Response of porcine granulosa cells isolated from primary and secondary follicles to FSH, 8-bromo-cAMP and epidermal growth factor *in vitro*

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A cell culture system was developed to study the function of porcine granulosa cells from primary and secondary follicles. Primary follicles were isolated from 1- to 3-day-old pigs. Secondary follicles were isolated from 50- to 60-day-old pigs. Follicles were isolated after a digestion for 15 min with 0.25% trypsin followed by 15 min with 1000 U DNAase. Follicles were plated at 100 primary follicles or 30 secondary follicles per well in 48-well plates and cultured in media containing 10% fetal bovine serum (FBS). During initial plating, follicles attached to the plate and cells spread from the point of attachment. This resulted in monolayer cultures of granulosa cells from primary or secondary follicles. On day 4 of culture, media were replaced with 0.5 ml media containing one of the following treatments: control (media only); 10% FBS; 100 ng FSH; 2 mmol 8-bromo-cAMP l^{-1} or 50 ng epidermal growth factor (EGF). Media and cells were harvested on day 6, after 2 days of treatment. FBS and EGF increased DNA in granulosa cell cultures from primary or secondary follicles ($P < 0.01$). Treatment with 8-bromo-cAMP increased DNA in granulosa cell cultures from primary but not from secondary follicles ($P < 0.05$). Conversely, treatment with FSH increased DNA in granulosa cell cultures from secondary but not from primary follicles ($P < 0.05$). Progesterone release was greater in granulosa cell cultures from primary and secondary follicles treated with 8-bromo-cAMP than in controls ($P < 0.001$); however, progesterone concentrations were not influenced by FBS, EGF or FSH in granulosa cells from primary or secondary follicles. In summary, EGF and 8-bromo-cAMP stimulated proliferation of granulosa cells from primary and secondary follicles, and 8-bromo-cAMP also stimulated progesterone secretion. In contrast, FSH had no effect on progesterone secretion or proliferation of granulosa cells from primary follicles, but stimulated proliferation of granulosa cells from secondary follicles.

Introduction

Development of primary follicles to the antral stage in pigs is a slow process that requires more than 80 days (Morbeck *et al.*, 1992). The mechanisms and factors involved in the growth of preantral follicles in pigs are not known. Growth and ovulation of porcine antral follicles can be stimulated with gonadotrophins (Paterson, 1982); however, the role of gonadotrophins in the development of preantral follicles is not known.

On the basis of studies with hypophysectomized rats and mice, there is some question as to whether gonadotrophins are necessary for the development of primary and early secondary follicles. In their review of research with rodents, Greenwald and Terranova (1988) suggested that although some preantral follicles develop in the absence of gonadotrophins, substantially fewer develop overall, indicating a requirement for gonadotrophins. In contrast, it appears that primary and secondary

follicles are not influenced by gonadotrophins in pigs. For example, the populations of primary and secondary follicles in the 35-day-old pig are not influenced by treatment with pregnant mares' serum gonadotrophin (PMSG) and hCG (Oxender *et al.*, 1979). In addition, the number of primary and early secondary follicles are normal at birth in pig fetuses killed by decapitation at 42 days *post coitum* (Colenbrander *et al.*, 1983), indicating that activation of primordial follicles and their subsequent development to early secondary follicles occurs independently of pituitary hormones in pig fetuses.

A primary reason for our poor understanding of the control of preantral follicle growth in pigs is the lack of a suitable system for studying preantral follicle development. Currently the most common method used is histological analysis, whereby follicles are counted and rates of atresia assessed (Esbenshade, 1987; Dufour *et al.*, 1988). This model has not proved suitable for the analysis of growth and differentiation of preantral follicles as manifested by granulosa cell proliferation, steroidogenesis and induction of gonadotrophin receptors.

Methods for the isolation and study of preantral follicles *in vitro* have been reported for hamsters (Roy and Greenwald, 1985), rats (Conway *et al.*, 1990), mice (Wang *et al.*, 1991), and

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rabbits (Maresh *et al.*, 1990). In addition, an enzymatic procedure has been reported for the isolation of primordial follicles from porcine ovaries (Greenwald and Moor, 1989). The effects of steroids, gonadotrophins and growth factors on thymidine incorporation and steroidogenesis have been examined with some of these culture systems (Roy and Greenwald, 1987, 1988, 1991a; Conway *et al.*, 1990).

The objectives of the current investigation were to establish an isolation procedure and a culture system for the study of the effect of FSH, 8-bromo-cAMP and EGF on granulosa cells from porcine preantral follicles.

Materials and Methods

Reagents and materials

Bovine insulin, selenium, and human transferrin (ITS), fibronectin from human plasma, 8-bromo-cAMP, antibiotics, NaHCO₃, media, trypsin from porcine pancreas, DNAase I from bovine pancreas, and BSA were purchased from Sigma Chemical Co. (St Louis, MO). Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT). Receptor grade murine EGF was purchased from Collaborative Research (Cambridge, MA). Ovine FSH (Sherwood *et al.*, 1970) was kindly provided by H. J. Grimek through W. L. Miller (Dept of Biochemistry, North Carolina State University) and was used because it was the most abundant supply of purified FSH readily available.

Isolation of preantral follicles and granulosa cells

Primary follicles (preantral follicles with one layer of granulosa cells) were isolated from newborn pigs (less than 5 days old, weight 1–2 kg) and secondary follicles (preantral follicles with more than one layer of granulosa cells) were isolated from older pigs (50–60 days of age, weight 15–20 kg). Pigs were sedated with a mixture of xylazine (10 mg kg⁻¹ body weight, i.m.) and ketamine hydrochloride (10 mg kg⁻¹ body weight, i.m.) and killed by captive bolt. Ovaries were removed through a mid-ventral incision and transported in ice-cold saline containing 200 U penicillin, 200 µg streptomycin and 0.75 µg amphotericin B ml⁻¹, pH 7.2–7.4.

Ovaries were rinsed once with sterile saline, placed in fresh sterile saline in a Petri dish and kept on ice during the isolation procedure. Ovarian tissue (40–60 mg wet weight) corresponding to one neonatal ovary or one-half of an ovary from a 50- to 60-day-old pig was then minced with scissors into four to five large pieces (2–3 mm diameter) in sterile 3 cm plastic Petri dishes containing Hank's balanced salt solution (HBSS; Ca²⁺ and Mg²⁺ free) with 0.25% trypsin, 0.25% BSA, 15 mmol Hepes l⁻¹ and 0.04% NaHCO₃, pH 7.4. The pieces of ovary were then teased with watch-maker's forceps into ≤1 mm pieces and transferred with a sterile, disposable plastic pipette to a 25 ml glass scintillation vial containing 5 ml of the HBSS–trypsin solution. The scintillation vial was shaken (140 oscillations min⁻¹) in a horizontal position in a 37°C water bath for 15 min and the tissue and media were then transferred to a sterile Petri dish (3 cm). The tissue was gently pipetted

through one wash of 10% FBS in Dulbecco's modified Eagle medium (DMEM): Ham's F12 containing 15 mmol Hepes l⁻¹, 0.12% NaHCO₃ and L-glutamine (DMEM:F12; 1:1) followed by two washes with serum-free DMEM:F12 in Petri dishes. The tissue was transferred to a new scintillation vial containing 5 ml DMEM:F12 with 1000 U DNAase ml⁻¹ and 0.25% BSA, and the digestion was completed by shaking in a 37°C waterbath for 15 min. After this digestion, the tissue and media were transferred to a sterile Petri dish.

Whereas most follicles were still encompassed in pieces of tissue following the trypsin digestion, and, therefore, required further digestion, a substantial number were floating free in the culture media in the Petri dish following the trypsin digestion. Further digestion of these follicles was inhibited by adding 2.5 ml DMEM:F12 containing 10% FBS and 2.5 ml of the DNAase solution to the culture dish and incubating at room temperature for 5–10 min.

Isolated follicles were harvested individually with precalibrated pipettes (<100 µm diameter for primary follicles and <300 µm diameter for secondary follicles). Approximately 100 primary follicles or 30 secondary follicles were obtained for each culture well.

Granulosa cells from antral follicles were also cultured as positive controls. Ovaries from prepubertal gilts (3–5 months of age) were obtained from a local abattoir and transported to the laboratory in ice-cold sterile saline containing antibiotics. Granulosa cells from 1–3 mm follicles (non-atretic based on visual appraisal) were aspirated aseptically with a 25-gauge needle and a 1 cm³ syringe. Follicular aspirates were pooled in a sterile, 15 ml conical tube on ice, centrifuged at 200 g for 10 min, washed once with sterile saline, centrifuged again and resuspended in 3 ml culture media. The number of viable cells was determined by trypan blue exclusion and was between 35 and 45%.

Culture conditions

To promote attachment of follicles and granulosa cells to the 48-well culture plates, each well was pretreated with 4 µg fibronectin in 120 µl PBS for 2 h. The fibronectin solution was removed with a sterile Pasteur pipette and replaced with 500 µl DMEM:F12 containing ITS (5 µg insulin, 5 µg transferrin and 5 µg selenium ml⁻¹) and 10% FBS. Preliminary studies demonstrated that FBS was necessary for establishment of the culture. After precoating with fibronectin, culture plates containing media were kept at 37°C in a humidified environment under 5% CO₂ in air. As follicles were isolated, they were placed in drops of media in a covered Petri dish. Once eight drops with 100 primary or 30 secondary follicles per drop were obtained, the follicles were transferred to the culture plates with a precalibrated pipette (<600 µm diameter). The procedure to isolate enough follicles for 48 wells (approximately 4800 primary follicles or 1400 secondary follicles) took 3–5 h.

Preliminary experiments illustrated that cultures of preantral follicles did not always grow to confluency and, therefore, were of lower density than were typical cultures of granulosa cells from antral follicles. As cell density influences both proliferation and differentiation of granulosa cells (May and Schomberg, 1984), granulosa cells from antral follicles were plated at two different densities: one to mimic the number of cells in the

follicle cultures (6×10^4 live cells per well) and one that would be similar to other studies with granulosa cells (3×10^5 live cells per well).

Cultures were maintained for 6 days, which consisted of three phases lasting 2 days each: an attachment phase, a growth phase and a treatment phase. Serum was used in all wells during the attachment phase only. After the attachment phase of culture (day 2), the media were removed and replaced with DMEM:F12 + ITS. Media were changed again after the growth phase (day 4) and replaced with DMEM:F12 containing compounds to be evaluated during the treatment phase. Media, harvested 2 days later (day 6), were centrifuged at 1700 *g* to remove debris and frozen for later analyses. After removal of media on day 6, 100 μ l of a solution containing 1% cholic acid and 0.1% lauryl sulfate was added to each well and allowed to incubate for 10 min to disrupt and lyse the cells, and 100 μ l of 2 mol NaCl l^{-1} was then added to each well for the DNA assay. Siliconized Pasteur pipettes were used to transfer cell lysates to test tubes and the samples were frozen for later analyses.

Treatments

At the beginning of the treatment phase (day 4), cells were assigned to receive one of five treatments in 0.5 ml media: controls (DMEM:F12 + ITS); 10% FBS; 100 ng FSH; 2 mmol 8-bromo-cAMP l^{-1} and 50 ng EGF. Doses of FSH and 8-bromo-cAMP were determined to be maximal by preliminary dose-response experiments in which 0.1–200 ng FSH ml^{-1} and 0.01–2.0 mmol 8-bromo-cAMP l^{-1} were tested in granulosa cell cultures from primary, secondary and antral follicles. The dose of EGF used was based on results from studies with preantral follicles from hamsters (Roy and Greenward, 1991a). Insulin, transferrin and selenium were included in the media for all treatments because insulin is needed in granulosa cell cultures (May and Schomberg, 1981). Each treatment group consisted of three to six wells per replicate, and the experiment was replicated three times.

Progesterone and DNA assays

Progesterone was determined by radioimmunoassay as reported by Stevenson *et al.* (1981) with the following modifications: (1) unextracted samples were assayed, (2) anti-progesterone antibody was used at a dilution of 1:90 000 and (3) 10 000 to 13 000 c.p.m. ^{125}I -labelled progesterone (Diagnostic Products Inc., Los Angeles, CA) were used. The sensitivity of the assay was 60 pg per tube and the intra- and interassay coefficients of variation were 12.5 and 16.3%, respectively. DNA concentration was determined with a DNA fluorometer (Hoefer Scientific, San Francisco, CA) using a previously described method (Labarca and Paigen, 1980). A control sample from granulosa cells was used as a measure of assay variation. Intra- and interassay coefficients of variation were 4.9 and 5.9%, respectively.

Statistical analyses

Data were analysed with a statistical model that included treatment, replicate and the treatment by replicate interactions (SAS Institute, Inc., 1984). Wells were averaged over all repli-

cates. DNA content was significantly affected by replicate and thus data for each replicate were converted to percentage of control. In contrast, progesterone was not influenced by replicate and data were expressed as ng μg^{-1} DNA. Statistical differences between means were determined using least significant difference tests.

Results

Characterization of isolated preantral follicles

The population of follicles in ovaries of neonatal pigs at birth consisted mainly of primordial follicles and primary follicles and relatively few secondary follicles containing no more than two layers of granulosa cells (Fig. 1a). Approximately four ovaries were needed to obtain 5000 primary follicles. Primary and early secondary follicles as they appeared following the isolation procedure are shown (Fig. 1b). The follicles appeared to have an intact basement membrane and one layer of granulosa cells. This was confirmed by randomly selecting follicles from a preliminary isolation embedding them in agar and processing them for histological analysis (Fig. 1c). Although none of the follicles examined histologically had more than one layer of granulosa cells, the presence of preantral follicles of a variety of sizes (as shown in Fig. 1b) coupled with the appearance of secondary follicles in ovarian cross-sections (Fig. 1a) indicate that a small percentage of the follicles used in this study may have been at the early secondary stage (no more than two layers of granulosa cells), whereas others may have been primordial. During the isolation of follicles, we could not differentiate between follicles with one versus two layers of granulosa cells. However, although primordial follicles were easily distinguished from other follicles, it was not possible to harvest primary follicles without inadvertently including some primordial follicles.

In contrast to neonatal pig ovaries, the population of follicles in ovaries of 50- to 60-day-old pigs consisted of a large number of secondary follicles containing multiple layers of granulosa cells (Fig. 1d). After the isolation procedure, groups of follicles (as they appear in Fig. 1e) were placed in culture wells. Two ovaries were needed to obtain sufficient follicles ($n = 1500$) for each replicate. The majority of isolated secondary follicles appeared to have intact basement membranes and did not appear to have any thecal or interstitial cells attached (Figs 1e, f). However, a small percentage of follicles in each culture contained cells on the outside of the basement membrane that were possibly thecal cells. It was not possible to obtain a population of follicles exclusive of follicles that were free of extra cells.

During the first 2 days of culture, follicles attached to the bottom of the plate and granulosa cells proliferated from the point of attachment. By day 6, the cells reached more than 75% confluency (Fig. 2). Intact follicles in culture either disintegrated following plating, so that there was no visible remnant of the follicle, or remained in their original form but were encircled by a monolayer of granulosa cells (Fig. 2).

Cell density for control wells was lowest for primary follicle cultures, similar between secondary and low density granulosa cell cultures (Table 1) and greatest for high density granulosa cell cultures from antral follicles ($P < 0.05$). Progesterone (μg^{-1} DNA) in control wells was highest for high density granulosa

Fig. 1. Photomicrographs of ovaries and follicles from pigs of two different ages. (a) Cross-section of an ovary from a neonatal pig. P: primordial follicle, I: primary follicle, II: secondary follicle. (b) Follicles isolated from an ovary of a neonatal pig. (c) Cross-section of a primary follicle isolated from an ovary of a neonatal pig. (d) Cross-section of an ovary from a 62-day-old pig. (e) Follicles isolated from an ovary of a 50- to 60-day-old pig. (f) Cross-section of secondary follicles isolated from an ovary of a 50- to 60-day-old pig. Scale bars (a) and (d) 200 μm ; (b) and (e) 50 μm ; (c) 30 μm and (f) 100 μm .

cell cultures, similar between low density and primary follicle cultures and lowest for secondary follicle cultures ($P < 0.05$).

Effects of FSH, 8-bromo-cAMP, serum and EGF on primary follicle cultures from neonatal pigs

Neither DNA (Fig. 3) nor progesterone secretion (Fig. 4) were influenced by FSH in primary follicle cultures. In contrast, 8-bromo-cAMP increased DNA by 40% ($P < 0.05$) and progesterone secretion by 430% ($P < 0.01$). Treatment with

either FBS or EGF resulted in greater increases ($P < 0.01$) in DNA content (70 and 80% increase, respectively); however, progesterone secretion was not affected by FBS or EGF.

Effects of FSH, 8-bromo-cAMP, serum and EGF on secondary follicle cultures from 50- to 60-day-old pigs

In contrast to the response by primary follicle cultures, FSH increased DNA content 20% in secondary follicle cultures (Fig. 5), although there was no effect on progesterone secretion

Fig. 2. Photomicrograph of a monolayer of granulosa cells from porcine primary follicle. Arrow indicates presence of follicle or remnant of follicle. Scale bar = 200 μ m.

Table 1. Amount of DNA and progesterone in control wells of primary, secondary and low and high density antral granulosa cell cultures of pigs

Type of culture	DNA (μ g well ⁻¹)	Progesterone (ng μ g ⁻¹ DNA)
Primary follicles	0.259 $\pm$ 0.026 ^a	4.04 $\pm$ 0.55 ^b
Secondary follicles	0.357 $\pm$ 0.025 ^b	2.22 $\pm$ 0.54 ^a
Low density GC	0.342 $\pm$ 0.024 ^b	4.49 $\pm$ 0.51 ^b
High density GC	0.805 $\pm$ 0.023 ^c	9.11 $\pm$ 0.50 ^c

Values are means $\pm$ SEM.

^{abc}Means with different letters within a column are significantly different ($P < 0.05$).

GC: granulosa cells from 1–3 mm follicles.

Fig. 3. Effects of fetal bovine serum (■), FSH (▨), 8-bromo-cAMP (▩), and epidermal growth factor (▧) on content of DNA in monolayer cultures of granulosa cells from porcine primary follicles (controls = □). Results are means $\pm$ SEM and there were three replicates per group. Different letters above bars denote significant differences between treatments ($P < 0.05$).

Fig. 4. Progesterone secretion in response to fetal bovine serum (■), FSH (▨), 8-bromo-cAMP (▩) and epidermal growth factor (▧) in porcine primary follicle cultures (controls = □). Results are means $\pm$ SEM and there were three replicates for each treatment. Different letters above bars denote significant differences between treatments ($P < 0.05$).

Fig. 5. Effects of fetal bovine serum (■), FSH (▨), 8-bromo-cAMP (▩) and epidermal growth factor (▧) on content of DNA in monolayer cultures of granulosa cells from porcine secondary follicles (controls = □). Results are means $\pm$ SEM and there were three replicates per group. Different letters above bars denote significant differences between treatments ($P < 0.05$).

(Fig. 6). Treatment with 8-bromo-cAMP failed to increase DNA, but resulted in a 330% increase in progesterone secretion ($P < 0.01$). FBS and EGF stimulated proliferation in a similar manner ($P < 0.01$); DNA increased by 70% in cultures treated with FBS versus 60% for cultures treated with EGF. Neither FBS nor EGF influenced progesterone secretion.

Effects of FSH, 8-bromo-cAMP, serum and EGF on granulosa cells from 1–3 mm follicles

Granulosa cells from 1–3 mm follicles were plated at two densities to serve as positive controls. While FSH and 8-bromo-cAMP stimulated proliferation of granulosa cells plated at high density, only FSH was effective in low density cultures (Fig. 7). Treatment with FBS or EGF resulted in maximal stimulation

Fig. 6. Progesterone secretion in response to fetal bovine serum (■), FSH (▨), 8-bromo-cAMP (▩) and epidermal growth factor (▧) in porcine secondary follicle cultures (controls = □). Results are means $\pm$ SEM and there were three replicates for each treatment. Different letters above bars denote significant differences between treatments ($P < 0.05$).

Fig. 8. Progesterone secretion in response to fetal bovine serum (■), FSH (▨), 8-bromo-cAMP (▩) and epidermal growth factor (▧) in porcine granulosa cell cultures from 1–3 mm follicles plated at low and high densities (controls = □). Results are means $\pm$ SEM and there were three replicates for each treatment. Different letters above bars denote significant differences between treatments ($P < 0.05$).

Fig. 7. Effects of fetal bovine serum (■), FSH (▨), 8-bromo-cAMP (▩) and epidermal growth factor (▧) on content of DNA in monolayer cultures of porcine granulosa cells from 1–3 mm follicles plated at low and high densities (controls = □). Results are means $\pm$ SEM and there were three replicates per group. Different letters above bars denote significant differences between treatments ($P < 0.05$).

of proliferation (Fig. 7) without an increase in progesterone secretion (Fig. 8). FSH and 8-bromo-cAMP caused maximal stimulation of progesterone secretion for low and high density cultures (Fig. 8).

Discussion

The present experiment examined control and function of porcine granulosa cells from primary and secondary follicles *in vitro*. The number of follicles isolated per ovary was maximized by collecting ovaries from pigs at two different ages. The neonatal pig provides an abundant source of primary follicles: primary follicles represent approximately 5% of 500 000 follicles at birth (Black and Erickson, 1968; Oxender *et al.*, 1979).

In 9- to 10-week-old pigs, secondary follicles represent approximately 20–25% of all follicles, whereas primary follicles make up only 3% of the 250 000 to 300 000 follicles present at that time (Black and Erickson, 1968; Oxender *et al.*, 1979). Between 100 and 200 days of age, growing follicles account for only 0.7% of the approximately 200 000 follicles (Dufour *et al.*, 1988; Greenwald and Moor, 1989). By using ovaries from pigs younger than 70 days, a large, relatively homogeneous population of preantral follicles was obtained.

Results of the present study illustrate that granulosa cells from primary follicles of neonatal pigs are not responsive to FSH, but are stimulated to proliferate and secrete progesterone by 8-bromo-cAMP. The large increase in progesterone secretion in response to 8-bromo-cAMP coupled with an increase in DNA content illustrates that primary follicles have an intact second messenger system distal to cAMP. These findings suggest that granulosa cells from primary follicles either lack, or have too few, FSH receptors to elicit a response or that their FSH receptors are uncoupled from the second messenger systems. An alternative explanation for the lack of response to FSH in these studies is that granulosa cells from preantral follicles cultured in a monolayer may not behave in the same way as granulosa cells *in vivo* or even as intact follicles *in vitro*. In addition, given the slow rate of growth of porcine preantral follicles (Morbeck *et al.*, 1992), it is possible that the 2-day treatment did not provide sufficient time for proliferation of the granulosa cells in response to FSH.

Analysis of FSH-binding sites with autoradiography has demonstrated the presence of FSH receptors in small preantral follicles of mice (Eshkol and Lunenfeld, 1972) and rats (Midgley, 1973). Roy *et al.* (1987) used radioreceptor analysis of isolated hamster follicles containing one to four layers of granulosa cells and obtained very low binding of FSH that was frequently undetectable. They then used autoradiography to substantiate the presence of the FSH receptor and found definite FSH-binding sites, although there were relatively few binding sites on follicles with one layer of granulosa cells. Indirect evidence suggests that

receptors for FSH are present and functional in primary follicles from hamsters, as tritiated thymidine incorporation into DNA increases in response to FSH (Roy and Greenwald, 1986). Roy and Greenwald (1988) also demonstrated increased DNA synthesis in response to 8-bromo-cAMP at all follicular stages, including primary follicles, in hamsters. The results of the present study indicate that there may be species differences in the role of FSH during early follicle development.

The findings of the present experiment suggest that the proliferation of granulosa cells from primary follicles is not stimulated by FSH. This observation is consistent with the finding that the number of primary and secondary follicles is normal at birth in decapitated pig fetuses (Colenbrander *et al.*, 1983). Whereas FSH could not stimulate proliferation of granulosa cells from primary cultures, EGF increased DNA content substantially and the degree of stimulation was similar to that obtained with serum. Neither EGF nor FBS stimulated progesterone secretion, which supports previous work indicating that the actions of EGF are proliferative and not differentiative (Hseuh *et al.*, 1981; Knecht and Catt, 1983; May *et al.*, 1988). Roy and Greenwald (1991a) found that EGF was as potent as FSH in stimulating thymidine incorporation in all stages of follicles, including those with one to four layers of granulosa cells. In addition, they found that EGF stimulated progesterone secretion in follicles with three or more layers of granulosa cells. The reason for the differences in the present study and the one by Roy and Greenwald (1991a) in terms of the effect of EGF on progesterone production may be that granulosa cells in monolayer culture behave differently from those in intact follicles.

In contrast to primary follicle cultures, secondary follicle cultures from 50- to 60-day-old pigs proliferated in response to FSH while progesterone secretion did not change. Treatment with 8-bromo-cAMP stimulated steroidogenesis but did not cause a statistical increase in DNA content. These results suggest that follicles acquire receptors for FSH as they develop into large secondary follicles and that the effect of FSH is primarily on follicle growth. However, attempts to stimulate follicular development in pigs younger than 60 days of age have been unsuccessful as measured by changes in percentages of primary and secondary follicles in response to various combinations of gonadotrophins (Casida, 1934; Oxender *et al.*, 1979). Exogenous gonadotrophins do not alter the proportion of primary and secondary follicles at 7 weeks of age but can induce ovulation beginning at about 9 weeks of age (Oxender *et al.*, 1979), at which time antral follicles begin to appear. However, as gonadotrophins were given for no more than 5 days and ovaries were examined 11 days after the initiation of treatment (Casida, 1934; Oxender *et al.*, 1979), duration of treatment may not have been sufficient to illicit a measurable response. According to growth rate calculations, a primary follicle requires more than 30 days to increase from one to three layers of granulosa cells (Morbeck *et al.*, 1992).

It is not clear why FSH caused an increase in DNA but did not influence progesterone secretion, whereas activation of its second messenger system (cAMP) resulted in an increase in progesterone secretion but did not influence DNA. However, a differential response to FSH and 8-bromo-cAMP for DNA content was obtained with granulosa cells from 1–3 mm follicles plated at low density, and FSH stimulated proliferation and

8-bromo-cAMP had no effect on DNA content. It is possible that the lack of proliferation in response to 8-bromo-cAMP is a function of density, as FSH and 8-bromo-cAMP stimulated proliferation of granulosa cells plated at high density. However, previous studies with granulosa cells plated at different densities illustrated that as density of granulosa cells increased, the proliferative response to FSH decreased (May and Schomberg, 1981).

Although we did not observe an increase in proliferation in secondary follicle cultures in response to 8-bromo-cAMP, progesterone secretion increased markedly. In contrast, the response to FSH was strictly proliferative and progesterone secretion was not influenced. There was a similar situation whereby FSH, but not cAMP, could stimulate proliferation in granulosa cells from antral follicles plated at low density. In addition, while 8-bromo-cAMP stimulated proliferation of high-density granulosa cell cultures, the response was not as great as seen with FSH. This dichotomy suggests that FSH stimulates proliferation through a second-messenger system other than cAMP in secondary follicles, and that other hormones, such as LH, may be acting through cAMP to stimulate steroidogenesis at this stage of follicular development. Activation of other second-messenger systems by gonadotrophins, including intracellular calcium mobilization (Asem *et al.*, 1987) and an increase in inositol triphosphate (Davis *et al.*, 1986) has been reported. The fact that FSH may act through different second-messenger systems presents the possibility that the role of FSH during early stages of follicular development is primarily proliferative, whereas during later stages of development, it becomes differentiative as well.

As was the case for primary follicle cultures, treatment with EGF and FBS increased DNA content in secondary follicle cultures. EGF was chosen after a preliminary study indicated that it was the most potent mitogen among basic fibroblast growth factor, insulin-like growth factor I and transforming growth factor β . It has subsequently been shown that porcine preantral and antral follicles possess receptors for EGF (Fujinaga *et al.*, 1992). Although it is likely that EGF is present in FBS, it is not known whether the effect of FBS can be attributed solely to EGF. Studies with porcine granulosa cells from 1–3 mm follicles have shown that although FBS stimulates tritiated thymidine incorporation more than does EGF, their combined effect is additive (May *et al.*, 1988). This additive effect is probably due to the presence of more than one growth factor, which presents a multifactorial stimulus for cell proliferation. An additive effect of growth factors on proliferation has been observed in preantral follicles (Roy and Greenwald, 1991a) and granulosa cells from antral follicles (May *et al.*, 1988).

While EGF in serum may act as an endocrine factor to stimulate granulosa cell proliferation *in vivo*, it is likely that EGF is produced within the ovary and acts in a paracrine/autocrine fashion. The proliferative actions of FSH, 8-bromo-cAMP or EGF on preantral follicles from hamsters are blocked by EGF antiserum (Roy and Greenwald, 1991b). This finding suggests that FSH increases DNA synthesis indirectly by stimulating EGF production by cultured follicles, which then acts directly in a paracrine/autocrine manner. Although FSH did not increase DNA content in primary follicle cultures, it is possible that it stimulated EGF production and, thereby, indirectly increased DNA content in secondary follicle cultures. However, although

prepro-EGF was detected in mouse ovaries (Roy and Greenwald, 1990) and EGF immunoreactivity was observed in hamster ovaries (Rall *et al.*, 1985), it is not known whether it is produced by porcine granulosa cells.

Taken together, these results suggest that growth of granulosa cells from primary follicles may not be controlled by FSH, and that intraovarian factors, possibly EGF, may represent the primary control of granulosa cell proliferation at this stage. Granulosa cells from secondary follicles proliferate in response to FSH and EGF; however, progesterone secretion is not influenced by either factor, suggesting that the main action of FSH during early stages of follicular development is proliferative.

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