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ABSTRACT We investigated the effects of theca cells or FSH on granulosa cell differentiation and steroid production during bovine early follicular growth, using a co-culture system in which granulosa and theca cells were cultured on opposite sides of a collagen membrane. Follicular cells were isolated from early antral follicles (2–4 mm) that were assumed to be in gonadotropin-independent phase and just before recruitment into a follicular wave. Granulosa cells were cultured under serum-free conditions with and without theca cells or recombinant human FSH to test their effects on granulosa cell differentiation. Messenger RNA levels for P450 aromatase (aromatase), P450 cholesterol side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), LH receptor (LHr), and steroidogenic acute regulatory protein (StAR) in granulosa cells were measured by real-time quantitative RT-PCR analysis. FSH enhanced aromatase mRNA expression in granulosa cells, but did not alter estradiol production. FSH also enhanced mRNA expression for P450scc, LHr, and StAR in granulosa cells, resulting in an increase in progesterone production. In contrast, theca cells enhanced aromatase mRNA expression in granulosa cells resulting in an increase in estradiol production. Theca cells did not alter progesterone production and mRNA expression in granulosa cells for P450scc, 3β-HSD, LHr, and StAR. The results of the present study indicate that theca cells are involved in both rate-limiting steps in estrogen production, i.e., androgen substrate production and aromatase regulation, and that theca cell-derived factors regulate estradiol and progesterone production in a way that reflects steroidogenesis during the follicular phase of the estrous cycle.

Key Words: aromatase; estrogen; follicle; granulosa cells; theca cells

INTRODUCTION

Although it is true that ovarian follicular growth is under the endocrine control of pituitary gonadotropins, we also know now that early follicular growth is independent of gonadotropins and under the control of local autocrine/paracrine systems (Gougeon, 1996; Driancourt et al., 2000; McGee and Hsueh, 2000; Nilsson and Skinner, 2001; Richards, 2001; Vitt and Hsueh, 2001; Monget et al., 2002). Recently, bovine follicular development has been classified into three phases according to its dependency upon FSH and/or LH (Ginther et al., 1996; Webb et al., 1999). They are: (1) basal growth through primordial, primary, secondary, and preantral follicles is believed to be absolutely independent of gonadotropins, (2) growth of preantral and early antral follicles up to 4 mm in diameter is thought to be somewhat responsive to FSH, but independent of gonadotropins. Actually, bovine follicles can grow to this size even when gonadotropin secretion is suppressed by hypothalamic stalk transection (Awotwi et al., 1984) or by administration of a GnRH agonist (Campbell et al., 1995; Gong et al., 1996), and (3) antral follicular growth beyond 4 mm is considered to be critically dependent on gonadotropins and includes recruitment (a cohort of follicles 4–5 mm in diameter emerges and begins to grow), selection (one follicle 8–9 mm in diameter develops from a wave of growing follicles and becomes the only follicle with ovulatory capacity), and ovulation.

A growing body of evidence indicates that steroidal and nonsteroidal factors produced by granulosa and theca cells influence differentiation and proliferation of both cell types on opposite sides of a basement membrane (Gougeon, 1996; Driancourt et al., 2000; McGee and Hsueh, 2000; Nilsson and Skinner, 2001; Richards, 2001; Vitt and Hsueh, 2001; Monget et al., 2002), which suggests the importance of granulosa-theca cell communication during folliculogenesis. To study the importance of the paracrine mechanisms between the theca

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and granulosa cells, we have developed a novel in vitro co-culture system (Kotsuji et al., 1990). By culturing with granulosa cells and theca cells on opposite sides of a collagen membrane, this co-culture model is designed to mimic the in vivo ovarian cellular environment (Fig. 1). In our culture system, the co-cultured cells have similar morphological and functional characteristics as follicular cells do in vivo (Kotsuji et al., 1990; Kotsuji and Tominaga, 1994). Reflecting the observation mentioned above, we have shown that the granulosa cells continue their estradiol production in our culture system (Yada et al., 1999), though bovine granulosa cells rapidly luteinize and lose the ability to produce estradiol in most cell culture systems. Using our model, we have demonstrated that theca cells play important roles on proliferation (Kotsuji et al., 1990; Kotsuji and Tominaga, 1994), differentiation (Kotsuji et al., 1990; Kotsuji and Tominaga, 1994; Yada et al., 1999), and apoptosis (Tajima et al., 2002) of granulosa cells during follicular maturation.

Follicular development is characterized by proliferation and functional differentiation of granulosa and theca cells. In situ hybridization studies indicated that expression of mRNAs for the gonadotropin receptors (FSH receptor [FSHr] and LH receptor [LHr]), key steroidogenic enzymes (P450 aromatase [aromatase], P450 cholesterol side chain cleavage [P450scc], 3β-hydroxysteroid dehydrogenase [3β-HSD]), and steroidogenic acute regulatory protein (StAR) in granulosa cells is dependent on the stage of follicular development, and that granulosa cell differentiation is characterized by the expression of these genes (Bao and Garverick, 1998; Webb et al., 1999). Prior to follicular recruitment, granulosa cells from early antral follicles (<4 mm) expressed FSHr mRNA but not the genes of steroidogenic enzymes. Expression of mRNAs for aromatase and P450scc was first detected in granulosa cells in the majority of early recruited follicles 4–6 mm in diameter. Therefore, the recruitment seems to be closely associated with the initiation of mRNA expression for aromatase and P450scc in granulosa cells. Expression of LHr and 3β-HSD mRNA in granulosa cells was first detected around the time of selection (8–9 mm in diameter) of the dominant follicle, indicating that the selection is associated with initiation of mRNA expression for LHr and 3β-HSD in granulosa cells. Tilly et al. (1992) reported that spontaneous luteinization of the granulosa cells after degeneration of the oocyte (i.e., follicular atresia) might stimulate P450scc, 3β-HSD, and StAR mRNA expression in granulosa cells in a similar way to the luteinization caused by the preovulatory LH surge. Thus, changes in gene expression are likely to be important to recruitment, selection, ovulation, and atresia in ovarian follicles.

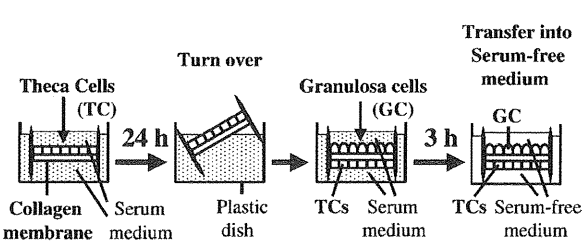


Fig. 1. Co-culture of granulosa and theca cells on a collagen membrane.

In recent years, it has generally been accepted that the local autocrine/paracrine systems play a central role during the gonadotropin-independent follicular growth. Therefore, we hypothesize that theca cells may stimulate the differentiation of granulosa cells at the early stage of follicular development. In this study, in order to test this hypothesis, we investigated how theca cells control the differentiation of granulosa cells at early antral follicles using our in vitro co-culture system.

MATERIALS AND METHODS

Preparations of Granulosa and Theca cells

Monotocous species, such as cattle, horses, and primates, are particularly useful models for studying mechanisms of follicular recruitment, selection, and dominance (Ginther et al., 1996; Fortune et al., 2001). In the present study, bovine ovaries were collected from heifers at a local abattoir and were transported to the laboratory in ice-cold buffered salt solution. The stage of estrous cycle was determined morphologically, as previously described by Ireland et al. (1980) and only ovaries with a regressing corpus luteum were used for this study. Both granulosa and theca cells were collected from early antral follicles (2–4 mm in diameter) that were assumed to be in the gonadotropin-independent phase and in the recruitable state (i.e., just before recruitment into a follicular wave for further development) (Ginther et al., 1996; Bao and Garverick, 1998; Webb et al., 1999).

Follicular cells were prepared from the ovaries under sterile conditions as described previously (Kotsuji and Tominaga, 1994). Briefly, granulosa cells were harvested by aseptic needle aspiration from follicles and washed three times in a culture medium consisting of Waymouth MB 752/1 medium (Invitrogen Corp., Carlsbad, CA), Hanks solution (Nissui Pharmaceutical Co., Tokyo, Japan), and fetal calf serum (FCS; 6:3:1, v:v:v) (Invitrogen) supplemented with streptomycin (100 μg/ml; Invitrogen) and penicillin (100 U/ml; Invitrogen). Washed cells were resuspended in culture medium, and cell viability was estimated at 30–42% by trypan blue exclusion.

For theca cell preparation, follicles with clear surfaces were cut into halves, and the theca interna layer was removed with fine forceps. Granulosa cells, together with a part of the theca cell layer, were removed by scraping with a scalpel under stereomicroscopy. The thin theca cell layer thus obtained was minced and then treated with a Hanks-Hepes buffer containing collagenase (2,150 U/ml, type 1; Sigma Chemical Co., St. Louis, MO), and DNase (100 U/ml; Sigma), 0.4% (v/v) bovine serum albumin (Sigma), and 0.2% (w/v) glucose (pH 7.4).

Cell dissociation was allowed to continue for 45 min at 37°C with continuous stirring at 800 rpm and with 0.25% (w/v) pancreatin (Sigma) in a Hanks-Hepes buffer for 7 min. Dispersed cells were washed three times and cell viability was estimated at 90–95% by trypan blue exclusion test.

Co-Culture of Granulosa and Theca cells on Collagen Membrane

Co-culture of granulosa and theca cells on a collagen membrane was prepared as previously described (Kotsuji and Tominaga, 1994) with minor modifications (Fig. 1). The membrane (thickness, 70 μm; Koken Co., Tokyo, Japan) was made of type 1 collagen and had an area of 8 cm^2. A supporting apparatus, to which a membrane was attached, was placed in a 6 cm plastic dish. Apical and basal chambers were separated, and materials less than 12.5 kDa were permeable through the collagen membrane.

Theca cells (5×10^5 or 1×10^6 viable cells per dish) were seeded onto a type 1 collagen membrane immediately after preparation in 1.5 ml culture medium consisting of Waymouth MB 752/1 medium, Hanks solution, and 10% FCS (6:3:1, v:v:v), supplemented with streptomycin (100 μg/ml) and penicillin (100 U/ml). To allow the theca cells to attach to the collagen membrane, cultures were maintained for 24 hr in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The membrane was then turned over and freshly prepared granulosa cells (1×10^6 viable cells per dish) were seeded on the opposite side (co-cultured). Following an additional 3 hr culture in a medium containing 10% FCS to facilitate the attachment of granulosa cells to the membrane, the medium was changed to serum-free Ham F-12 medium (Nissui) supplemented with insulin (2 μg/ml), transferrin (10 μg/ml), and testosterone (10^{-8} mol/L, as an aromatizing substrate) and the cells were cultured for up to 48 hr.

Experimental Design

To determine the effect of theca cells or FSH on granulosa cell differentiation, we prepared three groups. In GC group (as control), freshly prepared granulosa cells (1×10^6 viable cells per dish) alone were seeded on one side of a collagen membrane, and cultured for 3 hr under serum supplemented conditions to allow granulosa cells attachment onto the membrane. The cells were then transferred into serum-free Ham F-12 supplemented with insulin, transferrin, and testosterone, and cultured for up to 48 hr. In GC + FSH group, granulosa cells (1×10^6 viable cells per dish) alone were cultured on one side of a membrane under serum supplemented conditions for 3 hr. The cells were then transferred into serum-free Ham F-12 supplemented with insulin, transferrin, and testosterone, and treated with increasing concentrations of recombinant human FSH (0.01–1 IU/ml; Gonal-F; Serono, Geneva, Switzerland) for 48 hr. In GC + TC group, granulosa cells were cultured together with theca cells (5×10^5 or 1×10^6 viable cells) as described above.

The medium was collected from the apical and basal chambers separately and stored at −20°C for steroid assays. Cultured granulosa cells in each of the individual membranes were removed by trypsin treatment (1% trypsin and 0.2% EDTA in a 0.01 M phosphate buffer, pH 7.4) at 37°C for 5 min. Total cellular RNAs were extracted by the guanidium acid-isothiocyanate-phenol-chlorform method using TRIzol (Invitrogen), quantified by measuring the absorbance at 260 nm and stored at −80°C, pending analysis.

Steroid Assays

Estradiol and progesterone were measured in the culture media from the granulosa cell side by a double-antibody RIA (^{125}I). All samples were analyzed in the same assays, and the intra-assay coefficients of variation for estradiol and progesterone were 7.3 and 6.4%, respectively. Our preliminary study showed that theca cells alone produced negligible amounts of progesterone (<5% of that by granulosa cells), and did not produce estradiol (data not shown). This suggests that granulosa cells are the primary source of these hormones under these experimental conditions.

RT-PCR

RT-PCR analyses for bovine aromatase, P450scc, 3β-HSD, LHr, StAR, and 36B4 were performed on total RNAs from cultured granulosa cells using specific primers. 36B4 codes for acidic ribosomal phosphoprotein and is considered to be an efficient endogenous control gene, as previously described by Mazerbourg et al. (2001). 36B4 was measured to correct the differences in RNA concentration between samples. Bovine 36B4 genes were partially sequenced to design the specific primers presented in Table 1.

Our preliminary study showed that granulosa cells cultured with and without FSH or theca cells expressed negligible level of 17β-hydroxysteroid dehydrogenase, and did not express 17a-hydroxylase/17, 20-lyase mRNA (data not shown).

TABLE 1. Primers Used for Real-Time Quantitative RT-PCR

36B4	
Forward primer	GGC GAC CTG GAA GTC CAA CT
Reverse primer	GGA TCT GCT GCA TCT GCT TG
Aromatase	
Forward primer	GCC CAT GGT GAC CAT CTG T
Reverse primer	CAC ATA GCC CAA GTC ATT GCA
P450scc	
Forward primer	CTT CAT CCC ACT GCT GAA TCC
Reverse primer	GGT GAT GGA CTC AAA GGC AAA
3β-HSD	
Forward primer	GCC CAA CTC CTA CAG GGA GAT
Reverse primer	TTC AGA GCC CAC CCA TTA GCT
LH receptor	
Forward primer	TGG CTG GGA TTA TGA CTA TGG TT
Reverse primer	ATT TCC CGT GAT GGC TAG GAT A
StAR	
Forward primer	CCC AGC AGA AGG GTG TCA TC
Reverse primer	TGC GAG AGG ACC TGG TTG AT

In each case, RNAs were reverse transcribed in a final volume of 40 μl solution containing 1× First-Strand Buffer (3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl, pH 8.3), 500 μM each deoxynucleotide triphosphate (dNTP), 10 mM dithiothreitol, 200 U of SuperScript III RNase H free reverse transcriptase (Invitrogen), 200 ng random hexamers, and 2 μg total RNA. The target cDNAs of granulosa cells were amplified for 25 cycles (P450scc, 3β-HSD, 36B4) or 35 cycles (aromatase, LHr, StAR) (94°C for 20 sec, 60°C for 30 sec, and 72°C for 60 sec), using dNTP (0.2 mM) and 1.5 U of TaKaRa Ex Taq (Takara Shuzo Co., Kyoto, Japan) in a thermal cycler. Aliquots of PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining.

Real-Time RT-PCR

Real-time quantitative PCR analyses for bovine aromatase, P450scc, 3β-HSD, LHr, and StAR were performed on total RNAs from cultured granulosa cells, using an ABI PRISM 7700 sequence detection system instrument and software (PE Applied Biosystems, Foster City, CA). The analyses were performed using specific primers presented in Table 1. Each transcript level of target genes (aromatase, P450scc, 3β-HSD, LHr, and StAR) was normalized on the basis of the level of transcripts for the constitutive housekeeping gene product 36B4.

In each case, RNAs were reverse transcribed in a final volume of 20 μl solution containing 1× First-Strand Buffer (3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl, pH 8.3), 500 μM each dNTP, 10 mM dithiothreitol, 100 U of SuperScript II RNase H reverse transcriptase (Invitrogen), 100 ng random hexamers, and 1 μg total RNA. Amplification reaction was then performed using the SYBR Green PCR Master Mix kit (PE Applied Biosystems). The thermal cycling conditions were comprised of an initial denaturation step at 95°C for 10 min and 40 cycles at 95°C for 15 sec, and 60°C for 1 min. Levels of aromatase, P450scc, 3β-HSD, LHr, and StAR message were expressed as ratios to 36B4 mRNA values.

Statistical Analysis

Each study was repeated three times on different days. Data are presented as the mean ± SEM of the three experiments, each with three replicate culture dishes. ANOVA statistical analysis was performed. When a significant effect was detected ($P < 0.05$), Tukey–Kramer test was used for intergroup comparison. All statistical analysis was performed using a statistical analysis program (Stat View version 5.0; SAS Institute, Inc., Cary, NC).

RESULTS

Granulosa Cell Proliferation

To determine the effect of theca cells or FSH on granulosa cell proliferation, isolated granulosa cells were cultured with and without FSH or theca cells in serum-free medium for up to 48 hr, and granulosa cell numbers were determined with a hemacytometer. There was no significant difference in the number of granulosa cells among GC, GC + FSH, and GC + TC groups (data not shown).

Steroid Production by Granulosa Cells

To determine the effect of theca cells or FSH on steroid production by granulosa cells, isolated granulosa cells were cultured with and without FSH or theca cells in serum-free medium for up to 48 hr, and estradiol and progesterone in the culture media were measured.

FSH stimulation caused a dose-related increase in progesterone production by granulosa cells during 24 hr culture ($P < 0.05$) (Fig. 3), but it did not alter estradiol production even after 48 hr culture (Fig. 2). In contrast, co-culturing with theca cells significantly increased estradiol production by granulosa cells after 24 hr culture ($P < 0.05$) dependent on the theca cell number (Fig. 2), whereas it had no effect on progesterone production during 48 hr culture (Fig. 3).

Expression of Steroidogenic Enzymes, LH Receptor, StAR in Cultured Granulosa Cells

Granulosa cell differentiation is characterized by the induction of gene expression for the steroidogenic enzymes, LHr and StAR. To determine the effect of theca cells or FSH on granulosa cell differentiation, the mRNA levels for aromatase, P450scc, 3β-HSD, LHr, and StAR in the cultured granulosa cells were measured by conventional RT-PCR (Fig. 4A) and real-time quantitative RT-PCR (Fig. 4B) assays. These data were normalized by the levels of internal control, 36B4.

As shown in Figures 2 and 3, steroid production by granulosa cells reached the maximal level during 24 hr culture. In the preliminary RT-PCR study, a significant increase in mRNA expression of steroidogenesis related enzymes or proteins was observed by 0.1 IU/ml FSH and 5×10^5 theca cells under these experimental conditions (data not shown). Thus, further real-time RT-PCR assays were done using granulosa cells cultured with and without 0.1 IU/ml FSH or 5×10^5 theca cells in serum-free medium for 24 hr.

Figure 4A and B show that FSH significantly enhanced the mRNA levels for aromatase, P450scc, LHr, and StAR ($P < 0.05$), but not of 3β-HSD in granulosa cells. Theca cells also significantly increased aromatase mRNA expression ($P < 0.05$), while they did not alter P450scc, 3β-HSD, LHr, and StAR mRNA contents.

DISCUSSION

It is thought that, in vivo, FSH induces estrogen production by the granulosa cells of healthy antral follicles during the follicular phase. In the present in vitro study, recombinant human FSH enhanced aromatase mRNA expression in granulosa cells, but did not alter estradiol production. Our results are similar to those of previous studies that report that recombinant human FSH (Couzinet et al., 1988) or highly purified urinary FSH (Shoham et al., 1991) preparations were insufficient to induce optimal estradiol production in women with hypogonadotropic

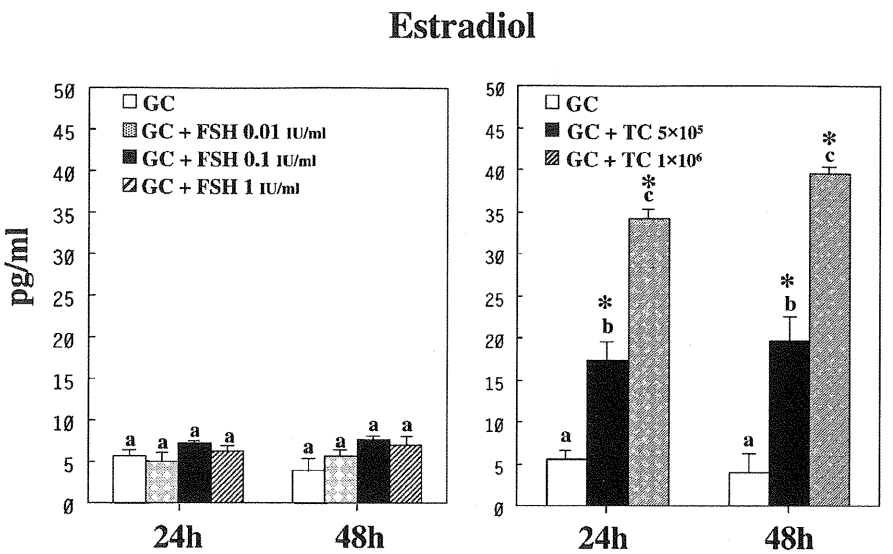


Fig. 2. Effect of FSH or theca cells on estrogen production by granulosa cells. Granulosa cells (1×10^6 viable cells per dish) were cultured (48 hr) with and without FSH (0.01–1 IU/ml) or theca cells (5×10^5 or 1×10^6 viable cells per dish) in serum-free medium, and the concentrations of estradiol in the culture media were measured by RIA. Data are the mean ± SEM of three different experiments with three replicate culture dishes. $^*P < 0.05$ versus GC.

hypogonadism, and that pure FSH stimulated growth of medium follicles but failed to increase serum estradiol concentrations in gonadotropin-releasing hormone immunized heifers (Crowe et al., 2001). We also demonstrated that FSH enhanced mRNA expression for P450scc, LHr, and StAR in granulosa cells, results in an increase in progesterone production. However, in vivo, progesterone production stimulated by FSH is not detected until the periovulatory period. These results suggest that although FSH promotes granulosa cell differentiation of early antral follicles in vitro, the stimulation of steroidogenesis by FSH may lead to the production of progesterone (i.e., premature luteinization), but not of estrogen.

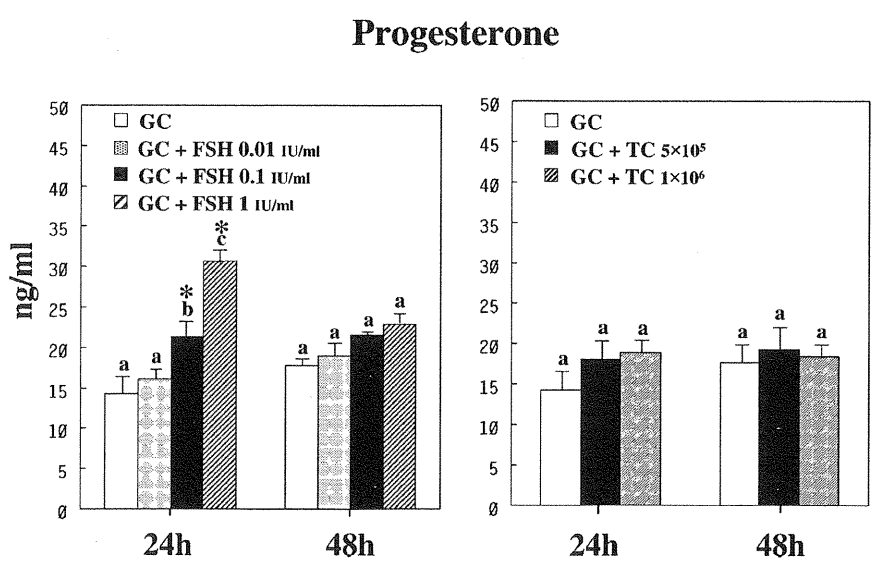
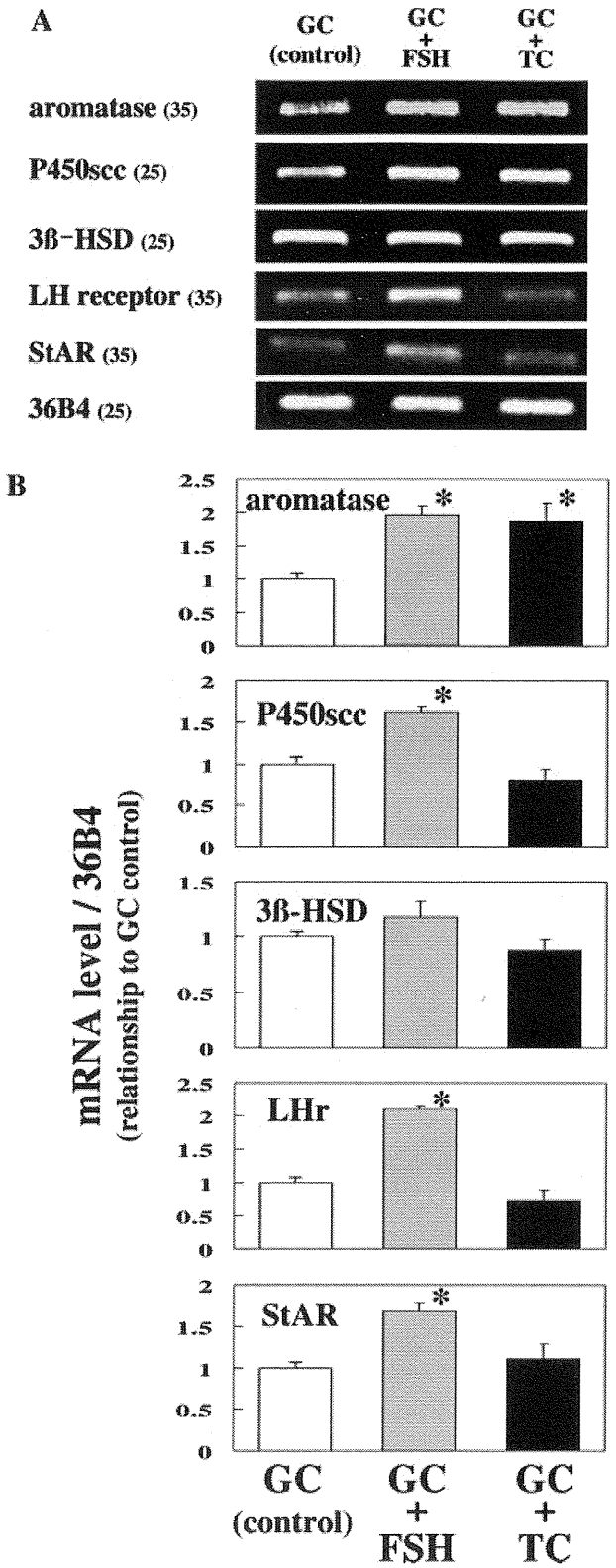


Fig. 3. Effect of FSH or theca cells on progesterone production by granulosa cells. Granulosa cells (1×10^6 viable cells per dish) were cultured (48 hr) with and without FSH (0.01–1 IU/ml) or theca cells (5×10^5 or 1×10^6 viable cells per dish) in serum-free medium, and the concentrations of progesterone in the culture media were measured by RIA. Data are the mean ± SEM of three different experiments with three replicate culture dishes. $^*P < 0.05$ versus GC.



In contrast, theca cells enhanced aromatase mRNA expression and increased estradiol production in granulosa cells. On the other hand, theca cells did not alter progesterone production and mRNA expression for P450scc, 3β-HSD, LHr, and StAR in the granulosa cells. Accordingly, theca cells must be involved in regulating the granulosa cell differentiation at early antral follicles. Our results suggest that theca cells differentially regulate estradiol and progesterone production by granulosa cells in a way that reflects steroidogenesis during the follicular phase of the normal estrous cycle.

What is the physiological significance of these theca cell activities? In cattle, follicular recruitment is considered to be gonadotropin-dependent and occurs when antral follicles grow larger than 4 mm in diameter (Ginther et al., 1996; Webb et al., 1999). However, the mechanisms by which recruitable follicles (<4 mm in diameter) grow to the recruitment stage (4 mm in diameter) and shift to FSH dependent development, remains unknown. In the present study, theca cells, but not FSH, enhanced aromatase mRNA expression and estradiol production in granulosa cells of the recruitable follicle. Richards (1980) reported that estradiol enhances granulosa cell responsiveness to FSH in rats. Segaloff et al. (1990) reported that estradiol is required for the induction of LHr on granulosa cells of rodents. Accordingly, locally produced estradiol may contribute in a coordinated manner to the intraovarian regulation of follicular development and seems to be essential for obtaining gonadotropin dependency. Therefore, we propose that theca cells promote granulosa cell responsiveness to FSH by enhancing the expression of aromatase mRNA and estradiol production in these cells, which, in turn, results in recruitable follicles becoming dependent on gonadotropins.

Numerous follicular factors, such as steroids, growth factors, cytokines, and extracellular matrix molecules have been shown to modulate follicular cells in a paracrine fashion, and responsiveness of the granulosa cells to these factors appears to differ with respect to species, developmental stage, and culture conditions (Gougeon, 1996; Driancourt et al., 2000; McGee and Hsueh, 2000; Nilsson and Skinner, 2001; Richards, 2001; Vitt and Hsueh, 2001; Monget et al., 2002). In our system, factors with relatively low molecular weight derived from the theca cells may play central roles

Fig. 4. Effect of FSH or theca cells on mRNA expression in granulosa cells. Granulosa cells (1×10^6 viable cells per dish) were cultured (24 hr) with and without FSH (0.1 IU/ml) or theca cells (5×10^5 viable cells per dish) under serum-free conditions. **A**: Conventional RT-PCR analyses for bovine aromatase, P450scc, 3β-HSD, LHr, and StAR were performed on total RNAs from the granulosa cells. The PCR cycles used for each factor are shown in parenthesis. **B**: Real-time quantitative RT-PCR analyses were performed using an ABI PRISM 7700 sequence detection system instrument and software (PE Applied Biosystems). Each transcript level of target genes (aromatase, P450scc, 3β-HSD, LHr, and StAR) was normalized on the basis of the level of transcripts for the constitutive housekeeping gene product 36B4. Data are expressed as relationship to control (untreated GC) and are presented the mean ± SEM of three different experiments. *$P < 0.05$ versus GC.

because only the materials less than 12.5 kDa can pass through the collagen membrane.

It is known that several growth factors expressed in theca cells stimulate the aromatase expression and estradiol production in granulosa cells, including insulin-like growth factors (IGF-I and IGF-II) (Adashi et al., 1985; Erickson et al., 1989; Christman et al., 1991; Yong et al., 1992; Mason et al., 1993; Hynes et al., 1996; Zhou et al., 1997; Spicer and Chamberlain, 1998; Monget and Bondy, 2000), transforming growth factor beta (TGF-β) (Ying et al., 1986; Bendell and Dorrington, 1988), and bone morphogenetic protein-4 (BMP-4) and BMP-7 (Shimasaki et al., 1999; Lee et al., 2001). However, none of these growth factors can directly regulate aromatase expression nor estradiol production by itself, and what they do is to enhance FSH-induced aromatase expression and estradiol production. Thus, our results showing theca cells stimulate aromatase mRNA expression and estradiol production in granulose cells without FSH seem to be unique.

In the present study, although culture media were supplemented with 10^{-8} M testosterone as an estrogen precursor beforehand, it is possible that an additional supply of androgen produced by theca cells plays an important role in stimulating aromatase expression and estradiol production in granulosa cells. Actually, in the preoptic area, testosterone has been shown to upregulate aromatase activity directly (Barthazart et al., 1990). The mechanisms of cell communication through the basement membrane are still unclear, and the factors involved in the granulosa-theca cell communication in the control of granulosa cell differentiation in our co-culture system remains to be identified. Regardless, we speculate that intraovarian regulators interact in a complex manner in the control of granulosa cell differentiation during early follicular development.

CONCLUSIONS

We have examined the influence of granulosa–theca cell interaction on the differentiation of granulosa cells during the gonadotropin-independent phase, using our co-culture system in which granulosa and theca cells were cultured on opposite sides of the collagen membrane. The results of the present study indicate that theca cells are involved in both rate-limiting steps in estrogen production, i.e., androgen substrate production and aromatase regulation. These results also suggest that theca cell-derived factors regulate estradiol and progesterone production in a way that reflects steroidogenesis during early follicular growth. Our previous studies suggest that theca cell-derived factors promote granulosa cell proliferation with the aid of serum (Kotsuji et al., 1990; Kotsuji and Tominaga, 1994) and suppress granulosa cell apoptosis under serum-free conditions (Tajima et al., 2002) at early antral follicle. Taken together, it can be concluded that granulosa–theca cell interaction is a major follicular constituent in the control of follicular growth, maturation, and atresia during the early stage of follicular development. Further investigation of the local cellular interactions are essential to better understand ovarian physiology, and shed new light on several reproductive problems such as poor response to gonadotropin stimulation, premature ovarian failure, and polycystic ovarian syndrome.

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