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STEM CELL FACTOR MODULATES THE EXPRESSION OF STEROIDOGENESIS RELATED PROTEINS AND FSHR DURING OVARIAN FOLLICULAR DEVELOPMENT

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1. ABSTRACT

Stem cell factor (SCF) is essential for the development of primordial follicles. By using cultured ovaries from neonatal rats, the effect of SCF on early follicular development was investigated. Steroidogenesis is a hallmark of follicular development. Expressions of three key protein factors in steroidogenesis, SF-1, StAR, and P450arom, were investigated using immunohistochemistry and *in situ* hybridization. SF-1 and StAR proteins were expressed in all ovarian cells. P450arom mRNA was localized exclusively in oocytes implying that estrogen might be synthesized by oocytes at this stage. SCF up-regulated the mRNA and protein expression of these proteins, suggesting SCF might promote the production of estrogen during this period of time. To study the differentiation status of follicular cells, the expression of FSHR and its response to SCF treatment was examined by using semi-quantitative RT-PCR. The results showed that SCF inhibited the expression of FSHR mRNA. It was also observed that SCF stimulated the expression of basic fibroblast growth factor (bFGF) in oocytes. Inactivation of bFGF by its neutralizing antibody resulted in a reversal of the inhibitory effect of SCF on the expression of FSHR. Therefore, the FSHR inhibitory effect of SCF could be mediated by bFGF. In summary, it seems that, at the early stages of follicular development, SCF might stimulate oocytes to produce estrogen while it inhibits the differentiation of granulosa cells that are the major sources of estrogen at the late stages of follicular development.

2. INTRODUCTION

During the first three days after birth in rats, primordial follicles are assembled and remain developmentally arrested until primary follicles are formed later (1, 2). Induction of primordial follicles to develop further is critical for successful female reproduction. However, the underlying mechanisms remain not well understood. Buccione *et al*. (3) have demonstrated that somatic cell and oocyte interactions play important roles in murine follicular development. Eppig and O'Brien (4) have successfully shown that oocytes developed in the cultured ovary are capable of undergoing *in vitro* fertilization. It is apparent that oocyte-somatic cell interactions could continue *in vitro* in the absence of the conventional *in vivo* endocrine milieu.

The physiological role of FSH in regulating follicle growth and differentiation *in vivo* is well established. However, FSH is unlikely to exert direct action on primordial follicle development when functional gonadotropin receptors have not yet developed in the follicular cells (5-8). As follicles reach the two-layer stage, granulosa cells start to express the receptor for FSH (FSHR), which confers folliculer responsiveness to pituitary gonadotropins (9).

Steroid hormones exert an important influence on follicular development. The synthesis of steroid hormones by the developing follicle is dependent upon the presence and activities of several key proteins, such as steroidogenic factor 1 (SF-1), steroidogenic acute regulatory protein (StAR), and cytochrome P450 aromatase (P450 arom) (10-12). SF-1 is an orphan nuclear receptor, and functions as a regulator of steroidogenic enzymes in gonadal and adrenal tissue (12). StAR promotes cholesterol movement to the

mitochondrial inner membranes, which is the rate-limiting step in steroidogenesis (13). Cytochrome P450 arom is directly involved in estradiol-17β (E_2) production (14). However, the expressions and regulation of SF-1, StAR, and P450 arom in the early stages of rat follicular development have not been well defined.

SCF is an important regulator of ovarian development (15). During ovarian organogenesis, receptor of SCF, c-kit is expressed in primordial germ cells, whereas SCF is expressed along their migratory pathway toward the genital ridge (15, 16). In postnatal rodent ovaries, the c-kit receptor is detected in theca cells and oocytes (17, 18), whereas SCF is detected in granulosa cells (18-20). SCF is required for the survival and proliferation of primordial germ cells in culture (21-23). In addition SCF stimulates the proliferation and differentiated (*i.e.* androstenedione production) of theca cells directly (24). These data suggest that SCF is a very important regulator of early stage follicular development.

Based on the above described expression of SCF/c-kit in immature rat ovaries, the actions of SCF in neonatal animals, and the progressive acquisition of steroidogenic capability and FSHR in follicular development, we started out to explore whether SCF participates in the regulation of the expression of SF-1, StAR, P450 arom, and FSHR, which are all key players in steroidogenesis in developing follicles.

3. MATERIALS AND METHODS

3.1. Reagents

Rabbit anti-rat bFGF (SC-79L) (SC-79), goat anti-rat steroidogenic factor-1 (SF-1) (sc-10976) were obtained from Santa-Cruz Biotechnology. Rabbit non-specific IgG, mouse anti-rat β-actin (A5441), Waymouth MB752/1, Taq DNA polymerase were purchased from Sigma. Superscript II reverse transcriptase was from Life Technologies. SCF were purchased from US Biological, Ins. SuperSignal® West Pico substrate was from PIERCE. TRIzol was from Life Technologies. dNTPs were from Gibco BRL.

3.2. Ovary cultures

Spague Dawley rats were obtained from Animal Facility of Institute of Zoology, Chinese Academy of Sciences. All experimental procedures were approved by the Animal Ethics Committees of both the Institute of Zoology and PRC. 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 in the absence of serum. The ovaries were cultured as previously described (25). Culture medium (2 ml of Waymouth MB 752/1 supplemented with 0.23 mM Pyruvic acid, 50mg/l of streptomycin sulfate, 75mg/l of penicillin G, 3mg/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 37ºC in a humidified atmosphere containing 5% CO_2, and the media were changed every 48h. Ovaries were cultured under these conditions for 5 days and appeared healthy.

3.3. Sample treatment

Ovaries were treated with: SCF (100ng/ml), SCF plus bFGF IgG antibody (1:100). Matched pairs of ovaries were separated and one used for treated cultures and the other for control cultures. The controls for the SCF plus bFGF IgG antibody treatment are ovaries treated with SCF plus non-specific rabbit IgG. Experiments were repeated three times.

3.4. Western blot analysis

Immunoblot was done as previously described (26). The tissues were homogenized in lysis buffer (5 mmol/L phosphate buffer, pH 7.2, containing 0.1% Triton X-100, 1 mmol/L 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 mmol/L, 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 transferred to the polyvinylidene difluoride membrane, the membranes were blocked with 5% nonfat milk/PBS for 1 h, followed by incubation at 20°C for 1 h with the primary antibodies (1:1000) in 5% milk/PBS. Beta-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 IgG (1:8000) in 5% milk/PBS for 1 h respectively. The membranes were washed in PBS three times 5 min for each, followed by 5 min of incubation with SuperSignal® West Pico substrate, then exposed on x-ray film. For negative controls, primary antibodies were replaced with normal IgG of the same concentration and origin.

3.5. Immunohistochemistry

Serial 5μm 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 hr. The sections were incubated with primary antibodies specific for SF-1 (0.5μg/ml), StAR (1μg/ml), bFGF (1μg/ml) in 10% goat serum at room temperature (RT) for 2 hr. Sections were then washed three times with PBS (10 min each) and incubated with biotin labeled secondary antibody (RT, 30 min). 3×10 min successive washes were followed by incubation with horseradish peroxidase-conjugated strepavidin (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.

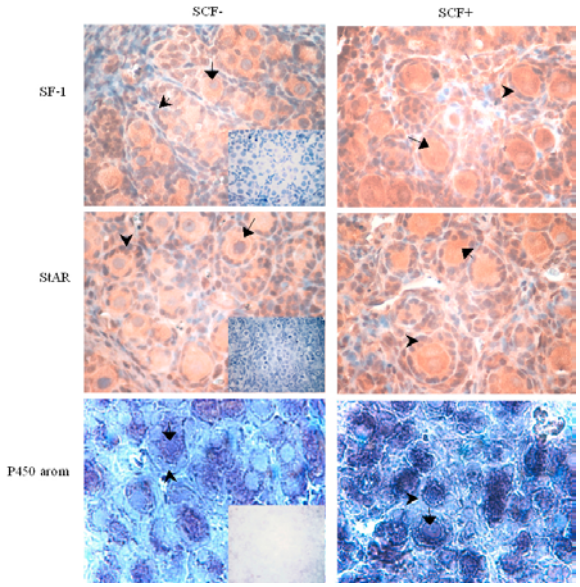

Figure 1. Expression and localization of SF-1, StAR protein, and P450 arom mRNA in cultured ovaries in the presence or absence of SCF SF-1 and StAR protein expression and localization were studied by using immunohistochemistry. Brown and blue colors represent target protein staining and background counter staining, respectively**.** P450arom mRNA expression and localization were detected by using *in situ* hybridization. Blue color represents the staining of P450arom mRNA. Oocytes and granulosa cells are indicated by arrows with wide head and arrow heads respectively. Insets are negative controls using pre-immune serum replacing the first antibody for immunohistochemistry and sense RNA probe for *in situ* hybridization. Magnification is 400×.

3.6. *In Situ* hybridization

Total RNAs from rat ovaries were prepared using TRIzol, a monophasic solution of phenol and guanidine isothiocyanate. Total RNA (5μg) was reverse transcribed into single-stranded cDNA using oligo dT primer and superscript II reverse transcriptase. Amplification of specific products was performed as previously described (27). Forty RT-PCR cycles were performed. Primers of P450 arom were as follows: 5'-ACTGTGCCTG TCAGTGCCAT-3'; 5'-ACCATTC GAAT AAGACCAG-3'. Probe labeling was based on the method of Liu *et al.* (28). The deparaffinized sections were treated with 4 μg/ml proteinase K for 15 min at 37°C and washed in PBS for 5 min. Sections were then fixed in 4% paraformaldehyde in PBS for 5 min and washed in PBS for 10 min. Before hybridization, the sections were dehydrated through a graded ethanol series and allowed to air-dry. The sections were prehybridized with 50% formamide and 2×SSC for 2 h at room temperature, then hybridized overnight with DIG labeled rat P450 arom cRNA probe in hybridization buffer (10 mmol/L Tris-HCl, PH 7.5, 2×SSC, 50%deionized formamide, 1×Demhardt's, 2.5 mmol/L DTT, 5%dextran sulfate, 250μg/ml yeast tRNA, and 0.5% SDS) at 53°C. After hybridization, the sections were thoroughly washed in 2×, 1×, and 0.1×SSC. The hybridization signals were visualized with anti-Dig AP-Fab and NBT/BCIP reagents. Sense probes were also included in each experiment as negative control. The presences of target mRNAs were indicated by blue color in the sections.

3.7. RNA extraction and semi-quantitative RT-PCR

Total RNAs were prepared using TRIzol, a monophasic solution of phenol and guanidine isothiocyanate. This reagent is an improvement over the single-step RNA isolation method developed by Chomczynski and Sacchi (29). The amount of RNA was estimated by spectrophotometry at 260 nm. Complementary DNAs were obtained from reverse transcription (RT) of 2 μg of total RNA using random hexanucleotides as primers (25 μM) in the presence of dNTPs (250 μM). Complementary DNAs (2 μl RT mixture) were amplified by polymerase chain reaction with Taq DNA polymerase (0.05U/μl), dNTP(250 μM) and specific FSHR oligonucleotide primers(10 μM) were asfollows:5'-CCACAAGCCAATACAAA-3'; 5'-AAGTC CAGCCCA ATACC-3'; PCR amplification was performed by first heating the mixture at 94°C for 5 min, followed by 30 cycles at 94°C for 30 sec, 55°C (melting temperature, °C) for 30 sec, 72°C for 50 sec. The reaction was last incubated at 72°C for 7 min. The amplified fragment is confirmed to be identical with expected fragment as 431 bp size. Amplification of β-actin gene served as the positive control. PCR products were verified by gel electrophoresis. Intensities of bands were estimated by densitometric scanning using the BioImage (Cheshire, U.K.) scanner. The data were expressed as the ratios of FSHR over β-actin.

3.8. Statistical analysis

Values were presented as mean ± SEM. The data were analyzed using one-way ANOVA as appropriate. P-values <0.05 were considered statistically significant.

4. RESULTS

4.1. Expression and SCF regulation of steroidogenic regulatory factors in ovaries of new born rats

The expression and localization of SF-1, StAR, and P450 arom in the ovaries cultured for 5 days with or without SCF treatment were studied by using immunohistochemistry and *in situ* hybridization. As shown in Figure 1, SF-1 was mainly expressed in the cytoplasm of oocytes, although it was also detected in granulosa cells. SCF treatment increased the expression of SF-1. In particular, oocyte nuclear staining was much stronger with SCF treatment. Similarly, StAR was expressed in all the cell types, and the expression levels were elevated upon SCF treatment with the elevation most apparent in oocytes. Notably, the expression of P450 arom mRNA was only detected in the oocytes of developing follicles, and SCF also enhanced its expression (Figure 1). The protein expression and SCF up-regulation of SF-1 and StAR were confirmed and quantified by Western blotting as shown in Figure 2.

4.2. FSHR mRNA expression was regulated by SCF in a bFGF dependent manner in cultured ovaries of new born rats

The expression of FSHR mRNA in new born rat ovaries cultured for 3, 4, 5 days with or without SCF treatment was measured by semi-QRT-PCR. In all cases, FSHR mRNA expression increased as culture time went on (Figure 3). Interestingly, exposure of ovaries to SCF resulted in significant decreases in FSHR mRNA level of

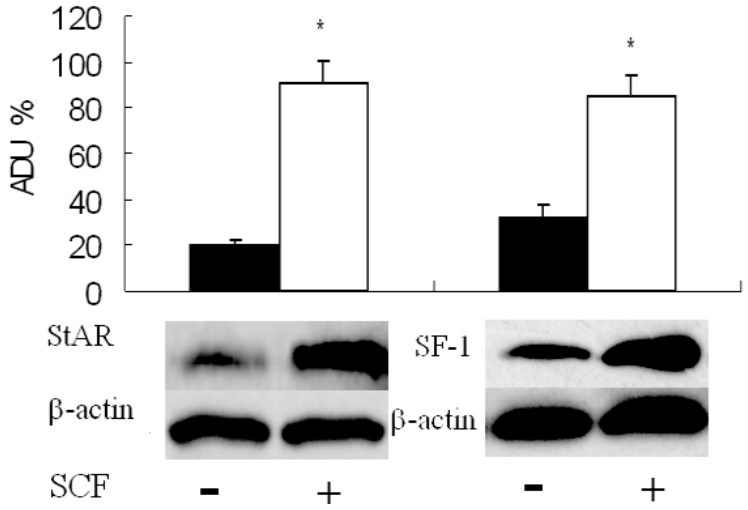

Figure 2. Western blot analysis of SF-1 and StAR proteins in cultured ovaries in the presence or absence of SCF Protein levels of SF-1 and StAR were quantified by using Western blot. β-actin expression was used as the internal control. Data were from at least three independent experiments. Each bar represents mean±S.E.M. (n=3). ADU (arbitrary densitometric unit) was defined as percentage of target protein densitometric value compared with β-actin. Statistical analysis was performed using the Student t-test (*P<0.01).

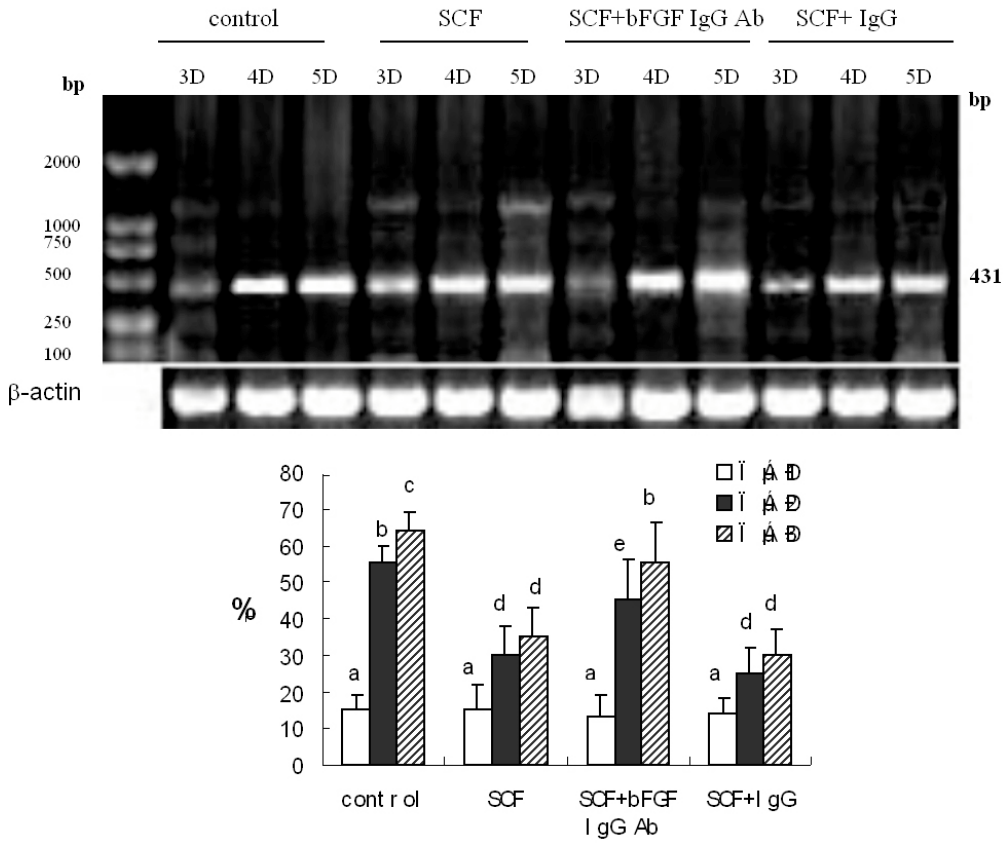

Figure 3. FSHR mRNA levels in D0 rat ovaries cultured for 3, 4, 5 days with different treatments A) FSHR mRNA levels as determined by semi-QRT-PCR in the ovaries untreated or treated with SCF, SCF plus bFGF IgG antibody, as well as SCF plus rabbit non-specific IgG B) Calculated FSHR mRNA values derived from three experiments. Bars represent mean±SEM. 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<0.05).

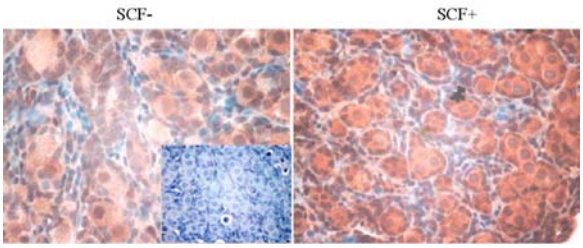

Figure 4. Immunohistochemical localization of bFGF in cultured ovaries in the presence or absence of SCF. Brown color represents staining of proteins to be analyzed, while blue color is background counter staining. Inset is negative control with a pre-immune serum replacing bFGF antibody. Magnification is 400×.

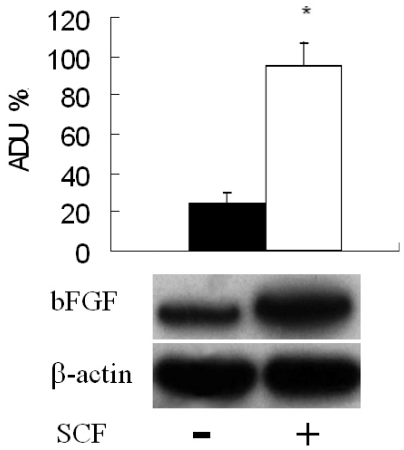

Figure 5. Western blot analysis of bFGF in cultured ovaries in the presence (+) or absence (-) of SCF Protein levels of bFGF were quantified by using Western blot. β-actin expression was used as the internal control. Data were from at least three independent experiments. Each bar represents mean±S.E.M. (n=3). ADU, arbitrary densitometric unit (defined as percentage of the densitometric value of β-actin). Statistical analysis was performed using the Student t-test. (*P<0.01)

day 4 and 5 culture, and such decreases were reversed significantly by bFGF antibody (Figure 3).

4.3. SCF induced expression of bFGF produced by oocytes

The expression of bFGF in the cultured ovaries was studied by using immunocytochemistry. bFGF was present in all the cell types with the highest level observed in the oocytes. SCF treatment increased bFGF expression in the oocytes (Figure 4). The result was also confirmed by western blotting as shown in figure 5.

5. DISCUSSION

Follicular development is a complex process consisted of both gonadotropin-independent and –dependent phases of growth (30-32). In the rat, most follicles are assembled after birth (1, 2). Following this organizational phase, subsets of newly formed primordial follicles begin to grow and undergo further differentiation that results in the acquisition of gonadotropin receptors and responsiveness to gonadotropins (1, 33, 34). Follicular development changes from gonadotropin-independent to gonadotropin-dependent.

An important observation in the present study was that steroidogenesis related proteins, SF-1, StAR were ubiquitously expressed while P450 arom was only expressed in oocytes of early developing follicles. The expressions of all three protiens in oocytes were up-regulated by SCF. In sheep, SF-1 was expressed in granulosa cells of primordial and small preantral follicles, whereas StAR mRNA was expressed in oocytes at all stages of follicular development (35). StAR mRNA has also been reported to be expressed in fetal human oocytes (36). In contrast, expression of StAR in cow is limited to theca cells in nonatretic antral follicles (11). P450arom was shown to be present in rat granulosa cells of the preovulatory follicles (14, 37). P450arom converts the theca cell derived androgens to E_2 in granulosa cells (38-40). In this study, we discovered that oocytes, as well as granulosa cells express SF-1 and StAR protein in cultured ovaries. P450arom mRNA was exclusively expressed in oocytes of early developing follicles. We can speculate that oocytes could secrete E_2 to support the growth of themselves as well as follicles during early follicular growth. SCF promotes oocyte growth and follicular development by elevating the expression of steroidogenesis related proteins.

The present study also showed that SCF inhibited the expression of FSHR mRNA in cultured ovaries. It seems that SCF inhibits granulosa cells differentiation and the follicular transition from gonadotropin-independent to –dependent phases. Since there is no expression of c-kit in granulosa cells, the action of SCF on formation of FSHR must be indirect. We hypothesized that SCF inhibits the formation of FSHR by inducing the production of proteins in oocytes as there is no morphologically distinguishable theca cells in the follicles of cultured ovaries at that stage.

Bidirectional signaling between oocytes and surrounding somatic cells is integral for the progression of preantral follicle development. Oocyte growth depends on somatic cells (41), in turn oocyte promotes granulosa cells proliferation and differentiation (42). Basic FGF is mainly localized to the oocyte of primordial and primary follicles (43-45), and this was confirmed by immunohistochemical data in our study too. Receptors for bFGF have been reported in rat granulosa cells (46). bFGF is one of several growth factors known to induce primordial follicle development, and regulates a wide range of ovarian functions including granulosa cells mitosis and apoptosis (42, 47, 48). The results that bFGF antibody partially reversed the inhibitory effect of SCF on the expression of FSHR mRNA to the normal level, and SCF increased the production of bFGF protein in oocytes implied that bFGF mediated the action of SCF on FSHR formation.

In conclusion, preliminary data in the present study indicated that a different E_2 production mechanism might well function in early developing follicles in comparison

with the traditional two-cell two-hormone mechanism in developed follicles. SCF could be a key player in this mechanism as it may promotes E_2 production by oocytes while inhibiting its production by granulose cells. Its action on granulose cells may be mediated by other factors such as bFGF. Such a hypothesis certainly needs more data to be verified. Currently, we are testing whether E_2 is really produced by ooctyes in primordial follicles.

6. ACKNOWLEDGMENTS

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