitary–ovarian axis and the resultant cyclicity of ovarian steroids. Our study revealed Ski expression was the highest at diestrus and the lowest at metoestrus in ovaries throughout the estrous cycle, suggesting a functional relationship with the hormones. Many hormones play essential roles in the control of ovarian estrus cycle, such as follicle stimulating hormone (FSH), luteinizing hormone (LH), estrogen, and progesterone. In addition, some factors such as, BMP-6, BMP-15, and GDF-9 derived from the oocytes can enhance estradiol secretion [4, 7] and suppress progesterone secretion [37]. During the estrous cycle, ovaries undergo structural and functional changes. The most significant and closely-regulated events in mammalian reproduction are the formation, maintenance, regression, and steroidogenesis of the follicles and CL [38, 39]. Hormones released by pituitary are well recognized as stimulators of follicular survival and growth [7, 37, 40–42]. The spatiotemporal features of Ski expression may be regulated by the presence of such hormones and the subsequent change in hormonal environment as well as the changes of TGF-β signaling.

Ski is always associated with SnoN, and Ski–SnoN heterodimers always have more dominant activity [20]. Although Ski and SnoN are highly homologous, their expression seems to be regulated by different mechanisms during follicular development [9]. SnoN expression is mainly found in the theca cells in mouse ovaries and in granulosa cells of the follicles that exhibited signs of atresia [10]. The different localizations of SnoN (theca cells and granulosa cells) from that of Ski (oocytes) indicate that they might not function as heterodimers in follicles. After ovulation, granulosa cells undergo luteinization

to form a CL, during CL formation, they have common localizations, both of them localize in CL cells, which implies that they might regulate CL formation and have the possibility to function as heterodimers during this time. Also it is intriguing that the expression of Ski converts from oocytes in primordial follicles to CL cells after ovulation in mice. The process of luteinization is widely regarded to be under the control of oocyte-derived luteinization inhibitors; it is considered that these inhibitors act to prevent luteinization and suppress progesterone synthesis until such a time that the oocyte is released at ovulation. Oocyte-derived BMP-6, BMP-15, and GDF-9 have the ability to act as inhibitors of luteinizing activity in cultured granulose cells, e.g., enhance estradiol secretion, inhibit progesterone production, promote granulosa cell survival by maintaining cell proliferation, and preventing premature luteinization and/or atresia [43, 44]. It is highly probable that the removal of these factors upon ovulation would have a significant effect on the remaining follicular cells and promote luteinization. At the same time, the disappearance of BMP-6, BMP-15, and GDF-9 inactivate TGF-β signaling, which might result in the reappearance of Ski expression in CL cells since inactivation of TGF-β signaling could lead to rapid activation of Ski [17, 35]. However, the translocation of Ski from oocytes in follicles to granulosa CL cells is unclear. Previous study found that Ski was expressed in granulosa cells of atretic follicles in rat [6], so the expression of Ski is also of species specificity.

Taken together, these results imply that Ski may be one of the key molecules involved in follicular development in mouse ovary. The study also provides additional evidence for the role of Ski in mouse ovarian function and follicular development.

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References

1. E.A. McGee, A.J. Hsueh, Initial and cyclic recruitment of ovarian follicles. Endocr. Rev. **21**, 200–214 (2000)
2. P.G. Knight, C. Glister, TGF-beta superfamily members and ovarian follicle development. Reproduction **132**, 191–206 (2006)
3. J.J. Eppig, Oocyte control of ovarian follicular development and function in mammals. Reproduction **122**, 829–838 (2001)
4. E.E. Nilsson, M.K. Skinner, Growth and differentiation factor-9 stimulates progression of early primary but not primordial rat ovarian follicle development. Biol. Reprod. **67**, 1018–1024 (2002)
5. J. Massague, D. Wotton, Transcriptional control by the TGF-beta/Smad signaling system. EMBO J. **19**, 1745–1754 (2000)
6. H. Kim, K. Yamanouchi, M. Nishihara, Expression of ski in the granulosa cells of atretic follicles in the rat ovary. J. Reprod. Dev. **52**, 715–721 (2006)
7. J.L. Juengel, K.P. McNatty, The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. Hum. Reprod. Update **11**, 143–160 (2005)
8. A.E. Drummond, M.T. Le, J.F. Ethier, M. Dyson, J.K. Findlay, Expression and localization of activin receptors, Smads, and beta glycan to the postnatal rat ovary. Endocrinology **143**, 1423–1433 (2002)
9. K. Luo, Ski and SnoN: negative regulators of TGF-beta signaling. Curr. Opin. Genet. Dev. **14**, 65–70 (2004)
10. W.W. Xu, X.B. Kong, L.G. An, C. Zhang, Relationship between SnoN expression and mouse follicular development, atresia, and luteinization. Zool Sci **26**, 66–73 (2009)
11. N. Nomura, S. Sasamoto, S. Ishii, T. Date, M. Matsui, R. Ishizaki, Isolation of human cDNA clones of ski and the ski-related gene, sno. Nucleic Acids Res. **17**, 5489–5500 (1989)
12. S. Pearson-White, R. Crittenden, Proto-oncogene Sno expression, alternative isoforms and immediate early serum response. Nucleic Acids Res. **25**, 2930–2937 (1997)
13. J.A. Reed, E. Bales, W. Xu, N.A. Okan, D. Bandyopadhyay, E.E. Medrano, Cytoplasmic localization of the oncogenic protein Ski in human cutaneous melanomas in vivo: functional implications for transforming growth factor beta signaling. Cancer Res. **61**, 8074–8078 (2001)
14. F. Zhang, M. Lundin, A. Ristimaki, P. Heikkila, J. Lundin, J. Isola, H. Joensuu, M. Laiho, Ski-related novel protein N (SnoN), a negative controller of transforming growth factor-beta signaling, is a prognostic marker in estrogen receptor-positive breast carcinomas. Cancer Res. **63**, 5005–5010 (2003)
15. K. Luo, S.L. Stroschein, W. Wang, D. Chen, E. Martens, S. Zhou, Q. Zhou, The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. Genes Dev. **13**, 2196–2206 (1999)
16. S. Akiyoshi, H. Inoue, J. Hanai, K. Kusanagi, N. Nemoto, K. Miyazono, M. Kawabata, c-Ski acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with smads. J Biol Chem **274**, 35269–35277 (1999)
17. Y. Sun, X. Liu, E. Ng-Eaton, H.F. Lodish, R.A. Weinberg, SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor beta signaling. Proc. Natl. Acad. Sci. USA **96**, 12442–12447 (1999)
18. C. Colmenares, E. Stavnezer, The ski oncogene induces muscle differentiation in quail embryo cells. Cell **59**, 293–303 (1989)
19. R. Li, R.J. Norman, D.T. Armstrong, R.B. Gilchrist, Oocyte-secreted factor(s) determine functional differences between bovine mural granulosa cells and cumulus cells. Biol. Reprod. **63**, 839–845 (2000)
20. S.B. Cohen, G. Zheng, H.C. Heyman, E. Stavnezer, Heterodimers of the SnoN and Ski oncoproteins form preferentially over homodimers and are more potent transforming agents. Nucleic Acids Res. **27**, 1006–1014 (1999)
21. V.M. Portela, G. Zamberlam, C.A. Price, Cell plating density alters the ratio of estrogenic to progestagenic enzyme gene expression in cultured granulosa cells. Fertil. Steril. **93**, 2050–2055 (2004)
22. M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29**, e45 (2001)
23. A. Aruffo, I. Stamenkovic, M. Melnick, C.B. Underhill, B. Seed, CD44 is the principal cell surface receptor for hyaluronate. Cell **61**, 1303–1313 (1990)
24. H. Lim, B.C. Paria, S.K. Das, J.E. Dinchuk, R. Langenbach, J.M. Trzaskos, S.K. Dey, Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell **91**, 197–208 (1997)

25. S.C. Thakur, K. Datta, Higher expression of hyaluronan binding protein 1 (HABP1/p32/gC1qR/SF2) during follicular development and cumulus oocyte complex maturation in rat. Mol. Reprod. Dev. **75**, 429–438 (2008)
26. S.A. McGrath, A.F. Esquela, S.J. Lee, Oocyte-specific expression of growth/differentiation factor-9. Mol. Endocrinol. **9**, 131–136 (1995)
27. E. Di Pasquale, P. Beck-Peccoz, L. Persani, Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am. J. Hum. Genet. **75**, 106–111 (2004)
28. J.A. Elvin, C. Yan, M.M. Matzuk, Oocyte-expressed TGF-beta superfamily members in female fertility. Mol. Cell. Endocrinol. **159**, 1–5 (2000)
29. S. Shimasaki, R.K. Moore, F. Otsuka, G.F. Erickson, The bone morphogenetic protein system in mammalian reproduction. Endocr. Rev. **25**, 72–101 (2004)
30. J. Dong, D.F. Albertini, K. Nishimori, T.R. Kumar, N. Lu, M.M. Matzuk, Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature **383**, 531–535 (1996)
31. H.E. McMahon, O. Hashimoto, P.L. Mellon, S. Shimasaki, Oocyte-specific overexpression of mouse bone morphogenetic protein-15 leads to accelerated folliculogenesis and an early onset of acyclicity in transgenic mice. Endocrinology **149**, 2807–2815 (2008)
32. G.F. Erickson, S. Shimasaki, The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. Reprod. Biol. Endocrinol. **1**, 9 (2003)
33. C. Yan, P. Wang, J. DeMayo, F.J. DeMayo, J.A. Elvin, C. Carino, S.V. Prasad, S.S. Skinner, B.S. Dunbar, J.L. Dube, A.J. Celeste, M.M. Matzuk, Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol. Endocrinol. **15**, 854–866 (2001)
34. K. Sugiura, Y.Q. Su, J.J. Eppig, Does bone morphogenetic protein 6 (BMP6) affect female fertility in the mouse? Biol. Reprod. **83**, 997–100433 (2010)
35. Y. Nagano, K.J. Mavrakis, K.L. Lee, T. Fujii, D. Koinuma, H. Sase, K. Yuki, K. Isogaya, M. Saitoh, T. Imamura, V. Episkopou, K. Miyazono, K. Miyazawa, Arkadia induces degradation of SnoN and c-Ski to enhance transforming growth factor-beta signaling. J Biol Chem **282**, 20492–20501 (2007)
36. X. Liu, Y. Sun, R.A. Weinberg, H.F. Lodish, Ski/Sno and TGF-beta signaling. Cytokine Growth Factor Rev. **12**, 1–8 (2001)
37. R.B. Gilchrist, L.J. Ritter, D.T. Armstrong, Oocyte-somatic cell interactions during follicle development in mammals. Anim. Reprod. Sci. **82–83**, 431–446 (2004)
38. R. Quintana, L. Kopcow, C. Sueldo, G. Marconi, N.G. Rueda, R.I. Baranao, Direct injection of vascular endothelial growth factor into the ovary of mice promotes follicular development. Fertil. Steril. **82**(Suppl 3), 1101–1105 (2004)
39. C. Stocco, C. Telleria, G. Gibori, The molecular control of corpus luteum formation, function, and regression. Endocr. Rev. **28**, 117–149 (2007)
40. S.M. Quirk, R.G. Cowan, R.M. Harman, C.L. Hu, D.A. Porter, Ovarian follicular growth and atresia: the relationship between cell proliferation and survival. J. Anim. Sci. **82**(E-Suppl), E40–E52 (2004)
41. T. Shimizu, B.C. Jayawardana, M. Tetsuka, A. Miyamoto, Differential effect of follicle-stimulating hormone and estradiol on expressions of vascular endothelial growth factor (VEGF) 120, VEGF164 and their receptors in bovine granulosa cells. J. Reprod. Dev. **53**, 105–112 (2004)
42. Y.X. Liu, Plasminogen activator/plasminogen activator inhibitors in ovarian physiology. Front. Biosci. **9**, 3356–3373 (2004)
43. S. Shimasaki, R.J. Zachow, D. Li, H. Kim, S. Iemura, N. Ueno, K. Sampath, R.J. Chang, G.F. Erickson, A functional bone morphogenetic protein system in the ovary. Proc. Natl. Acad. Sci. USA **96**, 7282–7287 (1999)
44. C. Glister, C.F. Kemp, P.G. Knight, Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. Reproduction **127**, 239–254 (2004)