In Vitro Antrum Formation of Oocyte-Cumulus-Granulosa Cell Complexes from Pig Early Antral Follicles

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Abstract: *Oocyte-cumulus cells connected to some of the parietal granulosa cells (OCG complexes) were mechanically obtained from pig early antral follicles 0.5–0.7 mm in diameter and were cultured for 8 days in collagen gels. The effects of FSH, dbcAMP, estradiol-17β (E_2) and EGF on antrum formation in the OCG complexes were studied. After 1 day of culture, every OCG complex formed a spherical compact structure. The OCG complexes which had undergone FSH and dbcAMP treatment then formed antra, resulting in antral follicle-like structures, but no antra were formed in the E_2, EGF-treated and control groups. Instead, in these groups the granulosa cells spread away from the oocytes. Proteins similar to those in pig follicular fluid were accumulated in the antral follicle-like structures of the OCG complexes induced by FSH and dbcAMP stimulation. These results indicate that mediated by cAMP rather than estrogen, FSH induces antrum formation in pig granulosa cells, and that EGF itself does not induce antrum formation in vitro.*

Key words: *Granulosa cell, Antrum formation, FSH, dbcAMP, Pig.*

In mammalian ovaries, primordial follicles continuously leave the non-growing pool as they are converted into primary follicles. Through a series of mitotic divisions of the granulosa cells, unilaminar primary follicles are converted into multilaminated secondary follicles, followed by the tertiary follicle stage [1]. Except in several families of small mammals (*Tenrecidae and Erinaceidae*), a single, large, fluid-filled antral cavity is formed in the tertiary follicles [2]. FSH secreted by the pituitary gland is necessary for ovarian folliculogenesis. The administration of FSH causes an increase in the number of antral follicles and their development in hypophysectomized rats [3, 4]. *In vitro*, FSH stimulates the preantral follicles to develop into antral follicles in hamster [5], in mouse [6, 7], and induces an antral follicle-like reorganization of the preantral follicles in the rat [8].

Antrum formation is thought to be attributable to the proliferation and differentiation of granulosa cells. In the rat, it has been determined that FSH receptors exist exclusively in the granulosa cells [9], and therefore the various effects of FSH on the follicles are thought to be implemented by the granulosa cells [10]. It has been suggested that the effect of FSH on the granulosa cells is mediated by cAMP [10, 11]; FSH activates adenylyl cyclase activity and promotes cyclic adenosine 3',5'-monophosphate (cAMP) accumulation [12], which in turn activates the cAMP-dependent protein kinase to modify the biochemical activity of the granulosa cells. Analogs of cAMP stimulate proliferation, progesterone secretion [13], and morphological changes in the cultured granulosa cells [14]. In cultured mouse primary follicles, dibutyryl cyclic adenosine monophosphate (dbcAMP) has been shown to stimulate granulosa cell proliferation [15]. Via cAMP and cAMP-dependent protein phosphorylation, FSH induces aromatase activity and regulates estrogen production in granulosa cells [9, 16]. Estrogen in turn promotes granulosa cell proliferation and augments the effect of FSH on rat ovarian antrum formation *in vivo* [4, 17] and *in vitro* [8].

It has also been suggested that, in addition to FSH, epidermal growth factor (EGF) may also be included in folliculogenesis. EGF is a single-chain polypeptide first purified from the submaxillary glands of the mouse [18]. Its mitogenic effects have been shown in the cultured granulosa cells of rabbits, humans and pigs [13, 19,

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20]. Evidence of EGF-stimulated DNA synthesis, including an increase in diameter and progesterone secretion from the cultured ovarian preantral follicles, has been found in the hamster [21] and the mouse [22]. In mouse preantral follicles cultured *in vitro*, EGF increases the number of antral and Graafian follicles under FSH-supplemented conditions [22].

Similar to the results in rodents, in large animals it has been shown that FSH stimulates the proliferation of pig granulosa cells from secondary follicles [13], and that EGF stimulates DNA synthesis and progesterone secretion of cultured ovarian preantral follicles in bovines [23], and in pigs [13]. In contrast with rodents, however, the direct effects of FSH, cAMP, estrogen, and growth factors on antrum formation have not been studied in large animals because the *in vitro* culture of follicles from large animals is much more difficult than in rodents. Pig preantral follicles take 10 days to develop to antral follicles in the ovary [24]. Hirao *et al.* [25] have made a culture system for pig oocyte growth in preantral follicles for 14–16 days, but the incidence of antrum formation and the rate of survival of the follicles were low (Hirao, personal communication). In our preliminary experiment, less than 10% of pig preantral follicles formed the antrum, whereas more than 50% of oocyte-cumulus-granulosa cell complexes collected from early antral follicles formed the antrum under the same culture condition. In this study, we investigated antrum formation of oocyte-cumulus-granulosa cell (OCG) complexes from pig early antral follicles under the direct stimulation of FSH, dbcAMP, estradiol-17β (E_2) and EGF. We also confirmed that the organized antra of the OCG complexes contain proteins similar to those in the ovarian follicular fluid of the pig.

Materials and Methods

Collection and culture of OCG complexes from pig early antral follicles

The ovaries were obtained from prepubertal pigs slaughtered at a local abattoir. They were washed with phosphate-buffered saline (PBS) three times. All preparatory steps before culture were carried out in pH 7.4 Waymouth MB 752/1 medium (Sigma Chemical Co., St Louis, MO, USA), supplemented with 336 μg/ml $NaHCO_3$, 100 μg/ml sodium pyruvate, 1 mg/ml bovine serum albumin (BSA: Intergen Co., NY, USA), 100 μg/ml kanamycin (Sigma Chemical Co.), and 5 mg/ml HEPES. The ovarian cortices which contained no large antral follicles were cut off with a surgical blade. Under a dissecting microscope, the early antral follicles were dissected from pieces of the cortices, and the tissues surrounding the follicles were torn off. Follicles 0.5–0.7 mm in diameter were selected, and the oocyte-cumulus cell complexes connected to a part of the parietal granulosa cells (OCG complexes) were collected with a pair of fine forceps and a needle. Collected OCG complexes were washed once and kept in the HEPES-buffered medium before culture.

The composition of the basic culture medium was as follows: Waymouth MB 752/1 supplemented with 5% heat-inactivated fetal calf serum (FCS: Bio Whittaker, Walkersville, ML, USA), 2.24 mg/ml $NaHCO_3$, 100 μg/ml sodium pyruvate, 2 mM hypoxanthine, and 10 μg/ml kanamycin. The culture media containing different concentrations of FSH from the pig pituitary gland (UCB-Bioproducts, Belgium), dbcAMP (Sigma Chemical Co.), E_2 (Sigma Chemical Co.), and human recombinant epidermal growth factor (EGF: Genzyme, Cambridge, MA, USA) were prepared and kept at 38.5°C in 5% CO_2 in humidified air before culture. According to the method described by Hirao *et al.* [25], the OCG complexes were divided randomly into several groups, rinsed once in the basic culture medium, and then suspended in a 0.3 ml collagen solution in culture dishes (# 1008, 35 × 10 mm, Falcon, Becton Dickinson Labware, NJ, USA). The collagen solution was prepared by mixing a 0.3% acid-soluble collagen solution (Cellmatrix type I-A, Nitta Gelatine Co. Ltd., Osaka, Japan), a ten-times-concentrated Waymouth MB 752/1 medium without bicarbonate, and 0.05 M NaOH containing 22 mg/ml $NaHCO_3$ as well as 47.7 mg/ml HEPES, at a ratio of 8:1:1 (v:v:v). The number of OCG complexes embedded in the gel was less than 10. After incubation for 20 min at 38.5°C to set the gels, 4 ml of culture media containing FSH (0, 10, 100 and 500 ng/ml), dbcAMP (0, 2, 4 and 8 mM), E_2 (0, 10^{-8} and 10^{-7} M) and EGF (0, 1, 10 and 100 ng/ml) were added to the culture dishes, and the gels were then floated in the media. The OCG complexes were cultured at 38.5°C in 5% CO_2 in humidified air for 8 days. During the culture period, half the volume of the media was replaced with fresh media every 3 days. The morphology of the OCG complexes was observed, and their diameters were measured every day under an inverted microscope connected to an ocular micrometer.

Analysis of fluid accumulating in OCG complexes by SDS-PAGE

The ovaries of prepubertal pigs collected at a local abattoir were kept in ice and transported to our laboratory. After the ovaries were washed three times with

ice-cold PBS, the early antral follicles 0.5–0.7 mm in diameter were dissected and washed three times with ice-cold PBS. Under an inverted microscope, the diameters of the antra were measured, and the volumes were calculated. They were then transferred onto a sheet of plastic film, and a volume of ice-cold PBS was added to make a 0.1% follicular fluid. The follicles were opened in PBS, the antral fluids with the PBS were aspirated with a micropipette and were pooled in Eppendorf tubes. They were centrifuged at 400 x g for 30 sec, and the supernatant was recovered and added to an equal volume of a 2 x SDS-PAGE sample buffer [26]. The samples were boiled for three min, and stored at −70°C before use.

After the OCG complexes were cultured for 3 days under stimulation with FSH or dbcAMP by the method described above, the diameters of the antra in the OCG complexes were measured. The collagen gels were then digested with 0.1% collagenase (Wako Pure Chemical Industries Ltd., Osaka, Japan) in Waymouth MB 752/1 at 38.5°C for 20 min. The released antral follicle-like structures were washed with ice-cold PBS three times, and samples of the fluid accumulating in the antral follicle-like structures were prepared by the method described above. The samples were run in 10.6% SDS-polyacrylamide gels. After electrophoresis, the gels were fixed, silver-stained, and photographed.

Statistical analyses

Results were combined from 7, 5, 5 and 5 independent experiments for FSH, dbcAMP, E_2 and EGF, respectively. The total number of OCG complexes was 29–31 in every group. The incidence of OCG complexes to form antrum was analyzed by χ^2-test. The mean diameters of OCG complexes were represented as the mean ± S.D. and analyzed by Student's *t*-test.

Results

Morphological change in OCG complexes

In the freshly collected OCG complexes from pig early antral follicles, the oocytes were surrounded by cumulus cells in contact with some of the parietal granulosa cells (Fig. 1). The typical morphological characteristics of the OCG complexes undergoing different stimulations during the culture period are shown in Fig. 2. After 1 day of culture, the parietal granulosa cells surrounded the oocytes completely, and every OCG complex became a compact spherical structure regardless of the kind of stimulation. No apparent morphological differences were observed between the control and treated groups of the OCG complexes. After 2 days of culture, no antrum formation occurred inside the OCG complexes in the control group. The granulosa cells then began spreading out into the collagen gels. Thus the compact area of the OCG complexes became smaller at the end of the culture period. In contrast with the control group, in the OCG complexes exposed to FSH and dbcAMP, a single cavity formed inside some of the complexes, which presented an antral follicle-like structure. The boundaries of the round OCG complexes were clear during the culture period. About 60% of the OCG complexes developed antral follicle-like structures at all concentrations of FSH (Fig. 3-A). The largest numbers of the antral follicle-like structures in the OCG complexes were observed after 3 days of culture. These structures appeared in 71, 61 and 58% of the OCG complexes at 10, 100 and 500 ng/ml FSH, respectively. At the end of the culture period, approximately 30% of the OCG complexes maintained their antral structures at all concentrations of FSH. There were no significant differences in antrum formation in the OCG complexes between the effects of differing concentrations of FSH ($p<0.01$). With the formation of antra in the OCG complexes, the diameter of the OCG complexes increased (Fig. 3-B). After 1 day of culture, the mean diameters of the OCG complexes were 542 ± 90, 581 ± 117 and 582 ± 98 μm in 10, 100 and 500 ng/ml FSH, respectively. The complexes were significantly larger than those in the control group (448 ± 65 μm, $p<0.01$). After 2 days of culture, with an increase in the proportion of OCG

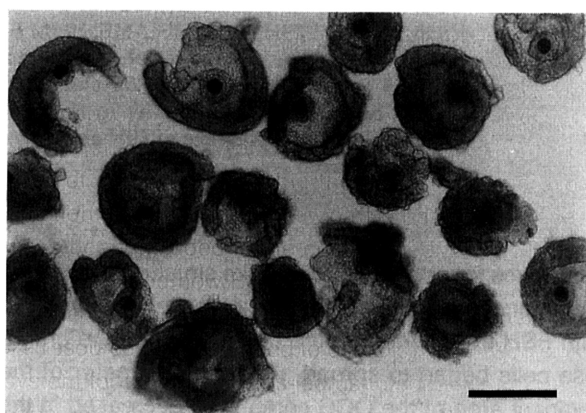

Fig. 1. Oocyte-cumulus-granulosa cell (OCG) complexes collected from pig early antral follicles of 0.5–0.7 mm in diameter before culture. Note that some of the parietal granulosa cells contact the oocyte-cumulus complex. The bar in the figure represents 500 μm.

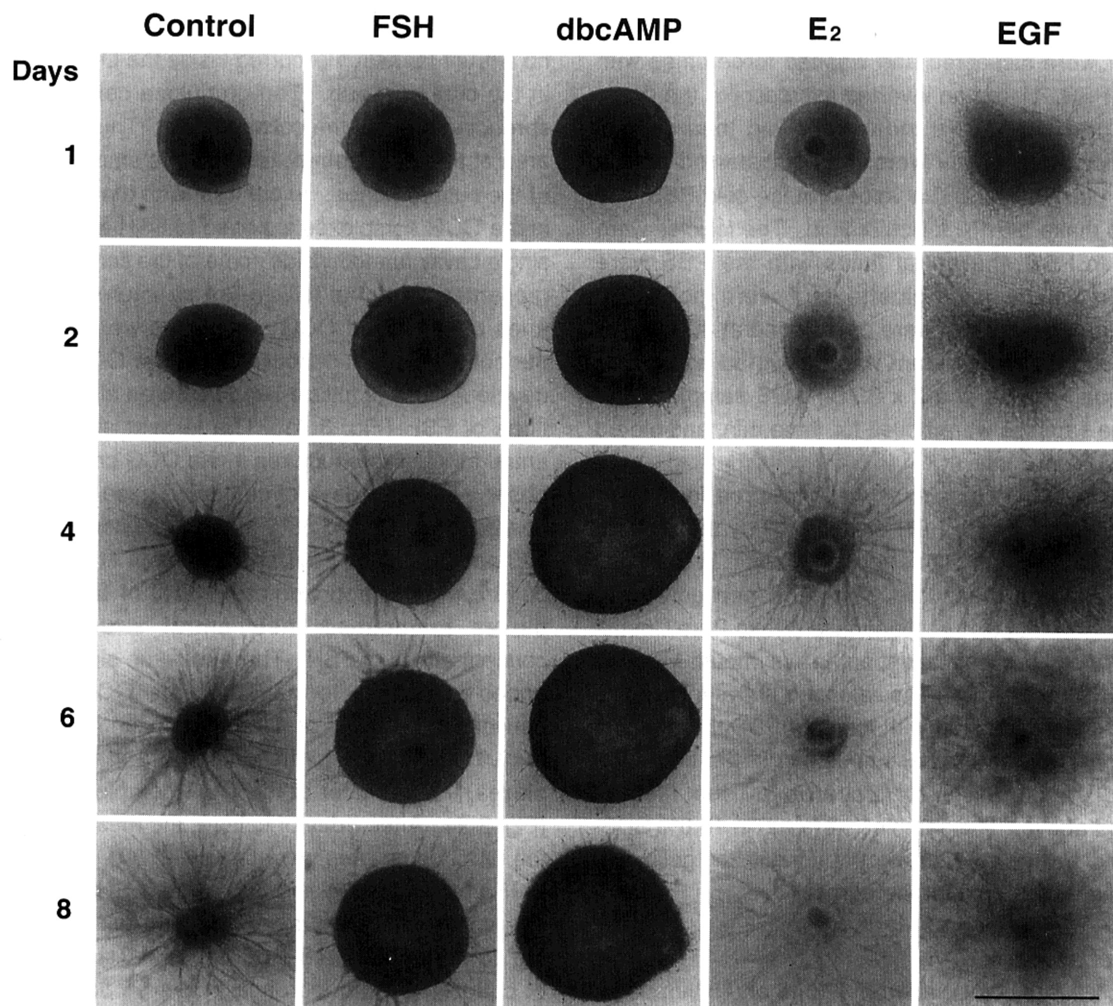

Fig. 2. Morphological changes in oocyte-cumulus-granulosa cell (OCG) complexes cultured in collagen gels in 5% FCS supplemented Waymouth MB 752/1. OCG complexes were cultured in the medium containing 10 ng/ml FSH, 4 mM dbcAMP, 10^{-7}M E_2, and 10 ng/ml EGF, and photographed after 1, 2, 4, 6 and 8 days of culture. Note the spherical compact structures which were formed in every OCG complex after 1 day of culture. After 2 days of culture, granulosa cells spread out, and the compact area of the OCG complex became smaller in the control group. A single antrum was observed inside OCG complexes in the presence of FSH and dbcAMP. In the presence of E_2 and EGF, granulosa cells spread in the collagen gel. The bar in the figure represents 500 μm.

complexes forming antral follicle-like structures, the mean diameters increased. No further increase was observed with FSH treatment. On the other hand, as the granulosa cells began to spread, the mean diameter of the compact area of the OCG complexes decreased in the control group.

In the presence of dbcAMP, most of the OCG complexes had antral follicle-like structures after 2 days of culture (Figs. 2 and 4-A). The percentages were higher in 4 and 8 mM dbcAMP (90 and 86%, respectively) than those found in 2 mM dbcAMP (29%, $p<0.01$). After 3 days of culture, the percentage of the OCG complexes forming antral follicle-like structures increased to 93% in 4 mM dbcAMP, which was significantly higher than those in 2 mM (38%, $p<0.01$) and 8 mM dbcAMP (55%, $p<0.01$). With a longer culture time, the antrum disappeared in some of the OCG complexes. At the end of the culture period, 12, 45 and 23% of the antral follicle-like structures were maintained in 2, 4 and 8 mM dbcAMP, respectively. As shown in Fig. 4-B, there was a significant increase in the mean diameters of the OCG complexes which coincided with antrum formation, as

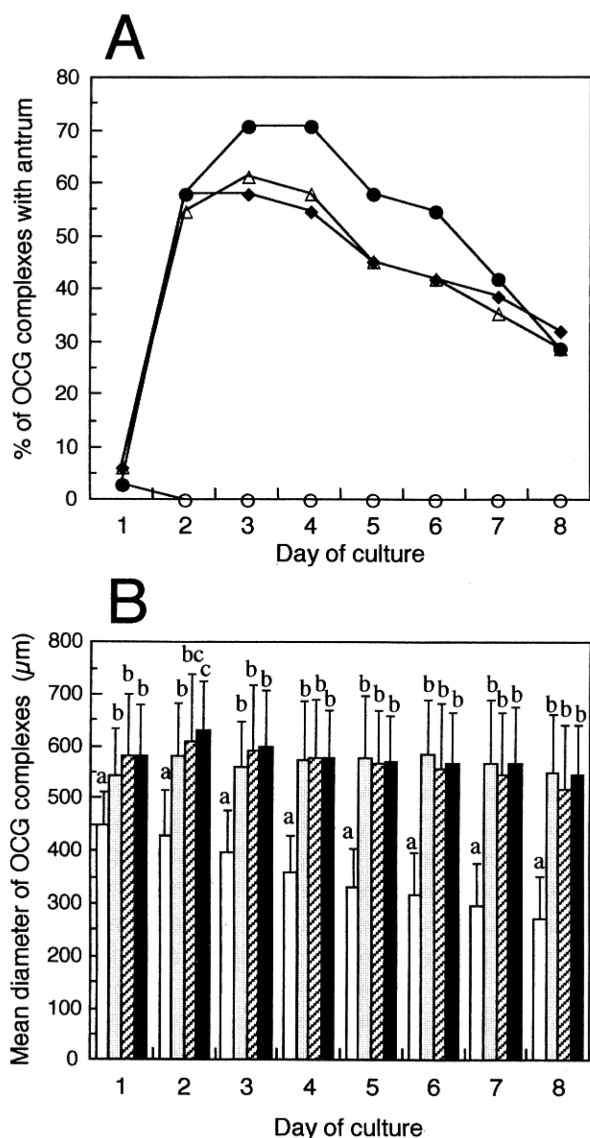

Fig. 3. Effect of FSH on antrum formation (A) and the mean diameters (B) of OCG complexes. OCG complexes from pig early antral follicles were cultured in media containing 0 (-○-, ▭), 10 (-●-, ▭), 100 (-△-, ▨) and 500 ng/ml FSH (-◆-, ■) for 8 days. The results are the combined results for 7 independent experiments. The numbers of OCG complexes were 31 in every group. Each column with a vertical bar in Fig. 3-B represents the mean ± S.D. Values within the figure with different superscripts are significantly different ($p<0.01$).

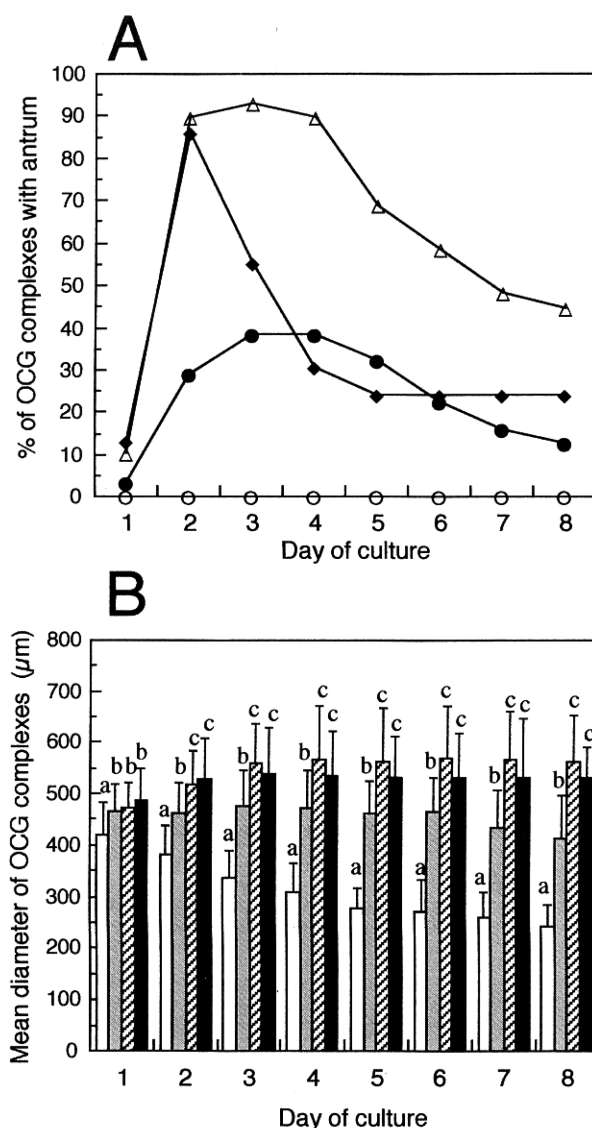

Fig. 4. Effect of dbcAMP on antrum formation (A) and the mean diameters (B) of OCG complexes. OCG complexes from pig early antral follicles were cultured in media containing 0 (-○-, ▭), 2 (-●-, ▭), 4 (-△-, ▨) and 8 mM dbcAMP (-◆-, ■) for 8 days. The results are the combined results for 5 independent experiments. The numbers of OCG complexes were 30, 31, 29 and 29, in 0, 2, 4 and 8 mM dbcAMP groups, respectively. Each column with a vertical bar in Fig. 4-B represents the mean ± S.D. Values within the figure with different superscripts are significantly different ($p<0.01$).

compared to the control group ($p<0.01$).

Antral follicle-like structures did not form in the OCG complexes in the presence of E_2 at concentrations of 10^{-8} (n=30) and 10^{-7} M (n=30), or EGF at 1 (n=30), 10 (n=29) and 100 ng/ml (n=30) (Fig. 2). In the presence of E_2, similar to the control groups, the parietal granulosa cells gradually spread out from the oocytes, and the compact area of the OCG complexes became smaller at the end of the culture period, but the morphology of the OCG complexes exposed to EGF was quite differ-

ent from that observed in the controls. The granulosa cells spread out and completely filled the collagen gel. As the granulosa cells spread out, the areas of compact OCG complexes disappeared, and the exact diameters of the OCG complexes could not be measured after 2 days of culture.

Fluid accumulating in the reorganized antral follicle-like structures

The culture medium showed three major bands which were derived from the supplemented fetal calf serum (Fig. 5). The molecular weights of the three corresponding proteins were 65, 63 and 55 kD. The proteins were contained in the follicular fluids of the early antral follicles in the pig ovaries. In addition to the proteins, the fluids contained other proteins whose molecular weights were 87, 71, 59, 50 and 45 kD. Bands similar to those of the proteins in the early antral follicles were also observed in the fluids from the antral follicle-like structures induced by FSH and dbcAMP stimulation.

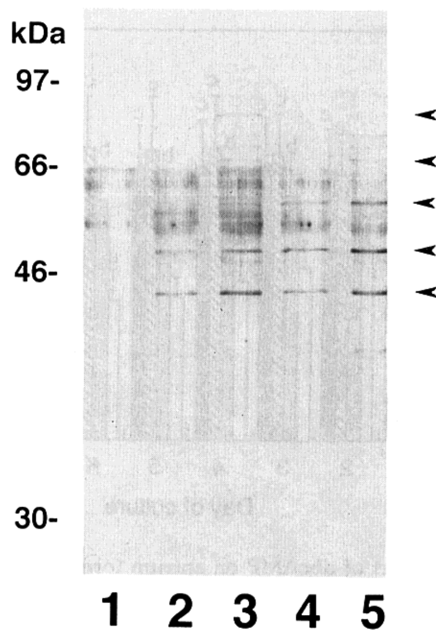

Fig. 5. Electrophoretic analysis of the fluids which accumulated in antral follicle-like structures of OCG complexes cultured *in vitro* for 3 days. Culture medium (Lane 1), ovarian follicular fluid collected from pig early antral follicles of 0.5–0.7 mm in diameters (Lanes 2 and 3), and fluids from antral follicle-like structures induced by FSH (Lane 4) and dbcAMP (Lane 5) were run in 10.6% SDS-polyacrylamide gels and silver stained. Molecular mass standards (kD) are shown on the left. Arrow heads on the right represent the proteins which are not contained in the culture medium but in the follicular fluids.

Discussion

Pig OCG complexes form antral follicle-like structures in response to stimulation by FSH and dbcAMP. Follicular fluid in the ovarian tertiary follicles is composed partly of secretions from the follicle cells (thecal cells and granulosa cells), and partly of exudates from plasma [27]. The fluid consists of nonhormonal components (inorganic components, carbohydrates, mucopolysaccharides, lipids and proteins), gonadotropins and steroid hormones. Although we did not examine any of the components other than the proteins, the fluid accumulating in the antral follicle-like structures of the cultured OCG complexes contained proteins which had molecular weights similar to those in the ovarian antral follicular fluid. This suggests that pig OCG complexes form antra *in vitro* upon stimulation by FSH and dbcAMP. When intact preantral follicles, including thecal cells, from mouse and hamster ovaries are cultured in the medium containing FSH, they develop into antral follicles [5, 6]. It has been reported that an adherent thecal layer is required for antrum formation [7, 28, 29]. Thecal cells stimulate DNA synthesis and the proliferation of granulosa cells by the production of a transforming growth factor [30, 31]. Since no thecal layer surrounded the parietal granulosa cells in the pig OCG complexes in the present study, it seems that it is not necessary for thecal cells to be present for the antra to be organized, if the OCG complexes are stimulated by FSH and dbcAMP. It also appears that the proteins which accumulate inside the antra possibly consist of secretions from the granulosa cells and transudates from the culture media.

Evidence of the effects of FSH on intact mouse and hamster preantral follicles developing into antral follicles *in vitro* [5, 6] has led to the idea that FSH is essential for the antrum formation of follicles. As the second messenger of gonadotropins, it is thought that cAMP transduces signals by gonadotropic stimulation to regulate granulosa cells [10, 11]. dbcAMP promotes granulosa cell proliferation of cultured mouse primary follicles [15], and 8-bromo-cAMP stimulates the proliferation and progesterone secretion of granulosa cells isolated from pig primary and secondary follicles [13]. Moreover, the effects of dbcAMP appear to be similar to those of FSH in inducing cultured flattened epithelioid

granulosa cells to transform into a spherical shape [14]. In the present series of experiments, dbcAMP induced pig OCG complexes to form antra containing proteins similar to those in FSH-induced antra. This indicates that cAMP may mediate the effects of FSH in inducing antrum organization of granulosa cells and the accumulation of antral fluid during ovarian follicular development. It suggested that FSH and cAMP induce aromatase activity and synthesis of estradiol in pig granulosa cells [32]. It has also been reported that estrogen induces ovarian antrum formation in the rat [4, 16], and augments the FSH-induced reorganization of unclosed antral follicle-like structures by rat granulosa cells *in vitro* [8]. In our experiment, E_2 itself did not induce antral formation in pig OCG complexes. This suggests that estrogen is not involved in ovarian antrum formation in pig granulosa cells downstream from cAMP.

EGF as well as FSH stimulates DNA synthesis and proliferation of pig granulosa cells from small antral follicles [13]. Pig follicular fluid contains EGF [33], and EGF receptors have been found in pig granulosa cells [34]. Studies of the gene expression of EGF and its receptors have suggested that EGF may have a paracrine and autocrine role in follicular growth and differentiation [35, 36]. In this study, EGF did not induce antrum formation of the pig OCG complexes. As seen by observation under the inverted microscope, the granulosa cells of pig OCG complexes proliferated and spread into the collagen gels in the EGF-supplemented medium, and no spherical structure with an antrum was formed. EGF has been reported to stimulate [^{3}H]-thymidine incorporation of hamster [21] and bovine [23] cultured follicles and to increase the number of follicles reaching the Graafian stage in the mouse preantral follicles [22]. In the present study, pig OCG complexes were mechanically dissected from the inside of early antral follicles. Since the complexes contained neither basement membrane nor thecal cells, it is thought that EGF-stimulated granulosa cells enter the S-phase, proliferate and then grow out into the collagen gels. On the other hand, it is possible that with FSH and dbcAMP stimulation, the granulosa cells differentiate to form a basement membrane, which in turn restricts the spreading of the granulosa cells.

From this study, we might conclude that FSH is necessary for pig follicular antrum formation. Mediated by cAMP rather than its downstream estrogen, FSH stimulates granulosa cell antrum organization as well as antral fluid secretion. EGF does not have any effect on pig follicular antrum formation.

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References

1) Greenwald, G.S. and Terranova, P.F. (1988): Follicular selection and its control. In: The Physiology of Reproduction (Knobil, E. and Neill, J.D., eds.), pp. 387–445, Raven Press, New York.
2) Mossman, H.W. and Duke, K.L. (1973): Comparative Morphology of the Mammalian Ovary, pp. 105–112, University of Wisconsin Press, Madison.
3) Greep, R.O., van Dyke, H.B. and Chow, B.F. (1942): Gonadotropins of the swine pituitary. I. Various biological effects of purified thylakentrin (FSH) and pure metakentrin (ICSH). Endocrinology, 30, 635–649.
4) Goldenberg, R.L., Vaitukaitis, J.L. and Ross, G.T. (1972): Estrogen and follicle stimulating hormone interactions on follicle growth in rats. Endocrinology, 90, 1492–1498.
5) Roy, S.K. and Greenwald, G.S. (1989): Hormonal requirements for the growth and differentiation of hamster preantral follicles in long-term culture. J. Reprod. Fert., 87, 103–114.
6) Qvist, R., Blackwell, L.F., Bourne, H. and Brown, J.B. (1990): Development of mouse ovarian follicles from primary to preovulatory stages *in vitro*. J. Reprod. Fert., 89, 169–180.
7) Nayudu, P.L. and Osborn, S.M. (1992): Factors influencing the rate of preantral and antral growth of mouse ovarian follicles *in vitro*. J. Reprod. Fert., 95, 349–362.
8) Gore-Langton, R.E. and Daniel, S.A.J. (1990): Follicle-stimulating hormone and estradiol regulate antrum-like reorganization of granulosa cells in rat preantral follicle cultures. Biol. Reprod., 43, 65–72.
9) Richards, J.S. and Midgley, A.R. Jr. (1976): Protein hormone action: a key to understanding ovarian follicular and luteal cell development. Biol. Reprod., 14, 82–94.
10) Hsueh, A.J.W., Adash, E.Y., Jonpbc, P.B.C. and Welsh, T.H. (1984): Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocrin. Rev., 5, 76–127.
11) Amsterdam, A. and Rotmensch, S. (1987): Structure-function relationships during granulosa cell differentiation. Endocrin. Rev., 8, 309–337.
12) Kolena, J. and Channing, C.P. (1972): Stimulatory effects of LH, FSH, and prostaglandins upon cyclic

3',5'-AMP levels in porcine granulosa cells. Endocrinology, 90, 1543–1550.
13) Morbeck, D.E., Flowers, W.L. and Britt, J.H. (1993): Response of porcine granulosa cells isolated from primary and secondary follicles to FSH, 8-bromo-cAMP and epidermal growth factor *in vitro*. J. Reprod. Fert., 99, 577–584.
14) Lawrence, T.S., Ginzberg, R.D., Gilula, N.B. and Beers, W.H. (1979): Hormonally induced cell shape changes in cultured rat ovarian granulosa cells. J. Cell Biol., 80, 21–36.
15) Carroll, J., Whittingham, D.G. and Wood, M.J. (1991): Effect of dibutyryl cyclic adenosine monophosphate on granulosa cell proliferation, oocyte growth and meiotic maturation in isolated mouse primary ovarian follicles cultured in collagen gels. J. Reprod. Fert., 92, 197–207.
16) Gore-Langton, R.E. and Armstrong, D.T. (1988): Follicular steroidogenesis and its control. In: The Physiology of Reproduction (Knobil, E. and Neill, J.D., eds.), pp. 331–385, Raven Press, New York.
17) Richards, J.S. (1975): Estradiol receptor content in rat granulosa cells during follicular development: modification by estradiol and gonadotropins. Endocrinology, 97, 1174–1184.
18) Cohen, S. (1962): Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J. Biol. Chem., 237, 1555–1562.
19) Gospodarowicz, D. and Bialecki, H. (1979): Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine and human origin. Endocrinology, 104, 757–764.
20) May, J.V., Frost, J.P. and Schomberg, D.W. (1988): Differential effects of epidermal growth factor, somatomedin-C/insulin-like growth factor I, and transforming growth factor-β on porcine granulosa cell deoxyribonucleic acid synthesis and cell proliferation. Endocrinology, 123, 168–179.
21) Roy, S.K. (1993): Epidermal growth factor and transforming growth factor-β modulation of follicle-stimulating hormone-induced deoxyribonucleic acid synthesis in hamster preantral and early antral follicles. Biol. Reprod., 48, 552–557.
22) Boland, N.I. and Gosden, R.G. (1994): Effects of epidermal growth factor on the growth and differentiation of cultured mouse ovarian follicles. J. Reprod. Fert., 101, 369–374.
23) Wandji, S.A., Eppig, J.J. and Fortune, J.E. (1996): FSH and growth factors affect the growth and endocrine function *in vitro* of granulosa cells of bovine preantral follicles. Theriogenology, 45, 817–832.
24) Telfer, E.E. (1996): The development of methods for isolation and culture of preantral follicles from bovine and porcine ovaries. Theriogenology, 45, 101–110.
25) Hirao, Y., Nagai, T., Kubo, M., Miyano, T., Miyake, M. and Kato, S. (1994): *In vitro* growth and maturation of pig oocytes. J. Reprod. Fert., 100, 333–339.
26) Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685.
27) Edwards, R.G. (1974): Follicular fluid. J. Reprod. Fert., 37, 189–219.
28) Boland, N.I., Humpherson, P.G., Leese, H.J. and Gosden, R.G. (1993): Pattern of lactate production and steroidogenesis during growth and maturation of mouse ovarian follicles *in vitro*. Biol. Reprod., 48, 798–806.
29) Telfer, E.E., Torrance, C. and Gosden, R.G. (1990): Morphological study of cultured preantral ovarian follicles of mice after transplantation under the kidney capsule. J. Reprod. Fert., 89, 565–571.
30) Bendell, J.J. and Dorrington, J. (1988): Rat thecal/interstitial cells secrete a transforming growth factor-β-like factor that promotes growth and differentiation in rat granulosa cells. Endocrinology, 123, 941–948.
31) Skinner, M.K. and Coffey, R.J. Jr. (1988): Regulation of ovarian cell growth through the local production of transforming growth factor-α by theca cells. Endocrinology, 123, 2632–2638.
32) Chan, W.K. and Tan, C.H. (1987): Induction of aromatase activity in porcine granulosa cells by FSH and cyclic AMP. Endocrin. Res., 13, 285–299.
33) Hsu, C.J., Holmess, S.D. and Hammond, J.M. (1987): Ovarian epidermal growth factor-like activity: concentration in porcine follicular fluid during follicular enlargement. Biochem. Biophys. Res. Commun., 147, 242–247.
34) Fujinaga, H., Yamoto, M., Nakano, R. and Shima, K. (1992): Epidermal growth factor binding sites in porcine granulosa cells and their regulation by follicle-stimulating hormone. Biol. Reprod., 46, 705–709.
35) Kennedy, T.G., Brown, K.D. and Vaughan, T.J. (1993): Expression of the genes for the epidermal growth factor receptor and its ligands in porcine corpora lutea. Endocrinology, 132, 1857–1859.
36) Singh, B., Rutledge, J.M. and Armstrong, D.T. (1995): Epidermal growth factor and its receptor gene expression and peptide localization in porcine ovarian follicles. Mol. Reprod. Dev., 40, 391–399.