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Murine Oocytes Suppress Expression of Luteinizing Hormone Receptor Messenger Ribonucleic Acid by Granulosa Cells[1]

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ABSTRACT

This study tested the hypothesis that murine oocytes participate in the establishment of granulosa cell phenotypic heterogeneity in preovulatory follicles. In these follicles, mural granulosa cells express LH receptors (LHR) and LHR mRNA, but expression of these molecules is low or undetectable in cumulus cells. Thus, the expression of LHR mRNA is a marker of the mural granulosa cell phenotype in preovulatory follicles. Cumulus cells expressed elevated steady-state levels of LHR mRNA when oocytes were microsurgically removed from oocyte-cumulus cell complexes, and this was prevented by paracrine factor(s) secreted by isolated oocytes. These factors also suppressed FSH-induced elevation of the level of LHR mRNA expression by mural granulosa cells isolated from small antral follicles, even when expression was augmented by culturing granulosa cells on components of basal lamina. Moreover, factor(s) secreted by oocytes suppressed the expression of LHR mRNA in mural granulosa cells isolated from preovulatory follicles already expressing elevated levels of these transcripts. The ability of oocytes to suppress the LHR mRNA expression by granulosa cells was developmentally regulated. Oocytes from preantral follicles and mature (metaphase II arrested) oocytes were less effective in suppressing expression than fully grown, germinal vesicle (GV)-stage oocytes. Furthermore, two-cell-stage embryos did not suppress LHR mRNA levels. Coculture of isolated oocytes with granulosa cells affected the synthesis of very few granulosa cell proteins detected by fluorography of two-dimensional gels after ^{35}S-methionine labeling. Thus, oocyte suppression of FSH-induced LHR mRNA expression is specific in both the suppressing cell type and the effects on granulosa cells. It is concluded that the default pathway of granulosa cell differentiation produces the mural granulosa cell phenotype, as represented by the expression of LHR mRNA. This pathway is abrogated by oocytes. Thus, oocytes play a dominant role in establishing the fundamental heterogeneity of the granulosa cell population of preovulatory follicles.

INTRODUCTION

Granulosa cells of preovulatory follicles are heterogeneous in organization, gene expression, and function. One population, the cumulus cells, are organized around the oocyte and another population, the mural granulosa cells, are associated with the basal lamina. In fact, several layers of cells in both the cumulus and mural granulosa cell populations often appear to be organized as a pseudostratified epithelium in direct contact with the oocyte or basal lamina, respectively [1, 2]. There are clear differences between mural and cumulus cells in both functions and fate. Just before ovulation, gonadotropins stimulate cumulus cells to produce and secrete hyaluronic acid that disperses the cumulus cells and embeds them in a mucus-like matrix. This process, called cumulus expansion or mucification [3–5], results in the ovulation of an expanded cumulus oophorus. Mural granulosa cells do not undergo expansion, but instead become luteal cells. Cumulus and mural granulosa cells also differ in the distribution of LH receptors (LHR) [6–8] and mRNA coding for LHR [9–11], steroidogenic capabilities [12–14] and mRNAs coding for cholesterol side-chain cleavage cytochrome P450 [15] and cytochrome P450 aromatase [11], IGF–1 mRNA [16], Müllerian inhibiting substance [17], lectin binding [18], and other uncharacterized molecules [19]. Thus, the two kinds of granulosa cells have distinct phenotypes in preovulatory follicles. This paper focuses on the role of oocytes in establishing these heterogeneous phenotypes.

The participation of granulosa cells in the development of oocytes is well documented [20, 21]. It is also clear that oocytes produce factors that can affect the function of granulosa cells. Oocytes secrete paracrine signals that enable cumulus cells to produce hyaluronic acid and undergo cumulus expansion in response to FSH stimulation [22–25], suppress progesterone production by granulosa cells [26–28], promote granulosa cell proliferation [29], and inhibit plasminogen activator production by granulosa cells [30]. Moreover, ovarian follicles fail to develop beyond the primary stage in mice that do not express growth differentiation factor–9, a member of the transforming growth factor-β family expressed only in oocytes [31], due to targeted deletion by homologous recombination [32]. Thus, it is clear that oocyte-granulosa cell communication is bidirectional and essential for both oocyte and follicular somatic cell function and development.

Expression of LHR is low or undetectable in the mural granulosa cell layer of small antral follicles, but increases dramatically after gonadotropin stimulation [33–37]. The occurrence of LHR in preovulatory follicles is usually greatest near the follicular basal lamina and decreases in a gradient away from the basal lamina; and these receptors are rarely, if ever, detected on cumulus cells [6–8, 36–38]. The regulation of LHR mRNA generally parallels that of the receptor itself [39–42], and the localization of LHR mRNA in preovulatory follicles is the same as that of the receptor [9–11]. Thus, expression of LHR or LHR mRNA differentiates the mural granulosa cell phenotype from that of cumulus cells in preovulatory follicles and may be considered a marker of the mural granulosa cell phenotype in these follicles.

Highest expression of LHR by granulosa cells near the follicular basal lamina, and lowest expression near oocytes, may reflect opposing influences of these two follicular components on the differentiation of granulosa cell phenotypes. Augmentation of the mural granulosa cell phenotype by components of basal lamina is consistent with the first part of this hypothesis [43, 44]. In the current study, the effect of paracrine signals from oocytes on granulosa cell differentiation was determined using expression of LHR mRNA

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as a marker of the phenotype of mural granulosa cell of preovulatory follicles. Two experimental paradigms were employed. In the first, we tested the hypothesis that microsurgical extirpation of the oocyte from within oocyte-cumulus cell complexes would result in increased steady-state expression of LHR mRNA by cumulus cells. In the second, we tested the hypothesis that isolated oocytes would suppress the steady-state level of expression of LHR mRNA in monolayer cultures of mural granulosa cells stimulated with FSH.

MATERIALS AND METHODS

Isolation of Oocytes, Preparation of Embryos, and Cultures of Cumulus Cells and Mural Granulosa Cells

Fully grown oocytes and oocyte-cumulus cell complexes were isolated from 22- to 24-day-old (C57BL/6J × SJL/J)F_1 mice, and growing oocytes were obtained from the preantral follicles of 12-day-old mice as described previously [45, 46]. For some experiments, oocytes were microsurgically removed from oocyte-cumulus cell complexes as described previously [22]. These oocyte-free complexes are referred to as oocytectomized (OOX) complexes. Two-cell and blastocyst-stage embryos were produced from in vitro-matured and -fertilized oocytes cultured as described previously [47].

Granulosa cells were isolated from antral follicles of 22- to 24-day-old female mice by puncturing follicles with a 24-gauge needle and collecting granulosa cells by centrifugation. The medium was M199, prepared exactly as described previously [48], except it was supplemented with 10% fetal bovine serum (HyClone, Logan, UT) rather than BSA; this medium is hereafter referred to as M199/FBS. Clumps of granulosa cells were resuspended, but not dispersed, and seeded into 48-well tissue culture dishes (Corning Inc., Corning, NY). They were cultured in 150 μl of M199/FBS for 24 h at 37°C in modular incubator chambers (Billups Rothenberg, Del Mar, CA) thoroughly infused with a gas mixture composed of 5% O_2:5% CO_2:90% N_2 (5:5:90 gas), which is necessary for maintaining oocyte viability [46, 49]. Attached cells were then washed with M199 supplemented with 3 mg/ml BSA (crystallized; ICN Biochemicals, Aurora, OH); this medium is hereafter referred to as M199/BSA. Subsequent cultures were carried out using 150 μl of M199/BSA supplemented as indicated for individual experiments with 100 μM 3-isobutyl-1-methylxanthine (IBMX; Aldrich Chemical Co., Milwaukee, WI), FSH, denuded oocytes, or combinations of these supplements. It is important to note that the ovine (o) FSH preparation used (NIDDK-oFSH-20) is highly purified and superpotent. According to the specifications provided by the NIDDK (Baltimore, MD), it is 175XNIH-oFSH-S1, or 4463 IU/mg. For some experiments, granulosa cells were cultured in wells coated with components of basal lamina, i.e., entactin, collagen IV, and laminin (ECL). These coated wells were prepared by adding a 20 μg/ml aqueous solution containing these components, purified from the Englebreth-Holm-Swarm mouse tumor, to the wells and incubating them for 1 h at 37°C as described by the supplier (Upstate Biotechnology, Lake Placid, NY). The wells were then washed thoroughly with M199/BSA. Small clumps of granulosa cells were then added to the wells and cultured overnight in M199/BSA. Unattached cells were then washed out of the wells, and 150 μl of one of several test media was added to each of the wells, with or without 2 oocytes/μl.

Preparation of Probes

The steady-state levels of LHR mRNA were assessed using RNase protection assays. For the preparation of probes for RNase protection assay, mRNA was prepared from mural granulosa cells isolated from eCG-primed 22-day-old (C57BL/6J × SJL/J)F_1 mice using Microfast track kits exactly as described by the manufacturer (Invitrogen Corp., San Diego, CA). Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD) was used to produce first strand cDNA that was subsequently amplified using the polymerase chain reaction (PCR) primer pairs described below. The PCR products were verified by sequence analysis. Design of PCR primers was based on published sequences of the mouse LHR gene [50] and ribosomal protein L19 gene (RPL19) [51], which is a "housekeeping" gene used as a reference for LHR mRNA expression. The primer pairs were as follows: LHR, 5′-GCGGGTCCCGGCTCTGAGACAGCT–3′ and 5′-AGTCGGGCGAGGCCAGCTCGA–3′ (producing a 165-base pair [bp] product); RPL19, 5′-GGGAAAAA-GAAGGTCTGGTTG–3′ and 5′-TTCAGCTTGTGGATG-TGCTC–3′ (producing a 386-bp product). Size and integrity of PCR products were confirmed by agarose gel electrophoresis, and the products were cloned into pCR-SCRIPT with pCR-SCRIPT TM SK(+) (Stratagene, La Jolla, CA). Individual clones were isolated and their identity and orientation were verified again by sequence analysis.

Plasmids were linearized with either *Not* I (when T7 RNA polymerase was used) or *Kpn* I (when T3 RNA polymerase was used); restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). Antisense RNA probes were generated incorporating [α-^{32}P]-CTP (New England Nuclear Research Products, Boston, MA) with either T7 or T3 RNA polymerases using MAXIscript kits (Ambion, Austin, TX). The LHR antisense RNA probe consisted of a 165-bp sequence from exon 1 of the LHR gene. All major LHR transcripts in mice and rats include exon 1 [52, 53], and therefore the RNase protection assay used here detects all the major transcripts.

RNase Protection Assay

The mRNA of samples to be subjected to RNase protection assay was isolated with a modified protocol using MicroFast Track kits from Invitrogen. Cells were lysed by addition of the lysis buffer to the 48-well dish and, after centrifugation of the mRNA bound to oligo(dT)-cellulose, exactly as described by the kit protocol, the supernatant solution was gently removed and 680 μl of binding buffer was added without disturbing the cellulose bed. The binding buffer-oligo(dT)-cellulose mixture was then placed into the spin column/microfuge tube provided and centrifuged at 4000 × *g* for 10 sec. Since sample size was very small, only one centrifugation step was necessary to separate the mRNA from genomic DNA. The binding buffer was decanted from the microcentrifuge tubes, the columns were returned to the centrifuge tubes, and 200 μl of the low-salt buffer was gently mixed into the cellulose bed of each tube using a micropipette tip. The tubes were centrifuged once again at 4000 × *g* for 10 sec. The low-salt buffer extracted non-polyadenylated RNA while polyadenylated mRNA bound to the oligo(dT)-cellulose was retained. The spin column was removed and placed in a fresh RNase-free tube, and 100 μl of elution buffer, heated to 65°C, was mixed into the oligo(dT)-cellulose bed and centrifuged for 10 sec at 4000 × *g*. The addition of elution buffer and the cen-

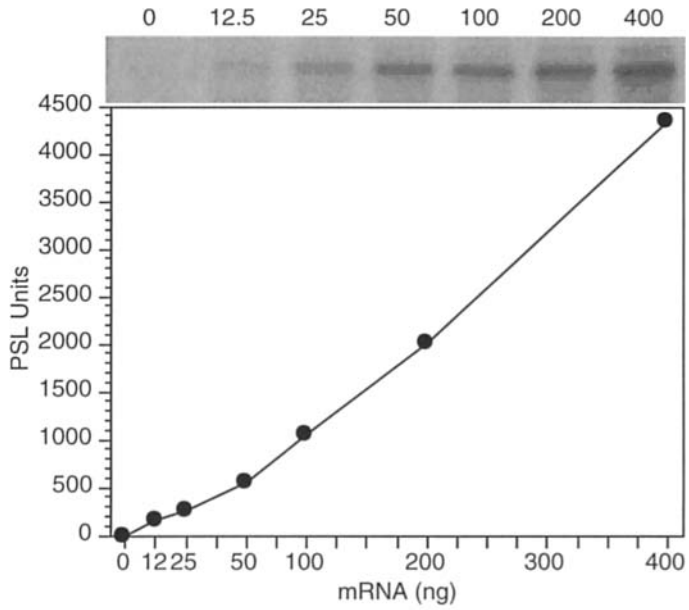

FIG. 1. Relationship between the amount of total mRNA and signal detected by RNase protection assay. Poly(A) mRNA was isolated from mural granulosa cells of eCG-primed mice, quantified by spectrophotometry at 260 nm, and serially diluted to produce the amounts indicated. The strength of the signal is indicated using arbitrary (PSL) units quantified using a Fuji Phosphor Imaging system.

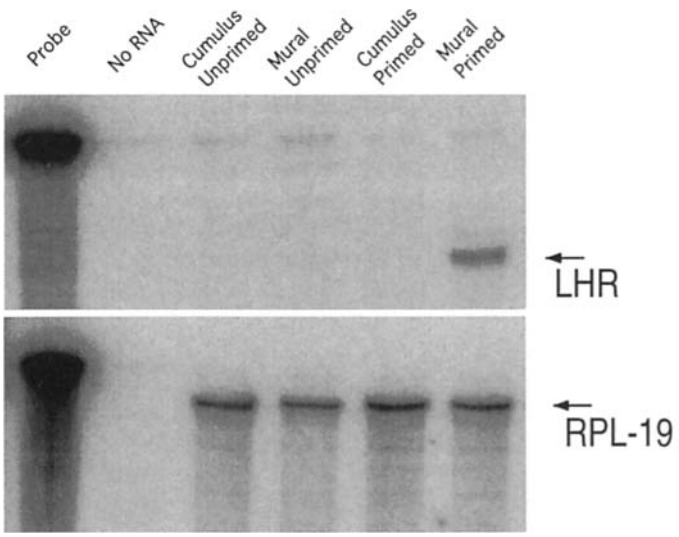

FIG. 2. Expression of LHR and RPL19 mRNA in mouse granulosa cells. Poly(A) RNA was isolated from mural granulosa cells and cumulus cells from eCG-primed or unprimed mice. LHR mRNA was detected by RNase protection assays only when the poly(A) RNA was isolated from the mural granulosa cells of eCG-primed mice.

trifugation steps were repeated twice, and therefore a total of 300 μl of elution buffer was added. The spin column containing the cellulose bed was then discarded, and mRNA in the centrifuge tubes was precipitated using the standard protocol provided by Invitrogen.

RNase protection assay was carried out using RPA–11 kits from Ambion. Protected RNA-RNA hybrids (LHR, 165 bp; RPL19, 385 bp) were analyzed by electrophoresis using a 6% urea-polyacrylamide gel. Gels were dried and exposed to Fuji phosphor imaging plates and quantified using the Fuji Phosphor 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. Data are expressed as the steady-state level of LHR mRNA normalized to RPL19 mRNA. All groups within an experiment were cultured and then assessed by RNase protection assay at the same time. A single sample was used for each group within an experiment due to the extensive effort required to produce some groups. Each experiment was repeated at least three times, and a representative experiment is shown.

Two-Dimensional (2D) Gel Electrophoresis

Granulosa cell monolayers were prepared in the 48-well plate as described above. The cells were cultured overnight in M199/FBS, and then the unattached cells were washed out of the wells and the medium was replaced with 150 μl of M199/BSA, IBMX, and 5 ng/ml FSH. The granulosa cells were cultured for 48 h with or without GV-stage oocytes, added at a concentration of 2 oocytes/μl. Before labeling, the medium was replaced by Whitten's medium [54] supplemented with 3 mg/ml polyvinylpyrrolidone (PVP; Sigma Chemical Co., St. Louis, MO), and oocytes were removed from the oocyte-supplemented wells. Granulosa cells were incubated in Whitten/PVP containing 1 mCi/ml of L-^{35}S-methionine (DuPont NEN, Wilmington, DE) for 1 h. After labeling, the wells were washed twice with Whitten/PVP and once with PBS/PVP, and the cells were lysed with 100 μl of 50 mM Tris-HCl (pH 8.0) buffer containing 0.3% SDS and 1% (v:v) 2-mercaptoethanol [55]. The lysates were transferred to microcentrifuge tubes and incubated at 100°C for 30 sec and stored at −80°C until use. The samples were prepared for and subjected to 2D-PAGE, using Mini-PROTEAN II Tube Cell (Bio-Rad, Hercules, CA) for the isoelectric focusing first dimension, and SE 250 Mighty Small II (Hoefer Scientific Instruments, San Francisco, CA) for the second dimension according to manufacturer's instructions. Second-dimension gels were soaked in a fixing solution of 50% methanol and 10% acetic acid for 1 h, and then treated with a fluorography enhancer (ENTENSIFY; DuPont NEN). The gel was then dried and exposed to film (REFLECTION; DuPont NEN) for fluorography to detect radiolabeled proteins.

RESULTS

Figure 1 illustrates the sensitivity of the RNase protection assay for the detection and quantification of the steady-state level of LHR transcripts using mRNA isolated from mural granulosa cells of eCG-primed mice (22–24 days old). Signal could be detected and quantified by phosphor imaging when ≥ 12.5 ng total granulosa cell mRNA was subjected to RNase protection assay.

Oocytes Suppress Expression of LHR mRNA by Cumulus Cells

Little or no LHR mRNA was detected in either cumulus or mural granulosa cells isolated from the small antral follicles of mice not primed with eCG. In contrast, eCG priming resulted in expression of LHR mRNA by mural granulosa cells, but there was no detectable expression by cumulus cells of eCG-primed mice (Fig. 2). To determine whether the low steady-state level of expression of LHR mRNA was related to the presence of oocytes, we ascertained whether microsurgical removal of oocytes from oocyte-cumulus cell complexes (OOX) promotes expression of the mural granulosa cell phenotype and loss of the cumulus cell phenotype. If it does, then OOX should induce

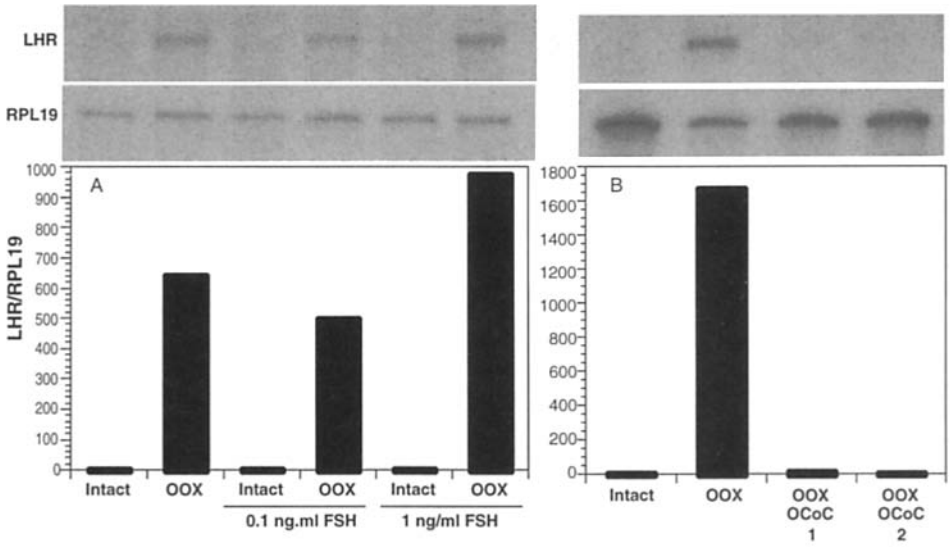

FIG. 3. **A**) Effect of oocytectomy (OOX) on the steady-state level of LHR mRNA expression by cumulus cells. There was little or no LHR mRNA expression detectable in intact complexes, but OOX promoted expression that was enhanced by FSH (1 ng/ml). **B**) Effect of oocyte coculture (OCoC) on the expression of LHR mRNA by OOX complexes. OOX complexes were cocultured with either 1 or 2 oocytes/μl throughout the culture period. All cultures received IBMX (100 μM) to maintain oocytes at the GV stage and received FSH (1 ng/ml) to enhance LHR mRNA expression as shown in **A**. Medium conditioned by cocultured oocytes completely suppressed LHR mRNA expression inducible by extirpation of the resident oocytes.

expression of LHR mRNA. As shown above, little or no LHR mRNA expression was detected in the intact oocyte-cumulus cell complexes (Fig. 3A). However, there was a dramatic elevation of the expression of LHR mRNA in the OOX complexes (Fig. 3A). Thus, cumulus cells can be induced to express a phenotypic characteristic of mural granulosa cells by OOX. The results suggest that oocytes suppress expression of LHR mRNA, a marker of the mural granulosa cell phenotype, in cumulus cells. This was tested further in the next experiment in which OOX complexes were stimulated with 1 ng/ml FSH in coculture with oocytes (either 1 or 2 oocytes/μl). The results, presented in Figure 3B, show that oocytes suppress expression of LHR mRNA below detectable levels in OOX complexes via a paracrine mechanism.

FSH-Induced Expression of LHR mRNA by Granulosa Cells Is Suppressed by Paracrine Factors from Fully Grown GV-Stage Oocytes

The effects of oocytes on FSH-induced steady-state levels of LHR mRNA in mural granulosa cells in vitro was tested. Mural granulosa cells from the small antral follicles of 22- to 24-day-old mice (not primed with eCG) were cultured in monolayers in 48-well dishes. Within 24 h, granulosa cells expressed clearly detectable levels of LHR mRNA in response to 1–5 ng/ml FSH in the presence of 100 μM IBMX (not shown). Maximum expression was observed by 48 h of culture. Addition of IBMX alone sometimes, but not always, promoted the expression of low but detectable levels of LHR mRNA (not shown). IBMX was added to maintain oocytes at the GV stage and was also added to cultures without oocytes for experimental balance. Coculture of 2 oocytes/μl (300 denuded GV-stage oocytes from antral follicles/150 μl of medium) suppressed the steady-state level of LHR mRNA by mural granulosa cells to below a detectable level (Fig. 4A). Moreover, coculture of 0.5 oocytes/μl (75 oocytes/150 μl) suppressed expression to approximately 20% of the control (FSH stimulated) level (Fig. 4A).

The cells of several of the mural granulosa cell layers appear organized as a pseudostratified epithelium [1]. Therefore, many of the mural granulosa cells probably contact the basal lamina. Others have shown that components of this extracellular matrix augment expression of the mural granulosa cell phenotype induced by gonadotropins [43, 44]. We therefore determined whether oocytes could prevent expression of this phenotype, as indicated by LHR mRNA expression, even in the presence of extracellular matrix components of basal lamina (ECL). Figure 4B illustrates that components of extracellular matrix augment expression of LHR mRNA by mouse mural granulosa cells in vitro, but even this matrix-enhanced expression of LHR mRNA was dramatically suppressed by paracrine signals from oocytes.

As shown above, mural granulosa cells from the preovulatory follicles of eCG-primed mice express LHR mRNA. In the next experiment, these mural granulosa cells already expressing LHR mRNA were isolated from preovulatory follicles 24 h after eCG priming. Small clumps of granulosa cells were incubated in drops of culture medium under paraffin oil, either in coculture with 2 oocytes/μl (GV-stage oocytes isolated from antral follicles) or not (control). As shown in Figure 5, there was a marked suppression of expression of LHR mRNA by mural granulosa cells cocultured with oocytes.

Production of LHR mRNA Suppressing Paracrine Factors by Oocytes Is Developmentally Regulated

The aim of the next series of experiments was to determine whether the secretion of LHR mRNA-suppressing factors by oocytes is developmentally regulated. In the first experiment, growing oocytes were isolated from preantral follicles and cocultured with FSH-stimulated granulosa cells. All cultures of granulosa cells from small antral follicles received both IBMX and 5 ng/ml FSH. There were three groups: granulosa cells cultured with 1) no oocytes, control; 2) fully grown oocytes from antral follicles, 2 oocytes/μl; 3) growing oocytes from preantral follicles, 4 oocytes/μl. The volume (and protein content) of oocytes from the preantral follicles is about half that of the oocytes from antral follicles. Therefore, the number of oocytes from preantral follicles was doubled to equalize the oocyte mass used to condition the medium [56]. After 48-h culture, oocytes were discarded and granulosa cells prepared for analysis of LHR mRNA. As shown in Figure 6A, oocytes from antral follicles suppressed FSH-induced expression of LHR mRNA to undetectable levels as described above. In contrast, oocytes from preantral follicles suppressed expression only to a steady-state level about half that of the control level. Thus, the ability of murine oocytes to secrete fac-

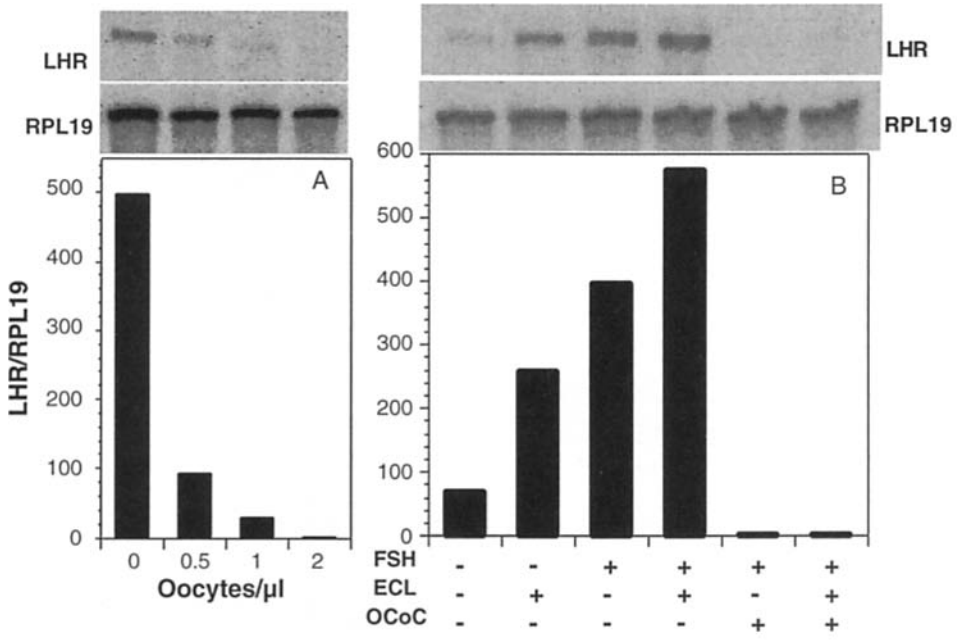

FIG. 4. **A**) Dose-dependent effect of oocytes on the steady-state level of LHR mRNA expression by granulosa cells. Approximately 10^5 granulosa cells were cultured in 48-well tissue culture dishes and stimulated with FSH (5 ng/ml) in the presence of IBMX. Granulosa cells were cocultured for 2 days with 0–2 oocytes/μl. Although significant suppression of LHR mRNA expression was observed with 0.5 oocytes/μl, a concentration of 2 oocytes/μl was required for complete suppression. **B**) Effect of FSH, components of extracellular matrix, and oocytes on the steady-state level of LHR mRNA in granulosa cells. The effect of extracellular matrix was determined by culturing the granulosa cells in wells coated with entactin, collagen IV, and laminin (ECL). The concentration of FSH was 5 ng/ml. The effect of oocytes on the steady-state expression of LHR mRNA was determined by coculturing granulosa cells with 2 fully grown GV-stage oocytes/μl. All groups were cultured for 2 days and contained 100 μM IBMX. Alone, both FSH and ECL increased the steady-state level of LHR mRNA, but the highest level of expression was when granulosa cells were treated with both FSH and ECL together. Nevertheless, coculture with oocytes (OCoC) completely abrogated the action of this combination of agonists on the steady-state level of LHR mRNA.

tor(s) that suppress LHR mRNA expression increases during oocyte growth and development.

In the second experiment, FSH-stimulated granulosa cells were cocultured with either GV-stage oocytes isolated from antral follicles, mature (metaphase II arrested) oocytes, two-cell-stage embryos, or blastocyst-stage embryos. All were cocultured at a concentration of 2/μl with granulosa cells. Mature oocytes suppressed the expression of LHR mRNA, but not to the same extent as GV-stage oocytes (Fig. 6B). In contrast, there was no suppression by two-cell-stage embryos, but, unexpectedly, some suppression by blastocyst-stage embryos was detected in every experiment (Fig. 6B).

Suppressive Effects of Oocytes on Granulosa Cell LHR mRNA Levels Is Partially Reversible

The next experiment was designed to determine whether the effect of oocytes in suppressing FSH-induced expression of LHR mRNA by mural cells is reversible. Mural granulosa cells from small antral follicles were cultured in ECL-coated 48-well dishes and stimulated with 5 ng/ml FSH in the presence of 100 μM IBMX. Some wells also contained 2 oocytes/μl. After 1-day culture, samples of the control (no oocytes) and oocyte-cocultured granulosa cells were assayed for expression of LHR mRNA. As expected, oocytes suppressed the expression of LHR mRNA (Fig. 7; lanes 1 and 4). After 1 day, oocytes were removed from parallel cultures and incubated for 1 or 2 additional days with fresh FSH and IBMX. Elevated expression of LHR mRNA was observed 1 day after removal of oocytes (Fig. 7, lane 5). Expression in granulosa cells 2 days after removal of oocytes was higher (Fig. 7, lane 6), but not equivalent to the level of expression of LHR mRNA in granulosa cells not cocultured with oocytes (Fig. 7, lane 3). Thus, although oocyte suppression of the steady-state level of LHR mRNA by mural granulosa cells was reversible, the level of expression achieved after reversal was not equivalent to that of granulosa cells not cocultured with oocytes.

Effect of Oocyte Paracrine Factors on Granulosa Cell Protein Synthetic Patterns

The data presented above show that oocytes secrete one or more paracrine factors that suppress the expression of LHR mRNA by oocytes. Expression of LHR mRNA was normalized to the expression of a housekeeping gene, RPL19. To further assess whether the suppression of LHR mRNA might reflect a generalized suppression of granulosa cell function, the activity of the granulosa cells was evaluated more globally by 2D-PAGE of ^{35}S-methionine-labeled proteins. Very few differences were detected by fluorography of the patterns of proteins synthesized by granulosa cells cultured with or without oocytes for 48 h (Fig. 8). Thus, paracrine factors from oocytes do not have a nonspecific suppressive effect on the overall pattern of protein synthesis by granulosa cells, though synthesis of undetected, less abundant, proteins may have been affected specifically.

DISCUSSION

Mural granulosa cells of preovulatory follicles express LHR and LHR mRNA, but expression of these molecules is low or undetectable in cumulus cells ([6–11] and the present study). The experiments presented here provide evidence of a mechanism that establishes this granulosa cell phenotypic heterogeneity and, therefore, the fundamental

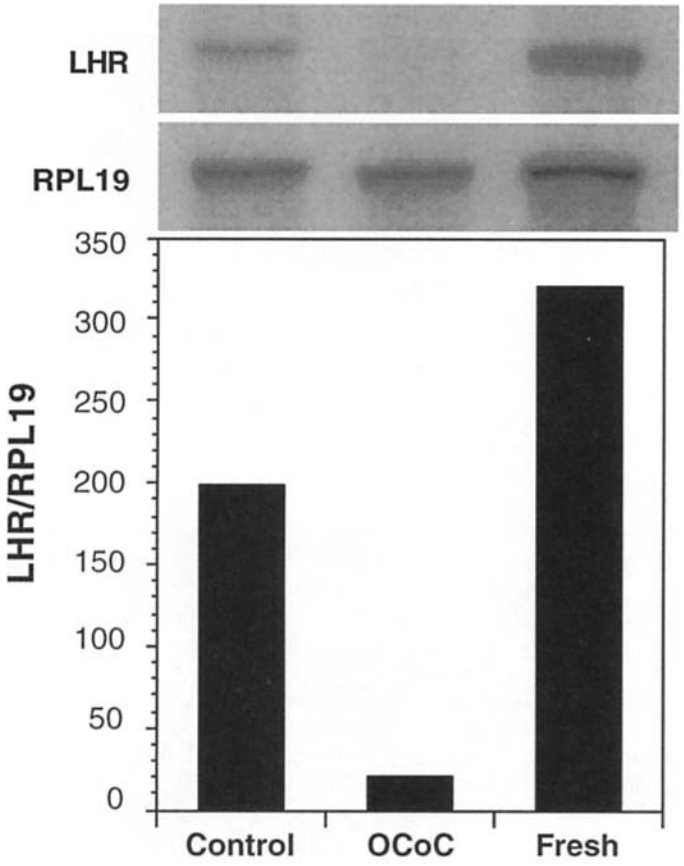

FIG. 5. Effect of oocytes on the steady-state level of LHR mRNA in mural granulosa cells. Mural granulosa cells were isolated from eCG-primed mice and cocultured with 2 oocytes/μl. LHR mRNA expression was high in freshly isolated (fresh), uncultured, mural granulosa cells, as shown also in Figures 1 and 2. Incubation of small clumps of these cells without oocytes (control) consistently resulted in decreased expression of LHR mRNA, but coculture with oocytes (OCoC) resulted in an almost complete suppression of expression. All cultured granulosa cells were treated with 100 μM IBMX. Thus, oocytes greatly decreased the steady-state level of expression of LHR mRNA in granulosa cells even when a high level of expression was already established in those cells.

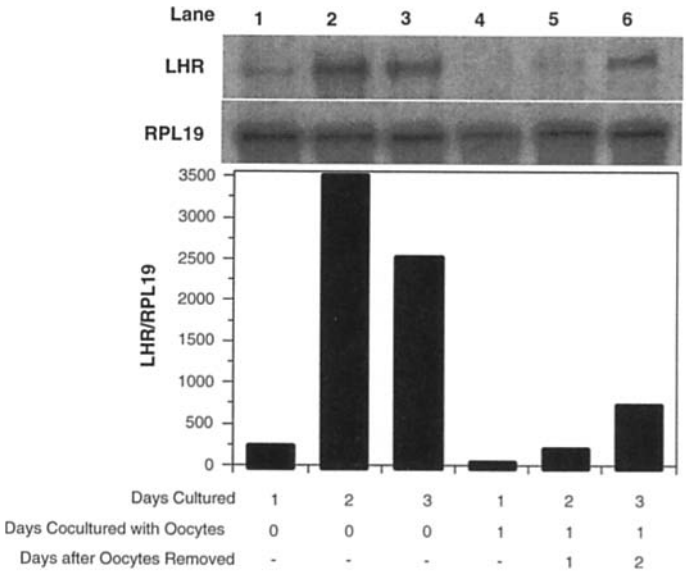

FIG. 7. Reversibility of oocyte suppression of LHR expression by granulosa cells. All groups of granulosa cells were isolated from unprimed mice and cultured in ECL-coated 48-well dishes, and were treated with FSH (5 ng/ml) and IBMX (100 μM). The concentration of oocytes was 2/μl. When granulosa cells were cultured without oocytes (lanes 1, 2, and 3), LHR mRNA was detectable after 1 day of culture; expression increased by 2 days of culture, but declined on Day 3. As expected, oocytes completely suppressed LHR mRNA expression; compare lanes 1 and 4. When the oocytes were removed after 1 day of coculture, the steady-state level of LHR mRNA expression was equivalent to that detected after 1 day of culture without oocytes; compare lanes 1 and 5. However, continued culture of granulosa cells for a second day after removal of oocytes did not produce a level of expression equivalent to 2 days of culture without any exposure to oocytes, although the level did continue to increase (compare lanes 2 and 6), showing that the effect of oocytes was at least partially reversible.

organization of preovulatory follicles. Central to this mechanism is the dominant influence of the oocyte on the pathway of differentiation of oocyte-associated granulosa cells. This influence is mediated by paracrine factors that suppress the expression of the mural granulosa cell phenotype as represented by the expression of LHR mRNA. The evidence supporting this mechanism includes the following.

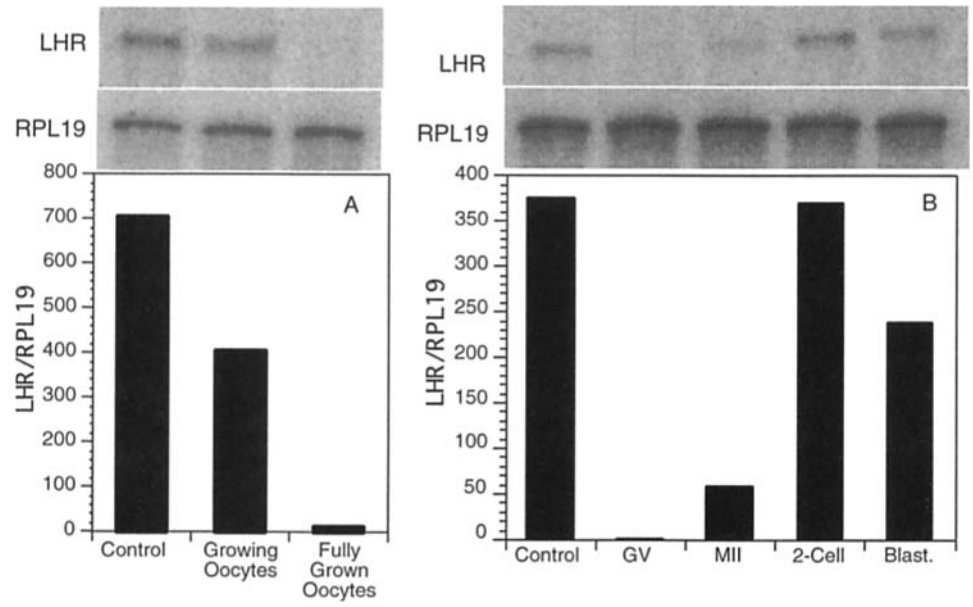

FIG. 6. The ability of oocytes to suppress the steady-state level of LHR mRNA by granulosa cells is developmentally regulated. All groups of granulosa cells were isolated from unprimed mice and then cultured in 48-well dishes and treated with FSH (5 ng/ml) and IBMX (100 μM) for 2 days, with or without coculture with denuded oocytes or preimplantation embryos. **A**) Growing oocytes isolated from preantral follicles suppressed the expression of LHR mRNA by cultured granulosa cells, but not to the same extent as fully grown oocytes isolated from antral follicles. The number of growing oocytes (n = 4 oocytes/μl) cocultured with granulosa cells was increased to be approximately equivalent to the volume of fully grown oocytes (n = 2 oocytes/μl). **B**) Although metaphase II-arrested oocytes (MII) suppressed the expression of LHR mRNA by granulosa cells, they did not suppress expression to the same extent as fully grown GV-stage oocytes. Two-cell-stage embryos did not suppress LHR mRNA expression by granulosa cells.

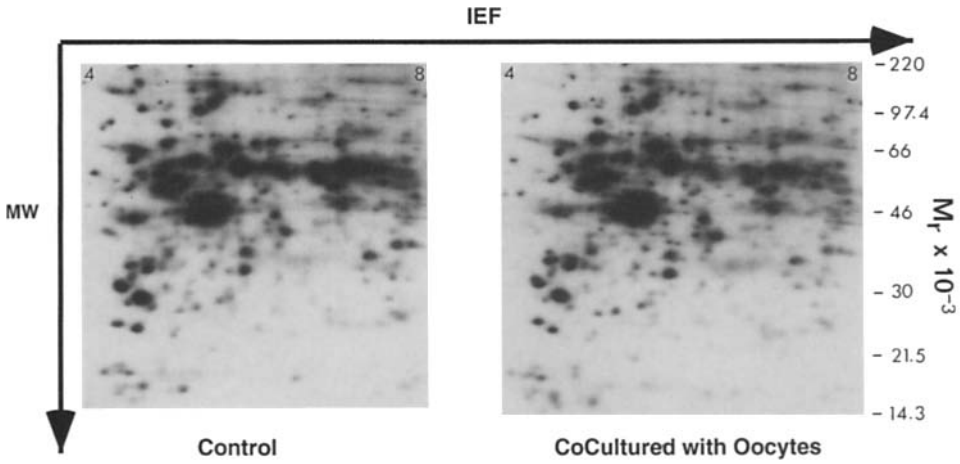

FIG. 8. Effect of oocytes on the pattern of proteins synthesized by granulosa cells. Granulosa cells were isolated from unprimed mice and cultured in 48-well dishes with FSH (5 ng/ml) and IBMX (100 μM) without (control) or with oocytes (2 fully grown oocytes/μl) for 2 days prior to labeling with ^{35}S-methionine. The remarkable similarity between the patterns of proteins resolved by 2D-PAGE shows that oocytes do not have a generalized nonspecific effect on granulosa cell function despite the ability of oocytes to dramatically suppress the steady-state level of LHR mRNA expression. The numbers in the upper left and right corners of the photographs indicate the approximate pH.

Cumulus cells expressed elevated steady-state levels of LHR mRNA when oocytes were microsurgically removed from oocyte-cumulus cell complexes, and paracrine factor(s) secreted by isolated oocytes prevented expression of LHR mRNA by cumulus cells after extirpation of the resident oocytes. Paracrine factor(s) secreted by isolated oocytes suppressed FSH-induced elevation of the steady-state level of LHR mRNA expression by mural granulosa cells isolated from small antral follicles even when expression of LHR mRNA was augmented by culturing the granulosa cells on components of basal lamina. Moreover, paracrine factor(s) secreted by oocytes suppressed the expression of LHR mRNA in differentiated granulosa cells already expressing elevated steady-state levels of these transcripts when isolated from the preovulatory follicles of eCG-primed mice.

The ability of murine oocytes to suppress FSH-induced expression of LHR mRNA by granulosa cells is dependent upon the stage of oocyte development. It appears that this ability increases with the stage of follicular development from which the oocytes were isolated. Growing oocytes isolated from preantral follicles were significantly less able to suppress LHR mRNA expression by granulosa cells than fully grown oocytes from antral follicles. Likewise, mature (metaphase II arrested) oocytes were less able to suppress LHR mRNA expression than GV-stage oocytes. Thus, the ability of the oocytes to suppress FSH-induced LHR mRNA expression is maximal when they are present in preovulatory follicles whose mural granulosa cells express LHR mRNA. Mouse cumulus cells developed LH receptors when cumulus expansion was stimulated with FSH in vitro [57]. However, in our study, cumulus cell LHR mRNA was not detected when intact complexes were stimulated with FSH. These differing results could be explained by the difference in maturation of the oocytes in the two studies. When LH receptors developed [57], the oocytes had resumed meiosis, whereas LHR mRNA was not detected in the present study in which the resumption of meiosis was prevented by IBMX. Taken together, the results indicate that maximal suppression of LHR mRNA, and the receptors themselves, is dependent upon the maintenance of the GV stage.

Two-cell-stage embryos did not suppress FSH-induced LHR mRNA expression by granulosa cells. Moreover, incubation of isolated oocytes with cultured granulosa cells caused very few changes in the pattern of protein synthesis detectable by 2D-PAGE and fluorography of ^{35}S-methionine-labeled granulosa cell proteins. Thus, oocyte suppression of LHR mRNA expression is specific in terms of both the suppressing cell type and the effects on granulosa cells. It is possible that oocytes induced changes in the synthesis of less abundant proteins not detected by this method and these could be changes important for the development of the cumulus cell phenotype.

FSH treatment of relatively undifferentiated granulosa cells, isolated from the small antral follicles of mice not primed with eCG, promotes expression of LHR mRNA regardless of whether the granulosa cells contact basal lamina. Therefore, the "default" program of granulosa cell differentiation is probably the program that leads to expression of the mural granulosa cell phenotype, as indicated by the expression of LHR mRNA. However, paracrine signals from oocytes abrogate this default pathway of differentiation in the granulosa cells closely associated with the oocytes, i.e., the cumulus cells. Such signals may not only suppress the mural granulosa cell phenotype but also promote differentiation of the cumulus cell phenotype. Experiments are underway to test this hypothesis, which is supported by the observation that mural granulosa cells in monolayer culture synthesize hyaluronic acid, a characteristic of cumulus cells, when stimulated with FSH in oocyte-conditioned medium [24].

The cells of several of the mural granulosa cell layers appear organized as a pseudostratified epithelium [1]. Therefore, many of the mural granulosa cells probably contact the basal lamina. The granulosa cells closest to the basal lamina probably have more contact with it than those closer to the antrum. Components of basal lamina augment expression of the mural granulosa cell phenotype. For example, culturing human or rat granulosa cells on dishes coated with an extracellular matrix derived from bovine corneal epithelium increased the production of LHR and progesterone in response to hCG compared with that by granulosa cells cultured on uncoated dishes [43, 44]. In the present study, some groups of granulosa cells were cultured in wells coated with components of basal lamina, i.e., entactin, collagen IV, and laminin (ECL). Even without the addition of FSH, the steady-state level of LHR mRNA was increased in granulosa cells cultured on ECL. Moreover, ECL augmented the stimulatory action of FSH on the level of LHR mRNA expression. Therefore, our results, together with those presented by others [43, 44], indicate that components of basal lamina enhance the expression of the mural granulosa cell phenotype. Nevertheless, despite this enhancement of differentiation of the mural granulosa cell phenotype by components of basal lamina, paracrine factors

secreted by oocytes were able to abrogate this pathway of differentiation. This observation suggests that oocytes produce dominant factors that can determine the pathway of granulosa cell differentiation.

In the experiments presented here, oocytes were cocultured with granulosa cells because the suppressing factor is apparently very labile and the continued presence of its source, the oocytes, was required for sustaining maximum activity (experiments not shown). Although there was probably some contact between the zonae pellucidae of the oocytes and some of the cultured granulosa cells, direct contact of oocytes with granulosa cells was extremely limited, if it occurred at all. Thus, it is concluded that the nature of the communication between the oocytes and granulosa cells that resulted in suppression of FSH-stimulated expression of LHR mRNA was via labile paracrine factor(s). It is not known whether the gradient of expression of LHR mRNA in mural granulosa cells in vivo is due to a diffusion gradient of this factor from the oocyte outward through mural granulosa cells, to the extent of contact between granulosa cells and basal lamina, or to a combination of both influences. The range of action of suppressing factor(s) from oocytes may actually be limited to cumulus cells because of the lability of the factor(s).

Oocyte secretions do not prevent FSH-stimulated LHR mRNA expression by inactivation of FSH. This conclusion is deduced from previous studies in which it was shown that one or more paracrine factors secreted by oocytes are required for FSH-induced cumulus expansion. These paracrine factors alone are insufficient to stimulate cumulus expansion; positive stimulation by FSH is also required [22–25]. Since the positive action of FSH is required to stimulate the expansion of OOX complexes in the presence of oocyte secretions, inactivation of FSH by oocyte secretions cannot occur. Moreover, medium removed from oocyte-granulosa cell cocultures at the termination of the experiments presented here still contained active FSH, as demonstrated by its ability to stimulate cumulus expansion after intact complexes and serum were added (data not shown). In addition to showing that oocyte secretions do not inactivate FSH, these experiments also indicate that the secretions do not affect the binding of FSH with granulosa cells. Thus, the action of the paracrine factor(s) preventing FSH-induced LHR mRNA expression is probably downstream of the gonadotropin-receptor interaction.

In conclusion, the results presented here suggest a mechanism for establishing the fundamental organization of granulosa cells in preovulatory follicles. The "default" program of differentiation is that of the mural granulosa cell phenotype in preovulatory follicles; the expression of LHR mRNA and LHR is probably a marker of this phenotype. Oocytes can preclude this pathway of differentiation via the secretion of paracrine regulatory factors. Thus, the granulosa cells closely associated with oocytes do not express the mural granulosa cell phenotype. There is probably a functional rationale for differential gene expression by mural granulosa cells and cumulus cells. Oocytes are highly dependent upon interactions with their companion somatic cells for a variety of processes, i.e., oocyte growth, maintenance of meiotic arrest, induction of maturation, etc., as reviewed in several papers [21, 58, 59]. The mural granulosa cell phenotype may be deficient in, or antagonistic to, the support of some aspects of oocyte development. It may not be the expression of LHR per se that is deleterious to oocyte development. Assuming that the expression of LHR is just one part of a larger program for differentiation and function of mural granulosa cells, it is possible that other aspects of that program may not be compatible with optimal oocyte development. Therefore, a mechanism may have evolved to influence the pathway of granulosa cell differentiation of the granulosa cells closely associated with oocytes in a way that would assure their own normal development. This mechanism may entail the secretion by oocytes of paracrine signals capable of suppressing the mural granulosa cell phenotype as shown in this study and, perhaps, by promoting development of the cumulus cell phenotype. Errors in this regulatory mechanism, whether instigated by oocytes or granulosa cells, may result in the production of defective oocytes or abnormal follicular development.

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