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Essential Role of the Oocyte in Estrogen Amplification of Follicle-Stimulating Hormone Signaling in Granulosa Cells

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The establishment of dominant ovarian follicles that are capable of ovulating fertilizable oocytes is a fundamental determinant of female fertility. This process is governed by pituitary gonadotropins as well as local ovarian factors. Within the follicle, estrogen acts in an autocrine/paracrine manner to enhance FSH action in the granulosa cells. These effects include the augmentation of P450aromatase expression and estradiol production. This feed-forward effect of estrogen is believed to play a key role in follicle dominance. Here we found the essential role of the oocyte in this physiological process using primary cultures of rat granulosa cells. In the presence, but not absence, of oocytes, estrogen amplified FSH-stimulated increases in mRNA expression of P450aromatase, FSH receptor, LH receptor, and inhibin α-, βA-, and βB-subunits as well as cAMP production. Thus, oocytes mediate the estrogen enhancement of FSH action in the granulosa cells. In comparison with FSH, cotreatment with estrogen and oocytes failed to amplify the stimulatory effects of forskolin or 8-bromoadenosine-cAMP on granulosa cell responses including P450aromatase mRNA expression and cAMP production, indicating that estrogen/oocytes amplify FSH action at a site upstream of adenylate cyclase. These findings support the novel conclusion that communication between the oocyte and granulosa cells plays a crucial role in mediating estrogen action during FSH-dependent folliculogenesis. (*Endocrinology* 146: 3362–3367, 2005)

IN MAMMALS, THE ABILITY of the ovary to produce dominant follicles that ovulate their oocytes at midcycle is the basis of female fertility. The classic model of the regulation of this process is based on the fundamental requirement of the anterior pituitary hormone, FSH. FSH binds to FSH receptors on the granulosa cells (GCs), and the cAMP signaling pathway activated by the FSH receptor leads to an increase in gene activity that is critical for GC cytodifferentiation and follicle dominance. Estrogen acts as a positive regulator of FSH action during the differentiation of the GCs (1, 2). Genetic studies in humans and mice have demonstrated that functional interactions between estrogen and FSH action are necessary for the establishment of dominant follicles in the ovary (3–6); however, the cellular and molecular mechanisms of how estrogen works to augment FSH action remain unknown.

Recent studies have introduced the new concept that communication networks between oocytes and somatic follicular cells are crucial not only for the growth and maturation of the oocyte but also for the proper mitosis and differentiation of surrounding somatic follicular cells (7–10). In this concept, the oocytes produce growth factors, most notably bone morphogenetic protein (BMP)-15 and growth and differentiation factor (GDF)-9, that can act on the GCs to regulate FSH activity and control GC proliferation (11–17). There is also evidence that GC-to-oocyte communication plays a critical role in folliculogenesis. For example, kit ligand, a GC-derived factor, and its receptor (c-kit), which is expressed in the oocyte, are necessary for follicle growth and development (18).

Given the importance of oocyte-somatic cell communication in follicle development, we wondered whether the oocytes might play a role in the mechanisms by which estrogen amplifies FSH action in the GCs. A striking finding of the present paper is that the oocyte is an obligatory mediator of the amplifying effects of estrogen on FSH action in GCs.

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Abbreviations: BMP, Bone morphogenetic protein; Br-cAMP, 8-bromoadenosine-cAMP; DES, diethylstilbestrol; ER, estrogen receptor; GC, granulosa cell; GDF, growth and differentiation factor; GPCR, G protein-coupled receptor; GRK, G-protein-coupled receptor kinase; GV, germinal vesicle; P450arom, P450aromatase.

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Materials and Methods

Reagents and supplies

Ovine FSH [National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-oFSH-S20] was provided by Dr. A. F. Parlow (National Hormone and Pituitary Program, Torrance, CA). Diethylstilbestrol (DES), forskolin, 8-bromoadenosine-cAMP (Br-cAMP), and 3-isobutyl-1-methylxanthine were purchased from Sigma-Aldrich Co. (St. Louis, MO). Female Sprague Dawley rats were from Charles River Laboratories (Wilmington, MA).

Primary coculture of rat GCs and oocytes

Female Sprague Dawley rats (21 to 22 d old) were implanted with SILASTIC capsules (Dow Corning, Corp., Midland, MI) containing 10 mg DES to increase GC number. Four days after DES implantation, GCs and oocytes were collected from the ovary and cultured in serum-free McCoy's 5a media. In the experiments that included oocytes in the cultures, oocytes were at a ratio of 1:5000 (oocytes to GCs), based on our

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previous observation of the numbers of oocytes and GCs extracted from DES-treated immature rats (19). For isolated GC cultures, the oocytes were removed using a 40-μm cell strainer as previously described (19). In experiments designed to investigate the dose-response effect of oocytes, denuded germinal vesicle (GV)-intact stage oocytes were individually collected by mouth pipetting under an inverted microscope and added to cultures of isolated GCs as indicated. The Institutional Animal Care and Use Committee at the University of California, San Diego approved all animal protocols.

Estradiol analysis

GCs (2×10^5 viable cells) were precultured with FSH (10 ng/ml), DES (5×10^{-8} M), and the indicated number of oocytes in 200 μl of McCoy's 5a medium for 72 h. The medium was then removed and replaced with fresh media containing androstenedione (10^{-7} M) as a substrate for aromatase. Five hours later the media were collected and estrogen levels were determined using our well-established RIA (19). DES up to 10^{-7} M showed no cross-reactivity with the estradiol RIA.

RNA extraction and RT-PCR

GCs (2×10^6 viable cells) were cultured in a six-well plate with 2 ml of McCoy's 5a medium containing indicated reagents. After 48 h culture, the medium was removed and total cellular RNA was extracted by isothiocyanate-acidphenol-chloroform methods using TRIzol (Invitrogen Corp., Carlsbad, CA) and quantified by measuring absorbance at 260 nm and stored at −80 C until assay. The steady-state levels of mRNA of P450 aromatase (P450arom) and the FSH receptor were analyzed by quantitative competitive RT-PCR as reported in our previous studies (16). The mRNA levels of the LH receptor and the inhibin subunits (α, βA, and βB) were measured by quantitative real-time RT-PCR using LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostics Co., Basel, Switzerland). Accumulated levels of fluorescence were analyzed by the second-derivative method after the melting-curve analysis, and then the expression levels of target genes were normalized to the expression level of L19 in each sample.

Measurement of cAMP production

GCs (1×10^5 viable cells) were cultured in a 96-well plate with 200 μl of serum-free medium containing a combination of FSH (10 ng/ml), DES (5×10^{-8} M), and 3-isobutyl-1-methylxanthine (0.1 mM). After 48 h, the conditioned medium was collected and the extracellular content of cAMP was measured by cAMP enzyme immunoassay kit (Sigma-Aldrich) after the acetylation of each sample.

Statistical analysis

Results are shown as mean ± SEM of at least three separate experiments, with triplicate determinations for each treatment. Differences between groups were analyzed for statistical significance using ANOVA (StatView 5.0 software, Abacus Concept, Inc., Berkeley, CA). $P \leq 0.05$ were accepted as statistically significant.

Results

It has been previously reported by Adashi and Hsueh (1) that estrogen amplifies FSH action in rat GCs cultured in serum free medium. In these studies, the GCs were collected by puncturing follicles using protocols established by Erickson and Hsueh (20). In this model, oocytes are incidentally included in the GC cultures at a ratio of about 1 oocyte per 5000 GCs as reported previously (19). Historically it was assumed that the contaminating oocytes did not affect the process of hormone-dependent GC cytodifferentiation. However, recent studies (21, 22) demonstrated that oocytes play crucial roles in regulating GC functions. We therefore investigated whether the presence of oocytes affects the estrogen effects on FSH action in GCs. For this purpose we separated oocytes from the GC/oocyte mixture, selected denuded GV-stage oocytes (Fig. 1A), and added these oocytes back to the isolated GC cultures. As shown in Fig. 1B, the addition of GV oocytes resulted in an oocyte dose-response increase in the levels of estradiol production by GCs cultured with FSH and DES. Increased estradiol accumulation occurred after the addition of as few as 10 oocytes, but a dramatic effect (~13-fold) was observed after the addition of 80 oocytes to these cultures.

To address the mechanism of the oocyte-GC interaction, we first determined whether P450arom mRNA expression is enhanced in GCs. In the absence of FSH, the basal levels of P450arom mRNA in the GCs were very low, and the levels were not changed by the addition of DES and/or oocytes (Fig. 2). As expected, expression of P450arom mRNA was markedly stimulated by treatment with FSH. No changes were observed in the expression levels when FSH was coincubated with either DES or oocytes alone. In contrast, the presence of both oocytes and DES significantly increased the ability of FSH to stimulate the expression of the P450arom mRNAs. Similar results were observed in the steady-state mRNA levels of FSH receptor (Fig. 2). The mRNA levels of the housekeeping gene, L19 (ribosomal protein), were not changed by any of these treatments. It is notable that because the combination of oocytes and DES did not increase FSH receptor mRNA expression in the absence of FSH, their ability to augment FSH action must occur at a site downstream of the FSH receptor.

To further document the combined role of oocytes and estrogen in augmenting FSH-dependent cytodifferentiation, we quantified mRNA levels of other major molecular mark-

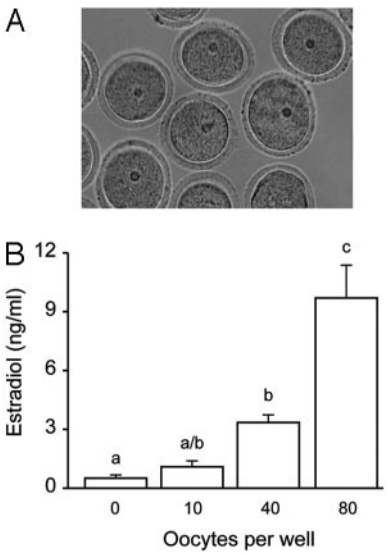

FIG. 1. Oocytes cause the DES amplification of FSH-induced estradiol production by GCs. A, A uniform population of GC-free oocytes at the GV stage was collected as described in *Materials and Methods*. B, GCs were seeded at 2×10^5 cells per well (200 μl final culture volume) together with FSH (10 ng/ml), DES (5×10^{-8} M), and the indicated number of GV oocytes. After a 72-h culture, the media were replaced with fresh media containing 10^{-7} M androstenedione, incubated for 5 h, and the media assayed for estradiol by RIA. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate treatments. *Bars with different letters* indicate that group means are significantly different at $P \leq 0.05$.

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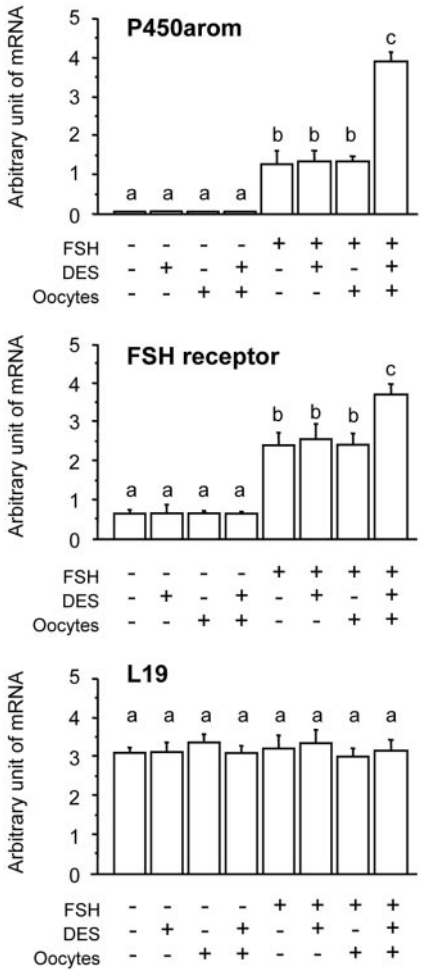

FIG. 2. Oocytes are required for DES to amplify FSH-induced P450arom and FSH receptor mRNAs in GCs. GCs (2×10^6 cells) were cultured with or without a saturating dose of FSH (10 ng/ml) and/or DES (5×10^{-8} M) in the presence and absence of oocytes (40 oocytes/well). After 48 h, steady-state levels of mRNA encoding P450arom, FSH receptor, and the housekeeping gene L19 were analyzed using quantitative competitive RT-PCR. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate treatments. *Bars with different letters* indicate that group means are significantly different at $P \leq 0.05$.

ers of GC differentiation by real-time PCR analysis. As seen in Fig. 3, DES was able to increase the FSH induction of the mRNAs encoding LH receptor, inhibin α-, inhibin/activin βA-, and βB-subunits; however, this effect occurred only in the presence of oocytes. These changes reflected the effects observed with respect to P450arom and FSH receptors shown in Fig. 2. The concept that emerges from these experiments is that oocytes must be present for estrogen to facilitate FSH action in the GCs.

We next investigated the effects of oocytes and DES on FSH-induced cAMP production. Consistent with the above mRNA results, the FSH stimulation of cAMP production by GCs was not significantly altered by treatment with either DES or oocytes alone, but cotreatment with DES plus oocytes caused a significant increase in cAMP accumulation (Fig. 4). Thus, it can be inferred that the cellular site of the DES/oocytes amplification of FSH action must be upstream of adenylate cyclase.

To analyze this further, we examined the effect of DES and oocytes on the induction of P450arom gene expression by forskolin and Br-cAMP, two agents known to increase intracellular cAMP. In contrast to FSH, cotreatment with oocytes and DES failed to further enhance the expression of P450arom mRNA induced by forskolin or Br-cAMP (Fig. 5). Furthermore, coculture with oocytes and DES did not enhance forskolin-induced cAMP production (Fig. 6). These data suggest that the target of DES/oocyte function might occur at a site upstream of adenylate cyclase

Discussion

The regulation of FSH responsiveness in GCs is critical for the establishment of dominant follicles and subsequent ovulation in mammals. Thus, elucidating the cellular and molecular mechanisms that regulate FSH action is important to understand female fertility. Estrogen and oocyte growth factors are known to play a pivotal role in the generation of FSH responses in GCs. In the present study, we made the novel discovery that the oocyte is required for estrogen to exert its positive effects on FSH-dependent GC differentiation. These results support the new concept that estrogen action in the follicular GCs is mediated by the oocyte.

Classic studies by Adashi and Hsueh (1, 2) and colleagues demonstrated that various native and synthetic estrogens interact with the FSH signaling pathways to amplify the FSH-dependent GC differentiation. However, it is clear from our results that estrogen can amplify FSH action in the presence, but not absence, of oocytes. Consequently, it can be concluded that the strong positive effect of estrogen on FSH action reported by Adashi and Hsueh is mediated by the oocytes that are present in the GC cultures. Using the mouse model, Vanderhyden and colleagues (23–25) demonstrated a role for the oocyte in enhancing FSH-induced estradiol synthesis by mouse cumulus GCs. Based on our present data, we propose that in Vanerhyden's experimental model, endogenous estradiol produced within the GC/oocyte cultures likely played a role in the ability of the oocytes to augment FSH action. Indeed, the observation by Adashi and Hsueh (1) that estradiol is equally effective as DES in amplifying FSH action provides experimental support for this hypothesis.

We found that the addition of DES/oocyte amplifies FSH-induced FSH receptor expression in the GCs. This result raises a question: to what extent does the amplifying effect involve the contribution of increased expression of FSH receptor? This may not be the case because the DES/oocyte does not potentiate the effects of forskolin and Br-cAMP, despite the fact that both of these stimulatory molecules increase FSH receptor expression (26). The fact that DES and oocytes together failed to increase FSH receptor expression in the absence of FSH (Fig. 2) supports our hypothesis that the target of DES/oocyte function is at some point downstream of FSH receptor expression and upstream of adenylate cyclase. Although the mechanism of oocyte/DES action remains unresolved, it is possible that it involves desensitization or internalization of FSH receptors. In this respect, there is recent evidence that FSH signaling is subject to reg-

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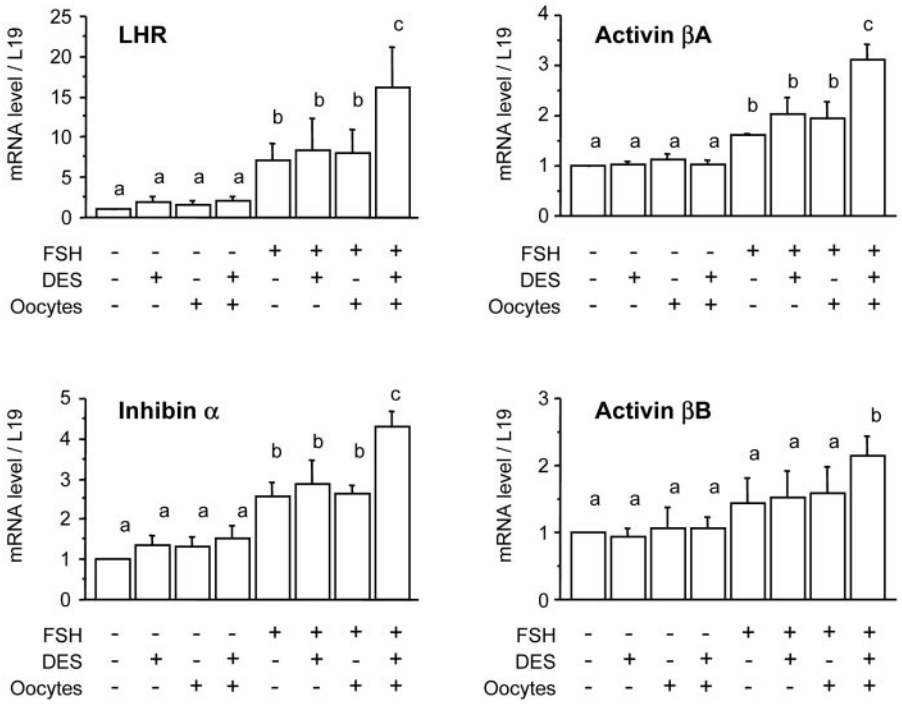

FIG. 3. Oocytes are required for DES to enhance the expression of FSH-induced mRNAs encoding LH receptor (LHR) and the inhibin/activin subunits. GCs were cultured as described in the legend of Fig. 2, and mRNA levels of the indicated genes were analyzed using quantitative real-time PCR. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate treatments. *Bars with different letters* indicate that group means are significantly different at $P \leq 0.05$.

ulation by the G protein-coupled receptor kinase (GRK)/β-arrestin system, a system that has been studied most extensively with respect to the regulation of β-adrenergic and rhodopsin receptor signaling. These studies have revealed that the general mechanism by which the GRK/β-arrestin system causes receptor desensitization involves the following steps: 1) upon binding of a ligand to its G protein-coupled receptor (GPCR), the cytoplasmic domain of the GPCR is phosphorylated by members of the GRK family, desensitizing the receptor by causing the uncoupling of the receptor from G proteins; and 2) after phosphorylation of the GPCR, β-arrestins can then interact with and internalize GPCRs by a clathrin-mediated mechanism (27, 28). The role of the GRK/β-arrestin system has been extended to the FSH receptor by recent studies that have used various strategies in rat primary Sertoli cells, a Sertoli cell line (MSC-1), and cell lines engineered to overexpress FSH receptors. These studies have shown that, in these models, GRK-2, -3, -5, and -6 as well

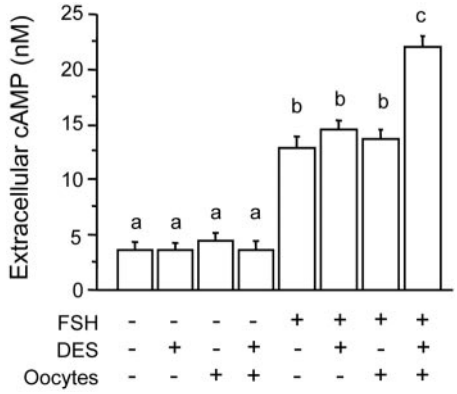

FIG. 4. Oocytes are required for DES to enhance FSH-stimulated cAMP accumulation. GCs were cultured as described in the legend of Fig. 2, and cAMP levels in the media were evaluated. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate treatments. *Bars with different letters* indicate that group means are significantly different at $P \leq 0.05$.

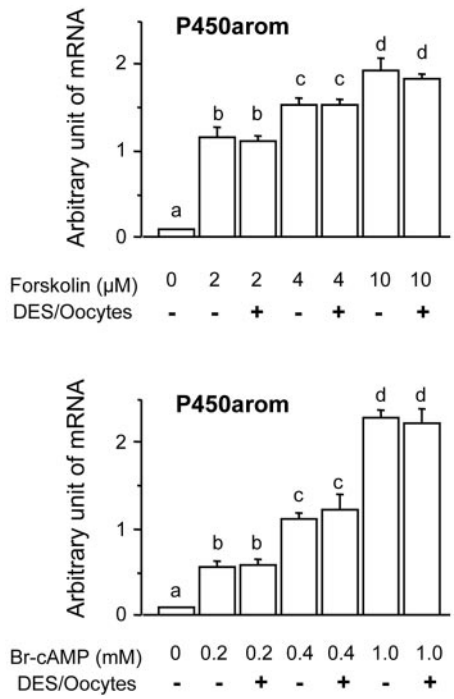

FIG. 5. Effects of DES and oocytes on the mRNA expression of P450arom induced by forskolin or Br-cAMP. GCs were cultured with or without DES (5×10^{-8} M) and oocytes in the presence of forskolin (*upper panel*) or Br-cAMP (*lower panel*). After 48 h, steady-state levels of mRNA encoding P450arom were analyzed using quantitative competitive RT-PCR. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate treatments. *Bars with different letters* indicate that group means are significantly different at $P \leq 0.05$.

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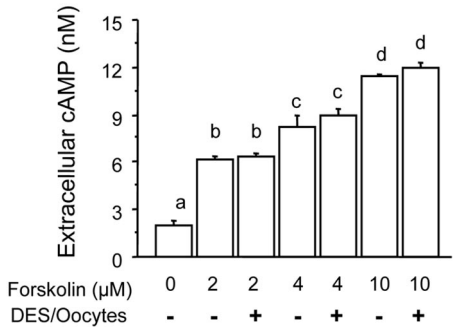

FIG. 6. Effects of DES and oocytes on cAMP production induced by forskolin. GCs were cultured as described in the legend of Fig. 5, and cAMP levels in the media were evaluated. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate treatments. *Bars with different letters* indicate that group means are significantly different at $P \leq 0.05$.

as β-arrestin-1 and -2 can interact with FSH receptors and impair FSH signaling (29–32). Accordingly, it is intriguing to hypothesize that this system could be involved in the mechanism of DES/oocyte action in GCs.

There has been extensive recent interest in the roles of oocyte-secreted TGFβ superfamily members, specifically BMP-6, BMP-15, and GDF-9, in the regulation of GC function. Based on studies using a similar rat GC experimental model, it was found that estradiol production is not affected by BMP-15 (15) or BMP-6 (26), whereas GDF-9 exhibits suppressive effects on estradiol synthesis (14) in the presence of FSH. Therefore, because oocytes act to augment estradiol synthesis under the conditions of the present study, it is unlikely that the above TGFβ superfamily members are directly responsible for the responses elicited by oocytes observed here. To the best of our knowledge, there have been no oocyte-secreted factors identified as of yet that have biological activities that account for the present results. Accordingly, future studies are required to identify the putative factor or factors secreted by oocytes that would have the steroidogenic actions observed in this study.

In summary, our discovery that oocytes are obligatory for estrogen-dependent increases in FSH action in GCs is an important new step toward understanding the physiological actions of these key hormones in the ovary. The current challenges are to understand how the oocyte contributes to this fundamental function and how the interactions are integrated into the dynamics of follicular growth, development, and atresia. This new mechanism of estrogen action in the ovary is now begging to be tested in other species including the human.

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