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# Direct Ovarian Effects of a GnRH Agonist in Hypophysectomized Immature Rats: Inhibition of Theca-Interstitial Luteinizing Hormone Receptor mRNA Expression and Induction of Interstitial Cell Differentiation

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**ABSTRACT**—The effect of a gonadotropin-releasing hormone (GnRH) agonist on luteinizing hormone (LH) receptor mRNA expression was examined histologically in the ovaries of immature hypophysectomized (HPX) rats by in situ hybridization. In the ovaries of HPX rats treated with diethylstilbestrol (DES) and pregnant mare serum gonadotropin (PMSG), LH receptor mRNA was expressed in the granulosa cells of mature follicles as well as the theca-interstitial cells. In DES-primed ovaries of rats treated with both GnRH agonist plus PMSG, many follicles were luteinized without ovulation, and the signal of LH receptor mRNA disappeared completely in the theca-interstitial cells as well as the luteinized cells, but remained in the granulosa cells of unaffected mature follicles. The complete suppression of the theca-interstitial LH receptor expression by GnRH agonist was also observed in HPX rats that received no other treatment. On the other hand, the coadministration of a GnRH antagonist with PMSG resulted in the hyperstimulation of follicular growth, accompanied by very strong expression of LH receptor mRNA in the granulosa cells as well as the theca-interstitial cells. In addition, morphological changes in the ovarian interstitial cells were also induced by the administration of GnRH agonist in HPX rats: loose connective tissue decreased and the interstitial cell mass markedly increased. The increase of the interstitial cells became more prominent when rats were treated with GnRH agonist and testosterone simultaneously. These results suggest that GnRH may be an important factor for modulating the interstitial cell function and differentiation in the rat ovary.

## INTRODUCTION

Recent studies have revealed that gonadotropin-releasing hormone (GnRH) exhibits many direct effects on the rat ovary, suggesting its capacity as a local ovarian factor (Hsueh and Jones, 1981). For example, as stimulatory effects, GnRH was demonstrated to act on mature follicles to induce ovulation (Hsueh *et al*., 1988) and meiotic maturation of oocytes (Hillensjö and LeMaire, 1980; Banka and Erickson, 1985). On the other hand, as inhibitory effects, GnRH was shown to inhibit the action of follicle stimulating hormone (FSH), such as the induction of luteinizing hormone (LH) receptor and aromatase activity in the premature follicles (Hsueh *et al*., 1980). The direct induction of apoptosis by GnRH was also demonstrated in the granulosa cells of growing follicles (Billig *et al*., 1994). Furthermore, Birnbaumer *et al*. (1985) demonstrated that a GnRH antagonist can potentiate the FSH effects on follicular development, implying the existence of endogenous GnRH which acts as a follicular atretic factor. GnRH was also shown to have direct effects on luteal cells: inhibition of steroidogenesis, LH receptor expression and LH receptor-mediated functions (Clayton *et al*., 1979; Harwood *et al*., 1980).

Recently, GnRH receptor cDNAs have been cloned from some mammals (Sealfon and Millar, 1995), and a high level of expression of the receptor transcripts was confirmed in the ovaries of rats (Kaiser *et al*., 1992; Kakar *et al*., 1994) and humans (Kakar *et al*., 1992). By in situ hybridization, we and other groups demonstrated strong expression of the GnRH receptor mRNA in the granulosa cells of atretic follicles and mature follicles, and moderate expression in the corpora lutea in rats (Bauer-Dantoin and Jameson, 1995; Kogo *et al*., 1995, 1999a; Whitelaw *et al*., 1995), consistent with the previously

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reported direct effects of GnRH in the rat ovary. Another major cell population expressing GnRH receptor is the interstitial cells (Kogo *et al.*, 1999a). Interestingly, we also found that the GnRH receptor mRNA is first expressed in the interstitial cells during ovarian development (Kogo *et al.*, 1999a), the expression being independent of any gonadotropic stimulation (Kogo *et al.*, 1999b). While the direct effects of GnRH on follicles and corpora lutea have been intensively studied, there are only a few reports on the direct effects of GnRH on the interstitial cells. Magoffin *et al.* (1981) reported that GnRH can inhibit steroid production in the interstitial cells, although the mechanism of the effect has not been clarified yet.

In the present study, we performed histological analysis of the direct effects of a GnRH agonist on the LH receptor mRNA expression in the ovaries of hypophysectomized (HPX) rats by in situ hybridization, and demonstrated that the agonist could completely suppress the theca-interstitial expression of the LH receptor mRNA, and cause morphological changes in the interstitial cells.

## MATERIALS AND METHODS

### Animals and hormone treatments

Animals were maintained under controlled conditions of light (12 hr of light, 12 hr of darkness; lights on at 6:00) and temperature (25±0.5°C) with free access to food pellets (CE-7; Japan Clea, Japan) and tap water (for hypophysectomized rats, sucrose was added at 5%). All experiments conformed to the regulations described in the NIH Guide to the Care and Use of Laboratory Animals. Twenty immature female Sprague-Dawley rats were anesthetized with pentobarbital (35–40mg/kg body weight) and hypophysectomized (HPX) at 22 days of age by the external auditory canal method (Koyama, 1962). Ten HPX rats were given 0.5mg diethylstilbestrol (DES, Sigma, USA) in 0.1ml of sesame oil every 12 hr between 22 and 25 days of age, and the remaining 10 HPX rats received no DES. Eight of 10 DES-treated rats were administered pregnant mare serum gonadotropin (PMSG, Teikokuzouki, Japan; 50U/0.1ml saline/injection) once at 48 hr after hypophysectomy, and three of them were subsequently treated with the GnRH agonist des-Gly$^{10}$, [D-Phe$^{6}$]-LHRH ethylamide (Sigma; 10 μg/0.1ml saline/injection) or the GnRH antagonist [Ac-3,4-dehydro-Pro$^{1}$, D-p-F-Phe$^{2}$, D-Trp$^{3,6}$]-LHRH (Sigma; 10 μg/0.1ml saline/injection) four times every 12 hr from 48 hr after hypophysectomy. The remaining two DES-primed HPX rats received no further treatment. Two or three rats without DES priming were treated with a GnRH agonist only, testosterone only (Sigma; 0.5mg/0.1ml sesame oil/injection) or combination of both four times every 12 hr from 48 hr after hypophysectomy. Two HPX rats were maintained without any other treatment. All rats were killed by decapitation at 26 days of age. The experimental schedules of these hormone treatments are illustrated in Fig. 1.

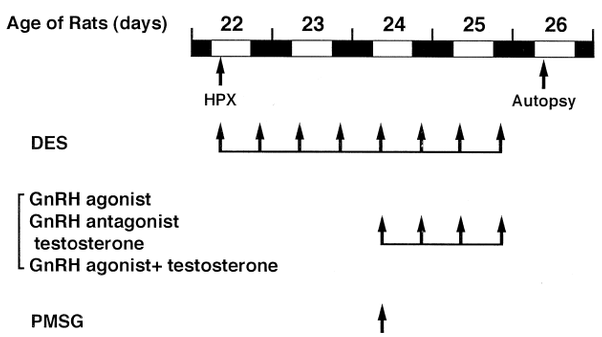


**Fig. 1.** Summary of hormone treatment schedule. Black and white bars indicate that the room was dark or light, respectively. Arrows indicate the timing of hormone administration (at 10:00 and 22:00). Twenty HPX rats were divided into DES-primed or HPX-only groups, 10 rats each. Eight DES-treated rats were treated with PMSG once at 48 hours after hypophysectomy, and three of them were subsequently treated with a GnRH agonist or a GnRH antagonist four times every 12 hr from 48 hr after hypophysectomy. Two or three HPX-only rats each were also treated with a GnRH agonist only, testosterone only, or combination of both four times every 12 hr from 48 hr after hypophysectomy.

### Preparation of riboprobes

Rat LH receptor cDNA fragment (codon 27 to 284; 774 base pairs) produced by Iizuka *et al.* (1996) was subcloned into pAM 18/19 (Amersham, UK). RNA probes labeled with $^{35}$S-UTP were synthesized by using bacteriophage SP-6 RNA polymerase (Paired Promoter SP-6 system, Amersham). The products were treated with RNase-free DNase (Promega, USA) to remove template DNA, then hydrolyzed to an average size of 150 bases and used for in situ hybridization.

### In situ hybridization

Immediately after autopsy, ovaries were fixed in 4% paraformaldehyde (electron-microscopic grade; Nakarai, Japan) in $Ca^{2+}$-, $Mg^{2+}$-free Dulbecco's phosphate-buffered saline (DPBS$^{-}$) at 4°C for 24 hr, then dehydrated through an ethanol series, cleared in xylene and embedded in Paraplast Plus (Sherwood, USA). Specimens were cut at 5-μm thickness and mounted on gelatin-coated glass slides. These sections were stored at 4°C and used for in situ hybridization within a week.

Sections were rehydrated through an ethanol series and DPBS$^{-}$, and pretreated before hybridization as previously described (Kudo *et al.*, 1994; Kogo *et al.*, 1995). Briefly, the sections were treated successively with 0.3% Triton-X 100-DPBS$^{-}$ (5 min), 0.2 M HCl (20 min), and 2 μg/ml proteinase K (37°C, 10 min). After postfixation with 4% paraformaldehyde-DPBS$^{-}$, the sections were immersed in 0.2% glycine-DPBS$^{-}$ (30 min, twice). After the pretreatment, the sections were dehydrated through an ethanol series, dried for 1 hr and incubated at 50°C for 16 hr with 50–100 μl/slide of probe-containing hybridization solution, of which the specific activity was $1.5\times10^{4}$ cpm/μl. After hybridization, slides were briefly rinsed with 50% formamide and 2× SSC, at 42°C, and incubated with 10 μg/ml RNase A (Sigma, USA) in RNase buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.5 M NaCl), at 37°C for 30 min. Then sections were washed twice in 2×SSC at 42°C for 30 min, then twice in 0.5×SSC at 42°C for 30 min, and dehydrated successively with 30%, 50%, 70% and 90% ethanol containing 0.3 M ammonium acetate, and 100% ethanol. After air drying, slides were dipped in autoradiographic emulsion (NR-M2, Konika, Japan) and exposed at 4°C for 10 to 14 days. After developing, Mayer's hematoxylin was used for counterstaining.

Hybridization with sense-strand probe was carried out in all of the ovaries as a negative control. None of the ovaries showed any significant signal when sense-strand probe was used. Serial sections not used for in situ hybridization were stained with hematoxylin and eosin for the histological determination of the ovarian components.

### Statistical analysis of ovarian weight

The effects of the administration of GnRH agonist or antagonist on the ovarian weight of DES- and PMSG-primed HPX rats were analyzed. The ovarian weights were expressed as the mean±SEM. The statistical differences of ovarian weights between control and treated rats were evaluated by Student's t-test.

## RESULTS

The treatment of DES-primed HPX rats with PMSG caused many follicles to grow and become Graafian follicles, in which the LH receptor mRNA was expressed in the granulosa cells (Fig. 2a, b, thick arrows). LH receptor mRNA was also expressed in the theca (Fig. 2b, arrowheads) and interstitial cells (Fig. 2b, thin arrow) irrespective of gonadotropin stimulation. In the ovaries of rats treated with PMSG and GnRH agonist simultaneously, the growth of many follicles was suppressed, and they were transformed to corpora lutea (CL)-like structures containing oocytes (Fig. 2c, asterisks) probably as a consequence of atresia. In these ovaries, the signal of LH receptor mRNA was intense only in the granulosa cells of the remaining mature follicles (Fig. 2d, arrows), and completely absent from the theca-interstitial cells (Fig. 2d, arrowhead and thin arrow) as well as the CL-like structures (Fig. 2d, asterisks). On the other hand, concomitant treatment with PMSG and the GnRH antagonist resulted in the hyperstimulation of follicular growth and formation of large antra in many follicles (Fig. 2e, asterisks). In these ovaries, LH receptor mRNA was strongly expressed in the granulosa cells of mature follicles (Fig. 2f, thick arrows), indicating that the antagonist treatment potentiated the PMSG-induced LH receptor expression. The theca (Fig. 2f, arrowheads) and interstitial cells (Fig. 2f, thin arrows) also strongly expressed the receptor mRNA. The ovarian weight of DES- and PMSG-treated HPX rats was significantly ($P<0.01$ by Student's t-test) decreased by the treatment with GnRH agonist (15±1 mg, n=6), and increased by the antagonist (72±3 mg, n=6) compared to the control (36±2 mg, n=4), confirming the results of the previous report by Birnbaumer *et al*. (1985).

The inhibitory effect of a GnRH agonist on the theca-interstitial LH receptor mRNA was also examined in HPX rats without DES and PMSG treatment. LH receptor mRNA was expressed in the theca cells (Fig. 3b, arrowhead), interstitial

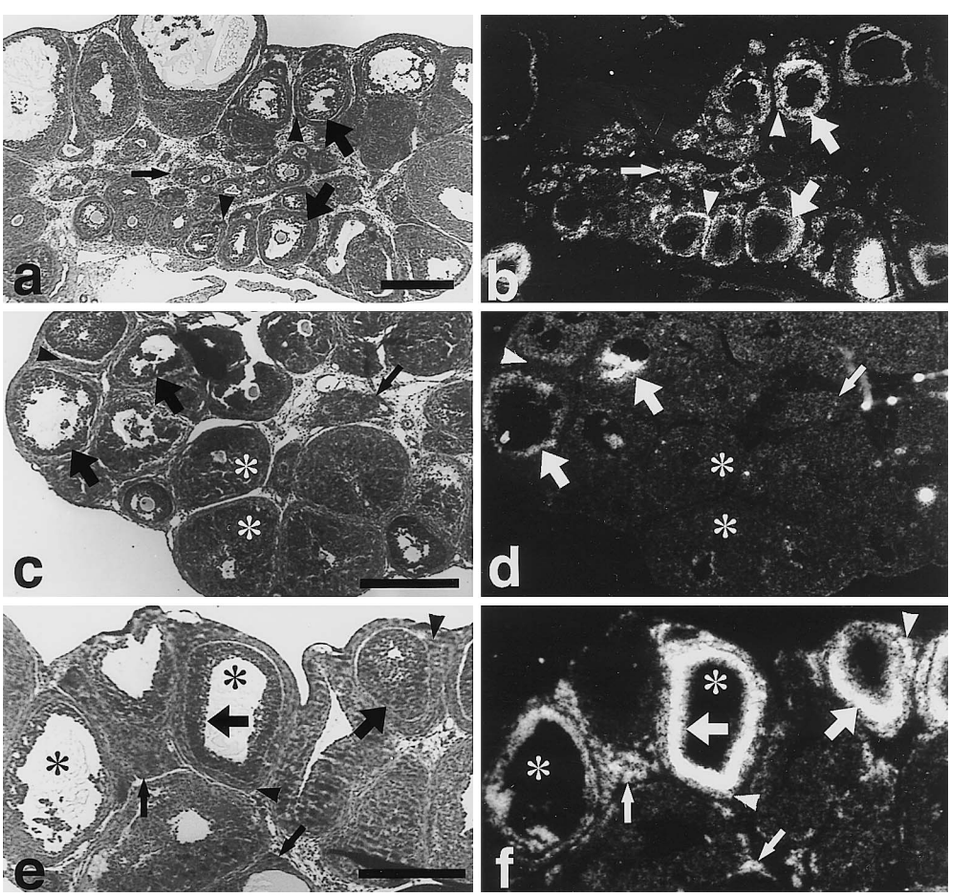


**Fig. 2.** Expression of LH receptor mRNA in ovaries of HPX immature rats treated with DES and PMSG in combination with a GnRH agonist or antagonist. Bright-field photographs of hematoxylin and eosin staining (**a**, **c**, **e**) and dark-field photographs of LH receptor mRNA signals (**b**, **d**, **f**). In the ovaries of rats treated with DES and PMSG (**a**, **b**), LH receptor mRNA was expressed in the granulosa cells (thick arrows) as well as in the theca (arrowheads) and interstitial cells (thin arrow). Concomitant treatment with a GnRH agonist (**c**, **d**) resulted in the complete suppression of the LH receptor expression in the theca (arrowhead) and interstitial cells (thin arrow) as well as the CL-like structures (asterisks), while the expression in the granulosa cells of the remaining mature follicles was not affected (thick arrows). Treatment with a GnRH antagonist (**e**, **f**) led to very strong expression of LH receptor mRNA in the granulosa cells (thick arrows) as well as the theca (arrowheads) and interstitial cells (thin arrows). Bars indicate 500 μm.

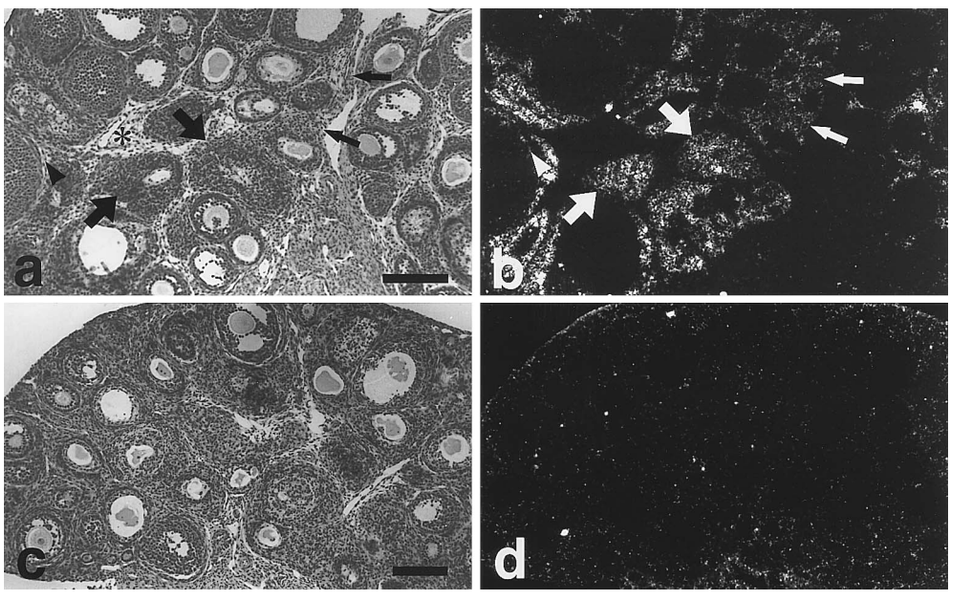


**Fig. 3.** Complete suppression of the LH receptor mRNA expression by treatment with a GnRH agonist in HPX-only rats. Bright-field photographs of hematoxylin and eosin staining (**a**, **c**) and dark-field photographs of LH receptor mRNA signals (**b**, **d**). In the ovaries of HPX rats (**a**, **b**), LH receptor mRNA was expressed in theca cells (arrowhead), interstitial cells (thin arrows) and interstitialized atretic follicles (thick arrows). The administration of GnRH agonist resulted in complete suppression of the LH receptor mRNA expression in the theca cells as well as the interstitial cells (**c**, **d**). Note the relatively greater interstitial cell mass in the GnRH agonist-treated ovary (**c**) compared to the ovary in the HPX-only rat, in which loose connective tissue is noticeable (**a**, asterisk). Bars indicate 150 μm.

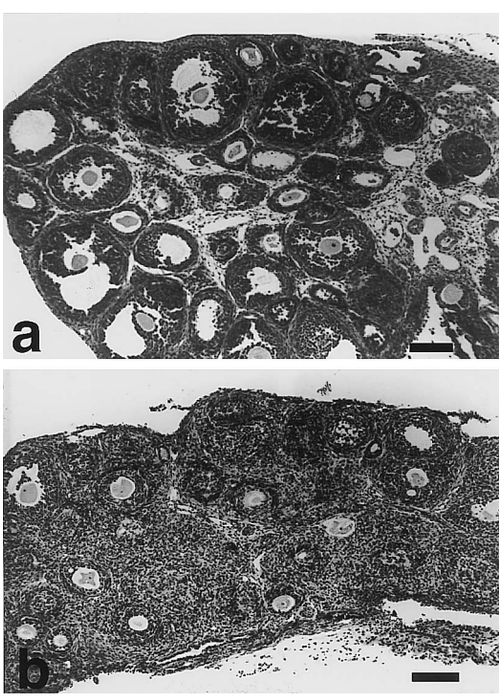


**Fig. 4.** Marked increase of the interstitial cell mass in the ovaries of HPX rats by simultaneous treatment with a GnRH agonist and testosterone. Bright-field photographs of hematoxylin and eosin staining. In the ovaries of rats treated with testosterone only, loose connective tissue was abundant between follicles (**a**). On the other hand, in the ovaries of rats treated simultaneously with GnRH agonist and testosterone, there was little loose connective tissue, and the interstitial cell mass was markedly increased, and almost completely filled the spaces between follicles (**b**). Bars indicate 150 μm.

cells (Fig. 3b, thin arrows) and interstitializing atretic follicles (Fig. 3b, thick arrows), but not in the granulosa cells. The administration of GnRH agonist resulted in complete suppression of the LH receptor mRNA expression in the theca-interstitial cells (Fig. 3d). In addition, histological changes of the interstitial tissue were also caused by the treatment with GnRH agonist. The ovaries of HPX rats contained relatively abundant loose connective tissue between follicles (Fig. 3a, asterisk), whereas the ovaries of HPX rats treated with GnRH agonist possessed considerably increased interstitial cell mass and little loose connective tissue between follicles (Fig. 3c). The increase of the interstitial cell mass was even more marked in ovaries of rats treated with both GnRH agonist and testosterone (Fig. 4). In the ovaries of HPX rats treated with testosterone alone (Fig. 4a), the interfollicular tissue was scanty and loose connective tissue was noticeable. In the ovaries of rats concomitantly treated with GnRH agonist and testosterone (Fig. 4b), the space between follicles was almost completely filled with a prominent interstitial cell mass in which LH receptor mRNA was not expressed (data not shown).

## DISCUSSION

A number of studies have demonstrated that GnRH inhibits LH receptor expression in FSH-stimulated growing follicles (Hsueh *et al*., 1980), mature follicles (Piquette *et al*., 1991), and corpora lutea (Harwood *et al*., 1980), suggesting that GnRH is a common suppresser of LH receptor expression in many ovarian cell populations. In the present study, we performed a histological examination of the effects of GnRH

agonist on the expression of ovarian LH receptor mRNA by in situ hybridization. The concomitant treatment of DES-stimulated HPX rats with PMSG and GnRH agonist caused many follicles to transform to CL-like structures showing no LH receptor expression. Although the CL-like structure is also formed as a consequence of follicular atresia, it is apparently different from the interstitial cells derived from theca cells of atretic follicles. While the interstitial cell mass is amorphous and in contact with loose connective tissues without boundaries, the CL-like structure is a spherical and relatively large cell mass having distinct boundaries. As the structure is indistinguishable from normal CL except for the remnant of oocytes, the cells in this structure appear to originate mainly from the granulosa cells. In contrast to the complete disappearance of the signal in the CL-like structures, the expression of LH receptor mRNA was still strong in the granulosa cells of the remaining mature follicles. These results suggest that the suppression of the FSH-induced LH receptor expression by a GnRH agonist (Hsueh *et al*., 1980) would be accompanied by cellular differentiation of granulosa to luteal cells. The present finding that a GnRH antagonist could potentiate FSH-induced LH receptor mRNA expression in the granulosa cells as well as follicular development supports the idea of the existence of an endogenous GnRH molecule in the rat ovary suggested previously by Birnbaumer *et al*. (1985). Recently, expression of the GnRH gene was reported in the rat ovary (Oikawa *et al*., 1990; Clayton *et al*., 1992; Goubau *et al*., 1992). However, whether these transcripts encode a protein capable of activating the GnRH receptor remains to be determined, and further studies on the identification of the ovarian GnRH molecule and its localization are needed.

Interestingly, we found that the administration of a GnRH agonist resulted in the complete suppression of the LH receptor mRNA expression in theca-interstitial cells. Magoffin *et al*. (1981) reported that GnRH can inhibit steroid production in the interstitial cells. Our results indicate that the suppression of androgen synthesis by GnRH occurs, at least in part, as a result of the suppression of LH receptor expression in these cells. The suppression of the LH receptor expression in theca cells as an direct effect of the GnRH was a surprise, because the GnRH receptor was not apparently expressed in the theca cells of most follicles (Kogo *et al*., 1999a). If the theca cells were not a direct target for GnRH, some paracrine factor(s) induced by GnRH in its target cells may be involved in the downregulation of thecal LH receptor expression. Although many cell populations expressed GnRH receptor mRNA in the rat ovary, one likely candidate for the source of this hypothetical paracrine factor is the interstitial cells, since the LH receptor downregulation was also observed in the ovaries of HPX rats, in which the GnRH receptor mRNA is exclusively expressed in the interstitial cells (Kogo *et al*., 1999b). It will be of interest to determine the biological significance of the GnRH action on the interstitial cells, since the receptor for GnRH is first expressed in the interstitial cells during neonatal ovarian development (Kogo *et al*., 1999a).

As theca-interstitial cells are responsible for the synthesis of the androgenic precursors required for estrogen production by the granulosa cells (Erickson *et al*., 1985), the acquisition of steroidogenic ability by theca-interstitial cells is a key step during follicular growth and maturation. There is a body of evidence demonstrating that LH is the principal hormone regulating the differentiation and steroidogenesis of theca cells (Erickson *et al*., 1985). Accordingly, the regulation of the thecal LH receptor expression is very important for the proper regulation of follicular development. Many growth factors have been demonstrated to modulate the LH receptor expression in theca-interstitial cells: insulin-like growth factor-I (Cara *et al*., 1990; Magoffin and Weitsman, 1994) and theca-cell differentiation factor (Magarelli *et al*., 1996) were demonstrated to enhance the LH receptor expression and androgen production in theca-interstitial cells, while hepatocyte growth factor (Zachow *et al*., 1997) was shown to suppress those functions. Our data indicate that GnRH can inhibit theca-interstitial function by downregulating the LH receptor expression and consequently suppressing androgen production directly or via some intraovarian paracrine factor(s).

In addition, an increase of the interstitial cell mass was observed in the ovaries treated with GnRH agonist, especially in rats treated with testosterone simultaneously. The mechanism of the GnRH effect on the interstitial mass increase is still unknown. In general, the interstitial cells in the ovary of rodents have been classified as primary and secondary, depending upon their source and the sequence of their appearance (reviewed in Guraya, 1978). The primary, which appear only during the neonatal period, originate from fibroblast-like cells, while the secondary, which appear and accumulate in mature ovaries, originate from the theca cells of atretic follicles. The present finding that GnRH treatment resulted in the loss of loose connective tissue and the increase of the interstitial cells in immature ovaries of HPX rats may indicate a functional role of GnRH in the differentiation of the primary interstitial cells in the developing ovaries, although further studies are needed to prove this. Moreover, considering the evidence of the induction of granulosa cell death by GnRH (Billig *et al*., 1994) and the GnRH receptor mRNA expression in the theca layer of advanced atretic follicles (Kogo *et al*., 1999a), it seems likely that GnRH is also implicated in the formation of secondary interstitial cells, which is accomplished by the removal of granulosa cells and the differentiaion of theca cells in atretic follicles. Taken together, the findings indicate that GnRH is a factor which is responsible for the formation of steroidogenic interstitial cells in the rat ovary.

In conclusion, the present results strongly suggest that GnRH may be an important factor involved in the regulation of follicular development and steroidogenic interstitial cell function by modulating the LH receptor mRNA expression and the differentiation of the interstitial cells in the rat ovary.

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