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Factors Affecting the Developmental Competence of Mouse Oocytes Grown In Vitro: Follicle-Stimulating Hormone and Insulin[1]

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ABSTRACT

This study was undertaken to test the hypothesis that FSH treatment of cultured oocyte-granulosa cell complexes promotes acquisition of competence to complete preimplantation embryo development. Oocyte-granulosa cell complexes were isolated from the preantral follicles of 12-day-old mice and cultured for 10 days in serum-free medium, supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml) and containing a highly potent preparation of FSH (0–5 ng/ml). Oocytes were matured and fertilized in vitro and embryos cultured to determine the frequency of development to the blastocyst stage. There was no effect of FSH on oocyte size, general morphology, or competence to resume meiosis. However, addition of FSH to medium containing insulin had a deleterious effect on the percentage of mature oocytes competent to develop to the blastocyst stage. Deletion of insulin from the medium for culture of oocyte-granulosa cell complexes prevented the deleterious effect of FSH, but FSH still did not promote acquisition of competence to complete preimplantation development. Culture of oocyte-granulosa cell complexes with FSH resulted in elevated expression of LH receptor (LHR) mRNA by granulosa cells and stimulated the production of functional LHRs, whether or not insulin was present. However, FSH-induced expression of LHR mRNA reached a maximum steady-state level by 4 days of culture in the presence of insulin, but this level was not reached until 10 days of culture without insulin. Granulosa cells encompassing growing mouse oocytes in vivo do not express LHR mRNA. Thus, expression of LHR mRNA by granulosa cells closely associated with growing oocytes in vitro indicates inappropriate or ambiguous development. In conclusion, conditions occurring during oocyte growth can have profound detrimental effects on oocyte developmental competence to complete preimplantation development, even when oocyte growth, general morphology, and competence to resume meiosis appear unaffected.

INTRODUCTION

Processes occurring during oocyte development establish the foundation for embryogenesis. For example, transcripts essential for early embryo development are produced and stored in dormant form throughout oocyte growth and are activated and translated during meiotic maturation and preimplantation development. Oocyte growth and development occur in an ovarian follicular environment characterized by complex cell-to-cell interactions mediated by gap junctional communication, as well as autocrine, paracrine, and endocrine signaling. These interactions drive continual changes in the organization and differentiation of follicular somatic cells. The dynamics of oocyte development are normally coordinated with follicular differentiation. This coordination, however, can be offset by accelerating follicular development with exogenous gonadotropins, or other factors driving follicular growth and differentiation. Precocious recruitment of small antral follicles into the pool of preovulatory follicles results in the ovulation of oocytes incapable of normal maturation, fertilization, or embryogenesis [1–3]. It is not clear how inappropriate follicular development and granulosa cell differentiation might affect the production of developmentally competent oocytes. One way to explore these relationships is through the use of systems that support oocyte development in vitro.

The system established in this laboratory to study oocyte growth and development in vitro consists of oocyte-granulosa cell complexes isolated from the preantral follicles of 12-day-old mice by collagenase digestion. Most, but not all, of the theca cells are removed, and the basal lamina encompassing the granulosa cells and oocytes is degraded. The oocytes at this stage are in mid-growth phase and incompetent to resume meiosis without further development. Between 200 and 300 oocyte-granulosa cell complexes are cultured attached to a collagen-impregnated membrane for 10 days, a period that spans the time of antrum formation and the acquisition of competence to resume meiosis and undergo fertilization and preimplantation development by oocytes in vivo. While initial studies were conducted using a medium supplemented with 5% fetal bovine serum (FBS) [4], it was found later that serum could be omitted if the serum protein fetuin was added to prevent precocious hardening of the zona pellucida during oocyte growth [5, 6]. Serum-free culture medium was supplemented with insulin, transferrin, and selenium (ITS) to promote more robust development of the complexes [5]. In this culture system, oocytes grow and become competent to undergo maturation, fertilization, and embryogenesis, but they are not equivalent in size or developmental competence to oocytes grown in vivo, particularly when the complexes are grown in serum-free media [4, 5, 7–9].

In the previous studies, the oocyte-granulosa cell complexes were cultured without gonadotrophic stimulation. Thus, the acquisition of competence to resume meiosis and undergo fertilization and embryogenesis in vitro does not require FSH or LH, though these gonadotropins, alone or together with other intrafollicular factors controlling growth and differentiation, might optimize the acquisition of developmental competence. In this study, we tested the hypothesis that FSH treatment of cultured oocyte-granulosa cell complexes promotes acquisition of competence to complete preimplantation embryo development. Oocyte-granulosa cell complexes were isolated from preantral follicles of 12-day-old mice and cultured in serum-free media for

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10 days with or without FSH, and effects on oocyte growth, maturation, and developmental competence were assessed.

MATERIALS AND METHODS

Reagents

FSH (ovine [o]FSH-20) and highly purified iodination grade LH (rLH-I-9) were generously provided by the National Hormone and Pituitary Program of the NIDDK (Rockville, MD). It is important to note that the preparation of FSH is highly potent. According to the specifications provided by the NIDDK, it is 175-strength NIH-oFSH-S1, or 4463 IU/mg, and without detectable LH contamination. ITS (insulin, 5 μg/ml; transferrin, 5 μg/ml; selenium, 5 ng/ml) was purchased from Collaborative Research (Bedford, MA). In those cultures in which insulin was omitted, transferrin and selenium were always present. Fetuin (1 mg/ml) was added to all media to prevent precocious hardening of the zona pellucida in the absence of serum fetuin [10]. The fetuin was purified, using the method of Spiro [11], by the Microchemistry Service of the Jackson Laboratory using FBS purchased from Sigma Chemical Co. (St. Louis, MO). The final preparations of fetuin were dialyzed extensively against embryo culture grade water before lyophilization.

Isolation and Culture of Oocyte-Granulosa Cell Complexes

The oocyte-granulosa cell complexes of 12-day-old $B6SJLF_1$ mice were isolated and cultured as described in detail previously [8]. Salient points to be reiterated are that the complexes are derived from preantral follicles having 2–3 layers of granulosa cells. Removal of most theca cells and degradation of the basal lamina by collagenase facilitate adherence of the complexes to collagen-impregnated membranes (Costar Transwell-COL membrane inserts; Costar Corp., Cambridge, MA). The culture medium was Waymouth medium MB752/1 supplemented with 0.23 mM pyruvic acid, 50 mg/L streptomycin sulfate, 75 mg/L penicillin G (Sigma), 3 mg/ml BSA (crystallized; ICN Biochemicals, Aurora, OH), ITS (Collaborative Research), and 1 mg/ml fetuin [10]. The oocyte develops within a ball of cells atop a stalk of granulosa cells that is attached to the membrane. Between 200 and 300 complexes, derived from 2 mice, were grown on each membrane, and care was taken to establish the cultures without contact between the complexes. Approximately 90% of the complexes in all groups survived through the 10-day culture period. Cultures were incubated at 37°C in modular incubation chambers (Billups Rothenberg, Del Mar, CA) thoroughly infused with a gas mixture composed of 5% O_2:5% CO_2:90% N_2. Cultures were fed every 2 days by replacement of approximately half of the medium in the compartment below the membrane. All control and experimental groups were isolated and cultured simultaneously for each experiment.

Oocyte Maturation and Fertilization In Vitro

After oocyte growth and development in vitro, the oocyte-granulosa complexes were dislodged from the membrane by sharply jolting the membrane insert with a snap of a finger against the side of the membrane. The complexes were collected and washed three times in fresh medium and allowed to mature for 17–18 h in 2.5 ml of medium supplemented with FSH (100 ng/ml) regardless of whether FSH was used for culture of the oocyte-granulosa cell complexes.

After the incubation for oocyte maturation, the granulosa cells were removed from oocytes by drawing the complexes in and out of a Pasteur pipette. The granulosa cell-free oocytes that had undergone germinal vesicle breakdown (GVB), indicative of the resumption of meiosis, were collected and washed three times in fertilization medium. The oocytes that had not undergone GVB were cultured for an additional 24 h in Minimum Essential Medium (MEM) without FSH to assess whether further culture without granulosa cells would allow additional oocytes to undergo GVB. The number of oocytes that underwent GVB with granulosa cells plus the number that underwent GVB subsequently without granulosa cells equaled the total number of oocytes that had developed competence to undergo GVB during the 10-day culture period. Ova were fertilized and preimplantation embryos were cultured as described previously [12–14].

Measurement of cAMP Production

Oocyte-granulosa cell complexes were removed from the membranes after 10 days culture and incubated in MEM containing either FSH (100 ng/ml), LH (1 μg/ml), or control medium for 3 h; all media contained 3-isobutyl-1-methylxanthine (100 μM). Intracellular cAMP levels in samples of 1–5 complexes was measured by ^{125}I RIA exactly according to the protocol provided by the manufacturer of the assay kits (DuPont NEN, Boston, MA). The experiment was repeated three times with triplicate samples in each experiment.

Evaluation of Expansion (Mucification) of Oocyte-Associated Granulosa Cells

Oocyte-granulosa cell complexes were removed from the membranes after 10 days of culture and incubated in MEM containing either 100 ng/ml FSH, 1 μg/ml LH, or control medium for 15 h. Since a component of serum is required for expansion [15, 16], media were supplemented with 5% FBS (HyClone, Logan, UT). Expansion was scored subjectively as described in detail previously [17]. Intermediate stages of expansion were rarely seen, so complexes were scored as either expanded or not. Data are presented as the percentage of expanded complexes in three independent experiments with at least 50 complexes scored in each experiment.

Measurement of Oocyte Size

The size of cultured oocytes was measured without allowing them to undergo maturation in vitro. The germinal vesicle-stage oocytes were denuded of their companion granulosa cells as described above and measured using the Image Explorer analysis system from Signal Analytics Corp. (Vienna, VA). The IP Lab Spectrum software of this system was set to measure the long and short diameter of the oocytes exclusive of the zona pellucida. The average diameter and oocyte volume were then calculated using Statview for Macintosh (Abacus Concepts, Berkeley, CA). At least 100 oocytes were measured in each of three independent experiments.

Measurement of LH Receptor (LHR) mRNA Steady State Expression

The steady-state expression of LHR mRNA in approximately 200 cultured complexes was determined by RNase protection assay exactly as described previously [18]. Pro-

tected RNA-RNA hybrids were analyzed by electrophoresis using 6% urea-polyacrylamide gels that were dried and exposed to Fuji phosphor imaging plates and quantified using the Fuji imaging system (Fuji Medical Systems USA, Stamford, CT). The background that was subtracted was the value of an area just below the protected target band and equal in area. The steady-state level of LHR mRNA expression was normalized to the expression of ribosomal protein L19 (Rpl-19) mRNA [19]. All groups within an experiment were cultured and assessed by RNase protection assay at the same time.

Presentation of Data and Statistical Analysis

Percentages, such as the percentage of 2-cell-stage embryos that developed to the blastocyst stage, or normalized levels of LHR mRNA, are presented as the mean percentage of at least three independent experimental replicates; variation between experiments is illustrated using the standard error of the mean. For evaluation of the differences between groups, data were subjected to arcsin transformation and ANOVA. When a significant F-ratio was defined by ANOVA, groups were compared through use of the Fisher's Protected Least-Significant-Difference post hoc test using StatView software; when $p \leq 0.05$, the difference was considered significant.

Experiments to measure cAMP levels were repeated three times with triplicate samples in each experiment. The average of the triplicate samples within an experiment was used in the calculation of the mean of the three independent experiments. Data are expressed as the mean cAMP level per complex, and variation between the three experiments is illustrated using the standard error of the mean. For evaluation of the differences between groups, data were subjected to ANOVA. When a significant F-ratio was defined by ANOVA, groups were compared using the Student-Newman-Keuls post hoc test; when $p \leq 0.05$, the difference was considered significant.

RESULTS

Effect of FSH on the Developmental Competence of Oocytes Grown In Vitro in Serum-Free Medium

As described above, the standard serum-free medium used in this laboratory for the culture of oocyte-granulosa cell complexes from preantral follicles is supplemented with ITS, because complexes cultured in serum-free medium without ITS are very fragile and difficult to maintain throughout the 10-day culture period. Therefore, in the first set of experiments, oocyte-granulosa cell complexes were cultured for 10 days in ITS-supplemented medium, with or without FSH, at concentrations ranging from 0.1 to 5 ng/ml. No consistent effect of FSH treatment was observed on oocyte growth; the final median oocyte diameter, excluding the zona pellucida, was 70–74 μm in all groups. Likewise, 80–90% of oocytes acquired competence to undergo GVB regardless of FSH concentration. Moreover, the morphology of oocytes was similar in all groups when they were examined by Nomarski interference optics (Fig. 1). In contrast, increasing concentrations of FSH during oocyte development in vitro had surprising deleterious effects on oocyte developmental competence. While FSH treatment of developing oocyte-granulosa cell complexes significantly reduced the percentage of oocytes competent to undergo fertilization and cleavage to the 2-cell stage (Fig. 2A), the most profound, and unexpected, effects were seen in the oocytes' competence to develop from the 2-cell stage to blastocyst stage (Fig. 2B). When oocyte-granulosa cell complexes were cultured in control medium (no FSH), 63% of the 2-cell stage derived from control oocytes developed to the blastocyst stage. This percentage was reduced to approximately 20% when the complexes were cultured with 0.5–5 ng/ml FSH (Fig. 2B).

Since the concentration of insulin in commercial ITS preparations is 5 μg/ml—approximately 10^3 times that measured in mouse plasma—and since insulin acts synergistically with FSH in promoting granulosa cell differentiation and function [20–22], the effect of FSH on the acquisition of oocyte developmental competence was assessed when oocyte-granulosa cell complexes were cultured in the absence of insulin. In this case, addition of FSH plus insulin to cultures of oocyte-granulosa cell complexes had slight, though significant ($p < 0.05$), deleterious effects on the acquisition of competence to undergo fertilization and cleavage to the 2-cell stage (Fig. 3A). However, FSH in the absence of insulin did not have a deleterious effect on the acquisition of competence to undergo the transition from 2-cell stage to blastocyst (Fig. 3B). Despite the fragility of the complexes, a higher percentage of oocytes grown in medium without either insulin or FSH were competent to undergo fertilization and cleavage to the 2-cell stage than was the case for oocytes grown in medium with insulin (Fig. 3A).

To investigate the relationship between FSH and insulin with respect to the acquisition of oocyte developmental competence in more detail, oocyte-granulosa cell complexes from preantral follicles were cultured for 10 days in control medium (no FSH or insulin), FSH (5 ng/ml) without insulin, insulin (5 μg/ml) without FSH, or 5 ng/ml FSH plus 0.05–5 μg/ml insulin. There was no difference in the acquisition of competence to undergo either fertilization and cleavage to the 2-cell stage or the transition from 2-cell stage to blastocyst when complexes were cultured in medium without either FSH or insulin, or with FSH alone (Fig. 4). However, consistent with the observation described above as seen in Figure 3A, a higher percentage of the oocytes cultured without either FSH or insulin cleaved to the 2-cell stage than when the complexes were cultured in medium with insulin alone (not shown). In contrast, competence to undergo both fertilization and cleavage to the 2-cell stage, as well as the transition from the 2-cell stage to blastocyst, was reduced in a dose-dependent manner when insulin was added in increasing amounts to medium containing 5 ng/ml FSH (Fig. 4).

The effects of insulin plus FSH could have been mediated by either insulin or insulin-like growth factor-1 (IGF-1) receptors or both [23, 24]. However, growing oocytes in FSH plus IGF-1 (10 ng/ml) did not have either deleterious or beneficial effects on oocyte developmental competence (data not shown). Moreover, culture of complexes with FSH and both IGF-1 and insulin together had the same deleterious effect on oocyte developmental competence as culture with FSH plus insulin (data not shown). Thus the deleterious effects of insulin, in combination with FSH, may be mediated via insulin receptors and not by IGF-1 receptors.

Effect of FSH on the Differentiation of Granulosa Cells Associated with Oocytes in Culture

In preovulatory follicles, cumulus cells, which are the granulosa cells closely associated with oocytes, express

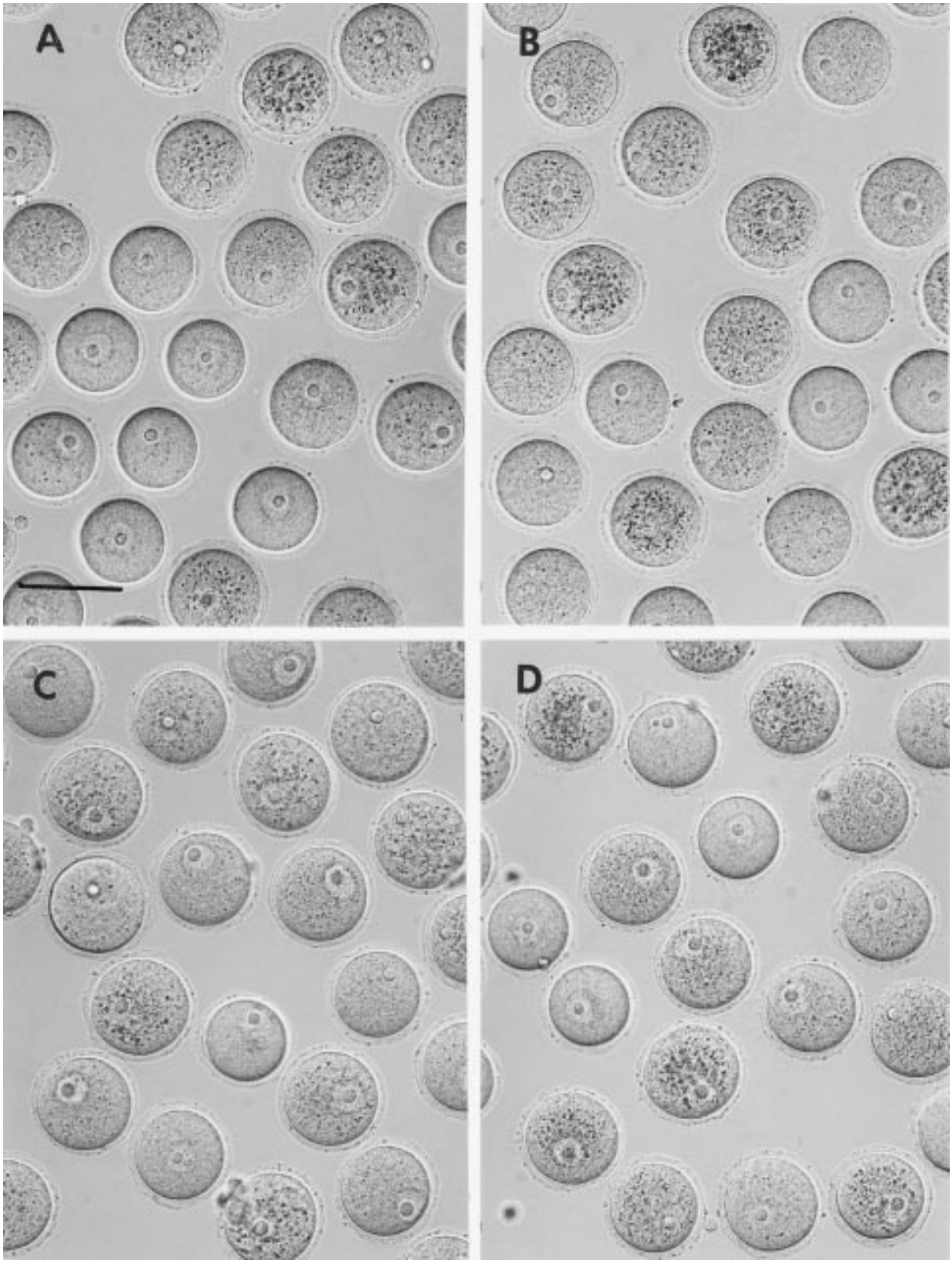

FIG. 1. General morphological appearance of oocytes grown in vitro as seen by Nomarski interference optics. Oocytes were grown in vitro for 10 days in medium containing **A**) insulin (5 μg/ml), **B**) insulin plus FSH (5 ng/ml), **C**) neither insulin nor FSH, **D**) FSH alone. Although oocytes grown in medium supplemented with both insulin and FSH (**B**) had significantly less competence to develop to the blastocyst stage after fertilization, they did not display morphological characteristics distinct from those of the other groups. Bar = 100 μm.

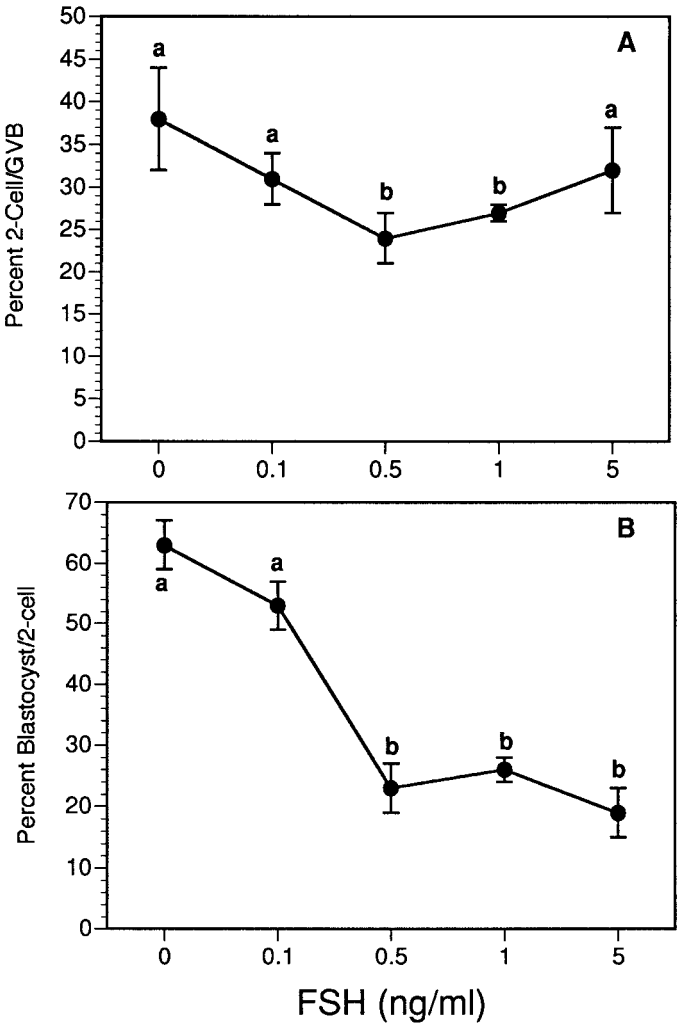

FIG. 2. Effect of FSH treatment of cultured oocyte-granulosa cell complexes on oocyte developmental competence. Oocyte-granulosa cell complexes isolated from preantral follicles of 12-day-old mice were cultured for 10 days in medium containing 0-5 ng/ml FSH, then matured and fertilized in vitro. All groups contained 5 μg/ml insulin during the 10-day culture. **A** Shows the percentage of mature oocytes that cleaved to the 2-cell stage, and **B** shows the percentage of 2-cell-stage embryos that developed to the blastocyst stage. Data are the mean ± SEM of four independent experiments. Where there are no common letters over the points, the groups are different at least at the 95% confidence level.

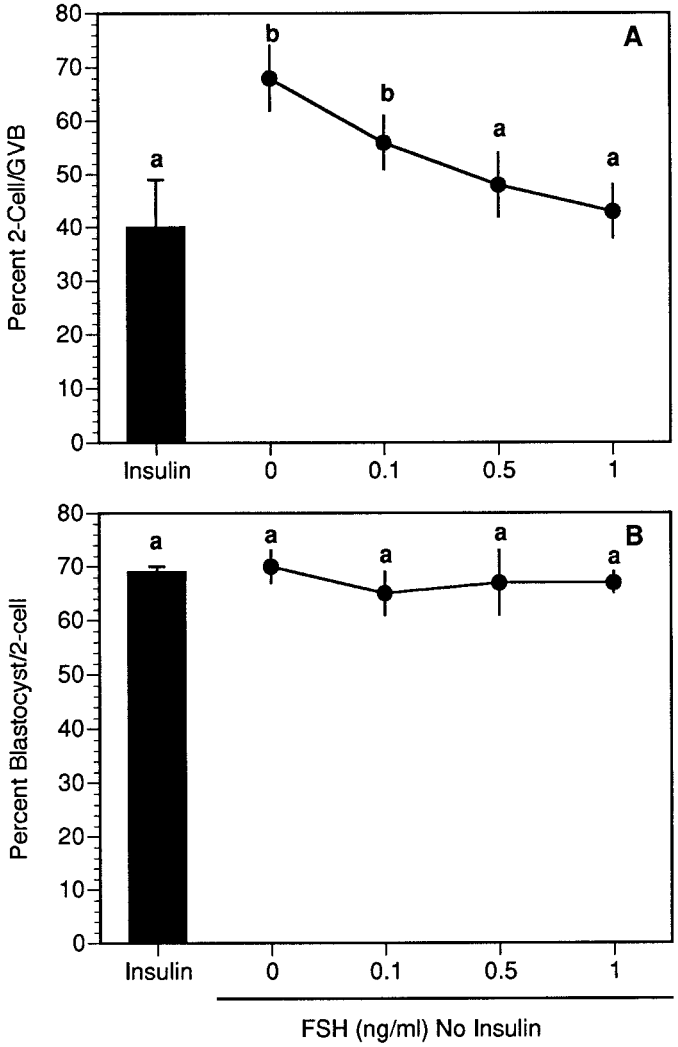

FIG. 3. Effect of FSH treatment without insulin on oocyte developmental competence. Oocyte-granulosa cell complexes isolated from preantral follicles of 12-day-old mice were cultured for 10 days in medium with 5 μg/ml insulin (histogram bar) or without insulin (line graph). Media without insulin contained 0-1 ng/ml FSH. After 10 days of oocyte growth and development in vitro, the oocytes were matured and fertilized in vitro. **A** Shows the percentage of matured oocytes that cleaved to the 2-cell stage, and **B** shows the percentage of 2-cell-stage embryos that developed to the blastocyst stage. Data are the mean ± SEM of four independent experiments. Where there are no common letters over the bars or points, the groups are different at least at the 95% confidence level.

several phenotypic differences from mural granulosa cells, which are associated with the follicle wall (reviewed in [18]). The functional reasons for the phenotypic differences between cumulus cells and mural granulosa cells are not known, though the oocyte clearly plays a role in establishing these differences [18, 25]. We hypothesized that some aspects of mural granulosa cell function may be antagonistic to oocyte development and that oocytes therefore prevent expression of the mural granulosa cell phenotype by their companion cumulus cells to avoid these deleterious effects [26]. Since FSH and insulin are known to act synergistically to promote granulosa cell differentiation [20–22], the possibility was tested that these agonists may act inappropriately in our culture system by driving mural granulosa cell-like development by cumulus cells. One marker differentiating mouse mural granulosa cells from cumulus cells is the expression of LHRs and LHR mRNA by mural granulosa cells in preovulatory follicles. Oocyte-cumulus cell complexes isolated from mouse preovulatory follicles do not undergo cumulus expansion or produce cAMP in response to highly purified LH [27, 28]. Therefore, the presence of functional LHRs was assessed by treating complexes after 10 days of culture with either highly purified LH (1 μg/ml), FSH (1 μg/ml), or control medium and 1) measuring cAMP production by RIA and 2) subjectively evaluating expansion (mucification) of the oocyte-associated granulosa cells. Neither cAMP production nor expansion was observed when oocyte-granulosa cell complexes were grown in medium without FSH for 10 days and then treated with either FSH or LH (Fig. 5). Increased cAMP production and expansion occurred in response to FSH when complexes were grown for 10 days in medium with 0.1 ng/ml or more FSH. More importantly, increased cAMP production and expansion occurred in response to highly purified LH when complexes were grown for 10 days in medium with 0.5 ng/ml or more FSH (Fig. 5). Thus,

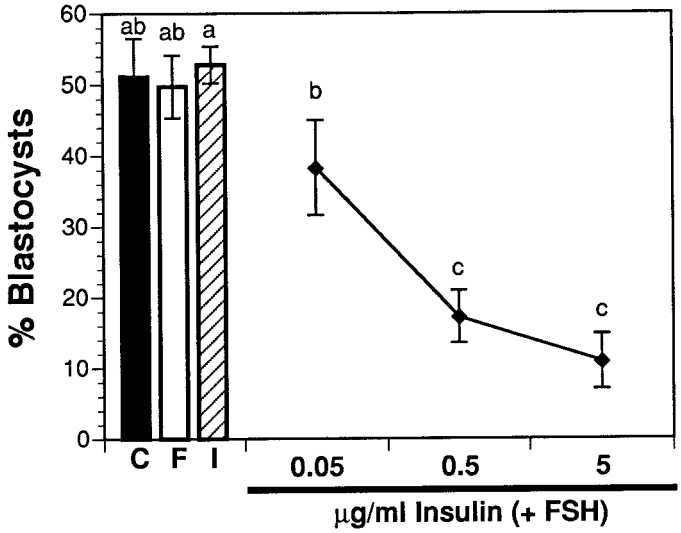

FIG. 4. Effect of insulin on oocyte developmental competence. Oocyte-granulosa cell complexes isolated from preantral follicles of 12-day-old mice were cultured for 10 days in medium with neither FSH nor insulin (C), FSH alone (5 ng/ml; F), insulin alone (5 μg/ml; I), or FSH (5 ng/ml) plus 0.05 to 5 μg/ml insulin (line graph). Data are the mean percentage of blastocysts that developed from 2-cell-stage embryos, ± SEM, in four independent experiments. Where there are no common letters over the bars or points, the groups are different at least at the 95% confidence level.

unlike cumuli oophori isolated from preovulatory follicles, the oocyte-associated granulosa cells (cumulus cells) grown in vitro with FSH produced functional LHRs, suggesting an inappropriate differentiation.

To determine whether the state of differentiation of oocyte-associated granulosa cells could be correlated with deleterious effects on oocyte developmental competence, complexes were cultured in control medium (no FSH or insulin), FSH (5 ng/ml) without insulin, insulin (5 μg/ml) without FSH, or 5 ng/ml FSH plus 0.05–5 μg/ml insulin for 10 days, and the steady-state level of LHR mRNA expression was then measured. Comparing results on LHR mRNA expression, shown in Figure 6, with data on oocyte developmental competence, shown in Figure 4, did not indicate a correlation. For example, in the absence of insulin, there was high steady-state expression of LHR mRNA and high developmental competence when complexes were cultured with FSH alone; but this level of LHR mRNA expression was the same as in the FSH plus 5 μg/ml insulin group wherein developmental competence was low. However, these data on granulosa cell expression of LHR mRNA were obtained after 10 days of culture, after much of the oocyte development in vitro had been completed. It seemed possible that, if inappropriate oocyte-associated granulosa cell differentiation or function is related to deficient oocyte-developmental competence, the stage of oocyte development sensitive to aberrant granulosa cell-oocyte communication might be earlier in the oocyte culture period. Therefore, the steady-state level of LHR mRNA expression was measured on Days 4, 6, 8, and 10 of culture in medium with 5 ng/ml FSH plus or minus 5 μg/ml insulin since, as shown in Figure 4, addition of insulin to medium containing FSH results in a dramatic decrease in the developmental competence of the oocytes. The steady-state level of LHR mRNA expression was low in complexes cultured for 6 days in medium containing FSH but not insulin (Fig. 7). In sharp contrast, there was the same high level of LHR

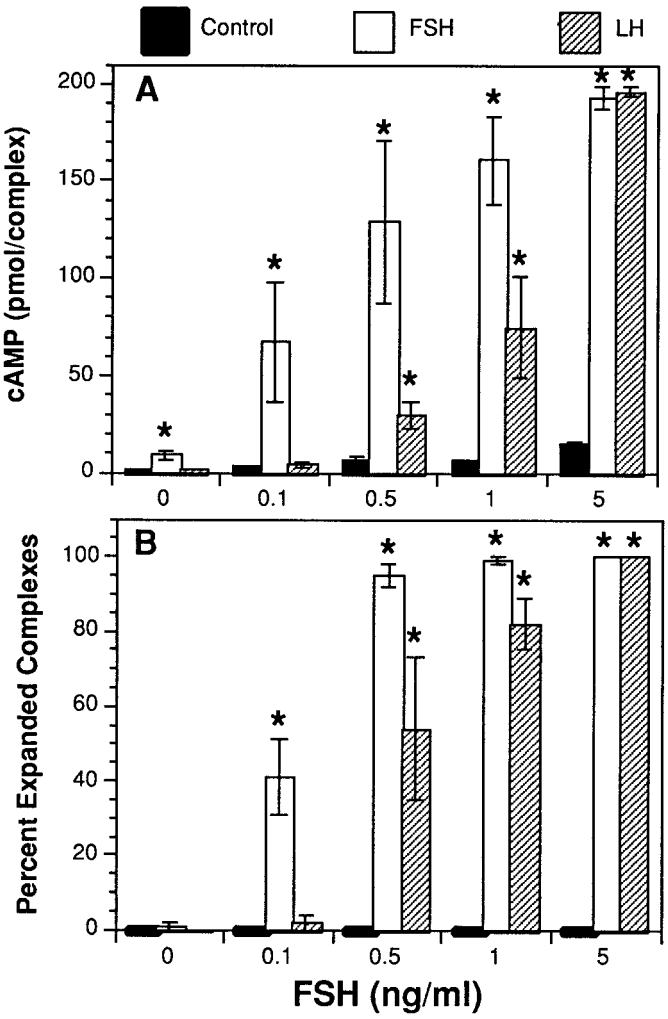

FIG. 5. Effect of FSH on the response of cultured oocyte-granulosa cell complexes to treatment with LH. Oocyte-granulosa cell complexes isolated from preantral follicles of 12-day-old mice were cultured for 10 days in medium containing 0-5 ng/ml FSH (x-axis) plus 5 μg/ml insulin. Complexes were then removed from the collagen-impregnated membranes and assessed in two ways for response to control medium or to highly purified LH or FSH: **A**) production of cAMP and **B**) expansion, or mucification. * Indicates a significant difference ($p \leq 0.05$) from the control.

mRNA expression at all the times measured from 4 to 10 days when complexes were cultured in medium containing insulin in addition to FSH (Fig. 7). Thus, insulin accelerated the time of onset of granulosa cell expression of LHR mRNA in response to FSH. Oocyte-associated granulosa cells did not, therefore, express an appropriate dynamics of differentiation coincident with oocyte growth and development in the presence of FSH and insulin.

DISCUSSION

This study was undertaken to test the hypothesis that FSH treatment of cultured oocyte-granulosa cell complexes isolated from preantral follicles will improve the quality of oocytes by promoting growth and a higher frequency of embryonic developmental competence. The results did not support this hypothesis. FSH treatment of cultured complexes did not significantly affect oocyte growth, the percentage of oocytes acquiring competence to resume meiosis, or the percentage of oocytes competent to undergo fertilization and preimplantation development. FSH or insulin

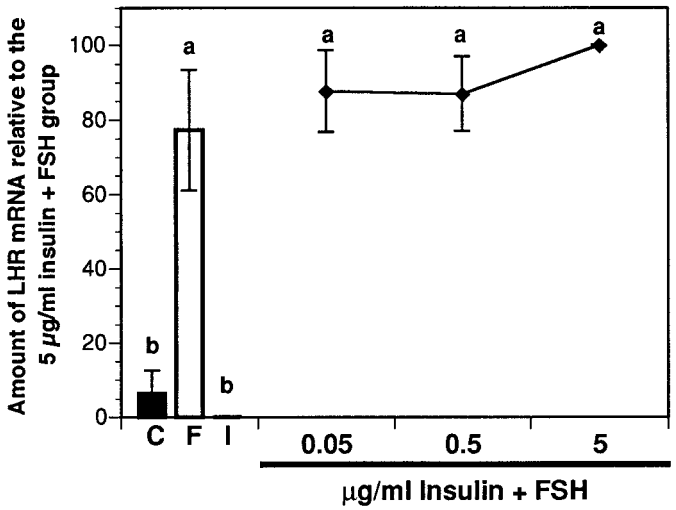

FIG. 6. Effect of FSH and insulin on the steady-state level of LHR mRNA expression by cultured oocyte-granulosa cell complexes. Complexes were cultured for 10 days in control medium without either FSH or insulin (C), medium containing 5 ng/ml FSH (F) or 5 μg/ml insulin (I), or medium containing 5 ng/ml FSH plus 0.05-5 μg/ml insulin (line graph). Data are the mean ± SEM of three independent experiments in which the level of LHR mRNA was normalized to that of Rpl-19. Data are presented as a percentage of the expression measured in the FSH plus 5 μg/ml insulin group. No common letters over the bars or points indicate a difference ≥ 95% confidence level from FSH plus 5 μg/ml insulin group.

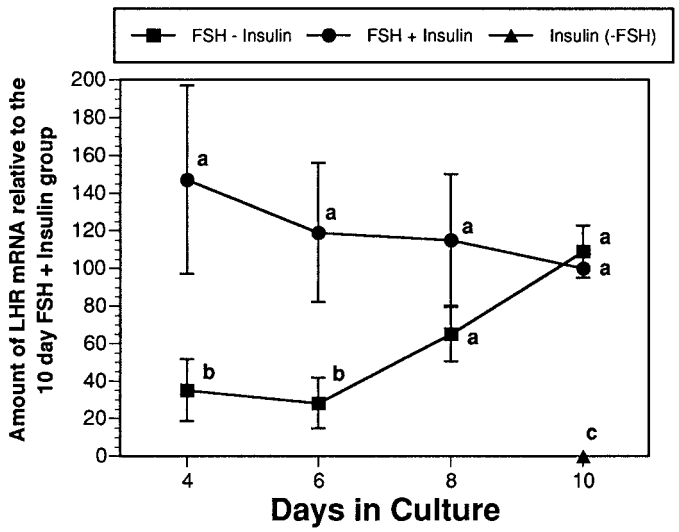

FIG. 7. Effect of insulin on FSH-induced expression of LHR mRNA by cultured oocyte-granulosa cell complexes. Complexes were cultured for 4, 6, 8, or 10 days in medium containing 5 ng/ml FSH in the presence or absence of 5 μg/ml insulin. Data are the mean ± SEM of three independent experiments in which the level of LHR mRNA was normalized to that of Rpl-19. Data are presented as a percentage of the expression measured in the FSH plus 5 μg/ml insulin group. No common letters near the points indicate a difference ≥ 95% confidence level from the 10-day FSH plus insulin group.

alone did not significantly affect competence to complete preimplantation development. However, treatment of complexes with both FSH and insulin produced an unexpected, highly deleterious effect on competence to undergo development from the 2-cell stage to the blastocyst.

FSH did not promote any positive or beneficial effects on oocyte growth in vitro, or on acquisition of competence to undergo maturation, fertilization, or preimplantation development. These results raise the question whether FSH plays any specific role in mammalian oocyte development. Oocytes isolated from early antral follicles of hypogonadal mice, which have undetectable levels of circulating gonadotropins [29], are fully grown and competent to undergo maturation, fertilization, and preimplantation development in vitro, albeit at frequencies lower than for oocytes from the larger antral follicles of littermate controls [30, 31]. Treatment of hypogonadal mice with eCG in vivo increases the percentage of oocytes that undergo maturation, fertilization, and development in vitro to levels equivalent to those for oocytes from littermate control mice [30]. It is well established that antral follicles undergo atretic degeneration in the absence of gonadotropic support. Thus, the only healthy antral follicles found in hypogonadal mice are small, since they only recently formed from secondary follicles [29]. Oocytes from small antral follicles of normal mice are less competent to complete maturation and preimplantation development than oocytes from large antral follicles [2, 4, 32]. Therefore, a probable explanation for the low frequency of competence of oocytes from hypogonadal mice to undergo preimplantation development is that the isolated oocytes were derived 1) from small healthy follicles that had only recently undergone antrum formation and 2) from degenerating follicles. In both cases, the oocytes would be expected to have poor potential for preimplantation development. The facts that oocytes in hypogonadal mice grow to normal size and that some of them become competent to undergo maturation and preimplantation development show that gonadotropins are not necessary for oocyte growth and acquisition of preimplantation developmental competence. This conclusion is supported by the in vitro studies reported here.

The absence of a beneficial effect of FSH was puzzling and unexpected. Indeed, in experiments not shown, we tested several different regimens of FSH treatments, such as different concentrations at different times during the 10-day culture period, and combinations of treatment with FSH and estrogen. Among these conditions we never observed a beneficial effect of FSH on the acquisition of developmental competence. Nevertheless, further testing of potential competence-promoting effects of FSH in combination with other hormones and growth factors is continuing. Supplementation of the culture medium with FBS did not prevent the deleterious effects of FSH on oocyte developmental competence [9], although the suppression was not as dramatic as that observed using serum-free medium. Perhaps no conclusion should be drawn from these negative results. Conditions of culture might preclude the possibility of FSH having a beneficial effect, or oocyte developmental processes might be able to compensate for the absence of FSH, or we may simply have not tested the appropriate combination or concentration of factors to demonstrate the ability of FSH to promote competence to complete preimplantation development. Alternatively, we must weigh the concept that FSH is not a factor that drives development and function of the peri-oocytic granulosa cell in a specific way that is beneficial to oocyte growth and acquisition of developmental competence. Rather, FSH may play only a general role in sustaining these processes in oocytes, i.e., by supporting overall follicular development and endocrine function and by preventing follicular degeneration.

By what mechanism(s) might the combination of FSH and insulin affect the acquisition of oocyte developmental competence in this culture system? Evidence on whether oocytes express FSH receptors is conflicting [33, 34],

though most studies do not detect FSH binding or expression of FSH receptor mRNA by oocytes [35, 36]. In contrast, there is general acceptance that the oocyte's companion granulosa cells express FSH receptors even in early stages of follicular development (see [35] for review). Thus, if FSH affects oocyte development, this action would probably be indirect and mediated via granulosa cells. Neither FSH nor insulin alone had a harmful effect on developmental competence; it was the combination of these two agents that was deleterious to subsequent embryogenesis. Since insulin acts synergistically with FSH in promoting granulosa cell differentiation and function [20–22], it seemed possible that this synergism might promote an inappropriate differentiation or function of oocyte-associated granulosa cells that was deleterious to oocyte development. Indeed, FSH plus insulin promoted a precocious and inappropriate differentiation of granulosa cells in cultures, and this was correlated with production of oocytes having low competence to complete preimplantation development. It is not likely that expression of LHR mRNA by granulosa cells directly affects oocyte development; but this expression probably occurs coordinately with, or affects, some other function of differentiated granulosa cells in a way that is deleterious to oocyte development.

Both gap junctions and paracrine signaling mediate bidirectional communication between oocytes and companion granulosa cells. Paracrine factors secreted by oocytes suppress FSH-induced elevation of the steady-state level of LHR mRNA expression by granulosa cells [18]. This probably explains, at least in part, the lack of expression of LHR and LHR mRNA by cumulus cells in preovulatory follicles. It was therefore unanticipated that oocytes grown in vitro were unable to suppress expression of LHR mRNA induced by FSH plus insulin in the cultured complexes. In experiments not shown, isolated oocytes grown in vitro were equivalent to oocytes grown in vivo in their ability to suppress FSH-induced elevation of LHR mRNA by mural granulosa cells in monolayer cultures. It is not known, however, whether this suppressing factor is produced and secreted by oocytes associated with granulosa cells during oocyte growth and development in vitro. In the assay system used to detect the LHR mRNA-suppressing factor, denuded oocytes were cocultured with monolayer cultures of granulosa cells at a concentration of 1–2 oocytes per microliter [18]. In the system for oocyte-growth and development in vitro used here, 200–300 complexes were grown in 4 ml of medium, a concentration of less than 0.1 oocyte per microliter. It is, therefore, possible that the LHR mRNA-suppressing factor(s) became diluted to ineffective concentrations in this culture system even if they were produced and secreted by the oocytes.

The deleterious effects of FSH plus insulin on oocyte developmental competence observed in this study cannot directly reflect the physiological function of FSH during normal follicular development, since developmentally competent oocytes are produced in vivo even in the environment of high FSH concentrations in neonatal mice [29, 37]. Compared to the environment of the simple culture system used here, the milieu in which FSH functions in vivo is one of complex interactions with many other regulatory factors. Nevertheless, the deleterious effects of FSH plus insulin on oocyte development obtained here, using an experimental culture system involving the minimum follicular and culture components needed to support oocyte development, may help unravel the complex interactions between cells essential for normal oocyte development. For example, this study shows that conditions occurring during oocyte growth can have profound effects on oocyte competence to complete preimplantation development. Interestingly, this deleterious effect occurs despite apparently unaffected oocyte morphology, growth, and competence to undergo GVB. In spite of these similarities, the oocytes must have important differences in molecules essential for later preimplantation development. Identification and characterization of these differences may provide information on the maternal molecules critical for completion of preimplantation development and perhaps an assay for oocyte quality useful in clinical or agricultural settings. Finally, the deleterious effects of combined FSH and insulin on oocyte developmental competence should discourage the implementation of clinical protocols that could result in inappropriate differentiation or function of granulosa cells during oocyte growth. Although these protocols might produce oocytes that appear morphologically normal and competent for meiotic maturation, fertilization, and even first cleavage, competence to continue preimplantation development could be jeopardized.

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