Hormonal regulation of preovulatory follicle maturation in the rat


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Summary. Oestrogen-priming of the hypophysectomized immature female rat promotes preantral follicle development in the absence of endogenous gonadotrophins and such an animal is useful for study of the intraovarian glycoprotein–steroidal hormone interactions which underlie morphological and functional development of the ovarian follicle. The present report identifies in vitro the functional characteristics (gonadotrophin binding and steroidogenesis) of granulosa cells harvested at different stages of follicular maturity following treatment with exogenous hormones in vivo. Preovulatory follicle maturation, induced by FSH, has been studied up until antrum formation and the acquisition by granulosa cells of the ability to respond directly to LH or hCG. Before the increases in available granulosa cell membrane LH/hCG receptors associated with the formation of follicular antra, effects of hCG or other hormones with interstitial cell stimulating activity are mediated via interactions with cells outside the lamina basalis. In-vivo studies with oestrogen-primed hypophysectomized immature rats indicate that androgens secreted by LH/hCG-stimulated thecal and/or interstitial cells may act directly on the preantral follicle to promote atresia. However, in-vitro studies have shown a stimulatory effect of androgen on FSH-responsive progesterone secretion by granulosa cells isolated from preantral follicles. These effects, if shown to operate within the ovary during the normal cycle, need not be mutually exclusive because FSH stimulation of granulosa cells in vivo may be a major determinant of follicular responses to androgen. The increase in follicle size and antrum formation accompanying FSH treatment in vivo are associated with (i) increases in the steroidogenic potential of isolated granulosa cells; (ii) the induction of granulosa cell LH/hCG receptors and of steroidogenic responsivity to hCG; and (iii) stimulation of granulosa cell aromatase activity. These observations highlight the critical role of FSH in the organization of preovulatory follicular morphology and function.

Introduction

During successive oestrous cycles, in the ovary of the rat a few well advanced preantral follicles undergo the final stages of maturation, culminating in antrum formation and the ability to respond to the ovulatory surge of LH. Granulosa cell differentiation during this period is characterized by intracellular modifications leading to increased steroidogenesis and by an increase in the number of available cell membrane LH receptors. While FSH plays a major role in these changes, several studies attest to the importance of intraovarian oestrogens acting

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locally to regulate follicular responses to gonadotrophins (e.g. Pencharz, 1940; Williams, 1940; Smith & Bradbury, 1963). Studies with hypophysectomized immature female rats given graded doses of diethylstilboestrol showed that oestrogen stimulated ovarian weight and that this response was the product of a reduction in follicular atresia and a concomitant stimulation of preantral follicle growth (Payne & Hellbaum, 1955). Goldenberg, Vaitukaitis & Ross (1972) showed a synergistic effect on ovarian weight when graded doses of FSH were administered following 2–4 days of diethylstilboestrol. In contrast to the predominance of large preantral follicles in ovaries of animals receiving diethylstilboestrol alone, numerous antral follicles were present following treatment with both hormones. The mechanism through which oestrogen exerted this effect entailed increased ovarian uptake of labelled FSH (Goldenberg et al., 1972). Granulose cells contain cytosolic and nuclear receptors for oestrogen (Richards & Midgley, 1976) and are thought to be the exclusive site of FSH receptors in the ovary (Zeleznik, Midgley & Reichert, 1974). An analysis of FSH receptor numbers per ovary and per granulosa cell from ovaries removed before and after treatment of hypophysectomized immature rats with graded doses of oestrogen revealed that oestrogens acted to increase FSH receptor activity principally by stimulation of granulosa cell proliferation (Louvet & Vaitukaitis, 1976).

Between 4 and 6 days after hypophysectomy and initiating treatment with oestrogen (at 21 days of age) the ovaries of oestrogen-primed immature rats contain a relatively homogeneous population of solid preantral follicles with multiple granulosa cell layers (Pl. 1, Fig. 1). Most of these follicles are greater than 200 μm in diameter (Louvet, Harman, Schreiber & Ross, 1975b) but rarely exceed 400 μm (Erickson & Hsueh, 1978). In the intact cyclic adult the Graafian follicles that will ovulate during the next oestrous develop from the preantral follicles which fall within this size range at the beginning of a normal cycle (Lane & Davis, 1939; Boling, Blandau, Soderwell & Young, 1941; Pederson, 1970). Moreover, there is evidence that between the onset of oestrous and ovulation it is the follicles within this category (250–349 μm) that are increasingly involved in the processes of atresia and degeneration (Mandl & Zuckerman, 1952). In view of these considerations, a systematic appraisal of the gonadotrophic and steroidal hormone interactions associated with the processes of antrum formation and atresia in the follicles of oestrogen-primed ovaries of hypophysectomized rats may provide information relevant to the events which take place during preovulatory follicle maturation. Similar studies with intact cyclic animals suffer from the constraints imposed by steroid hormones feeding back on pituitary gonadotrophin secretion.

In the present report, we summarize and discuss major hormonal interactions which underlie altered follicular morphology and granulosa cell function in the ovaries of oestrogen-primed hypophysectomized immature rats.

**Studies pertaining to the role of LH in preovulatory follicle maturation**

Human chorionic gonadotrophin (hCG) has been used instead of LH in these studies because it binds to the same receptors as LH, is available in a highly purified form and is more stable than LH after radioiodination. All the experiments refer to rats hypophysectomized at 21 days of age.

In preantral follicles, LH binds to and acts specifically on thecal and interstitial cells (Zeleznik et al., 1974), but does not bind to the granulosa cells (Channing & Kammerman, 1973; Lee, 1976; Stouffer, Tyrey & Schomberg, 1976; Hillier, Zeleznik & Ross, 1978).

Treatment of hypophysectomized immature females with oestrogen significantly increases the weight of the ovaries, but administration of hCG to such animals inhibits the oestrogen-stimulated ovarian weight gain (Louvet, Harman & Ross, 1975a; Louvet et al., 1975b): maximal inhibition is induced by a dose of 0.3 m.i.u. per animal (Text-fig. 1). Since the effect was specific for hCG and human LH and could not be reproduced by treatment with an FSH preparation devoid of significant interstitial cell-stimulating activity, it was suggested that gonadotrophic stimulation of ovarian thecal and/or interstitial tissue mediated this response.
Histological studies revealed that the reduction in ovarian weight induced by low doses of hCG was accompanied by interstitial cell stimulation, a reduction in follicle size, and a marked increase in the incidence of follicular atresia. Since co-administration of specific chemical or biological antagonists of androgen action reversed the last two effects of hCG, it was suggested that androgens secreted by interstitial and/or thecal cells in response to hCG act locally to inhibit the effect of oestrogen on follicle growth (Louvet et al., 1975b). More recent experiments demonstrated the anti-oestrogenic action of testosterone directly, showing both time- and dose-dependent reductions in oestrogen-stimulated ovarian weight (Text-fig. 1) and promotion of follicular atresia (Hillier & Ross, 1979).

Text-fig. 1. Effects of (a) hCG and (b) testosterone on ovarian weight in oestrogen-primed hypophysectomized rats. Mean paired ovarian weight (±1 s.e.m.) at 27 days of age is plotted as a function of the total dose of hormone/animal, administered as twice daily s.c. injections during the 4 days before death. Hypophysectomy and s.c. placement of a 1 cm Silastic capsule containing diethylstilboestrol was performed at 21 days of age. Data from Louvet et al. (1975b) and Hillier & Ross (1979).

Consistent with the aforementioned finding that granulosa cells from oestrogen-primed hypophysectomized rats had a limited ability to bind $^{125}$I-labelled hCG, the addition of hCG (10–1000 mi.u./ml) to granulosa cell culture medium had no effect on progesterone secretion by these cells (Hillier, Knazek & Ross, 1978). On the other hand, the addition of testosterone or 5α-dihydrotestosterone (but not oestradiol or diethylstilboestrol) to the culture medium resulted in dose- and time-dependent increases in progesterone production (Lucky, Schreiber, Hillier, Schulman & Ross, 1977). This effect (Text-fig. 2) was inhibited in the presence of the non-steroidal antiandrogen, SCH-16423 (Hillier et al., 1977), a derivative of flutamide which inhibits ovarian nuclear uptake of tritiated testosterone in vivo (Zeleznik, Hillier & Ross, 1979), presumably by inhibiting the nuclear translocation of cytoplasmic testosterone–receptor complexes within granulosa cells (Schreiber & Ross, 1976). Androgen has also been shown to stimulate progestagen production by cultured granulosa cells from mature antral follicles of cyclic rats and pigs (Nimrod & Lindner, 1976; Schomberg, Stouffer & Tyrey, 1976). The ability to secrete progestagens and respond in this fashion to androgens in vitro may therefore represent a biochemical characteristic of the preantral granulosa cell which is retained throughout preovulatory follicle maturation in vivo.
Text-fig. 2. Dose- and time-dependency of testosterone-stimulated progesterone production by granulosa cells from the preantral follicles of oestrogen-primed hypophysectomized rats. Granulosa cells were taken from 27-day-old animals which were hypophysectomized and received (s.c.) a 1 cm Silastic implant containing diethylstilboestrol at 21 days of age. Each value represents the mean ± 1 s.e.m. of determinations on 4 replicate cultures. Data from Hillier et al. (1977).

The potential interactions between androgens and FSH during the stimulation of steroidogenesis are of importance. In contrast to our own findings, Armstrong & Dorrington (1976) reported that granulosa cells isolated from preantral follicles in oestrogen-primed hypophysectomized rats produced “minimal” amounts of progesterone regardless of whether androgen was present in the culture medium. However, incubations in the presence of FSH stimulated progesterone production and this effect was augmented when testosterone or 5α-dihydrotestosterone was included with FSH in the culture medium. We have re-examined this interaction and the results (Text-fig. 3) confirm that testosterone and FSH have independent stimulatory effects on progesterone production during a 48 h period of culture (approximately 5-fold and 16-fold, respectively, in the experiment shown), whereas co-incubations in the presence of both hormones elicit a synergistic response (94-fold relative to controls). In addition we found that the stimulatory and synergistic effects of testosterone were inhibited in the presence of the specific antiandrogen, SCH-16423, thereby emphasizing the specificity of the response to testosterone. These data suggest that this highly specific response to androgen may be important because of the augmentation of steroidogenesis induced by FSH rather than the induction of steroidogenesis per se.

The foregoing observations prompt the following speculations. The degree to which granulosa cells in preantral follicles have been or are being exposed to FSH may be a major determinant of the follicular response to androgens of thecal and/or interstitial cell origin. Inadequate exposure may encourage an atretic response to androgen while appropriate stimulation with FSH may facilitate enhanced sensitivity of androgen-responsive steroido
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SCH-16423
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Text-fig. 3. Interaction between testosterone- and FSH-responsive progesterone secretion by granulosa cells isolated from the ovaries of 27-day-old oestrogen-primed hypophysectomized rats which were hypophysectomized and received (s.c.) a 1 cm Silastic capsule containing diethylstilboestrol at 21 days of age. The additions to the medium were nothing or various combinations of \(10^{-7}\) m-testosterone (T), 100 ng hFSH (LER-8/116)/ml and \(10^{-5}\) M-SCH-16423 (a-a-a-trifluoro-2-methyl-4'-nitro-m-lactotoluidide). Each value represents the mean ± 1 s.e.m. of determinations on 4 replicate cultures. See Hillier et al. (1977) for methods.

genesis. Concomitant increases in the activity of granulosa cell aromatase enzyme(s) (see later) would also serve to increase the metabolism of aromatizable androgens to oestrogens, and thereby protect the follicle from the atretic effect of androgen. Although recent studies in vitro with isolated and recombined thecal and granulosa cells from preovulatory follicles suggest that an interaction between follicular granulosa cells and theca may be operational during FSH-stimulated progesterone synthesis (Makris & Ryan, 1977), definitive evidence for either the atretic action or the steroidogenic action of androgen constituting a physiologically operational mechanism during normal cyclic follicle maturation is lacking.

Studies pertaining to the role of FSH in preovulatory follicle maturation

In the rat ovary FSH receptors are located exclusively on granulosa cells (Zeleznik et al., 1974). The importance of local effects of ovarian oestrogens to follicular responses elicited by FSH was indicated earlier (see above). FSH plays a central role in the induction of granulosa cell steroidogenesis and the increased number of available LH receptors which characterize the advanced stages of granulosa cell differentiation during preovulatory follicle maturation in the cyclic adult. Classical morphological correlates of FSH stimulation during this period are increased follicle size and the development of follicular antra: each of these effects have been observed after treatment of oestrogen-primed hypophysectomized rats with exogenous FSH (Pl. 1, Fig. 2).

Although molecular mechanisms underlying antrum formation remain obscure (Zachariae, 1959) studies with oestrogen-primed hypophysectomized rats have revealed a dose-dependent stimulation of \(^{35}\)SO\(_4\) incorporation into ovarian proteoglycans by FSH in vivo (Mueller et al., 1978). Isolated granulosa cells in tissue culture were also able to secrete proteoglycans and to respond directly to exogenous FSH with an increase in \(^{35}\)SO\(_4\) incorporation.
Gonadotrophin-induced steroidogenesis in follicular cells is mediated by increases in the intracellular concentration of 3′,5′-cAMP which is thought to initiate a complex sequence of subcellular biochemical events which culminate in the induction or activation of one or more of the rate-limiting enzymic steps in steroidogenesis (Kolena & Channing, 1972; Marsh, 1976). Granulosa cells obtained from the preantral follicles of oestrogen-primed hypophysectomized rats contain a membrane-associated FSH-sensitive adenylate cyclase system and progesterone secretion by these cells is stimulated by FSH in vitro (Text-fig. 4; Hillier et al., 1978).

Text-fig. 4. Relationship between membrane-associated FSH-sensitive adenylate cyclase activity in (●) and progesterone secretion by (O) granulosa cells isolated from the preantral follicles of 27-day-old oestrogeen-primed hypophysectomized rats. The animals were hypophysectomized and received (s.c.) a 1 cm Silastic capsule containing diethylstilboestrol at 21 days of age. Adenylate cyclase activities in a crude membrane fraction were determined by the procedure of Salomon, Londos & Rodbell (1974) but with a 10 min reaction sequence. Progesterone production represents the accumulation of immunoreactive progesterone in culture medium during a 48 h incubation of cell suspensions. The responses (mean of triplicate incubations) are shown as a function of hFSH concentration. The hFSH preparation used (LER 8/116) contained approximately 900 i.u. FSH and 6 i.u. LH/mg. Calculation of the molar concentration of hFSH was made assuming the biological activity of 'pure' FSH to be 4000 i.u./mg and the mol. wt to be 35 000. The half-maximal value of both responses (indicated by the broken lines) was elicited by the same concentration of FSH (5.3 × 10^{-10} M 'pure' hFSH; i.e. 82.4 ng LER 8/116 ml). Data from Hillier et al. (1978).

Direct evidence for the importance of FSH in the induction of granulosa cell steroidogenesis during the course of antrum formation was adduced from studies of granulosa cells collected after treatment with exogenous FSH (Table 1). In these experiments suspensions of granulosa cells isolated from control animals (injected with saline) did not produce measurable amounts of progesterone (<0.8 pg/μg cell protein) during 90 and 240 min incubations, even when hFSH or dibutyryl cAMP were added to the culture medium at concentrations known to stimulate steroidogenesis during longer incubations. On the other hand, treatment with FSH for 24 h before removal of the granulosa cells (sufficient to stimulate early antrum formation as shown in Pl. 1, Fig. 2) led to readily detectable basal secretion of progesterone (no additions to culture medium) which was stimulated 3.4- and 1.6-fold after 240 min in the presence of FSH and dibutyryl cAMP, respectively. Short-term incubations of whole follicles gave similar results.
Table 1. Effect of FSH treatment in vivo on early basal, FSH- and dibutyryl cAMP-responsive progesterone production (mean ± s.e.m. for 3 determinations) by isolated rat granulosa cells

<table>
<thead>
<tr>
<th>Treatment in vivo</th>
<th>Addition to culture medium</th>
<th>Progesterone production (pg/µg cell protein)</th>
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<tr>
<td></td>
<td></td>
<td>1.5 h</td>
</tr>
<tr>
<td>Saline</td>
<td>None</td>
<td>ND</td>
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<tr>
<td></td>
<td>hFSH</td>
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<td></td>
<td>dbcAMP</td>
<td>ND</td>
</tr>
<tr>
<td>oFSH (24 h)</td>
<td>None</td>
<td>48.7 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>hFSH</td>
<td>67.3 ± 16.1</td>
</tr>
<tr>
<td></td>
<td>dbcAMP</td>
<td>42.7 ± 4.5</td>
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</tbody>
</table>

Granulosa cell suspensions were prepared from the ovaries of 27-day-old oestrogen-primed hypophysectomized immature rats receiving s.c. injections of saline or 100 µg oFSH in saline at 24 and 12 h before death. The accumulation of immunoreactive progesterone in culture medium was determined following 1.5 and 4 h of incubation in medium containing no additions and containing 500 ng hFSH (LER 8/116)/ml or 1 µm dibutyryl cAMP (dbcAMP). See Hillier et al. (1978) for Methods. ND = not detectable (<0.8 pg/µg cell protein).

(Zeleznik, Keyes, Menon, Midgley & Reichert, 1977). FSH-sensitive adenylate cyclase activities in granulosa cell membranes from control and FSH-treated animals are alike (Hillier et al., 1978). Thus the action of FSH on steroidogenesis is mediated by the activation or sensitization of a biochemical step(s) located distally to the primary membrane effector interaction and the locus of cAMP production (Hillier et al., 1978). Histochemical studies of steroidogenic enzymes in the ovaries of adult rats during the oestrous cycle suggest that 3β-hydroxysteroid dehydrogenase activity in the granulosa cells of maturing follicles increases as the cycle proceeds, becoming apparent in some of the larger growing follicles on the 2nd day of dioestrus and increasing markedly by pro-oestrus (Pupkin, Bratt, Weisz, Lloyd & Balogh, 1966). Moreover, Zeleznik et al. (1974), also using histochemistry, reported that granulosa cell 3β-hydroxysteroid dehydrogenase activity and antrum formation was stimulated following the treatment of immature rats with FSH. Collectively, these observations imply that FSH may be responsible for the induction or activation of one or more of the major rate-limiting steroidogenic enzymes present in the granulosa cells of preovulatory follicles.

The increased steroidogenic capacity which granulosa cells acquire during preovulatory follicle maturation in the cyclic mammal is associated with an increased ability to bind 125I-labelled hCG (Channing & Ledwitz-Rigby, 1975). Granulosa cells harvested from large preovulatory follicles undergo morphological 'luteinization' when placed into culture and basal levels of progestagen secretion are substantial. Progestagen secretion is further increased when LH or hCG are included in the culture medium (Channing, 1970).

The critical role of FSH in the induction of granulosa cell LH/hCG receptors was illustrated by the studies of Zeleznik et al. (1974) showing that binding of 125I-labelled hCG to the granulosa cells of immature rat ovaries was markedly stimulated after treatment with rat FSH in vivo for 2 days. An examination of the relationship between FSH-induced LH/hCG receptors and the steroidogenic capacity of granulosa cells of oestrogen-primed hypophysectomized rats showed that early antrum formation following treatment with FSH for 24 h was associated with marked increases in both steroidogenic potential and binding of 125I-labelled hCG in vitro (Hillier et al., 1978). The 9-fold increase in hCG binding induced by FSH treatment in vivo was shown to reflect the acquisition of functional LH/hCG receptors since it was accompanied by the ability of isolated granulosa cells to respond to low concentrations of hCG with striking increases in progesterone production (Text-fig. 5).

Oestrogens are clearly important for the early stages of normal follicular development and facilitating follicular responses elicited by FSH. Although the primary cellular source of
oestrogen in the ovarian follicle has yet to be identified, preovulatory Graafian follicles are considered to be responsible for the large quantities of oestrogens secreted by the ovary into the peripheral circulation during oestrus (Baird & Fraser, 1975; Makris & Ryan, 1975). The classical experiments of Falck (1959) led to the conclusion that both theca and granulosa cells were required for ovarian oestrogen synthesis. Support for this hypothesis was provided by experiments in which intraovarian and peripheral blood levels of oestradiol were measured in hypophysectomized immature rats given androgens and FSH. Armstrong & Papkoff (1976) showed that ovarian and peripheral blood oestradiol levels were elevated when graded doses of testosterone were followed with a fixed dose of FSH, but no such increments were found when 5α-dihydrotestosterone (a non-aromatizable androgen) was given with the FSH. These results suggested that LH and hCG enhance ovarian synthesis of androgen which is aromatized to oestrogens. Experiments with tissue isolated from follicles of rats in pro-oestrus have confirmed that theca but not granulosa cells secrete aromatizable androgen and that this response is stimulated by LH in vitro (Fortune & Armstrong, 1977). In addition, Dorrington, Moon & Armstrong (1975) showed that FSH stimulated the secretion of oestradiol when granulosa cells from preantral follicles were incubated with testosterone, further suggesting that FSH stimulates aromatase activity in granulosa cells which can then convert androgens secreted by cells outside the lamina basalis into oestrogens.

Substantial increases in granulosa cell aromatase activity accompany preovulatory follicular development during the normal oestrous cycle: the highest activity is associated with mature Graafian follicles on the day of pro-oestrus (Hillier, van den Boogaard, Reichert & van Hall, 1980). The relationship between FSH-induced follicular maturation and granulosa cell aromatase activity in oestrogen-primed hypophysectomized rats was established as follows. Granulosa cell suspensions were prepared from the ovaries of animals treated with saline (controls) or treated with FSH for 12 and 24 h respectively before death. Replicate aliquots of
**Fig. 1.** Preantral follicular morphology in the ovary of a 27-day-old oestrogen-primed hypophysectomized immature rat. Hypophysectomy and subcutaneous implantation of a 1 cm Silastic capsule containing diethylstilboestrol was performed at 21 days of age. H & E, ×220.

**Fig. 2.** Early antrum formation following treatment as above plus a subcutaneous injection of 100 µg ovine FSH (LER-1698) in saline administered 24 and 12 h before death at 27 days of age. H & E, ×220.

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each cell suspension were incubated for 48 h in culture medium containing no additions (controls), testosterone (10⁻⁷ M), human FSH (100 ng/ml) or a mixture of the two hormones. At the end of the incubation the concentration of immunoassayable oestradiol in spent culture medium was determined. The results (Text-fig. 6) show that early stages of antrum formation induced by 24 h of treatment with FSH (Pl. 1, Fig. 2) are associated with dramatic increases in the ability of isolated granulosa cells to aromatize testosterone. Under the experimental conditions employed, the concentration of testosterone, rather than FSH, added to the culture medium appeared to be the rate-limiting factor. However, the limited conversion of testosterone to oestradiol by cells from the control group (preantral follicles) was stimulated approximately 3-fold in the presence of FSH in vitro. The intermediate period of FSH treatment in vivo (12 h) resulted in a 5-fold increase in oestradiol accumulation in the presence of testosterone alone (relative to cells isolated from saline-treated controls) and was further increased 1.5-fold when FSH was included in the culture medium. In similar experiments with oestrogen-primed hypophysectomized rats, Erickson & Hsueh (1978) observed that after 48 h of FSH treatment (when follicular morphology was similar to that of preovulatory follicles of adult animals on the morning of pro-oestrus) granulosa cell aromatase activities had increased to levels which were comparable to those observed for granulosa cells isolated from the fully mature Graafian follicles of cyclic adults.

Text-fig. 6. Effect of FSH-treatment in vivo on isolated granulosa cell aromatase activity. Granulosa cell suspensions were prepared from the ovaries of 27-day-old oestrogen-primed hypophysectomized rats injected with saline (9 g NaCl/l) or 100 µg oFSH (LER-1698) in saline at 12 h or 24 and 12 h before death. Additions to the culture medium were none or combinations of 10⁻⁷ M-testosterone (T) and 500 ng hFSH (LER-8/116)/ml. Each value represents the mean ± 1 s.e.m. of determinations on triplicate incubations. See Hillier et al. (1978) for methods.

The induction of granulosa cell aromatase activity would seem, therefore, to constitute a further functional correlate of FSH acting during the course of normal preovulatory follicle maturation.

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