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Morphological Assessment of the Effect of Growth Hormone on Preantral Follicles from 11-Day-Old Mice in an *in Vitro* Culture System

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The present study was carried out to verify that the cells attached to the outside of the basement membrane of mechanically isolated follicles are theca cells and to evaluate the effect of growth hormone (GH) on these cells. Preantral follicles, 100–140 μm in diameter, were mechanically isolated from 11-day-old BDF1 hybrid immature mice, divided randomly into two groups, and cultured *in vitro.* One group was treated with 0.1% collagenase immediately after mechanical isolation in an attempt to remove theca cells attached to the outside of the basement membrane. The other group was untreated. Morphological examination revealed that 86.1% of mechanically isolated follicles before collagenase treatment had at least one theca cell around the basement membrane on the single section. However, after collagenase treatment no theca cells remained on the basement membrane of the follicles. Androstenedione secretion as a result of stimulation by 100 ng/ml hCG was significantly higher in the culture medium of the follicles with theca cells than in those of collagenase-pretreated follicles ($p < 0.0001$), indicating that the cells attached to the outside of the basement membrane were actually functional theca cells, not interstitial cells. To elucidate the effect of GH on theca cells, preantral follicles cultured in the presence of 1.0 mIU/ml GH were morphologically examined. Preantral follicles mechanically isolated from immature mice showed significant proliferation of not only granulosa cells but also theca cells in the presence of GH. In particular, theca cells, which remained dotted on the basement membrane in a small number just after isolation, proliferated and finally formed complete layers after the culture with GH. This is the first report that GH induced the proliferation of theca cells to form morphologically complete layers around the preantral follicle from 11-day-old mice.

Key Words: **growth hormone; theca cell; *in vitro* follicle culture.**

An isolated granulosa cell culture system has added greatly to the knowledge of the function of these cells, but the results obtained by using this system are not directly applicable to intact follicles because the isolated granulosa cells do not proceed through normal development (1, 2). Follicles consist of an oocyte, granulosa cells, and theca cells, and each component is mandatory for normal folliculogenesis that is characterized by morphological changes as well as functional development. An *in vitro* follicle culture system has been developed in an attempt to overcome problems inherent in granulosa cell culture systems, and indeed important information that had not been clarified in granulosa cell culture systems has been disclosed by this system (3–5). Because of the interaction of all three components, follicles can attain morphological development and steroidal hormone production (6–13). Many reports have been made since this new culture method was developed, but few studies have focused on theca cells. The presence of theca cell layers in mechanically isolated follicles was indicated in a previous study (14), but whether these cells represented true theca cells or stromal cells remains undetermined. This is because it is technically difficult to reserve theca cells on preantral follicles in the very early stage of folliculogenesis in this system. In this study, we assessed theca cells on mechanically isolated preantral follicles in an attempt to verify that these cells were functional theca cells. Moreover, we tried to elucidate the effect of GH on theca cells from a morphological point of view. This is of particular interest since this hormone has received considerable attention entirely

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in the context of the function of theca cells, such as androstenedione secretion or localization of the GH receptor (15, 16). The *in vitro* follicular culture system can greatly help in understanding how theca cells proliferate around a follicle in the early stages of folliculogenesis. Using this system we found that GH plays an important role in the proliferation of theca cells to form theca layers around isolated preantral follicles.

MATERIALS AND METHODS

Chemicals. Collagenase was purchased from Wortington Biochemical Corporation (NJ). Recombinant human activin A (rh activin A) was prepared as described previously (17). Recombinant human growth hormone (rhGH) was obtained from Saizen, Ares-Serono Laboratories (Switzerland). All other chemicals were of analytical grade or the highest quality commercially available.

Animals. Female BDF1 hybrid mice were purchased from Japan Charles River Inc. (Tokyo, Japan), and housed in a temperature and light-controlled room with a 14L:10D photoperiod in accordance with the principles of the Animal Science Center of the Gunma University School of Medicine. Food and water were given *ad libitum*. Eleven-day-old female mice were sacrificed by cervical dislocation for the experiments.

Follicle culture and isolation of follicles. The preantral follicle culture was prepared as described previously (18). Ovaries were removed aseptically from freshly killed 11-day-old mice and placed in Falcon deep well dishes (multiwell tissue culture plate:6-well, flat bottom) filled with the culture medium (DMEM, GIBCO BRL, Tokyo, Japan). After removing surrounding tissue, the capsule of an ovary was peeled off and follicles were carefully isolated from the ovary by using 27-gauge needles attached to 1-ml syringes, then, preantral follicles 100–140 μm in diameter were visually collected and transferred into serum free DMEM supplemented with 6.25 mg/ml of insulin, 6.25 mg/ml of transferrin, 6.25 ng/ml of selenious acid, 5.35 mg/ml of linoleic acid and 0.15% BSA. Ten to 30 follicles per well were cultured in a 6-well polystyrene dish (FALCON, Lincoln Park, NJ) containing 1.0 ml of the culture medium with or without 100 ng/ml hCG, 1.0 mIU/ml GH, or 100 ng/ml activin A and cultured in a humidified chamber with 5% CO_2 in the air at 37°C for 12–96 h according to the particular experiments as described in the results.

Light and electron microscopy. Removed ovaries and cultured follicles were fixed in 2.0% paraformaldehyde-2.5% glutaraldehyde in 0.1 mol phosphate buffer (pH 7.4) at 4°C for 2 h. After washing in the same buffer containing 7% sucrose, the follicles were arranged on fluorine-coated watchglasses and embedded in 1% agar. The specimens were postfixed in 1% osmium tetroxide in the same phosphate buffer for 1.5 h at 4°C, dehydrated through graded alcohol, placed in propylene oxide and embedded in Quetol 812. Serial sections of 1 μm thick were cut on an LKB8800 Ultratome III with glassknives, stained with 0.5% toluidine blue and examined by light microscopy. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and examined in a transmission electron microscope (JEOL, 2000CX).

Functional verification of theca cells. To verify that the cells attached to the basement membranes of preantral follicles were functional theca cells, mechanically isolated follicles were placed into a culture medium containing 0.1% collagenase for 1–2 h with occasional vigorous pipetting in an attempt to remove theca cells. These enzymatically treated follicles and nontreated follicles were cultured in the medium with 100 ng/ml hCG for 12 h and the secretion of androstenedione was measured. Size of the follicles, estradiol, and immunoreactive (IR) inhibin secretion as a result of stimulation by activin A (100 ng/ml) for 4 days were evaluated, respectively.

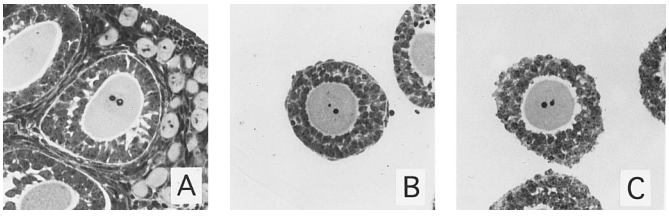

FIG. 1. A preantral follicle about 100 μm in diameter in the mouse ovary (A), mechanically isolated (B), and collagenase treated after mechanical isolation (C). Intact preantral follicles have 2–3 layers of granulosa cells and 1–3 layers of theca cells around the basement membrane. Although an oocyte, granulosa cells, and a basement membrane are reserved intact, theca cells are only partially reserved by mechanical isolation (B). Collagenase treatment removed the basement membrane and theca cells (C). Epon/Toluidine blue. Magnification, ×200.

Hormone assays. Inhibin concentrations in the culture medium were assayed by double antibody RIA using rabbit antiserum against bovine follicular fluid inhibin as described previously (5). Estradiol concentrations were measured by direct RIA using antiestradiol antiserum kindly supplied by Dr. W.F. Crowley, Jr., and a radioactive tracer of oestradiol-6-(*O*-carboxymethyl)oximino-(2-[^{125}I]iodohistamine) (Amersham, Buckinghamshire, UK). Androstenedione levels were determined using a RIA kit (Diagnostic Systems Laboratories, Webster, TX) after extraction by diethyl ether. The assay sensitivity was 0.5 pg/ml for inhibin, 0.9 pg/ml for estradiol and 5.0 pg/ml for androstenedione. The intraassay coefficients of variation were 4.8, 2.4, and 4.0% for inhibin, estradiol, and androstenedione, respectively.

The effect of GH on theca cells. Separated follicles were dispersed and cultured in 6-well polystyrene dishes containing 1.0 ml of the culture medium with or without 1.0 mIU/ml GH, as this dose of GH has been shown to have a linear increase in follicle size for 4 days (19). After 2–4 days in culture, the follicles were fixed, stained, and examined under light and electron microscopy. Theca cells on preantral follicles were counted on the section of maximum diameter. Follicles that were surrounded by theca cells all around the basement membrane were regarded as follicles with a complete theca layer. The number of total follicles was calculated and the ratio of follicles with complete theca cell layers to total follicles was calculated.

Statistics. Results were expressed as mean ± SE. Statistical analysis of hormone value was performed by one-way ANOVA, followed by Scheffe's multiple comparison test. The ratio of theca cell formation was analyzed by Fisher's exact probability test. $p < 0.05$ was considered statistically significant.

RESULTS

Figure 1 shows morphological features of the preantral follicle located in the ovarian tissue (A), mechanically isolated follicles (B) and collagenase treated follicles (C). The mechanically isolated follicles retained theca cells outside the basement membrane (B), while after collagenase treatment, follicles lost both the basement membrane and theca cells (C). Since mechanical isolation causes some damage to theca cells outside the basement membrane, the reservation rate of theca cells per follicle was calculated. Of 165 primary follicles examined, 23 follicles (13.9%) did not have any theca cells on the examined section, but the remaining ones

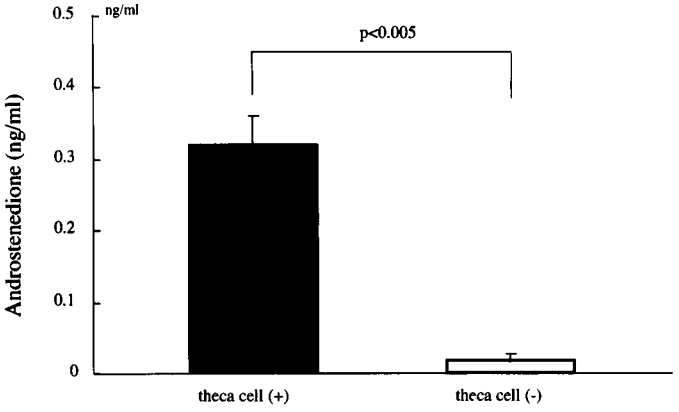

FIG. 2. Androstenedione secretion from primary follicles as a result of stimulation by 100 ng/ml of hCG for 12 h. Theca (+), mechanically isolated follicles; theca (−), collagenase treated follicles after mechanical isolation. Each group consisted of 30 follicles and the experiment was repeated three times.

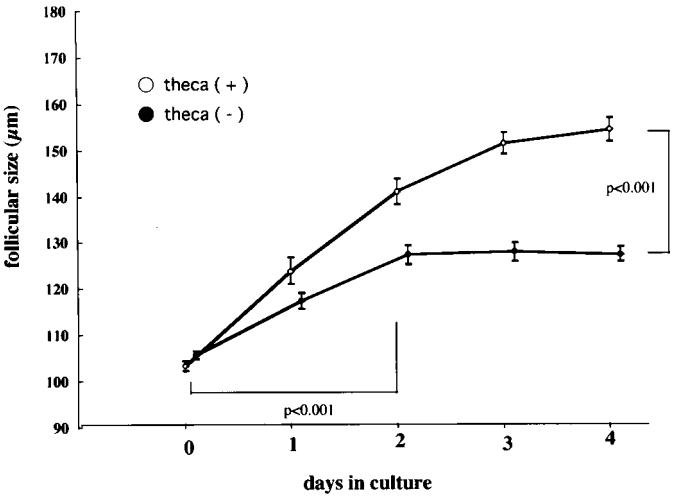

FIG. 3. Changes in follicular size of preantral follicles. Ten follicles were cultured in the presence of 100 ng/ml activin A for 4 days, and the experiment was repeated three times. Means were obtained from 30 follicles. Theca (+), mechanically isolated follicles; theca (−), collagenase treated follicles after mechanical isolation. Follicular sizes of both groups became significantly larger after 2 days ($p < 0.001$) and the size of the follicles with theca cells was significantly larger than those without theca cells ($p < 0.001$) on day 4.

(86.1%) had more than 1 theca cell around the basement membrane.

To confirm whether these cells outside the basement membrane were functionally identical with theca cells, androstenedione secretion was compared after stimulation by 100 ng/ml hCG in mechanically isolated (theca +) follicles and those treated with collagenase (theca −) (Fig. 2). The androstenedione level of the preantral follicles with theca cells was 0.319 ± 0.041 ng/ml, while that of preantral follicles treated with collagenase was undetectable, indicating that the former group of cells was functionally theca cells.

Changes in follicular size during 4 days of culture with 100 ng/ml of activin A with and without collagenase treatment are shown in Fig. 3. As can be seen, both groups showed a significant increase in follicular size, but the growth of the follicles was significantly higher in preantral follicles with theca cells, than in those without theca cells. Estradiol and IR-inhibin secretion of these two groups as a result of stimulation by activin A is shown in Fig. 4. Preantral follicles with theca cells showed both estradiol and IR-inhibin secretion. Although IR-inhibin secretion was shown from preantral follicles without theca cells, estradiol was not detected, suggesting that theca cells are mandatory for estradiol secretion of preantral follicles.

The morphological changes of preantral follicles cultured with or without 1.0 mIU/ml of GH are shown in Fig. 5. As shown in Fig. 5B, preantral follicles cultured for 4 days with the medium alone (control) did not show any difference in size of follicles or in appearance of the theca layer in comparison with those immediately after isolation (Fig. 5A). However, preantral follicles cultured for 4 days in the presence of 1.0 mIU/ml of GH showed an increase in the number of granulosa cells as well as theca cells (Fig. 5C), indicating that GH causes proliferative effects on both granulosa cells and theca cells. It is also interesting to note that reserved theca cells proliferated and completely surrounded the basement membrane and finally formed 2–3 theca layers around follicles (Fig. 5D). Chronological changes in the number of theca cells on the largest section of follicles showed that theca cells significantly increase in number on day 1 and reach a plateau by day 2 (Fig. 6). Moreover, 31% of follicles ($n = 68$) stimulated by GH

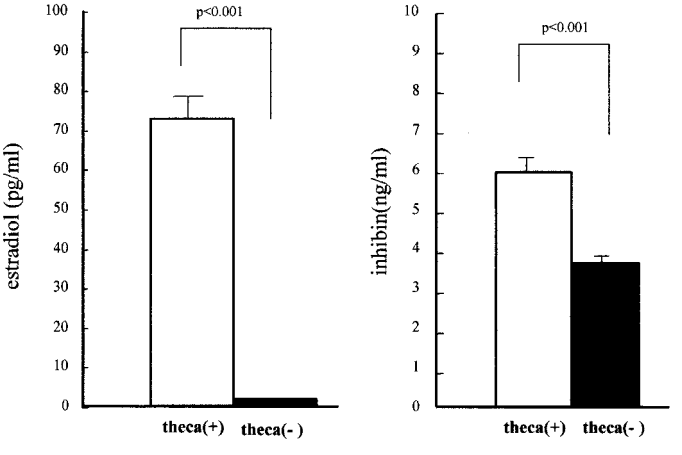

FIG. 4. Estradiol and IR-inhibin secretions from primary follicles as a result of stimulation by 100 ng/ml activin A for 4 days. Theca (+), mechanically isolated follicles; theca (−), collagenase treated follicles after mechanical isolation. Ten follicles of each group were cultured in a 6-well polystyrene dish containing 1.0 ml of the culture medium with or without 100 ng/ml activin A in a humidified chamber with 5% CO_2 in the air. Results were obtained from three experiments.

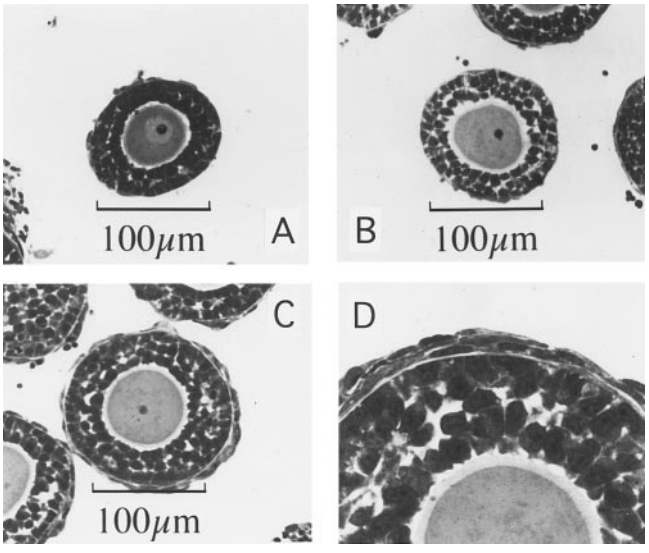

FIG. 5. Morphological changes of preantral follicles cultured with or without 1.0 mIU/ml GH. Preantral follicles immediately after isolation (A) and at 4 days after culture with medium alone (B). Preantral follicles cultured for 4 days in the presence of 1.0 mIU/ml GH (C). The magnified figure of theca layers (D). Epon/Toluidine blue. Magnification, ×100 (A, B, C), ×400 (D).

formed complete theca layers whereas none of the control group ($n = 20$) formed any layers (Fig. 7).

Figure 8 shows morphological features of theca cells by electron microscope. The theca cells were abundant in mitochondria and rough endoplasmic reticulum and in many lipid droplets (A). Mitosis of theca cells was sometimes observed in follicles cultured with GH (B). Granulosa cells also proliferate in the presence of GH and the mitosis of granulosa cells can be observed as well as theca cell proliferation (C).

DISCUSSION

The present study has demonstrated that mechanical isolation of small preantral follicles from immature mice can retain cells outside the basement membrane and that these cells are in fact theca cells. It is generally accepted that granulosa cells of early preantral follicles do not possess LH receptors but that theca internal cells contain specific LH receptors and can respond to LH throughout follicular development (20, 21). Because the mechanically isolated follicles secreted a significant amount of androstenedione or estradiol in comparison to collagenase-treated follicles as a result of stimulation by hCG or activin A, it is concluded that the cells attached outside of the basement membrane are theca cells. Electron microscopic studies also showed that these cells have a flattened shape and that the cytoplasm contained many mitochondria, rough endoplasmic reticulum, free ribosomes and lipid droplets. These features are compatible with the electron microscopic descriptions of the early stage of theca cells from sheep or rats (22, 23). Thus it is suggested that the cells attached outside the granulosa cell layers are theca cells from both a functional and morphological point of view.

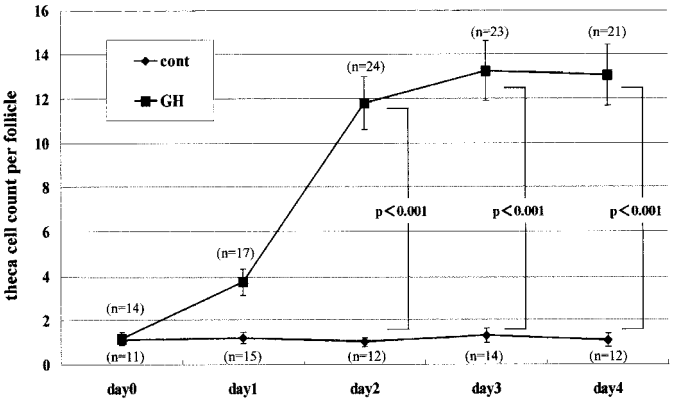

FIG. 6. Changes in the number of theca cells of preantral follicles cultured with GH and the medium alone. Follicles cultured with the medium alone did not show any increase in the number of theca cells, while those cultured with GH showed a significant increase. Values in parentheses, numbers of follicles examined.

In this study, activin A was employed in order to stimulate follicular growth, because preantral follicles from immature mice do no respond to FSH (18). Preantral follicles from immature mice increase in size and secrete estradiol and IR-inhibin as a result of stimulation by activin A (5, 18). As shown in Fig. 3, however, preantral follicles without theca cells could increase in size, but the degree of growth was signifi-

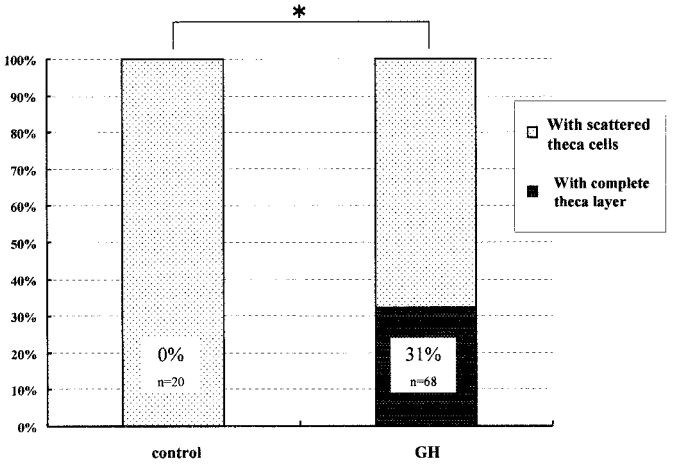

FIG. 7. The ratio of follicles with complete theca cell layers. Follicles whose basement membranes were surrounded by theca cells were regarded as follicles with complete theca layers while those whose basement membranes were not completely surrounded by theca cells were regarded as follicles with scattered theca cells. 31% of follicles ($n = 68$) stimulated by GH for 2–4 days formed complete theca layers whereas none of the control group ($n = 20$) formed any layers. *$p < 0.005$ vs control.

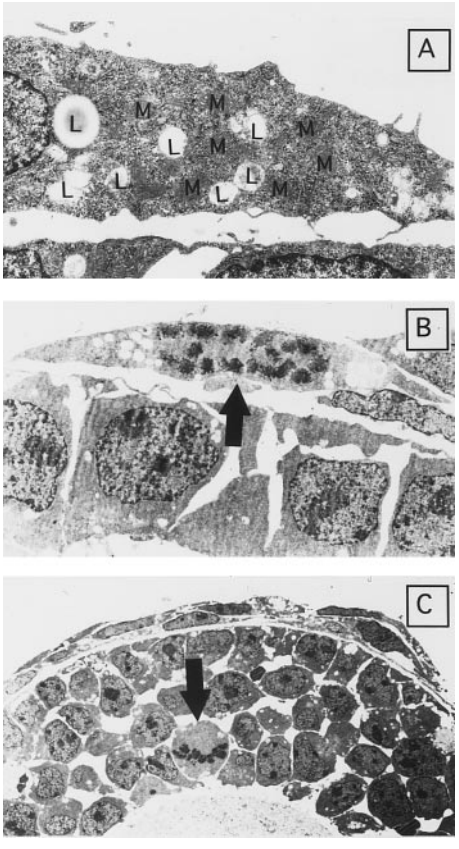

FIG. 8. Electron microscopic features of theca and granulosa cells of follicles cultured for 4 days with GH. The theca cells were abundant in mitochondria and rough endoplasmic reticulum and many lipid droplets (A, magnification ×9300). Mitosis of theca cells was sometimes observed in follicles cultured with GH (B, magnification ×4500). Karyomitosis in the granulosa cell (C, magnification ×900).

cantly smaller than that seen in preantral follicles with theca cells. Moreover, preantral follicles without theca cells secrete less IR-inhibin as a result of stimulation by activin A as shown in Fig. 4, suggesting that the presence of the theca cells may affect the growth of granulosa cells. It has been reported that collagenase treatment of preantral follicles does not damage the follicle viability since these follicles can become fertile (14), and so the decreased follicular growth of preantral follicles without theca cells is likely to be due to the loss of interaction between granulosa cells and theca cells (6–13). However the present study did not aim to clarify the factor(s) that are involved in the interaction between theca cells and granulosa cells. As can be seen in Fig. 5, we have shown that GH stimulates the proliferation of partially reserved theca cells and leads to the formation of complete layers outside the basement membrane. Electron microscopic findings clearly demonstrated that flattened shaped cells pile up around the basement membrane and that these cells contain many lipid droplets (Fig. 6A), indicating that these cells are endocrine cells. Apa *et al.* (16) have shown that GH can stimulate androstenedione secretion from rat theca-interstitial cells without requiring hCG, and we have shown that GH stimulates estradiol secretion from preantral follicles *in vitro* (5). Thus these two results suggest that GH causes functional development of theca cells. However, Apa *et al.* (16) also stated in their report that GH did not affect cellular proliferation, which is inconsistent with our result but this could be attributable to the difference between a conventional cell culture system and an *in vitro* follicular culture system. The present study could not clarify whether these findings were due to the direct effect of GH on theca cells or the indirect action of unidentified growth factors produced from granulosa cells. Although recently studies have demonstrated the presence of GH receptors in the ovary, it is still controversial whether theca cells have GH receptors. Lobie *et al.* showed GH receptor/binding protein on adult rat theca cells (15), while Sharara *et al.* could not find GH receptor mRNA in human theca cells (24). Moreover, GH has lactotroic action, but our previous study (5) could not find any changes in follicular growth in a culture of 10–1000 ng/ml of PRL, suggesting less possibility of involvement of PRL. In addition, electron microscopic examination in Fig. 6 reveals kariomitosis in both cells and no evidence of hypertrophy was observed, indicating that an increase in follicular size is due to an increased number of granulosa cells and theca cells.

In conclusion, the present study has proved for the first time since *in vitro* follicular culture systems were developed, that cells outside the basement membrane of mechanically isolated preantral follicles are functional theca cells. Also we have shown for the first time that GH plays an important role in the proliferation of theca cells to form theca layers around isolated preantral follicles in the early stage of folliculogenesis.

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