

Modulation of In Vitro DNA Synthesis in the Chicken Ovarian Granulosa Cell Follicular Hierarchy by Follicle-Stimulating Hormone and Luteinizing Hormone

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ABSTRACT Folliculogenesis in domestic hens appears to be controlled by numerous factors, particularly the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The involvement of LH in follicular steroidogenesis has been described in some detail; however, the specific role of FSH has remained elusive. In 3 experiments, the effects of ovine (o)- or chicken (c)-derived FSH (oFSH, cFSH) or LH (oLH, cLH) were evaluated on in vitro DNA synthesis [³H-thymidine (³H-TdR) incorporation], indicative of cellular proliferation, of granulosa cells from F1, F3, or F5-6 preovulatory follicles. In experiment 1, oFSH or cFSH stimulated ($P < 0.05$) and oLH or cLH decreased DNA synthesis by F1 granulosa cells. In experiment 2, oFSH resulted in concentration-related changes in DNA synthesis by F5-6 granulosa cells; however, no significant

changes were observed in F1 or F3 granulosa cells. No effect of oLH was observed on granulosa cell proliferation from any of the follicles. Similar to oFSH, cFSH resulted in concentration-related increases in DNA synthesis in granulosa cells from F5-6 follicles with smaller magnitude changes in proliferation of F1 or F3 granulosa cells. Granulosa cells from F5-6 or F3 follicles had small increases in DNA synthesis in response to cLH. These data support the proposed role for FSH in granulosa cell proliferation, possibly contributing to follicle growth, and suggest that in vitro ³H-TdR incorporation by granulosa cells may provide a sensitive and selective bioassay for chicken gonadotropin preparations. Furthermore, data suggest that proliferative responsiveness of granulosa cells to FSH or LH may differ depending on position of follicles in the preovulatory hierarchy.

(Key words: chicken, granulosa, proliferation, follicle-stimulating hormone, luteinizing hormone)

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INTRODUCTION

The ovary of the domestic hen consists of a hierarchy of preovulatory follicles and groups of prehierarchical follicles of various sizes and maturities. The most obvious group of larger follicles forms the preovulatory hierarchy and is composed of 5 to 7 yolk-filled follicles in the processes of rapid yolk accumulation and cellular maturation in preparation for ovulation. Although the steroidogenic profile of the follicles in the preovulatory hierarchy has been partially characterized and reviewed in detail by others (Cunningham, 1987; Etches, 1990; Johnson, 1990), the factors involved in the maintenance of the follicular hierarchy and growth of follicles remains unclear. The involvement of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), in some facet of the development

or maintenance of the follicular hierarchy is undeniable. Although LH has been documented to play a substantial role in the steroidogenic capacity of the most mature follicles in the hierarchy, the contributions of FSH, either to steroidogenesis or follicular growth, are less understood.

Follicle-stimulating hormone is postulated to play a role in follicular growth and maintenance of the follicular hierarchy. In vivo experimentation involving administration of FSH to hens has resulted in findings supportive of this role (Johnson, 1986; Bahr and Palmer, 1989; Palmer and Bahr, 1992). Investigators have reported induction of follicles into the preovulatory hierarchy, increased deposition of yolk, a decrease in atretic follicles, and the rapid growth onset of small follicles in response to administration of FSH to laying hens (Johnson, 1986; Bahr and Palmer, 1989; Palmer and Bahr, 1992). These

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Abbreviation Key: cFSH = chicken follicle-stimulating hormone; cLH = chicken luteinizing hormone; FSH = follicle-stimulating hormone; ³H-TdR = ³H-thymidine; LH = luteinizing hormone; oFSH = ovine follicle-stimulating hormone; oLH = ovine luteinizing hormone.

findings suggest a role for FSH in follicle growth, particularly in the small, yellow, prehierarchal follicles and immature follicles in the preovulatory hierarchy.

In the intense growth stages of folliculogenesis, the follicle not only accumulates enormous amounts of yolk, but the follicular cells, particularly the granulosa cells, are documented to change in quantity, distribution, structure, and function (Bellairs, 1965; Gilbert et al., 1980; Bahr et al., 1983; Cunningham, 1987; Marrone and Crissman, 1988; Marrone et al., 1990). Granulosa cells change from a pseudostratified organization of columnar cells to a single organized layer of cuboidal cells (Bellairs, 1965; Marrone et al., 1990) and increase an estimated 5-fold in number (Gilbert et al., 1980). In progression from the immature follicles in the preovulatory hierarchy (F5, F6) to the mature follicle in the F1 position, granulosa cells appear to differentiate from a highly proliferating state to a less proliferative nature (Marrone et al., 1990; Tischkau et al., 1997). Interestingly, this decrease in proliferation of granulosa cells as the follicle matures is correlated with a decrease in the number of receptors for FSH localized on granulosa cells (Ritzhaupt and Bahr, 1987). Although these 2 independent findings have not been directly associated with one another, it could be suggestive for a role of FSH in granulosa cell proliferation. In a 72-h cell culture evaluating the effects of human FSH and human LH on actual granulosa cell counts in vitro, Yoshimura and Tamura (1988) observed an increase in numbers of granulosa cells collected from the F1 and F2 follicles in combined culture. Other researchers have more recently reported increased granulosa cell proliferation in the presence of ovine FSH (Davis et al., 2001) or human pituitary FSH (Schmierer et al., 2003) in in vitro culture systems.

Collectively, these data are suggestive of a functional role of FSH in regulation of the follicular hierarchy, specifically with influence on follicular growth and a possible contribution by granulosa cell proliferation. The ability to closely evaluate the effects of FSH on follicular growth or granulosa cell proliferation in the domestic hen has been hindered by the scarcity of purified chicken preparations of FSH. The purpose of the present experiments was to evaluate the effect of ovine or chicken-derived FSH or LH on in vitro DNA synthesis of granulosa cells from follicles of different maturity in the hen ovarian hierarchy (F1, F3, and F5–6).

MATERIALS AND METHODS

Birds

Single-comb White Leghorn hens² (45 wk of age) maintained on a photoperiod of 18L:6D were used for

all experiments. Hens were housed individually in layer batteries and provided a commercial layer ration that met or exceeded NRC (1994) guidelines, and water was provided ad libitum. The approximate time of ovulation for hens was determined by visually monitoring oviposition for several days prior to the experiments.

Isolation and Culture of Granulosa Cells

The basic cell culture medium for all granulosa cell cultures consisted of Eagle's minimum essential medium³ modified with Earles salts, L-glutamine, and 25 mM HEPES. The complete cell culture medium contained 2.2 g/L sodium bicarbonate,³ 1 g/L BSA,³ 100 units/mL penicillin, and 100 µg/mL streptomycin.⁴ The final pH of the medium was adjusted to 7.4 with sodium hydroxide.

On the day of each experiment, 12 hens with a palpable hard shell egg in utero were killed by cervical dislocation approximately 6 h prior to ovulation. The F1, F3, F5, and F6 follicles were collected from each hen and immediately placed in complete cell culture medium. The granulosa cells from the follicles of different hierarchical maturity were isolated and cultured separately with the exception of granulosa cells from F5 and F6 follicles, which were combined for isolation procedures and culture. The granulosa cell layer was isolated from follicles as previously described (Zakar and Hertelendy, 1980). Granulosa cells were dispersed by trypsin³ digestion (1 mg/mL) at 37°C for 20 min; trypsin inhibitor³ (1 mg/mL) was then added for an additional 5 min at 37°C. The resulting cell suspension was filtered through nylon mesh⁵ to remove large clumps of cells and debris. The filtered cell suspension was pelleted by centrifugation at 120 × g for 5 min. Cells were washed by resuspension in fresh cell culture medium (4°C) and repelleted by centrifugation. A total of 3 washes were performed prior to assessment of cell viability and number. Immediately following the final washing step, a 100 µL aliquot of the cell suspension was subjected to the live-dead staining procedure of Freshney (1983), and the final concentration of cells in suspension was adjusted to 4 × 10⁶ viable cells/mL for the DNA synthesis assay with fresh complete culture medium.

Analysis of DNA Synthesis

The effects of chicken FSH⁶ (cFSH, USDA-cFSH-I-1), chicken LH⁶ (cLH, USDA-cLH-I-3), ovine FSH⁷ (oFSH; NIDDK-oFSH-19-SIAFP), or ovine LH⁷ (oLH, NIADDK-oLH-25) on DNA synthesis by granulosa cells from the F1, F3, or F5–6 follicles were assessed in 96-well polystyrene tissue culture plates.⁸ Granulosa cells, gonadotropin, and ³H-thymidine⁹ (³H-TdR; specific activity 6.7 Ci/mmol and 1 mCi/mL) were added to treatment wells in 50-µL volumes with the volume of complete cell culture medium adjusted to obtain a total of 250 µL in all wells (6 replicate wells/treatment group). In all experiments, cells were exposed to gonadotropin (FSH or LH)

²Feather Crest, Bryan, TX.

³Sigma Chemical Co., St. Louis, MO.

⁴Gibco BRL, Life Technologies Inc., Grand Island, NY.

⁵Nytex, Tetco Inc., Elmsford, NY.

⁶USDA Animal Hormone Program, Beltsville, MD.

⁷National Hormone and Pituitary Program, NIDDK, Rockville, MD.

⁸Becton Dickinson and Co., Lincoln Park, NJ.

⁹ICN Pharmaceuticals Inc., Costa Mesa, CA.

for 16 h prior to addition of ^3H -TdR. Following these initial incubation periods, cells were pulsed with ^3H -TdR (1.0 $\mu\text{Ci}/\text{mL}$) to label cells actively synthesizing DNA and were incubated for an additional 8 h, for a total culture period of 24 h at 41°C in an environment containing 5% CO_2 and saturated humidity. At termination of the culture period, cells were harvested onto filter mats¹⁰ using a semi-automatic 12-well cell harvester¹⁰ (Model 7000DGA). Following the first harvest, a 50- μL volume of a trypsin (0.8 mg/mL) and EDTA³ (2 mg/mL) solution was added to each well, and plates were incubated for 10 min at 41°C and 5% CO_2 to allow for dispersion of adherent cells from wells. Redispersed cells were then harvested onto filter mats. Filter mats were dried, the 2 filter discs (from the first and second harvest) corresponding to each well were placed into 7-mL polypropylene scintillation vials,¹¹ and 5 mL of an aqueous-based scintillation counting cocktail¹² (Ultima Gold) was added. Radioactivity was counted for 2 min in a standard liquid scintillation counter¹² (Minimaxi Tricarb model B4430). Counts were automatically quench corrected, and data are reported as disintegrations per min.

Experimental Protocol

For all 3 experiments, groups of cells from the F1, F3, or F5–6 follicles not exposed to FSH or LH were included to represent basal levels of DNA synthesis in these pools of isolated granulosa cells. Three replications were conducted for each experiment. In experiment 1, granulosa cells from the F1 follicle were incubated alone or in the presence of oFSH, oLH, cFSH, or cLH at concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50, or 100 ng/mL. The effects of oLH or oFSH on DNA synthesis by granulosa cells from the F1, F3, or F5–6 follicles were evaluated in experiment 2 with granulosa cells exposed to oFSH or oLH at concentrations of 1, 5, 10, 50, or 100 ng/mL. In experiment 3, granulosa cells from the F1, F3, or F5–6 follicles were exposed to cFSH or cLH at the following concentrations: 1, 5, 10, 50, or 100 ng/mL.

Statistical Analysis

Means of individual treatment group ^3H -TdR incorporation data from 3 replicate trials of each experiment were analyzed using the general linear model procedure for analysis of variance (SAS Institute, 1988). Statistically different ($P < 0.05$) means were further separated using Duncan's multiple range test (SAS Institute, 1988). Significance was considered at $P < 0.05$.

RESULTS

In experiment 1, the relative efficacy and potency of the selected mammalian (ovine) or avian (chicken) FSH

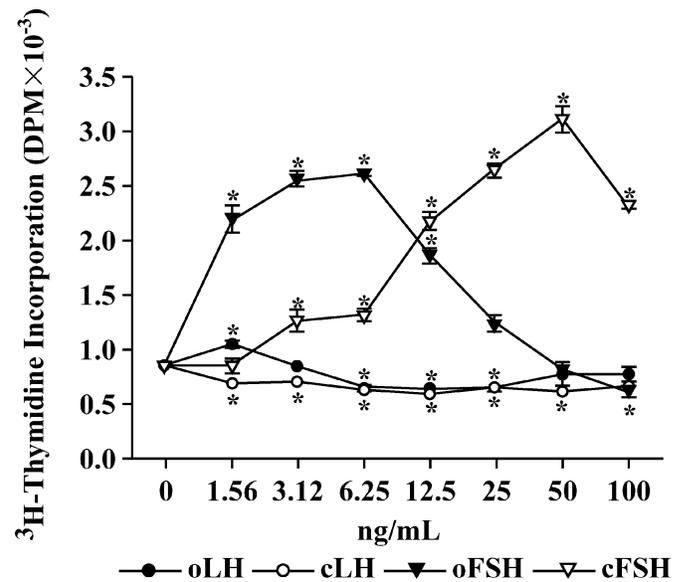


FIGURE 1. Effect of ovine- (o) or chicken- (c) derived follicle-stimulating hormone (FSH) or luteinizing hormone (LH) on DNA synthesis by granulosa cells from F1 chicken ovarian follicles. Figure 1 represents treatments of granulosa cells cultured in medium alone (basal) or in the presence of the gonadotropins. Each point represents mean disintegration per minute (DPM) \pm SE for each treatment group from 3 replicate trials. *Indicates significant difference ($P < 0.05$) from basal within gonadotropin treatment.

or LH on in vitro DNA synthesis by granulosa cells from the F1 follicle were examined (Figure 1). Whether from ovine or chicken origin, FSH increased in vitro DNA synthesis by F1 granulosa cells in a concentration-related manner as compared with basal levels of synthesis. Although oFSH increased DNA synthesis at concentrations from 1.56 to 12.5 ng/mL, the most marked increases (approximately 3- to 4-fold above basal) were observed with cFSH at concentrations of 25 or 50 ng/mL, with cFSH concentrations from 3.12 to 100 ng/mL causing lesser increases. Whereas increases in DNA synthesis occurred at lower concentrations of oFSH, the largest changes in DNA synthesis as compared with basal levels were observed in response to cFSH. The changes in granulosa cell proliferation resulting from oLH or cLH were less consistent. The only increase in DNA synthesis in granulosa cells from the F1 follicle in the presence of LH was observed with 1.56 ng/mL oLH, whereas oLH from 6.25 to 25 ng/mL resulted in decreases in DNA synthesis, as did all concentrations of cLH.

In experiment 2, comparison of the effects of oFSH or oLH on granulosa cell DNA synthesis using pools of F1, F3 or F5-6 granulosa cells revealed differential effects of oFSH or oLH (Figures 2A and 2B). Granulosa cells derived from F1 follicles did not significantly increase their DNA synthesis in response to oFSH. Exposure of F3 granulosa cells to oFSH at the selected concentrations did not result in any observable changes in DNA synthesis (Figure 2A). In contrast to the lack of response observed with granulosa cells from F3 follicles, oFSH caused increases in DNA synthesis in granulosa cells

¹⁰Skatron Instruments Inc., Sterling, VA.

¹¹Kimble Glass, Vineland, NJ.

¹²Packard Instrument Co. Inc., Meridian, CT.

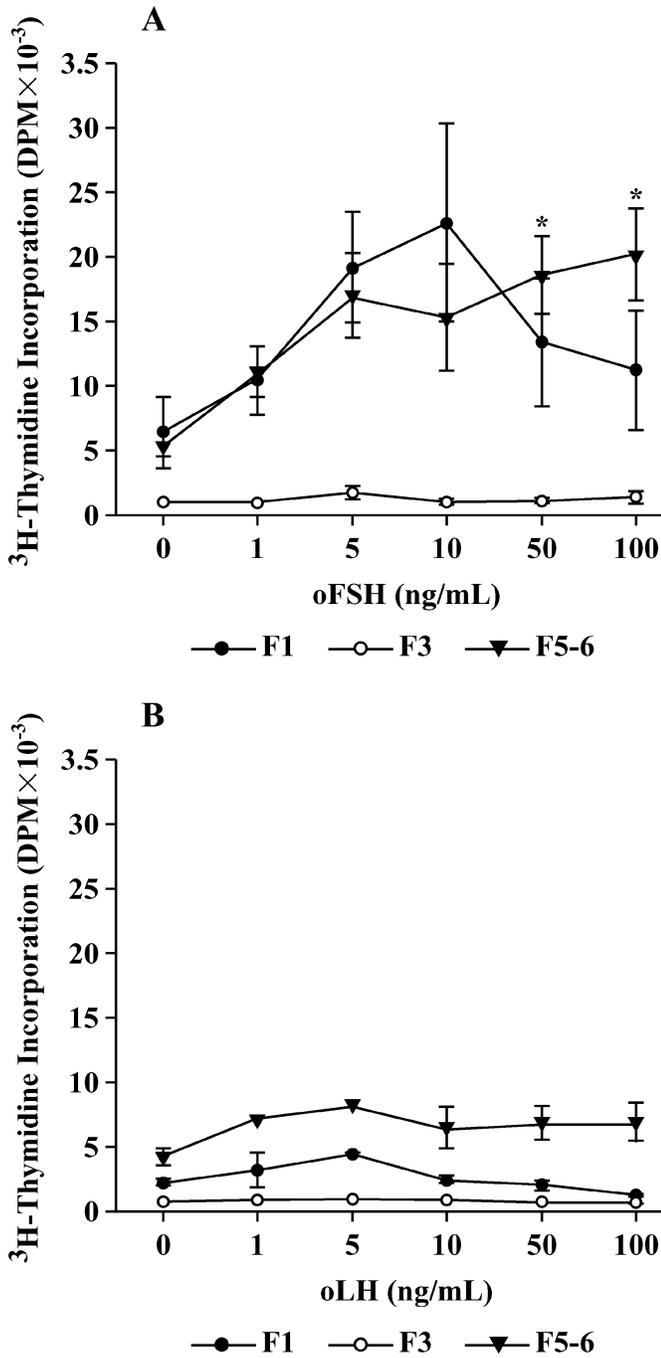


FIGURE 2. Effect of ovine-derived follicle-stimulating hormone (oFSH) or luteinizing hormone (oLH) on DNA synthesis by granulosa cells from the F1, F3, or F5-6 chicken ovarian follicles. Panels A and B represent pools of granulosa cells collected from F1, F3, or F5-6 ovarian follicles and cultured in the presence of oFSH or oLH, respectively, or incubated in medium alone (basal). Each point represents mean disintegrations per minute (DPM) ± SE for each treatment group from 3 trials. *Indicates significant difference ($P < 0.05$) from basal within follicular stage cell group.

from F5-6 follicles at 50 or 100 ng/mL (Figure 2A). No concentration of oLH evaluated in this experiment had effects on DNA synthesis of granulosa cells from F1, F3, or F5-6 follicles (Figure 2B).

Similar to the results indicating a stimulatory action of the mammalian-derived gonadotropins on DNA syn-

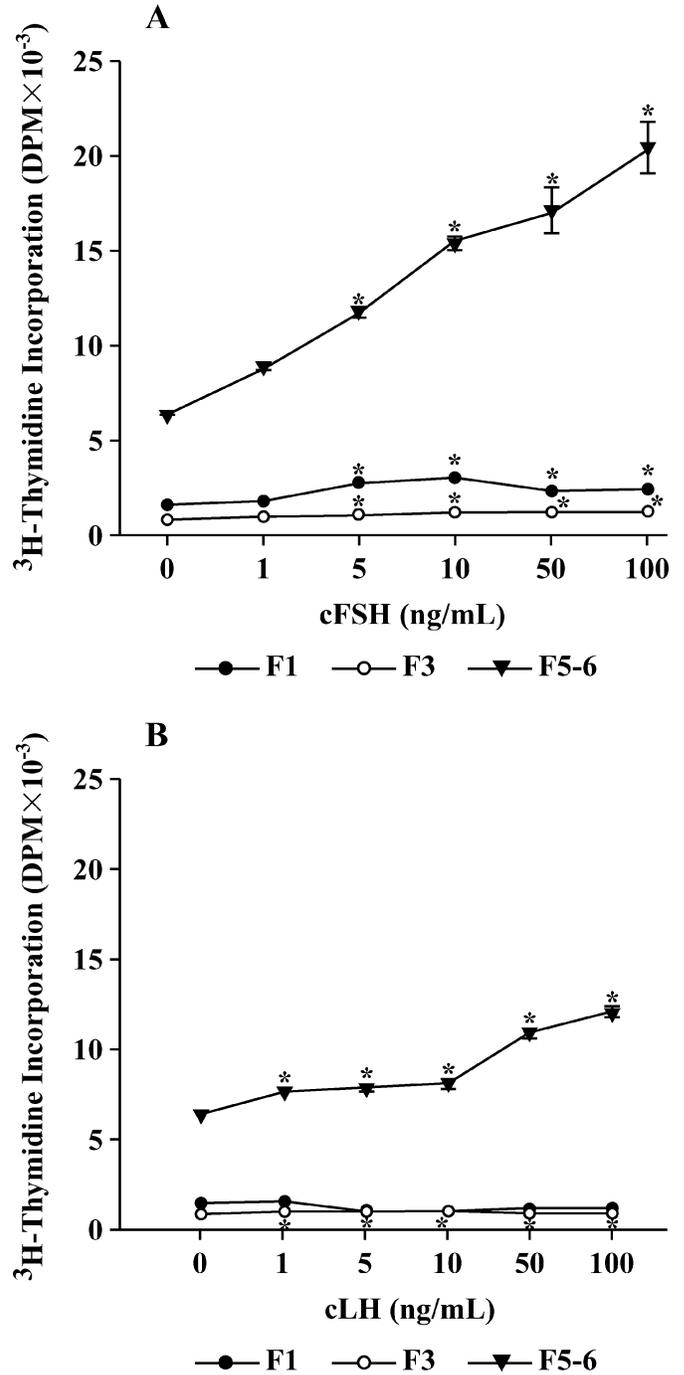


FIGURE 3. Effect of chicken-derived follicle-stimulating hormone (cFSH) or luteinizing hormone (cLH) on DNA synthesis by granulosa cells from the F1, F3, or F5-6 chicken ovarian follicles. Panels A and B represent pools of granulosa cells collected from the F1, F3, or F5-6 ovarian follicles and cultured in the presence of cFSH or cLH, respectively, or in medium alone (basal). Each point represents mean disintegrations per minute (DPM) ± SE for each treatment group from 3 replicate trials. *Indicates significant difference ($P < 0.05$) from basal within follicular stage cell group.

thesis of granulosa cells from F1 or F5-6 avian ovarian follicles, cFSH caused concentration-related significant increases in DNA synthesis by granulosa cells isolated from F1, F3, or F5-6 follicles in experiment 3 (Figure 3A). Increases in proliferation were observed at evaluated cFSH concentrations ranging from 5 to 100 ng/

mL. In response to *in vitro* exposure to cFSH, a 2-fold increase in DNA synthesis in F1 granulosa cells and more than a 3-fold increase in DNA synthesis by granulosa cells from the F5–6 follicles were observed. The proliferative response to cFSH in F3 granulosa cells was of lesser magnitude than that observed with F1 or F5–6 follicular-derived granulosa cells. Although changes in DNA synthesis were also observed with *in vitro* exposure of the granulosa cells to cLH (Figure 3B), the responses were of much smaller magnitude as compared with those shown with cFSH. The increases in DNA synthesis with cLH in experiment 3 were at concentrations from 1 ng to 100 ng/mL cLH with granulosa cells from F3 or F5–6 follicles. No changes were observed in F1 granulosa cell proliferation at any cLH concentration.

DISCUSSION

In avian species, follicular development refers not only to growth of follicular mass but also to the development and differentiation of the follicular cells in a varying hormonal environment. It is increasingly apparent that the control of the follicular hierarchy and folliculogenesis is a very complex physiological system. The mechanisms that regulate the follicular hierarchy including the recruitment of follicles, the maintenance of the follicular hierarchy, and the growth and development of follicular cells have not been identified. There has been an increasing amount of research in recent years in an attempt to determine the mechanism and factors involved in the proliferation and differentiation of granulosa cells that contribute to folliculogenesis. It has long been postulated that FSH, with weak steroidogenic properties in the domestic hen, may play a substantial role in the growth of avian ovarian follicles and maintenance of the follicular hierarchy possibly through granulosa cell proliferation and maturation. Although not yet directly associated, reports are indicative of a role of FSH in granulosa cell proliferation, with perhaps differential effects on granulosa cells from follicles of different follicular maturity.

The results from the present study indicate *in vitro* FSH- or LH-induced modulation of DNA synthesis in individual pools of granulosa cells collected from F1, F3, or F5–6 follicles in the avian ovarian follicular hierarchy. Additionally, the modulation of granulosa cell DNA synthesis by FSH or LH appears to be differential and dependent on the position or maturity of the follicle in the hierarchy. Several previous studies have described changes in the structural and proliferative state of the granulosa cells as the follicle undergoes the most intense phase of growth in the 7 d prior to its ovulation (Marrone et al., 1990; Tischkau et al., 1997). In general, from the present study it appears that granulosa cells are in different basal proliferative rates through the follicular hierarchy. In experiment 3, granulosa cells from F5–6 follicles had higher basal levels of proliferation than granulosa cells collected from F1 follicles, whereas in experiment 2 basal levels of the F1 or F5–6 isolated cells

were similar. In experiments 2 and 3 in this study, basal levels of DNA synthesis were markedly lower in granulosa cells from F3 follicles than from granulosa cells from the other follicles, F1 or F5–6, evaluated. Interestingly, granulosa cells from the F3 follicles, having low basal levels of DNA synthesis, were also the least responsive to treatment with either cFSH or oFSH under the conditions used in these experiments. It is interesting to note that this decreased basal and FSH-stimulated DNA synthesis in granulosa cells from the F3 follicle, as compared with granulosa cells from the F5–6 or F1 follicle, occurs in the follicular hierarchy when the follicles are differentiating from androgen biosynthesis to progesterone production (Bahr et al., 1983). The most obvious differential proliferation between granulosa cells taken from follicles of different maturity was observed in experiment 3 with the chicken-derived gonadotropins. Although, cFSH resulted in significant increases in DNA synthesis in F1, F3, and F5–6 granulosa cells, the most pronounced effects were on cells from the F5–6 follicles, which is supported by previous reports of increased expression of cFSH receptor mRNA (You et al., 1996) and increased rates of proliferation with granulosa cells of immature follicles as compared with those closer to ovulation in the preovulatory hierarchy (Marrone et al., 1990; Tischkau et al., 1997). Somewhat in contradiction to the previous studies are the results from experiments 1 and 2 evaluating oFSH. Granulosa cells from the F1 follicle in experiment 1 appeared to be more sensitive to lower concentrations of oFSH than of cFSH, and in experiment 2, F1 granulosa cells responded to lower concentrations of oFSH than did cells from F5–6 follicles, in contrast to the results with cFSH in experiment 3. These results, although still indicating differential proliferation of granulosa cells in response to *in vitro* stimulation by FSH, may indicate differences in the preparations of the ovine- and chicken-derived gonadotropins used in the present experiments. The difference in preparations could possibly allow for stimulation with lower concentrations of oFSH from cross-reaction with receptors in less pure preparations of the hormone. The effects of LH on DNA synthesis by granulosa cells were different at the selected stages of follicular maturity. Regardless of animal origin, LH had less consistent effects on DNA synthesis by granulosa cells from the F1, F3, or F5–6 follicles.

In addition to FSH- or LH-induced differential proliferation rates of granulosa cells from follicles of different maturity in the hierarchy, variability in responsiveness to the gonadotropins was observed between experiments for granulosa cells from the same maturation of follicle in the hierarchy. Granulosa cells isolated from preovulatory follicles in the hierarchy are collected from an environment consisting of a milieu of endocrine, paracrine, neural, and other physiological regulators. These factors alone could contribute to the observed differences in basal granulosa cell proliferation rates between experiments. Furthermore, with variation in basal proliferation rates, inconsistency in the degree of respon-

siveness to FSH or LH could result. The exact *in vivo* hormonal environment, particularly the influence of hormonal surges associated with ovulation, may influence the degree of *in vitro* responsiveness to LH or FSH and subsequently the large variation in mean thymidine incorporations, as observed in experiment 2.

The elucidation of the role that FSH plays in the follicular hierarchy has been partially hindered by the scarcity of pure chicken-derived preparations of this gonadotropin. This study may indicate that differences between mammalian and avian preparations of this hormone are minor, at least in terms of the pattern of response generated by *in vitro* exposure to FSH in this system. Furthermore, results suggest that concentrations of oFSH of less than 10 ng may be the most stimulatory to granulosa cell proliferation. This is important in that other research has typically involved the use of higher concentrations for *in vitro* granulosa cell cultures. With many of the previously used and currently existing preparations of avian FSH there has been a question as to the purity of the preparation. They have been continually plagued by contamination with LH or other factors that may influence experimental results. However, the more pure preparations of cFSH that are available, such as the preparation used in these experiments (0.2% \times PRC-AE1-s-1 LH by radioimmunoassay; Krishnan et al., 1992), provide excellent reference and comparative parameters for the actions of these hormones. These experiments indicate that FSH and LH are capable of *in vitro* modulation of DNA synthesis by granulosa cells from the F1 or F5-6 follicles, with little effects on granulosa cells from F3 follicles. From these experiments, it appears that the modulation of DNA synthesis by FSH or LH is dependent on the hierarchical position of the follicle, particularly in consideration of the effects of cFSH or cLH. Previous studies have shown that as follicles progress through the hierarchy towards the F1 position, the granulosa cells change from a highly proliferative state to a less proliferate state as the follicles near ovulation (Tischkau et al., 1997). In experiment 3, granulosa cells from the F5-6 follicles had higher basal proliferation rates and responded to cFSH with greater than 3-fold increases in DNA synthesis as compared with responses of less magnitude with F1 or F3 granulosa cells. These data are suggestive of a role of these gonadotropins in granulosa cell proliferation. In agreement with previously reported and hypothesized roles for LH or FSH in the follicular hierarchy development, from these studies it appears that FSH generally causes more stimulation of DNA synthesis than does LH. Collectively, these *in vitro* data are suggestive of a role of FSH and, to a lesser extent LH, in maintenance or modulation of granulosa cell proliferation in folliculogenesis. Furthermore, it appears from these *in vitro* studies that responsiveness in DNA synthesis of the granulosa cells to these gonadotropins differs as the follicle progresses through the hierarchy. Although the independent *in vitro* effects of FSH or LH on DNA synthesis by granulosa cells were evaluated in these experi-

ments, possible interactions of these gonadotropins were not presently evaluated. When considering the known differential effects of FSH or LH on steroidogenesis and follicular growth of the ovarian follicular hierarchy, these gonadotropins may in future studies be shown to have differential actions on granulosa cell DNA synthesis when both are present in this *in vitro* system. Further experiments are necessary to determine the role of these gonadotropins, both independently and in combination, in folliculogenesis, particularly with regard to follicle growth and avian granulosa cell proliferation and differentiation.

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