Functional and ultrastructural characteristics of two types of rat granulosa cell cultured in the presence of FSH or transforming growth factor α (TGF- α)

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Granulosa cells were isolated from 15-day-old, 25-day-old or PMSG-primed rats and were separated by Percoll gradient (20 to 60%) into five fractions. The cells in fraction 2 were mostly small cells (6.96–9.57 μm) and fractions 3 and 4 had a relatively high population of large cells (10.96–13.05 μm) which were sorted to collect a pure population of large cells. Aliquots of small or large cells were cultured separately in serum-free defined DMEM/F-12 medium containing 50 ng FSH ml^{-1} , or 10 ng transforming growth factor α (TGF- α) ml^{-1} for 3 days. In PMSG-primed rats, the large cells produced 3.2-fold more progesterone than did small cells (with FSH/TGF α : 255 ± 35.0 versus $77.32 \pm 14.5 \times 10^{-6}$ ng, day 1). Large and small cells from 25-day-old rats produced similar amounts of progesterone (FSH/TGF α : 65.68 ± 9.6 versus $78.25 \pm 12.3 \times 10^{-6}$ ng, day 1). In 15-day-old rats, large and small cells produced very low concentrations of progesterone (FSH/TGF α : 4.69 ± 1.2 versus $2.66 \pm 1.0 \times 10^{-6}$ ng, day 1). Large cells from PMSG-primed rats had characteristics of steroidogenic cells, i.e. smooth endoplasmic reticulum and well-developed mitochondria with tubular cristae compared with small cells, whereas small and large cells from 25- and 15-day-old rats contained the regularly occurring organelles without the endoplasmic reticulum of the smooth variety and mitochondria with lamellar cristae. This study shows that the heterogeneity of granulosa cells is related to size, metabolic response to FSH, TGF- α or to both factors and morphological features, all of which may be associated with the transition from preantral to preovulatory stages of follicle differentiation.

Introduction

Rat granulosa cells have been shown to consist of two distinct cell types that can be separated according to size (Telfer *et al.*, 1988; Hartshorne, 1990; Vanderhyden *et al.*, 1990; Rao *et al.*, 1991). Rao *et al.* (1991) consider that the two types of granulosa cell differ in their degree of differentiation, and that this is independent of the cell cycle within the follicle. The different characteristics of the two cell types seem to be important for follicular function(s); however, little is known concerning the function of these subpopulations of granulosa cells and whether they are related to the stages of follicular development. There is some evidence that mural granulosa cells contain more LH receptors than do the cumulus antral cells (Amsterdam *et al.*, 1975; diZerega and Hodgen, 1980). Buccione *et al.* (1990) and Salustri *et al.* (1990) have pointed out that cumulus cells undergo FSH-dependent mucification, whereas mural granulosa cells do not.

Functional differences could be investigated if these cells were separated into relatively pure populations. Density gradient centrifugation (Lahteenmaki *et al.*, 1982; Magoffin and Erickson, 1988) or flow cytometry (Hatfield and Hymer, 1986a; 1986b) is useful for separation and purification of living cells

and have been applied to many types of cell including granulosa cells (Hirshfield *et al.*, 1988), luteal cells (Ohara *et al.*, 1987) and Leydig cells (Gale *et al.*, 1982). Separation methods such as unit gravity sedimentation or Percoll gradient have yielded large cell preparations that are usually contaminated with small cells (Koos and Hansel, 1981). The combined use of density gradient and flow cytometry allows the isolation of fractions of highly purified luteal cells (Alila *et al.*, 1988). We applied the techniques of density gradient and flow cytometry to rat granulosa cells in an attempt to separate them into subpopulations and examine further their structural and physiological features.

The specific aims of the present study were therefore (1) to apply the techniques of density gradient and flow cytometry to rat granulosa cells and (2) to assess the steroidogenic ability and morphological features of the two types of rat granulosa cell isolated from preantral, antral or preovulatory stage follicles cultured in the presence of FSH or transforming growth factor- α (TGF- α) or both factors.

Materials and Methods

Granulosa cell collection

Sprague-Dawley rats were purchased from Taconic, Germantown, NY. Five rats were killed by cervical dislocation

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in each experiment when they were 15 and 25 days old, or injected with PMSG (15 iu per rat in 0.15 ml saline; Sigma, St Louis, MO) when they were 23 days old and killed 50–52 h after the injection. Each experiment was repeated five times. Ovaries were removed and immediately immersed into McCoy's 5A medium (Irvine Scientific, Santa Ana, CA). The ovaries were punctured with fine needles and transferred into 2.0 ml non-enzymatic cell dissociation solution (Sigma) for 10 min; this method prevents the dissociation of thecal and stromal cells. In PMSG-primed rats, only large follicles (0.3–0.5 mm) were punctured. The ovaries were then transferred to McCoy's 5A containing 0.5 mol sucrose l^{-1} (Fisher Scientific, Pittsburgh, PA), 1.8 mmol EGTA l^{-1} (Sigma) and 0.25% BSA (Sigma) at pH 7.4 and incubated at room temperature for 5 min. The ovaries were pressed to release the granulosa cells into McCoy's 5A. The cell suspension was centrifuged at 200 *g* for 10 min and the pellet resuspended in McCoy's 5A. Cell viability was checked by Erythrosin B exclusion using a haemocytometer (Philips, 1973). Isolated granulosa cells were suspended in 1.5 ml McCoy's 5A ($4\text{--}5 \times 10^6$).

Percoll gradient and cell separation

For preparation of the Percoll gradients, nine parts of Percoll (Pharmacia LKB Biotechnology, Piscataway, NJ) were mixed with one part of tenfold concentrated McCoy's 5A medium to give an osmolality 310 mosmol kg^{-1} . This mixture was diluted with McCoy's 5A containing 0.25% BSA to make various concentrations of Percoll solution. Two millilitres of 60% Percoll solution was pipetted into a sterile $18 \times 100\text{ mm}^2$ plastic centrifuge tube (Nalge Company, Rochester, NY). Two millilitres of 50% Percoll solution were then carefully layered on top of the 60% Percoll solution, followed by 2 ml each of 40, 30, and 20% Percoll solution. Five tubes of Percoll gradient were used for preparations from 15-day-old rats, ten tubes for 25-day-old rats and 15 tubes for PMSG-treated rats. Up to 1.5 ml of the crude cell suspension containing $3\text{ to }5 \times 10^6$ cells ml^{-1} was layered onto the top of each gradient. The tubes were then centrifuged at 400 *g* for 10 min at 4°C.

Fraction collection

Fractions were collected from the interphase of Percoll layers from the top of the tube using a Pasteur pipette. Sixty to eighty per cent of cells were recovered. Three fractions were pooled and diluted in 3–5 ml isotonic McCoy's 5A medium and centrifuged at 200 *g* for 10 min. Finally, each fraction was resuspended and an aliquot (50 μ l) of each fraction was used for cell counting using a haemocytometer.

Flow cytometry

After separation by Percoll gradient, cells were analysed on forward angle light scatter (FALS; $1.0\text{--}19^\circ$) and light scatter (LS; 90°) signals by an EPICS 750 series cell sorter (Coulter Electronics, Hialeah, FL) with an argon ion laser (Coherent Laser Division, Palo Seto, CA) tuned to the 488 nm line at 200 mW. The FALS and 90° LS positions were adjusted to channels 3 to

64. The flow cytometer simultaneously sorted two populations of cells at a rate of 1000 cells s^{-1} with each sorting lasting 2–3 h. After sorting, cells in each fraction were recounted on a haemocytometer and cell viability determined.

One-parameter analysis and cell sorting

Distribution of small and large cells was demonstrated by FALS single parameter. A number of 10 000 cells were counted in each fraction for analysing cell distribution. To determine the standard setting, a gate was placed on the nadir between the two populations of cells from 25-day-old rats. This gate setting was used for cell distribution of samples from 15-day-old and PMSG-primed rats.

Quadrant analysis

Distribution of pooled total cells was demonstrated by FALS- 90° LS dual parameter. The FALS gate setting was the same place as one-parameter analysis and the 90° LS gate was placed on the centre of the maximum density of small cell population from 25-day-old rats. The gate settings were used for cell distribution of samples from 15-day-old and PMSG-primed rats. In this study, quadrants 1 and 2 contained high granularity of small and large cell populations, respectively, and quadrants 3 and 4 contained low granularity of small and large cell populations, respectively.

Determination of cell size

Cell diameters were recorded using a light microscope equipped with a micrometer.

Cell culture

The serum-free medium of Anderson and Lee (1993) for granulosa cell culture was used in this study. Attachment-promoting substrate (E-C-L; UBI, Lake Placid, NY) was prepared by pretreating the surface of the culture wells. Solutions of E-C-L (20 μ g in 400 μ l H_2O) were added to eight-well culture dishes (Nunc Inc., Naperville, IL) and incubated at 37°C for 24 h. Aliquots were removed by aspiration before the addition of medium to the well. Small or large cells ($0.15 \times 10^6\text{ ml}^{-1}$) were incubated in 0.5 ml DMEM/F12 medium containing 5 μ g fibronectin ml^{-1} , 5 ng selenium ml^{-1} , 40 ng hydrocortisone ml^{-1} , 2 μ g insulin ml^{-1} , 10 μ g transferrin ml^{-1} , 5 pg triiodothyronine ml^{-1} , 10 μ g low-density lipoprotein ml^{-1} (LDL), 30 μ g high-density lipoprotein ml^{-1} (HDL) and 10 μ g gentamicin ml^{-1} at 37°C under 5% CO_2 in air. All reagents were from Sigma except fibronectin (UBI, Lake Placid, NY). Steroid production is affected by the number of cells present; similar numbers of cells from each population were therefore cultured. Media from these cultures were removed 24 h later by gentle aspiration and replaced with 50 ng FSH ml^{-1} (oFSH-NIH-S9) or 10 ng transforming growth factor- α ml^{-1} (TGF- α , rat; Bachem California, Torrance, CA) or a combination of both. The rationale for selecting FSH and TGF- α was from observation by Yeh *et al.* (1991), Anderson and Lee (1993), and Yeh *et al.* (1993). Granulosa cells were

cultured for 3 days and the media were collected every 24 h for progesterone assay.

Determination of progesterone production

Culture media were assayed for progesterone by radioimmunoassay as described by Anderson *et al.* (1987). The sensitivity limit of the assay was 5 pg per tube. Values are expressed per 10^6 cells. The intra- and interassay coefficients of variation were 6.3% and 12.0%, respectively.

Ultrastructural analysis

In some experiments, sorted cell fractions were collected for ultrastructure analysis. Cells were fixed with 2.5% glutaraldehyde (EMS, Fort Washington, PA) in 0.1 mmol sodium cacodylate l^{-1} (NaCaC) buffer for 15 min, post-fixed with 1% osmium tetroxide (OsO_4) and processed according to Anderson *et al.* (1987). Thin sections were made and examined with a Phillips 300 electron microscope.

Statistical analysis

Steroid concentration in culture media, cell number and cell diameter were analysed by analysis of variance and Bonferroni test (Snedecor and Cochran, 1982). Differences were considered significant when $P < 0.05$.

Results

Cell separation by Percoll gradient

By calibrating the density of the granulosa cells at various positions in the Percoll gradient (data not shown), solutions of Percoll with the specific gravity adjusted to 1.029 to 1.076 $g\ ml^{-1}$ (20 to 60% Percoll) allowed granulosa cells to be separated into five fractions. No granulosa cells were separated beyond this range of densities. To determine the distribution of cells in the gradient, the number of cells from each fraction was recorded under a light microscope (Fig. 1). The diameters of granulosa cells were measured with a light microscope and the diameter of the two types of cell appeared to be constant among three groups (Table 1). Fraction 1 was discarded because it was composed mainly of damaged cells. Fraction 2 consisted of mainly small cells ($94.1 \pm 4.3\%$ in 15-day-old rats, $84.5 \pm 4.5\%$ in 25-day-old rats and $91.0 \pm 7.2\%$ in PMSG-primed rats) and these cells were cultured without sorting. Large cells were separated into incompletely resolved fractions; however, fractions 3 and 4 contained relatively large numbers of large cells ($39.1 \pm 3.5\%$ in 15-day-old rats $65.0 \pm 5.0\%$ in 25-day-old and $42.1 \pm 4.2\%$ in PMSG-primed rats). These fractions were sorted to obtain populations of purified large cells. Fraction 5 had relatively few cells. Blood cells formed a pellet at the bottom of the tubes and this was discarded.

Flow cytometry

The FALS signal is assumed to reflect cell size, whereas the $90^\circ LS$ signal reflects internal structure such as cytoplasmic

Fig. 1. The forward angle light scatter (FALS) analysis of small (upper panel) and large (lower panel) cell populations from (a) 15-day-old, (b) 25-day-old and (c) PMSG-primed rats. The FALS gate was set at channels 3–64 and the plots were generated from 10 000 cells. The gate was placed on channel 28 (I) for collecting further purified large cells.

Table 1. Comparison of diameters of small and large rat granulosa cells

Animals	Small (μm)	Large (μm)
15-day-old	8.84 ± 0.71	11.18 ± 0.80
25-day-old	8.95 ± 0.56	11.27 ± 1.00
PMSG-primed	8.96 ± 0.60	11.05 ± 0.66

Cell diameter measured by a micrometer.

Values are means $\pm$ SD diameter of 200–250 cells.

Fig. 2. Forward angle light scatter (FALS)-90° light scatter (LS) distribution (channel setting: FALS-23, 90°LS-33). The plots were generated from 10 000 granulosa cells from (a) 15-day-old, (b) 25-day-old and (c) PMSG-primed rats. The heavy dots represent most cells. Regions 1–2 and 3–4 are defined by 90°LS and contain ridges of high and low cytoplasmic granularity. Regions 1–3 and 2–4 are defined by FALS and contain ridges of small and large cells. A lower ridge (channel 15; 90°LS) in region 3 was eliminated because it consisted of cellular debris or red blood cells.

Table 2. Composition of large cells in total population of rat granulosa cells

Animals	Large cell (%)	Total cells (10 ⁶ per rat)
15-day-old	10.85 ± 1.13	0.61 ± 0.08
25-day-old	15.70 ± 2.50	6.76 ± 0.22
PMSG-primed	12.50 ± 3.10	10.81 ± 1.04

Each value represents the mean ± SD of measurements of two to three replicates.

Table 3. Quadrant analysis of granularity of rat granulosa cells

Animals	Small cells			Large cells		
	Low	High	High:low	Low	High	High:low
15-day-old	6324	1306	0.21	612	672	1.10
25-day-old	5288	1979	0.37	399	739	1.85
PMSG-primed	4582	4455	0.97	54	678	12.56

Small and large cells in regions (1), (2), (3) and (4) from Fig 2.

secretory granule content (Shapiro, 1985). One-parameter analysis showed the small cell (fraction 2) and large cell populations (fractions 3 and 4; Fig. 2). When cells from 25-day-old rats were used as a standard, the patterns of cell distribution from 15-day-old and PMSG-primed rats were different. The gate was placed on channel 23 (FALS) for calculating the percentage of small and large cell populations. In unseparated preparations of granulosa cells, the percentage of large cells represented 10.85 ± 1.13%, 15.70 ± 2.50%, and 12.50 ± 3.10% of total cells from 15-, 25-day-old and PMSG-primed rats, respectively (Table 2). To avoid cross-contamination with

small cells, the gate was placed on channel 28 for collecting further purified large-cell populations. The purity of large cells obtained by cell sorting was 83.6% in 15-day-old, 87.9% in 25-day-old, and 84.6% in PMSG-primed rats. The FALS–90°LS distribution analysis was performed on total pooled granulosa cells to determine whether there was a shift in 90°LS (cytoplasmic granularity) among the three groups of granulosa cell. The cell distribution patterns are shown (Fig. 3). For standard setting, the gates were placed on the cell distribution from 25-day-old rats for determining the percentage of cells that lay within regions 1–4. The FALS gate was placed on channel 23 (equivalent to one-parameter analysis) and the 90°LS gate was placed on channel 33. Cellular debris and erythrocytes were excluded from analysis by placing the gate on channel 15 in quadrant 3. In 25-day-old rats, small and large cells with high:low granularity were 0.37 and 1.85 (Table 3). Compared with 25-day-old rats, there were fewer small and large cells with high:low granularity in 15-day-old rats. However, the number of large cells with high granularity was dramatically increased in PMSG-primed rats compared with that of 25-day-old rats (12.56 versus 1.85). These results indicated a shift in small and large cells with increased granularity from 15-day-old to PMSG-primed rats.

Progesterone production by granulosa cells

Functional integrity of the granulosa cells was demonstrated by secretion of progesterone into the culture medium (Fig. 3). For all groups, small or large cells cultured in basic medium for 3 days did not further increase their progesterone secretion, although the basal rate of production from large cells was greater than from small cells in PMSG-primed rats. Incubation in the presence of FSH or TGF-α increased the amount of progesterone. Throughout the culture period, FSH plus TGF-α induced the highest concentrations of progesterone in all groups. Small and large granulosa cells from 15-day-old rats did not respond to the stimulants and secreted low concentrations of progesterone (FSH/TGF-α 4.69 ± 1.2 versus 2.66 ± 1.0 × 10⁻⁶ ng, day 1). In 25-day-old rats, incubation in the presence of FSH, TGF-α or both resulted in increased concentration of progesterone with both types of cell producing similar amounts of progesterone (FSH/TGF-α 65.68 ± 9.6 versus

Fig. 3. Progesterone production of small (left) and large (right) cells from (a) 15-day-old, (b) 25-day-old and (c) PMMSG-primed rats. Granulosa cells were cultured with (▨) 50 ng FSH ml⁻¹, (▩) 10 ng TGF- α ml⁻¹ or (■) a combination of both for 3 days; (□) control.

$78.25 \pm 12.3 \times 10^{-6}$ ng, day 1). A different profile of progesterone production was obtained in the PMMSG-treated group; large cells produced 3.2 times more progesterone than did small cells on day 1 ($P < 0.05$).

Ultrastructure analysis

Morphological integrity of granulosa cells was observed by electron microscopy. Ultrastructural studies revealed differences between the small and large cells. The intracellular organelles involved in steroidogenesis are lipid droplets, mitochondria, lysosomes and smooth endoplasmic reticulum. At the time of harvest and separation by Percoll, there were few structural differences between the two types of cells (Fig. 4a, b). The small cells had many more lipid droplets in close association with mitochondria. Cytoplasmic changes and development of organelles associated with steroidogenesis were confirmed only in cultured granulosa cells from PMMSG-primed rats. The abundant smooth endoplasmic reticulum (SER), well-developed mitochondria and lipid droplets were observed in large cells (Fig. 5a, b). After 3 days of culture, the large cells but not the small cells contained mitochondria with tubular cristae when cultured in the presence of FSH plus TGF- α (Fig. 6a, b). This morphological change in mitochondrial structure may represent luteinization of granulosa cells since mitochondria with tubular

cristae are features of the corpus luteum (Anderson and Little, 1985).

Large and small granulosa cells from 15-day-old rats were structurally homogeneous (Fig. 7a, b).

Discussion

Our study shows that ovarian follicles of 15-day-old, 25-day-old and PMMSG-primed rats have populations of large and small granulosa cells the morphological and physiological features of which are related to their response to FSH, TGF- α or both substances. These characteristics may be related to the transition from the preantral to the preovulatory stage of follicle differentiation.

The heterogeneity in granulosa populations is related to cell location within the follicle (Magnusson *et al.*, 1982). The two granulosa cell populations are functionally different from each other in that the mural granulosa cells contain higher concentrations of Δ^5 -3 β -hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase activity (Zoller and Weisz, 1979). Amsterdam *et al.* (1975) found that labelled-binding sites for hCG are located over the mural granulosa cells adjacent to the basement membrane, but that labelling decreased towards the inner layers of the granulosa cells in the follicle. Using the method of [³H]thymidine incorporation, Hirshfield (1986) found

Fig. 4. (a) A small granulosa cell and (b) a large granulosa cell from a PMMSG-primed rat follicle. ER: rough endoplasmic reticulum; M: mitochondria; L: lipid droplets; GC: Golgi complex; N: nucleus; $\times 8400$.

that mural granulosa cells were more differentiated than antral granulosa cells.

A high degree of purity of the cell populations is necessary to study the individual function of the two types of granulosa cell. We have used a combination of density gradients and flow cytometry. Prior separation by Percoll gradient allowed purification of small-cell populations (84.5–94.1%). Separation by Percoll gradient eliminated cellular debris and erythrocytes, minimized the sorting time, and enhanced the recovery of granulosa cells. In large-cell preparations (fractions 3 and 4) obtained by Percoll gradient, large cells contained 39.1–65.0% of the total number of cells, whereas the purity of the large cells obtained by flow cytometry was 83.6–87.9%. In agreement

with the method described by Alila *et al.* (1988), this combined method improved the purity of large-cell preparations. However, compared with their large luteal cell preparations (90–99%), the purity of large granulosa cells in our study was low, possibly due to the narrow separation between the small-cell diameter range of 6.96–9.57 μm and the large-cell diameter range of 10.96–13.05 μm .

The FALS-90°LS distribution analysis by flow cytometry measures the granularity of cells as well as relative cell size. Since 90°LS signals reflect cytoplasmic elements, such as certain organelles, the increased signals indicated a higher degree of maturation (Rao *et al.*, 1991). This analysis determined whether the granularity of granulosa cells was changed among the three

Fig. 5. A small portion of (a) a small cell and (b) a large cell from a PMSG-primed rat follicle cultured for 1 day. SER: smooth endoplasmic reticulum; ER: rough endoplasmic reticulum; L: lipid droplet; M: mitochondria; $\times 48\,000$.

experimental groups. A shift in small and large cells with increased granularity from 15-day-old to PMSG-primed rats was observed. Large cells from PMSG-primed rats dramatically increased in granularity compared with those from 25-day- and 15-day-old rats. Electron microscopy allowed the structural composition of these granulosa cells to be examined, but the results showed few structural differences among these granulosa cells. Changes in cytoplasmic structures within the granulosa cells were observed after they were placed into culture.

The functional integrity of the granulosa cells was demonstrated by secretion of progesterone during culture. The two

subpopulations of granulosa cell responded differently to FSH and TGF- α stimulation. Unpublished observations from our laboratory revealed that TGF- α stimulated protein synthesis. As shown in the present study, TGF- α promoted the production of progesterone. It is possible that certain differentiated states of granulosa cells respond differently to TGF- α when in combination with FSH. The synthesis of TGF- α by rat granulosa cells (Yeh *et al.*, 1991; 1993) or theca cells (Kudlow *et al.*, 1987) suggests a paracrine and autocrine role in the regulation of granulosa cell function. TGF- α promotes cell proliferation (Skinner and Coffey, 1988) and may have a role in modulating hormone action on granulosa cells (Adashi *et al.*, 1987). As with

Fig. 6. A small portion of (a) a small cell and (b) a large cell from a PMSG-primed rat follicle cultured for 3 days. ER: rough endoplasmic reticulum; SER: smooth endoplasmic reticulum; DB: dense bodies; M: mitochondria; $\times 48\,000$.

epidermal growth factor (EGF) (Jones *et al.*, 1982), TGF- α plus FSH can enhance progesterone production by granulosa cells (Yeh *et al.*, 1991; 1993). The regulatory role of TGF- α in the growth and differentiation of granulosa cells may occur within the ovary (Kudlow *et al.*, 1987). The results of the study reported here demonstrate a differential control of progesterone production in small and large granulosa cells from PMSG-treated rats. Large cells are maximally responsive to stimulation by FSH plus TGF- α on day 1 of culture as indicated by progesterone secretion. Electron microscopy of the granulosa cells indicated regional differences in cell morphology. The abundant smooth endoplasmic reticulum, well-developed mitochondria and lipid droplets observed in large cells are characteristics of high steroidogenic activity (Amsterdam and Rotmensch, 1987).

After 3 days of culture, the large cells, but not the small cells, contain mitochondria with tubular cristae that were observed in the presence of FSH plus TGF- α . Such morphological changes are consistent with luteinization of the granulosa cells. As FSH alone did not induce this apparent luteinization in serum-free medium (Orly *et al.*, 1980), FSH plus TGF- α can induce or enhance luteinization of the large granulosa cells. These results together with the progesterone production indicate that large cells are functionally differentiated cells. As the large cells account for only 10.85–15.70% of the total granulosa cell population, the mechanism by which large cells are involved in follicular function is not known. Rodgers *et al.* (1985) suggested a paracrine interaction between subpopulations of luteal cells from sheep. It is possible that separation of cells into subpopu-

Fig. 7. A small portion of (a) a small cell and (b) a large cell from a 15-day-old rat follicle cultured for 3 days. ER: rough endoplasmic reticulum; M: mitochondria; MT: microtubule; L: lipid droplets; $\times 48\,000$.

lations might alter interactions between the two cell types that influence steroidogenic responses. In contrast, small and large cells from 25-day-old rats respond in a very similar way to FSH and TGF- α stimulation. Granulosa cells from 15-day-old rats were not responsive to these stimulations and secreted very low concentrations of progesterone. These observations correlate with our electron microscope analysis which demonstrated that small and large cells are structurally homogeneous cells. The steroidogenic potential of these granulosa cells could be due to degrees of differentiation; granulosa cells from 15-day-old rats are preantral, from PMSG-treated rats are preovulatory and from 25-day-old rat follicles are intermediate. It is most likely that the states of the microenvironments within all follicles are quite different and would be expected to affect granulosa cell

differentiation during follicle development. Depending upon the stage of follicular development, granulosa cells vary markedly in their response to hormonal stimuli and their ability to secrete steroid hormones (Channing, 1970). Our findings have shown that metabolic and morphological features of large cells are associated with follicular development. The question is therefore raised as to whether there is movement of cells between the two subpopulations. The possibility that small cells are precursors of large cells cannot be ruled out. However, it is unlikely that all of the large cells arise from the small cells, as the percentage of large cells is not significantly different among granulosa cells from the different stages of follicles. In agreement with the observations of Rao *et al.* (1991), this finding could indicate that the two types of granulosa cell are

independent populations and have different cell cycles. It is also possible that the two populations may be a constant feature since there are also two populations of luteal cells not only in the corpus luteum of rats (Wilkinson *et al.*, 1976) but also in the corpora lutea of other species (Fitz *et al.*, 1982). However, Alila and Hansel (1984) claimed that the small cells of the bovine corpus luteum are of 'thecal origin'. It must be emphasized that functional differentiation of granulosa cells during transition from the preantral to preovulatory follicle stage is to be expected and that the metabolic properties of small and large cells correlate with the stages of follicle development.

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