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Signal transduction of stem cell factor in promoting early follicle development

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Abstract

Stem cell factor (SCF), another alternative name is kit ligand, is essential for the development of early follicles. However, the underlying molecular mechanism remains to be defined. By using cultured ovaries that are rich in primordial follicles, the action of SCF (kit ligand) on early follicular development and the activated signal transduction pathways were investigated. SCF (kit ligand) promoted early follicle development. PKC and MEK but not PKA were involved in the signal transduction of SCF (kit ligand) as indicated by results using their specific pharmacological inhibitors. SCF (kit ligand) also enhanced the phosphorylation of two MEK substrates, Erk1 and 2 (Erk1/2) in thecal-interstitial cells where PKC might play an important role indicated by results using its inhibitors. SCF (kit ligand) elevated the expression of steroidogenic factor 1 (SF-1) in thecal-interstitial cells probably through a pathway that consists of Erk1/2. These results suggest that SCF (kit ligand) promotes follicular growth by stimulating the function of thecal-interstitial cells through the Erk1/2 pathway.

Keywords: Ovary; PKC; Erk1/2; SF-1

1. Introduction

During the first three days after birth in rats, primordial follicles are assembled and remain developmentally arrested until primary follicles are formed later (Hirshfield, 1991; Jin and Liu, 2003). Primordial follicles are characterized by oocytes surrounded by a single layer of squamous pregranulosa cells while primary follicles contain oocytes encircled by a single layer of cuboidal granulosa cells. Subsequently, secondary follicles are developed with oocytes surrounded by two to three layers of cuboidal granulosa cells. The physiological role of FSH in regulating follicle growth and differentiation in vivo is well established. However, FSH is unlikely to exert a direct action on primordial follicles given that its receptors have not yet developed at this stage. In fact, follicles do not express functional FSH receptors until the secondary stage of follicular development (O'Shaughnessy et al., 1997; Oktay et al., 1997; Liu et al., 2000). Although, some growth factors in the ovary stimulate follicle growth and differentiation in vitro (Elvin et al., 2000; Webb et al., 1999), the underlying molecular mechanism has not been well characterized.

Stem cell factor (SCF) (kit ligand) is an important regulator of ovarian development in embryos and adults (Matsui et al., 1990). In post-natal rodent ovaries, SCF (kit ligand) is detected in granulosa cells while its receptor c-kit is expressed in thecal-interstitial cells and oocytes (Yoshida et al., 1997; Motro and Bemstein, 1993; Laitinen et al., 1995; Ismail et al., 1996). SCF (kit ligand) is required for the survival and proliferation of primordial germ cells in culture (Dolci et al., 1991; Godin et al., 1991; Parrott and Skinner, 1999). In addition, SCF (kit ligand) stimulates the proliferation and differentiation of thecal-interstitial cells and the growth of ovarian stromal-interstitial cells (Parrott and Skinner, 1997, 2000).

In several cell types, binding of SCF (kit ligand) causes c-kit receptor dimerization and phosphorylation. The activated receptor in turn phosphorylates different substrates representing distinct signal pathways such as PI3K/AKT and Ras/MEK/MAPK pathways (Blume-Jensen et al., 1998;

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Rameh and Cantley, 1999; Ueda et al., 2002). The activation of these pathways results in changes of cells in their survival, proliferation, differentiation, and chemotactic behaviors. Whether these molecules and pathways are activated in follicular cells by SCF (kit ligand) remains unknown.

Steroidogenic factor 1 (SF-1) is an orphan nuclear receptor (Teruo et al., 2001; Mellon and Bair, 1998) and plays a major role in regulating the expression of genes essential for gonadal development and sexual differentiation (Abraham and Natarajagounder, 1997). However, regulation of the expression of SF-1 has not been well defined.

The present study was designed to examine the ability of SCF (kit ligand) to promote follicular development in cultured ovaries at the early stage of folliculogenesis. We focused on investigating the possible signal pathway(s) triggered by SCF (kit ligand) through identifying typical protein molecules activated. We have demonstrated that SCF (kit ligand) activates protein kinase C (PKC) and the extracellular signal-regulated kinases 1 and 2 (Erk1/2). SCF (kit ligand) also induced the expression of SF-1 in thecal-interstitial cells through the Erk1/2 pathway.

2. Materials and methods

2.1. Reagents

Mouse anti-rat PCNA antibody Ab-1, GF109203X-(203290) was purchased from Calbiochem, Inc.; rabbit anti-rat p44/42 MAPK(9102), phospho-p44/42 MAPK(9201) were from Cell Signaling Technology, Inc.; goat anti-rat steroidogenic factor-1 (SF-1) (sc-10976), biotin labeled secondary antibodies, horseradish peroxidase-conjugated streptavidin were obtained from Santa-Cruz Biotechnology, Inc.; HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG were from vector Inc.; mouse anti-rat β-actin (A5441), Waymouth MB752/1, H89(B1427), U0126(U120), PMA (P8139) were purchased from Sigma. SCF were purchased from US Biological, Ins., SuperSignal® West Pico substrate was from PIERCE.

2.2. Ovary culture

Sprague–Dawley rats were obtained from Animal Facility of the Institute of Zoology, Chinese Academy of Sciences. The day when the rats were born was designated as D0. The ovaries were removed on D0 and immediately placed in ice-cold Waymouth medium MB752/1. Tissue adhering to the ovary was removed using the beveled edge of a 21-gauge needle. Each ovary was transferred to a Costar Transwell membrane which had been cut out of the Costar Transwell membrane insert and been floated on the media. The ovaries were cultured as previously described (John and Marilyn, 1996). Briefly, culture medium (2 ml of Waymouth MB 752/1 supplemented with 0.23 mM pyruvic acid, 50 mg/l of streptomycin sulfate, 75 mg/l of penicillin G, 3 mg/ml of BSA) was added to the culture dish compartment below the membrane, and the ovaries were covered by a thin film on the floating filter. Ten ovaries per floating filter were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, and the media were changed every 48 h. Ovaries cultured under these conditions appeared healthy.

2.3. Treatments

Ovaries were treated with the following agents and their combinations: SCF (100 ng), PMA (100 nM), SCF plus GF109203X (2 μM), SCF plus U0126 (20 μM), SCF plus H89 (10 μM). Matched pairs of ovaries were separated and one used for control cultures and the other for treated cultures. Experiments were repeated three times.

2.4. Western blot

Western blot was done as previously described (Zhou et al., 2002). The tissues were homogenized in lysis buffer (5 mM phosphate buffer, pH 7.2, containing 0.1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 1 mg/l chymostatin) and the protein content of the supernatant from centrifugation was determined by spectrophotometer, using bovine serum albumin as a standard. Sample lysates were mixed with the loading buffer (final concentration, 62.5 mM, 1,4-dithiothreitol, 5% sodium dodecyl sulfate (SDS), and 10% glycerol), boiled for 8 min, separated by SDS–polyacrylamide gel electrophoresis (30 μg total protein/lane). After electrophoretic transfer to the polyvinylidene difluoride membrane, the membranes were blocked with 5% non-fat milk/PBS for 1 h, followed by incubation at 20 °C for 1 h with the primary antibodies for PCNA, P44/42 MAPK, phospho P44/42 MAPK, respectively (1:500) in 5% milk/PBS. β-actin was used as a loading control. The membranes were washed three times, 5 min for each, in 5% milk/PBS and incubated with HRP-conjugated goat anti-rabbit IgG (1:5000) or goat anti-mouse IgG (1:2000) in 5% milk/PBS for 1 h. The membranes were washed in PBS three times for 5 min for each, followed by 5 min of incubation with SuperSignal® West Pico substrate, and then exposed on X-ray film. For negative controls, primary antibodies were replaced with normal IgG of the same concentration and origin.

2.5. Immunohistochemistry

Serial 5 μm sections of the ovarian tissue were deparaffinized, and rehydrated through degraded ethanol. Antigen retrieval was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0) at 98 °C for 20 min and cooling at room temperature for 20 min. Non-specific binding was blocked with 10% (v/v) normal goat serum in PBS for 1 h. The sections were incubated with primary antibodies specific for P44/42 MAPK (1:200), photo P44/42 MAPK (1:200) respectively in 10% goat serum at RT for 2 h. Sections were then washed three times with PBS (10 min each) and incubated

with biotin labeled secondary antibody (goat anti-rabbit IgG, RT, 30 min). 3 × 10 min successive washes were followed by incubation with horseradish peroxidase-conjugated streptavidin (RT, 30 min), sections were developed with diaminobenzidine for the same amount of time, and then dehydrated in ethanol and mounted. Sections incubated with normal IgG instead of primary antibody served as negative controls.

2.6. Statistical analysis

Values shown in all the figures are given as the mean ± S.E.M. The data were analyzed using one-way ANOVA as appropriate. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Erk1/2 and PKC are involved in primordial follicles development promoted by SCF

Number of follicles at different stages of development was counted in untreated ovaries and ovaries treated with different agents. Ovaries cultured for 8 days without any treatment contained only primordial and primary follicles (Fig. 1A). Ovaries treated with SCF for the same time period contained less number of primordial and more primary follicles. Notably, secondary follicles were also observed in ovaries treated with SCF (Fig. 1A and B).

Pharmacological inhibitors and activators of intracellular proteins potentially activated by SCF were used to identify the signal pathways activated by SCF in ovarian cells. Addition of protein kinase A (PKA) inhibitor, H89, to SCF-treated ovaries did not change the numbers of different follicle types in SCF-treated ovaries, suggesting that the PKA pathway is not activated by SCF (Fig. 1A and B). In contrast both PKC inhibitor GF109203X and MEK inhibitor, U0126, changed the numbers of different follicle types in SCF-treated ovaries significantly. Therefore, the PKC and MEK pathways are likely to be activated by SCF. It was also observed that a PKC activator PMA was able to change the numbers of different follicle types in a similar way as SCF. This suggests that the activation of PKC pathway leads to follicular development.

One important aspect of follicular development is the active proliferation of granulosa cells. Proliferating cell nuclear

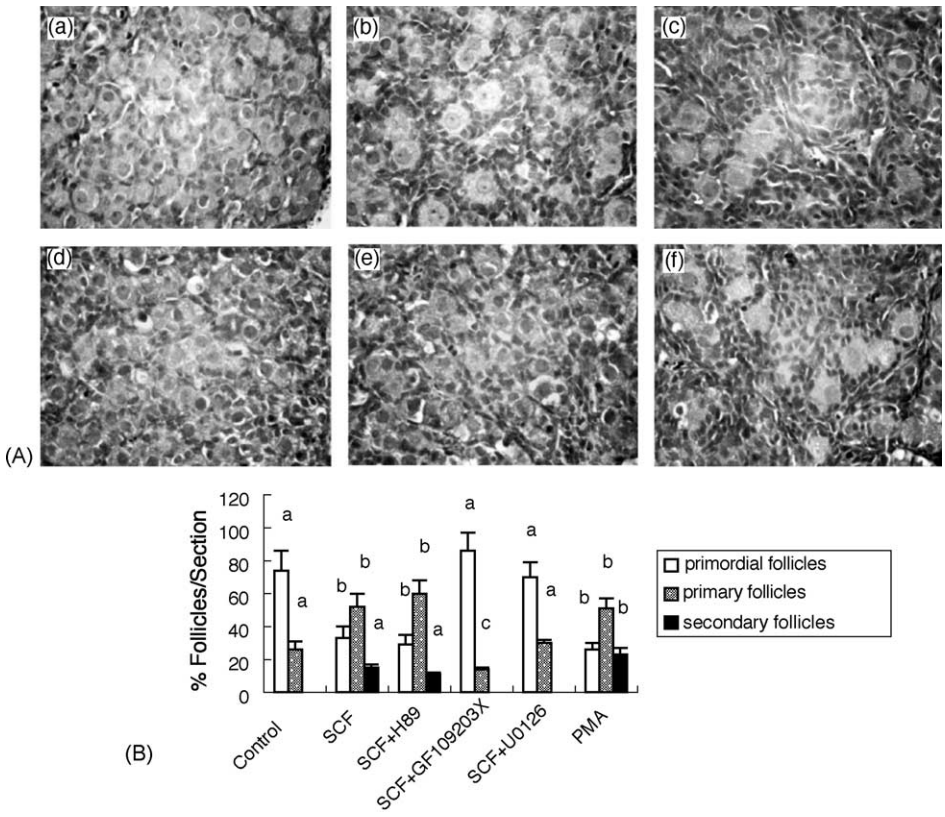

Fig. 1. Induction of follicular development by SCF, (A) Ovaries were cultured for 8 days in the presence of the following agents and their combinations: a, nothing; b, 100 ng/ml SCF; c, 100 ng/ml SCF + 10 μM H89; d, 100 ng/ml SCF + 2 μM GF109203X; e, 100 ng/ml SCF + 20 μM U0126; f, 100 nM PMA. Ovarian sections were stained with hematoxylin to show follicles at different developmental stages. Each microscopic picture was taken from a typical experiment that was repeated at least three times. (B) Numbers of primordial follicles, primary follicles and secondary follicles were counted. All follicles were counted in serial cross-sections at the widest portion of the ovary. Percentages of the numbers of each category over the total number were plotted. Statistical analysis was performed using ANOVA followed by the Student Newman–Keuls multi-range test. Bars with different letters label values that are significantly different ($P < 0.01$). Data are presented as mean ± S.E.M.

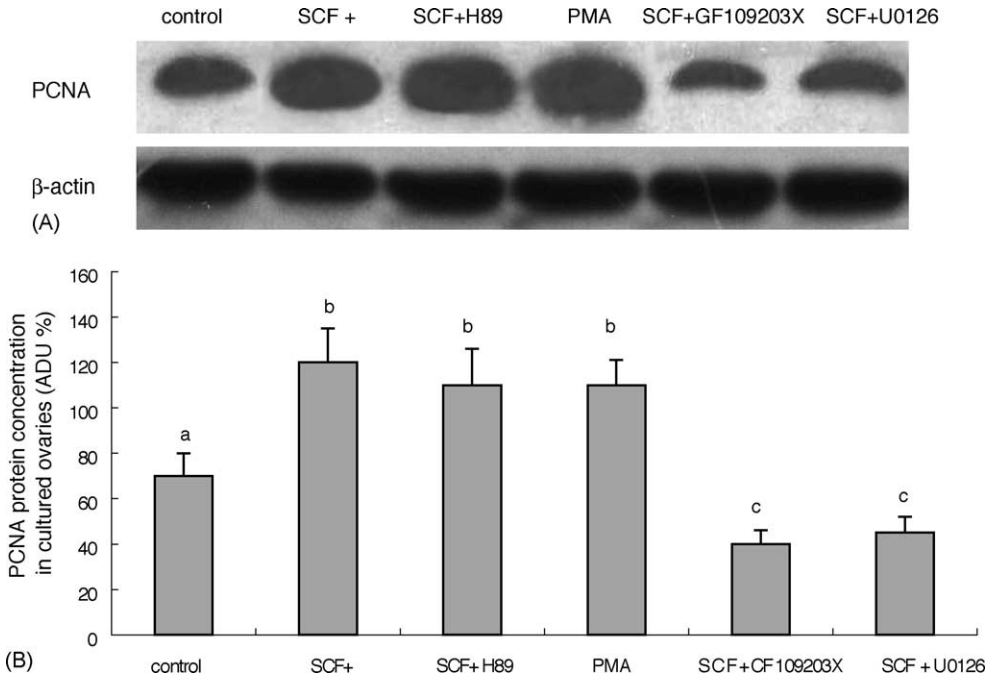

Fig. 2. Follicular development as indicated by the expression of PCNA protein in response to SCF and other treatments in cultured ovaries, (A) Western blot analysis of PCNA protein levels in cultured ovaries without any treatment (control) or ovaries treated with different agents and their combinations. (B) Quantitative analysis of the staining signals in the Western blotting experiments. Results were based on at least three independent experiments. Values are mean ± S.E.M ADU, arbitrary densitometric unit (defined as percentage of the densitometric value of β-actin). Statistical analysis was performed using ANOVA followed by the Student Newman–Keuls multi-range test. Bars with different letters indicate statistically significant differences ($P < 0.01$).

antigen (PCNA) is a typical molecular marker used to indicate the proliferation of cells. Its protein level was measured by using Western blot, and the results showed that SCF increased its expression significantly. H89 had no effect on SCF-induced PCNA expression. PMA up-regulated the expression of PCNA in a similar way as SCF. GF109203X and U0126 reduced SCF-induced PCNA expression (Fig. 2).

3.2. SCF induces phosphorylation of Erk1/2 through PKC signal pathway

To further identify signaling molecules activated by SCF, total protein levels and phosphorylation status of two Erk proteins Erk1 and 2 were checked by Western blot. Phosphorylations of the Erk1/2 were up-regulated significantly by SCF, while the total protein levels remained unchanged (Fig. 3). The up-regulation of phospho-Erk1/2-protein expression by SCF was reversed back to the normal level by PKC inhibitor GF109203X (Fig. 3). To further confirm that PKC activation leads to Erk1/2 phosphorylation, protein levels of phosphorylated Erk1/2 upon PMA treatment were compared with that of SCF-treated group. Similar increases were observed for these two groups (Fig. 3). The total protein levels in all groups remained unchanged.

The protein distribution and phosphorylation were also studied by immunohistochemistry. Proteins of the Erk1/2 were distributed in the cytoplasm and nuclei of all cell types including oocytes, granulosa cells and thecal-interstitial cells. Interestingly, phosphorylated Erk1/2 were detected in both treated and untreated ovaries, and were only seen in thecal-interstitial cells (Fig. 4). PMA by itself induced a similar change in expression pattern of phosphorylated Erk1/2 as SCF.

3.3. SCF induces the expression of SF-1

Orphan nuclear receptor SF-1 plays a major role in regulating the expression of genes essential for gonadal development and sexual differentiation (Abraham and Natarajagounder, 1997). Immunohistochemistry study was conducted to check whether its expression was regulated by SCF (Fig. 5). The results showed that it was mainly expressed in the cytoplasm of oocytes, and some staining was also observed in granulosa cells and thecal-interstitial cells. The expression of SF-1 in all cells increased in response to SCF treatment. SF-1 signal also appeared in the nuclei upon SCF treatment. Addition of MEK inhibitor U0126 to SCF-treated ovaries mainly reduced the SF-1 staining in thecal-interstitial cells.

4. Discussion

Previous report has shown that SCF (kit ligand) promotes the initiation and progression of primordial follicle development in ovaries (Parrott and Skinner, 1999). In somatic cells, binding of SCF (kit ligand) to its membrane receptor c-kit

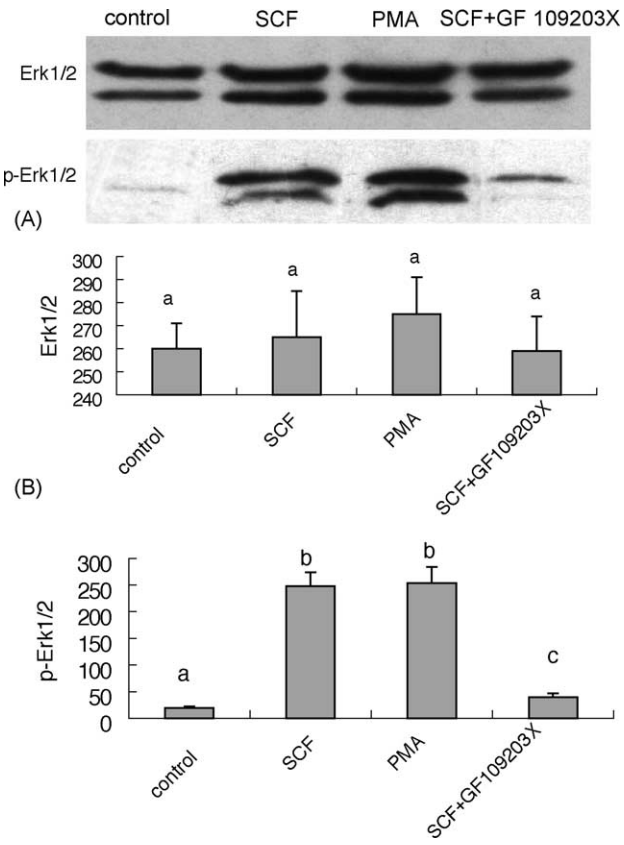

Fig. 3. Phosphorylation of Erk1/2 in ovaries cultured for 4 days with different treatments. (A) Western blot analysis of phospho-Erk1/2, and Erk1/2. (B) Quantitative analysis of Erk1/2 and phosphorylated Erk1/2 in the Western blotting analysis. Results were based on at least three independent experiments. Values are the mean ± S.E.M. Statistical analysis was performed using ANOVA followed by the Student Newman–Keuls multi-range test. Bars with different letters indicate statistically significant differences ($P < 0.01$).

induces rapid receptor dimerization (Blume et al., 1991) and activation of its intrinsic tyrosine kinase activity. A number of intracellular substrates are subsequently phosphorylated and signal transduction elicited (Tsai et al., 1993; Herbst et al., 1991; Lev et al., 1991). Signaling from the c-kit receptor is indispensable for primordial germ cells growth as shown by both in vivo (Donovan, 1994) and in vitro studies (Dolci et al., 2001; Godin et al., 1991). However, the signal pathways that mediate the function of SCF (kit ligand) have not been defined.

In this study, we have developed a system to examine the signal pathways induced by SCF (kit ligand). We took advantage of the available specific pharmacological inhibitors and activators, and found that two signal transduction pathways are involved in SCF-induced early follicle development. The PKC inhibitor and MEK inhibitor, but not the PKA inhibitor significantly inhibited the stimulation of SCF (kit ligand) on follicular development and PKC activator PMA by itself could also promote follicular development resembling the effect of SCF (kit ligand).

One prominent aspect of follicles development is the proliferation of granulosa cells, a process heralded by the expression of PCNA (Oktay et al., 1995). SCF (kit ligand) increased the level of PCNA, and such an increase could be completely abolished by PKC inhibitor and MEK inhibitor, but not PKA inhibitor. PKC activator PMA stimulated the expression of PCNA.

The activation of mitogen-activated protein kinases (MAPKs) is a key event in many cellular processes, including proliferation, differentiation, and apoptosis (Davis, 1993). There are three main classes of MAPK, Erks (Boulton et al., 1990; Hunter, 1995), c-Jun NH_2-terminal protein kinases (JNKs) (Kyriakis et al., 1994; Derijard et al., 1994), and p38-MAPK (Han et al., 1994; Lee et al., 1994; Jiang et al., 1996; Mertens et al., 1996; Wang et al., 1997; Goedert et al., 1997). Erks are preferentially activated in response to growth factors, cytokines. JNKs and p38-MAPKs are activated in response to a variety of cell stresses (Lopez-Ilasaca, 1998). Our other study indicated that Erk1 and 2 appeared to be the only MAPK that was activated by SCF (kit ligand); JNKs and p38-MAPKs were not activated by SCF (kit ligand) (data not shown). PKC signal transduction has been implicated in the regulation of a wide variety of cellular processes, including cell growth and cell cycle progression, differentiation, survival/apoptosis, and transformation (Black, 2000; Nishizuka, 1992; Dekker and Parker, 1994; Clemens et al., 1992). The present study implicates the PKC signal pathway in mediating the effect of SCF (kit ligand) on follicular development.

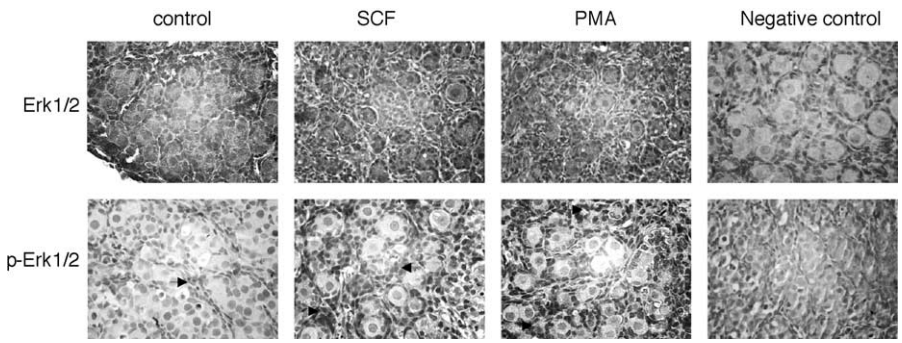

Fig. 4. Immunohistochemical localization of Erk1/2 and phosphorylated Erk1/2 in ovaries cultured for 4 days. Proteins to be analyzed are stained brown while the background counter staining is blue. Arrows indicate the thecal-interstitial cells. Magnification is 400×.

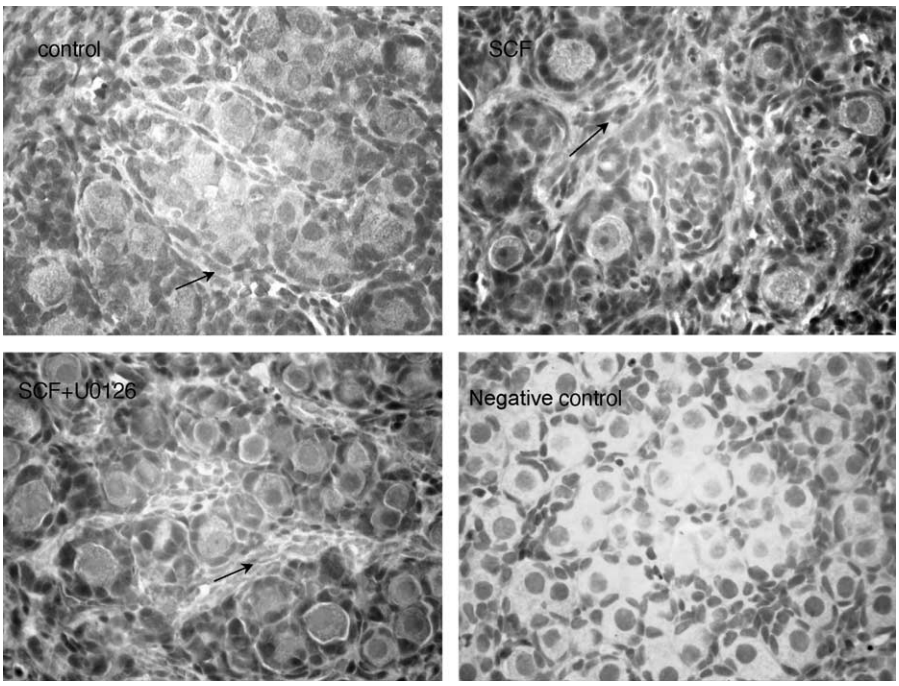

Fig. 5. Immunohistochemical localization of SF-1 in the ovaries cultured. Brown color represents the staining of proteins to be analyzed; blue color is background counter staining. Arrows indicate the thecal-interstitial cells. Magnification is 400×.

Our study demonstrates clearly that SCF (kit ligand) activates Erk1/2 is largely associated with the PKC pathway. To our knowledge, the present report is the first to demonstrate a link between PKC, Erk1/2 activity, and SCF-induced follicular development. Previous researches have demonstrated that cAMP could promote follicular development by stimulating the production of progesterone and androstenedione (James and Shyamal, 2000). FSH enhanced the development of follicles possibly through the PKA pathway (Yu et al., 2003). Interestingly, our study indicates that c-kit-induced follicular development is probably not mediated by the PKA pathway.

Erk activity can lead to cell differentiation, influence both nuclear and cytosolic events. Phospho-Erks translocate to the nucleus where they phosphorylate transcription factors and thus regulate gene expression (Su and Karin, 1996). Our data also showed that Erk1/2 activated by SCF (kit ligand) and PMA were located in thecal-interstitial cells.

Differentiated thecal-interstitial cells produce androstenedione and progesterone to regulate follicle development (James and Shyamal, 2000). SCF (kit ligand) can stimulate thecal-interstitial cell differentiation as measured by androstenedione production (Parrott and Skinner, 1997; Chris et al., 2001). The synthesis of steroid hormones by the developing follicle is dependent upon the presence and activities of several key proteins, such as SF-1, steroidogenic acute regulatory protein (StAR), and cytochrome P450 aromatase (P450arom) (Godin et al., 1991). Studies using SF-1 null mice indicated that the expression of the gene is critical for the differentiation of the gonadal ridge (Luo et al., 1994). SF-1 regulates the expression of *StAR* gene and steroidogenic P450 enzymes (Wehrenberg et al., 2001; Stocco and Clark, 1996; Morohashi et al., 1995; Zhang and Mellon, 1996). Besides that, SF-1 is an essential factor in adrenal and gonadal development and for the proper functioning of the hypothalamic–pituitary–gonadal axis through genetic ablation experiments in mice (Hammer and Ingraham, 1999). The present study indicated that SCF (kit ligand) up-regulated the expression of SF-1 in all cell types, including oocytes, granulosa cells and thecal-interstitial cells, and induced the translocation of SF-1 from cytoplasm into the nucleus. Induction of SF-1 by SCF (kit ligand) in thecal-interstitial cells could be abolished by MEK inhibitor, U0126. Our results are consist with previous study that phosphorylation-dependent SF-1 activation is likely mediated by the MAPK signaling pathway, and this single modification of SF-1 and the subsequent recruitment of nuclear receptor cofactors couple extracellular signals to steroid and peptide hormone synthesis (Hammer et al., 1999; Babu et al., 2000). In conclusion, SCF (kit ligand) may promote early follicular development by using MEK/Erk signal pathway and transcription factor SF-1 in thecal-interstitial cells.

Acknowledgements

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